Chemical genomics of cancer chemopreventive dithiolethiones

Quynh T.Tran1,2,3, Lijing Xu1,3, Vinhthuy Phan1,4, Shirleen B.Goodwin2,4, Mostafizur Rahman3,3, Victor X.Jin2, Carrie H.Sutter2,3, Bill D.Roebuck5, Thomas W.Kensler6, E.Olusegun George1,3,4 and Thomas R.Sutter2,3,6

1Department of Mathematical Sciences and 3Department of Biology, University of Memphis, Memphis, TN 38152, USA, 2Department of Cancer Drug Discovery and Development, University of Memphis, Memphis, TN 38152, USA, 4Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, USA and 5Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA.

2To whom correspondence should be addressed. Tel: +1 901 678 8391; Fax: +1 901 678 2458; Email: tsutter@memphis.edu

3H-1,2-dithiole-3-thione (D3T) and its analogues 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (OLT) and 5-tert-butyl-3H-1,2-dithiole-3-thione (TBD) are chemopreventive agents that block or diminish early stages of carcinogenesis by inducing activities of detoxication enzymes. While OLT has been used in clinical trials, TBD has shown to be more efficacious and possibly less toxic than OLT in animals. Here, we utilize a robust and high-resolution chemical genomics procedure to examine the pharmacological structures–activity relationships of these compounds in livers of male rats by microarray analyses. We identified 226 differentially expressed genes that were common to all treatments. Functional analysis identified the relation of these genes to glutathione metabolism and the known pharmacological relationships that are present in the chemical analogues in this chemical series.

Introduction

The chemicals 3H-1,2-dithiole-3-thione (D3T), 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (OLT) and 5-tert-butyl-3H-1,2-dithiole-3-thione (TBD) are dithiolethione class chemopreventive agents that have been shown to inhibit chemical toxicity and carcinogenicity in many target organs (1). They continue to be under preclinical and clinical investigations for use as cancer chemopreventive agents in humans (2). The three compounds are known to activate the Kelch-like erythroid cell-derived protein with Cap ‘n’ collar homology (ECH)-associated protein 1–nuclear factor, erythroid derived 2-related factor 2 (Nrf2)-signaling pathway (3,4). Kelch-like ECH-associated protein 1 sequesters Nrf2 through binding to the N-terminal regulatory domain of Nrf2 in the cytoplasm (5). Administration of sulfhydryl reactive compounds such as diethylmaleate, sulforaphane or D3T abolishes Kelch-like ECH-associated protein 1 repression of Nrf2 activity, facilitating the translocation and accumulation of Nrf2 in the nucleus (4,6,7). As a transcription factor, Nrf2 binds to the antioxidant response element, forms heterodimers with small Maf (v-maf, avian musculoaponeurotic fibrosarcoma oncogene homolog) proteins and regulates the expression of antioxidant response element-containing genes (6,7). The induction of these cytoprotective proteins enhances resistance against electrophiles, oxidative stress and chemical toxicants (8).

Several studies of OLT have evaluated the pharmacodynamics of this compound in cancer chemoprevention (9). Additional clinical trials were conducted in Qidong, Jiangsu Province, People’s Republic of China (10–12). These trials were randomized, placebo-controlled and double-masked studies. In a Phase IIa chemoprevention trial, high dose OLT (500 mg/week) inhibited bioactivation of aflatoxin B1 (AFB1), whereas sustained low dose OLT (125 mg/day) increased conjugation of AFB1 epoxide, leading to increased rates of excretion of aflatoxin–mercapturic acid in urine (12). TBD is a recent lead dithiolethione compound for cancer chemoprevention. In 2003, Roebuck et al. (2) compared the cancer chemopreventive potency of several dithiolethione analogues in male F344 rats. In this study, D3T, OLT and TBD were shown to attenuate the hepatic burden of AFB1-induced preneoplastic lesions. Relative potency estimates were obtained by comparing the doses of dithiolethiones required to reduce the focal volume percent to 0.1% of the liver volume. D3T proved to be the most potent agent, whereas TBD was equipotent with OLT. Furthermore, it was suggested that TBD may be a more useful chemopreventive agent than OLT because of features that may make it less toxic, specifically the difference in its wavelength absorption and the lack of a pyrazine ring, as well as relative ease and yield of synthesis (2) (see Figure 1A for chemical structures).

