Modeling pro-death signaling pathways in cancer hepatocytes using multi-combinatorial treatments of inhibitors and stimuli

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***Abstract*—Cell death in hepatocellular carcinoma (HCC) cells as well as in other cell types is driven by a complex signaling transduction network comprised of several different pathways. In this study, we measured cell death of the HCC cell line C3A and we quantify the contribution of several signaling pathways using a reverse approach; namely instead of measuring the activity of the intracellular signal, we correlated the inhibition of that signal to the cell death. To achieve that, cells were treated with 6 stimuli and 7 inhibitors in a multi-combinatorial manner. Approximately 400 observations were made with the simultaneous treatment of up to 2 inhibitors with up to 3 stimuli. A modified linear regression model was developed to predict cell death as indicated by lactate dehydrogenase (LDH) activity. The correlation coefficients of this model were used to quantify the role of each pathway on HCC cell death. Our results are in good agreement with the literature; caspase 8 was revealed as the strongest pro-death mediator whereas the Akt pathway was shown to be the most pro-survival signal. MEK/ERK pathway had a dual role depending on the applied stimuli. Experimentally, the present method is fast, cost efficient, and easy. The coupling of the experiments to a mathematical model allowed us to quantify the contributions of a broad spectrum of pathways on cellular behavior. Additionally, such approach has a significant advantage in cases where measurements of signaling activities (i.e. from cell lysates) are experimentally impractical. For example, using the current methodology we might be able to monitor chondrocyte signaling transduction within its native environment: the articular cartilage extracellular matrix which presents a major challenge in cartilage biology.**

I. Introduction

HEPATOCELLULAR carcinoma (HCC) is the most common type of primary liver cancer and the third leading cause of cancer death worldwide [1]. Despite its well-defined aetiology (i.e. viral infection via hepatitis B and C, alcohol abuse, or exposure to certain toxic compounds such aflatoxin) there are no satisfactory therapeutic approaches. Traditional treatments for HCC include surgical resection, radiotherapy, chemotherapy, or liver transplantation and are characterized by poor outcomes as indicated by the median survival which is less than 6 months. The molecular basis of the disease lays on genomic aberrations or epigenetic mechanisms that result to a dysregulation in the balance between survival and apoptotic signaling pathways with a significant advantage on the latter [2-4].

There is an intense research on the molecular pathways that lead to a survival advantage of transformed hepatocytes and thus, to the onset of hepatocarcinogenesis [4]. Genomic or transcriptomic analysis have revealed dozens to hundreads of genes correlated to liver tumorgenesis [5] that can be heterogenic in nature even within adjacent tumor nodules of the same individual [6]. On the other hand, protein–based approaches have focused on several different pathways that might be responsible for the initiation and progression of HCC and therefore are pharmacologically interesting: Akt, ERK, NFκB, p38, JNK, STAT3 and caspases are among the main pathways that have been studied on the context of HCC cell death [4, 7-9]

In this work we combined experimental and computational approaches to evaluate the effects of several signaling transduction nodes in the cell death of C3A, an HCC cell line that has been derived from HepG2 cells based on a strong hepatocyte phenotype (high albumin production, high production of alpha fetoprotein, and gluconeogenesis activity) [10]. Traditional protein-based biological methods are usually based on phosphorylation measurements as a surrogate of the signaling transduction activity which is responsible for a specific cell behavior. In the present study we evaluated a reverse approach; instead of measuring the phosphorylation activity, we blocked the activity of up to two different pathways under the stimulation of up to 3 different cytokines related to hepatocyte physiology. A simple model based on linear regression was developed in order to deconvolute the contribution of each signal on the cell death phenotype. The results of the model are in good agreement with the literature. We were able to extract known cell behavior such as a strong pro-death signal that passes through the Caspase 8 signaling protein and a strong pro-survival signal via the PI3K/Akt pathway. Furthermore we we were able to unveil unknown signaling responses on C3As such as the dual role of MEK/ERK pathway which

