Published in final edited form as:

Chem Res Toxicol. 2014 January 21; 27(1): 17–26. doi:10.1021/tx400247g.

An Approach to Investigate Intracellular Protein Network Responses

Holly N. Currie[†], Julie A. Vrana[†], Alice A. Han[†], Giovanni Scardoni[‡], Nate Boggs[§], and Jonathan W. Boyd^{†,*}

[†]Department of Chemistry, West Virginia University, 217 Clark Hall, Prospect Street, Morgantown, West Virginia 26506, United States

[‡]Center for BioMedical Computing, University of Verona, Istituti Biologici, Blocco A, Strada le Grazie 8, 37134, Verona, Italy, EU

[§]The Johns Hopkins Applied Physics Laboratory, 11100 Johns Hopkins Road, Laurel, Maryland 20723, United States

Abstract

Modern toxicological evaluations have evolved to consider toxicity as a perturbation of biological pathways or networks. As such, toxicity testing approaches are shifting from common endpoint evaluations to pathway based approaches, where the degree of perturbation of select biological pathways is monitored. These new approaches are greatly increasing the data available to toxicologists, but methods of analyses to determine the inter-relationships between potentially affected pathways are needed to fully understand the consequences of exposure. An approach to construct dose-response curves that use graph theory to describe network perturbations amongst three disparate mitogen-activated protein kinase (MAPK) pathways is presented. Mitochondrial stress was induced in human hepatocytes (HepG2) by exposing the cells to increasing doses of the complex I inhibitor, deguelin. The relative phosphorylation responses of proteins involved in the regulation of the stress response were measured. Graph theory was applied to the phosphorylation data to obtain parameters describing the network perturbations at each individual dose tested. The graph theory results depicted the dynamic nature of the relationship between p38, JNK, and ERK1/2 under conditions of mitochondrial stress, and revealed shifts in the relationships between these MAPK pathways at low doses. The inter-relationship, or crosstalk, amongst these 3 traditionally linear MAPK cascades was further probed by co-exposing cells to deguelin plus SB202190 (JNK and p38 inhibitor) or deguelin plus SB202474 (JNK inhibitor). The cells exposed to deguelin plus SB202474 resulted in significantly decreased viability, which could be visualized and attributed to the decrease of ERK1/2 network centrality. The approach presented here allows for the construction and visualization of dose-response curves that describe network perturbations induced by chemical stress, which provides an informative and sensitive means of assessing toxicological effects on biological systems.

*Corresponding Author. Jonathan Boyd, PhD, C. Eugene Bennett Department of Chemistry, West Virginia University, 217 Clark Hall, Prospect Street, Morgantown, WV 26506, Phone: (304)-293-4741, Fax: (304)-293-4904, Jonathan.Boyd@mail.wvu.edu.

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Radiality dose-response curves for AKT, HSP27, IKBa, p53, and p90RSK. Euclidean distance tables for deguelin alone, deguelin in combination with SB202190, and deguelin in combination with SB202474. This material is available free of charge via the internet at <http://pubs.acs.org>.

Keywords

Network; Perturbation; Graph Theory; Crosstalk; MAPK Signaling; Dose-response

INTRODUCTION

Modern toxicological evaluations have evolved to consider toxicity as a perturbation of biological pathways or networks.^{1–4} As such, toxicity testing approaches are shifting from common endpoint evaluations to pathway based approaches, where the degree of perturbation of select biological pathways is monitored.^{3, 4} These new approaches are greatly increasing the information available to toxicologists, but it is imperative to bear in mind that all pathways are related, and that actions directed at specific locations can radiate throughout a network, affecting distant locations.² Monitoring particular cellular signal transduction cascades has already been used to increase our understanding of the mode-of-action of many toxicants,^{3–7} but we propose that it can also be used to reveal and understand changes in protein network connectivity that are responsible for the orchestration of the biological system's response to chemical insults.

The signaling proteins that have been most closely associated with chemical exposure stress are the mitogen-activated protein kinases (MAPKs).^{3, 6, 8–11} MAPKs are a group of signaling proteins that respond to various extracellular stimuli/stresses to produce a wide range of cellular responses, including growth, differentiation, apoptosis, and the stress response.¹² The deregulation of MAPK pathways has been associated with various diseased states, and a recent review has highlighted that the networked response of MAPKs under exposure conditions is much more complicated than previously thought.¹³ There are seven known separate families of mammalian MAPKs, and the most extensively studied of these groups are the ERK1/2, JNK, and p38 kinase pathways.¹² ERK1/2 pathway activation is generally associated with cell growth, proliferation, and survival, while p38 and JNK signaling pathways are typically associated with stress response and apoptosis.¹² These three MAPK families have generally been thought to be involved in separate linear signaling cascades, since each MAPK is activated by different upstream kinases, which are directed by distinct extracellular stimuli.^{11, 14} However, it has been shown that crosstalk between these MAPK pathways does exist, and that crosstalk between these pathways may be crucial to their ability to coordinate cellular stress responses including survival and inflammation.^{14–16} A network depicting the modular organization of MAPK signaling is shown in Figure 1. It has been suggested that under normal physiological conditions MAPK signaling pathways are linear with no crosstalk, but that pathological conditions and stresses, such as exposure to xenobiotics, may drive crosstalk between these pathways.^{13, 17–19} The ability to appropriately capture the exposure-induced relationship between distinct MAPK signaling pathways provides a different view of pathway based toxicity testing, and could yield valuable insight into the underlying biological response to chemical insults.

Central to pathway perturbation based toxicity testing is the ability to determine concentration ranges that lead to adverse health effects over time,³ which requires approaches that are capable of assessing the effects that chemical exposures have on entire networks on a dose-to-dose basis. Graph theory has proven to be a valuable mathematical modeling tool for gaining insights into the topological organization of networks across various disciplines.^{20–23} In graph theoretical analyses, networks are depicted as graphs where objects in the network are represented as nodes, and the pair wise relations between the objects are represented by lines referred to as edges. Based on the connectivity of the graphs, statistical attributes that characterize the structure and behavior of networked systems can be calculated (for more detailed information see^{20, 22–25}). Since its development

in the 18th century, graph theory has found application in the study of many networks including social, computer, economic, and, most recently, biological networks.^{21, 26–32} While advances in high-throughput data collection techniques have led to a rapid increase in the application of graph theory analyses to the study of biological signaling networks, it has yet to be used in toxicology, and specifically has never been applied to dose.