Here, we apply a robust chemical genomics approach to compare and contrast the gene expression activities of D3T, OLT and TBD. We show that this procedure is simultaneously able to identify treatment-responsive genes and the known pharmacological relationships that are common among, and distinct between, the treatment groups. Furthermore, we show that the distinct gene clusters, indicative of unique structure–activity relationships, capture the activities of additional pharmacophores present in the chemical analogues in this chemical series.

Materials and methods

Animal treatments

Male Fischer F344 rats (80–100 g) were obtained from Harlan Laboratories (Indianapolis, IN) at 6 weeks of age. The chemicals and procedures for animal care were described previously in detail (13). Animals were randomly assigned into four groups of four animals for each treatment by gavage with 100 μl of vehicle (saturated sucrose), D3T (0.3 mmol/kg body wt), OLT (0.3 mmol/kg body wt) or TBD (0.3 mmol/kg body wt), once every other day over 5 days. Animals were killed 24 h after the third dose and the liver tissues were snap-frozen for analysis.
Total RNA was isolated from the frozen liver tissue using STAT-60 (Tel-Test, Friendswood, TX). Affymetrix RG-U34A chips (containing 8799 probe sets) were used to measure the messenger RNA levels as described previously (13). The Affymetrix dataset was submitted to the Gene Expression Omnibus database, GSE8882. Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) was performed as described previously (13). The Ct values for all genes were normalized to that of β-actin, and the relative value for the control samples was set as one arbitrary unit.

Cytochrome P450 1A2 inhibition assays
Cytochrome P450 (CYP) 1A2 inhibition assay for 7-ethoxyresorufin O-deethylase activity, 1 ml each, contained 5 pmol of human microsomal CYP1A2 (BD Biosciences, San Jose, CA), 1.4 mM reduced nicotinamide adenine dinucleotide phosphate and 0.2 μM of the substrate, 7-ethoxyresorufin in 0.1 M KPO4 buffer, pH 7.4. D3T, OLT and TBD were dissolved in dimethyl sulfoxide and added at concentrations of 0, 1, 3, 10, 30 and 100 μM. The reactions were incubated for 15 min at 37°C, stopped by the addition of 2 ml of ice-cold methanol, vortexed and then centrifuged for 5 min at 4302 g. The formation of product was determined fluorometrically against a standard of resorufin, using a Varian Cary Eclipse fluorescence spectrophotometer (EL 0206-5717) with an excitation and emission wavelength of 530 and 585 nm, respectively.

Data analysis
Microarray data were preprocessed by GC-Robust Multi-array Average (14). Affymetrix Microarray Suite 5.0 was used to detect the presence and absence of gene expression (15). Genes that were present in 75% of any treatment group were selected using a Perl program. The Kruskal–Wallis (KW) procedure, permutation tests and the recursive procedure were implemented using JAVA. In the
current Affymetrix technology, one gene may be represented by more than one probe set. For these redundant probe sets, only the probe sets with the most numbers of smallest pairwise comparison $P$-values were kept for further analysis. Principal component analysis (PCA) was performed according to Raychaudhuri et al. (16). Eigen values for PCA were calculated using Cluster 3.0 (17). SigmaPlot 10.0 (Synergy Software, Inc., Chicago, IL, 2002) was used to produce three-dimensional PCA plots with color-coded clusters. Analysis of variance (ANOVA) was performed using a National Institute on Aging Array Analysis Tool (18). Gene ontology (GO) enrichment analysis was performed using Gene Ontology Enrichment Analysis Software Toolkit (19). The significant of each GO class was determined by its $P$-value using default criteria. Biological network and pathway analysis were performed using ingenuity pathway analysis software (Ingenuity System, Redwood City, CA). Common transcription factor–binding sites (TFBSs) in promoters of coregulated genes were identified using conserved and overrepresented transcription factor binding sites (CORE_TF) (20). Ensembl gene IDs were used as input into CORE_TF. A 5′ flanking region of 2 kb upstream to 1 kb downstream of the transcription start site was designated as an experimental promoter set since most of TFBSs are located within these regions. A random set of 3000 promoters from rat species was selected to be compared with the experimental set in searching for overrepresented TFBSs (20).

