UNIVERSITY OF CALIFORNIA, SAN DIEGO

PathInsight: A Novel Tool for Modeling Biomolecular Pathways

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

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The Thesis of Aarya Venkat is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2017

DEDICATION

To my father who came to America with nothing, yet ensured his children had everything.
To my mother who raised me to pursue what I wanted, not what my community expected.

EPIGRAPH

[The true chemical philosopher] sees man an atom amidst atoms fixed upon a point in space; and yet modifying the laws that are around him by understanding them; and gaining, as it were, a kind of dominion over time, and an empire in material space, and exerting on a scale infinitely small a power seeming a sort of shadow or reflection of a creative energy… animated by a spark of the divine mind.

Sir Humphry Davy

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ABSTRACT OF THE THESIS

PathInsight: A Novel Tool for Modeling Biomolecular Pathways

by

Aarya Venkat

Master of Science in Chemistry

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Professor Michael Gilson, Chair

Depicting biochemical relationships and predicting their consequences is an important facet of systems biology and pharmacology research. In particular, the ability to model the effects of small molecular binders in cell signaling pathways would be a useful as a tool to predict the effects of drugs or drug candidates.

PathInsight is a new Cytoscape tool whose aim is to simplify pathway complexity and provide a new method with which to model and analyze biological pathways. It models the effects of a molecular binder, such as a drug or naturally occurring protein ligand, downstream a pathway and annotates the affected proteins or genes with simple visuals indicating whether the downstream product has been activated, inhibited, or unaffected. Additionally, PathInsight includes support for Systems Biology Graphical Notation, further aiding in the comprehensibility of biological pathways.

Introduction

Visualizing and modeling biological pathways is an important capability for biomedical scientists seeking to understand a network's biochemical relationships (Arakawa et al., 2005). Pathway diagrams may be used as an overview for planning gene knockouts or predicting the effects of ligand interactions, or as teaching tools. Several pathway databases like Reactome and KEGG contain digital images or XML files of various biological pathways (Arakawa et al., 2005; Joshi-Tope et al., 2005). These pathways are fundamentally composed of nodes (proteins, drugs, or other compounds or biomolecules) and edges (connections between one node and another). While useful in understanding how individual nodes relate to one another, these diagrams do not dynamically represent the downstream effects (Gilman and Arkin, 2002) in a pathway when changing initial conditions or perturbing pathways, such as in the case of adding drug-like compounds or modeling mutations or knockouts of specific proteins. Programmatically predicting and modeling cause-and-effect in biological pathways is essential to biochemists and pharmacologists (Chindelevitch et al., 2012) seeking to comprehend the dynamic nature of biological pathways.

There are several quantitative approaches for analyzing pathways, including time-series analyses (Martini et al., 2014), fuzzy logic algorithms (Terfve et al., 2012), and Bayesian algorithms (Isci et al., 2011), and such methods are implemented in a few programs. For example, Ingenuity Pathway Analysis (IPA) by Qiagen has a quantitative causal analysis tool (Krämer et al., 2014), which uses an existing dataset and perform a Fisher's Exact Test on each node to generate a scoring system that it

uses to predict the flow of the pathway. The issue with these quantitative methods is that they require pre-determined or experimental data with, and this data is not always available. Moreover, the IPA software requires a commercial license to use. Attempts have been made at open-source alternatives to IPA, such as SimBoolNet (Zheng et al., 2010) and Cytocopter (Terfve et al., 2012). SimBoolNet allows boolean values to propagate downstream, declaring nodes as activated or inhibited in given pathways. Cytocopter is an application based on the biostatistics package CellNOptR (Terfve et al., 2012) for the R programming language. It performs the same general function as SimBoolNet, but it uses quantitative fuzzy logic algorithms and can perform time-series analyses on given pathways, so long as an SBML file is presented. Unfortunately, both plugins are now obsolete, as they no longer work with the current Cytoscape 3 architecture.

Although such quantitative pathway modeling techniques have great potential utility, simply being able to track what components of a pathway are expected to be affected by a targeted perturbation, and what other components downstream will be affected in turn, can be invaluable for biomedical scientists. Although such a qualitative readout may not be as detailed as that provided by a quantitative model, it has far broader applicability, as it can be used even when there are insufficient data to perform quantitative analyses. Until a curated set of experimental interaction data can be provided for every pathway, there will be value in having a less quantitative method to analyze biological networks.

