Scaffold Ranking and Positional Scanning Identify Novel Neurite Outgrowth Promoters with Nanomolar Potency

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S Supporting Information

ABSTRACT: Central nervous system (CNS) neurons typically fail to regrow their axons after injury. Injuries or neuropathies that damage CNS axons and disrupt neuronal circuitry often result in permanent functional deficits. Axon regeneration is therefore an intensely pursued therapeutic strategy for numerous CNS disorders. Phenotypic screens utilizing primary neurons have proven successful at identifying agents that promote axon regeneration *in vivo*. Here, we report the screening of mixture-based combinatorial small molecule libraries in a phenotypic assay utilizing primary CNS neurons and the discovery of neurite outgrowth promoters with low nanomolar potency.

KEYWORDS: Nerve repair, axon regeneration, neurite outgrowth, phenotypic screening

The failure of central nervous system (CNS) neurons to regenerate damaged axons poses a barrier for functional recovery.^{1,2} Traumatic brain injury, for example, can produce lifelong psychological and cognitive deficits,³ and spinal cord injury can result in permanent paralysis.⁴ In addition to mechanical injury, CNS axons can be damaged by degenerative neuropathies including glaucoma,⁵ multiple sclerosis,⁶ Alzheimer's disease, and Parkinson's disease. Promoting axon regeneration is therefore a highly pursued therapeutic strategy with clinical applicability in a wide range of indications.^{7–10}

Suppression of axon regeneration is mechanistically complex,^{9,11} making it difficult to tackle using target-based drug discovery approaches. In recent years, phenotypic screening has emerged as an alternative approach for discovering safe and efficacious drugs.^{12–14} This approach tests compounds directly on cells or tissues, thereby circumventing challenges related to identifying effective drug targets.¹⁵ We have previously developed and optimized a phenotypic assay using primary neurons to screen for agents that can promote neurite outgrowth.^{16,17} The assay allowed us

to identify genes¹⁸ as well as small molecules^{11,16,19} that promote axon regeneration *in vivo*.

Although phenotypic screening provides a powerful approach for identifying biologically active compounds, the added burden of handling, imaging, and data processing typically imposes a lower throughput compared to target-based biochemical screening.¹⁷ The majority of neurite outgrowth screens reported in the literature were carried out with relatively small compound libraries comprising <2000 compounds.^{19–22} Recently, a neurite outgrowth assay was scaled up to screen a compound library comprised of ~50,000 compounds.²³ In the current proof-of-concept study, we applied a mixture-based and scaffold ranking approach to a neurite outgrowth screen, which allowed us to interrogate hundreds of millions of unique compound structures and identify a novel group of hit compounds suitable for lead development (Figure 1). Hits were confirmed by resynthesis

Received: September 17, 2018

Accepted: September 24, 2018

Published: September 24, 2018

Figure 1. Approach used to identify active compounds. Starting with just 79 samples comprised of >400 million compounds in the Scaffold Ranking Library. The specific Positional Scanning Library (PSL) 2275 was selected based on its activity. PSL 2275 contains 116 samples, and the results from screening this library were used to select 27 compounds that were then synthesized (a process known as deconvolution). The screening results of the 27 individual crude samples led to the purification and additional testing of nine of the compounds.

Figure 2. Prioritization of active scaffolds. Scaffold ranking data at the 0.16 $\mu\text{g/mL}$ treatment concentration compared to positive control (ML-7). Values are neurite total length relative to DMSO control (%NTL).

and validation in the phenotypic assay. One compound, 2539-14, promoted neurite outgrowth by 2–3-fold relative to control with remarkable potency (13 nM). The success of our approach demonstrates the ability of mixture-based combinatorial libraries to identify novel compounds with nanomolar activity in cell-based assays.

