PERSPECTIVE

Exploiting kinase polypharmacology for nerve regeneration

The human central nervous system (CNS) has a markedly poor capacity for regenerating its axons following injury. This appears to be due to two main causes: 1) a developmentally regulated decline in regenerative capacity within mature CNS neurons, and 2) the presence of biological components that constitute barriers to axon regeneration (*e.g.*, growth-inhibitory molecules). Intrinsic alterations have been elucidated by studies that show causative links between developmental changes in gene expression programs and growth-signaling states on one hand, and changes in regenerative capacity on the other (Moore et al., 2009; Park et al., 2010). In addition to these neuron-intrinsic factors, several molecular species that are native to the CNS microenvironment, such as myelin associated proteins, and others that are secreted by injury-activated astrocytes, such as chondroitin sulfate proteoglycans (CSPGs), exert extrinsic inhibitory influences on growing axons (Young, 2014).

Given the various factors that negatively influence axon regrowth in the adult CNS, achieving clinically relevant regeneration to promote recovery from traumatic injury has been difficult. Experimental manipulation of individual inhibitory factors, or their mediators, has resulted in some improvement of regeneration/sprouting (Young, 2014). However, manipulations that simultaneously target intrinsic growth capacity while also blocking inhibition from extrinsic factors appear to produce the most pronounced effects *in vivo* (Lee et al., 2014). Development of effective therapies thus requires agents that simultaneously modulate multiple sources of regeneration failure. This could be achieved with drugs that engage multiple targets (polypharmacology) within various relevant signaling networks. The use of multi-target drugs to treat complex polygenic disorders is not a new concept; however, the lack of appropriate methodologies has hindered systematic exploitation of polypharmacology (Peters, 2013). Interestingly though, it appears that polypharmacology is important for the therapeutic efficacy of many approved drugs (Peters, 2013).

For the past few years, we worked to identify small molecule compounds that can engage multiple targets to promote axon regeneration. We focused our investigations on kinases as targets for several reasons. Kinases are involved in regulating most if not all cellular processes. They are well-established and readily druggable targets, with numerous therapeutic applications ranging from neurology to cancer. Moreover, the homology of kinase catalytic domains gives rise to kinase inhibitor promiscuity (Metz et al., 2011), making it possible to discover drugs with multiple "intended" kinase targets. Numerous kinases have been shown to play a role in regulating axon growth. However, predicting the most effective pharmacological targets (and target combinations) requires detailed knowledge of time-dependent properties of all nodes within relevant signaling networks, which at present is not feasible. Thus, we utilized phenotypic screening, which does not require *a priori* target hypotheses. Phenotypic screening tests compounds on whole cells rather than individual drug targets, and enables the discovery of compounds with desired biological effects, including those with favourable polypharmacology. We developed an assay that utilizes primary neurons from rat brains and used it to identify kinase inhibitors that strongly promote neurite outgrowth (Al-Ali et al., 2013). This technique by itself, however, does not provide direct information on the identity of the cellular targets through which the compounds exert their biological effects. Without this information, it is not possible to rationally select compounds with the best polypharmacology. To overcome this problem, we made use of another popular drug discovery technique, target-based screening. Target-based screening assays a single functional molecule (in our case a kinase) against a large number of compounds. We assayed several