The present project advances the state of the art in modeling and interpreting pathways by introducing PathInsight, a novel plugin application for the network visualization tool Cytoscape (Shannon et al., 2003), which enables qualitative modeling of cause-and-effect relationships in biomolecular pathways. Cytoscape is a program for creating and modifying network relationships. The base program allows creative freedom in designing pathways and visualizing network relationships. Additionally, Cytoscape has a large variety of third-party plugins that perform a variety of design improvements, qualitative and quantitative analyses, and other generally useful functions. The goal of PathInsight is to equip Cytoscape users with a plugin tool to predict downstream consequences of pathway perturbations, due, for example, to activation or inhibition of a protein by a drug-like molecule. PathInsight gives users an easy user-interface to use for modeling such cause-and-effect relationships.

The key capabilities of PathInsight are as follows. Once the user has built or imported a pathway, he or she can set the background conditions for the pathway, including details like the presence of specific cytokines or hormones, then modify the activity level of one or more nodes to model the consequence of adding inhibitors and/or activators, and propagate the consequences through the pathway, visualizing how nodes downstream are affected by the perturbation(s). It is also worth noting that PathInsight allows pathways to be represented in Systems Biology Graphical Notation (SBGN) (Le Novère et al., 2009), a recent type of standardized graphical representation useful for consolidating pathway depictions into a single consistent and

recognizable format. The only other application that offers SBGN functionality in Cytoscape's App Store is CySBGN, which no longer works with the Cytoscape 3 architecture. PathInsight is therefore currently the only Cytoscape application to offer SBGN functionality.

PathInsight pairs well with the BindingDB (Liu et al., 2007) plugin for Cytoscape, a module that pulls protein-ligand binding data from the BindingDB website to enumerate which proteins in a network of interest may be targeted by drugs or drug-like compounds. This information may then be analyzed with PathInsight to predict possible consequences of adding specific compounds to the biological system.

Here I describe PathInsight and illustrate its application to the JAK signaling pathway, chosen for its clinical significance (Igaz et al., 2001). I consider perturbations to several different states of this pathway, where the state is determined by, for example, which hormones or cytokines are present at the time of the signaling cascade. By performing multiple cause-and-effect models, each of these states may be modeled and compared with each other. Visualizing these changes whether in comparing different pathway states or predicting the effects of a small molecule binder may be an important facet for system biology research.

Methods

Software Tool

The fundamental concept of PathInsight is as follows. Before the network of interest is perturbed, PathInsight considers all nodes to have some baseline level of activity. The user can then activate or inhibit one or more of these nodes, relative to this baseline. If a node is classified as activated or inhibited by the user, it gets a +1 or -1 value, respectively. The user may also classify edges as activating or inhibiting in character. The effects of any given node can then be propagated down the pathway. Summative values are added to each node based on whether a node immediately upstream gives an activating or inhibiting input to it. For example, if a node downstream is given an activating input, a +1 value is added to its baseline value. Similarly, if three nodes activate a single node, and one node inhibits the same node, its value then becomes +2. If it is not known whether a given edge is activating or inhibiting, then downstream nodes are not assigned a numerical value, but are instead marked as perturbed with a "?". Nodes downstream of a node marked "?" are similarly assigned "?", to indicate a perturbation of unknown character. Values are propagated by the PathInsight tree traversal algorithm, which is run in a series of steps, whose number is user-defined. In each step, any perturbations present propagate downstream by one edge to the next set of nodes. Carrying out multiple propagation steps shows the user the series of steps by which the initial perturbation spreads through the network.

PathInsight version 1.0.8 is a plug-in created with the Cytoscape 3 OSGi (Open Services Gateway Initiative) framework built on Java 8 for the Cytoscape 3.4

API. In addition to supporting the concepts of activation, inhibition, unknown perturbation, and propagation, PathInsight also includes tools to build and modify a network. In particular, users may easily create, modify, annotate, and model multiple nodes simultaneously in a network with the following tools available on its simple user interface: Node Label, Edge Label, SBGN, Node Analysis, Phosphorylation, Reset Values, and KEGG Prepare, as now described.