The advantages of scaffold ranking libraries and the methods by which they are constructed have been previously described.²⁴ Briefly, scaffold ranking libraries are arranged so that each sample contains close structural analogues based on a single core scaffold. In our study, the most complex library (library 2388) consisted of over 5 million compounds, whereas the least diverse (library 2058) consisted of just over 2000. We used our previously established phenotypic assay with primary mammalian neurons to screen for compounds that promote neurite outgrowth.¹⁶ *In vivo* differentiated neurons best recapitulate the gene and protein expression profiles of actual neuronal subpopulations^{17,25} and are therefore better suited than cell lines or *in vitro*-differentiated cells for purposes of drug discovery and target validation. The assay typically has a Z-factor ≥ 0.7 and is quite sensitive for identifying promoters of neurite outgrowth. Seventy nine different mixtures, representing over 400 million unique structures, were screened

at six doses ranging from 10 $\mu\text{g/mL}$ to 3.2 ng/mL. Scaffold ranking data were acquired, and the most active scaffolds (Figure 2) were considered for subsequent testing of the corresponding positional scan library.²⁴ Several scaffold samples that contained an imidazoline branched to a heterocycle (1276 and 2275 branched to a cyclic thiourea, 1319 to a cyclicurea, and 2137 to a diketopiperazine) produced large neurite total length relative to control (%NTL) across a wide concentration range; 1276 and 2275 are two different synthesis lots of the same scaffold mixture, providing clear evidence for the reproducibility of the chemistry and screening assay.

On the basis of the results of the scaffold ranking screen (Figure 2), mixture 2275 was selected for positional scanning to identify individual compounds that promote neurite outgrowth. Positional scanning samples from library 2275 contain either 1258 or 1665 dihydroimidazolyl-butyl-cyclic thiourea compounds systematically arranged into 116 subset mixtures (Table S1). Samples were tested in the same manner as the scaffold ranking step at six doses ranging from 10 $\mu\text{g/mL}$ to 0.003 $\mu\text{g/mL}$ (Table S1). The higher doses of 10 and 2 $\mu\text{g/mL}$ for the majority of samples were toxic. Samples with broad activity profiles and low apparent toxicity were selected for

Table 1. Average %NTL Data for 27 Individual Compounds at Six Doses (ng/mL)

