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Deconstructing the *Iboga* Alkaloid Skeleton: Potentiation of FGF2-induced Glial Cell Line-Derived Neurotrophic Factor Release by a Novel Compound

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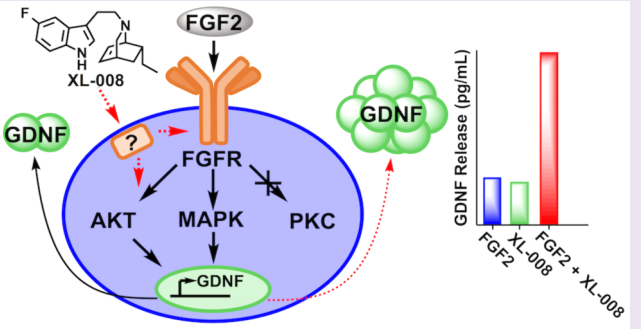
Supporting Information

ABSTRACT: Modulation of growth factor signaling pathways in the brain represents a new experimental approach to treating neuropsychiatric disorders such as depression, anxiety, and addiction. Neurotrophins and growth factors exert synaptic, neuronal, and circuit level effects on a wide temporal range, which suggests a possibility of rapid and lasting therapeutic effects. Consequently, identification of small molecules that can either enhance the release of growth factors or potentiate their respective pathways will provide a drug-like alternative to direct neurotrophin administration or viral gene delivery and thus represents an important frontier in chemical biology and drug design. Glial cell line-derived neurotrophic factor (GDNF), in particular, has been implicated in marked reduction of alcohol consumption in rodent addiction models, and the natural product ibogaine, a substance used traditionally in ritualistic ceremonies, has been suggested to increase the synthesis and release of GDNF in the dopaminergic system in rats. In this report, we describe a novel *iboga* analog, XL-008, created by unraveling the medium size ring of the ibogamine skeleton, and its ability to induce release of GDNF in C6 glioma cells. Additionally, XL-008 potentiates the release of GDNF induced by fibroblast growth factor 2 (FGF2), another neurotrophin implicated in major depressive disorder, increasing potency more than 2-fold (from 7.85 ± 2.59 ng/mL to 3.31 ± 0.98 ng/mL) and efficacy more than 3-fold. The GDNF release by both XL-008 and the FGF2/XL-008 mixture was found to be mediated through the MEK and PI3K signaling pathways but not through PLCγ in C6 glioma cells.



Isolated from the West African shrub *Tabernanthe iboga*, the natural product ibogaine and the other members of the ibogamine alkaloid family have traditionally been used in religious ceremonies, likely due to their dissociative effects observed at high doses.[1,2] In recent decades, however, ibogaine has been investigated as an experimental therapeutic for treating substance use disorders (SUDs), with evidence for suppression of craving and self-administration of diverse drugs of abuse in humans (e.g., alcohol, opioids, and cocaine) for extended periods of time (weeks to months),[3] as well as reduction of acute opioid withdrawal symptoms.[4] These clinical findings (mostly uncontrolled clinical studies and anecdotal reports)[3] have been recapitulated in animal models.[5−7]

Unfortunately, despite decades of ongoing interest, ibogaine's molecular mechanism of action remains undefined. Ibogaine has been reported to bind to, and/or show functional activity at, many central nervous system (CNS) receptors with micromolar potency, including the *N*-methyl-D-aspartate receptor (NMDAR), the dopamine and serotonin transporters, mu-opioid receptor, sigma 2 receptor, 5-HT2a, acetylcholine receptors, ERG channels, and others,[8−11] which, combined with its hallucinogenic effects, makes ibogaine a controversial treatment option. The complex pharmacology of ibogaine (and its metabolite noribogaine)[12,13] continues to be studied: while ibogaine has been shown to block NMDA receptors in different brain tissues in the range of 3−10 μM,[14−16] it does not appear to activate the mu-opioid receptor, suggesting an indirect mechanism of action for ibogaine's effects on opioid withdrawal.[17] In addition, the inhibition of human ERG channels by ibogaine at ∼4 μM may account for the heart arrhythmias associated with ibogaine usage.[11,18] Therefore, there have been efforts to isolate the key therapeutic mechanism(s) from the dissociative and other potentially dangerous side effects.[19] Most notably, the ibogaine analog 18-methoxycoronaridine (18-MC) was developed in this spirit as an antagonist of $\alpha3\beta4$ nicotinic receptor with much improved selectivity for this molecular target over other CNS receptors when compared to ibogaine.[20] 18-MC is effective at reducing self-administration in rodents of several addictive substances, including morphine, cocaine, ethanol, and nicotine, and thus $\alpha3\beta4$ nicotinic receptor antagonism is considered an important

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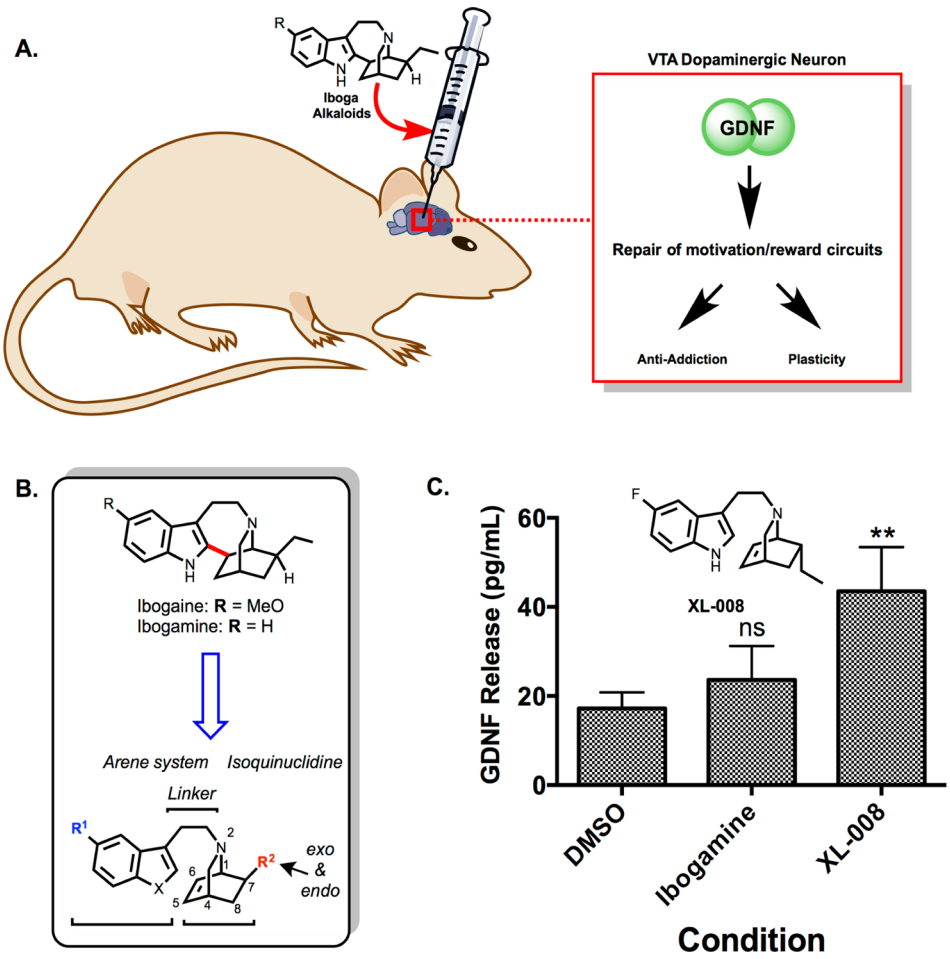