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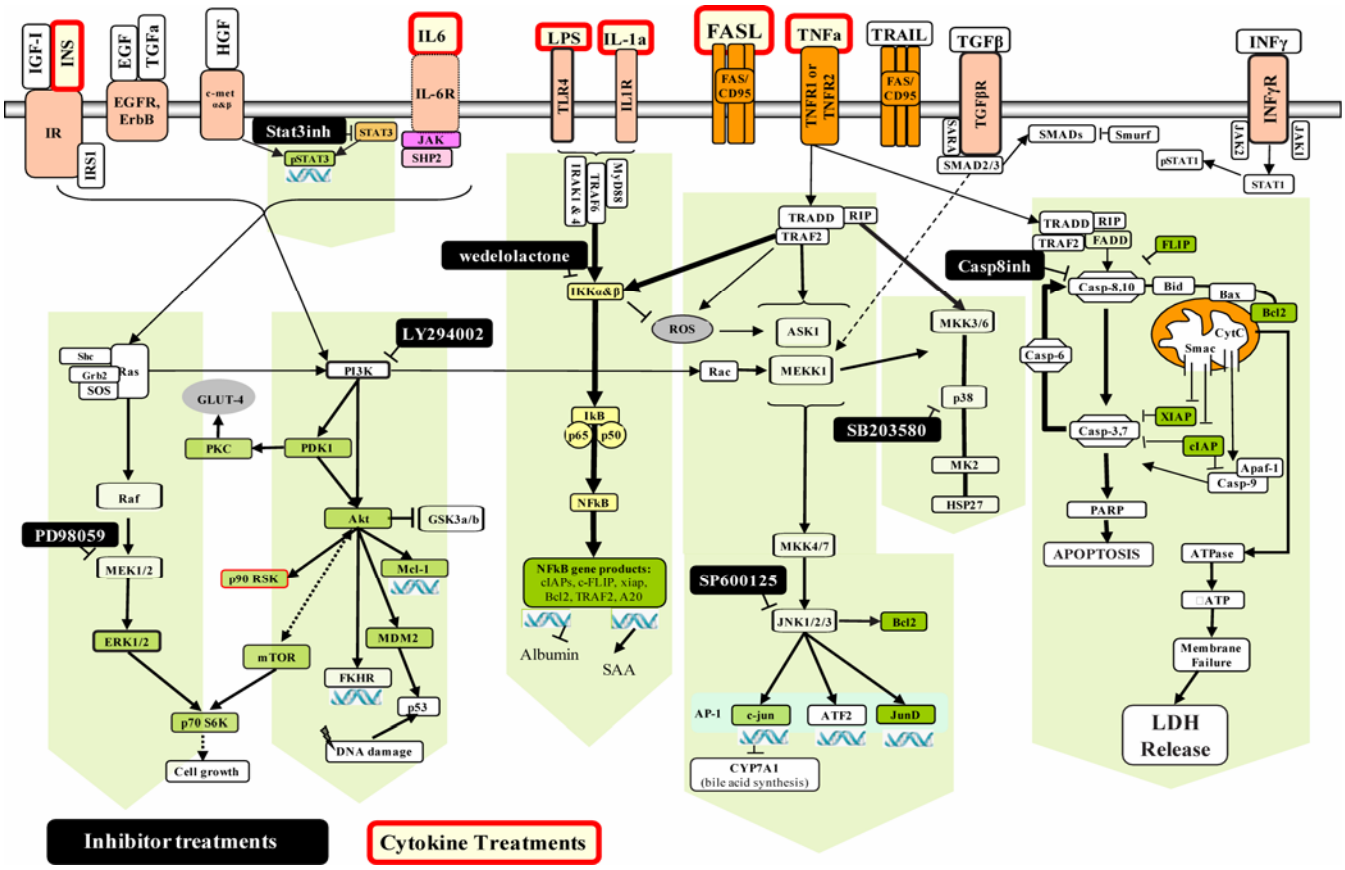

Fig. 1. Signaling network of hepatocytes treated with multi-combinatorial stimuli comprised of 6 different cytokines (boxes with red borders) and 7 inhibitors (black boxes). Inhibitors were selected so they block diverse pathways (i.e. MEK1/2, AKT, JNK1/2, NFκB, p38, Caspase 8, and Stat3)

can transducer both pro-survival and pro-death signals that depend on the applied stimuli. Our approach poses several advantages and limitations as explained in the discussion section.

