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Predicting *In Vivo* Anti-Hepatofibrotic Drug Efficacy Based on *In Vitro* High-Content Analysis

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Abstract

**Background/Aims:** Many anti-fibrotic drugs with high *in vitro* efficacies fail to produce significant effects *in vivo*. The aim of this work is to use a statistical approach to design a numerical predictor that correlates better with *in vivo* outcomes.

**Methods:** High-content analysis (HCA) was performed with 49 drugs on hepatic stellate cells (HSCs) LX-2 stained with 10 fibrotic markers. ~0.3 billion feature values from all cells in >150,000 images were quantified to reflect the drug effects. A systematic literature search on the *in vivo* effects of all 49 drugs on hepatofibrotic rats yields 28 papers with histological scores. The *in vivo* and *in vitro* datasets were used to compute a single efficacy predictor (*E*ₚ*p*req*ᵣ*). This optimized relationship was independently validated using *in vivo* data from two different contexts (treatment of DMN rats and prevention of CCL₄ induction). A linear *in vitro-in vivo* correlation was consistently observed in all three contexts. We used *E*ₚ*p*req*ᵣ* values to cluster drugs according to efficacy; and found that high-efficacy drugs tended to target proliferation, apoptosis and contractility of HSCs.

**Conclusions:** The *E*ₚ*p*req*ᵣ* statistic, based on a prioritized combination of *in vitro* features, provides a better correlation between *in vitro* and *in vivo* drug response than any of the traditional *in vitro* markers considered.

Introduction

Liver fibrosis, a disease of excessive extracellular matrix (ECM) accumulation, is a common downstream response to repeated liver injury, caused by factors such as hepatitis B or C virus infection, excessive alcohol consumption, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, or drugs and toxins such as azathioprine [¹], D-galactosamine [²] or low doses of paracetamol [³]. In current clinical practice, the most effective anti-fibrotic treatment is indirect: to target the underlying cause(s) of injury, as removal of primary insults may lead to spontaneous regression of fibrosis. For example, lamivudine, which blocks hepatitis B virus replication, can result in fibrosis resolution [⁴]. However, fully activated hepatic stellate cells (HSCs), besides being a major source of fibrotic ECM [⁵], also secrete a broad range of chemokines and cytokines for self-perpetuating fibrosis in the absence of primary insults [⁶]. As a result, indirect treatment by removing the underlying irritant is not effective in a significant population of liver fibrosis patients.

Current drug discovery efforts for direct anti-fibrotic therapies have primarily targeted activated HSCs. Over recent years, the focus in drug discovery research has shifted from cell-free approaches based on molecular targets, to cell-based systems-biology based approaches, in an effort to increase success rates and reduce the overhead costs of drug development [⁷]. Since multiple complex pathways are involved in fibrogenesis, it is important to study the anti-fibrotic effects of a drug in the cellular context. Several high-throughput *in vitro* screenings have been performed...
previously on HSCs or fibroblast cells. Xu et al. (2007) established a quantitative screening platform based on TGF-β1 dependent fibroblast nodule formation [8]. Using this system, 8 out of 21 herbal extracts were found to have anti-fibrotic activities [9]. In other studies, HSC proliferation and apoptosis were used to assess the direct effects of drugs on HSC [10,11]. Collagen expression is another indicator commonly used in high-throughput systems [12,13]. These studies together with conventional low-throughput in vitro and in vivo studies have identified a diverse group of positive chemicals. The most promising ones, such as losartan, pioglitazone and Fuzheng Huayu tablets, have entered phase IV clinical trials [14].

Despite numerous efforts in anti-fibrotic drug discovery, there is no anti-fibrotic drug approved by the U.S. Food and Drug Administration. Many candidate drugs for fibrosis have failed in preclinical or clinical trials. One of the reasons is that in vitro data have poor correlation with in vivo drug effects due to the complicated pathophysiological background of hepatic fibrogenesis. As a result, drugs with high in vitro efficacies based on simple biochemical assays may fail to produce significant in vivo effects [15]. Despite the different levels of complexity between the in vitro and in vivo systems, previous studies from other fields such as drug dissolution [16,17], have demonstrated that optimized design of in vitro systems can result in better correlation with in vivo data [18,19].

In the present study, we quantitatively assessed and compared end-point anti-fibrotic drug responses from in vitro and in vivo models. A high-content analysis (HCA) system was established that provides a strong positive correlation with the in vivo drug responses. A drug efficacy predictor (E_predict) was computed and optimized to have a high positive correlation with the in vivo drug efficacy (E_vivo) extracted from studies using rat carbon tetrachloride (CCL4) treatment models. This positive correlation was validated with two additional validation datasets from rat CCL4 preventive and dimethylnitrosamine (DMN) treatment models. Moreover, a linear in vitro-in vivo relationship was consistently observed in all three datasets, suggesting that the E_predict value can also be used to rank drug efficacy and generate predictions. Drugs with higher E_predict were observed to exert their primary effects by targeting HSC proliferation, apoptosis or contractility, which are consistent with previous anti-fibrosis strategies.