Figure 1. GDNF release and addiction. (A) Glial cell line-derived neurotrophic factor (GDNF) is a small protein that is synthesized and secreted in glial and neuronal cells. It has been shown to protect dopaminergic neurons in the brain and is linked to many brain disorders. Ibogaine, an alkaloid natural product isolated from *Tabernanthe iboga*, has shown antiaddictive properties, possibly mediated through the induction of GDNF release in the reward circuits of the brain. It was suggested that the GDNF release repairs neuronal circuits altered by the development of the drug dependent state (supported by reduction of alcohol consumption in rodents). (B) Disconnection of the heteroarene and isoquinuclidine systems of the *iboga* skeleton reveals a novel class of *iboga* analogs. (C) One such analog, XL-008, is a superior releaser of GDNF in comparison to the *iboga* alkaloid ibogamine, when tested at a 10 μM concentration after 24 h. Data represent mean ± SD of biological replicates in one experiment from $n = 4$ independent experiments. One-way ANOVA followed by Dunnett's Multiple Comparisons Test is shown ($^{**}p < 0.01$).

mechanism of action of ibogaine and 18-MC.[20] However, clinical efficacy of 18-MC has not yet been reported. Others have also developed acyclic ibogaine analogs that show binding to some of the same targets, including dopamine and serotonin transporters, the kappa-opioid receptor, and the NMDA receptor; however, these compounds have apparently not been pursued further.[21]

We were inspired by an intriguing mechanistic hypothesis that links iboga alkaloids to modulation of neurotrophic factor signaling systems. Namely, ibogaine was shown to induce glial cell line-derived neurotrophic factor (GDNF) expression in the ventral tegmental area (VTA) of rats, and it was suggested that GDNF activates an autocrine loop, leading to the increased and long-term synthesis and release of GDNF, which in turn repairs the function of the VTA-ventral striatum reward system (Figure 1A).[22] Although this hypothesis does not elucidate a primary molecular target, it offers a larger physiological picture and a rationale for the long-term effects of *iboga* alkaloids. In support of this mechanistic hypothesis, GDNF infusion to the VTA leads to reduced self-administration of alcohol and cocaine in rats.[22−25] However, it was also suggested that the role of GDNF in addiction may be more complex, as GDNF enhances the incubation of cocaine cravings during the first few weeks of withdrawal.[23−25]

In this context, we chose to explore novel *iboga* analogs in an attempt to discover superior releasers of GDNF that were also structurally distinct from ibogaine, thus providing a drug-like alternative to direct GDNF administration or viral gene delivery in the treatment of neuropsychiatric disorders.

GDNF is an important signaling protein in the CNS[26−30] that belongs to the GDNF family of ligands (GFL), together with other members such as neurturin,[31] persephin,[32] and artemin.[33] GDNF signals through the transmembrane receptor tyrosine kinase known as "rearranged at transfection" (Ret). Activation of Ret by GDNF involves the formation of a tetrameric complex containing two molecules of Ret and two molecules of GDNF family receptor α (GFRα; GFRα1 in particular for GDNF).[34] The activated Ret/GFRα complex can then trigger intracellular signaling through the MEK, PI3K, and PLCγ pathways, leading to a spectrum of cellular effects such as modulation of differentiation, survival, proliferation, and plasticity of neurons.[35]

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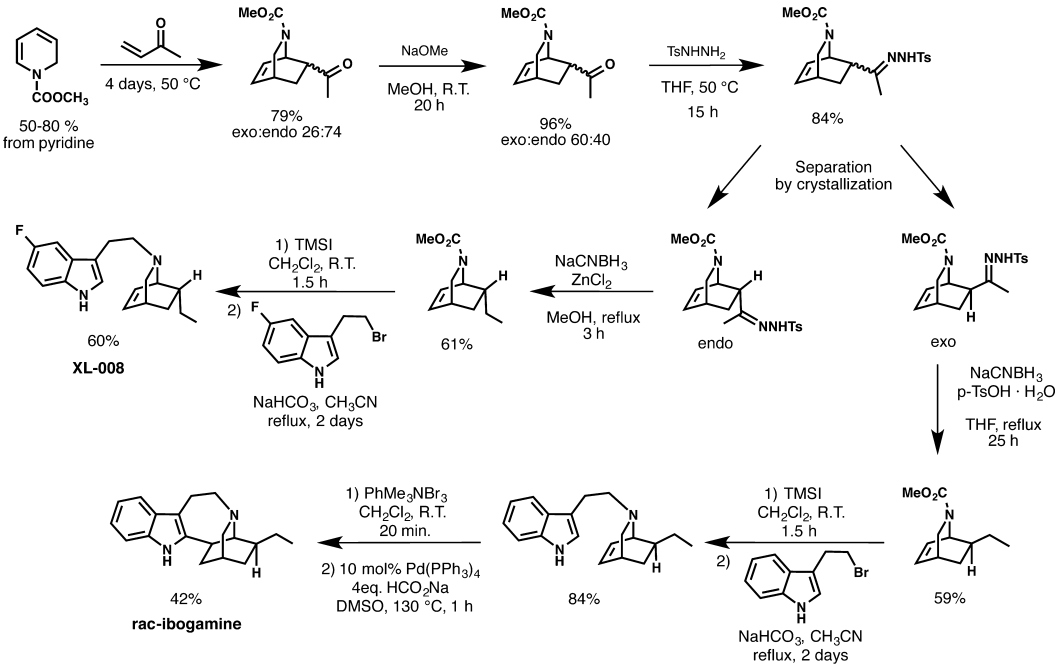
Scheme 1. Synthesis of XL-008 and Ibogamine



