A Novel Statistical Algorithm for Gene Expression Analysis Helps Differentiate Pregnan X Receptor-Dependent and Independent Mechanisms of Toxicity

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Abstract

Genome-wide gene expression profiling has become standard for assessing potential liabilities as well as for elucidating mechanisms of toxicity of drug candidates under development. Analysis of microarray data is often challenging due to the lack of a statistical model that is amenable to biological variation in a small number of samples. Here we present a novel non-parametric algorithm that requires minimal assumptions about the data distribution. Our method for determining differential expression consists of two steps: 1) We apply a nominal threshold on fold change and platform p-value to designate whether a gene is differentially expressed in each treated and control sample relative to the averaged control pool, and 2) We compared the number of samples satisfying criteria in step 1 between the treated and control groups to estimate the statistical significance based on a null distribution established by sample permutations. This method captures group effect without being too sensitive to anomalies as it allows tolerance for potential non-responders in the treatment group and outliers in the control group. Performance and results of this method were compared with the Significant Analysis of Microarrays (SAM) method. These two methods were applied to investigate hepatic transcriptional responses of wild-type (PXR+/+) and pregnane X receptor-knockout (PXR−/−) mice after 96 h exposure to Cmp013, an inhibitor of β-secretase (β-site of amyloid precursor protein cleaving enzyme 1 or BACE1). Our results showed that Cmp013 led to transcriptional changes in hallmark PXR-regulated genes and induced a cascade of gene expression changes that explained the hepatomegaly observed only in PXR+/+ animals. Comparison of concordant expression changes between PXR+/+ and PXR−/− mice also suggested a PXR-independent association between Cmp013 and perturbations to cellular stress, lipid metabolism, and biliary transport.

Introduction

Microarrays are now the preferred technology in many biological applications ranging from functional characterization of genes and pathways to classification of disease signatures for diagnostic and prognostic purposes. Within the field of toxicogenomics, genome-wide gene expression data have been widely used to assess potential toxicity as well as to elucidate mechanisms of toxicity of drug candidates [1–4]. The growing number of applications and wide adoption of microarray data have in turn fueled the development of analysis methods devised to extract information from these datasets [5]. While earlier studies were often complicated by technical inconsistencies, microarray data have become significantly more reliable and reproducible [6,7]. In fact, analysis of reference mRNA obtained from mixed rat tissues processed over a multi-year period by Amgen and several external facilities has consistently shown relatively high sensitivity and specificity [8,9]. However, even with highly improved technology, the microarray community continues to struggle with the analysis, interpretation, and extraction of biologically relevant knowledge from the large volume of expression measurements. Much work has been invested in developing models and algorithms for these purposes and their levels of complexity have tended to increase over time. Unfortunately, however, the increased level of algorithmic complexity does not always translate to improved biological understanding [10]. In particular, many model-driven methods often assume certain distributions for the data that are either not true or not easily verifiable. Furthermore, while most existing statistical models perform well with simulated data, they often are too sensitive to what is generally considered an acceptable level of biological variation.

Our goal here is to devise a method that requires fewer assumptions about intensity distribution of genes and therefore can be described with an intuitive mathematical model. The method is...
meant to capture group effects without being too sensitive to anomalies in a small subset of subjects. Such tolerance is necessary because biological variation is typically large due to uncontrollable variables resulting both from inherent heterogeneity and technical procedures during the course of experiments. Therefore, it may not be desirable to penalize large variations in the amplitude of gene expression as long as the changes relative to the control group are in the same direction across the majority of animals. Our approach leverages the platform p-value [11] and fold change cutoffs to designate whether changes in a gene constitute biologically differential expression between each treated sample relative to the vehicle-control pool. The significance of the group effect for the gene is then estimated by comparing the number of changed samples observed between the two groups. In particular, the algorithm involves two steps:

1. A nominal threshold of 1.25 fold change and platform p-value [11] of 0.1 were used to designate whether a gene displays differential expression in each treated and control sample relative to the averaged control pool.

2. False discovery rate was estimated based on the probability of encountering the observed number of differentially expressed samples in the treated group, given the total number of observed differentially expressed samples in both groups, under the empirically determined distribution derived from the null hypothesis that the differentially expressed samples are equally distributed in both groups.

Thresholds on fold change and p-values were set to values we believed would likely translate to biological significance from our experience with similar toxicology studies. Performance of this method was compared with the popular microarray analysis method Significant Analysis of Microarrays (SAM) [12]. The two methods were applied to investigate the role of pregnane X receptor (PXR) in hepatic toxicities induced by CMP013, a small molecule inhibitor of β-secretase (BACE1) enzyme. BACE1, the first of the two proteases that cleaves amyloid precursor protein, is believed to be a prime drug target for Alzheimer’s disease [13–15]. Early toxicology screening with CMP013 in rats revealed prominent effects in the liver including hepatomegaly (liver weight nearly 2x above control) with histological correlates of increased mitotic figures, vacuolation, and hepatocellular hypertrophy.