In this study, a graph theory-based approach was used to analyze MAPK networks from a toxicological perspective in human hepatocellular-carcinoma derived cells (HepG2) exposed to increasing doses of the mitochondrial inhibitor deguelin.³³ Graph theory was used to calculate centrality parameters describing the networked protein response at each dose based on the levels of phosphorylation of 8 proteins (ERK1/2, JNK, p38, AKT, HSP27, I κ B α , p53, and p90RSK) related to mitochondrial stress. Euclidean distances between pair wise protein responses were used as weighted edge values for graph theory analysis. Dose-response curves consisting of protein centrality parameters were constructed, and these curves were able to identify doses in which alterations in network signaling occur. The dose-response curves also allowed identification of shifts in individual protein centralities that accompany toxic exposures. The approach used in this study is advantageous in comparison to methods that investigate signaling via the compilation of responses observed across a time course, because it is capable of revealing network perturbations that are induced by individual doses. An additional advantage of this approach is that centrality parameters can be calculated based on weighted edge values rather than the binary existence or absence of an edge that is typical of network analyses, eliminating the need for set threshold values. Overall, this study demonstrates the utility of graph theory in assessing the effects of chemical stress on network signaling between MAPK pathways. Such network based assessments provide a quantitative means of assessing chemical exposures using dose-response parameters to describe perturbations.

EXPERIMENTAL PROCEDURES

Deguelin was obtained from Sigma Aldrich (St. Louis, MO). SB202190, and SB202474 were obtained from Calbiochem (San Diego, California). RPMI-1640, sodium pyruvate, HEPES, L-glutamine, fetal bovine serum, and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Bio-plex beads, lysis buffer, antibodies, and reagents necessary for determination of relative phosphorylation were obtained from Bio-rad (Hercules, CA).

Cell culture

HepG2 cells were cultured in RPMI-1640, supplemented with 1 mM sodium pyruvate, 5 mM HEPES, 2 mM L-Glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere at 37°C, 5% CO₂ and passaged at 80 % confluence.

Dosing

Cells were seeded into clear-bottom, black-sided 96-well plates at a density of 4×10^4 cells per well and allowed to grow for 24 h. Media was then aspirated from wells, and cells were challenged with varying concentrations of single and mixed compounds in fresh media. Deguelin was prepared in dimethyl sulfoxide (DMSO) to concentrations of 0.001 μ M to 100 μ M. SB202190 and SB202474 were prepared in DMSO to their manufacturer specified IC₅₀ concentrations of 350 nM and 34 nM, respectively.

MTT Viability Assay

After 400 min of exposure to single compounds or mixtures of compounds, cell viability was determined using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. This 400 min time point was chosen based on a previous study showing that changes in cellular oxygen consumption point to critical signaling events occurring at 400 min of exposure to deguelin³⁴. The MTT assay was performed according to the manufacturer's protocol. The assay is based on the reduction of tetrazolium MTT to colored formazan by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. Briefly, MTT reagent was added to the wells of the microplate, and after two hours of incubation at 37°C, intracellular formazan crystals were solubilized with the provided detergent solution. Absorbance values were obtained using the Safire2 microplate reader (Tecan US, Raleigh, NC) with a measurement wavelength of 570 nm and a reference wavelength of 700 nm, read from the bottom. From this, relative viability was determined by subtracting the absorbance of blank wells (consisting of cell culture media, the highest dose of the inhibitor being tested, all MTT reagents, but no cells) from all treatments and controls. We next determined the ratio of the blank-corrected absorbance values for wells containing treated cells relative to the wells containing controls (consisting of everything in the treated wells, except the inhibitor).

Bio-Plex Multiplex Immunoassay Methods

HepG2 cells were plated at 500,000 cells per well in 12-well tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) growth media (10% heat-inactivated FBS) supplemented with 0.1% fatty acid-free bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 24 h. Cells were then treated with increasing doses of deguelin (0.001, 0.01, 0.1, 1.0, 10, 100 μ M) in 1% DMSO alone, or in binary combination with the IC₅₀ concentrations of SB202190 (350nM) or SB20474 (34nM). After an incubation period of 400 min, cells were lysed with lysis buffer (Bio-Rad, Hercules, CA) supplemented with 500 μ M phenylmethanesulfonylfluoride (PMSF) (Sigma, St. Louis, MO) and phosphatase inhibitors (Bio-Rad, Hercules, CA). Total protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Beads containing antibodies against phosphorylated ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), AKT (Ser473), HSP27 (Ser78), I κ B α (Ser32/Ser36), JNK (Thr183/Tyr185), p38MAPK (Thr180/Tyr182), p53 (Ser15), and p90RSK (Thr359/Ser363) were obtained from Bio-Rad (Hercules, CA). Protein phosphorylation was quantified using the multiplexed bead-based assays and the Bio-Plex suspension array system according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Relative fluorescence values were calculated from the raw fluorescence levels after subtracting background (no cells) and normalizing to control cells receiving only 1% DMSO. All experiments were performed in duplicate and error bars reflect the standard error of the mean (SE).

Data Analysis

Two-way ANOVA's with Bonferroni's post tests were performed using GraphPad Prism version 5.03 for Windows, GraphPad Software (San Diego, California). Euclidean distances (E) were determined with SAS JMP (Carey, NC) using the equation

$$E(\nu, \omega) = \sqrt{\sum_{i=1}^n ((Relative\ Phosphorylation_{\nu_i}) - (Relative\ Phosphorylation_{\omega_i}))^2} \quad (1)$$

Where ν and ω represent the 2 protein nodes for which the distance between is being calculated, and n represents the number of replicates for which phosphorylation data was collected. All Euclidean distances were multiplied by 100 to facilitate network parameter

calculations. The node centrality parameter radiality was calculated using a modified version of the Cytoscape plug-in CentiScaPe.³⁵ The CentiScaPe plug-in was modified so that the parameter calculation used the Euclidean distance values as edge weights. Node centralities are complex topological parameters allowing quantitative local measurement of the position of a node relative to other nodes, and can be used to infer relative node importance in global network organization.³⁶ Thus, the centrality index calculation allows categorization of nodes in a network according to their specific regulatory relevance with respect to other nodes in a network. There was no set threshold value for the existence of an edge, and thus all distance values were used in the calculation of radiality. The node centrality parameter, radiality ($C_{rad}(v)$), is calculated using the equation

$$C_{rad}(v) = \frac{\sum_{\omega \in N} (\Delta_G + 1 - dist(v, \omega))}{n - 1} \quad (2)$$

where Δ_G is the network diameter (longest path length connecting any 2 nodes in the network), n is the number of nodes in the network, and $dist(v, \omega)$ is the shortest possible summation of Euclidean distance weighted edge lengths connecting node v to node ω . Δ_G represents the compactness of the network, and can be interpreted as a relative estimate of protein communication. To interpret centrality from $C_{rad}(v)$: if the radiality of a node, v , is high, then the node is generally close to all other nodes in the network, and therefore is considered central.³⁶ Radiality parameters were normalized to the average radiality values for all proteins in the network, making proteins with a radiality higher than 1, more central than average, and proteins with radiality lower than 1, less central than average.

