



The implementation of drug reposition for alcoholic hepatitis based on a sub-pathway integration strategy

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Background: Alcoholic hepatitis (AH) is one of the most severe forms of liver disease. The therapies which are currently available are not entirely effective, and thus novel therapies are urgently needed. However, the development of these novel therapies is limited due to incomplete information about the molecular mechanisms involved in AH.

Methods: The microarray data (GSE28619) was downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) between the AH and the control samples were identified using the significant analysis of microarrays (SAM) method. Metabolic sub-pathways were identified using the SubpathwayMiner R package. Cell Counting Kit-8 (CCK-8) was used to evaluate the cell viability of AML-12 cells treated with different concentrations of ethanol or riboflavin. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were employed to show the hepatocyte function.

Results: A total of 1,041 genes were determined to be expressed differentially. We then identified 11 metabolic sub-pathways that could be involved in the development of AH. This was followed by a final integrated analysis of the sub-pathways involved in AH as well as the sub-pathways involved in the drug-affected cases. The final integration results led to the identification of 64 small molecular drugs. A potential novel drug (riboflavin) involved in the fatty acid metabolism pathway was identified for further investigation. Riboflavin at the 60 nM for 24 h could reverse ethanol-induced AML-12 cell injury and could markedly decrease ALT and AST activity. The decrease in the activities of these two enzymes was observed in a dose-dependent manner when it was compared to ethanol alone, which suggests that riboflavin has a protective effect against liver cell injury caused by alcoholism.

Conclusions: To summarize, the candidate agents which are identified in the present study might give practitioners insight into the development of novel therapeutic approaches for AH.

Keywords: Drug reposition; alcoholic hepatitis (AH); sub-pathway; metabolic network

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Introduction

The most serious form of alcoholic liver disease (ALD) is alcoholic hepatitis (AH), most commonly called AH (1). It is a clinical syndrome that is usually characterized by damage to hepatocellular tissues, steatosis, and pericellular

fibrosis (2). Typically, AH is associated with alcohol misuse and usually exhibits progressive jaundice and inflammatory liver injuries (2,3). Severe cases have poor short-term prognosis, and if observed for 28 days, a death rate of around 30–50% (4,5). In addition to this, there is yet no

definitive pathological process responsible and thus a lack of developed targeted therapy (6).

Considerable progress has been made toward the characterization of immune response for this disease. These responses include the development of both pro- and anti-inflammatory cytokines in the blood, trafficking of immune cells to the liver, and determining the mechanism of cell death (3,7-9). However, the unavailability of animal models for studying AH has limited further progression in these types of studies (5). Recent research has shown that the drugs targeting inflammatory cytokines such as tumor necrosis factor (TNF) may play an essential role in AH, and thus, drugs interfering with TNF were tested in patients (8-10). Unfortunately, the results observed from these studies were disappointing as they led to an increased risk of fatal bacterial infections in patients treated with TNF (11,12). Therefore, targeted strategies for the treatment of AH are still unavailable.

It is known that the *de novo* development of drugs is time-consuming, costly, and has no guarantee for success. Meanwhile, another innovative approach, referred to as drug repositioning, has been developed due to advances in computational biology and the availability of public databases (13). The Gene Expression Omnibus (GEO) and the connectivity map (CMap) databases provide considerable microarray datasets which were obtained either under diseased conditions or under the effects of drugs (14). In this study, a pathway-based method responsible for the drug repositioning of AH was conducted by using a bioinformatics analysis, which was followed by experimental validation. Initially, the metabolic mechanisms of AH were explored, which was followed by the identification of potential small molecular drugs. The drugs were selected in such a way that they could target metabolic sub-pathways which undergo dysregulation during the disease's development and progression. Finally, via *in vitro* study, our data indicated that the treatment of riboflavin, one of the agents we identified, could reverse ethanol-induced AML-12 cell injury. In brief, our research may offer significant insight into the potentially effective therapies for AH.