In vivo data suggested that CMP013 might be an agonist for PXR and we hypothesized that this nuclear receptor was at least partially responsible for the potent hepatic effects noted in 4-day rat toxicology studies. To further evaluate the role of PXR in mediating mechanisms of toxicity by CMP013, a subsequent 4-day toxicology study with CMP013 was carried out with wild type (C57Bl/6) and PXR-knockout (C57Bl/6NTac) mice (Table 1) to confirm that mice respond to CMP013 in a manner similar to rats.

![Effect of CMP013 on liver weight](Image)

**Figure 1. Effect of CMP013 on liver weight of wild type and PXR-knockout mice.** Wild type mice showed similar liver weight increase as previously observed in Sprague Dawley rats; such increase was absent in the knockout strain.

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Following confirmation of CMP013-mediated hepatic effects in C57Bl/6 mice (Figure 1), gene expression data was generated from liver tissue of these animals for mechanistic investigation. Specifically, the data from this knock-out study allowed us to differentiate PXR-dependent and independent mechanisms of this BACE1 inhibitor in mediating the observed hepatotoxic effects.

### Materials and Methods

**Ethics Statement**

All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and all animal work was approved by the Amgen’s Institutional Animal Care and Use Committee under IACUC protocol 2008-00174.

**In vivo study**

Two groups of mice, C57Bl/6 (PXR+/+) and PXR-knockout C57Bl/6NTac (PXR−/−), were administered by oral gavage either CMP013 or vehicle (2% HPMC/1% Tween 80 in DI water, pH 2.2, adjusted with methanesulfonic acid) according to dose levels outlined in Table 1. Food (irradiated Harlan Teklad rodent maintenance diet) and water were available ad libitum during the study except for the last 3–4 h prior to necropsy during which animals were fasted and only water was available. Actual food intake and water consumption were not monitored for individual animals during the course of study. At 96 h, all animals

| Strain | Test Article Dose level (mg/kg/day) | Dose volume (mL/kg) | Concentration (mg/mL) |
|--------|-----------------------------------|---------------------|-----------------------|
| C57Bl/6 (WT) Vehicle | 0 | 10 | 0 |
| C57Bl/6 (WT) CMP013 | 150 | 10 | 15 |
| C57Bl/6NTac (PXR-KO) Vehicle | 0 | 10 | 0 |
| C57Bl/6NTac (PXR-KO) CMP013 | 150 | 10 | 15 |

All animals were 9-week old males at initiation of treatment. Mice were dosed via oral gavage every 24 h and euthanized at 96 h. Each of the following groups contains 5 animals.

2% HPMC/1% Tween 80 in DI water, pH 2.2, adjusted with methanesulfonic acid.

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were euthanized via CO2 asphyxiation followed by exsanguination. Liver samples were promptly collected and frozen until ready for RNA extraction.

RNA Isolation
Total RNA was isolated from pieces of mouse liver according to the RNeasy extraction procedure (Qiagen, Valencia, CA). Tissues were homogenized in QiAazol lysis buffer using the GenoGrinder 2000 homogenizer (SPEX SamplePrep, Metuchen, NJ). Samples were processed on the Qiagen BioRobot Universal system according to the manufacturer’s instructions. An on-column DNase digestion was performed to remove any residual genomic DNA contamination. RNA concentration and yield were measured spectrophotometrically using the Nanodrop instrument. Quality of the nucleic acid samples was evaluated with the RNA 6000 Nano chip kit (Agilent Technologies, Expert software). Quality of samples was verified with distinct ribosomal 18S & 28S peaks, low baseline, and high RIN values (PXR+/−: 9.3-10; PXR−/−: 8.7-9.6).

Gene expression data generation
Liver RNA from individual mice was profiled separately on the Affymetrix GeneChip® platform without technical replication. Microarray profiling was performed by Cogenics (Morrisville, NC). Briefly, 1 μg of total RNA was reverse transcribed to double stranded cDNA with the BioarrayTM Single-Round RNA Amplification and Labeling Kit and biotinylated cRNA was generated using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY). For each sample, 10 μg of biotinylated cRNA spiked with hybridization controls (bioB, bioC, bioD and cre) was hybridized to an Affymetrix Mouse 430_2 microarray for 16 h at 45°C. Following hybridization, arrays were washed and stained in an Affymetrix GeneChip Fluidics Station and scanned with a GeneChip Operating Software (GCOS) and Quality Reporter. All data were MIAME compliant and raw data (cel files) have been deposited to a MIAME compliant database, GEO, accession GSE23780.

Gene expression analysis

Analysis with the proposed method. Log-ratios of gene expression data and associated platform p-values [11] were generated in the Rosetta Resolver System (Rosetta Biosoftware, Seattle, WA, version 7.2) for all profiles relative to strain-matched vehicle-treated controls. For each Affymetrix sequence (or probe set), log-ratio was defined to be the log_{10} of the intensity ratio of each animal (either treated with vehicle or CMP013) to the mean intensity of that sequence across the five profiles in the corresponding vehicle control group. To identify differentially expressed sequences due to CMP013 treatment, we carried out a two-step non-parametric statistical analysis, which was applied to data from wild type (WT) and PXR-knockout (PXR-KO) mice separately. This analysis was performed in the programming language R.