RESULTS

Phosphorylation Response and Viability

The relative phosphorylation levels (normalized to control cells that received less than 1% DMSO) for ERK1/2, JNK, p38, AKT, HSP27, I κ B α , p53, and p90RSK were measured following 400 min exposure to increasing doses (0.001, 0.01, 0.1, 1, 10, and 100 μ M) of deguelin alone (Figure 2A). The most exaggerated response to treatment with deguelin alone was observed for p38 at the 10 μ M dose. At the 10 μ M dose of deguelin, p38 levels were 4.9 ± 0.1 times greater than controls. SB202190, a combination p38 and JNK inhibitor, was examined and the relative phosphorylation levels for the 8 proteins were determined in cells treated with its single IC₅₀ concentration of 350 nM (Figure 2B). SB202190 was chosen due to the availability of a negative control, SB202474. SB202474 is shown to exert the same effects as SB202190 on cellular signaling, except for inhibition of p38.^{37–39} The 350 nM dose of SB202190 reduced p38 phosphorylation to 0.61 ± 0.02 times that of control cells. Cells were exposed to the IC₅₀ dose of SB202474 (34 nM), and relative p38 phosphorylation levels were 1.2 ± 0.1 times that of control cells (Figure 2B). Two-way ANOVA comparing the 8 proteins relative phosphorylation responses to the IC₅₀ doses of SB202190 and SB202474 shows that the only statistical difference at $p < 0.01$ was for p38. The IC₅₀ doses of SB202190 and SB202474 decreased relative JNK phosphorylation to 0.416 ± 0.004 and 0.519 ± 0.004 , respectively. Relative I κ B α phosphorylation decreased to 0.328 ± 0.025 following exposure to the IC₅₀ dose of SB202190 and to 0.329 ± 0.001 following exposure to SB202474 at its IC₅₀ concentration.

To probe the network response to deguelin, cells were exposed to increasing doses of deguelin in combination with the 350 nM dose of SB202190, and relative protein phosphorylation was determined (Figure 2C). The increased p38 phosphorylation observed in response to deguelin alone was inhibited by SB202190 (Figure 2C) with the level of p38 phosphorylation at the 10 μ M dose of deguelin only reaching 1.1 ± 0.01 times that of control treated cells. Cells were then exposed to increasing doses of deguelin in combination with

34 nM SB202474, and relative protein phosphorylation was measured (Figure 2D). In cells treated with 10 μ M deguelin in combination with 34 nM SB202474, the measured level of phosphorylated p38 was 2.5 ± 0.1 times greater than control cells. Statistically significant differences in protein phosphorylation responses of cells treated with each inhibitor treatment are listed in Table 1.

Figure 3 depicts relative viability (normalized to control cells that received less than 1% DMSO) for cells treated with the inhibitors for 400 min. The only treatments that led to statistically significant ($p < 0.01$) changes in relative viability in comparison to controls were for cells treated with 34 nM SB202474 in combination with 10 or 100 μ M doses of deguelin. HepG2 relative viability was reduced to $62 \pm 3\%$ when treated with 10 μ M deguelin in combination with 34 nM SB202474 and $34 \pm 3\%$ when treated with 100 μ M deguelin in combination with 34 nM SB202474.

Euclidean Distances and Radiality

Deguelin Alone—To obtain network edge weight values for graph theory analysis, Euclidean distances between each pair of proteins were calculated based on their relative phosphorylation responses (normalized to control cells that received less than 1% DMSO) to each individual dose of deguelin. The Euclidean distance values (in order of shortest distance to longest) calculated based on the phosphorylation levels of proteins treated with increasing doses of deguelin alone are shown in Table S1 of the Supporting Information. A low distance value means that the protein responses to the specific conditions are similar, providing evidence of a connection or relationship between the two proteins. Conversely, a high distance value means that the protein responses are not similar, and that there is little evidence of a relationship between the two proteins. The distances between ERK1/2, p38, and JNK were all relatively high (all distances being greater than the average distance value) at the 0.001, 0.01, and 0.1 μ M doses of deguelin. At the 1.0 μ M dose of deguelin, the distance between p38 and ERK1/2 decreased to less than the average protein distance for all proteins, while the p38-JNK and JNK-ERK1/2 distances were well above average. At the 10 μ M dose of deguelin the distance between p38 and ERK1/2 increased and was much greater than average, while the distance between JNK and ERK1/2 decreased to below average. The p38-JNK distance remained high at the 10 μ M dose. At the 100 μ M dose of deguelin, the distance between p38 and ERK1/2 decreased to less than average; the JNK-ERK1/2 distance was near average; and the p38-JNK distance was above average. Three graphs depicting the characteristics of the different network responses to the 3 inhibitor treatments at the 10 μ M dose of deguelin are shown in Figure 4.

Normalized radiality parameters for p38, ERK1/2, and JNK are depicted in Figure 5. Radiality concisely describes each protein's simultaneous phosphorylation mediated relation to all other proteins in the network, and was calculated as described in the methods section (above). Fluctuations in radiality values indicate that the inhibitor treatment is causing the network relationships between signaling proteins to change. Radiality remains relatively constant in the dosing range of 0.001 to 0.1 μ M for JNK, p38, and ERK1/2 MAPKs, suggesting that these proteins are acting in separate, independent pathways at these doses. JNK radiality is lower than both p38 and ERK1/2 in the 0.001 to 0.1 μ M range. At doses of deguelin 1 μ M and above, the normalized radiality parameters for p38, ERK1/2, and JNK change: JNK radiality is increased while p38 and ERK1/2 radiality is decreased at the 1 μ M dose; JNK radiality remains high at the 10 and 100 μ M doses, while p38 radiality remains low at the 10 and 100 μ M doses. ERK1/2 radiality is increased at the 10 μ M dose to a level close to that of JNK, and is slightly decreased at the 100 μ M dose. The normalized radiality parameters for AKT, I κ B α , p53, p90RSK, and HSP27 remain relatively constant across the entire dosing range (see Figure S1 of Supporting Information for dose-response curves).