GDNF and other neurotrophic factors such as brain-derived neurotrophic factor (BDNF), fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF) have become increasingly recognized for their important role in mood disorders and addiction.[36−39] Recent evidence indicates that there is cross-talk between different neurotrophins and growth factors; for example, it was shown that fibroblast growth factor 2 (FGF2) induces GDNF release in C6 glioma cells as well as in human neuroblastoma and glioblastoma cell lines, via activation of FGF receptor 1 (FGFR1).[40,41] The fibroblast growth factor system, currently comprised of four fibroblast growth factor receptors (FGFRs) and 18 fibroblast growth factors (FGFs), make up a complex system that plays critical roles in the development, maintenance, and regeneration of CNS tissues.[36,42−44] Additionally, the FGF system has been directly implicated in neuropsychiatric disorders such as depression and anxiety. Post-mortem analysis of brains of patients with major depressive disorder showed decreased levels of both FGF2 and FGFRs.[45] Therefore, the FGF system and its connection to GDNF release is an interesting area to pursue in the search for novel mechanisms mediating neuroplasticity and neurorestoration. As proteins are not typically able to cross the blood−brain barrier, small molecules capable of modulating neurotrophic factor signaling *in situ* represent a frontier in the treatment of complex neuropsychiatric diseases.[46−51]

Herein, we describe the novel *iboga* analog XL-008, its synthesis, and its ability to induce GDNF release from C6 glioma cells. Not only does XL-008 induce GDNF release on its own, but it also greatly potentiates the GDNF release by FGF2. Additionally, the GDNF release by FGF2 and XL-008 was found to depend on activation of the MEK and PI3K pathways of signal transduction but not the PLCγ pathway.

RESULTS AND DISCUSSION

Synthesis of Compounds. Compound XL-008 and ibogamine were prepared in racemic form according to a divergent Diels−Alder strategy as previously described (Scheme 1).[52] Briefly, the isoquinuclidine fragment was synthesized by a Diels−Alder reaction between a protected dihydropyridine and methyl vinyl ketone. The 7-acetyl group was then reduced via the tosylhydrazone to provide both the *endo*- and *exo*-isoquinuclidine fragments. These were deprotected and alkylated with bromoethylindoles to provide *N*-heteroarylalkylisoquinuclidines, including XL-008. For ibogamine, the *endo*-isoquinuclidine was epimerized and reduced by a similar sequence. Treatment of the appropriate intermediate with trimethylphenylammonium tribromide afforded selective bromination at the indole 2 position. This crude aryl bromide intermediate was then cyclized under reductive Heck conditions to provide *rac*-ibogamine.

GDNF Release from C6 Glioma Cells. It has been previously established that GDNF release can be measured in the growth medium of conditioned C6 rat glioma cells using conventional ELISA with basal levels between 6 and 81 pg/mL.[53] C6 glioma cells, a model for astrocytes, are known to express the mRNA of GDNF, as well as Ret and GFRα1, and can therefore be utilized for observing GDNF release induced by different compounds.[54] To assay the GDNF release induced by novel compounds, C6 cells were incubated with test compounds for 24−48 h, and GDNF was then detected in the conditioned media with picogram sensitivity using a standard sandwich-style ELISA. Extensive optimization of cell culture and release conditions was performed in order to obtain reproducible experiments (see Methods below). XL-008 was initially identified as a superior releaser of GDNF in a screen of ibogamine analogs. In particular, its release far surpassed that of ibogamine (Figure 1C), indicating the importance of the key disconnection in the *iboga* skeleton between the isoquinuclidine and indole 2-position to form "acyclic" analogs (Figure 1B and also see Supporting Information, Figures S1 and S5). We noted that the 48-h release experiments initially conducted (Figure S1A), which allowed for the production of significant GDNF, were stressful to the cells and resulted in marked cytotoxicity (Supporting Information, Figures S1 and S2). Therefore, all

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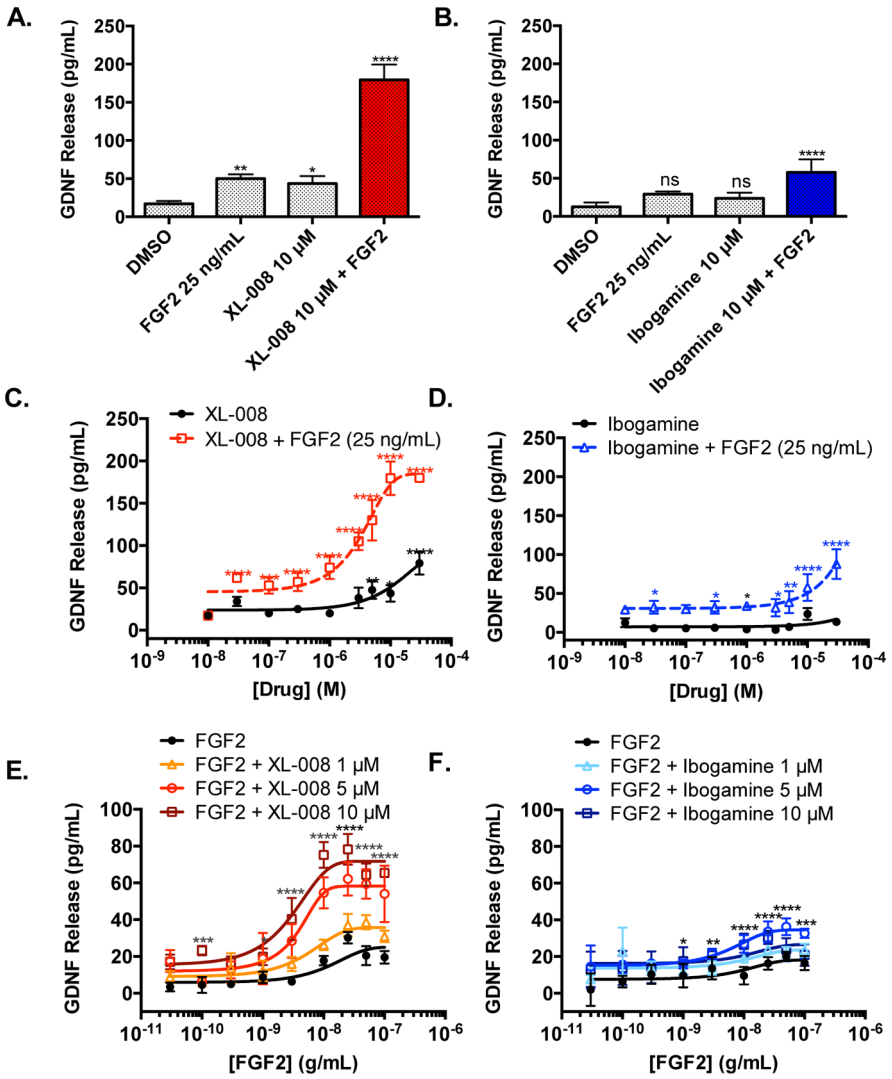