Step 1: Counting the number of samples in which a sequence i showed differential expression based on platform p-value and fold change. For each sequence, we identified the number of samples that satisfy |log-ratio| ≥ 0.097 (equivalent to a fold change cutoff of 1.25) and platform p-value ≤ 0.1. The number of animals passing this criterion was counted separately for the vehicle and the CMP013-treated groups (Equation 1).

\[
N_{ij}^{v} = \max \begin{cases} |S| & \text{where } S = \{n \mid P_{n}^{i} \geq 0.097 \land P_{n}^{t} \leq 0.1\} \end{cases}
\]

\[
N_{ij}^{t} = \max \begin{cases} |R| & \text{where } R = \{n \mid P_{n}^{i} \leq -0.097 \land P_{n}^{t} \leq 0.1\} \end{cases}
\]

where \(P_{n}^{i}\) is the log-ratio intensity of gene i measured in animal n, \(P_{n}^{t}\) is the associated p-value of gene i, and \(N_{ij}^{v}\) represents the number of animals satisfying the above conditions for sequence i in the vehicle group. \(N_{ij}^{t}\) is defined similarly for animals in the treatment group. |S| represents the number of animals in which sequence i was potentially up-regulated relative to the control pool, while |R| represents the number of animals in which that sequence was potentially down-regulated. For each of WT or PXR-KO dataset, this step produced two vectors of length equal the number of sequences on the GeneChip® (Figure 2). In cases where |S| = |R|, i.e., the genes show increased and decreased expression in equal number of animals, such expression change was designated non-interpretable and step 2 was not necessary.

Step 2: Estimate of statistical significance for group difference between treatment and vehicle. A gene is identified as having significantly altered expression by CMP013 treatment if a significantly greater number of animals in the treatment group satisfy the criteria in step 1 as compared to those in the control group. In other words, we need to evaluate the probability of getting a pairing of \(N_{ij}^{v}, N_{ij}^{t}\) by chance. The null hypothesis for a sequence i is that, for a given \(N_{ij}^{Total} = t\), the numbers of animals that satisfied conditions in step 1 are equally distributed between the vehicle (\(N_{ij}^{v}\)) and treated group (\(N_{ij}^{t}\)) (Equations 2–4). The values for \(t\) range from 0–9 because conditions for significance are set relative to the mean of log-ratio |log-ratio| ≥ 0.097/\(2^{t}\) (Figure 2).

\[P_{n}^{i} = \max \begin{cases} |S| & \text{where } S = \{n \mid P_{n}^{i} \geq 0.097 \land P_{n}^{t} \leq 0.1\} \end{cases}
\]

\[P_{n}^{t} = \max \begin{cases} |R| & \text{where } R = \{n \mid P_{n}^{i} \leq -0.097 \land P_{n}^{t} \leq 0.1\} \end{cases}
\]

where \(P_{n}^{i}\) is the log-ratio intensity of gene i measured in animal n, \(P_{n}^{t}\) is the associated p-value of gene i, and \(N_{ij}^{v}\) represents the number of animals satisfying the above conditions for sequence i in the vehicle group. \(N_{ij}^{t}\) is defined similarly for animals in the treatment group. |S| represents the number of animals in which sequence i was potentially up-regulated relative to the control pool, while |R| represents the number of animals in which that sequence was potentially down-regulated. For each of WT or PXR-KO dataset, this step produced two vectors of length equal the number of sequences on the GeneChip® (Figure 2). In cases where |S| = |R|, i.e., the genes show increased and decreased expression in equal number of animals, such expression change was designated non-interpretable and step 2 was not necessary.

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the control group and thus the maximal significant samples from this group is 4.

\[ E(N_{i \text{CMP013}} | N_{i \text{Total}} = t) = \frac{1}{2t} \]  

\[ N_{i \text{Total}} = N_{i \text{Vehicle}} + N_{i \text{CMP013}} \]  

\( \{0, 1, 2, 3, 4, 5, 6, 7, 8, 9\} \)

A sequence \( i \) is deemed significantly differentially expressed by CMP013 if \( N_{i \text{CMP013}} \) is equal or larger than a cutoff, which is determined by controlling the false discovery rate (FDR). A null distribution was created by permuting the profile labels among vehicle and treated groups 252 times (exhaustive combinations) and re-computing step 1 on this randomized data. For a given number of significant samples \( j \) in treated group, with \( N_{j \text{Total}} = t \), the FDR is given by Equation 4.

\[ \text{FDR}_{ij} = \frac{\text{median}_{\text{over all permutations}} \left( \frac{\sum_{k \geq j} \hat{n}_{ik}^{\text{perm}}}{\sum_{k \geq j} \hat{n}_{ik}^{\text{obs}}} \right)}{\text{median}_{\text{over all permutations}} \left( \frac{\sum_{k \geq j} \hat{n}_{ik}^{\text{perm}}}{\sum_{k \geq j} \hat{n}_{ik}^{\text{obs}}} \right)} \]  

The denominator in the fraction represents the number of genes that would be considered significant from observed data; the numerator represents the number of genes considered significant from permutation. Sequences with \( \text{FDR}_{ij} \leq 0.05 \) were considered statistically significant (Table 2).