Methods

Identification of gene expression using microarray data

Microarray data for GSE28619 were obtained from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) provided by the National Center of Biotechnology Information (NCBI). In this profile, hepatic gene expression was assessed in patients with AH (n=15) and controls with

normal livers (n=7) using the Affymetrix Human Genome U133 Plus 2.0 Array platform (6). The data obtained from microarray experiments and the probe annotation files were then downloaded for the analysis. Here, 2 methods were used for the identification of genes undergoing differential expression, commonly known as DEGs (differentially expressed genes), and the overlapping gene set was considered as the final DEGs of interest. At first, if 1 gene matched more than 1 probe, the expression value of this gene was computed by taking the average expression value of all the corresponding probes. We then converted each probe into a gene by using the Entrez Gene IDs, and the fold change was calculated for all the genes. The genes that showed a higher than 2 or lower than 0.5-fold change was selected. A total of 1,123 genes were obtained. Next, we used the SAM method to identify DEGs, and genes with a q-value <0.01 were selected (4,337 genes were obtained). Finally, the overlapping genes between the 2 sets of data (1,040 genes) were considered as DEGs.

Identification of AH-related metabolic sub-pathways

SubpathwayMiner R package, which is a pathway identification method known to be flexible for sub-pathway determination was used (15) to determine which sub-pathways were perturbed during AH. This software firstly converted the pathway structure data present in the Kyoto Encyclopedia of Genes and Genomes (KEGG) to undirected R graph objects. Thereafter, the enzymes were regarded as nodes. The 2 nodes which belonged to a single reaction were connected by an edge in an undirected graph. Finally, SubpathwayMiner could divide the entire pathway into sub-pathways using the "k-clique" method, which is defined as a sub-graph in which the distance between any 2 nodes is no greater than a parameter, k, in social network analysis (15). Here, k=3 was the parameter set, which suggests that the gap between the enzymes in a single sub-pathway was lower than 3.

After our DEGs were put into SubpathwayMiner, the results obtained indicated the significantly enriched sub-pathways of AH. A P value <0.01 was considered as the cutoff criterion for statistically significant metabolic sub-pathways.

The connections between potential drug targets and sub-pathways involved in metabolism

For the identification of the novel small molecular drugs

that might affect important sub-pathways involved in AH, we considered the data on the global associations which were observed between drugs and the metabolic sub-pathways provided by Li and coworkers (16). Li and colleagues analyzed microarray data from cells that were pre-treated with small molecular drugs determined from the Cmap database and thus identified the DEG set for every drug using a cutoff with a $|\log(\text{fold change})| > 1$ (16). Then, the sub-pathways which were affected by drugs were identified using the SubpathwayMiner software package (P value < 0.01) (16). Subsequently, a total of 3,925 associations were obtained between the drugs and the metabolic sub-pathways, that were affected, comprising 488 drugs and 403 metabolic sub-pathways. Out of the obtained 403 sub-pathways, 6 sub-pathways (corresponding to 64 drugs) were hampered by AH as well.

Cell culture

AML-12 cells were used for this study, and these cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in F12 medium in the presence of 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The culture medium and the antibiotics were purchased from Invitrogen (USA) while the FBS was purchased from Invitrogen (Carlsbad, CA, USA). The cells used for the study were grown and kept under humidified conditions in the presence of 5% CO_2 at a temperature of 37 °C.

Cell Counting Kit-8 (CCK-8) cell viability assay

AML-12 cells were treated with different concentrations of ethanol (0, 50, 75, 100, 150, 200) mM or riboflavin (Sigma, USA) at the concentrations of 30 or 60 nM. Cells were seeded in 96-well plates at a density of 2×10^3 per well and cultured for 24 h. Cell viability was assessed by the Cell Counting Kit-8 (CCK-8, Dojindo, Japan).

Biochemical assays

The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercial kits that were procured from Nanjing Jiancheng Bioengineering Institute located in Nanjing, China. The amount of ethanol in the blood was then determined by a kit which was procured from BioVision Inc. (San Francisco, CA, USA). The experiments using the procured kits were

performed as per the manufacturer's instructions.

