Small-molecule agonists of the RET receptor tyrosine kinase activate biased trophic signals that are influenced by the presence of GFRα1 co-receptors

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Abstract

Glial-cell line-derived neurotrophic Factor (GDNF) is a growth factor that regulates the health and function of neurons and other cells. GDNF binds to GDNF family receptor alpha 1 (GFRα1), and the resulting complex activates the RET receptor tyrosine kinase and subsequent downstream signals. This feature restricts GDNF activity to systems in which GFRα1 and RET are both present, a scenario that may constrain GDNF breadth of action. Furthermore, this co-dependence precludes the use of GDNF as a tool to study a putative functional cross-talk between GFRα1 and RET. Here using biochemical techniques, TUNEL staining, and immunohistochemistry in murine cells, tissues, or retinal organotypic cultures, we report that a naphthoquinone/quinolinedione family of small molecules (Q compounds) acts as RET agonists. We found that, like GDNF, signaling through the parental compound Q121 is GFRα1-dependent. Structural modifications of Q121 generated analogs that activated RET irrespective of GFRα1 expression. We used these analogs to examine RET–GFRα1 interactions and show that GFRα1 can influence RET-mediated signaling and enhance or diminish AKT Ser/Thr kinase (AKT) or extracellular signal–regulated kinase (ERK) signaling in a biased manner. In a genetic mutant model of retinitis pigmentosa, a lead compound, Q525, afforded sustained RET activation and prevented photoreceptor neuron loss in the retina. This work uncovers key components of the dynamic relationships between RET and its GFRα1 co-receptor and provides RET agonist scaffolds for drug development.

Introduction

Naphthoquinones are structurally and biologically diverse molecules of natural and synthetic origin, that have been studied extensively as drug leads (1). The mechanisms of action are quite varied and the biological outcomes range on a spectrum, with many variables governing the balance between cytotoxicity and cytoprotection (2). Activities include wide and non-selective inhibition of protein tyrosine phosphatases (PTPs) (3); but there are examples of selective PTP inhibition (4). Recent work with the natural quinones shikonin (5) and plumbagin (6) indicated that this class can generate a wide range of cytotoxic or protective effects, from the regulation of inflammatory and stress mediators such as TNFa and pMAPK, to the activation of pro-survival signals through growth factors and their receptors.

Hence, analogs of naphthoquinone scaffolds may be used to potentially generate more selective activity. We focussed on activation of neurotrophic receptors to regulate neuronal health and function after stress or injury (7). Specifically, we studied mechanisms related to Glial cell line-Derived Neurotrophic Factor (GDNF), a growth factor that was tested in trials for Parkinson’s (8). GDNF therapy has also been proposed for retinal degenerative diseases, and showed early promise in animal models of Retinitis Pigmentosa (RP) (9-11). RP is a blinding condition resulting from the progressive loss of photoreceptors. Since over 300 different mutations (e.g. in the Rodopsin gene) can give rise to the RP phenotype, there is a need for a broad-spectrum neuroprotective strategy (12).

So far, GDNF therapy has failed clinically. The reasons for slow therapeutic progress include poor pharmacokinetics, stability, and distribution. This means it must be given continuously in order to be efficacious, and the potential for side-effects is a concern. Another problem is related to the way by which GDNF activates trophic signals. GDNF must bind to a receptor GFRα and only then it can activate a receptor...
tyrosine kinase RET which mediates the trophic signals. This requirement by GDNF of a co-receptor narrows the breadth of responding cells to those that can access both subunits. A small molecule selective activator of RET, irrespective of GFRα expression, might circumvent all these therapeutic problems. Moreover, such molecules could be used as chemical-biology tools to evaluate RET-GFRα functional interactions.