Deguelin in Combination with SB202190—Cells were treated with SB202190 in combination with deguelin to further probe the alterations in p38, ERK1/2, and JNK network centrality observed in response to treatment with deguelin alone, as our initial results show that SB202190 partially inhibits both p38 and JNK (Figure 2B). Table S2 of the Supporting Information shows the calculated distance values for each pair of proteins in response to treatment with deguelin in combination with SB202190. When cells were dosed with 350 nM SB202190 in combination with the lower doses of deguelin (0.001 – 0.1 μ M) the p38-ERK1/2, JNK-ERK1/2, and p38-JNK distances were shorter in comparison to when cells were treated with deguelin alone. At the 1 μ M dose of deguelin, the distance between p38 and JNK was the shortest distance between any of the 3 MAPKs (JNK, p38, and ERK1/2). At the 10 μ M dose the distances between all three MAPKs increased. At the 100 μ M dose of deguelin, the distance between p38 and ERK1/2 was much shorter in comparison to the JNK-ERK1/2 and p38-JNK distances.

Figure 5B shows the normalized radiality dose-response curve for cells treated with deguelin in combination with SB202190. The normalized radiality values observed for deguelin plus SB202190 were relatively constant at the lower doses of deguelin (0.001 – 0.1 μ M). ERK1/2 and JNK radiality values remained relatively constant (ERK1/2: 1.06–1.12; JNK: 1.17–1.20) from 0.001 to 1 μ M and decreased at 10 and 100 μ M. At 10 μ M deguelin ERK1/2 radiality was 0.32, and at 100 μ M it was 0.50. JNK radiality was 0.95 and 0.91 at the 10 and 100 μ M doses, respectively. p38 radiality remained relatively constant (0.82–0.94) from 0.001 to 0.1 μ M deguelin with values less than both ERK1/2 and JNK. At the 1 μ M dose of deguelin, p38 radiality increased to 1.17, a value greater than both ERK1/2 and JNK (1.06 and 1.15, respectively). At the 10 and 100 μ M doses of deguelin, p38 radiality was 1.04 and 0.56, respectively.

Deguelin in Combination with SB202474—Cells were treated with SB202474 in combination with deguelin to further probe the alterations in p38, ERK1/2, and JNK network centrality, as our initial results show that SB202474 partially inhibits JNK, but not p38 (Figure 2B). Table 3 of the Supporting Information shows the distance values for each pair of proteins in response to treatment with deguelin in combination with SB202474. The distances between p38 and ERK1/2 were shorter than the JNK-ERK1/2 and p38-JNK distances for the 0.001, 0.01, and 0.1 μ M doses. The p38-ERK1/2 distance increased dramatically at the 1 μ M dose of deguelin while the p38-JNK distance decreased dramatically. At the 1 μ M dose of deguelin, the p38-JNK distance much less than the p38-ERK1/2 distance and the JNK-ERK1/2 distance. At the 10 and 100 μ M doses, the distances between all three MAPK's were relatively high.

Figure 5C shows the normalized radiality dose-response curve for treatment with deguelin in combination with SB202474. There is little change in the radiality values for any signaling proteins for the 0.001, 0.01, and 0.1 μ M doses of deguelin (JNK: 1.13–1.18; ERK1/2: 1.07–1.08; p38: 0.94–0.99). JNK radiality remains relatively constant all the way up to the 10 μ M dose. At the 10 and 100 μ M doses of deguelin JNK radiality was 1.15 and 0.93, respectively. The 1 μ M dose of deguelin indicates a point of slight change for ERK1/2 and p38. At the 10 and 100 μ M doses of deguelin, ERK1/2 radiality decreased to 0.23 and 0.13, respectively. p38 radiality was 0.81 at the 10 μ M dose of deguelin and 1.11 at the 100 μ M dose. Overall, the radiality curves for deguelin in combination with SB202474 are similar to the radiality curves for deguelin in combination with SB202190. The curves do differ in the p38 and ERK1/2 radiality parameters at the 10 and 100 μ M doses of deguelin. This aligns with the viability curves for the 2 treatments, showing that treatment with deguelin in combination with SB202474 leads to significantly decreased ($p < 0.01$) viability at the 10 and 100 μ M dose of deguelin while treatment with deguelin in combination with SB202190 does not lead to significantly decreased viability at any doses tested.

DISCUSSION

With regard to toxicity testing, there is a current call to action for the modernization of approaches that are used to quantitatively assess the health risks associated with chemical exposure.^{1, 3, 40} Specifically, there is support for the integration of pathway based toxicity assays into current methods to provide a better understanding of how even low-dose exposures perturb biological processes in human cells and tissues.⁴ The major focus of this modernization has been on monitoring the upregulation of proteins in specific pre-defined pathways.^{1, 3–5, 40, 41} It is now understood that protein pathways are members of larger dynamic networks that are highly interconnected, and not always linear.²⁹ One way of integrating pathway-based methods of monitoring toxicity would be to incorporate quantitative graph theory parameters that describe the relevance or centrality of proteins within the network framework following chemical exposure. In the work presented here, a simple data-driven approach was used to obtain such parameters, describing MAPK signaling network perturbations that occur following chemically induced mitochondrial stress. Importantly, network parameters were assessed at each individual inhibitor dose, which allows for a clear association between dose and perturbation response.

Deguelin was chosen as the inhibitor of interest in this study due to its ability to induce mitochondrial stress, which is an important target for toxicity assays. Deguelin inhibits NADH:ubiquinone oxidoreductase (complex I of the electron transport chain), AKT, and HSP90, and exhibits anticancer activity.^{42–46} The dosing range of deguelin (0.001 to 100 μ M), to which HepG2 cells were exposed in this study, is comparable to the levels of deguelin found in the liver of mice following oral administration of doses that lead to significantly reduced tumor multiplicity, volume, and load.⁴⁷ Mitochondria have a significant impact on cellular physiology due to their roles in ATP production, reactive oxygen species (ROS) generation, Ca^{2+} homeostasis, and cell death.^{48, 49} Alterations in the signaling networks involved in the regulation of these mitochondrial functions have been implicated as contributing factors to many diseases including cancer.^{49–51} Further, mitochondria integrate a multitude of signaling events, contribute to cellular death via mitochondrial membrane permeability, and are common targets of both therapeutic and environmental xenobiotics.^{52–57}

The first step of any network analysis is to choose what defines network membership. For biological networks, all molecules within a cell or even within an entire organism could constitute one network. The chosen network boundaries depend on the specific application of the analysis. For this proof-of-concept study, we chose to define our network as 8 proteins that are related to both mitochondrial stress and cell death, and therefore are relevant when studying the toxicological effects of deguelin. Euclidean distances between relative protein phosphorylation levels were used to describe relations between signaling proteins following exposure to increasing doses of deguelin (independent of time). The Euclidean distance is a simple metric for describing similar responses when analyzing biological networks.^{21, 58, 59} Graph theory was applied to the Euclidean distances to obtain the protein node centrality parameter, radiality, and describe the relationship between the traditionally distinct (i.e. linear) ERK1/2, p38, and JNK MAPK pathways. Dose-response curves were constructed for each protein where radiality was the response (Figure 5). Radiality is a centrality parameter that was originally developed by Valente and Foreman in 1998 for the analysis of social networks.⁶⁰ Centrality parameters can be interpreted in various ways, and the interpretation often depends on the specific application of the analysis.⁶¹ Radiality as calculated here, concisely and simultaneously describes each protein's phosphorylation mediated relation to all other proteins in the network. Mechanistically, this means that a protein/node with low radiality is not highly integrated within the network, and is not central to network

communication. Therefore the other proteins are more central to this network's response for a given exposure condition.