Materials and Methods

Cell culture

The human HSC cell line LX-2 was obtained as a generous gift from Dr. Scott Friedman (Mount Sinai Hospital, NY). The cells were cultured in Dulbecco’s modified eagle medium with 1000 mg/L glucose (Biolips Shared Facilities, Singapore) and 10% heat inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and incubated in 37°C in a humidified atmosphere with 95% air/5% carbon dioxide.

Drug preparation

45 anti-fibrotic drugs and 4 non-specific control compounds not related to fibrosis were included in this study. The stock solution of each drug was prepared by dissolving the drug in dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA) at the maximum solubility of a drug unless the solvent is specifically indicated in the manufacturer’s information sheet. The highest working concentration of each drug was determined as the IC50 value from a cell viability assay (Table S1) and was dispensed in the second column of a 96-well plate (Nunc, Roskilde, Denmark). 10 other working concentrations were prepared by 2-fold serial dilution from the highest concentration in the same 96-well plate from column 3 to column 12. The first column of each plate was used as a drug-free control column.

Drug treatment

LX-2 cells were seeded in 96-well glass-bottom optical plates (Matrical bioscience, Spokane, Washington). The seeding density was 0.007 million in 100 μl medium per well, allowing cells to reach 70% confluence after a 3-day incubation. 24 hours after cell seeding, the culture medium was removed and fresh medium with drug was added and the cells were further incubated for 48 hours before the viability assay or staining was performed.

Cell viability assay

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), according to the manufacturer’s instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega). MTS reagent was prepared by mixing minimum essential medium (Gibco, Grand Island, NY, USA), FBS and CellTiter One solution at a ratio of 9:1:2 just before the assay. 120 μl of the prepared reagent was added to each well and the plates incubated for 60 minutes in a 37°C incubator. At the end of the incubation, 100 μl of the medium was transferred to a new 96-well plate and the absorbance read at 490 nm. All readings were corrected with blank controls (MTS reagent incubated for 1 hour in 37°C in empty wells). All conditions were duplicated per experiment and all experiments were performed twice. The average values were used to determine the IC50 values and the highest drug working concentrations were set to be close to the IC50 values.

Cell staining

Ten markers of fibrosis (Table S3) were included in this study and they were studied using 7 staining sets. We used 5 Cellomics Hitkits to track changes in cell proliferation (BrdU cell proliferation kit), apoptosis (Multiparameter apoptosis 1 kit and Caspase 3 activation kit), cell shape (Multiparameter apoptosis 1 kit), oxidative stress (Oxidative stress 1 kit) and cytokine activities (Smad3 and phospho-CREB activation kit). Five samples and their duplicates were separately stained using the 5 kits. The staining steps were carried out according to the manufacturer’s instructions (Thermo Fisher Scientific, Rockford, Illinois) with the exception of the nuclear staining procedure. For all the staining protocols in this study, nuclei were separately stained (Hoechst 33258 diluted 1:1000) after secondary antibody staining and incubated for 10 minutes under room temperature before the cells were washed and subjected to image acquisition.

In addition, two samples and their duplicates were separately stained with collagen type III antibody or double-stained with matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) antibodies. LX-2 cells were fixed in pre-warmed 3.7% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) in 37°C for 10 minutes and permeabilized with 1% Triton X-100 (Thermo Fisher Scientific, Rockford, Illinois) at room temperature for another 10 minutes before blocking with 10% BSA (Sigma, Canada). After 30 minutes blocking, the cells were incubated with either anti-collagen III antibody (diluted 1:1000, Santa Cruz Biotechnology) or a mixture of the MMP-2 and TIMP-1 antibodies (anti-MMP-2 antibody was diluted 1:1000, Santa Cruz Biotechnology; anti-TIMP-1 antibody was diluted 1:1000, Santa Cruz Biotechnology) for 2 hours at room temperature. After washing, the cells were incubated with fluorescein-conjugated affinity purified anti-rabbit IgG (H&L) (goat) (diluted...
and determining the cellular pathways that are common among data sets respectively (described in the result section 4).

**Image acquisition**

Images were acquired using Cellomics ArrayScan VTI (Thermo Scientific) controlled by vHCS™ Scan software version 6.1.4 (Build 6133). All images were taken with a LD PlanﾅNeofluar 20× air objective. 16 high-resolution images (1024×1024 pixels) were taken per well, which captured about 1000 to 2000 cells per experimental condition.

**Image processing and statistical analysis**

There are about 100 cells captured per image. Image segmentation and feature extraction were performed with a modified evolving generalized Voronoi diagrams algorithm [20], in which individual cells were identified and 25 or 16 cytological features were extracted per cell, for samples with 3-channel or 2-channel staining respectively. These features described cellular shape, protein distribution and content. A complete list of cytological features is shown in Table S2. The efficacy predictor \( E_{\text{pred}} \) was computed using Matlab R2009a with image processing and statistical toolboxes (material S1). In short, the raw data from \( \text{in vitro} \) experiments consists of multiple dimensions that include multiple cells in each treatment condition, drug concentrations, cellular features, and fibrotic markers. We reduced the data complexity in a 3-step statistical process (described in the material S1) and derived a SAUC value per fibrotic marker per drug. Subsequently, the \( E_{\text{pred}} \) score was computed by a linear combination of the SAUC values. The optimized weight for each fibrotic marker in the linear combination was calculated and validated independently with the training and validation \( \text{in vivo} \) data sets respectively (described in the result section 4).