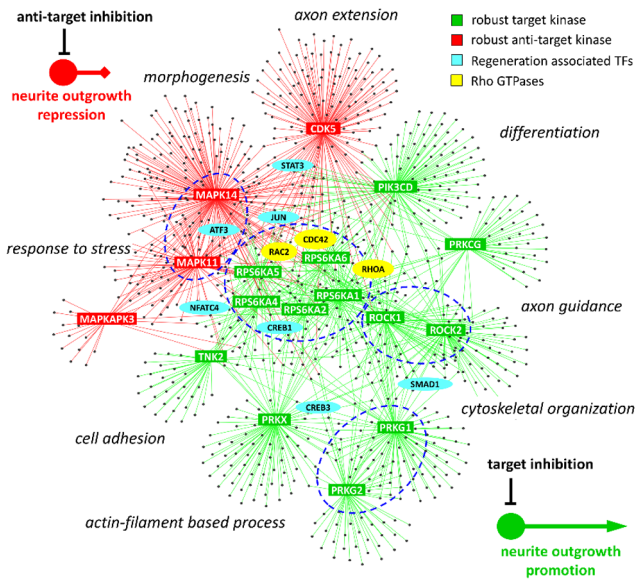

Figure 1 First-neighbor protein interaction network for top ranked target and anti-target kinases.
The network was modeled in Cytoscape using annotations from the STRING 9.1 protein-protein interactions database; black dots represent proteins interacting directly with one or more node kinases. Green nodes represent target kinases, while red nodes represent anti-target kinases. Blue ovals represent several well-known regeneration associated transcription factors (TFs), and yellow ovals are Rho GTPases central to cytoskeletal rearrangement. Inhibition or knockdown of targets promotes neurite outgrowth, while inhibition or knockdown of anti-targets represses neurite outgrowth. Clustering is based on shared first-neighbors. Dashed circles depict kinases with a high degree of pharmacological linkage. Compounds that inhibit multiple targets and no anti-targets strongly promote neurite outgrowth in primary neurons.

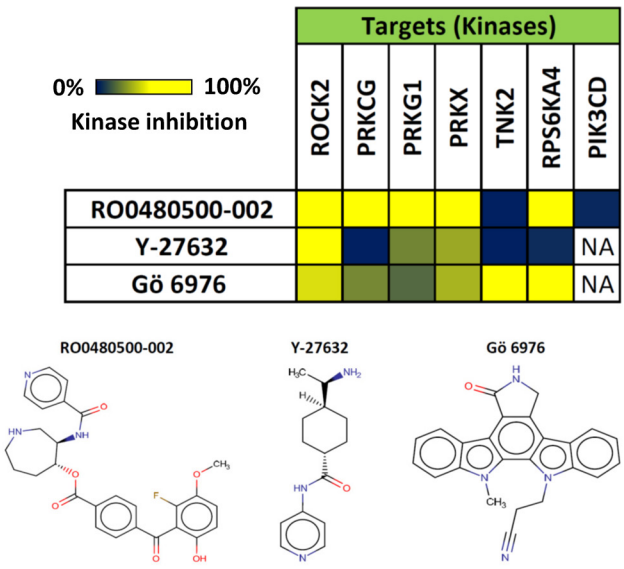

Figure 2 Kinase inhibitors previously reported to promote axon growth *in vivo* exhibit poly-pharmacology.
In addition to RO0480500-002, the two compounds Y-27632 and Gö6976, previously shown to promote axon growth *in vivo*, exhibit polypharmacology against several deconvolved targets. RO0480500-002 was assayed at 0.1 μM (Al-Ali et al., 2015), while Y-27632 and Gö6976 were assayed at 0.5 μM (Anastassiadis et al., 2011).

hundred compounds (previously screened in the phenotypic assay) against more than two hundred kinases to obtain the compounds' inhibition profiles towards those kinases. Using machine learning and information theory to relate the compounds' kinase inhibition profiles to their influence on neurite outgrowth, we were able to identify kinases that can serve as targets for promoting neurite outgrowth. We also identified kinases whose inhibition represses neurite outgrowth, and thus their targeting should be avoided (anti-targets) (Al-Ali et al., 2015).