Figure 2. FGF2-induced GDNF release in C6 cells is potentiated by *iboga* analog XL-008. (A) Fibroblast growth factor 2 (FGF2)-induced GDNF release is greatly enhanced by XL-008 in C6 cells after a 24-h treatment time. (B) Ibogamine gives a much smaller induction effect in comparison to XL-008. (C) FGF2 (25 ng/mL) potentiates the dose response of XL-008 from an EC_{50} > 15 μM to 6.17 ± 2.40 μM (n = 4). (D) This effect is only additive on the dose response of ibogamine. (E) The dose response curve of FGF2 is potentiated by XL-008 in a dose-dependent manner. (F) The effect on the FGF2 curve is less pronounced in the presence of ibogamine. Data represent mean ± SD of biological replicates in one experiment from n > 4 independent experiments. One-way ANOVA with Dunnett's Multiple Comparisons Test are shown (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

GDNF release experiments were conducted using a 24-h treatment. While the GDNF release was not as high at this time point, the GDNF release from XL-008 was readily measurable (and statistically significant), while cytotoxic effects were minimized.

Potentiation of FGF2-Induced GDNF Release. In addition to studying the effects of XL-008 on GDNF release from C6 cells, we were also interested in investigating any potential GDNF release caused by growth factors, FGF2 in particular (see the introduction section for the rationale). FGF2 afforded robust GDNF release consistent with previous reports, and thus we explored the potential interactions of XL-008 and FGF2 on GDNF release.[40,55] A competition experiment was performed in which XL-008 and FGF2 were coincubated to determine if their GDNF releasing effects were additive, and surprising results were obtained. The GDNF release induced by FGF2 (25 ng/mL) was greatly increased in the presence of 10 μM XL-008 (Figure 2A). In comparison, ibogamine's effects on the GDNF release induced by FGF2 were only additive (Figure 2B). Not only does XL-008 induce release of GDNF independently, but it also potentiates the GDNF release by another pharmacologically relevant target, FGFR. While both FGF2 and XL-008 each induce a statistically significant release of GDNF alone, their effect together is almost 2-fold higher than additive.

Intrigued by this apparent potentiation, the effect of FGF2 (25 ng/mL) on the GDNF release elicited by varying concentrations of XL-008 was measured. FGF2 both increases the efficacy of GDNF release by XL-008 in C6 glioma cells and potentiates the dose response curve, shifting the EC_{50} from more than 15 μM to 6.17 ± 2.40 μM (n = 4), a greater than 2-fold increase in potency (Figure 2C). Due to the cytotoxic effects of treatment with higher concentrations of XL-008, a full dose response curve for XL-008 alone was not obtained. Concentrations greater than those measured (i.e., more than 30 μM) were highly toxic even in the 24-h treatment, as

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determined by visual observation, lactate dehydrogenase (LDH) assay, and water-soluble tetrazolium (WST-1) assay; therefore only an approximate EC_{50} has been noted for comparison. The effects of FGF2 on ibogamine's GDNF release were less pronounced. Ibogamine trends toward GDNF release but does not reach statistical significance. However, in the presence of FGF2, ibogamine increases GDNF release in a statistical manner, albeit only adding to the efficacy of this release rather than potentiating it, as in the case of XL-008 (Figure 2D, $n = 3$).

The potency of GDNF release induced by FGF2 was then measured in the presence of a range of concentrations of both ibogamine and XL-008. XL-008 increased the potency of FGF2-induced GDNF release more than 2-fold, from an EC_{50} of 7.85 ± 2.59 ng/mL to 3.31 ± 0.98 ng/mL with 10 μM XL-008 ($n = 5$, Figure 2E). Statistical analysis with Tukey's multiple comparisons test showed that this change in potency was statistically significant when compared to the EC_{50} of FGF2 alone ($^*p < 0.01$). The shift in the potency can also be seen with lower concentrations of XL-008; 5 μM XL-008 shifts the EC_{50} to 4.45 ± 2.68 ng/mL. Again, the effect is weaker with ibogamine. Lower concentrations of ibogamine, such as 1 and 5 μM, have little effect on the GDNF release induced by FGF2, but there is some increase in GDNF release by a 10 μM cotreatment of ibogamine, which shifts the EC_{50} to 4.15 ± 2.22 ng/mL (Figure 2F). This shift in potency is not statistically significant, unlike that with XL-008, which further highlights the superiority of XL-008 as a potentiator of GDNF release by FGF2. The results of Figure 2 are summarized in Table 1.

Table 1. Summary of GDNF Release Data

Treatment	EC_{50}
ibogamine	NS
ibogamine + FGF2 (25 ng/mL)	>15 μM
XL-008	>15 μM
XL-008 + FGF2 (25 ng/mL)	6.17 ± 2.40 μM
FGF2	7.85 ± 2.59 ng/mL (0.482 ± 0.159 nM)
FGF2 + ibogamine 1 μM	6.87 ± 2.08 ng/mL (0.422 ± 0.128 nM)
FGF2 + ibogamine 5 μM	6.24 ± 2.88 ng/mL (0.383 ± 0.177 nM)
FGF2 + ibogamine 10 μM	4.15 ± 2.22 ng/mL (0.255 ± 0.136 nM)
FGF2 + XL-008 1 μM	6.87 ± 3.00 ng/mL (0.422 ± 0.184 nM)
FGF2 + XL-008 5 μM	4.45 ± 2.68 ng/mL (0.273 ± 0.165 nM)
FGF2 + XL-008 10 μM	3.31 ± 0.98 ng/mL[a] (0.203 ± 0.060 nM)

[a]One-way ANOVA with Tukey's Multiple Comparisons Test shows $^*p < 0.01$ for FGF2 + XL-008 10 μM when compared to FGF2 alone. NS means "not significant." GDNF, glial-cell-line-derived neurotrophic factor; FGF2, fibroblast growth factor 2.