### Statistical methods

To ensure robustness, we used the KW rank procedure, which does not require assumptions of data distribution. To account for the small number of replicates, exact $P$-values of the KW tests were computed using a permutation resampling method (21) to perform 10,000 $P$-values permutations. For each gene with a significant KW test, post-hoc analyses were performed to identify the pairs of treatments with significantly different expression values using the Wilcoxon rank-sum tests. Since the number of replication per treatment group is small, exact $P$-values of the Wilcoxon tests were computed by a recursive procedure (22). False discovery in the multiple testing was controlled with the false discovery rate (FDR) procedure (23). Based on the results of the post-hoc pairwise comparisons, we clustered the gene responses in relation to treatment effects by applying a hierarchical procedure developed by Sutter et al. (24). Specifically, for testing $H_0: \mu_a = \mu_b$ versus $H_1: \mu_a < \mu_b$, where $\mu_a$ denotes the expected value of the expression of gene $g$ under treatment $a$, we use the recurrence relation (22) to compute the exact $P$-value $P_{\mu_a}(r, l)$, where $r$ is the rank sum of the $n$ expression values of gene $g$ under treatment $a$ and $m$ is the number of replications under treatment $b$. If $P_{\mu_a}(r, l) \leq \alpha$, then $\mu_a < \mu_b$, and a 1 was assigned to the result of the comparison (upregulated genes). Another possibility was $H_1: \mu_a > \mu_b$. The $P$-value for this test was computed by $1 - P_{\mu_a}(r - 1)$. If this $P$-value was not $>\alpha$, then $\mu_a > \mu_b$, and a 0 was assigned (downregulated genes). When both $P_{\mu_a}(r)$ and $1 - P_{\mu_a}(r - 1)$ were $>\alpha$, the null hypothesis was accepted and a 1 was assigned to the result (no change).

### Results and discussion

#### Clustering of microarray data

Normal probability plots and Kolmogoroff–Smirnov tests (25,26) demonstrated that the raw gene expression values, as well as the log-transformed expression values, were not normally distributed (data not shown). Therefore, a non-parametric procedure was used to test the differences among the expression levels of each gene under four treatment conditions. After controlling for FDR, 431 significant probe sets were identified. A recursive procedure was then applied to detect differences occurring between any two treatments. An illustration of this analysis is shown for one gene (supplementary Table I is available at Carcinogenesis Online), Gstpl. After resolving the redundant probe sets, the number of significant genes was reduced from 431 to 367 genes. The genes were then clustered according to their responses to the treatments. For four treatments, there were six pairwise comparisons and 219 possible clusters (24). A 1 was assigned to all insignificant comparisons and 2563 probe sets had the 111111 patterns of pairwise comparisons. The remaining 367 genes were binned into 64 additional clusters (Table I).

#### Comparison with ANOVA

By comparison, the non-parametric and ANOVA methods appeared to detect similar differences in gene expression (supplementary Table II is available at Carcinogenesis Online), with 93% of the probe sets identified by both methods. However, the ANOVA method identified many more significant probe sets (761 versus 431). As the assumption of normality was not supported, we explored this issue in more detail by RT–PCR. From the results of each procedure, we selected the five genes whose FDR $P$-values were closest to the cutoff threshold (supplementary Table III is available at Carcinogenesis Online). RT–PCR was performed on each sample, and Pearson correlation analysis was performed between the microarray and RT–PCR results. We observed a strong correlation between the expression values for the genes identified by the non-parametric method ($r = 0.8576$, $P$-value = 0.0001) (supplementary Figure 1A is available at Carcinogenesis Online). However, for the genes selected from ANOVA, the correlation of the expression values between microarray and RT–PCR was low ($r = 0.0879$, $P$-value = 0.446) (supplementary Figure 1B is available at Carcinogenesis Online). These results support the robustness of the non-parametric method and provide confidence in the identification and clustering of genes by this procedure, thereby reducing biological noise in the subsequent functional analysis of this data.