The graph theory results provided an integrated view of how low doses of deguelin perturb normal MAPK signaling, and show that between the 0.1 and 1 μM doses of deguelin, there exists a likely threshold dose where perturbation occurs. This is easily visualized by the dose-response curves for p38, ERK1/2, and JNK radiality following exposure to deguelin (Figure 5A). The MAPK centralities remain relatively constant for doses lower than 1 μM , but at doses 1 μM and higher there is a switch where JNK radiality becomes higher, p38 radiality decreases, and ERK1/2 radiality varies. The normalized radiality values at low doses are indicative of distinct, linear responses, while the normalized radiality responses at higher doses may be representative of a response that has crossed a threshold that leads to changes in the relationships between signaling proteins. The observed trends in the dose-response radiality curves provide “snap shots” of signaling pathway alterations that occur when cells perceive that an applied stress has reached a level in which a change in the mechanism of response to the stress is necessary. For all proteins other than ERK1/2, JNK, and p38, radiality remained relatively constant across the entire dosing range (Figure S1), thus supporting the concept of this type of analysis for the identification of perturbations to normal network signaling. It is important to note that none of the doses of deguelin tested significantly reduced HepG2 viability after 400 min of exposure (Figure 3A). Exposure to deguelin for 24 h at doses higher than 1 μM does however lead to significantly decreased HepG2 viability (data not shown). Therefore, the analysis of the 400 min data revealed alterations in network signaling induced by deguelin at a point where toxicity as determined by the MTT was not yet observed. This demonstrates the utility of such data driven methods for assessing biological effects on a dose-to-dose basis, and determining significant doses capable of altering network signaling before viability is compromised. The critical doses identified in this study may capture points where cells are teetering between initiating death and promoting survival, and the application of graph theory helps to illuminate shifts in the balance between MAPK pathways.

In this study, SB202190 and SB202474 were selected as secondary inhibitors (dosed in combination with deguelin) to further investigate the balance between the MAPK signaling pathways following chemically induced stress. We demonstrated that SB202190 and SB202474 exert the same effects on all of the proteins studied, except p38 (Figure 3B). Both compounds inhibit JNK, thus allowing comparison of partial JNK inhibition to partial JNK and p38 inhibition during deguelin exposure. Comparison of the Euclidean distances and radiality parameters obtained for cells treated with deguelin in combination with SB202190 (Table S2 of Supporting Information) to cells treated with deguelin in combination with SB202474 (Table S3 of Supporting Information) show similar results up to the 1 μM dose of deguelin. Under both sets of conditions JNK radiality remains relatively high across the entire dosing range (Figure 5A and B). At the 10 and 100 μM doses of deguelin, partial inhibition of p38 by SB202190 leads to a decrease in p38 radiality while SB202474 does not have this same effect. ERK1/2 radiality decreases for both sets of conditions at these doses, but more so with deguelin in combination with SB202474. The only significant decreases in viability observed for any of the treatments in this study were observed for the 10 and 100 μM doses of deguelin in combination with SB202474 (Figure 3A). Comparison of the dose-response radiality curves for all three treatments (Figure 5) shows that the unique feature for deguelin in combination with SB202474 is that ERK1/2 radiality is extremely low (effectively removed from the network) while p38 radiality is high. Treatment with deguelin alone, or in combination with SB202190, leads to decreased radiality for p38 and comparatively higher radiality values for ERK1/2. This indicates that for cellular viability to be decreased in the cells tested, both p38 and JNK must maintain a central role within the network, while ERK1/2's centrality is simultaneously diminished. ERK1/2 has previously

been shown to promote survival under conditions of stress when apoptosis is not favorable.^{62–64} When apoptosis is favorable, both JNK and p38 signaling suppress the survival promoting activity of ERK1/2 via crosstalk, thereby inducing apoptosis.^{13, 65–68} The dynamic balance between these three pathways has been shown to be a key determinant as to whether cells survive a threat or die.^{11, 13, 67} Specifically, the sustained activation of JNK and p38 signaling along with ERK1/2 pathway inhibition has been shown to be required for the induction of apoptosis,⁶⁷ and the results of this study clearly support this notion. In addition to allowing the visualization of p38, ERK1/2 and JNK signaling dynamics, the analysis presented also captured the p53 response to stress; p53 radiality increases in a dose dependent manner in cells exposed to deguelin in combination with SB202190 or SB202474 (Figure S1). It is well known that p53 is activated following a variety of cellular stresses playing a pivotal role in the stress response,⁶⁹ which provides further validity to the proposed analysis approach to characterize networked cellular responses.

In agreement with previous studies, our observations show that crosstalk between p38, ERK1/2, and JNK signaling pathways is a complex and dynamic process that is difficult to capture.^{13, 15, 70, 71} The approach presented here was able to extract information reflecting the dynamic nature of MAPK signaling and the central influence that the balance between ERK1/2, JNK, and p38 signaling crosstalk has on whether or not cells maintain viability following chemical insult. Simple inspection of the relative phosphorylation responses to each set of inhibitor treatments in Figure 2, as well as the significant differences between these responses in Table 1, cannot provide such an integrated description, thus demonstrating the utility of graph theory based methods for assessing toxicological actions more broadly by reflecting a networked response. The work presented here is well suited for identifying xenobiotic doses that induce changes in protein networks before typical apical toxicological endpoints are reached. The calculated graph theory parameters provide an integrated way of describing the degree of perturbation induced by specific doses of toxic compounds in an *in vitro* human model by providing an informative and sensitive means of assessing chemical effects on biological systems. These types of approaches have the ability to highlight low-dose exposure induced changes at the molecular level, and may provide an enhanced understanding of toxicological actions. In the future, the method presented in this study could be expanded to include multiple exposure times, in addition to multiple doses. The analysis of network changes over a sequential time series could also provide a means to monitor network responses to xenobiotic exposures that contribute to adaptation. This, along with an increased number of protein targets could provide a rich depiction of the cells response to xenobiotic exposure, and represents a potential enhancement to current methods used to study toxic mechanisms of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING SOURCES

This work was supported by the Johns Hopkins University Applied Physics Laboratory Independent Research and Development Program and the Defense Advanced Research Projects Agency (DARPA), NIH IDeA Networks of Biomedical Research Excellence (INBRE), U.S. Army Research Laboratory, and the U.S. Army Research Office (W911NF-12-1-0165).