Statistical analysis

The data obtained from these experiments were analyzed using SPSS 19.0 (Chicago, IL, USA). The results of the experiments are stated as the mean values, along with the standard deviation (SD) obtained from the multiple sets of experiments. A comparison between multiple groups was conducted by use of the analysis of variance (ANOVA) and Student-Newman-Keuls method. Differences were statistically significant at a P value < 0.05 .

Results

Analysis of DEGs between AH and healthy controls

We obtained the mRNA expression profiles of AH samples and normal control samples from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). The GSE28619 dataset, which was also available in the GEO database, was downloaded, and then the DEGs between AH patients and controls were identified by fold changes and the SAM method (6). A total of 1,041 genes were determined to be expressed differentially (*Figure 1*; fold change > 2 or < 0.5 and q-value < 0.01 ; see Methods).

Exploration of the AH mechanism based on sub-pathway enrichment analysis

For the exploration of the metabolic mechanisms of AH, the Subpathwayminer was used to identify AH-involved metabolic sub-pathways (15). After annotating the DEGs onto the metabolic sub-pathways ($k=3$), 11 enriched metabolic sub-pathways were identified which corresponded to 8 total metabolic pathways having a strict cutoff P value (P value < 0.01) as shown in *Table 1*. As a result of these experiments, 11 metabolic sub-pathways were identified as being related to AH development.

AH drug repositions

Next, we identified candidate agents that could potentially target AH-related metabolic sub-pathways, using the dataset of global associations observed between the drugs and the metabolic sub-pathways published by Li and colleagues (16) (see the Methods).

After the integration of the 11 AH-related sub-pathways

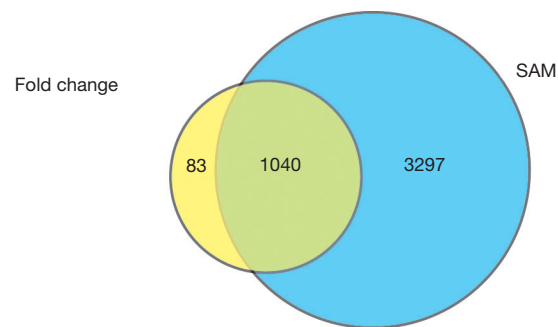


Figure 1 A Venn diagram of DEGs identified by the two methods. Overlapped genes are shown. DEG, differentially expressed gene.

Table 1 Enriched sub-pathways and their corresponding entire pathways

Sub-pathway ID	Pathway name	P value	Gene number
Path:00071_2	Fatty acid metabolism	2.97E-06	20
Path:00120_3	Bile acid biosynthesis	0.004102891	8
Path:00120_4	Bile acid biosynthesis	0.004102891	8
Path:00120_5	Bile acid biosynthesis	0.000741794	7
Path:00232_1	Caffeine metabolism	0.000847133	12
Path:00232_2	Caffeine metabolism	0.000847133	12
Path:00260_5	Glycine, serine and threonine metabolism	0.005276321	6
Path:00361_2	gamma-Hexachlorocyclohexane degradation	0.007718836	15
Path:00521_5	Streptomycin biosynthesis	0.005010718	3
Path:00590_3	Arachidonic acid metabolism	0.003407715	19
Path:00641_1	3-Chloroacrylic acid degradation	0.007653709	7

and the 403 sub-pathways affected by drugs, the overlapping sub-pathways, which were both related to AH and affected by small molecular drugs, were obtained. The drug molecules which could affect the sub-pathway are potential therapeutic candidates to target the related pathways of AH. Sixty-four small molecules were identified in total (Table 2).

This was followed by the building of a bipartite network as per the predicted drugs and the overlapping metabolic sub-pathways by the integration of the above relationships, as stated in Figure 2. We found that some drugs could perturb up to 5 metabolic sub-pathways which corresponded to 4 complete pathways (fatty acid metabolism, caffeine metabolism, gamma-hexachlorocyclohexane degradation, caffeine metabolism, and arachidonic acid metabolism), whereas others could only perturb 1 sub-pathway (glycine, serine, and threonine metabolism; right part of Figure 2).