ID	R1	R2	R3	%NTL						%NTL- Standard Deviation					
				500	250	125	62.5	31.25	15.625	500	250	125	62.5	31.25	15.625
1	R-methyl	4-methylpentyl	2-methylbutyl	323.0821	209.15761	169.097341	155.0100924	115.5767987	92.44827971	5.2709954	7.3885423	2.261664	14.151836	11.914561	5.270995
2	R-methyl	4-methylpentyl	cyclopentyl-methyl	323.72109	264.80688	213.9162061	175.8850392	137.8846234	113.8061117	3.5483016	8.6244396	7.698132	32.185286	4.289216	3.548302
3	R-methyl	4-methylpentyl	butylbenzene	346.66283	257.3265	160.4343899	124.2111001	100.4613202	116.4117479	30.408191	6.3437253	6.796014	12.394780	14.216656	30.408191
4	R-methyl	cyclohexyl-ethyl	2-methylbutyl	357.54938	246.07334	196.1175308	142.5948141	121.3965298	102.3249713	12.360192	6.8182926	3.523633	3.232298	4.973617	12.360192
5	R-methyl	cyclohexyl-ethyl	cyclopentyl-methyl	304.43872	245.7679	250.699371	188.0422309	157.6452815	104.6541522	1.7636448	31.205603	7.385534	31.764833	21.345639	1.763645
6	R-methyl	cyclohexyl-ethyl	butylbenzene	242.65772	246.59483	209.7047191	122.018414	122.1099329	100.7929003	12.408552	5.3853636	4.357328	13.313592	1.314701	12.408552
7	R-methyl	4-methyl-1-cyclohexylmethyl	2-methylbutyl	293.62204	266.73461	166.1863223	150.0102366	134.9413843	97.32619944	7.9380786	4.6434464	11.099452	21.195990	8.836402	7.938079
8	R-methyl	4-methyl-1-cyclohexylmethyl	cyclopentyl-methyl	322.29816	257.34782	230.7497307	168.0611376	128.2971117	94.60966785	5.0339717	15.628605	31.880428	29.485739	9.024231	5.033972
9	R-methyl	4-methyl-1-cyclohexylmethyl	butylbenzene	296.90361	237.62399	70.33424199	117.7309907	101.9251833	88.36185892	9.4115954	4.1469395	70.334242	10.322279	4.112369	9.411595
10	R-isobutyl	4-methylpentyl	2-methylbutyl	239.52923	258.66096	253.9587593	222.5542903	179.7558182	122.8281621	17.299776	20.31385	0.468236	19.135715	14.632779	17.299776
11	R-isobutyl	4-methylpentyl	Cyclopentanecarboxylic acid	256.36926	263.49788	273.3912203	266.4046258	215.0559784	134.8346999	6.0929323	40.738799	6.581123	21.652649	7.432304	6.092932
12	R-isobutyl	4-methylpentyl	butylbenzene	239.93079	265.56508	237.1185178	194.1006078	145.2857722	127.4064317	5.0083755	18.725387	14.962291	11.902408	7.683705	5.008376
13	R-isobutyl	cyclohexyl-ethyl	2-methylbutyl	264.0755	253.08893	252.9294817	261.8880756	178.9196693	158.8791005	29.443838	30.203472	2.826873	21.301928	36.245769	29.443838
14	R-isobutyl	cyclohexyl-ethyl	cyclopentyl-methyl	248.71789	277.19762	282.9150886	270.9736881	256.472534	194.8725246	33.016137	14.586666	10.581237	25.506102	4.837398	33.016137
15	R-isobutyl	cyclohexyl-ethyl	butylbenzene	246.78065	267.29395	260.8199883	199.2794512	137.4720531	113.9612714	24.356916	26.900073	41.861946	46.991842	15.218418	24.356916
16	R-isobutyl	4-methyl-1-cyclohexylmethyl	2-methylbutyl	264.44043	236.02199	268.1565132	253.5669413	216.2756979	131.1003696	13.796698	17.748353	13.474113	11.761564	19.933507	13.796698
17	R-isobutyl	4-methyl-1-cyclohexylmethyl	cyclopentyl-methyl	263.00665	238.46383	311.5685882	253.6395713	255.3516473	169.9221451	33.300826	0.7894018	1.737584	1.956086	15.151787	33.300826
18	R-isobutyl	4-methyl-1-cyclohexylmethyl	butylbenzene	224.36658	240.03794	256.8514866	246.7166295	197.0158481	144.870476	23.911183	14.955846	12.950289	30.757558	17.091528	23.911183
19	R-Phenyl	4-methylpentyl	2-methylbutyl	276.25479	265.84416	263.6607954	252.7745046	179.4409708	121.3929879	9.9933357	17.304838	2.439153	14.557842	12.238589	9.993336
20	R-Phenyl	4-methylpentyl	cyclopentyl-methyl	272.97836	277.93357	249.1534291	279.6409084	234.3128473	173.9107775	21.776102	9.3820135	8.897463	22.600713	20.806482	21.776102
21	R-Phenyl	4-methylpentyl	butylbenzene	225.06979	253.89739	259.07153	172.3561029	170.1791776	113.7643469	14.570885	47.363494	42.364482	20.025369	47.653071	14.570885
22	R-Phenyl	cyclohexyl-ethyl	2-methylbutyl	228.05645	221.91013	245.5278075	257.1510252	200.793887	139.0359432	13.825795	11.322513	3.714754	15.728117	11.598258	13.825795
23	R-Phenyl	cyclohexyl-ethyl	cyclopentyl-methyl	185.88496	232.41659	261.904324	260.4807582	251.5205655	182.2844591	37.796239	29.035858	31.799198	20.512943	35.145231	37.796239
24	R-Phenyl	cyclohexyl-ethyl	butylbenzene	183.52552	221.82219	254.168245	251.5972888	208.8912553	143.7146278	29.549168	26.323877	23.072290	35.728950	48.714376	29.549168
25	R-Phenyl	4-methyl-1-cyclohexylmethyl	2-methylbutyl	279.80133	255.91244	301.9048879	296.6891253	256.2123179	190.0098371	11.836739	37.953877	7.453368	8.692699	15.542365	11.836739
26	R-Phenyl	4-methyl-1-cyclohexylmethyl	cyclopentyl-methyl	229.55988	270.81582	286.2245388	305.5905216	271.9280071	251.0286065	24.672074	33.500694	0.902997	14.274474	15.150711	24.672074
27	R-Phenyl	4-methyl-1-cyclohexylmethyl	butylbenzene	221.2261	244.67794	269.0841487	237.5208908	174.2660118	184.9133382	5.1243629	7.1557964	24.576674	32.254130	28.405536	5.124363

retesting. These samples were tested at three doses ranging from 500 to 0.2 ng/mL (Table S2). The data show a range of dose-dependent activities at all variable R positions. This differentiation in activity is vital for successful deconvolution and structure–activity relationship (SAR) studies. On the basis of these data, three different functionalities were selected from each of the diversity positions. First, we prioritized the samples that produced a significant increase in %NTL over a broad concentration range. Then, we prioritized compounds based on chemical diversity. For example, in the R3 position, cyclohexyl-ethyl (2275-107), cyclohexyl-methyl (2275-105), cyclopentyl-methyl (2275-112), and cyclohexyl-propyl (2275-114) have chemically similar functionalities, and they all displayed similar activity profiles. Thus, rather than choosing all four functionalities, we selected cyclopentyl-methyl to represent this class. By selecting three functionalities at each position, we prioritized 27 individual compounds (3 functionalities $\times$ 3 positions) for synthesis.