**Analysis with SAM.** SAM analysis was carried out using the method `sam` in the Bioconductor [16] package `siggenes`. The input was a data frame containing log2 intensity of 10 profiles (vehicle and treatment) in either the WT or PXR-KO dataset. The class label was a vector of 10 elements, assigning zeros for vehicle and ones for treatment profiles. The default method `d.stat` (a modified t-test) as defined by Tusher et. al. [12] was applied as the test statistics. The output of `sam` was a table containing a list of 10 delta cutoffs, the number of genes deemed statistically significant at these cutoffs and associated FDR. We used this table to fine tune cutoffs, the number of genes deemed statistically significant at these cutoffs and associated FDR. We used this table to fine tune delta values so that the final FDR was <10% below the desired FDR of 0.05. The resulting gene lists for both WT and PXR-KO data were used for pathway analysis.

**Pathway Analysis**

Pathways associated with differentially expressed genes identified by our proposed algorithm and SAM were analyzed using the Tox Analysis function in IPA (Ingenuity Systems, Redwood City, CA) [Application version: 8.0, Build: 82437; Content version: 2602]. Canonical pathways and tox list (list of genes associated with toxicities as determined by IPA) with p-value ≤0.05 were deemed significantly perturbed pathways.

**Results**

Identification of differential expressed genes due to CMP013 treatments

**Results obtained with current method.** The Mouse Affymetrix 430_2 GeneChip® contains 43,037 probe sets, which we refer to as sequences in this paper. The principle component analysis (46% explained, Figure 3) of these sequences, based on quantile-normalized intensity data, showed that separation was observed for animals treated with vehicle vs. CMP013. In fact, the effect of CMP013 treatment appeared much larger than the effect of knocking out PXR, highlighting the fact that this compound modulated a relatively large number of genes in mouse liver.

Log-ratio data and associated platform p-values for all sequences were generated in Rosetta Resolver (version 7.2). Results with the current method showed that CMP013 led to 4,213 and 3,369 differentially expressed sequences in the livers of WT and PXR-KO mice, respectively (Figure 4, Data S1). In the WT model, the majority of sequences differentially expressed by CMP013 were changed in all five animals in the treatment group. In the PXR-KO model, however, the majority of differentially expressed sequences were changed in only three animals in the treatment group. This suggested that the transcriptional response to CMP013 in PXR-KO mice was less homogeneous than that in WT mice. We speculated that the difference in group behaviors between the knockout and WT strains was a result of different compensatory mechanisms each knockout animal developed to compensate for the absence of PXR regulation. There was not sufficiently strong evidence to support this hypothesis in the current study, but we are investigating mouse strains with knockout of other nuclear receptors to determine if similar behaviors are exhibited. Nevertheless, our method is particularly well-suited for studies in both preclinical and clinical settings where subject-to-subject variation is relatively large. Overall, we

|   | WT | 0 | 1 | 2 | 3 | 4 | 5 |
|---|----|---|---|---|---|---|---|
| A |    |   |   |   |   |   |   |
| 0 | 1  |   |   |   |   |   |   |
| 1 | 1  | 0.558 |   |   |   |   |   |
| 2 | 1  | 0.827 | 0.227 |   |   |   |   |
| 3 | 1  | 0.925 | 0.500 | 0.058 |   |   |   |
| 4 | 1  | 0.975 | 0.781 | 0.183 | 0.001 |   |   |
| 5 | 1  | 0.993 | 0.983 | 0.484 | 0.003 | 0 |   |
| 6 | 1  | 0.993 | 0.990 | 0.692 | 0.044 | 0 |   |
| 7 | 1  | 0.980 | 0.980 | 0.843 | 0.144 | 0 |   |
| 8 | 1  | 0.947 | 0.947 | 0.842 | 0.333 | 0 |   |
| 9 | 1  | 1   | 1   | 1   | 0.5 | 0 |   |

|   | PXR-KO | 0 | 1 | 2 | 3 | 4 | 5 |
|---|--------|---|---|---|---|---|---|
| B |        |   |   |   |   |   |   |
| 0 | 1     |   |   |   |   |   |   |
| 1 | 1     | 0.632 |   |   |   |   |   |
| 2 | 1     | 0.831 | 0.225 |   |   |   |   |
| 3 | 1     | 0.897 | 0.473 | 0.028 |   |   |   |
| 4 | 1     | 0.938 | 0.808 | 0.080 | 0.001 |   |   |
| 5 | 1     | 0.973 | 0.948 | 0.404 | 0.005 | 0 |   |
| 6 | 1     | 0.979 | 0.973 | 0.642 | 0.046 | 0 |   |
| 7 | 1     | 0.977 | 0.977 | 0.826 | 0.217 | 0 |   |
| 8 | 1     | 1   | 1   | 0.923 | 0.385 | 0 |   |
| 9 | 1     | 1   | 1   | 1   | 0.5 | 0 |   |

Each value \( F_{ij} \) in the table indicates the FDR for a gene found to be differentially expressed (based on fold change and platform p-value cutoffs) in \( j \) samples of the CMP013 treatment group out of \( i \) samples that are differentially expressed in both groups. Underlined values correspond to cases where the genes would be considered statistically significant at FDR ≤0.05. FDR = 0 corresponds to events that were not observed in permutated data.

