Discovery of Small-Molecule Ligands of Retinoblastoma Protein (Rb1) using Artificial Intelligence

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Abstract

Retinoblastoma protein (Rb1) is robustly expressed in adult sensory neurons and axons after nerve injury. Tumor suppressor pathways such as Rb1 may offer novel targets capable of altering the plasticity of post-mitotic adult neurons. Work in the Zochodne laboratory has shown that Rb1 knockdown using siRNA enhances neurite outgrowth from adult sensory neurons *in vitro* and improves indices of regeneration *in vivo* **[1,2]**. Rb1 knockdown impairs its inhibitory binding to the divergent transcription factor E2F1, facilitating neuron growth. In this study, using the AtomNet® model **[3]**, a deep convolutional neural network for structure-based drug discovery, we screened millions of compounds that might interrupt Rb1-E2F1 binding. We discovered several small drug-like molecules validated by surface plasmon resonance binding assay and a functional assay measuring the disruption of Rb1-E2F1 association. Encouragingly, among candidates identified, several promote outgrowth of PC12 cells that can be differentiated into neuronal like cells with nerve growth factors and enhance the outgrowth of adult sensory neurons.

Background

Knockdown of Rb1 improves the regeneration of peripheral axons following injury. Work in the Zochodne laboratory has shown that Rb1 knockdown (KD/KO) using siRNA enhances neurite outgrowth from adult sensory neurons *in vitro* and improves indices of regeneration *in vivo* **[1,2]**. The addition of siRNA to E2F1, a critical target of Rb1, eliminated the impact of Rb1 siRNA on outgrowth and branching in rat DRG neurons, indicating a role for E2F1 in neuronal plasticity. Plasticity is achieved in part through upregulation of neuronal PPARU; its antagonism inhibits Rb1 siRNA plasticity, whereas a PPARU agonist increases growth. In an in vivo regenerative paradigm following complete peripheral nerve trunk transection, direct delivery of Rb1 siRNA prompts increased outgrowth of axons from proximal stumps and entrains Schwann cells to accompany them for greater distances. Similarly, Rb1 siRNA delivery following a nerve crush improves behavioral indices of motor and sensory recovery in mice.

There are no current therapies available to reverse the neurological damage from axon damage in peripheral nerve trauma or peripheral neuropathies. This target may also have relevance in CNS axonal damage. A small molecule inhibitor which prevents Rb1 inhibition of E2F transcriptional signaling can be a first-in-class therapy for neuronal regeneration.

Experimental Methods

Biochemical assay

GST-tagged Rb1-AB domain was cloned and immobilized on glutathione resin. Each binding reaction contained 0.1 mg of GST-Rb1 (1.2 µM) and 0.1 mg of MBP-tagged E2F1 (1 µM) in 1 ml of binding buffer (20 mM Tris-HCl, 200 mM NaCl, 20 mM KCl, 0.5% Triton X100, pH 8). Bind for an hour with constant nutation, then wash with three times, and samples were analyzed by SDS-PAGE, coomassie blue staining and quantification. The compound concentration was fixed at 10 µM. The same experiment was run three times.