In the present work we report the development of novel substituted naphthoquinones along with their corresponding quinoline forms, and show that they act as selective agonists for the RET tyrosine kinase receptor, activating the downstream effectors Akt and Erk. Some agents are agonistic regardless of whether or not the GFRα1 receptor is present. For other agents, GFRα1 receptor expression causes biased RET signaling with differential effects upon pAkt and pErk activation, and this can be regulated positively or negatively by the reported GFRα1 modulator XIB4035. A GFRα1–independent RET agonist Q525 was neuroprotective in a mouse model of RP, generating trophic signals in vivo within the Muller glial cell population, thus validating RET as a druggable therapeutic target, and suggesting potential utility for therapy of neurodegenerative diseases.

**Results**

**Identification of novel naphthoquinone RET activators/modulators**

All compounds and structures are summarized in Table 1 in the order that they are mentioned. Biochemical assays revealed that the chlorinated methoxy 1,4 naphthoquinone Q121 activated pAkt and pErk in MG87 RET/GFRα1 cells. Q121 induced RET phosphorylation in a dose-dependent manner (blots were probed using mAb 4G10 for total p-Tyr residues) (Fig 1A). Compound Q128 with an (–OH) substituting the (–OCH3) group of Q121 was inactive (Fig 1A), indicating the functional relevance of the –OCH3 adduct. These data provide a rationale for further substitutions of the core structure at this position.

To further evaluate RET phosphorylation by Q121 as well as its potential requirement of GFRα1 expression (which GDNF requires), immunoprecipitation experiments were performed. Phospho-RET was significantly increased by treatment of 100 µM. However, in MG87 RET cells lacking GFRα1, there were no increases in RET phosphorylation detected above background vehicle control (Fig 1C). These data indicate that Q121, like GDNF, requires GFRα1 to signal through RET.

Compounds coded Q101, Q105, Q112, Q141, Q143, Q151, and Q1047 were then synthesized (See Materials and Methods, Supplemental Data) and evaluated in biochemical assays. Significant increases in pAkt and pErk were observed with compounds Q105, Q112, Q141, Q151, and Q1047, while Q101 and Q143 were inactive (Fig 1C). However, counter-assays using MG87 cells expressing the NGF receptor TrkA tyrosine kinase (instead of RET) showed significant activation of pAkt and pErk even though RET is absent. This indicates that these quinone derivatives lack selectivity (Fig 1D).

In spite of poor RET selectivity, compounds such as Q151 remained interesting because they induced RET phosphorylation whether or not the GFRα1 co-receptor was present (Fig 1E). These data suggest that the modifications to the side-chain of position A (Table 1) allows GFRα1-independence.

One concern with these agents is that significant cytotoxicity was observed in the 1-10 µM range when these compounds were screened via the MTT survival assay; this was therefore addressed in further analogs.
Chlorine substitution affects potency and toxicity, and generates GDNF-modulating compounds

To address cytotoxicity, we evaluated substitutions of the chlorine atom at position B (Table 1) because the ring-associated chlorine can enhance chemical reactivity and oxidative toxicity (2). Compounds Q2003 and Q2004, bearing a hydrogen in place of the chlorine, were prepared.

Biochemical assays revealed these compounds to be significantly less potent, with non-significant pAkt/pErk activation even when assayed at a high µM concentration range (data not shown). Moreover, in biological assays measuring cell survival, both Q2003 and Q2004 displayed weak trophic activity which was non-selective and was detectable in MG87 RET/GRFa1 as well as in MG87 TrkA cells. In MTT assays Q2003 and Q2004 supported cell viability to 8-18% of the survival induced by optimal growth factor concentrations (GDNF for RET/GRFa1-expressing cells and NGF for TrkA-expressing cells) (Fig 1F and 1G).

While Q2003 and Q2004 lacked potency and selectivity, they remained interesting because their activity was additive in MG87 RET/GRFa1 with sub-optimal doses of GDNF (Fig 1F), and this effect was not observed in MG87 TrkA with sub-optimal doses of NGF (Fig 1G).

Therefore, although the survival induced by Q2003 and Q2004 can be RET-independent (i.e. the compounds afford survival to MG87 TrkA cells), the RET/GDNF axis is involved in the functional outcome because the agents potentiate GDNF trophic action but they do not potentiate NGF trophic action.