Pathway Specificity of FGF2-Induced GDNF Release. After identifying the novel potentiation of FGF2-induced GDNF release from C6 cells, the mechanism of this process was studied. Significant effort was dedicated to the use of conventional methods for observing the various signal transduction pathways involved in GDNF release. First, Western blotting was employed to measure activation of the ERK1/2 pathway and RET, the latter being the kinase involved in GDNF activation of GFRα1. Due to the tumorogenic nature of this glioma cell line and probable overexpression of many receptors, the basal levels of ERK1/2 activation in Western blotting were high, making acute signaling events impossible to detect in a reliable manner. Additionally, the ERK1/2 pathway was found to be extremely sensitive to movement, temperature, and even the vehicle control DMSO, further masking any pathway activation that might have otherwise been observed via Western blot. Considering these difficulties, we turned to more sensitive techniques for signaling pathway observation, such as cell-based ELISA for measuring both ERK1/2 and AKT phosphorylation. Unfortunately, our results were again confounded by the high basal levels of kinase activation. Therefore, more indirect methods for elucidating the pathway of potentiation were utilized.

Small molecule inhibitors were chosen that could pharmacologically block the different possible pathways of signal transduction activated in XL-008-induced potentiation of GDNF release. For the three major pathways of signal transduction, protein kinase B (PKB or AKT), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC), inhibitors were chosen that have been shown to be selective and effective at blocking activation through these proteins.[56] The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor LY294002 (20 μM),[55,57] the mitogen-activated protein kinase kinase (MEK1/2) inhibitor U0126 (10 μM),[22,58] and the phospholipase C (PLC-γ) inhibitor U73122 (2 μM)[47,58] were used as upstream inhibitors of the AKT, MAPK, and PKC pathways, respectively. Furthermore, due to the possible involvement of the FGF2 receptors, the FGFR inhibitor PD173074 (1 μM)[59−61] was chosen for treatment. Additionally, the potential involvement of other growth-factor-activated receptor tyrosine kinases (RTKs) was studied. The dual platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) inhibitor KRN633 (1 μM)[58,62] was used in these experiments to determine whether transactivation by these receptors was involved in the GDNF signaling. The structures and targets of the inhibitors used are summarized in Table 2.

The GDNF release induced by FGF2 alone was dependent on the AKT and MAPK pathways but not the PKC pathway (Figure 3A), which is in agreement with previously reported results.[48,55,58] Additionally, the GDNF release was dependent upon FGFR but not PDGFR or VEGFR (also confirming the selectivity of the RTK inhibitor). Similar trends were noted in the GDNF release of XL-008 alone; however, interestingly, the GDNF release from XL-008 does show some dependence on the PDGFR and VEGFR pathways (Figure 3B), suggesting a possible mechanism for GDNF release that may be unique when compared to FGF2-induced GDNF release. Importantly, the potentiation of FGF2 by XL-008 was dependent on the same pathways as FGF2 alone (Figure 3C). While the exact target responsible for XL-008-induced GDNF release is unknown, it is clear that the signaling involved in the potentiation is connected to that of FGFR. As a control, the GDNF release induced by the inhibitors alone was measured. This showed that they did not release any statistically significant amount of GDNF when compared to the DMSO vehicle control (Figure 3D). A recent report has also commented on the selectivity of certain protein kinase inhibitors used here. Inhibitors U0126 and LY294002 were screened against a panel of 70−80 protein kinases for off-target activity. The selectivity of U0126 for the MAPK pathway was confirmed, and while LY294002 had some noticeable activity at off-target proto-oncogene kinases (including PIM1 and PIM3), it still remains the better AKT inhibitor choice over wortmannin for longer experiment durations.[56] We are therefore confident in the involvement of the observed signaling in GDNF release.

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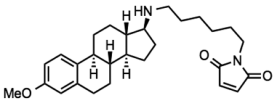
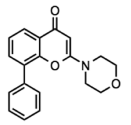
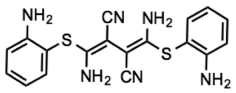
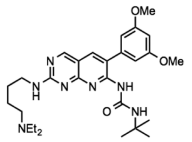
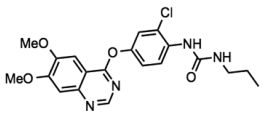
Table 2. Pharmacological Inhibitors of GDNF Release

Inhibitor	Structure	Target
U73122[a]		PLCγ
LY294002[b]		PI3K
U0126[c]		MEK-1/2
PD173074[d]		FGFR-1, FGFR-3
KRN633[e]		VEGFR, PDGFR

[a]Refs 47 and 58. [b]Refs 55 and 57. [c]Refs 22 and 58. [d]Refs 59−61. [e]Refs 58 and 62. PLCγ, phospholipase C; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; MEK-1/2, mitogen-activated protein kinase kinase; FGFR-1, fibroblast growth factor receptor 1; FGFR-3, fibroblast growth factor receptor 3; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor.

A proposed model for the signaling involved in FGF-induced GDNF release from C6 cells is portrayed in Figure 4. XL-008 acts through a target that either directly amplifies the signaling events leading to increased GDNF production or transactivates FGFRs to further increase GDNF production through the relevant pathways. Transactivation of FGFRs by XL-008 is a less likely target for the increased production of GDNF since the FGFR inhibitor PD173074 does not fully block GDNF release of XL-008 on its own (see Figure 3B). It is also possible that XL-008 directly activates FGFRs to lead to the observed results. However, the lack of complete inhibition by PD173074 makes this conclusion less likely. The likelihood of direct FGFR agonism is further diminished by the inability of XL-008 to elicit phosphorylation of FGFR1 in FGFR1-HEK cells (see Supporting Information, Figure S4). Additionally, XL-008 does not cause release of FGF2 itself (see Supporting Information, Figure S6). Therefore, the direct molecular target by which XL-008 exerts its GDNF modulating effects remains to be identified and most likely occurs downstream of FGFR1.

Cell Viability and Toxicity Effects of XL-008/FGF2. Given the protective and proliferative effects known for FGF2 alone, it was important to confirm that potentiation of FGF2-induced GDNF release also retained some of these same cellular effects. We examined whether there is a correlation between GDNF release and cytotoxicity in the 24-h release experiments.