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identified 20% more differentially expressed sequences due to CMP013 treatment in the WT model as compared to the PXR-KO model. In addition, most of these sequences appeared to be strain-specific: 76% of sequences changed in the WT model were found only in this model and 70% of sequences changed in the PXR-KO model were unique to that model. About 18% of the total number of differentially expressed sequences in WT mice were similarly modulated (either up-regulation or down-regulation) in PXR-KO mice; these sequences and associated genes were considered to represent PXR-independent effects.

Comparison with results obtained with SAM. SAM returned 5,202 differential expressed sequences in CMP013-exposed WT mice and 2,300 sequences in the PXR-KO mice (Figure 4, Data S1). That is, at a false discovery rate of 5%, SAM identified more sequences than our proposed method in the WT model, and we identified more sequences than SAM in the PXR-KO model. In designing our analysis, we wanted to give considerations to both biological and statistical significance. The key difference between the proposed method and SAM is that our method used a threshold approach to determine biological significance and group effect to determine statistical significance determination. In addition, we did not penalize large variation of changes within the same treatment group as long as the changes were in the same direction. As a result, though SAM returned approximately 20% more statistically significant sequences than our method in the WT model; the vast majority of them (85%) did not satisfy a fold change cutoff of 1.25. While such changes were sufficiently homogenous among animals in the same treatment group to achieve statistical significance; the increase or decrease might be too small to warrant biological difference. On the other hand, the proposed method identified 1,214 additional sequences that were not returned by SAM. These sequences were more likely to represent significant biological differences because their expression changes, though varied from animal to animal, were in the same direction. Furthermore, since there were more non-responders in the PXR-KO group, the current method was more sensitive than SAM and was able to return many sequences which did not express large differential expression in 1–2 animals. In other words, the SAM method is less sensitive when group behavior is less consistent. Heat maps of sequences identified only by the current method or SAM further illustrate the differences in results of the two methods (Figure 5 C–F). Panels C and E show that sequences found by our method have clear differential expression (due to fold change cutoff) as compared to the control group even when the magnitude of difference is relatively small. In contrast, panels D and F show that a portion of sequences identified by SAM do not display visible differential expression. That is, sequences identified only by our

Differentially expressed genes in CMP013-treated C57Bl/6 (WT) and C57Bl/6NTac (KO) mice

![Figure 3. Principle component analysis. The first three principle components are based on log2 intensity and shown as four groups: (□) WT, (O) PXR-KO, black: vehicle, red: CMP013 treatment. doi:10.1371/journal.pone.0015595.g003](#)

![Figure 4. Differentially expressed genes in CMP013-treated C57Bl/6 (WT) and C57Bl/6NTac (PXR-KO) mice. “CMP013 vs. vehicle” represents genes identified as differentially expressed due to CMP013 treatment; “Changed in WT model only” represent genes that are likely mediated by PXR in response to compound treatment; and “Changed in the same direction in two models” represents sequences that modulated by the compound independent of PXR regulation. doi:10.1371/journal.pone.0015595.g004](#)
method cover a larger ranges of (unidirectional) fold changes, whereas those identified only with SAM were likely sequences that had less than 1.25 fold change but were consistent among all five animals in the treatment group. As noted earlier, sequences in this latter category could appropriately be considered to have statistically significant differences, but such differences may not be sufficient to constitute biological significance. It is, of course, up to the investigator to decide how much change would constitute biological difference, and the approach presented in this paper is designed to be amenable to such modification.

Transcriptional effects of BACE1 inhibitor CMP013

We grouped CMP013-modulated sequences into two categories: A) sequences uniquely changed in the WT model, i.e., PXR-
dependent effects and B) sequences showing concordant changes between the WT and PXR-KO models, i.e., PXR-independent response to CMP013 treatment. Sequences in group A were found to be involved in PXR-mediated pathways, as their expression was modulated only in WT mice and unchanged in the knockout model (Figure 5A). Examination of this group of pathways in relation to histopathology findings provided a clearer understanding of PXR’s role in the dramatic hepatocellular hypertrophy observed in WT mice. Sequences in group B reflected properties of the compound that did not depend on the presence of PXR (Figure 5B). These groups of sequences were transferred to IPA where they were mapped to known genes and associated pathways. It should be noted, however, that only about 50% of sequences were mapped to annotated genes and pathways in IPA, and thus our interpretation of these data was limited by this annotation. When there were multiple sequences mapped to the same gene symbol, the sequence with the largest fold change was assigned to that gene.