Node Label lets users begin preparing their networks for modeling by selecting a node, or multiple nodes, and clicking the nested menu items “Activated” or “Inhibited”, which annotate the node with a “1” or “-1” value respectively. Alternatively, users may select “Reset” to set a node as having a value of zero. These changes assign the starting perturbation of the baseline system, which will subsequently be propagated through the network.

Edge Label performs for edges functions analogous to those of “Node Label” for nodes, allowing an edge to be marked as Activating, Inhibiting, or Indeterminate. An Activating edge activates a node downstream if the upstream node is activated, and is represented by a line that end in a circle (Fig 1). Note that an Activating edge will, in effect, inhibit a node downstream, if its upstream node is inhibited. Similar considerations apply to an Inhibiting edge, which is represented by a line that ends with a perpendicular line Fig 1). An Indeterminate edge, represented by a diamond terminator, can only put its downstream node into an indeterminate state.

The **Systems Biology Graphical Notation (SBGN)** tool allows the user to quickly and precisely transform a selected network into SBGN format. For Cytoscape 3, there

are no other applications that allow the SBGN format, so users keen on using this well-developed notation will find this useful. This tool allows the user to designate nodes as simple chemicals, macromolecules, or genes, and to create process nodes that represent a process, such as translocation, transcription, or dimerization. This informative notation makes for a network description that is relatively easy to read and comprehend. Additional symbols have been added or changed within PathInsight to provide users a wider range of freedom in visualization, while enhancing pathway clarity. Fig. 1 outlines these changes with respect to the original notation.

Node Analysis executes a tree traversal algorithm performed in one, two, or N-steps, as selected by the user, once the user has finished designating Nodes and Edges with the Label tools above. The user selects how many steps he or she wishes to perform: one, two, or some larger number N. For one step, the algorithm seeks out neighbors of the nodes labeled as perturbed and annotates them with a numerical value or "?", indicating, respectively, activation, inhibition or indeterminate perturbation. If additional steps were requested, this process is iterated for the desired number of steps, each time modifying the states of the downstream neighbors of the nodes that were perturbed in the prior step. If a node receives, for example, two activating perturbations from two upstream nodes, the consequences will be summed to provide a count of the net degree of activation or inhibition. Figure 2 illustrates the step-wise propagation of a network perturbation in a simplified example network.