To assess the basis of toxicity by some compounds, MTT assays were performed with cells growing in serum, to assess negative effects on growth/survival over 72 hrs. At 1 µM, both Q2003 and Q2004 (lacking the chlorine) showed no signs of causing cell death (Fig 2). In contrast, Q1047, identical to Q2003 but including the chlorine, was significantly toxic. Toxicity was comparable for MG87 RET/GRFa1 and MG87 TrkA cells; indicating that the toxicity is from a RET-independent mechanism.

Knowing that the presence of the chlorine is tied with potency, we designed structural modifications that preserve that position but reduce toxicity. Synthesis and evaluation of Q525 showed that is significantly less toxic than Q1047. Q525 was selected for further studies.

In summary, simple side-chain substitutions of the naphthoquinone core resulted in RET-activating compounds, and although lacking RET selectivity the compounds did not require GRFa1 co-expression. The chlorine is associated with the potency of the compounds, but also with the toxicity. Removal of the chlorine generated less potent compounds, and while RET selectivity was still not achieved, the compounds were considerably less toxic and had the ability to cooperate with GDNF. These observations prompted further chemical design of the naphthoquinone core in efforts to improve RET selectivity.

Quinoline analogs are RET activators with improved selectivity and GRFa1-independence

To improve upon this first-generation of molecules, we synthesized quinoline derivatives bearing identical side chains to their naphthoquinone counterparts. This effort generated new chemical entities (NCEs) with a nitrogen at position C (Table 1). These compounds are chemically distinguished from known inhibitors of protein tyrosine phosphatases (PTPases), to exclude this as a possible factor in the poor selectivity of the first-generation molecules.
Q525 (and Q508) were screened in biochemical assays. Q525 was active in MG87 RET/GFRα1 cells across a broad range of concentrations, and generated large and significant increases in pAkt/pErk (Fig 3A, quantified in 3B). The compound maintained a high degree of selectivity for RET, as no significant increases in pAkt and pErk were observed in MG87 TrkA cells (Fig 3C, quantified in 3D).

The improved selectivity of the quinoline series (e.g. Q525) is exemplified by lack of selectivity in Q1047 (the naphthoquinone form of Q525) which activates signals in MG87 TrkA cells (Fig 2C). Additionally, analogs Q1041 and Q1048 bearing a quinoxaline motif with N-substitutions at both positions C and D (Table 1) were synthesized and found to be inactive at all concentrations tested.

Together, these data suggest that the N-substitution in the ring system has a significant impact on compound selectivity, generating agents that maintain RET activity and GFRα1-independence.

**Differential signaling profiles through pAkt and pErk in combination with the GFRα1 modulator XIB4035**

We used the GFRα1-independent RET agonists as chemical-biology tools, to study the influence of the GFRα1 co-receptor upon RET signaling. We evaluated the quinone/quinoline pairs Q1047/Q525, and Q112/Q508 in combination with XIB4035 which is a reported GFRα1 modulator with no agonistic activity on its own (19).

Sub-optimal doses (10 µM) of Q1047 (non-selective agonist) or Q525 (selective RET agonist) generated low but significant increases in both pAkt and pErk, compared to control vehicle or to 4 µM XIB4035. A combination with 4 µM XIB4035 caused a reduction by 50% of the pAkt that was induced by Q112 or Q508 alone (Fig 4C) while leaving pErk unaffected (Fig 4B).

Hence, a GFRα1 modulator XIB4035 alters the signals induced by RET-agonists, even when these agonists do not require GFRα1 to signal on their own. In one case (Q1047 or Q525) it enhances pErk signals but does not affect pAkt, and in another case (Q112 or Q508) it decreases pAkt signals but does not affect pErk. The end result for both families of compounds is a pErk signaling bias, however this is likely achieved through different mechanisms that involve a ligand-mediated regulation of RET by the GFRα1 receptor. Also, it is of interest that N-substitution in the ring of structurally related quinone/quinoline pairs does not affect regulation of signaling by XIB4035.