**PXR-dependent transcriptional effects.** Parallel treatment of wild type and PXR-KO mice with CMP013 allowed us to elucidate transcriptional responses that were dependent on PXR regulation. Knockout of the nuclear hormone receptor PXR completely prevented the CMP013-induced increases in liver weights, hepatocellular hypertrophy and mitotic activity. Comparison of gene expression data obtained from two groups of animals provided molecular evidence for many of these changes. In particular, CMP013 increased the expression of 3,206 genes exclusively in WT mice, many of which were indicative of PXR/CAR agonism properties of this compound. In particular, there was increased expression of hallmark P450 genes including Cyp3a4 (4.3 fold) and Cyp2b6 (88 fold). We additionally observed induction of genes encoding phase 1 and 2 enzymes such as aldehyde dehydrogenase (1a1, 1a7, 1b1, and 18a1, 1.5-2 fold), glutathione S-transferase alpha (Gsta4, 4.5 fold; Gsta5, 11 fold), and inhibitor of kappa light polypeptide gene enhancer in B-lymphocytes (Ikzf1, 2.9 fold). Collectively, these data suggested PXR-dependent transcriptional effects. We identified 754 sequences that were perturbed in the same direction in both WT and PXR-KO mice treated with CMP013. These genes associated with these sequences were mapped to five biological processes. In addition to this shared gene set, we observed groups of differentially expressed genes that were unique in each model of mice, but were mapped to these same five pathways (Table 3).

**PXR-independent transcriptional effects.** We identified 754 sequences that were perturbed in the same direction in both WT and PXR-KO mice treated with CMP013. These genes associated with these sequences were mapped to five biological processes. In addition to this shared gene set, we observed groups of differentially expressed genes that were unique in each model of mice, but were mapped to these same five pathways (Table 3).

A notable common response to CMP013 between WT and PXR-KO mice was the down-regulation of genes involved in fatty acid metabolism. In particular, decreased expression was observed for key enzymes in β-oxidation including acyl-CoA dehydrogenase (Acads, -1.4 fold in both models), which catalyzes the initial step in each cycle of β-oxidation; and acetyl-CoA acyltransferases (Acaa1 and Acaa1b, both genes: -1.3 fold in WT, -1.6 fold in PXR-KO), which catalyze the final step of the β-oxidation cycle. We additionally observed transcriptional decrease in isoforms of these genes as well as related genes in fatty acid metabolism; these genes were unique to each mouse model. In the WT model, decreased expression of -1.3-3 fold was noted for key enzymes in β-oxidation including acyl-CoA dehydrogenase (Acads, -1.4 fold in both models), which catalyzes the initial step in each cycle of β-oxidation; and acetyl-CoA acyltransferases (Acaa1 and Acaa1b, both genes: -1.3 fold in WT, -1.6 fold in PXR-KO), which catalyze the final step of the β-oxidation cycle. We additionally observed transcriptional decrease in isoforms of these genes as well as related genes in fatty acid metabolism; these genes were unique to each mouse model. In the WT model, decreased expression of -1.3-3 fold was noted for key enzymes in β-oxidation including acyl-CoA dehydrogenase (Acads, -1.4 fold in both models), which catalyzes the initial step in each cycle of β-oxidation; and acetyl-CoA acyltransferases (Acaa1 and Acaa1b, both genes: -1.3 fold in WT, -1.6 fold in PXR-KO), which catalyze the final step of the β-oxidation cycle. We additionally observed transcriptional decrease in isoforms of these genes as well as related genes in fatty acid metabolism; these genes were unique to each mouse model.
Comparison of differentially expressed genes identified by the proposed method and SAM.

| Gene                      | WT   | PXR-KO |
|---------------------------|------|--------|
| Fatty acid metabolism     |      |        |
| ACA81                    | −1.368 | −1.368 | −1.619 | −1.619 |
| ACA81B                   | −1.337 | −1.337 | −1.642 |      |
| ACA82                    | −1.318 |        |        |        |
| ACA88                    | −1.545 | −1.545 | −1.622 | −1.485 |
| ACAD5                    | −1.411 |        | −1.427 |        |
| ACAD5B                   | −2.616 | −2.616 | −1.856 |        |
| ABC2                     | 1.927 | 1.927 |      |        |
| ABC21                    | 3.761 | 3.177 | 2.89  | 2.89   |
| ABC3                     | 2.432 | 2.432 | 1.325 | 1.325  |
| CYP7B1                   | −1.876 | −1.876 |        |        |
| CYP8B1                   | −1.933 | −1.933 |        |        |
| Cholesterol biosynthesis  |      |        |
| DHCR7                    | 1.692 | 1.692 |      |        |
| FDFT1                    | 2.498 | 1.862 | 1.345 | 1.345  |
| FBP1                     | 1.366 |        |      |        |
| HMGCR                    | 2.631 | 2.631 | 1.55  |        |
| HMGCS1                   | 10.515 | 10.515 | 2.985 |        |
| ID1                      | 1.544 | 1.445 | 1.815 | 1.815  |
| LSS                      | 2.303 | 2.303 |      |        |
| MVD                      | 5.342 | 5.342 |      |        |
| MVK                      | 2.633 | 2.633 | 2.968 | 2.968  |
| SQLE                     | 1.945 | 1.945 | 1.242 |        |
| Oxidative Stress         |      |        |
| DNAJ12                   | 1.439 |        |      |        |
| DNAJB9                   | 2.367 | 2.367 | 1.962 | 1.962  |
| DNAJC2                   | 1.965 | 1.965 | 1.533 |        |
| FMO1                     |        |        | 1.547 | 1.547  |
| GPX2                     | 3.464 | 3.464 |      |        |
| GSR                      | 3.065 | 3.065 |      |        |
| HMOX1                    |        |        | 2.848 | 2.848  |
| KEAP1                    | 1.445 | 1.445 | 1.268 |        |
| NQO1                     | 2.392 | 2.392 | 1.741 | 1.741  |
| PRDX1                    |        |        | 1.35  |        |
| TXN                      | 1.413 | 1.36  |      |        |
| TXNRD1                   |        |        | 1.423 |        |
| Endoplasmic reticulum signaling | |        |
| ATF4                     |        |        | 1.64  |        |
| ATF6                     | 1.308 | 1.811 | 1.811 |        |
| EIF2AK3                  | 1.416 | 1.61  | 1.61  |        |
| MAPK8                    | 1.557 | 1.686 |      |        |
| MBTPS2                   | 1.369 | 1.663 |      |        |
| Bile acid signaling      |      |        |
| ABCB1                    | 3.761 | 3.177 | 2.89  | 2.89   |
| ABCB2                    | 1.927 | 1.927 |      |        |
| ABCB3                    | 2.432 | 2.432 | 1.325 | 1.325  |
| CYP7B1                   | −1.876 | −1.876 |        |        |
| CYP8B1                   | −1.933 | −1.933 |        |        |
| CYP27B1                  |        |        |        |        |