Compounds with R-methyl in the R1 position (compounds #1–9, Table 1) had good activity at the highest dose (500 ng/mL) but did not maintain activity at lower doses compared to compounds with other R1 substitutions (compounds #10–27). Some preference for the cyclopentyl methyl in R3 is observed within this series and holds for all compounds (compounds 5 and 8). There is an obvious drop in activity for the R-isobutyl series (compounds 10–18) when an aromatic group (butylbenzene) is in the R3 position (compounds 12, 15, and 18). The substitution of an aromatic group (R-phenyl) did not decrease activity, however, when introduced in the R1 position (compounds 19–27). The R2 substitution of 4-methyl-1-cyclohexylmethyl within this series yielded the most potent compounds. The R-phenyl series maintained the preference for the cyclopentyl methyl substitution in the R3 position with the compound having these two substitutions (compound 26) showing the highest potency. Nine individual compounds from the set of 27 were purified and retested in the

neurite outgrowth assay. All nine compounds induced outgrowth by at least 200% relative to controls (Table 2). Five compounds (compounds 14, 16, 23, 25, and 26) were retested over an extended dose range and verified to have nanomolar potency (Figure 3).

Table 2. Calculated Concentrations for the Doses Needed to Induce a %NTL of 200% (ED200%) and 300% (ED300%) for the Pure Individual Compounds^a

compd ID	ED200% (nM)	ED300% (nM)
8	130	282
14	4	>543
16	10	53
17	4	25
18	25	461
23	7	91
25	5	54
26	2	>520
27	17	>471

^aValues were calculated by use of linear interpolates of each compound's dose response curve.

Mixture-based synthetic libraries are highly effective tools for generating novel lead compounds in a fraction of the time and cost required to screen the equivalent amount of individual compounds.²⁴ The approach has been successfully applied to a variety of assay types and was recently used to identify binders of the nicotinic acetylcholine receptor.²⁶ Here, we applied it to a phenotypic cell-based assay utilizing primary neurons and identified compounds with remarkable potencies for inducing neurite outgrowth.

Given the high potencies of the compounds discovered here, which are comparable to, or even exceed, the range of potencies for extracellular receptor ligands previously discovered using this screening approach,²⁶ it is tempting to speculate

Figure 3. Neurite total length (%NTL) relative to DMSO in hippocampal neurons treated with pure 2539 compounds at the indicated concentrations for 2 DIV.

that our hits may be acting through an extracellular receptor. However, previous studies by our group and others have identified promoters of neurite outgrowth that exert their effects via intracellular targets (e.g., lipid metabolizing enzymes and kinases) with submicromolar potency.^{19,23} This suggests that the hits reported in this study could also be exerting their effects via intracellular targets. Identification of targets by affinity purification followed by mass spectrometry is likely appropriate here and will be pursued in future studies to identify the putative mechanisms of action of these compounds.

The structure–activity relationship conclusions generated from the scaffold ranking library (i.e., that a branched imidazoline is a favored scaffold) and the positional scanning library (i.e., identifying key R group functionalities) indicate that the hit compounds are amenable to further optimization as they progress into more advanced in vivo and preclinical studies. The scaffold ranking library data indicate that the cyclic urea moiety could be modified as needed based on future efficacy or toxicity results.

In conclusion, we screened over 400 million compounds via the use of mixture-based libraries and identified novel and potent promoters of neurite outgrowth. Characterizing the

physicochemical properties of these compounds, such as their membrane permeability, as well as identifying their high affinity binding partners, will be important for elucidating their mechanism(s) of action and potentially revealing novel drug targets for promoting CNS axon regeneration.