Two independent measurements of cell viability were performed. LDH is a cytosolic enzyme that is released upon cell lysis. Simple colorometric detection can be used to measure the amount of LDH present in conditioned media, which directly correlates with the integrity of the cell membrane and, therefore, with cell health.[63] Another measure of cell viability is the WST-1 cell assay, which measures cell viability or cytotoxicity using colorometric detection of a formazan dye that forms as a result of intact, metabolizing mitochondria.[62] These two assays were used together to identify the cytotoxic effects, if any, occurring concurrently with GDNF release.

The GDNF release induced by increasing concentrations of XL-008 in the presence or absence of FGF2 (25 ng/mL) following 24-h treatment in C6 cells showed no cytotoxic effects as measured by LDH release (Figure 5A). Therefore, unlike the 48-h treatment, there is no correlation between GDNF release and cytotoxicity under these experimental conditions. Furthermore, the addition of the selected inhibitors to the induction conditions with FGF2, XL-008, or FGF2/XL-008 resulted in no cytotoxicity as measured by LDH release (Figure 5B). Only the addition of inhibitors alone sometimes caused a statistically significant release of LDH when compared to DMSO, as seen for inhibitor U0126 (which also shows the protective effects of FGF2/XL-008). The WST-1 assay further supported these results (Figure 5C), showing that, in general, treatments caused increased metabolism (a measure of cell viability) that was statistically significant when compared to DMSO, consistent with increased production of GDNF. The only exception was with LY294002 pretreatment, which consistently displayed cell viability-reducing effects. As the PI3K/AKT pathway has been widely connected to cell survival and proliferation,[64,65] the reduced cell viability caused by LY294002 was not surprising. Interestingly, it was found that actual levels of cell proliferation, as measured through bromodeoxyuridine (BrdU) incorporation, were not increased during these treatments, indicating that the increases in cell viability observed were independent of proliferation and likely occur via a metabolism-boosting effect (see Supporting Information, Figure S7). The LDH release and cell viability were also measured for the dose response of FGF2 in the absence or presence of increasing concentrations of XL-008. Again, there were few cytotoxic effects under these treatment conditions as measured by LDH release (Figure 5D). Only the highest concentrations of FGF2 in the presence of XL-008 resulted in elevated LDH levels in some experiments, but the overall LDH release from the "toxic" treatments was much lower than for those seen in the 48-h experiments (~12% LDH activity at 24 h compared to >30% at 48 h). In addition, the WST-1 assay showed that cell viability was enhanced with increasing concentrations of both FGF2 and XL-008 (Figure 5E). These results indicate that the combined treatment of FGF2 with XL-008, in fact, promotes cell viability and metabolism at each concentration tested, showing protective effects under these experimental conditions. Similar LDH and WST-1 data and trends were observed for ibogamine and can be found in the Supporting Information (Figure S3). Consistent with potentiation of GDNF release, XL-008 also enhanced the viability effects of FGF2, most importantly even at concentrations where FGF2 alone had no effect (sub-nanomolar concentrations, Figure 5E). These results indicate that compound XL-008 potentiates the effects of FGF2 signaling, in the latter case using cell viability as a distinct cellular readout. In this assay, the viability effects were dependent on the PI3K/AKT pathway (Figure 5C). In the 48-h treatment experiments, however, trends in GDNF release correlated with cytotoxic effects as measured by LDH release

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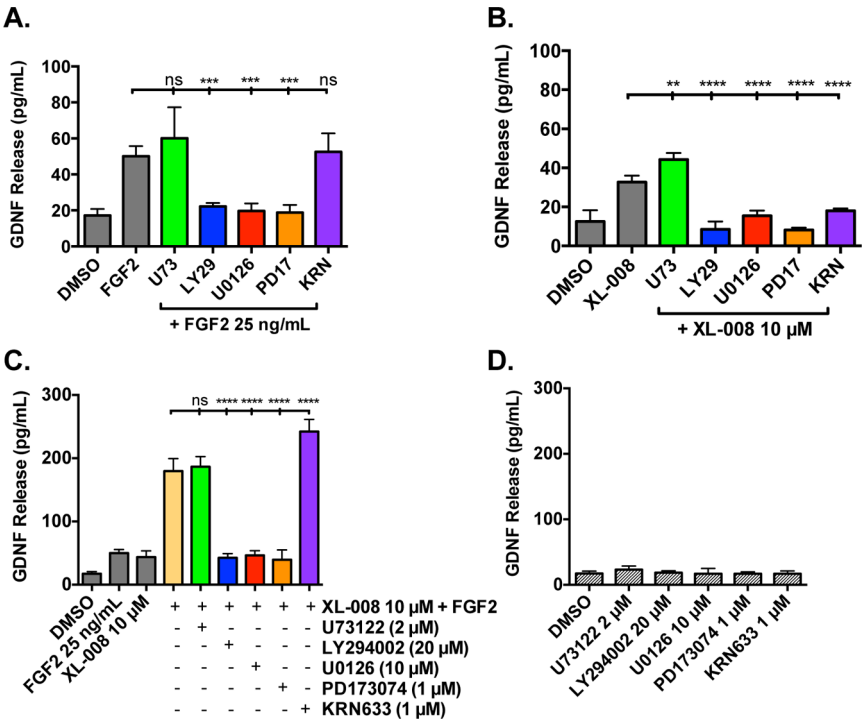

Figure 3. Pathway specific potentiation of FGF2-induced GDNF release by *iboga* analogs. (A) The GDNF release by FGF2 in C6 glioma cells after 24 h is mediated by the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Pretreatment of cells with inhibitors for 1 h (30 min for U0126, as reported[59]) indicates involvement of PI3K (LY294002, 20 μM), MAPK (U0126, 10 μM), and fibroblast growth factor receptor (FGFR; PD173074, 1 μM) pathways but not the phospholipase C (PLCγ) (U73122, 2 μM) and platelet-derived growth factor receptor/vascular endothelial growth factor receptor (PDGFR/VEGFR) (KRN633, 1 μM) pathways. (B) The GDNF release by XL-008 in C6 glioma cells after 24 h shows similar pathway activation to that seen from FGF2 alone with the exception of some inhibition by PDGRF/VEGFR inhibitor KRN633. (C) The GDNF release by XL-008/FGF2 in C6 glioma cells after 24 h shows similar pathway activation to that seen from FGF2 alone. (D) No GDNF release is observed in the presence of the inhibitors alone in C6 glioma cells after 24 h. Data represent mean ± SD of biological replicates in one experiment from $n = 9$ independent experiments. One-way ANOVA followed by Dunnett's Multiple Comparisons Test is shown (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