ABBREVIATIONS

MAPK	mitogen-activated protein kinase
HepG2	human hepatocellular-carcinoma derived cells
DMSO	dimethyl sulfoxide
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
FBS	fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
BSA	Bovine Serum Albumin
PMSF	phenylmethanesulfonylfluoride

References

1. Collins FS, Gray GM, Bucher JR. Toxicology. Transforming environmental health protection. *Science*. 2008; 319:906–907. [PubMed: 18276874]
2. Andersen ME, Dennison JE. Mechanistic approaches for mixture risk assessments—present capabilities with simple mixtures and future directions. *Environ. Toxicol. Pharmacol.* 2004; 16:1–11. [PubMed: 21782689]
3. Bhattacharya S, Zhang Q, Carmichael PL, Boekelheide K, Andersen ME. Toxicity testing in the 21st century: defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. *PLoS One*. 2011; 6:e20887. [PubMed: 21701582]
4. Judson RS, Kavlock RJ, Setzer RW, Hubal EA, Martin MT, Knudsen TB, Houck KA, Thomas RS, Wetmore BA, Dix DJ. Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chem. Res. Toxicol.* 2011; 24:451–462. [PubMed: 21384849]
5. Simmons SO, Fan CY, Ramabhadran R. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. *Toxicol. Sci.* 2009; 111:202–225. [PubMed: 19567883]
6. Kultz D. Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.* 2005; 67:225–257. [PubMed: 15709958]
7. Reif DM, Martin MT, Tan SW, Houck KA, Judson RS, Richard AM, Knudsen TB, Dix DJ, Kavlock RJ. Endocrine profiling and prioritization of environmental chemicals using ToxCast data. *Environ. Health Perspect.* 2010; 118:1714–1720. [PubMed: 20826373]
8. Kong AN, Yu R, Lei W, Mandlekar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis. The role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. *Restor. Neurol. Neurosci.* 1998; 12:63–70. [PubMed: 12671299]
9. Kong AN, Yu R, Chen C, Mandlekar S, Primiano T. Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. *Arch. Pharm. Res.* 2000; 23:1–16. [PubMed: 10728649]
10. Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. *Biochem. Pharmacol.* 2002; 64:765–770. [PubMed: 12213568]
11. Matsuzawa A, Ichijo H. Stress-responsive protein kinases in redox-regulated apoptosis signaling. *Antioxid. Redox. Signal.* 2005; 7:472–481. [PubMed: 15706095]
12. Cargnello M, Roux PP. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiol. Mol. Biol. Rev.* 2011; 75:50–83. [PubMed: 21372320]
13. Junttila MR, Li S-P, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB. J.* 2008; 22:954–965. [PubMed: 18039929]
14. Singh RP, Dhawan P, Golden C, Kapoor GS, Mehta KD. One-way cross-talk between p38(MAPK) and p42/44(MAPK). Inhibition of p38(MAPK) induces low density lipoprotein receptor expression through activation of the p42/44(MAPK) cascade. *J. Biol. Chem.* 1999; 274:19593–19600. [PubMed: 10391894]

15. Shen YH, Godlewski J, Zhu J, Sathyanarayana P, Leaner V, Birrer MJ, Rana A, Tzivion G. Cross-talk between JNK/SAPK and ERK/MAPK pathways: sustained activation of JNK blocks ERK activation by mitogenic factors. *J. Biol. Chem.* 2003; 278:26715–26721. [PubMed: 12738796]
16. Xiao YQ, Malcolm K, Worthen GS, Gardai S, Schiemann WP, Fadok VA, Bratton DL, Henson PM. Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *J. Biol. Chem.* 2002; 277:14884–14893. [PubMed: 11842088]
17. Noselli S, Perrimon N. Signal transduction. Are there close encounters between signaling pathways? *Science.* 2000; 290:68–69. [PubMed: 11183153]
18. Zhang H, Shi X, Hampong M, Blanis L, Pelech S. Stress-induced inhibition of ERK1 and ERK2 by direct interaction with p38 MAP kinase. *J. Biol. Chem.* 2001; 276:6905–6908. [PubMed: 11238443]
19. Wang Z, Yang H, Tachado SD, Capó-Aponte JE, Bildin VN, Koziel H, Reinach PS. Phosphatase-Mediated Crosstalk Control of ERK and p38 MAPK Signaling in Corneal Epithelial Cells. *Invest. Ophthalmol. Vis. Sci.* 2006; 47:5267–5275. [PubMed: 17122112]
20. Albert R, Barabasi A-L. Statistical mechanics of complex networks. *Rev. Mod. Phys.* 2002; 74:47–97.
21. Pavlopoulos G, Secrier M, Moschopoulos C, Soldatos T, Kossida S, Aerts J, Schneider R, Bagos P. Using graph theory to analyze biological networks. *BioData Min.* 2011; 4:10. [PubMed: 21527005]
22. Newman MEJ. The Structure and Function of Complex Networks. *SIAM Rev.* 2003; 45:167–256.
23. Christensen C, Albert R. Using Graph Concepts to Understand the Organization of Complex Systems. *Int. J. Bifurcation Chaos.* 2007; 17:2201–2214.
24. Bollobas, B. *Graph Theory: An Introductory Course.* NY: Springer-Verlag; 1979.
25. Boccaletti S, Latora V, Moreno Y, Chavez M, Hwang DU. Complex networks: Structure and dynamics. *Phys. Rep.* 2006; 424:175–308.
26. Jeong H, Tombor B, Albert R, Oltvai ZN, Barabasi AL. The large-scale organization of metabolic networks. *Nature.* 2000; 407:651–654. [PubMed: 11034217]
27. Grindrod P, Kibble M. Review of uses of network and graph theory concepts within proteomics. *Expert Rev. Proteomics.* 2004; 1:229–238. [PubMed: 15966817]
28. Barabasi AL, Oltvai ZN. Network biology: understanding the cell's functional organization. *Nat. Rev. Genet.* 2004; 5:101–113. [PubMed: 14735121]
29. Albert R. Scale-free networks in cell biology. *J. Cell Sci.* 2005; 118:4947–4957. [PubMed: 16254242]
30. Huber W, Carey VJ, Long L, Falcon S, Gentleman R. Graphs in molecular biology. *BMC Bioinf.* 2007; 8:S8.
31. Koschutski D, Schreiber F. Centrality Analysis Methods for Biological Networks and Their Application to Gene Regulatory Networks. *Gene Regul. Syst. Biol.* 2008; 2:193–201.
32. Ma'ayan A. Insights into the Organization of Biochemical Regulatory Networks Using Graph Theory Analyses. *J. Biol. Chem.* 2009; 284:5451–5455. [PubMed: 18940806]
33. Kim WY, Chang DJ, Hennessy B, Kang HJ, Yoo J, Han SH, Kim YS, Park HJ, Seo SY, Mills G, Kim KW, Hong WK, Suh YG, Lee HY. A novel derivative of the natural agent deguelin for cancer chemoprevention and therapy. *Cancer Prev. Res. (Phila).* 2008; 1:577–587. [PubMed: 19139008]
34. Boyd J, Vrana JA, Williams HN. In vitro approach to predict post-translational phosphorylation response to mixtures. *Toxicology.* 2012
35. Scardoni G, Petherlini M, Laudanna C. Analyzing biological network parameters with CentiScaPe. *Bioinformatics.* 2009; 25:2857–2859. [PubMed: 19729372]
36. Scardoni, G.; Laudanna, C. Centralities Based Analysis of Complex Networks. In: Zhang, Y., editor. *New Frontiers in Graph Theory.* 2012. InTech.
37. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Keys JR, Land vatter SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher SM, Livi GP,