**Automation**

During activation, HSCs undergo phenotypic changes such as increasing proliferation and ECM production (Fig. 1A) [21]. Many of these changes are potential therapeutic targets. We followed 5 such changes (Fig. 1B) with 10 chosen markers (Fig. 1C) using an HCA system that can be divided into 4 components: sample preparation, automated image acquisition, image processing and statistical analysis (Fig. 1D). All the sample preparation procedures including cell culture, drug preparation, drug treatment, cell viability assay and immunofluorescence staining were automated using a JANUS™ automated liquid handling system (Perkin Elmer).

**Results**

We have developed an HCA-based quantitative assessment screen that uses the \( E_{\text{pred}} \) value to correlate \( \text{in vitro} \) and \( \text{in vivo} \) anti-fibrotic drug responses. Subsequently the \( E_{\text{pred}} \) value was used in two applications: predicting \( \text{in vivo} \) drug efficacy from \( \text{in vitro} \) data, and determining the cellular pathways that are common among the more effective anti-fibrotic drugs.

**All 10 markers of fibrosis captured drug-induced changes in LX-2 cells**

In HCA, cells were treated with the 49 drugs at 11 concentrations, stained for 10 markers of fibrosis (Table S3), and imaged using automated microscopy. Cellular features such as the extent of changes in shape and marker intensity were then quantified for assessing the anti-fibrotic efficacies of the drugs.

Drug-induced changes can be clearly detected in the datasets; for example, glycyrrhizin caused an increase in apoptosis (i.e., increase in the caspase 3 level and decrease in the mitochondrial membrane potential \( \Delta \Psi_m \), measured by MitoTracker Red) and a decrease in four other markers: proliferation (i.e., bromodeoxyuridine (BrdU) positive cells), oxidative stress (i.e., dihydroethidium (DHE) intensity), collagen (i.e., collagen type III intensity), and TIMP-1 (i.e., TIMP-1 intensity). The Smad3 marker for TGF-β1/fibrosis signaling was also studied. The ratio between nuclear and cytoplasmic intensities for Smad3 decreased with drug treatment, demonstrating reduced nuclear translocation and reduced activation of the protein. This suggests that glycyrrhizin can down-regulate the TGF-β1 signaling pathway. Furthermore, the total Smad3 level increased in cells treated with anti-fibrotic drugs; previous work showed that Smad3 is required for inhibiting HSC proliferation [22] (images in Fig. 2A).

**Changes in fibrotic markers \( \text{in vitro} \) are consistent with \( \text{in vivo} \) drug response**

We used a modified evolving generalized Voronoi diagrams algorithm to identify individual cells from the images. 5 nuclear features and 11 cytoplasmic features per marker were extracted from each cell. These features quantitatively described cellular characteristics such as cell shape, protein expression levels and protein localization in the nucleus and cytoplasm (Table S2).

The cellular features from cells treated with various drug concentrations were normalized and combined to create a single SAUC score per fibrotic marker per drug (material S1). The SAUCs vary positively with the anti-fibrotic effects of a drug on the 10 markers. Briefly, we converted the cellular feature values into a Kolmogorov-Smirnov score [23] or ratio depending on whether a feature value has a unimodal or bimodal distribution. Both Kolmogorov-Smirnov score and ratio vary from -1 to 1 and the sign of the Kolmogorov-Smirnov score [23] or ratio depends on whether a feature value has a unimodal or bimodal distribution. Both Kolmogorov-Smirnov score and ratio vary from -1 to 1 and the combined result was termed the KR value (Fig. 2A). A negative KR value represents a decreasing feature value (e.g. intensity) compared with the control, while a positive one represents increasing feature value. The KR values exhibit drug concentration-dependent changes shown by the color intensities in the heatmaps. The extent of changes of cells stained with a particular marker is then computed from the KR values and termed the SAUC score, which is the sum of the sign corrected area under the curve from a plot of KR values versus drug concentrations. The sign of the SAUC value was used in ranking anti-fibrotic effects (material S1).

Each drug has 10 SAUC values corresponding to the 10 markers of fibrosis. \( \text{in vitro} \) drug effects can be assessed based on these values, and the results could be correlated to \( \text{in vivo} \) response. For example, oxymatrine exhibited a higher efficacy than colchicine, as oxymatrine treated rats had lower histopathological scores, smaller collagen area in the liver tissue, and lower concentrations of the serum markers such as hyaluronic acid and procollagen III compared with colchicine treated rats [24]. From our HCA results, the SAUC values for at least half of the markers showed a higher value for oxymatrine than colchicine (Fig. 2B). In order to have a more quantitative comparison of the drug efficacies, our goal is to consolidate the 10 SAUC values into a single index as a drug efficacy predictor that is positively correlated with an \( \text{in vivo} \) drug efficacy index.