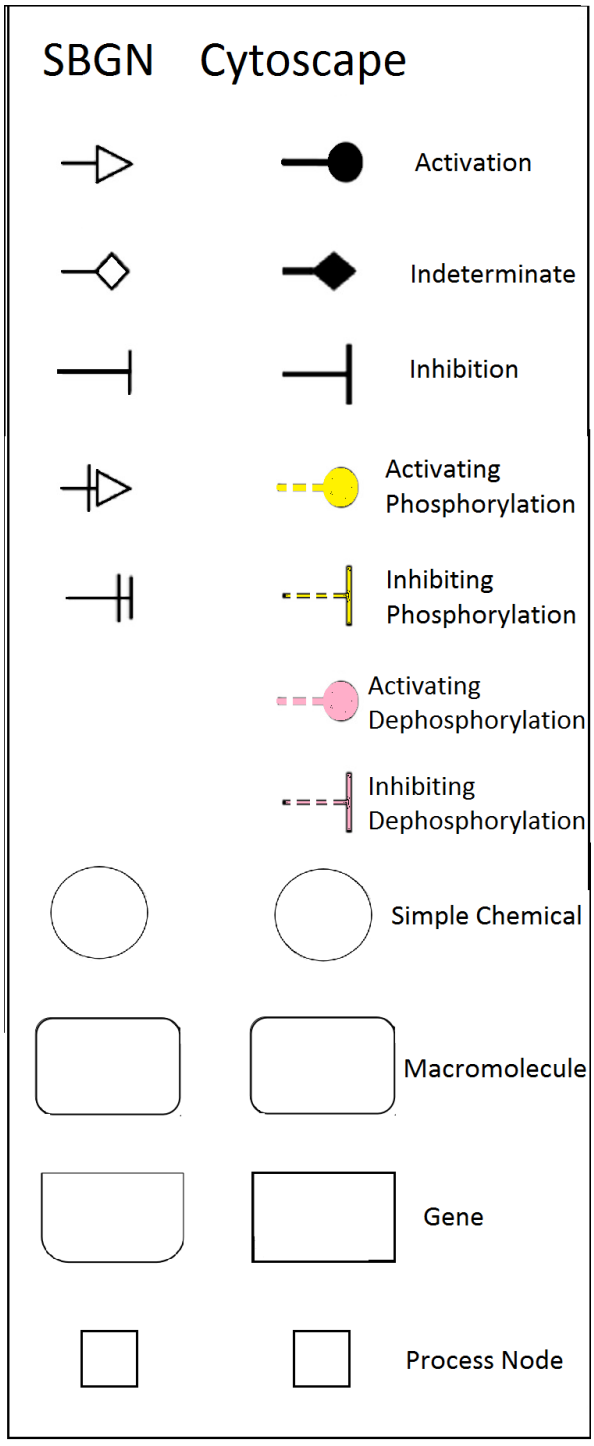
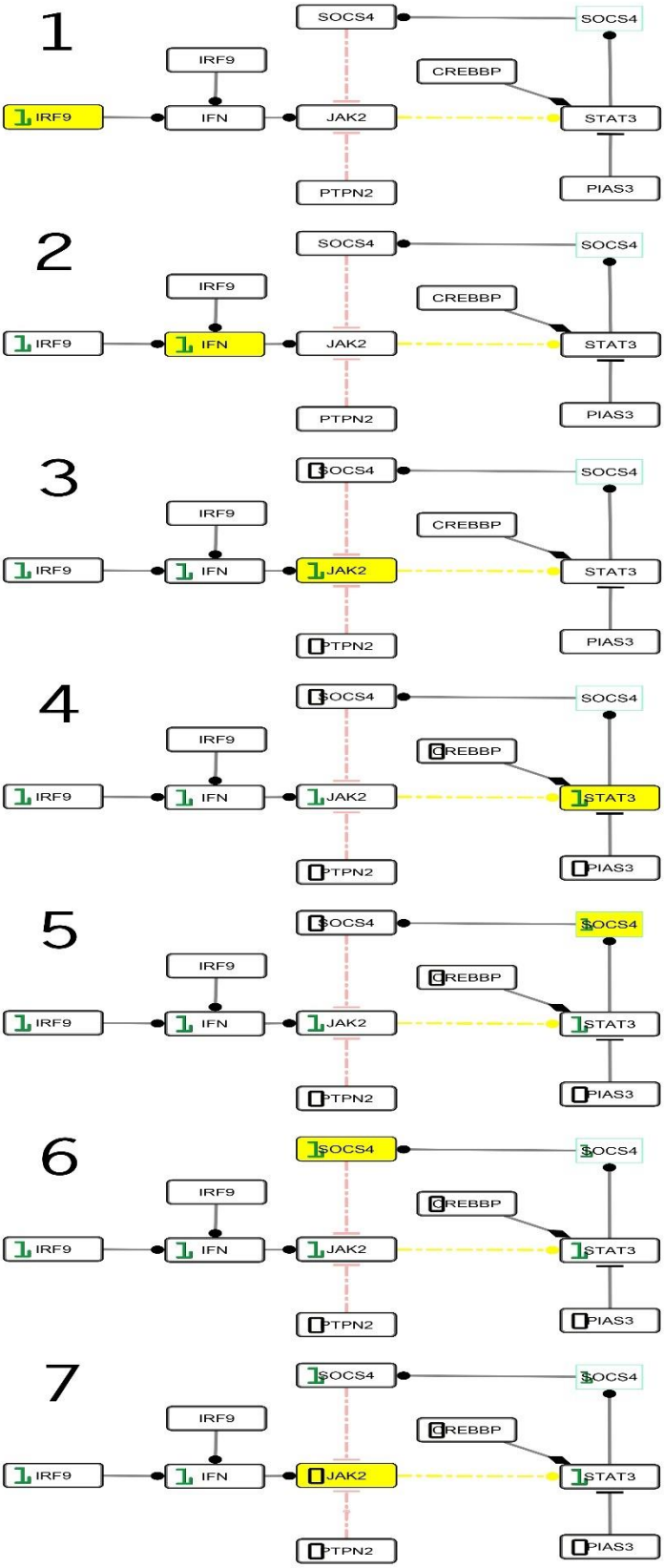


Figure 1: Legend for pathways in the following figures. Systems Biology Graphical Notation versus PathInsight's notation for each node and edge representation. A few additional symbols have been added for purposes of clarity.