### PCA

The resolution of our chemical genomics approach was revealed by comparison with PCA, a statistical technique for dimension reduction in multivariate data sets, often used to cluster genes with similar expression profiles (18,27). PCA allows one to observe graphically the key variables of the differences in the gene observations. In this study, experimental treatments were the variables. The percentage variance from PCA showed that the first three components captured most of the information about the observed variability in this experiment. Shown in Figure 1B is the plot of the first three principal components for genes in clusters containing more than six genes, in order to show a clearer view of the data. In Figure 1B, PCA identified only two large clusters that appeared close to one another. However, if we color code these genes (Figure 1C) using the clusters identified by our method (Table I), we observe that the two major groups of genes identified by PCA are identified by our method as upregulated (red and gray tones) and downregulated (green and yellow tones) genes, relative to control. For example, clusters 211001 (dark red) and 011221 (yellow) represent genes showing increased or decreased expression in response to only treatment with D3T. Moreover, we observe that the distinct clusters identified by our procedure tend to group in distinct regions of the PCA plot. This observation indicates a general concordance of these two methods, with our method producing a much finer cluster, expressing an explicit pharmacological relationship between responses and treatments (Figure 1C). For example, clusters 122221 (blue) and 100001 (cyan) represent genes that, although D3T is the most efficacious chemical among the three (2), respond only to OLT and TBD. This unique ability to identify distinct clusters in a series of closely related chemical structures clearly demonstrates the strength of our procedure for chemical genomic applications.

### Common pharmacodynamic action of D3T, OLT and TBD

The shared structure–activity relationships of the three dithiolethiones (Figure 1A) was observed in the clusters containing the most responsive genes, clusters 1–8 and 54–65 (patterns 222xxx and 000xxx) consisting of 68 upregulated genes and 158 downregulated genes, respectively (Table I). As D3T, the parent compound, is known to activate the Nrf2 pathway, this observation was expected. Included in the upregulated genes were those known to be mechanistically important to the prevention AFB1 carcinogenesis, including glutathione S-transferase (GST) Yc2 subunit and AFB1 aldehyde reductase, as well as other well-known Nrf2-regulated genes including NAD(P)H quinone oxidoreductase 1 (Nqo1), Gstpl and uridine diphosphate glucuronosyltransferase 1 family, polypeptide A6 (UGT1A6) (26,28).

Functional analysis of the 226 genes in the 222xxx and 000xxx clusters by GO enrichment (19) identified metabolic process and...
Table I. The clusters of genes whose patterns identify responses to treatments