II. Materials and Methods

A. Experimental Procedure

A hepatocellular carcinoma cell line C3A, purchaced by ATCC, was maintained in Eagle's Minimum Essential Medium with 10% serum for up to 5 passages. In the first day of the experiment, cells were plated on collagen type I coated 96-well plates with 100µl phenol-free Williams' Medium E (WEM, Sigma-Aldrich) supplemented with 1.0 mM L-Glutamine (Gibco), 100 nM Dexamethasone (Sigma-Aldrich), 5µg/ml Insulin (human, Sigma-Aldrich), 5µg/ml Transferrin (from human serum, Roche, and 5µg/ml Sodium Selenite (Sigma-Aldrich). Cells were cultured overnight and then starved for 6 hours in 180ul of WEM media with L-Glutamine and dexamethozone, pretreated with inhibitors, and stimulated with cytokines. Cytokine or inhibitor reagents were prepared at 20x concentration and spiked at 5% of the total volume (10µl). Conditioned medium samples were collected at 24 hours following cytokine/inhibitor stimulation and lactate dehydrogenase (LDH) release was measured as a marker of cell death.

TABLE I
CYTOKINE TREATMENTS

Cytokine	Name	Conc.
FASL	Fas ligand	100 ng/ml
TNFα	Tumor necrosis factor alpha	10 µg/ml
IL6	Interleukin 6	100 ng/ml
INS	Insulin	500 nM
LPS	Lipopolysaccharide	10 µg/ml
IL1α	Inteleukin 1 alpha	20 ng/ml

TABLE II
INHIBITOR TREATMENTS

Inhibitor	Name	Conc.
p38i	SB203580	10µM
Stat3i	Inhibitor Peptide	25µM
PI3Ki	LY294002	30µM
JNKi	SP600125	30µM
Casp8i	Z-IETD-FMK	50µM
IKKi	Wedelolactone	20µM
MEKi	PD98059	30µM

Tables I & II: Cytokines and inhibitors used in the multicombinatorial experiment.

B. Cell death Assay

Cell death was measured by lactate dehydrogenase (LDH) activity in supernatants using the CytoTox-ONE Homogeneous Membrane Integrity Assay according to manufacturer's recommendations (Promega, Madison, WI). All LDH measurements were fold-change normalized to the no-treatment control at 24 hours post-cytokine/inhibitor stimuli. Despite the fact that LDH is a sensitive assay for C3A (because of the inheritably large intracellular amounts of LDH in liver cells), the assay cannot distinguish the two modes of death that can occur, namely necrosis and apoptosis. To better quantify necrosis versus apoptosis modes of death, more specific assays are needed for the apoptosis pathways such as cleaved PARP or assays for the activity of Caspase3/7 or Caspase8.

C. Multi-combinatorial treatmens

Cells were treated with a multicombinatorial treatment comprised by 7 inhibitors with 6 cytokines (Fig. 1 and Tabels I and II). For each treatment, cell death was measured by LDH assay. A total of 368 different observations were made using up to 2 inhibitors and 3 cytokines simultaneously.

D. Linear Regression Analysis

To capture the effects of both cytokines and inhibitors, a modified version of a simple linear regression model was implemented. The main assumption was that the two types of independent input variables (i.e. the *i-th* cytokine and *j-th* inhibitor) under *k* conditions (i.e. number of experiments) are linearly correlated with a dependent output variable Y (i.e. LDH release) via a weight coefficient w_{ij}. In our study, k=368(=16x23) conditions have been generated comprising by the combinatorial treatments of 16 different cases of cytokine stimuli with 23 different cases of inhibitor sets. To apply the linear regression, the LDH data were normalized to the basal level of LDH release (blank treatment). Initial data had shown that LDH measurement are linear within our range of measurements (data not shown).