**RET activators are not inhibitors of Protein Tyrosine Phosphatases**

Several naphthoquinones have been reported as inhibitors of PTPases, some non-selective (3) and some with a degree of selectivity (4). RET activation could potentially stem from inhibition of PTPases, and we evaluated this possibility.

Select compounds were tested in assays of PTPase activity to evaluate their impact upon the enzymatic activity of five PTPase enzymes (LAR, PTP-sigma, PTP-1B, MKPX, and SHP-1) as described (4). The compounds did not affect the activity of purified PTPases compared to DMSO.
vehicle, while the positive control Sodium Orthovanadate showed significant inhibition of >80% (Table 2). The 40 µM compound concentrations evaluated in these PTPase assays are at least 3-fold higher than the compound concentrations that induce biological signals in cultured cells, and likely much higher than compound concentrations inside the cell, where PTPases are present.

In particular we note lack of inhibition by the quinone/quinoline pairs Q1047/Q525, and Q112/Q508, none of which affected any of the five PTPases tested. Moreover, the fact that XIB4035 (a GFRα1 modulator with no reported link to PTPases) regulates pErk signals induced by Q1047/Q525, and Q112/Q508 (Fig 4) is consistent with the notion that these agents are not PTPase inhibitors.

**Q525 is neuroprotective by activation of Müller Glial cells**
We selected Q525 for pharmacological evaluation in vivo in the transgenic mutant RHOP347S mice (expressing a mutant rhodopsin gene that causes Retinitis Pigmentosa). These mice undergo rapid photoreceptor apoptosis which peaks in vivo between post-natal days 18-22 (20). The rate of photoreceptor apoptosis is fully replicated ex vivo in cultured whole retinal explants from these mice, allowing rapid testing of many compounds and doses.

Retinal explants were prepared from 18-day old mice and incubated for 24 hours with either compounds or vehicle, followed by quantification of TUNEL staining. Representative confocal images of the central retinal region are shown (Fig 5A). Q525 at a 20 µM dose affords robust preservation of the photoreceptors, as indicated by a 40% reduction in TUNEL+ cells in this layer (Fig 5B). In controls, the inactive compound Q143, assayed previously (Fig 1C) was indistinguishable from vehicle-treated retinas. The data support the notion that RET activation by Q525 may be a disease-modifying strategy for degenerative conditions like RP.

To understand the neuroprotective mechanism of action, we studied activation of biochemical pathways in the retina. Mice (n=3) received intravitreally administered test compound in one eye and control vehicle in the contralateral eye. Eyecups were collected 1 hour later and examined by immunohistochemistry to quantify pErk and pAkt signals. Results show that the Q525 treated eyes had elevated levels of pErk and pAkt by 1.8 and 2.0-fold respectively, while no significant elevations in pErk or pAkt were observed in animals injected with the inactive Q143 (Fig 5E-F). This is consistent with biochemical assays using cell lines (Fig 1 and 3). The pAkt and pErk signals colocalized with the Müller cell marker CRALBP. This indicates that the Müller glial cells are among the primary cellular targets for the RET agonists, and these cells are known to express RET and to support the health of photoreceptors.

**Discussion**

We discovered a series of new chemical entities, naphthoquinone/quinoline derivatives with agonistic activity at the RET receptor tyrosine kinase. Employing them as biological tools, we explored functional regulation between RET and its co-receptor GFRα1. A summary of screening and biochemical data is provided in Table 3.

We demonstrated that although these agents do not require the presence of GFRα1, their signaling profiles can be nuanced by the presence and bound-state of GFRα1. The regulatory functions that the GFRα subunits exert over RET provides additional points to consider when using RET agonists for therapy. We also showed the neuroprotective efficacy of a lead agent, Q525, in a model of
RP neurodegeneration; further validating RET as a potential therapeutic target.