| Cluster | Pattern* | Number of genes |
|---------|----------|-----------------|
|         | CD CO CT DO DT OT |                  |
| 1       | 2 2 2 2 2 1 | 3               |
| 2       | 2 2 1 2 1 1 1 | 5               |
| 3       | 2 2 2 1 1 1 27 |     |
| 4       | 2 2 2 1 0 1 8 |     |
| 5       | 2 2 2 1 0 0 1 |     |
| 6       | 2 2 2 0 1 2 1 |     |
| 7       | 2 2 0 1 1 9 |     |
| 8       | 2 2 0 0 0 1 14 |    |
| 9       | 2 2 1 2 1 0 1 |     |
| 10      | 2 2 1 1 0 1 2 |     |
| 11      | 2 2 1 0 1 1 2 |     |
| 12      | 2 2 1 0 0 1 8 |     |
| 13      | 2 1 2 1 1 2 1 |     |
| 14      | 2 1 2 1 0 1 2 |     |
| 15      | 2 1 2 0 1 2 2 |     |
| 16      | 2 1 2 0 1 1 3 |     |
| 17      | 2 1 2 0 0 1 3 |     |
| 18      | 2 1 1 0 1 1 1 |     |
| 19      | 2 1 1 0 0 1 5 |     |
| 20      | 1 2 2 2 2 1 9 |     |
| 21      | 1 2 2 2 1 1 3 |     |
| 22      | 1 2 2 1 1 2 1 4 |    |
| 23      | 1 2 1 2 1 1 1 |     |
| 24      | 1 2 1 2 1 1 1 |     |
| 25      | 1 1 2 2 2 2 2 |     |
| 26      | 1 1 1 1 1 1 2563 | |
| 27      | 1 1 0 1 0 1 1 |     |
| 28      | 1 0 0 1 0 0 2 |     |
| 29      | 1 0 0 0 1 1 1 |     |
| 30      | 1 0 0 0 1 1 1 |     |
| 31      | 1 0 0 1 0 0 2 |     |
| 32      | 1 0 0 1 0 0 2 |     |
| 33      | 1 0 0 0 1 1 4 |     |
| 34      | 1 0 0 0 1 2 12 |    |
| 35      | 1 0 0 0 1 1 3 |     |
| 36      | 1 0 0 0 1 1 3 |     |
| 37      | 0 2 2 2 1 1 3 |     |
| 38      | 0 2 1 2 2 1 1 |     |
| 39      | 0 2 0 2 1 0 1 |     |
| 40      | 1 1 2 2 2 1 1 |     |
| 41      | 1 1 1 2 2 1 9 |     |
| 42      | 0 1 1 2 2 0 1 |     |
| 43      | 0 1 0 2 2 1 11 |    |
| 44      | 0 1 0 2 2 1 11 |    |
| 45      | 0 1 0 2 1 0 11 |    |
| 46      | 0 1 0 1 1 1 4 |     |
| 47      | 0 1 0 1 1 1 4 |     |
| 48      | 0 0 2 1 2 2 1 |     |
| 49      | 0 0 2 1 2 2 1 |     |
| 50      | 0 0 1 2 2 1 1 |     |
| 51      | 0 0 1 2 2 1 1 |     |
| 52      | 0 0 1 1 1 1 2 |     |
| 53      | 0 0 1 1 1 1 2 |     |
| 54      | 0 0 0 2 2 1 9 |     |
| 55      | 0 0 0 2 1 1 29 |    |
| 56      | 0 0 0 1 2 1 0 1 |     |
| 57      | 0 0 0 1 2 2 1 |     |
| 58      | 0 0 0 1 2 1 6 |     |
| 59      | 0 0 0 1 1 2 1 |     |
| 60      | 0 0 0 1 1 1 74 |    |
| 61      | 0 0 0 1 1 1 0 6 |     |
| 62      | 0 0 0 1 1 1 12 |     |
| 63      | 0 0 0 1 0 0 4 |     |
| 64      | 0 0 0 0 0 0 1 13 | |
| 65      | 0 0 0 0 0 0 1 2 |     |

*CD, control versus D3T; CO, control versus OLT; CT, control versus TBD; DO, D3T versus OLT; DT, D3T versus TBD; OT, OLT versus TBD.

Chemical genomics of dithiolethiones

The efficacies of OLT and TBD are similar and both weaker than D3T

As the test compounds were administered in equal molar doses, a lower efficacy of OLT, relative to D3T and TBD, was observed in clusters 13–17 and 42–47 (patterns 212xxx and 010xxx) containing 48 genes (Table I). These genes did not change in response to OLT, indicating that OLT was less efficacious in the response of these genes, a finding consistent with the study of Roebuck et al. (2) for other end points. Of the observed clusters, a large portion (three of five in clusters 13–17 and four of six in clusters 42–47) showed no response to either OLT or TBD. This observation is also consistent with the reported efficacies of these compounds, which indicates that OLT and TBD are weaker than the parent compound, D3T (2). Similarly, clusters showing the greater efficacy of D3T were also observed, i.e. the 16 genes that responded only to D3T (patterns 211xxx and 011xxx). Finally, there were 23 genes that responded to OLT and D3T, but not to TBD (patterns 221xxx and 001xxx). Collectively, these data support the current understanding that D3T is the most efficacious of the three compounds, with the observed efficacies of D3T ≫ TBD ≫ OLT.