- White JR, Adams JL, Young PR. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 1994; 372:739–746. [PubMed: 7997261]
38. Ho AK, Price L, Mackova M, Chik CL. Potentiation of cyclic AMP and cyclic GMP accumulation by p38 mitogen-activated protein kinase (p38MAPK) inhibitors in rat pinealocytes. *Biochem. Pharmacol.* 2001; 62:1605–1611. [PubMed: 11755113]
39. Karahashi H, Nagata K, Ishii K, Amano F. A selective inhibitor of p38 MAP kinase, SB202190, induced apoptotic cell death of a lipopolysaccharide-treated macrophage-like cell line, J774.1. *Biochim. Biophys. Acta.* 2000; 1502:207–223. [PubMed: 11040446]
40. Krewski D, Acosta D Jr, Andersen M, Anderson H, Bailar JC 3rd, Boekelheide K, Brent R, Charnley G, Cheung VG, Green S Jr, Kelsey KT, Kerkvliet NI, Li AA, McCray L, Meyer O, Patterson RD, Pennie W, Scala RA, Solomon GM, Stephens M, Yager J, Zeise L. Toxicity testing in the 21st century: a vision and a strategy. *J. Toxicol. Environ. Health B Crit. Rev.* 2010; 13:51–138. [PubMed: 20574894]
41. NRC. Toxicity Testing in the 21st Century: A Vision and a Strategy. Washington, DC: 2007.
42. Okun JG, Lümnen P, Brandt U. Three Classes of Inhibitors Share a Common Binding Domain in Mitochondrial Complex I (NADH:Ubiquinone Oxidoreductase). *J. Biol. Chem.* 1999; 274:2625–2630. [PubMed: 9915790]
43. Murai M, Ishihara A, Nishioka T, Takao Yagi a, Miyoshi H. The ND1 Subunit Constructs the Inhibitor Binding Domain in Bovine Heart Mitochondrial Complex I. *Biochemistry.* 2007; 46:6409–6416. [PubMed: 17474759]
44. Schuler F, Casida JE. Functional coupling of PSST and ND1 subunits in NADH:ubiquinone oxidoreductase established by photoaffinity labeling. *Biochim. Biophys. Acta.* 2001; 1506:79–87. [PubMed: 11418099]
45. Degli Esposti M. Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim. Biophys. Acta.* 1998; 1364:222–235. [PubMed: 9593904]
46. Degli Esposti M, Ghelli A, Ratta M, Cortes D, Estornell E. Natural substances (acetogenins) from the family Annonaceae are powerful inhibitors of mitochondrial NADH dehydrogenase (Complex I). *Biochem. J.* 1994; 301:161–167. [PubMed: 8037664]
47. Lee HY, Oh SH, Woo JK, Kim WY, Van Pelt CS, Price RE, Cody D, Tran H, Pezzuto JM, Moriarty RM, Hong WK. Chemopreventive effects of deguelin, a novel Akt inhibitor, on tobacco-induced lung tumorigenesis. *J. Natl. Cancer Inst.* 2005; 97:1695–1699. [PubMed: 16288123]
48. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *Biochem. J.* 2011; 435:297–312. [PubMed: 21726199]
49. Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? *Trends Cell Biol.* 2008; 18:165–173. [PubMed: 18296052]
50. Modica-Napolitano JS, Singh KK. Mitochondria as targets for detection and treatment of cancer. *Expert Rev. Mol. Med.* 2002; 4:1–19. [PubMed: 14987393]
51. Kroemer G. Mitochondria in cancer. *Oncogene.* 2006; 25:4630–4632. [PubMed: 16892077]
52. Boyd J, Saksena A, Patrone JB, Williams HN, Boggs N, Le H, Theodore M. Exploring the boundaries of additivity: mixtures of NADH: quinone oxidoreductase inhibitors. *Chem. Res. Toxicol.* 2011; 24:1242–1250. [PubMed: 21749117]
53. Schmidt CW. Unraveling Environmental Effects on Mitochondria. *Environ. Health Perspect.* 2010; 118:A292–A297.
54. Scatena R, Bottoni P, Botta G, Martorana GE, Giardina B. The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. *Am. J. Physiol. Cell Physiol.* 2007; 293:C12–C21. [PubMed: 17475665]
55. Sherer TB, Richardson JR, Testa CM, Seo BB, Panov AV, Yagi T, Matsuno-Yagi A, Miller GW, Greenamyre JT. Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. *J. Neurochem.* 2007; 100:1469–1479. [PubMed: 17241123]
56. Green DR, Kroemer G. The Pathophysiology of Mitochondrial Cell Death. *Science.* 2004; 305:626–629. [PubMed: 15286356]
57. Wallace KB, Starkov AA. Mitochondrial targets of drug toxicity. *Annu. Rev. Pharmacol. Toxicol.* 2000; 40:353–388. [PubMed: 10836141]