Screening and optimization of novel derivatives

The finding that a relatively simple naphthoquinone Q121 was a weak RET agonist but required GFRα1 expression was surprising, given its extremely small size in comparison to GDNF and the large surface of interactions of the receptor complex. Large growth factors like GDNF are typically envisioned as mediating a ‘bridge’ between receptor components, bringing them in close proximity through multiple contact points and stabilizing an active conformation. It is unlikely that small molecules are capable of fully replicating this. Literature suggests that RET and GFRα1 associate in a ligand-independent manner (21), and it is therefore possible that our agents cause RET conformational changes through a preformed receptor complex.

The substitution of the methoxy group for a hydroxyl abrogated RET activity, and clearly implicated this position for synthesis of analogs. However, further screening of these naphthoquinone derivatives, while biochemically interesting, did not possess the RET selectivity worthy of pursuit. It is unclear how the compounds activate pAkt and pErk in non-RET expressing cells, but the potential molecular targets of the quinone family are broad. Their GFRα1 independence was unexpected, but highlights one of the many strengths in small molecule development in that minor structural modifications can have major biochemical impacts. This is again reflected by the impact of the chlorine to hydrogen substitution, yielding mildly pro-survival, GDNF-modulating effects in vitro.

In order to differentiate the core structure from those reported previously as being phosphatase inhibitors (4), we made additional modifications to the ring system, generating novel quinolinediones which demonstrated much improved characteristics. The quinoline scaffold has a rich pharmacological background, particularly in regard to anti-cancer therapeutics (22) and in fact a family of RET kinase inhibitors with this motif exists (23). Others also reported a series of quinolines with neurotrophic activity (24), but their molecular target and signals remain unknown.

Significance of GFRα1-dependent signaling bias

Biased signaling is a relatively new concept in the field of pharmacology, and even more recent is the notion that this property can be exploited to improve therapeutic efficacy (25). Some of the first biased ligands were synthetically derived and it was largely unknown whether this type of signaling occurred endogenously as an added regulatory mechanism. The chemokines and their receptors are one such example that reveal the presence and utility of signaling bias at the biological level, as a means to fine-tune responses to a wide array of stimuli (26).

Our findings with the compounds in combination with XIB4035 demonstrate a unique aspect of ligand bias working through a co-receptor, and there are few examples of this in the literature (27,28).

The compounds reported display a balanced activation of both pAkt and pErk pathways through RET by themselves, but become biased in the presence of a GFRα1 modulator. In contrast GDNF activation of the RET tyrosine kinase functions solely in conjunction with a membrane bound or soluble co-receptor GFRα, a feature of this receptor that is conserved among vertebrates (29).

It is tempting to speculate that GFRαs may be a gatekeeper of signaling bias. Through shifting their expression levels and patterns, GFRαs may allow a single ligand to generate a series of orchestrated responses within the same tissue. Ligand/GFRα complexes may also control RET distribution into and out of
lipid rafts, as is the case when comparing persephin versus GDNF (30), and this could dictate which adaptor proteins are most accessible to the activated kinase. It is also known that receptor-receptor interactions on the membrane can create allosteric regulatory sites at which ligands may bind to alter the functional outcome (31). Studies with constitutively active p75 mutants reveal that very slight changes in subunit positioning affect the resulting signaling profile, and therefore suggest that different ligands could stabilize unique conformations of the receptor complex to drive signaling bias (32).

Although we did not address this within the present work, soluble GFRas can also be liberated from the membrane surface, and the biological roles of this process are understudied but are likely to be diverse (33-35). This could provide another way for bias-control via the RET/GFRa complex.

Additionally, there may be other endogenous ligands such as GDF15 (36) or ligands of GFRa that can modulate biased signals through RET to mediate important processes. In that regard, it would be interesting to use GFRa modulators in combination with RET agonists but unfortunately XIB4035 cannot be used in vivo in the retina due to poor solubility and high toxicity (19). There are GFRa modulators under development (e.g. XIB4035 analogs) that eventually can be tested to answer this question.