Potential pathway of riboflavin in the metabolism of purines

For the prediction of the potential mode of action of riboflavin in AH, the DEGs for AH and the DEGs for riboflavin were mapped to the pathway responsible for the metabolism of purines (path: 00230) by using the “user data mapping” tool which is available in the KEGG database (<http://www.genome.jp/kegg/>) (Figure 3). The DEGs of riboflavin were obtained from the supplementary datasets, which were provided by Li and colleagues (16). The DEGs of riboflavin and AH are indicated by blue and red rectangles, respectively, in Figure 3.

Protective effects of riboflavin on alcoholic liver injury

The effect of riboflavin was determined in liver injury which is induced by alcohol usage. We first explored

Table 2 Potential drugs and their information

Drug ID	Drug name	Number of overlaps
	Raubasine	5
	Pipenzolate bromide	5
	Azaperone	5
DB00338	Omeprazole	5
	Tiapride	5
	Etanidazole	5
DB01438	Phenazopyridine	5
DB00692	Phentolamine	5
DB00759	Tetracycline	5
DB00714	Apomorphine	5
DB01392	Yohimbine	5
	Acemetacin	5
DB00553	Methoxsalen	5
DB00448	Lansoprazole	5
DB00734	Risperidone	5
DB02187	Equilin	5
DB00904	Ondansetron	5
DB00140	Riboflavin	5
DB00450	Droperidol	5
DB03467	Naringenin	5
	Butamben	5
DB01097	Leflunomide	5
DB00605	Sulindac	5
DB03585	Oxyphenbutazone	5
Lisuride	Lisuride	5
	Thiocolchicoside	5
DB00292	Etomidate	5
DB00624	Testosterone	5
DB00780	Phenelzine	4
DB04815	Clioquinol	4
DB00396	Progesterone	4
DB01058	Praziquantel	3
DB00270	Isradipine	3
DB00564	Carbamazepine	3
	Enilconazole	3

Table 2 (continued)**Table 2** (continued)

Drug ID	Drug name	Number of overlaps
DB04571	Trioxysalen	3
DB00646	Nystatin	3
DB00613	Amodiaquine	3
DB01428	Oxybenzone	3
DB00875	Flupentixol	2
DB00461	Nabumetone	2
	Cefalonium	2
DB07402	Azapropazone	2
DB00975	Dipyridamole	1
	Dihydroergocristine	1
	Cyproterone	1
	Dequalinium chloride	1
DB08800	Chloropyramine	1
DB01156	Bupropion	1
DB00656	Trazodone	1
	Fenbendazole	1
	Alexidine	1
DB08984	Etofenamate	1
DB04216	Quercetin	1
DB00877	Sirolimus	1
	Todalazine	1
DB01017	Minocycline	1
DB00320	Dihydroergotamine	1
DB01153	Sertaconazole	1
DB00575	Clonidine	1
DB00639	Butoconazole	1
DB02877	Ttnpb	1
	Spiperone	1
DB06803	Niclosamide	1

whether treatment with alcohol can induce AML-12 cell injury. We evaluated the cell viability of alcohol at different concentrations (0, 50, 75, 100, 150, 200) mM for 24 h. As observed in *Figure 4A*, the cell viability of the used cells was found to decrease in a dose-dependent manner. We also determined the levels of AST and ALT, which was the