58. Pržulj N. Biological network comparison using graphlet degree distribution. *Bioinformatics*. 2007; 23:e177–e183. [PubMed: 17237089]
59. Pržulj N, Corneil DG, Jurisica I. Modeling interactome: scale-free or geometric? *Bioinformatics*. 2004; 20:3508–3515. [PubMed: 15284103]
60. Valente TW, Foreman RK. Integration and radiality: Measuring the extent of an individual's connectedness and reachability in a network. *Social Networks*. 1998; 20:89–105.
61. Borgatti SP, Everett MG. A Graph-theoretic perspective on centrality. *Social Networks*. 2006; 28:466–484.
62. Blank JL, Gerwins P, Elliott EM, Sather S, Johnson GL. Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. *J. Biol. Chem*. 1996; 271:5361–5368. [PubMed: 8621389]
63. Karandikar M, Xu S, Cobb MH. MEKK1 binds raf-1 and the ERK2 cascade components. *J. Biol. Chem*. 2000; 275:40120–40127. [PubMed: 10969079]
64. Waetzig V, Herdegen T. MEKK1 controls neurite regrowth after experimental injury by balancing ERK1/2 and JNK2 signaling. *Mol. Cell. Neurosci*. 2005; 30:67–78. [PubMed: 16006144]
65. Junttila MR, Ala-aho R, Jokilehto T, Peltonen J, Kallajoki M, Grenman R, Jaakkola P, Westermarck J. p38alpha and p38delta mitogen-activated protein kinase isoforms regulate invasion and growth of head and neck squamous carcinoma cells. *Oncogene*. 2007; 26:5267–5279. [PubMed: 17334397]
66. Li SP, Junttila MR, Han J, Kahari VM, Westermarck J. p38 Mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogenactivated protein/extracellular signal-regulated kinase kinase1,2. *Cancer. Res*. 2003; 63:3473–3477. [PubMed: 12839928]
67. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*. 1995; 270:1326–1331. [PubMed: 7481820]
68. Liu Q, Hofmann PA. Protein phosphatase 2A-mediated cross-talk between p38 MAPK and ERK in apoptosis of cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol*. 2004; 286:H2204–H2212. [PubMed: 14962831]
69. Murray-Zmijewski F, Slee EA, Lu X. A complex barcode underlies the heterogeneous response of p53 to stress. *Nat. Rev. Mol. Cell Biol*. 2008; 9:702–712. [PubMed: 18719709]
70. Black EJ, Walker M, Clark W, MacLaren A, Gillespie DA. Cell transformation by v-Jun deactivates ERK MAP kinase signalling. *Oncogene*. 2002; 21:6540–6548. [PubMed: 12226757]
71. Friedman A, Perrimon N. A functional RNAi screen for regulators of receptor tyrosine kinase and ERK signalling. *Nature*. 2006; 444:230–234. [PubMed: 17086199]

Figure 1. Modular organization of MAPK signaling pathways. Modified version republished with permission of Federation of American Societies For Experimental Biology, from Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival, Melissa R. Junttila, Song-Ping Lie, and Jukka Westermarck, 22, 2008; permission conveyed through Copyright Clearance Center, Inc.

Figure 2.

Phosphorylation response to inhibitor treatments. HepG2 cells were exposed to inhibitor treatments for 400 min. Shown are the phosphorylation levels relative to the vehicle control (1% DMSO) as determined by multi-plexed bead immunoassay. Observed data points represent the average of two replicates. Error bars represent the standard error of the mean. Figure A shows the phosphorylation response to increasing doses of deguelin alone. Figure B shows the phosphorylation response to 34 nM SB202474 and the phosphorylation response to 350 nM SB202190. Bars marked with (–) on Figure B represent significantly different ($p < 0.01$) responses when comparing the response to SB202190 to the response to SB202474. Figure C shows the phosphorylation response to increasing doses of deguelin in combination with 350 nM SB202190. Figure D shows the phosphorylation response to increasing doses of deguelin in combination with 34 nM SB202474.

Figure 3.

Relative viability at 400 min as determined by the MTT assay. (A) HepG2 cells were treated with increasing doses of deguelin alone, increasing doses of deguelin in combination with a constant 350 nM dose of SB202190, or increasing doses of deguelin in combination with a constant 34 nM dose SB202474. Data are presented in terms of percent viability of the vehicle control (1% DMSO). Observed data points represent the average of three independent experiments. Error bars represent the standard error of the mean. Statistically significant ($p < 0.01$) changes in viability in comparison to control treated cells are marked with (*). (B) Box-and-whisker plots for cells treated with SB202190 at its single IC_{50} dose, 350 nM or SB202474 at its IC_{50} value, 34 nM. Whisker ends represent the minimum and maximum values.

Figure 4.

Network graphs following exposure to a 10 μ M deguelin dose of deguelin alone, in combination with SB202190, and in combination with SB202474. Edge color and thickness are scaled to represent the Euclidean distance between proteins. Red thick lines correspond to short distances and green thin lines correspond to long distances. Node colors are scaled to represent the calculated radiality for each node. Nodes with high radiality values are shown in red and nodes with low radiality values are shown in green.

Figure 5.

Normalized radiality dose-response curves for JNK, ERK1/2, and p38. Radiality parameters are normalized to the sum of radiality parameters for all measured proteins.

Table 1

Significant Differences in Phosphorylation Response^a

	Dose Deguelin (μM)						
	0.001	0.01	0.1	1	10	100	
Deguelin vs Deguelin + 350 nM SB202190							
AKT	***	***	***	***	***	***	
ERK1/2	**	**		***	**		
JNK	***	***	**		***	***	
p38				***	***	***	
HSP27		**			***	***	
IκBa	***	***	***	***	***	**	
p53							
p90RSK				***			
Deguelin vs Deguelin + 34 nM SB202474							
AKT	***	***	***	***	***	***	
ERK1/2	**			***	***	***	
JNK	**				***	***	
p38				***	***	***	
HSP27	***	***			***	***	
IκBa	***	***	***	***	***	***	
p53							
p90RSK			***	***	***	***	
Deguelin + 350 nM SB202190 vs Deguelin + 34 nM SB202474							
AKT	***	***	**				
ERK1/2					***	***	
JNK							
p38						***	
HSP27							

	Dose Deguelin (μM)					
	0.001	0.01	0.1	1	10	100
Deguelin vs Deguelin + 350 nM SB202190						
IκBα						
p53						
p90RSK	**			**	***	***

(a) Statistically significant differences in relative protein phosphorylation when comparing different inhibitor treatments.

(***) indicates $p < 0.001$,

(**) indicates $p < 0.01$,

(*) indicates $p < 0.05$.