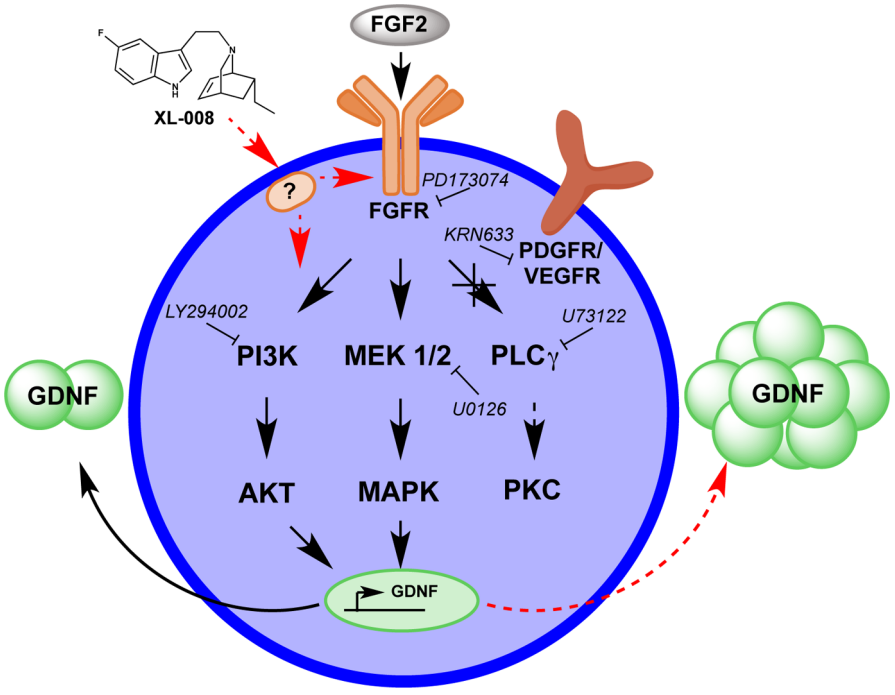

Figure 4. Schematic representation of signaling pathways involved in potentiation of FGF2-induced GDNF release by XL-008. Pharmacological inhibition of XL-008/FGF2 reveals pathway specificity through MAPK and AKT.

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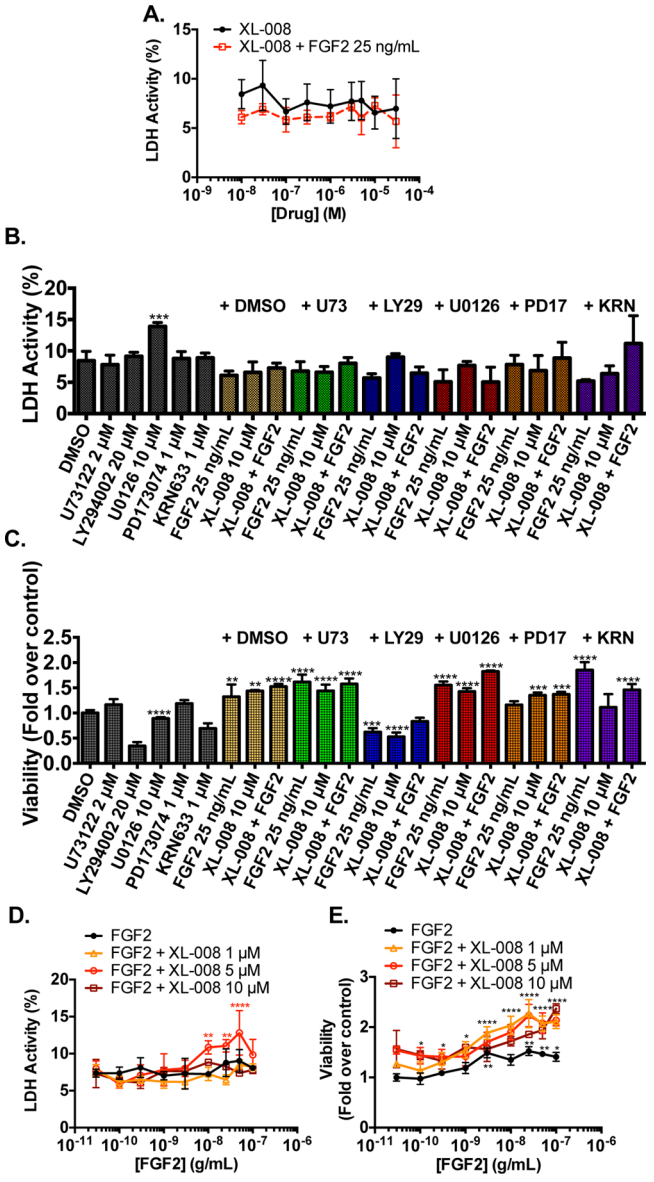

Figure 5. Cell viability and cytotoxicity studies. Potentiation of FGF2-induced GDNF release by XL-008 also shows cell viability enhancing effects and little to no cytotoxicity as measured by lactate dehydrogenase (LDH) release and cell viability assays. (A) LDH release after 24 h of XL-008 in the presence of FGF2 (25 ng/mL) reveals no cytotoxic effects when compared to the DMSO vehicle control. (B) LDH release in the presence of XL-008/FGF2 and the kinase inhibitors also indicates no cytotoxic effects, with the exception of a small LDH release in the presence of ERK inhibitor U0126 alone. (C) Cell viability measurement by tetrazolium (WST-1) assay shows minor cytotoxic effects in the presence of kinase inhibitors. (D) LDH release of varying concentrations of FGF2 in the presence of XL-008 is increased only at higher concentrations of FGF2/XL-008 potentiation mixtures. (E) Cell viability as measured by the WST-1 assay reveals no cytotoxic effects from the 24-h treatment at increasing concentrations of FGF2/XL-008, where cell viability and metabolism is increased (nearly 2-fold). Data represent mean ± SD of biological replicates in one experiment from $n > 4$ independent experiments. One-way ANOVA followed by Dunnett's Multiple Comparisons Test is shown ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

(Supporting Information, Figure S1), providing additional reasons to avoid this extended treatment time for GDNF release.