Figure 2: States of a simple network before and following six successive steps of the PathInsight algorithm. State 1 is the initial state, where the interferon cytokine, IRF9, is activated, and in turn activates the Interferon receptor (IFN). In the subsequent steps (States 2-7), this activates JAK2, which activates STAT3 via phosphorylation. STAT3, uninhibited by the STAT regulator PIAS3, transcribes the SOCS4 gene, whose cognate protein dephosphorylates and inhibits JAK2 to stop further STAT3 phosphorylation, ending the loop. Changes in each step are highlighted in yellow. See Figure 1 for definitions of symbols.


1
SOCS4
SOCS4
IRF9
CREBBP
IRF9
IFN
JAK2
STAT3
PTPN2
PIAS3
2
SOCS4
SOCS4
IRF9
CREBBP
IRF9
IFN
JAK2
STAT3
PTPN2
PIAS3
3
SOCS4
SOCS4
IRF9
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PIAS3
7
SOCS4
SOCS4
IRF9
CREBBP
IRF9
IFN
JAK2
STAT3
PTPN2
PIAS3

KEGG Prepare automates much of the network preparation if a network is imported from the CyKEGGParser application, creating activating or inhibiting edges based on KEGG data, while **Phosphorylation** uses KEGG data to color these edges yellow or purple representing phosphorylation or dephosphorylation.

Illustrative Cases

I used the clinically important JAK pathway as a test case for PathInsight. JAK2 is a phosphorylating protein that can be bound to several types of membrane receptors; Interleukin, Interferon, and Erythropoietin (Epo) receptors are specifically covered in these cases. When one of these membrane proteins is bound by a cognate cytokine or hormone, including, respectively, interleukins, interferons, and Epo cytokines, it undergoes a conformational change activating an attached JAK2 dimer. Upon activation, JAK2 undergoes a conformational change that reveals phosphorylating domains(Babon et al., 2014; Feng et al., 1997) which phosphorylate a pair of STAT proteins; the specific STAT proteins phosphorylated depends on the cytokine that was initially bound(Igaz et al., 2001), such as Epo cytokines leading to STAT5 phosphorylation. Each of these STAT proteins may perform a variety of actions, including upregulating or downregulating transcription of apoptotic genes. In the first illustrative case, the Epo cytokine binds to the Epo receptor, activating JAK2 and phosphorylating proteins downstream in the JAK2 pathway. The proteins phosphorylated are STAT5, PI3KR5 and the GRB2 complex.

Additionally, the Cytoscape BindingDB plugin was used to annotate JAK2 with small molecules having significant affinity for this protein, based on its UniProt ID. Of these compounds, the drug Lestaurtinib was chosen and modeled as a demonstration of the multiple functions of PathInsight. Lestaurtinib is a drug that inhibits STAT5 dimer transcription, as well as the Akt/mTOR and Ras/Raf pathways, by inhibiting the activation of JAK2 upon ligand binding of the membrane receptor to which JAK2 is attached(Furumoto and Gadina, 2013). In the second illustrative case, PathInsight was used to model the effects of Lestaurtinib on the JAK2 pathway in the presence of Epo, and is hence compared with the real world effects of Lestaurtinib to determine the effectiveness and accuracy of PathInsight.

Results

Pathways were set up initially using KEGGParser, a Cytoscape application that loads pathways from the KEGG catalogue. KEGG only contains generic pathways, in that similar pathways are grouped together without any distinguishing features, thus delving into the literature was necessary to make specific pathways, particularly representing the effects of JAK2 on the STAT5, Ras/Raf, and Akt/mTOR pathways.