EXPERIMENTAL PROCEDURES

Rabbit anti- β III (T2200) was purchased from Sigma-Aldrich; Alexa Fluor 488 cross-linked Goat anti-Rabbit (A11034) antibodies were purchased from Life Technologies. Poly-D-Lysine (P7886-500MG) and sterile dimethyl sulfoxide (DMSO) (D2650) were purchased from Sigma-Aldrich. Hippocampal tissue was incubated in Hibernate E from BrainBits supplemented with NeuroCult SM1 (05711) from StemCell Technologies. Neurons were cultured in NbActive4 media from BrainBits.

Compound mixtures were screened in our previously described neurite outgrowth assay.^{16,17,19} Briefly, dissociated embryonic (E18) rat hippocampal neurons were immediately seeded in 96-well plates (coated overnight with PDL at 0.5 mg mL⁻¹ in HBSS and washed 5 times with PBS) at 1800 cells per well in 150 μ L of NbActive4 and allowed to adhere for 2 h before treatment. Compound mixtures were prediluted in culture media at 4 $\times$ final concentration and equilibrated in a CO₂ incubator for at least 1 h prior to treatment, after which 50 μ L of compound solution was moved onto the cells to a final experiment volume of 200 μ L. Cells were incubated for 48 h after which plates were fixed, immunostained, and imaged in a Cellomics ArrayScan VTI robot. Images were automatically traced using the Neuronal Profiling Bioapplication (version 3.5). Mean neurite total length was calculated as a percentage of vehicle controls in each plate (%NTL). Conditions that reduced valid cell counts by more than 50% of control were considered toxic. ML-7 was used as the positive control, and experiments with Z-factor > 0.5 were considered valid. Each compound mixture was tested at least twice using independently prepared neuronal cultures on separate weeks. Dose–responses for individual hits were obtained using a similar assay format.

The compounds were synthesized as described in Scheme 1 using standard Boc chemistry. The solid-phase synthesis was performed using the “tea-bag” methodology.²⁷ The desired product was cleaved from the solid support resin and extracted using 95% acetic acid. Samples were then repeatedly frozen and lyophilized in 50% acetonitrile and water three times prior to analysis. During purification, the peak corresponding to the desired product with calculated *m/z* was isolated and concentrated. Compounds were purified using a Shimadzu Prominence preparative HPLC system

Scheme 1. Synthetic Route for Library 2275 and Individual Compounds

consisting of LC-8A binary solvent pumps, an SCL-10A system controller, an SIL-10AP auto sampler, an FRC-10A fraction collector, and a Shimadzu SPD-20A UV detector. The wavelength was set to 214 nm. Chromatographic separations were obtained using a Phenomenex Gemini NX-C18 preparative column (5 μ m, 150 mm $\times$ 21.2 mm i.d.). The column was protected by a Phenomenex C18 column guard (5 μ m, 15 mm $\times$ 21.2 mm i.d.). Prominence prep software was used for detection and collection parameters. The mobile phases for HPLC purification were HPLC grade obtained from Sigma-Aldrich and Fisher Scientific. The mobile phase A consisted of water with 0.1% trifluoroacetic acid, and mobile phase B consisted of acetonitrile with 0.1% trifluoroacetic acid. The initial setting was set to 2% mobile phase B and was gradually increased over time to achieve ideal separation for each compound. See the [Supporting Information](#) for details on the synthesis of compounds 8, 14, 16, 17, 18, 23, 25, 26, and 27.

¹H NMR spectra were obtained utilizing the Bruker 400' Ascend (400 MHz). NMR chemical shifts were reported in δ (ppm) using the δ 2.50 signal of DMSO-*d*₆ as an internal standard. The ACD/NMR Processor Academic Addition Software package was utilized to aid in the integration and assignment of peak tables. Confirmation of the desired product was obtained by reverse-phase LCMS analysis using a Shimadzu 2010 LCMS system consisting of an LC-20AD binary solvent pump, a DGU-20A degasser unit, a CTO-20A column oven, an SIL-20A HT auto sampler, and an SPD-M20A diode array set to scan 190–600 nm. Separation was achieved using a Phenomenex Gemini C18 column (5 μ m, 50 mm $\times$ 4.6 mm i.d.) protected with a Phenomenex C18 column guard column (5 μ m, 4 $\times$ 3.0 mm i.d.), mobile (A) water with 0.1% formic acid and mobile phase (B) acetonitrile with 0.1% formic acid. A gradient of 5–95% mobile phase B over 6 min was run. See [Supporting Information](#) for LCMS and ¹H NMR spectral data for compounds 8, 14, 16, 17, 18, 23, 25, 26, and 27.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](#) at DOI: [10.1021/acsmedchemlett.8b00425](#).