The presence or absence of cytokines (i.e. $[\text{Cytokine}]_i$) were modeled with 1 and 0 respectively. When an inhibitor was present, the corresponding pathway is blocked and thus the $[\text{PATHWAY}]_j$ is 0 (assuming 100% inhibition). The default value of the $[\text{PATHWAY}]_j$ is 1. Cell death was calculated as:

$$LDH_k = a + \sum w_{ij} \cdot [CYTOKINE]_{ik} \cdot [PATHWAY]_{jk} \quad (1)$$

The equation (1) (with i=6 cytokines and j=7 inhibitors) has 42 unknowns $w_{i,j}$ that can be calculated by the overdetermined system of k=368 observations. The constant a corresponds to the basal level of LDH release. A MATLAB solver for linear systems that minimizes the sum of least square errors was used to calculate the weight factors $w_{i,j}$..

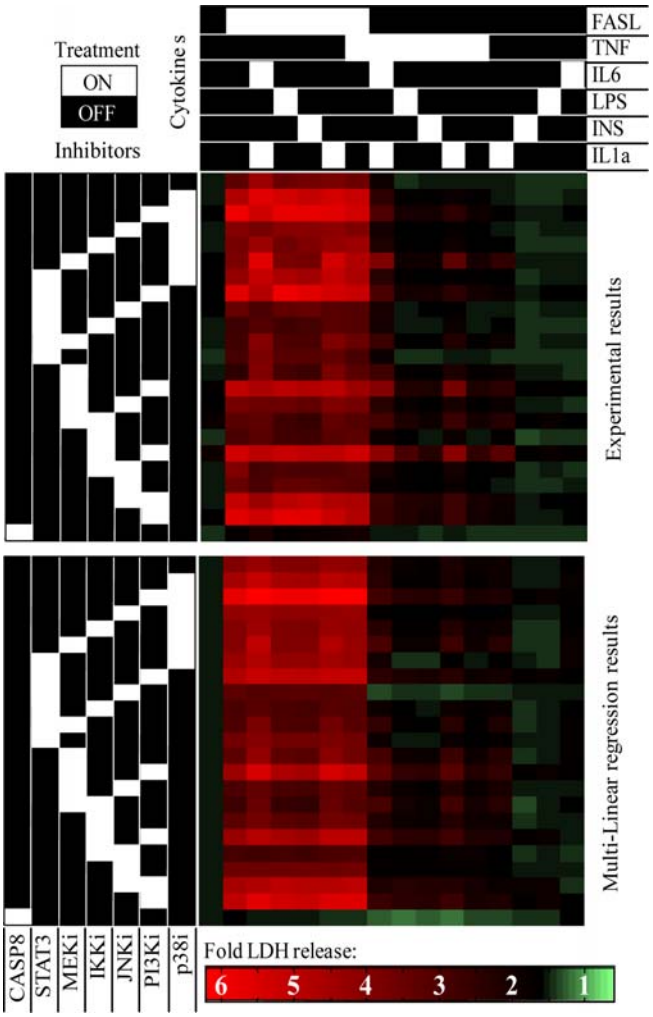

Fig. 2. Hepatocellular Carcinoma cell death measured by LDH release and induced by a multicombinatorial treatment with several cytokines and inhibitors (Black: treatment absent, White: treatment present). Modified linear regression analysis (bottom panel) is in good agreement with the experimnetal results (top panel).

III. Results

The C3A cell line exhibited cell death under several different treatments (Fig. 2, top panel). FASL but not TNFa was a main driving force for cell death (red area in the top panel). Blocking the pro-survival Akt pathway with a PI3K inhibitor also caused an increase in cell death (increased red colored horizontal stripes that coincide with PI3K presence). On the other hand, blocking the caspase pathway upstream with the caspase 8 inhibitor protected cells from cell death (last horizontal stripe).

Despite the simplicity and the assumptions of the modified linear regression model, we were able to capture well the experimental data as shown in Fig. 2 (bottom panel) and in Fig. 3.