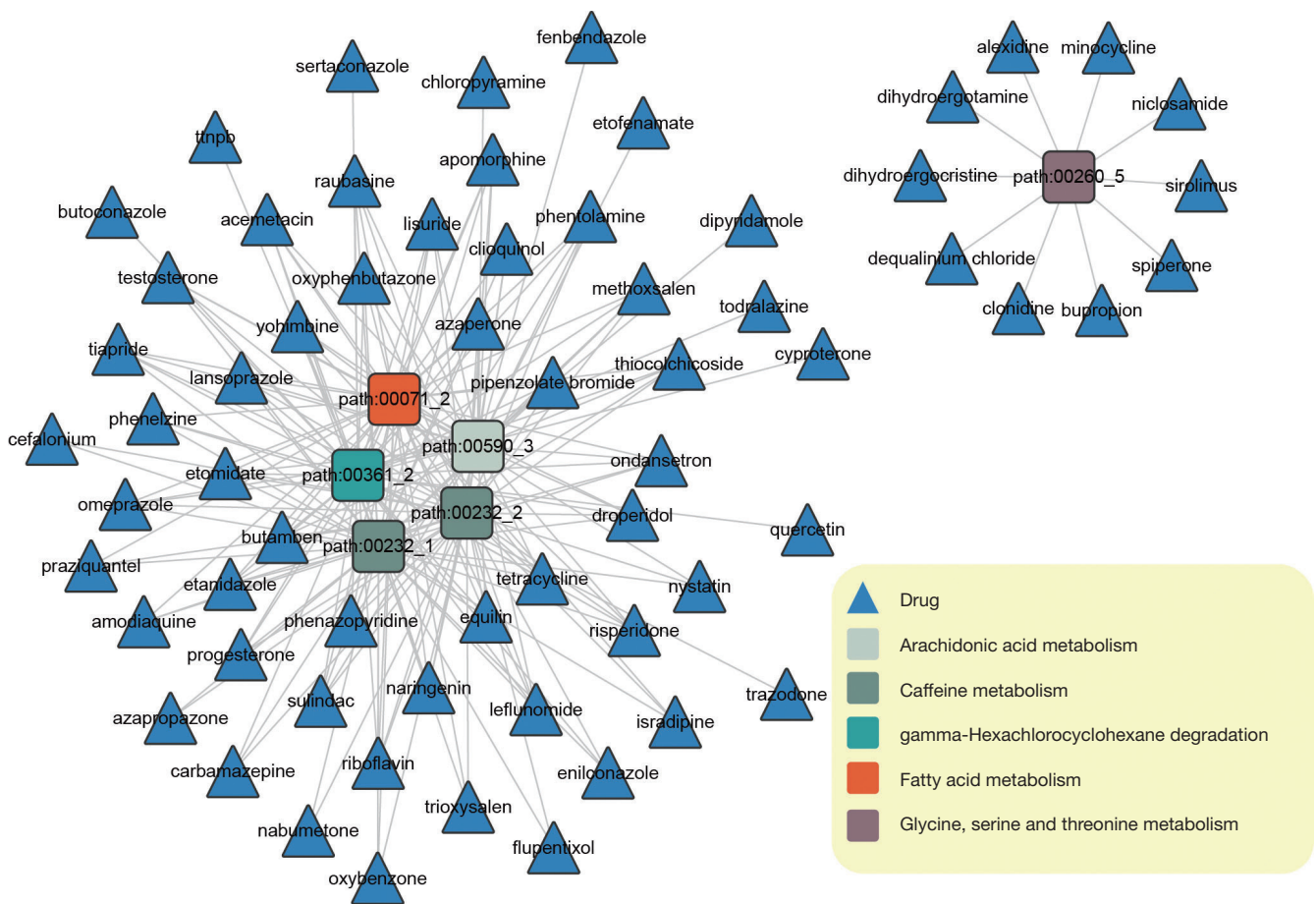


Figure 2 Small molecular drugs and their perturbed sub-pathways in AH. The drugs and sub-pathways are represented by triangular and rectangular nodes, respectively. Sub-pathways of 1 color belong to 1 entire pathway. A complete description of the entire pathway is shown on the left of the figure. AH, alcoholic hepatitis.