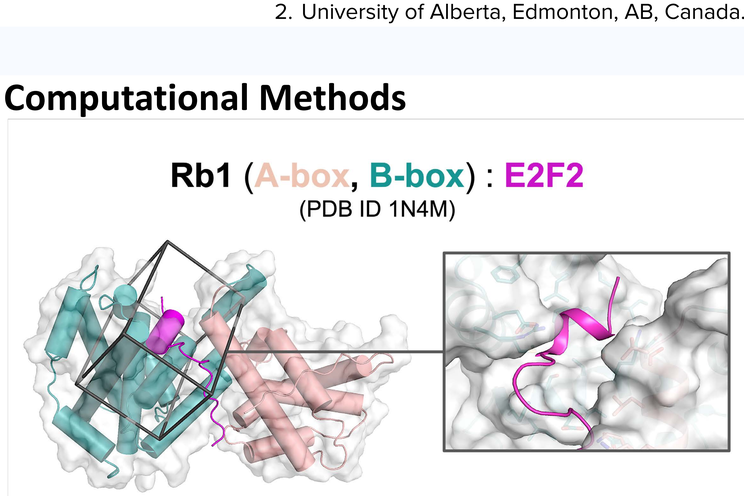
PC-12 cell-based assay

PC12 cells can be differentiated into neuronal like cells with 50 ng/ml NGF. PC12 cells grown in the presence of 10 µM compounds (0.1% DMSO) show that some compounds promote outgrowth after two days of growth. To generate the images PC12 cells were fixed and stained: nuclei (blue, DAPI), F-actin (Alexafluor-488 phalloidin). The bar on the images is ~ 10 µm (magnification of all images are the same).

Primary adult sensory neuron outgrowth

Adult rat L4-6 dorsal root ganglia (DRGs) were harvested for dissociated *in vitro* cultures in standard media, exposed for 24h to Rb1 siRNA, carrier or compounds at 2-10 µM (n=4 cultures/compound/dose) then fixed and stained for βIII tubulin by immunohistochemistry. Neurite outgrowth, sprouting and branching were analyzed by NeuroMath software. [NC-carrier control; Rb1 KD/KO is an siRNA positive control.]

Computational Methods

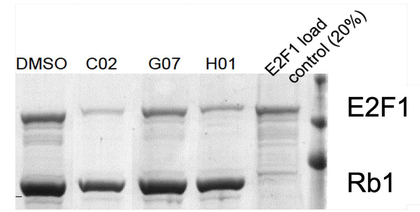


Results and Discussion

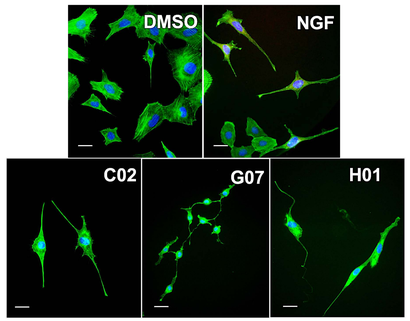
Hits disrupt Rb1 : E2F1 protein-protein interaction in biochemical assay

Compound	% Inhibition	SEM	IC50 (µM)
DMSO	0	0	
C02	31.7	3.7	21.6
G07	16.9	4.8	49.0
H01	39.9	6.8	15.0

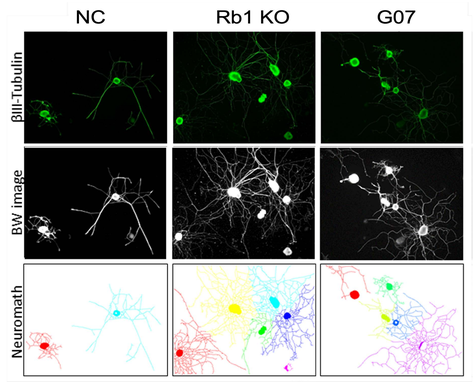
The assay was run at 10 µM compound concentration (n=3). The IC50 values were estimated via IC50 = [*C*]/%inh. – [*C*], where [*C*] is the compound concentration.



Hit compounds promote outgrowth of PC12 cells after two days equivalent to the control, NGF



Hit compounds stimulate growth of the primary adult sensory neurons similar to the control, Rb1 knock-out (KO)



- Preliminary results from the surface-plasmon resonance studies indicate binding of the hit compounds to Rb1.
- Studies to measure the impact of C02 and H01 on the growth of the primary adult sensory neurons are planned.
- A fluorescence-polarization-based functional assay is under development as an orthogonal method to test the inhibition of the Rb1 : E2F1 protein-protein interaction by the compounds.
- Validated hits of interest are planned to advance to hit expansion to identify more potent analogs.
- *In vivo* studies are planned to test the neurite outgrowth in mice.

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