Unique OLT and TBD gene clusters indicate novel pharmacodynamic action

While the common clusters discussed above contained 89% of the statistically significant genes, the remaining 11% of the responding genes identified two unique sets of clusters, 20–23 and 31–35 (patterns 122xxx and 100xxx), containing 40 genes responding to OLT and TBD, but not to D3T (Table I). Two of the most highly induced genes in this set were Cyp2b6 and Cyp2b19, elevated 11- and 7-fold, respectively. Two previous studies have shown that OLT induces members of multiple families of CYP, especially the CYP1A and Cyp2b6 families in livers of rat (24,30). Furthermore, it has been proposed that the induction of these genes may be related to the inhibitory properties of OLT toward hepatic CYP (31). One explanation for this observed activity of OLT for CYP enzyme induction is its unique structure, compared with the unsubstituted parent dithiolethione, D3T (24). As shown in Figure 1A, OLT contains a 5-pyrazinyl substituent on the dithiolethione ring. This nitrogen-containing, electron-withdrawing ring is believed to make OLT an inhibitor of hepatic CYP (24). To explore this property in light of the previously identified OLT cluster associated with inhibition of CYP (24), we determined the relative potencies of D3T, OLT and TBD for inhibition of CYP1A2-catalyzed 7-ethoxyresorufin-O-deethylase (Figure 1B). The rank order of inhibitor potency was OLT ≫ TBD ≫ D3T, with half maximal inhibitory concentration values estimated as 0.2, 12.8 and >100 μM, respectively. Although the relatively high and multiple doses of 0.3 mmol/kg body wt did not...
permit discrimination of the potential differences between OLT and TBD in this regard, our results do show that the dose–response relationships for enzyme inhibition and Nrf2 activation by these chemicals are quite different. If this unique action of OLT and TBD proves to be an important consideration in their use as prophylactic agents, then further studies to explore the dose–response relationships and mechanism of this response could be performed.

In order to further explore this unique activity, functional analysis of these 40 genes was performed (Table II). The top four significant biological functions were related to molecular transport, including the mitochondrial fatty acid oxidation pathway. PPARs play important roles in the regulation of carbohydrate, lipid and protein metabolism, inflammation and adipocyte differentiation. PPARs are highly expressed in liver where it stimulates fatty acid oxidation and ketogenesis (43). It also regulates the peroxisomal and mitochondrial fatty acid oxidation pathway. PPARγ is expressed at low levels in the liver, but can be upregulated by PPARγ activators. These same activators can suppress carcinogenesis in animal models (43,44).

In mice, OLT has been shown to increase the expression of Cyp2b10 by a constitutive androstane receptor-mediated mechanism (45), which could account for the observed induction of Cyp2b6 messenger RNAs here. However, the constitutive androstane receptor TFBS was not significantly represented in the 40 gene set. In rat hepatocyte H4IE cells, OLT has been shown to increase the expression of the GSTA2 via enhanced binding of the CCAAT/enhancer-binding protein (CEBP) β (46). Although the CEBPβ TFBS was not significantly represented in the 40 gene set, a recent study shows that 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), increased the expression of GSTA2, which was lost by deletion of either the CEBPβ or PPAR-Responsive enhancers, indicating that these factors interact in the regulation of this gene (48).

In another study, 15d-PGJ2 was shown to activate Nrf2, as well as PPARγ (49), demonstrating that such chemicals may activate multiple nuclear receptors in the liver, adding to the complexity of their effects. Combined genetic and chemical genomic studies will be necessary to understand the mechanisms of action of such chemicals. The information and procedures described here should aid in the design and analysis of such studies.