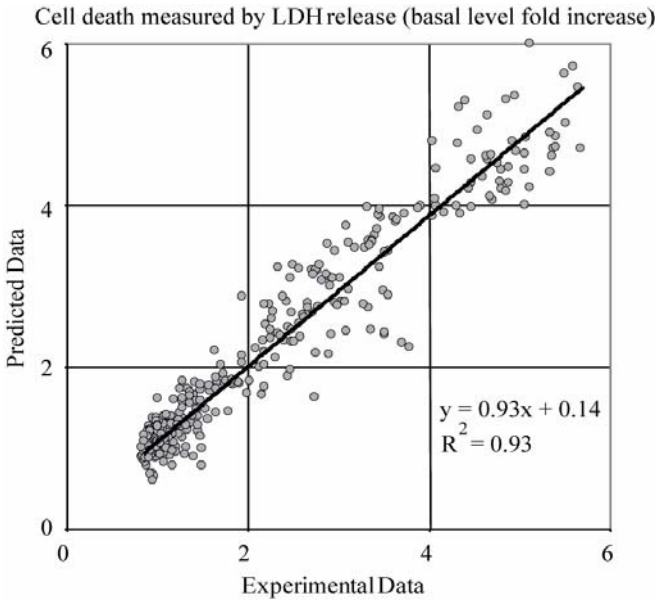

Fig. 3: Experimental and modeled results with a modified linear regression approach are in good agreement.

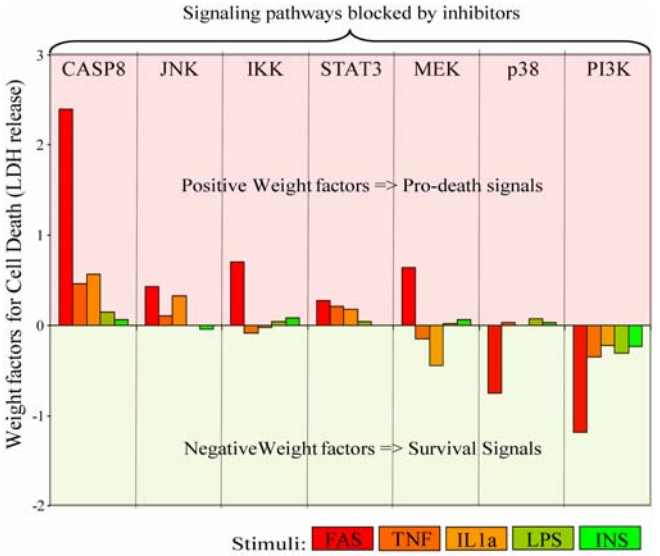

Fig. 4. Contribution of each signal on the cell death of the C3A HCC cell line. Positive weights corresponds to pro-death signals induced by different stimuli. Thus only pro-death information passes via the caspase 8 protein. On the other hand, negative weight corresponds to pro-survival survival signlas. PI3K exhibits only pro-survival signal even under a FASL treatment.

The weighing factors w_{ij} are shown in Fig. 4. The results, as revealed by the weight factors, are in good agreement with the literature. The caspase pathway, blocked by the upstream caspase 8 inhibitor, correlated to a strong pro-death response specially when triggered by the FASL death receptor (Fig. 4) [11, 12]. TNFa and IL1a also induced some cell death via the caspase pathway but in a significantly smaller intensity. On the other hand, the Akt pathway, blocked by the PI3K inhibitor, correlated to a strong pro-survival response, as indicated by the negative values of the weigh factors w_{ij} (Fig. 5, last column). Surprisingly, FASL (red bars in Fig. 5) did induce not only a pro-death responses via Caspases but also a strong compensatory survival mechanism via the PI3K suggesting a delicate balance between pro-survival and pro-death pathways [4]. Additionally, the NFkB pathway activity, know as main inflammatory regulator which can be block by the IKK inhibitor, also conveyed strong Fas-mediated pro-death signals [13].

Some less intuitive results were revealed for the MEK/ERK pathway which can convey pro-survival signals when stimulated by IL1a or pro-death signals on a FASL induced mechanism. Even though such dual mechanism is not known to our knowledge, our results are in good agreement with the cell death response that a MEK inhibitor can induce in HCC [14, 15].