**An \( \text{in vivo} \) anti-fibrotic drug efficacy index ranks drugs based on their \( \text{in vivo} \) effects**

Different weights will be assigned to the SAUC values to reflect the relative importance of each of the markers towards the overall efficacy. The weights should be chosen so that the overall index...
can reflect the \textit{in vivo} response of a drug. Before we can do that, we need a numerical measure of the \textit{in vivo} drug efficacy. Previous work that involved multiple drugs in a single \textit{in vivo} study carried out the drug efficacy comparison by assessing the extent of fibrosis in liver biopsy samples, as well as the level of surrogate serum markers for liver fibrosis such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Such an approach does not summarize the experimental results into a drug efficacy index for direct comparison and ranking of drugs within a single \textit{in vivo} study or between studies. Here we analyzed the literature and an \textit{in vivo} drug efficacy index was computed based on histological scores.

Most of the \textit{in vivo} studies reported in the literature were carried out in rat models. Although numerous such papers are available, there is no standard method to compare these results. To compare the \textit{in vivo} drug efficacies, we have established an \textit{in vivo} index based on pathologist-graded histological scores, which are considered a gold standard for quantifying the extent of fibrosis. A systematic search was performed on the reported \textit{in vivo} effects of all 49 drugs on hepatofibrotic rats. The search yielded 28 papers from 1986 to 2009 with pathologist-graded histological scores, using CCl4, TAA, DMN, cisplatin, pig serum, high calorie diet or bile duct ligation induced fibrotic rats (Table S4). These studies can be further divided into preventive or treatment models, depending on whether a drug is given since the first injection of hepatotoxin or after liver fibrosis has been established.

To define a formula for \textit{in vivo} drug efficacy, we attempted to combine the histological score of fibrotic animals without drug treatment ($S_c$) and the histological score of drug treated animals ($S_t$). The \textit{in vivo} efficacy of a drug is expected to be positively correlated with the changes in histological scores between the control and drug-treated biopsy samples ($S_c - S_t$). In addition, the drug efficacy may also be positively dependent on the fibrosis severity, as there are observations that individuals with more advanced fibrosis are less likely to respond to treatment, hence these patients require drugs with higher efficacy [15].
quantitative in vivo efficacy index ($E_{in\,vivo}$) was computed as shown below:

$$E_{in\,vivo} = S_c \times (S_c - S_t)$$

Both $S_c$ and $S_t$ were linearly correlated to a 0–4 scale, which is a commonly used range for histological scores in several fibrosis scoring systems such as Metavir, Knodell and Ludwig [25]. If histological scores of a drug from multiple studies were available, the highest $E_{in\,vivo}$ value was chosen.

The severity of fibrosis induced by different hepatotoxins varies (e.g., $E_{in\,vivo}$ for silymarin is 0.8 for DMN treatment model, 3.1 and 6 for CCl$_4$ treatment and preventive models); hence the indices are only comparable within the same fibrosis model. Subsequent correlation analysis was conducted using studies with long-term (>3 week) drug treatment, and fibrotic models with at least 3 drugs. The in vivo results satisfying these criteria are summarized in Table 1. CCl$_4$ preventive and treatment models have 5 drugs in common; we found that three of these drugs: silymarin, malotilate and pioglitazone have the same relative ranking in both models while PCN and taurine didn’t follow the ranking. Interestingly subsequent analysis showed that both PCN and taurine were outliers in the in vitro-in vivo correlation plots.

The calculated $E_{in\,vivo}$ is an attempt to capture the therapeutic efficacy of drugs on human patients. There are relatively few studies suitable for directly comparing drug effects on human patients due to variations in experimental design. In one example, two similar clinical studies using colchicine and silymarin on patients with cirrhosis due to any primary insults showed that colchicine led to 75% 5-year survival rate [26], while silymarin led to 58% 4-year survival rate [27]. $E_{in\,vivo}$ agrees with these reports that colchicine has a higher value (5.7) than silymarin (0.8) (Table 1).