Table II. GO and functional analyses of the 226 genes regulated by D3T, OLT and TBD and the 40 genes regulated by OLT and TBD only

| A | GO ID | GO term | q | m | t | K | P-value |
|---|---|---|---|---|---|---|---|
| GO:0008152 | Metabolic process | 62 | 3872 | 8799 | 75 | 1.7 × 10^-10 |
| GO:0006412 | Translation | 16 | 216 | 8799 | 75 | 3.8 × 10^-10 |
| GO:0004364 | Glutathione transferase activity | 8 | 31 | 8799 | 75 | 4.8 × 10^-10 |

q, the number of probe sets in the list responding to D3T, OLT and TBD and associated with the GO term; m, number of probe sets on the chip associated with the GO term; t, total number of probe sets on the chip; K, number of probe sets in the input list for GO enrichment analysis.

| B | Canonical pathway | P-value | D3T-, OLT- and TBD-regulated genes |
|---|---|---|---|
| Metabolism of xenobiotics by CYP | 5.0 × 10^-11 | GSTa3, GSTm1, GSTp1, UGT2B4, UGT1A6, CYP2C18, MGST2, CYP2A12, UGT2B17, GST1 |
| NRF2-mediated oxidative stress response | 1.9 × 10^-09 | AKR7A2, GSTa3, AKR7A3, GSTm1, GSTp1, AKRI1A, FTL, MGST2, Nqo1, GST1 |
| Xenobiotic metabolism signaling | 8.5 × 10^-09 | GSTa3, GSTm1, GSTp1, FTL, UGT1A6, Nqo1, GST1, MGST2, UGT2B4, UGT2B17, GST1, MAOA |
| Glutathione metabolism | 1.7 × 10^-08 | GSTa3, GSTm1, GSTp1, MGST2, IDH2, GST1 |

| C | Biological functions | P-value | OLT- and TBD-regulated genes |
|---|---|---|---|
| Molecular transport | 3.6 × 10^-4 | Ifrd1, SLC34A1, Nr0b2, COMT, Slc37a4, Adh1, Acat1, HSP90AA1, Slc25a10, Cyp26b, Pck1 |
| Carbohydrate metabolism | 5.4 × 10^-4 | Slc37a4, Slc25a10, Pck1, SDS |
| Drug metabolism | 8.1 × 10^-4 | SLC34A1, CYP2C18, COMT, Adh1, Cyp2b6, HSD17B2 |
| Small molecule biochemistry | 8.1 × 10^-4 | SLC34A1, Adh1, Pck1, Hac1, Hmgcs2, FAH, Ifrd1, ATPF1, Nr0b2, CYP2C18, BCKDHA, COMT, Slc37a4, Acat1, Slc25a10, Cyp2b6, SDS, HSD17B2, LTB4DH, Cyp2b19 |

TFBSs for PPARγ and PPARα, respectively (supplementary Table V is available at Carcinogenesis Online). These genes include Nr0b2, Pck1, Slc37a4, Acat1, Slc25a10, Adipor2, Adh1, Hmgcs2 and Hac1 (also known as phytanoyl-CoA 2-hydroxylase 2, Phyh2), which were associated with lipid metabolism. Studies have shown that Acat1, Slc25a10, Adipor2 and Hmgcs2 are PPARα-regulated genes (38,40), whereas Nr0b2 and Pck1 are mediated by PPARγ (41,42). The PPAR nuclear receptors play important roles in the regulation of carbohydrate, lipid and protein metabolism, inflammation and adipocyte differentiation. The in vivo and in vitro action of OLT and TBD and the 40 genes regulated by OLT and TBD only are presented in Table II. The top four significant biological functions were molecular transport, including the mitochondrial fatty acid oxidation pathway. PPARs play important roles in the regulation of carbohydrate, lipid and protein metabolism, inflammation and adipocyte differentiation.
Table III. The top three significant overrepresented matrices found in the promoters of the 40 genes regulated by OLT and TBD using CORE_TF

| Name of matrix | P-value | Number of experimental promoters | Number of random of random promoters | Frequency of hits in the random data |
|---------------|---------|-----------------------------------|--------------------------------------|-------------------------------------|
| PPARG_03      | 2.1 × 10^{-08} | 1521                              | 2944                                 | 0.52                                |
| PPAR_DRIQ_02  | 1.3 × 10^{-07} | 1135                              | 2944                                 | 0.39                                |

*Frequency of hits in the random data.

Supplementary materials

Supplementary Tables I-V and Figure 1 can be found at http://carcin.oxfordjournals.org/

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