Below are five biological processes and associated genes that were commonly modulated in both WT and PXR-KO mice after 96 h treatment with CMP013. Values in each row are averaged fold change of the gene across all five animals in the treatment group. Fold change values of a gene may differ between the proposed method and SAM if each method identifies a different Affymetrix sequence corresponding to the same gene as significant.

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| Gene                      | WT   | PXR-KO |
|---------------------------|------|--------|
| SLC01A2                   | 11.494 | 7.756  |
accumulation of unfolded proteins. Cells responded by decreasing the rate of protein translation to prevent the accumulation of unfolded proteins (via the action of Perk and its target eukaryotic initiation factor 2, eIF2) and activating regulated intramembrane proteolysis [19] (via the action of Atf6 and S2p). There appears to be an interesting relationship between ER stress and increased cholesterol biosynthesis. Previously, Lee et al. reported that ER stress inhibited the synthesis of Insig-1 protein, which in turn stabilized Srebp2 and increased cholesterol biosynthesis. Previously, Lee et al. reported that ER stress inhibited the synthesis of Insig-1 protein, which in turns stabilized Srebp2. The authors used cultured cells transfected with human Insig-1 and Insig-2 and herpes simplex virus Srebp2; here we observed a similar phenomenon in vivo.

Lastly, there were perturbations in bile acid homeostasis associated with CMP013 exposure in both WT and PXR-KO models. Alteration to biliary transport was noted with increased expression of the canicular transporters Abcb1 (or Mrkl, 3.8 fold), the basolateral transporter Abcc3 (or Mrp3, 2.4 fold), and the basolateral uptake transporter Slco1a2 (or Oatp, 11 fold). Increased expression of Mrp3 observed in the PXR-KO model suggested that another nuclear receptor was compensating for PXR in regulating bile acid homeostasis. In WT mice, we further observed increased expression of the canicular transporter Abcc2 (Mrp2, 1.9 fold). In PXR-KO mice, Cyp7b1, encoding oxysterol 7alpha-hydroxylase, which converted cholesterol to bile acids, decreased 1.5 fold in expression. Cyp8b1, encoding the enzyme sterol 12-alpha-hydroxylase, which controls the balance between cholic acid and chenodeoxycholic acid secreted into the bile, decreased 1.9 fold in expression. These transcriptional changes indicated that CMP013 perturbed the transport and recycle of bile acids and their conjugates in WT mice and affected the biosynthesis and metabolism of bile acids in the knock-out strain. Together, the changes were suggestive of a cholestatic response induced by the compound [21].