### Table 1. Indexing of anti-fibrotic drugs from in vivo data.

| Animal models                      | Drugs                  | histological score (DMN alone) ($S_c$) | histological score (with drug) ($S_t$) | $E_{in\,vivo} = (S_c - S_t)\times S_c$ |
|------------------------------------|------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| DMN induced fibrosis (treatment)   | silymarin [40]         | 2                                     | 1.6                                   | 0.8                                   |
| DMN induced fibrosis (treatment)   | thalidomide [41]       | 1.56                                  | 0.89                                  | 1                                     |
| DMN induced fibrosis (treatment)   | tetrandrine [40]       | 2                                     | 1.3                                   | 1.4                                   |
| DMN induced fibrosis (treatment)   | colchicine [42]        | 3.8                                   | 2.3                                   | 5.7                                   |
| CCl$_4$ induced fibrosis (treatment) | silymarin [43]        | 3.4                                   | 2.5                                   | 3.1                                   |
| CCl$_4$ induced fibrosis (treatment) | 5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN) [44] | 3.84                                 | 2.8                                   | 4                                     |
| CCl$_4$ induced fibrosis (treatment) | malotilate [45]       | 3.76                                  | 2.67                                  | 4.1                                   |
| CCl$_4$ induced fibrosis (treatment) | rosmarinic acid [43]  | 3.4                                   | 2.1                                   | 4.4                                   |
| CCl$_4$ induced fibrosis (treatment) | pioglitazone [46]     | 4                                     | 2.63                                  | 5.5                                   |
| CCl$_4$ induced fibrosis (treatment) | taurine [47]          | 3.33                                  | 1.33                                  | 6.7                                   |
| CCl$_4$ induced fibrosis (preventive) | PCN [44]              | 3.6                                   | 3.68                                  | ~0.3                                  |
| CCl$_4$ induced fibrosis (preventive) | taurine [48]          | 3.03                                  | 1.87                                  | 3.5                                   |
| CCl$_4$ induced fibrosis (preventive) | melatonin [49]       | 3.38                                  | 2.25                                  | 3.8                                   |
| CCl$_4$ induced fibrosis (preventive) | oxymatrine [50]      | 3.76                                  | 2.43                                  | 5                                     |
| CCl$_4$ induced fibrosis (preventive) | silymarin [51]       | 4                                     | 2.5                                   | 6                                     |
| CCl$_4$ induced fibrosis (preventive) | malotilate [45]      | 2.91                                  | 0.76                                  | 6.3                                   |
| CCl$_4$ induced fibrosis (preventive) | EGCG [52]            | 3.58                                  | 1.5                                   | 7.4                                   |
| CCl$_4$ induced fibrosis (preventive) | pioglitazone [46]    | 4                                     | 1.94                                  | 8.2                                   |

All data are taken from the literature using dimethylnitrosamine (DMN) treatment, carbon tetrachloride (CCl$_4$) treatment, or CCl$_4$ preventive fibrotic rat models. Histological scores are linearly converted to a scale from 0 to 4. $E_{in\,vivo}$ is established as shown. Drugs under each animal model are sorted according to increasing $E_{in\,vivo}$. Silymarin, malotilate and pioglitazone have the same relative ranking in CCl$_4$ treatment and preventive models.

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correlation test [28] against $E_{\text{in vitro}}$ from CCl4 fibrosis model. One outlier was allowed in the analysis, as the sample size is relatively small. The Spearman's rank correlation coefficient rho ranges from 0 to 1, where 1 means perfect rank correlation (excluding the outlier), and 0 means the opposite order. The optimized weight for each marker was determined to be the value with the highest frequency occurrence out of all cases which achieved rho = 1 (Fig. S8). High weight implies high importance of the marker towards a strongly positive correlation. The optimized weights yielded the following efficacy predictor ($E_{\text{predict}}$) computed as the linear combination of the 10 optimized weights with the $\Delta$AUC values:

$$E_{\text{predict}} = SAUC_{\text{DHE}} + SAUC_{\text{collagen III}} + 2 \times SAUC_{\text{mitochondrial membrane potential}} + 2 \times SAUC_{\text{temp}} + 1 + 2 \times SAUC_{\text{creb}} + 2 \times SAUC_{\text{smad3}}$$

A greater $E_{\text{predict}}$ represents a higher drug efficacy and all negative values were assigned to 0 as no efficacy. We also incorporated an additional step to identify drugs with non-specific effects that cause an increase in collagen expression (material S2 and Fig. S3); their $E_{\text{predict}}$ values were assigned to 0 as no efficacy. We also incorporated an additional step to identify drugs with non-specific effects that cause an increase in collagen expression (material S2 and Fig. S3); their $E_{\text{predict}}$ values were assigned to 0 as no efficacy.

To validate that $E_{\text{predict}}$ is a robust anti-fibrotic drug efficacy predictor that can correlate with the $in vitro$ data from other rodent fibrosis models different from the training dataset; we tested the ability of $E_{\text{predict}}$ to correlate with two “blind” $in vitro$ datasets. We drew two additional correlation plots of $E_{\text{predict}}$ against $E_{\text{in vitro}}$ from DMN treatment (Fig. 3B) and CCl4 preventive models (Fig. 3C). $E_{\text{predict}}$ was kept the same as computed for the CCl4 treatment model. A positive correlation as well as a linear relationship between $E_{\text{predict}}$ and $E_{\text{in vitro}}$ was again observed in both plots. To further prove that this relationship does not depend on the choice of the training set of data, similar results were obtained if DMN treatment or CCl4 preventive models were used as the training dataset instead of the CCl4 treatment model (data not shown).

Fig. 3D and E demonstrate how rho varies with the number of markers and the number of cytological features, respectively. Both curves reach a plateau before or at our experimental configuration of 10 markers and 16 features per cell, showing that our study design is sufficient for the anti-fibrotic correlation study. We next test the robustness of the experimental configuration by shuffling the weights in the $E_{\text{predict}}$ formula; Fig. 3F shows the plot for the percentage distribution of rho for all possible combinations of the 3 weights and 10 markers. There is a 23% chance of rho being equal to 1, which is significantly higher than the random control (5% chance of rho being equal to 1) in which the relative ranks were randomized before applying the Spearman’s rank correlation test. This demonstrates that a positive correlation between the $in vitro$ and $in vivo$ indices can be achieved even if the optimized set of weights is not used, implying that the weighting procedure of our system is not vulnerable to high background noise. The $in vitro$ $\Delta$AUCs have good predictive value alone, and the $E_{\text{predict}}$ weighting of the $\Delta$AUCs optimizes their correlation and augments their predictive power.