Synthesis, LCMS, and ¹H NMR data for compounds 8, 14, 16, 17, 18, 23, 25, 26, and 27 and average %NTL data for the 116 mixture samples that make up library 2275 ([PDF](#))

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Funding

This work was supported by grants from the Department of Defense (W81XWH-13-1-077) and the National Institutes of Health (HD057632) to J.L.B. and V.P.L., the Wallace H Coulter Center (University of Miami) to J.L.B., V.P.L., and H.A.A., and the Florida Drug Discovery Acceleration Program by the State of Florida, Department of Health to G.D., R.G.S., R.A.H., J.C.D., and M.A.G. V.P.L. holds the Walter G. Ross Distinguished Chair in Developmental Neuroscience.

Notes

The authors declare the following competing financial interest(s): H.A.A., V.P.L., J.L.B., and M.A.G. are inventors on a provisional patent application covering the compounds reported in this manuscript.

■ ACKNOWLEDGMENTS

We thank Y. Shi for her help the cell imaging and high content analysis. We thank T. Slepak and Y. Martinez for their help with the primary cell culture.

■ ABBREVIATIONS

CNS, central nervous system; NTL, neurite total length

■ REFERENCES

- (1) Huebner, E. A.; Strittmatter, S. M. Axon Regeneration in the Peripheral and Central Nervous Systems. *Results Probl. Cell Differ.* **2009**, *48*, 339–351.
- (2) Cafferty, W. B. J.; McGee, A. W.; Strittmatter, S. M. Axonal Growth Therapeutics: Regeneration or Sprouting or Plasticity? *Trends Neurosci.* **2008**, *31* (5), 215–220.
- (3) DeKosky, S. T.; Ikonovic, M. D.; Gandy, S. Traumatic Brain Injury–Football, Warfare, and Long-Term Effects. *N. Engl. J. Med.* **2010**, *363* (14), 1293–1296.
- (4) Silva, N. A.; Sousa, N.; Reis, R. L.; Salgado, A. J. From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury. *Prog. Neurobiol.* **2014**, *114*, 25–57.
- (5) Vrabec, J. P.; Levin, L. A. The Neurobiology of Cell Death in Glaucoma. *Eye* **2007**, *21*, S11–S14.
- (6) Haines, J. D.; Inglese, M.; Casaccia, P. Axonal Damage in Multiple Sclerosis. *Mt. Sinai J. Med.* **2011**, *78* (2), 231–243.
- (7) Medana, I. M.; Esiri, M. M. Axonal Damage: A Key Predictor of Outcome in Human CNS Diseases. *Brain* **2003**, *126* (3), 515–530.
- (8) Fitzgerald, J.; Fawcett, J. Repair in the Central Nervous System. *J. Bone Jt. Surg., Br. Vol.* **2007**, *89* (11), 1413–1420.
- (9) Young, W. Spinal Cord Regeneration. *Cell Transplant* **2014**, *23* (4), 573–611.
- (10) Chang, E. E.; Goldberg, J. L. Glaucoma 2.0: Neuroprotection, Neuroregeneration, Neuroenhancement. *Ophthalmology* **2012**, *119* (5), 979–986.
- (11) Al-Ali, H.; Beckerman, S. R.; Bixby, J. L.; Lemmon, V. P. In Vitro Models of Axon Regeneration. *Exp. Neurol.* **2017**, *287*, 423–434.
- (12) Kotz, J. Phenotypic Screening, Take Two. *Sci. Exch.* **2012**, *5* (15), 380.
- (13) Zheng, W.; Thorne, N.; McKew, J. C. Phenotypic Screens as a Renewed Approach for Drug Discovery. *Drug Discovery Today* **2013**, *18* (21–22), 1067–1073.
- (14) Al-Ali, H. The Evolution of Drug Discovery: From Phenotypes to Targets, and Back. *MedChemComm* **2016**, *7* (5), 788–798.
- (15) Peters, J.-U. Polypharmacology - Foe or Friend? *J. Med. Chem.* **2013**, *56* (22), 8955–8971.
- (16) Al-Ali, H.; Schürer, S. C.; Lemmon, V. P.; Bixby, J. L. Chemical Interrogation of the Neuronal Kinome Using a Primary Cell-Based Screening Assay. *ACS Chem. Biol.* **2013**, *8* (5), 1027–1036.
- (17) Al-Ali, H.; Blackmore, M.; Bixby, J. L.; Lemmon, V. P. High Content Screening with Primary Neurons. *Assay Guid. Man.* **2013**.
- (18) Moore, D. L.; Blackmore, M. G.; Hu, Y.; Kaestner, K. H.; Bixby, J. L.; Lemmon, V. P.; Goldberg, J. L. KLF Family Members Regulate Intrinsic Axon Regeneration Ability. *Science* **2009**, *326* (5950), 298–301.
- (19) Al-Ali, H.; Lee, D. H.; Danzi, M. C.; Nassif, H.; Gautam, P.; Wennerberg, K.; Zuercher, B.; Drewry, D. H.; Lee, J. K.; Lemmon, V. P.; et al. Rational Polypharmacology: Systematically Identifying and Engaging Multiple Drug Targets to Promote Axon Growth. *ACS Chem. Biol.* **2015**, *10* (8), 1939–1951.
- (20) Usher, L. C.; Johnstone, A.; Erturk, A.; Hu, Y.; Strikis, D.; Wanner, I. B.; Moorman, S.; Lee, J. W.; Min, J.; Ha, H.-H. H.; et al. A Chemical Screen Identifies Novel Compounds That Overcome Glial-Mediated Inhibition of Neuronal Regeneration. *J. Neurosci.* **2010**, *30* (13), 4693–4706.
- (21) Ma, T. C.; Campana, A.; Lange, P. S.; Lee, H.-H.; Banerjee, K.; Bryson, J. B.; Mahishi, L.; Alam, S.; Giger, R. J.; Barnes, S.; et al. A Large-Scale Chemical Screen for Regulators of the Arginase 1