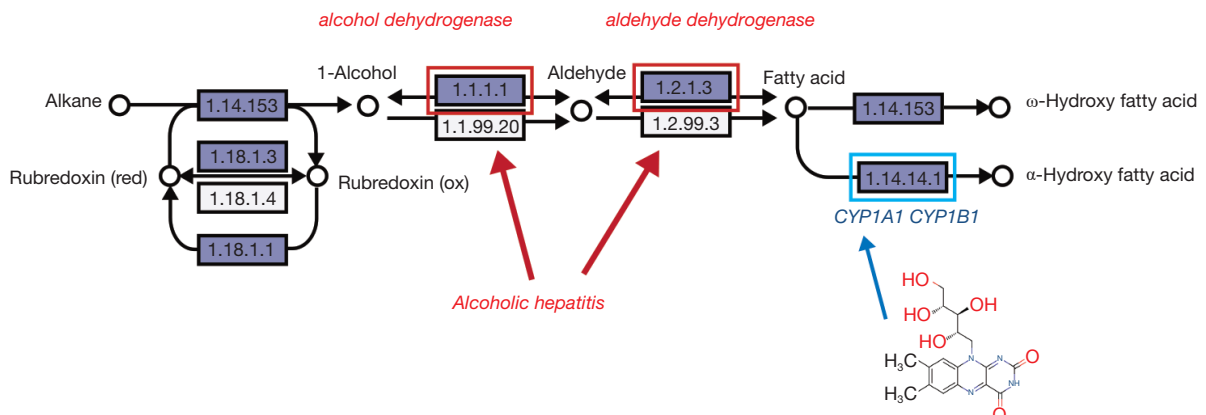


Figure 3 Enzymes that were perturbed due to AH and by riboflavin. Enzymes that were perturbed due to AH and by riboflavin are indicated by red and blue rectangles, respectively. AH, alcoholic hepatitis.

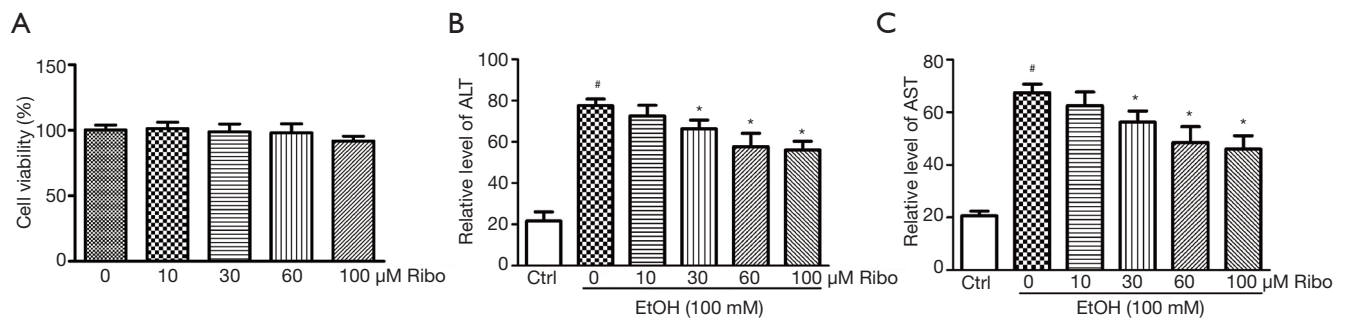


Figure 4 Protective effects of riboflavin on alcoholic liver injury. (A) Cell viability was determined by CCK-8 assay; (B) serum levels of alanine aminotransferase (ALT); (C) serum levels of aspartate aminotransferase (AST). The data are presented as the mean \pm SD of 3-6 independent experiments. Statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc test and represented as follows: #, $P < 0.05$ vs. the control group and *, $P < 0.05$ vs. the ethanol group.

standard of liver injury. Our experiments also showed that the level of AST or ALT was decreased in a dose-dependent manner (Figure 4B,C). This was followed by stimulation of AML-12 cells with 100 mM EtOH for 24 h, which could serve as an alcoholic *in vitro* model for the study.

To identify the regulatory effect of riboflavin towards ethanol-induced AML-12 injury, we detected the cell viability of AML-12 treated with riboflavin (0, 10, 30, 60, 100) nM for 24 h. As displayed in Figure 4B,C, the results show that, compared to the control group, the levels of ALT and AST, and the activity of these enzymes were higher in the alcohol group. However, compared to ethanol alone, riboflavin treatment significantly lowered ALT and AST activity in a dose-dependent manner, thereby suggesting that riboflavin shows a protective effect against liver injury caused by alcohol. Collectively, the results from these experiments indicate that riboflavin is an effective molecule in protecting ethanol-induced liver cell injury.