The $in vivo$ histological scores can be estimated from $E_{\text{predict}}$

The linear relationship observed in all the three correlation plots may be used to generate predictions of $in vivo$ drug efficacies based on $in vitro$ measurements. Since all the $in vivo$ data from long-term drug treatment studies have been used to build or validate the $in vitro-in vivo$ correlation, we now turn to short-term drug treatment ($<3$-week treatment including single injection) as another source of information for validating the predictive capability of $E_{\text{predict}}$. One such study is available, concerning sulfasalazine. We would like to use $in vitro$ $E_{\text{predict}}$ values generated from HCA to predict $in vivo$ histological scores. Since $E_{\text{predict}}$ was optimized with data from long-term studies, the predicted histological scores should be similar to long-term drug treatment outcomes. The histological scores from short-term studies are expected to be slightly higher than our prediction, because prolonging the treatment with the same drug used in the short-term studies may further improve the fibrotic status and decrease the histological scores.

The $E_{\text{predict}}$ value of sulfasalazine is 39437; using the linear relationship from the CCl4 treatment model (equation in Fig. 3A), the $E_{\text{predict}}$ value is calculated to be 5.8. Assuming the histological score of rat livers with CCl4 induced fibrosis and no anti-fibrotic treatment is 3.0 (same as in [29]), a long-term treatment with sulfasalazine is predicted to reduce the fibrosis histological score to 1.1. A short-term study on rat CCl4 treatment model reported that a single injection of sulfasalazine reduced the fibrosis score to 1.5 compared with 3.0 in untreated CCl4-only livers [29]. The results agreed with our expectation, showing that the $in vivo$ histological scores can be estimated from $E_{\text{predict}}$.

High-efficacy drugs tend to target proliferation, apoptosis and contractility of HSCs

All drugs were grouped into 3 categories based on their $E_{\text{predict}}$ values. The negative (n) group was defined to include all drugs with $E_{\text{predict}}$ equivalent to 0. Seven drugs with the highest $E_{\text{predict}}$ values were placed into the very positive (vp) group. The rest of the drugs were in the positive (p) group. Before proceeding to quantitative analysis, we firstly remark on some trends and background about the categorized drugs. The n group has 16 drugs including 6 anti-oxidants, two HMG-CoA reductase inhibitors, simvastatin and lovastatin, and all 4 non-specific background about the categorized drugs. The n group has 16 drugs including 6 anti-oxidants, two HMG-CoA reductase inhibitors, simvastatin and lovastatin, and all 4 non-specific control compounds not related to fibrosis. Tranilast from the p group showed anti-fibrotic effects in renal and liver fibrosis [30,31], and it has a relatively high $E_{\text{predict}}$ value of 19594. It has been reported as a positive drug in another high-throughput screening study [8]. In the vp group, glycyrrhizin, a herbal extract from licorice, showed positive effects on patients with hepatitis C [32]. Pioglitazone is another highly effective drug in the vp group that has been subjected to multiple advanced stage clinical studies [14]. It is one of the peroxisomal proliferator activated receptor gamma ligands, which have overall higher efficacies on human patients than colchicine, interferon gamma, and angiotensin receptor blockers [15].

The mean $\bar{K}$R values of the average intensity for fibrosis markers were represented as boxplots for the n, p and vp groups of drugs (Fig. 4A). Fewer outliers (red plus) were observed in the plot for the vp group compared with that for all the drugs (n+p+vp), showing that drugs with high efficacies have similar cellular effects and probably have similar cellular targets.

A principal component analysis (PCA) was carried to detect the set of markers that carry the most information, which could reflect the importance of the underlying pathways. We found that the top 4 principal components built from $\Delta$AUC values from drugs in the vp group explained more than 95% of the cumulative variance in
Table 2. List of $E_{\text{predict}}$ values for all the drugs.