Promoter Identifies the Soy Isoflavone Daidzeinas a Clinically Approved Small Molecule That Can Promote Neuronal Protection or Regeneration via a CAMP-Independent Pathway. *J. Neurosci.* **2010**, *30* (2), 739–748.

(22) Koprivica, V.; Cho, K. S.; Park, J. B.; Yiu, G.; Atwal, J.; Gore, B.; Kim, J. A.; Lin, E.; Tessier-Lavigne, M.; Chen, D. F.; et al. EGFR Activation Mediates Inhibition of Axon Regeneration by Myelin and Chondroitin Sulfate Proteoglycans. *Science (Washington, DC, U. S.)* **2005**, *310* (5745), 106–110.

(23) Li, H.; Kuwajima, T.; Oakley, D.; Nikulina, E.; Hou, J.; Yang, W. S.; Lowry, E. R.; Lamas, N. J.; Amoroso, M. W.; Croft, G. F.; et al. Protein Prenylation Constitutes an Endogenous Brake on Axonal Growth. *Cell Rep.* **2016**, *16* (2), 545–558.

(24) Houghten, R. A.; Pinilla, C.; Giulianotti, M. A.; Appel, J. R.; Dooley, C. T.; Nefzi, A.; Ostresh, J. M.; Yu, Y.; Maggiora, G. M.; Medina-Franco, J. L.; et al. Strategies for the Use of Mixture-Based Synthetic Combinatorial Libraries: Scaffold Ranking, Direct Testing in Vivo, and Enhanced Deconvolution by Computational Methods. *J. Comb. Chem.* **2008**, *10* (1), 3–19.

(25) Daub, A.; Sharma, P.; Finkbeiner, S. High-Content Screening of Primary Neurons: Ready for Prime Time. *Curr. Opin. Neurobiol.* **2009**, *19* (5), 537–543.

(26) Wu, J.; Zhang, Y.; Maida, L. E.; Santos, R. G.; Welmaker, G. S.; LaVoi, T. M.; Nefzi, A.; Yu, Y.; Houghten, R. A.; Toll, L.; et al. Scaffold Ranking and Positional Scanning Utilized in the Discovery of NAChR-Selective Compounds Suitable for Optimization Studies. *J. Med. Chem.* **2013**, *56* (24), 10103–10117.

(27) Houghten, R. A. General Method for the Rapid Solid-Phase Synthesis of Large Numbers of Peptides: Specificity of Antigen-Antibody Interaction at the Level of Individual Amino Acids. *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82* (15), 5131–5135.