The JAK signaling pathway is a critical component of a variety of cellular functions and pathologies, including immunological responses, differentiation, proliferation, apoptosis, and oncogenesis. However, activation of this pathway produces different consequences, depending on the extracellular signals present. Here, PathInsight is used to model two cases. The first focuses on the action of erythropoietin (Epo) at its cognate Epo Receptor, which triggers JAK2 to phosphorylate STAT5, as well as activate the Ras/Raf and Akt/mTOR pathways, leading to gene transcription and regulation. The second case considers how this process is affected by the JAK2 inhibitor Lestaurtinib.

Case I: Figure 3 illustrates the effects of Epo binding to its receptor, where green "1"s indicate activated nodes predicted by PathInsight after propagation for six steps. Upon binding, the Epo receptor undergoes a conformational change, activating JAK2 and revealing JAK2's kinase domain, which phosphorylates STAT5a/b (Funakoshi-Tago et al., 2010, p. 5; Gilmour et al., 1995) . These STAT proteins form a heterodimer and upregulate members of the bcl-2 family, promoting cell survival. The STAT5 complex also is involved in a negative feedback loop: it induces transcription

of the CIS gene, leading to the eventual formation of a cytokine-inducible SH2 protein that inhibits STAT5a/b, by competitively binding to the JAK2 recruitment domains (Croker et al., 2008; Matsumoto et al., 1997), reducing further transcription of the bcl-2 family. PathInsight visualizes the effect of this negative feedback loop on the STAT5 dimer with a zero, as it is originally activated, as annotated at first with a green “1”, by JAK2 but then is inhibited downstream by the product of the CIS gene, changing the “1” to a “0”. Also phosphorylated by JAK2 are the SHP2/Grb complex, which leads into the Ras/Raf pathway promoting differentiation, and the PI3K protein necessary to promote the Akt/mTOR pathway involved in the transition from the G1 to S phase in the cell cycle. The upregulation of these pathways is represented by the green “1”. In this manner, the PathInsight plugin helps the user identify the qualitative downstream consequences of an upstream perturbation.

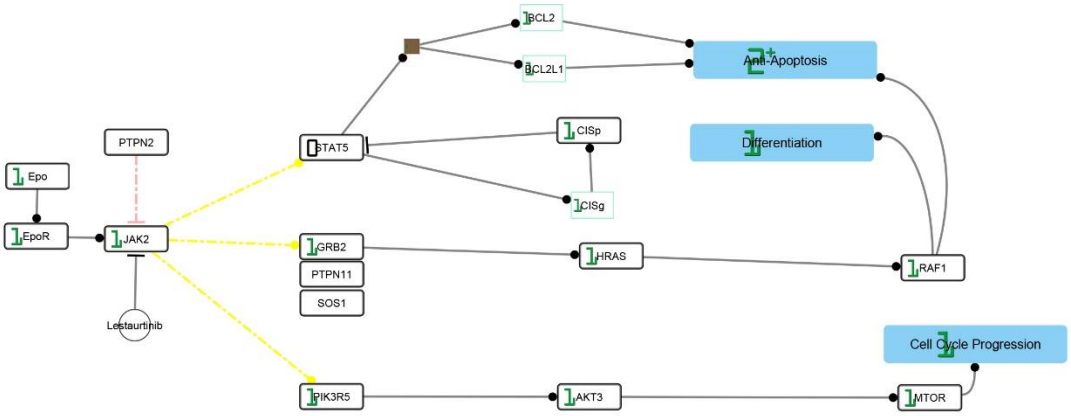

Figure 3. Illustration of Case I, Epo activation of the JAK2 pathway. The diagram illustrates the state of the network following six steps of the algorithm, upregulating anti-apoptosis genes, genes involved in the progression of the cell cycle, and genes involved in differentiation, all represented by the blue pathway nodes. Erythropoietin, which is annotated with a green "1" symbolizing activation, binds to the Epo receptor. Its value is propagated to the receptor, activating it, which undergoes a conformational change activating JAK2. JAK2 phosphorylates the PI3KR5 subunit, the GRB2 complex, and the STAT5 dimer, annotating each of these with a green "1". The STAT5 dimer activates and transcribes members of the bcl-2 family promoting cell survival, but STAT5 also transcribes the CIS gene, whose protein causes STAT5 inhibition, thus annotating STAT5 with a "0". The effects of the initial cytokine also lead to activation of the GRB2 complex, which activates the rest of the nodes in the RAS/RAF pathway, and PI3KR5, which activates Akt and MTOR, upregulating transcription of genes involved in the cell cycle.