Typically, a prioritized drug target is a single functional protein. The goal of target-based discovery campaigns is often to identify compounds that potently interact with the assayed target to elicit a desired therapeutic response. However, due to structural similarities arising from homology or parallel evolution, various proteins can show a high degree of similarity in the identity of compounds that they bind (Metz et al., 2011). This means that inhibiting a given target with a compound will frequently also inhibit other proteins that have a topologically similar binding pocket. Thus, as the similarity between binding pockets increases, so does the likelihood for co-inhibition. Proteins with highly similar binding pockets can be said to be pharmacologically linked (*i.e.*, it is difficult to engage one without also engaging the other). This is especially true for catalytically active kinases, given their shared lineage and evolutionarily conserved requirement for ATP binding. To account for this, we extended the concept of drug target from that of a single kinase to a group of pharmacologically linked kinases. Thus, a kinase was considered a robust target only if it was pharmacologically linked to other kinases that also behave as targets, or at least do not behave as anti-targets. Since most small molecule inhibitors will engage all members in a group of pharmacologically linked kinases, this criterion ensures that the overall effect of inhibiting a robust target group will be positive (pharmacologically linked targets and anti-targets will tend to counteract one another). We also identified several pharmacologically linked anti-target groups whose inhibition correlated with strong negative effects on neurite outgrowth. We prioritized the ten most robust target and anti-target groups (based on the outcome of our phenotypic screen), and selected a single member from each group for prioritizing lead compounds. For example, the activity of a compound against rho kinase 2 (ROCK2) was used to represent the activity of that compound against the corresponding group of pharmacologically linked kinases (in this case, ROCK1 and ROCK2). Activated CDC42 kinase 1 (TNK2), rho-associated kinase-II (ROCK2), PI3-kinase δ (PIK-3CD), protein kinase C γ (PRKCG), ribosomal protein S6 kinase α-4 (RPS6KA4), cGMP-dependent protein kinase G 1 (PRKG1), and cAMP-dependent protein kinase X (PRKX) were selected as representatives of robust target groups, while p38α MAP kinase (MAPK14), MAP kinase-activated protein kinase 3 (MAPKAPK3), and cyclin-dependent-like kinase 5 (CDK5) were selected as representatives of robust anti-target groups (**Figure 1**). Using these representative targets and anti-targets, we identified a compound with exemplary polypharmacology, which inhibits 5 out of the 7 robust target groups and does not affect the robust anti-target groups. Amongst its targets, RO0480500-002 inhibits both PKC and ROCK, kinases known to mediate repression of axon growth by myelin and CPSGs in the CNS (Young, 2014). RO0480500-002 also inhibits the growth regulatory S6 kinases, which have been shown to limit intrinsic neuronal capacity for axon growth and regeneration (Hubert et al., 2014). Moreover, RO0480500-002 inhibits PRKG1 and PRKX, two kinases involved in the regulation of cell migration and cytoskeletal rearrangement. Our phenotypic assays showed that this compound promotes neurite outgrowth both from hippocampal neurons and from postnatal cortical neurons (Al-Ali et al., 2015). Importantly, RO0480500-002 has beneficial effects on descending motor axons *in vivo* following spinal cord injury.

This new appreciation for favourable polypharmacology among kinase inhibitors may shed light on earlier results. Previous studies had shown that two kinase inhibitors, Gö6976 and Y-27632, promote axon regeneration *in vivo*. Y-27632 was described as a ROCK inhibitor (Fournier et al., 2003), while Gö6976 was described as a PKC inhibitor (Wang et al., 2013). Interestingly, kinase profiling of these two compounds reveals that each of them also inhibits several robust targets identified by our method (**Figure 2**), raising the possibility that their polypharmacology may have contributed to their *in vivo* efficacies. Interestingly, the compounds share no chemical similarity despite having somewhat similar target polypharmacology. This underscores the idea that desirable polypharmacology is not necessarily restricted to particular chemical scaffolds. With the expanded view of a polypharmacology profile (as opposed to just a single target), lead compounds with desirable polypharmacology and improved pharmacokinetic/pharmacodynamic properties can be discovered more easily, even if they share no chemical similarity to the original hit compounds. The polypharmacology profile elucidated in this study provides such a platform for discovering and developing effective multi-target drugs for neurodegenerative applications.

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