Conclusions. The induction of growth factor synthesis and release and/or potentiation of growth factor signaling with small molecule therapeutics presents a novel approach for treating neurological, neurodegenerative, and psychiatric disorders.[50,51] In this report, we have demonstrated that the new compound XL-008 is able to increase GDNF release on its own in the well-established glial cell model, as well as potentiate the release of GDNF induced by FGF2. The potentiation of the FGF2 signaling was also confirmed by increased cell viability (cell phenotype readout). Although the molecular target for XL-008 was not identified, we were able to pinpoint the key kinase signaling pathways involved in the induction of GDNF release and cellular viability using pharmacological tools (kinase inhibitors). We also demonstrated that XL-008 does not activate FGFR1, employing ELISA for receptor phosphorylation and FGFR1 inhibitors, consistent with a mechanistic model where XL-008 acts downstream of FGFR1. The downstream effects of FGF2-induced signaling have been previously connected with many desirable physiological, cellular, and behavioral outcomes spanning a wide temporal spectrum, such as modulating neuronal spiking dynamics, inducing neurogenesis, and exerting antidepressant and anxiolytic effects.[37,66] Therefore, the identification of a small molecule that potentiates FGF2 is relevant to the search for new therapeutic leads. Future studies will be aimed at examining this new *iboga* analog and related compounds in brain tissue and *in vivo*.

METHODS

Chemical synthesis of XL-008 and ibogamine was carried out as reported previously and can be found in the Supporting Information.[52]

Reagents. Recombinant rat fibroblast growth factor basic (FGF2, 400-29) was purchased from Peprotech. Protease inhibitor cocktail (P8340) and phosphatase inhibitor cocktail 2 (P5726) were purchased from Sigma-Aldrich. KRN633 was purchased from Selleck Chemicals. LY294002 was purchased from Cayman Chemical Company. PD173074 was purchased from Biotang, Inc. U0126 was purchased from Alfa Aesar, and U73122 was purchased from MP Biomedicals, LLC.

Cell Culture. Rat C6 glioma cells were purchased from the American Type Culture Collection (CC-107) and maintained in Dulbecco's Modified Eagle Medium (Life Technologies; 10569) with 5% (v/v) fetal bovine serum (FBS, Atlanta Biologicals) and 100 U mL^{-1} of penicillin and streptomycin (Life Technologies). Cells were incubated at 37 °C with 5% CO_2 humidified atmosphere. The GDNF release in C6 cells is highly variable from one experiment to another and even from one passage to the next. Additionally, as a glioma cell line, C6 cells are highly susceptible to phenotypic drift, which can lead to varying expression levels of receptors and growth factors of interest. For the purposes of these experiments, it was found that if the C6 cells were maintained and used between a strict set of passages, experiments (though variable) provided reliable trends in GDNF release that were highly reproducible. Therefore, all data presented here show a single representative experiment of many independent replicate trials. C6 glioma cells were used between passages 41−42.

GDNF Release Experiments. Into a 96-well plate were added C6 cells at a density of 25 300 cells/well in full growth medium (see above). Cells were allowed to adhere for 24 h at 37 °C. Cells were then serum-starved with media containing 0.5% FBS (low serum) for an additional 24 h. Low serum media was refreshed prior to starting the experiment. Compounds were added in 50 μL of low serum media to obtain a final volume of 200 μL/well. All inhibitors were added for 1 h in advance with the exception of U0126, which was pretreated for 30 min, as reported.[59] Treatments were performed in quadruplicate. Cells were incubated at 37 °C for 24 h. Experiments were terminated by removing the conditioned media from each well and storing them at

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−80 °C until analyzed. GDNF was detected using a standard sandwich-style ELISA kit purchased from Promega Corporation following the manufacturer's instructions. Briefly, monoclonal anti-GDNF antibodies were captured onto a 96-well Nunc Immulon Immunoassay plate at a dilution of 1:1000 in carbonate coating buffer (25 mM sodium bicarbonate, 25 mM sodium carbonate, pH 8.2) overnight at 4 °C. After removing the monoclonal antibody, wells were blocked with 1X block and sample buffer for 1 h at RT (200 μL/well). A GDNF standard curve was created by serially diluting the recombinant human GDNF standard in 1X block and sample buffer to a concentration range of 0−1000 pg mL^{-1}. To each sample well was added 100 μL of conditioned media from above and the standard curve (in duplicate), and plates were incubated for 6 h with shaking at RT. After washing five times with TBST (150 mM sodium chloride, 10 mM Tris·HCl, 10 mM Tris base, 0.05% Tween 20, pH 7.6), wells were incubated with antihuman polyclonal GDNF antibodies (1:500) in 1X block and sample buffer overnight at 4 °C. Following an additional five washes with TBST, wells were incubated with antichicken IgY-HRP conjugate antibody (1:250) for 2 h with light shaking. After a final five washes, TMB One (100 μL/well) was added to each well and allowed to develop in the absence of light until there were clear differences in color between the highest and lowest concentrations of the standard curve. Wells were then quenched with 1 M HCl (100 μL/well), and the plates were read at an absorbance wavelength of 450 nm using a BioTek Synergy H1 plate reader.

LDH Cytotoxicity Assay. The lactate dehydrogenase cytotoxicity assay (Promega) was performed following the manufacturers instructions. Briefly, following compound treatment, conditioned media were removed, and untreated wells were washed twice with phosphate buffered saline. To untreated wells was added 40 μL of low serum media supplemented with lysis buffer provided in the kit (1:10), protease inhibitor cocktail (1:100), and phosphatase inhibitor cocktail 2 (1:100). Cells were lysed at 37 °C for 1 h. Cell lysates were diluted with 160 μL of conditioned media and used as 100% cytotoxicity in the LDH standard curve. Lysates were serially diluted down to 6.25% cytotoxicity with low serum media filling the last. The standard curve was added in duplicate to a 96-well plate followed by the conditioned media from each treated well at 50 μL/well. To each well was added 50 μL of the reconstituted substrate mix, and the plates were allowed to develop in the dark until differences were seen in the standard curve. The wells were quenched with 50 μL of stop solution, and the plates were read at an absorbance wavelength of 490 nm.

WST-1 Cell Viability Assay. After compound treatment, conditioned media were removed and replaced with 75 μL of warm low-serum media. To each well was added 5 μL of WST-1 Cell Proliferation Reagent (Roche Applied Science), and the cells were incubated at 37 °C for no more than 1 h. Plates were briefly shaken prior to reading the absorbance at 450 nm. Treatments were compared to vehicle control.

Statistical Analysis. Data analysis was performed using Graphpad Prism 6 Software (San Diego, CA). Conditions are expressed as mean ± SD and were subjected to ANOVA followed by either Dunnett's or Tukey's Multiple Comparisons Test with a significant level of $p < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00678.

Figures S1−S7 and synthetic procedures (PDF)

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Notes

The authors declare no competing financial interest.

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