Case II: Using the JAK2 UniProt accession ID, the BindingDB application was used to search and produce SMILES strings for all compounds capable of binding to JAK2. Of the compounds given, Lestaurtinib, a JAK inhibitor, was chosen as it has a high binding affinity and has experimentally proven inhibitory effects on STAT5 phosphorylation, thus reducing transcriptional activity of all nodes downstream (Gaikwad et al., 2007; Thiant et al., 2017). JAK inhibitors prevent JAK phosphorylation of STAT proteins by binding to the active site of the kinase domain (Furumoto and Gadina, 2013). In this case (Fig 4), Lestaurtinib binds to JAK2 and prevents the phosphorylation of STAT5a and STAT5b, despite the simultaneous binding of the Epo cytokine to JAK2's upstream receptor. By inhibiting JAK2, Lestaurtinib prevents everything downstream of JAK2 from being phosphorylated, and hence from being activated. The rest of the pathway is at an unperturbed level of activity. Assuming every node operates at some baseline, Lestaurtinib's binding to JAK2 prevents a signaling cascade from perturbing these nodes from their baseline.

It is worth noting that the precise, quantitative outcome, when both Epo and Lestaurtinib are present, is not certain, and will presumably depend in part of the concentrations of these two bioactive molecules. Thus, the PathInsight outputs should note be interpreted as implying zero change to the system. Nonetheless, the results with and without Lestaurtinib may be compared to understand how this compound is likely to modulate the effects of Epo.

The modeled effects of Lestaurtinib in PathInsight are reflected in the research of Dr. Elizabeth Hexler's team who found that Lestaurtinib's inhibition of JAK2

phosphorylation, in the context of Epo receptor activation, led to demonstrable reduction of phosphorylation, suppressing the STAT5, PI3-K/Akt, and Ras/Raf pathways (Hexner et al., 2008). Given the anti-apoptotic and cell survival focused nature of these pathways, mutations make them susceptible to tumors and hematopoietic disorders (Um and Lodish, 2006). By downregulating these pathways, Lestaurtinib reduces tumorigenesis and the effects of receptor or JAK2 mutations (Diaz et al., 2011; Hexner et al., 2008; Iyer et al., 2010).

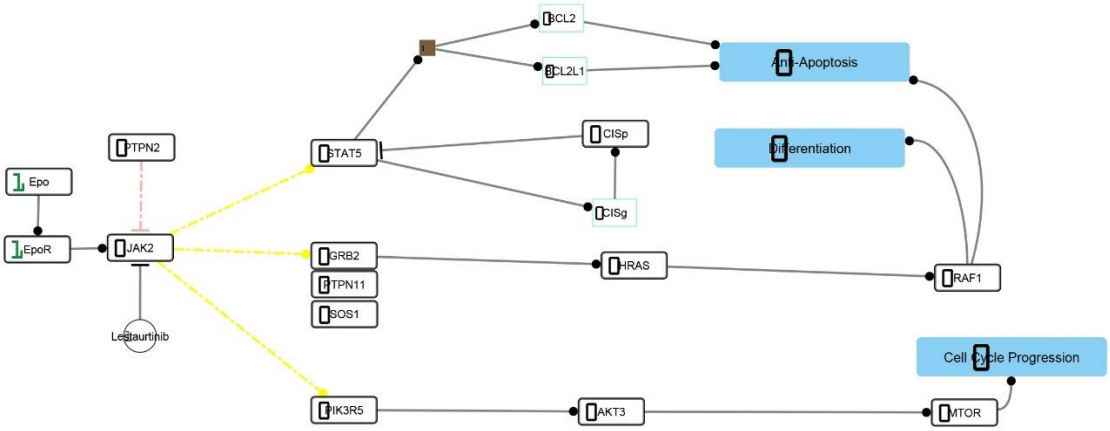

Figure 4. Illustration of Case II, the JAK2 pathway perturbed simultaneously by Epo and by the JAK inhibitor Lestaurtinib, and propagated for six steps. Both Lestuartinib and the Epo receptor were activated, visually represented on both of these nodes by the green "1". These values were propagated simultaneously, causing the effect of Epo receptor activation of JAK2 to be cancelled by JAK2, reducing the phosphorylation of all nodes downstream. STAT5, the GRB2 complex, and PI3KR5 are all not phosphorylated and further propagate zero values downstream, having neither activating nor inhibiting effects on proteins and genes regulating anti-apoptosis, differentiation, and cell cycle progression.