IV. Discussion

In this work we evaluated the contribution of several pathways on the hepatocellular carcinoma cell death using a reverse approach; namely instead of measuring the activity of each protein and correlating to cell death, we inhibited their activities of several pathways on a multi-combinatorial manner and we correlated their absence of signal to cell death. A modified linear regression model was able to evaluate the contribution of each pathway to cell phenotype. Our results were in good agreement with the published literature.

Our multi-combinatorial approach has several advantages compared to standard biological techniques such as ease of use, low experimental cost, and a broad spectrum of investigated signals that is limited by the number of inhibitors used. The LDH assay can be performed on 96 and 384 well plates and the total dataset which evaluated 7 signals (via inhibitors) under 6 cytokines required less than 4 96well plates. On the other hand, standard biological approaches that measures the phoshorylation of proteins is performed either by western blots or standard immunoassays (i.e. ELISA, in-cell western, immunohistochemistry) and are biased to high abundance proteins because of the inability to detect low copy number proteins. However, signal transduction can occur with a very small number of proteins and thus, the use of inhibitors offers a more balanced approach since inhibitor activity is usually not correlated to the protein abundance.

For understanding signaling transduction, most of the measurements of phosphorylation activity are performed in cell cultures on plates or in cells in suspension because cells can be lysed. However most mammalian cell types are embedded in an extracellular matrix which prevent cell lysis. A main advantage of the use of inhibitors is the ability to study signal transduction in cells within their natural environment. This is especially true for articular cartilage, which is an avascular, aneural connective tissue with a single population of cells know as chondrocytes. Thus, this approach, might allow to study chondrocyte signaling

transduction, as well as any other cell type in their extracellular matrix, without the need to isolate the cells from the natural environment [16, 17].

A main limitation of the experimental setup is the off target behavior of most of the commercially available inhibitors driven by their low affinity and low selectivity. Such off target behavior poses an uncertainty on the correlation coefficients that can be due to off target effects. Significant improvements on the design of the new generation inhibitors with nanomolar or picomolar affinities and with known kinase profiles will improve the specificity of the inhibitors and thus the accuracy of the approach. Furthermore, the [PATHWAY] value is either 0 or 1, implying that the inhibitor is blocking 100% the targeted signal. A more accurate approach would be to perform an IC50 curve for the inhibition of the downstream target and replace the "0" value by the actual inhibitory action at the specific inhibitor concentration. In the current work, we applied saturated level of inhibitors in order to be closer to 100% inhibition (albeit with more off target effects).

Several assumptions were made for the modified linear regression model. First, the model has only an interaction factor (i.e. [CYTOKINE]*[PATHWAY]) which assumes that a stimuli is transdusing the signals only via the 7 available nodes (inhibitor blocked pathways) and the effect of all other signals has been averaged into the constant α (eq.1). Even thought the inhibitors have been selected because there are the main downstream signals of the stimuli, there should be several other inhibitors that if included, they can increase the accuracy of our predictions. In addition, there is no main effect factor for [PATHWAY] which implies that inhibitors cannot cause cell death by themselves (an observation that is right in our current setting, as shown in the first column of Fig 2). Even though there is no explicit main factor for [CYTOKINE], the effect of cytokines only can be simulated because [PATHWAY] is "1" when no inhibitors are present (first row of Fig 2). In general, more sophisticated analyses can be performed in the future, but the simple linear regression approach together with a simple multicombinatorial experimental design can provide clear and testable observations.

In the paper, we presented an alternative approach to quantify signaling transduction networks. The approach is based on simple multi-combinatorial experiments with inhibitors and stimuli and is coupled to a simple linear regression model. We applied our approach for the quantification of pro-death and pro-survival signals in HCC cells and several known and unknown interactions were found. This approach can easily be extended to more signals (by including more inhibitors) and more predictions (by including more readouts). Such approach can be proved particularly useful for cases where standard biological techniques for protein activity (e.g. phosphorylation events via western blot, or ELISA) cannot be performed (i.e. in chondrocytes embedded in cartilage tissue).

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