Discussion

The present study describes a novel algorithm for analyzing gene expression data and its application in studying the mechanism of toxicity of a drug candidate. Results with the proposed method were compared with analysis obtained with SAM to identify strengths and weaknesses associated with the new approach. First, a compelling aspect of our approach is that it is straightforward in its assumptions and therefore would be relatively easy for investigators to determine if the method is suitable for their experiments or questions. The threshold procedure in step 1 is simple and investigators can easily substitute an alternate cutoff that constitutes biological significance in their experiment. In the current study, by allowing a small cutoff on fold change, we increase our sensitivity for subtle expression changes because it is important, in toxicological assessment, to maintain a low false negative rate, even at the expense of false positives. This low threshold facilitates the discovery of signaling and regulatory genes which can produce a large downstream effect with a small change in expression. In fact, as most regulatory proteins are low threshold facilitates the discovery of signaling and regulatory genes which can produce a large downstream effect with a small change in expression. In fact, as most regulatory proteins are regulated by post-translational modification, only a small transcriptional increase is necessary to supplement the allosteric changes of the proteins. As compared to results obtained with SAM, the current method produced improved results in at least two pathways. For example, none of the genes in the ER stress response were found with SAM in WT mice. These genes have relatively small fold change (1.25 – 1.5), but the combined increase of multiple genes in this pathway strongly suggests that the ER stress response is not insignificant. The second example involves the cholesterol biosynthesis pathway in PXR-KO mice (Table 2).
difference. We understand that failure to discover these genes is perhaps a drawback of the current method. However, given the uncertainty associated with magnitude of expression changes as discussed above, it is unlikely that all of these genes are associated with true signals. It is noteworthy to mention that if such genes were associated with a true signal, one may still be able to realize the associated biological response by the expression of other genes involved in the same pathway. We therefore feel that the elimination of such genes does not necessarily impact our overall interpretation of the experiment. Our best recommendation in these situations would be to apply our analysis method in conjunction with an algorithm similar to SAM, i.e. using two methods that have complementary approaches, so that one can obtain a clear picture of the overall transcriptional response.

In summary, we describe a novel non-parametric statistical method for the analysis of gene expression data for studies in which conventional variance-based analysis methods result in suboptimal results. Indeed, the benefit of our method is substantiated for datasets from preclinical or clinical studies where subject-to-subject variations are relatively large. The method is straightforward in its assumptions and allows investigators to specify criteria for both biological significance and statistical significance. In the mouse-knockout example described here, the application of this method allowed us to unravel the molecular mechanisms associated with hepatic toxicities induced by an inhibitor of \( \beta \)-secretase in the presence and absence of the nuclear hormone receptor PXR.

### Supporting Information

#### Data S1

Differentially expressed genes resulting from CMP013 treatment in wild type and knockout mice.

(XLS)

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### Author Contributions

Conceived and designed the experiments: RD CA TW JB CQ HH. Performed the experiments: YC LC CD SM. Analyzed the data: AM SV NE GC CS MHG YH. Wrote the paper: AM.

### References

1. Searfoss GH, Ryan TP, Jolly RA (2003) The role of transcriptional analysis in pre-clinical toxicology. Curr Mol Med 3: 53–64.
2. Blomme EA, Yang Y, Waring JF (2009) Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. Toxicol Lett 186: 22–31.
3. Hamadheh HK, Bushel PR, Jayadev S, DiStefano O, Bennett L, et al. (2002) Prediction of compound signature using high density gene expression profiling. Toxicol Sci 67: 232–240.
4. Hamadheh HK, Bushel PR, Jayadev S, Martin K, DiStefano O, et al. (2002) Gene expression analysis reveals chemical-specific profiles. Toxicol Sci 67: 219–231.
5. Gant TW, Zhang SD, Taylor EL (2009) Novel genomic methods for drug discovery and mechanism-based toxicological assessment. Curr Opin Drug Discov Devel 12: 72–80.
6. Rammels R, Beyer RP, Bhattacharya S, Boorman GA, Boyles A, et al. (2005) Standardizing global gene expression analysis between laboratories and across platforms. Nat Methods 2: 351–356.
7. Draghici S, Khatri P, Eklund AC, Szallasi Z (2006) Reliability and reproducibility issues in DNA microarray measurements. Trends Genet 22: 101–109.
8. Mongan A, Higgins M, Phe S, Althari C, Hamadheh H (2008) Assessment of repeated microarray experiments using mixed tissue RNA reference samples. Biotechniques 45: 203–209.
9. Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, et al. (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24: 1151–1161.
10. Walker MS, Hughes TA (2008) Messenger RNA expression profiling using DNA microarray technology: diagnostic tool, scientific analysis or un-interpretable data? Int J Mol Med 21: 13–17.
11. He YD, Dai H, Schade EE, Cavet G, Edwards SW, et al. (2003) Microarray standard data set and figures of merit for comparing data processing methods and experiment designs. Bioinformatics 19: 956–965.
12. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 98: 5116–5121.
13. Vassar R (2001) The beta-secretase, BACE: a prime drug target for Alzheimer’s disease. J Mol Neurosci 17: 157–170.
14. Vassar R, Kovacs DM, Yan R, Wong PC (2009) The beta-secretase enzyme BACE in health and Alzheimer’s disease: regulation, cell biology, function, and therapeutic potential. J Neurosci 29: 12787–12794.
15. Citron M (2001) Human beta-secretase and Alzheimer’s disease. Expert Opin Ther Targets 5: 341–348.
16. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Deting M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.
17. Weintraub S, Prater CA, Dean DC (1992) Retinoblastoma protein switches the E2F site from positive to negative element. Nature 359: 259–261.
18. Reed SI (1997) Control of the G1/S transition. Cancer Surv 29: 7–23.
19. Lai E, Teodoro T, Volchuk A (2007) Endoplasmic reticulum stress: signaling the unfolded protein response. Physiology (Bethesda) 22: 193–201.
20. Lee JN, Ye J (2004) Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1. J Biol Chem 279: 45257–45265.
21. Trauner M, Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev 83: 633–671.