Discussion

Biomolecular systems are intricate and difficult to understand, and a variety of modeling and informatics tools have been created to assist in the visualization and interpretation of biological pathways. The first step in pathway and network comprehension was cataloguing pathways, achieved by organizations like KEGG, Reactome, and Wikipathways. The second step was visualizing these pathways and making them interactive and modifiable, accomplished by Cytoscape, Qiagen and a small number of other bioinformatics programs. Tertiary steps involve being able to input or derive meaningful data from the pathways, performed by many apps in the Cytoscape App Store, by Ingenuity Pathway Analysis, and several statistical programs.

In the scope of modeling the effects of biomolecules on pathways, both Ingenuity Pathway Analysis and the Cytoscape application Pathway Signal Flow Calculator (Nersisyan et al., 2015) can perform quantitative analyses to determine the effects of a perturbed pathway. While quantitative efforts in pathway analysis offer the possibility of high accuracy, they require data not often available for many pathways studied. Even when available, these datasets are sometimes incomplete, thus damaging the level of accuracy given by a quantitative tool. The lack of available quantitative data makes a qualitative tool like PathInsight a necessity when modeling complex pathways, as it provides an option to analyze and understand these pathways without requiring quantitative parameters. Removing this quantitative need may reduce the sophistication and accuracy of the pathway, but in doing so, it lowers the technological

and computational barrier required for scientists wanting to do pathway prediction and modeling. As demonstrated in the results, these pathways can resemble experimental evidence. Thus, PathInsight fills a niche role in the absence of quantitative data, as it is more generally applicable and can still be of great value in interpreting the implications of a pathway and how it may be perturbed.

PathInsight provides a novel capability to build or modify a biological pathway and model a variety of conditions for comparison. Science educators or medical students may take a healthy pathway and change certain nodes to understand the mechanism or molecular implications of some diseased state. PathInsight strives to improve the clarity of biological pathways by simplifying interpretations when modeling. As demonstrated in the illustrative cases considered here, the program can help users predict the downstream consequences of perturbations to a pathway. In combination with the BindingDB plugin, it supports a capability of identifying proteins that can be bound by known inhibitors, and then predicting the network consequences and thus potential effects and side-effects. It can also be used to model the consequences of inhibiting a protein with a potential new inhibitor, thus testing whether the protein could represent a useful drug target.

It is also worth noting that using PathInsight can pose challenges, because of the difficulty in correctly setting up a pathway of interest. Pathways acquired from KEGG or other sources are not always accurate, or may have been averaged, to represent the most common interactions in a pathway. Modifying these pathways to accurately reflect the literature relevant to a specific project is necessary to properly

model a pathway of interest. No other program is different in this regard. Whether one is using PathInsight or another software, building and modifying a pathway, based on available literature data, is a key step in ensuring that the model reflects the best current state of knowledge.

Future improvements to PathInsight may involve better algorithmic processing for loop calculations, so negative feedback loops, for example, will stop after a period, if the modeling is still ongoing. Additionally, it will be useful to modify the KEGG Prepare function to support pathway catalogues beyond KEGG, such as WikiPathways. Another function being added is improving drug discovery support by programmatically pulling UniProt accession IDs from the pathway allowing ligand binding data to be added via the BindingDB application. This allows binding activity data to be present while modeling the effects of small molecular binders. Finally, it may be worth exploring modeling the phenotypic consequences of molecular perturbations of key pathway components to create a holistic map of effects from gene to phenotype. With these changes, PathInsight will mature into a capable and useful plugin for research and education in pharmacology, systems biology, and biomedicine.

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