| Drugs                          | $E_{\text{predict}}$ |
|--------------------------------|----------------------|
| taxifolin                     | 0                    |
| taurine                       | 0                    |
| curcumin                      | 0                    |
| resveratrol                   | 0                    |
| silymarin                     | 0                    |
| minoxidil sulphate            | 0                    |
| simvastatin                   | 0                    |
| genistein                     | 0                    |
| lovastatin                    | 0                    |
| PTK787/ZIK2258 (PTK/ZK)       | 0                    |
| Y27632                        | 0                    |
| rotenone                      | 0                    |
| AG1295                        | 0                    |
| paclitaxel                    | 0                    |
| aphidicolin                   | 0                    |
| nocardazole                   | 0                    |
| pentoxifylline                | 5175                 |
| matrine                       | 5295                 |
| astragaloside IV              | 5496                 |
| thalidomide                   | 6263                 |
| colchicine                    | 6487                 |
| TGF-β inhibitor V             | 6974                 |
| gliotoxin                     | 7086                 |
| S-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN) | 8203 |
| camostat mesylate             | 8231                 |
| imatinib mesylate             | 8454                 |
| oxymatrine                    | 8528                 |
| pirfenidone                   | 8837                 |
| minoxidil                     | 9069                 |
| AG1296                        | 9154                 |
| somatostatin                  | 10057                |
| MG132                         | 10669                |
| tetrandrine                   | 10747                |
| telmisartan                   | 11467                |
| malotilate                    | 12941                |
| melatonin                     | 13728                |
| fasudil HCl                   | 14295                |
| olmesartan medoxomil          | 15959                |
| silybin                       | 18138                |
| TGF-β inhibitor III           | 18315                |
| tranilast                     | 19594                |
| epigallocatechin gallate (EGCG) | 19704         |
| bortezomib                    | 20147                |
| rosmarinic acid               | 21435                |
| berberine chloride            | 21983                |
| staurosporine                 | 25015                |
| glycyrrhizin                  | 25728                |
| pioglitazone                  | 35226                |
| sulfasalazine                 | 39437                |

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Discussion

Suppressing collagen production or reducing HSC viability represents an important anti-fibrotic drug screening strategy. Single-parameter in vitro studies have relatively poor correlation with in vivo drug efficacy; while multi-parameter in vitro studies are easy to perform but difficult to interpret. In this paper, we take a systematic statistical approach to the problem of correlating multi-parameter HCA screening against published in vivo drug effects. Our HCA system includes 10 fibrotic markers and 16 imaging features per marker, which follow changes in reactive oxygen species, TGF-β, proliferation, apoptosis, collagen regulation and cell contractility. Using a limited subset of the in vivo literature, we compute an optimized interpretation of the in vitro data, called $E_{\text{predict}}$ to predict in vivo drug efficacy. Then we test the performance of the $E_{\text{predict}}$ values on two different subsets of the in vivo literature. We find that $E_{\text{predict}}$ is able to identify drugs with anti-fibrotic effects, and also be able to distinguish drugs with moderate and high efficacies.

Studies of in vitro-in vivo comparative efficacy can help select promising categories of drugs to be given priority in the drug discovery pipeline. However, it is challenging to perform such...
analysis from limited in vivo literature. The preclinical and clinical results of many drugs are often lacking, incomplete or inconclusive. Even when in vivo data is available, the histological scores may not be assessed, while other serum markers such as ALT and AST may not directly reflect fibrosis severity [34]. Another important concern is the inter-observer variability by pathologists doing histological examination of biopsy samples [35]. This intrinsic baseline error seems to be well tolerated and low enough not to mask the linear relationship between data from in vitro cell culture and in vivo rat models in this study.

In this study the \( E_{\text{predict}} \) value was derived from HCA and a limited training set of in vivo data, but its magnitude showed strong positive correlation with most of the available in vivo scores from fibrotic rat models, including blinded data sets that were reserved for validation purposes. The level of the in vitro efficacy was assessed by the overall effect of a drug on multiple pathways and partially reflects the complex in vivo response. It is interesting to see that a linear relationship with \( R^2 = 0.9 \) exists between the in vitro and in vivo data for CCl4 and DMN fibrotic treatment models; while a weaker linear correlation (\( R^2 = 0.54 \)) was observed for the

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**Figure 3. Correlation between \( E_{\text{predict}} \) and \( E_{\text{in vivo}} \)**

(A) Optimization of \( E_{\text{predict}} \). \( E_{\text{predict}} \) is computed as a weighted combination of the features with weights optimized using Spearman’s rank correlation test to best correlate with \( E_{\text{in vivo}} \) from the CCl4 treatment model. (B, C) Blind validations of in vitro-in vivo correlation between \( E_{\text{predict}} \) and \( E_{\text{in vivo}} \) from two independent datasets containing DMN treatment and CCl4 preventive models respectively. The linear relationship is highlighted using linear regression lines in all (A, B and C). The equations of the linear regression lines and the \( R^2 \) values are computed without considering the outliers in the graphs (i.e., taurine in A, colchicine in B and PCN in C). (D) The relationship between the average rho value and the number of markers. (E) The relationship between average rho and the number of features per marker. Error bars represent standard deviation. (F) The percentage distribution of rho is plotted for all possible combinations of the 3 weights and 10 markers. The random control was done by randomizing the relative ranks of the in vivo drug efficacies for the Spearman’s rank correlation test.

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Figure 4. Distinctive characteristics of the negative (n), positive (p), and very positive (vp) groups of drugs. (A) The average intensities of the 10 makers for all drugs (n+p+vp), all positive (p+vp) drugs and the vp group of drugs are shown in the boxplots, where inter-quartile ranges are represented by boxes. Whiskers represent 1.5 times the inter-quartile range and any data (outliers) beyond the whiskers are shown using red +. (B) Principal component analysis (PCA) is done using data from the vp group. The top 4 principal components explain 95% of the cumulative variance in the data.
CCL4 preventive model. In the latter, fibrosis causing agents such as CCL4 and drugs were given together to rats. As a result, many of the drugs showing positive effects are protecting hepatocytes from toxins or preventing HSC activation, rather than inducing fibrosis regression. Since an activated HSC cell line is used in our screening platform, it is more closely mimicking the treatment model; hence a stronger linear relationship exists for both CCL4 and DMN treatment models. Furthermore, anti-oxidants work by preventing HSC activation induced by free radicals. This group of drugs can be considered preventive drugs, more than treatment drugs, which agrees with our result that most of the anti-oxidants have lower Eσ values.