Discussion

Microarray analysis is a high-throughput screening technology that is considered an essential tool for analyzing gene expression patterns and providing possible targets for improvements in therapeutic interventions. In the present study, microarray data of laparoscopic liver biopsy specimens of AH patients were used to explore the basic mechanisms for the development of AH. We used strict criteria for the identification of DEGs; i.e., genes comprising of a fold change >2 or <0.5 and a q -value <0.01 using the SAM method. As a result, 1,040 DEGs were identified. With the use of the SubpathwayMiner software, it was indicated that there were 11 metabolic sub-pathways,

which corresponded to a total of 8 pathways, and were dysregulated during AH. The potential small molecules for AH were then predicted by the integration of these sub-pathways that were related to AH and the drugs used. Finally, a total of 64 small molecules that might perturb the development of AH were identified.

Unlike other methods, the sub-pathway approach focuses on a certain local area of the entire pathway, suggesting that it can identify more subtle changes that are always neglected by the analysis of complete pathways. This may, therefore, be a more suitable and more flexible approach for understanding drug responses (15,16). In the present study, 11 sub-pathways, which corresponded to 8 complete KEGG metabolic pathways, were identified. A few of these sub-pathways have been reported to play important roles in AH (Table 1). For example, the most significant sub-pathway detected was shown to be involved in fatty acid metabolism (path:00071_2; P value = 2.97^{-16}). It is known that the accumulation of fat in the form of lipid droplets within hepatocytes can result in hepatic steatosis, which is a hallmark of liver diseases like AH (17,18). Furthermore, dietary fatty acids and obesity have been shown to affect the degree of fat accumulation within the liver (17,18). Therefore, fatty acid metabolism appears to play a key role in the development of AH. The “caffeine metabolism” pathway (path: 00232) was also identified to be among the affected sub-pathways. Studies have indicated that caffeine clearance is enhanced during AH compared with controls (19). The “arachidonic acid metabolism” (path: 00590) path was also found to be broadly distributed in AH, suggesting there to be abnormal microsomal elongation activity in the liver cells (20).

We also identified a group of drugs with possible

therapeutic efficacy for the disease. The findings of the studies in the current study culminated in 64 small molecules being identified. Some molecules share affected sub-pathways with AH, and it is understood that some of these agents are active against AH (testosterone) and other potentially new agents. Testosterone, a steroid sex hormone, has the ability to robustly inhibit hepcidin transcription associated with the development of liver disorders such as liver disease (21).

Decades ago, testosterone was shown to be efficacious in the removal of hepatic fat, which is variably present among patients with acute AH (22). Riboflavin, also known as vitamin B2, is an easily absorbed, water-soluble micronutrient with a critical role in maintaining human health. A deficiency of riboflavin has been reported among patients with a histologically normal liver but not among other groups (23). However, there have been no direct reports of the therapeutic management of riboflavin in AH patients, and the underlying mechanisms are still unclear. To investigate the potential mechanisms of action of riboflavin on AH, we annotated the DEGs of AH and riboflavin in the most significant pathway “fatty acid metabolism” (Figure 3). Annotation of the DEGs for AH identified alcohol-metabolizing enzymes, including aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH), whose genetic mutation was associated with the development of acute AH (24).

Furthermore, the DEGs for riboflavin included CYP1B1 and CYP1A1, which participate in the same or consecutive reactions with ADH and ALDH. Our data showed that riboflavin treatment could reverse ethanol- induce AH and markedly decrease ALT and AST activity. The enzymatic activity was decreased in a dose-dependent manner in comparison to the group with ethanol alone, which suggests that riboflavin exerts a protective effect against liver injury caused by alcohol. These findings imply the potential therapeutic effect of riboflavin on AH by targeting critical alcohol-metabolizing reactions.

In this study, we identified 64 novel drugs, which are small molecules in nature, with the potential to target AH-involved sub-pathways. We also illustrated the potential mechanisms of the activity of these novel drugs on AH. Although our work needs further experimental verification, the potential molecules identified in this study provide valuable knowledge in the development of novel therapeutic approaches for AH.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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