The ability of cell culture models to predict in vivo drug effects is limited by many fundamental constraints. For example, drugs might be able to improve liver fibrosis by improving vascular flow or liver architecture, such as the angiotensin II receptor antagonists losartan and candesartan. Some drugs are metabolized by hepatocytes into secondary compounds with different effects; and such effects cannot be foreseen by hepatocytes into secondary compounds with different effects; and such effects cannot be foreseen in vivo using HSC monocultures. This study investigated the effects of drugs on HSCs only. Our system is not suitable to substitute for animal trials, but we recommend it for prioritizing the selection of drugs to enter animal trials.

Interestingly, we have observed promising pieces of evidence that the Eσ-score can potentially be correlated with data from human clinical trials. For example, the group of drugs with relatively high Eσ-scores (e.g. pioglitazone [36] and glycyrrhizin [37]) gave more promising results in human clinical trials than the group of drugs with low Eσ-scores (e.g. colchicine [38] and silymarin [39]). Furthermore, drugs with lower Eσ-scores generally have fewer in vivo publications than drugs with higher Eσ-scores. Such relationship may be partially due to the fact that the hepatic stellate cell line used in this study is from human origin.

In conclusion, our anti-fibrotic drug screening platform is able to index and rank drugs according to their in vitro efficacy. The in vitro index system positively correlates with the in vivo histological scores, which shows that our in vitro cell-based system has some predictability of the in vivo drug response. Furthermore, drugs with higher efficacies are found to exert their effects through directly modulating HSC proliferation, apoptosis or contractility.

Supporting Information

Figure S1 Correlation between SAUC and Eσ in vivo for rat CCL4 treatment model.
(TIF)

Figure S2 Pie charts showing the chance of occurrence of weights in all cases where the Spearman’s rank correlation coefficient r achieves 1 for the training set of data. The optimized weight for each marker is the value with the highest occurrence indicated with a * in each pie chart, which implies the relatively higher importance of the marker towards contributing to a stronger positive correlation.
(TIF)

Figure S3 Images and quantification of hepatic stellate cells LX-2 with collagen III immuno-fluorescence staining. Cells are treated with (A) pioglitazone, (B) EGCG, or (C) aphiidicolin at the indicated concentrations for 48 hours (blue: nuclei; green: collagen III). The amount of collagen III in the cytoplasmic region is quantified and represented as the percentage of total collagen III intensity with respect to the control without drug treatment. Error bars represent standard deviation from 2 replicate datasets.
(TIF)

Table S1 List of drugs and their highest working concentrations.
(DOC)

Table S2 List of cellular features according to staining sets. 10 fibrotic markers were studied using 7 staining sets. S1: Cellomics BrdU cell proliferation kit (BrdU). S2: Cellomics multiparameter apoptosis kit 1 (F-actin, mitochondrial membrane potential, ΔΨm). S3: Cellomics caspase 3 activation kit (caspase 3). S4: Immunofluorescence staining of collagen III (collagen III). S5: Immunofluorescence staining of MMP-2 and TIMP-1 (MMP-2, TIMP-1). S6: Cellomics oxidative stress 1 kit (DHE). S7: Cellomics Smad3 and phospho CREB activation kit (Smad3, pCREB). Ch1: channel 1 for nuclear staining (blue), Ch2: channel 2 for protein staining (red or green for two-channel images; green for three-channel images). Ch3: channel 3 for protein staining (red for three-channel images). The nuclear region is defined by the Ch1 object mask. The cytoplasmic region that is positive for protein staining is defined by Ch2 (or Ch3) object mask. Nuclei were stained in all 7 staining sets. Since nuclear features (features 1 to 5) are similar regardless of the protein stainings in channel 2 and 3, they are only considered once in S1. S1, S3, S4 and S6 were double-stained with one nuclear dye (Ch1) and one dye for a marker protein (Ch2). They do not have features related to Ch3.
(DOC)

Table S3 List of references for the 10 markers of fibrosis.
(DOC)

Table S4 List of papers with pathologist-graded histological scores on fibrotic rats from 1986 to 2009.
(DOC)

Table S5 Mechanisms of action of the drugs. All 49 drugs are classified based on their mechanisms of action from the literature.
(DOC)

Material S1 Computing Eσ from cellular feature values.
(DOC)

Material S2 Method to identify drugs with non-specific effects from in vitro HCA analysis.
(DOC)
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Author Contributions

Conceived and designed the experiments: BZ. Performed the experiments: BZ LT XM. Analyzed the data: BZ YW REW PTCS HY LTK. Contributed reagents/materials/analysis tools: WY. Wrote the paper: BZ.

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