Our findings have therapeutic relevance, especially in disease where RET expression remains stable, but GFRa levels vary considerably (37,38). A RET agonist may provide benefits in some diseases where dynamic changes occurring at GFRa in vivo are difficult to assess or control. As more work is done to validate RET and its co-receptors as targets, the ability to take advantage of signaling bias to yield highly specific outcomes with lower side-effects may be a promising strategy.

**Materials and Methods**

**Commercially available compounds**

Compounds **Q121** (CAS Registry Number 6956-96-3), **Q128** (CAS Registry Number 1526-73-4) and **Q151** (CAS Registry Number 84348-90-3) are known and described.

**Chemistry and Synthesis of novel naphthoquinone derivatives**

All chemicals were purchased from Aldrich, J&K or Otava. Reactions were monitored by TLC using aluminum backed silica gel plates (Aldrich, silica gel matrix with fluorescent indicator), products visualized under UV light (254 and 365 nm). Flash chromatography purifications were performed in columns using Silica Gel 60A, 230-400 mesh (ACP). NMR spectra were recorded on Bruker 400 MHz spectrometer. Mass spectra were obtained with a Bruker Mass Spectrometer AmaZon SL Direct Infusion and Bruker UltraFlextreme MALDI-TOF. Chemical synthesis, Mass spectrometry (MS), and Nuclear Magnetic resonance (NMR) spectra and other characterization of the compounds are presented in the Supplemental.

**Cell Lines**

MG87 RET are murine MG87 fibroblasts stably transfected with RET proto-oncogene cDNA (13), and were cultured in DMEM containing 10% FBS, 2 mM L-Glutamine, 10 mM HEPES, 100 U/ml Penicillin/Streptomycin, and 2 µg/ml puromycin. MG87 RET cells were transfected with a GFRa1 cDNA construct containing Blasticidin resistance to generate the MG87 RET/GFRa1 cell line, and were cultured in the same media with the addition of 5 µg/ml Blasticidin S. MG87 TrkA cells were transfected with human TrkA cDNA, and cultured in DMEM 10% FBS supplemented with 250 µg/ml G418. MG87RET/GFRa1 cell lines were stably
transfected with PathDetect Elk-1 system (Stratagene) harboring Luciferase reporter under the control of Erk activity. Cells were cultured in DMEM, 10% FBS, 15 mM HEPES pH 7.2, 100 µg/ml normocin (Invivogen), 2 µg/ml puromycin, 500 µg/ml (14). Growth factors GDNF and NGF were purchased from Peprotech (catalogue 450-10, 450-01).

**RET phosphorylation assays**
Phosphorylation of RET was assessed as previously described (15). MG87 RET cells were plated on 35 mm tissue culture dishes, left to attach to the surface overnight and then transfected with 4 µg/dish of GFRα1-expressing plasmid using Lipofectamine 2000 (Invitrogen) for DNA delivery as described by manufacturer. Next day cells were starved for 4 hr in serum-free DMEM containing 15 mM Hepes, pH 7.2 and treated with 1% DMSO vehicle, compounds, or GDNF (200 ng/ml) for 15 min. Cells were lysed using RIPA-modified buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% TX-100, 10% glycerol, EDTA-free EASYpack, protease inhibitor cocktail (Roche), 1 mM Na3VO4, 2.5 mg/ml of sodium deoxycholate). Cleared lysates were immunoprecipitated with 2 µg/ml of anti-RET C-20 (Santa-Cruz Biotechnology, Inc., sc-1290) and beads conjugated with protein G (Thermo Fisher Scientific, Cat # 10004D). Eluted samples were resolved on 7.5% SDS-PAGE and total phosphotyrosine residues were then probed in Western blots using the 4G10 antibody (Millipore). To confirm equal loading, membranes were re-probed with anti-RET C-20 antibodies (1:500, Santa-Cruz Biotechnology, Inc.).

**pAkt, pErk1,2 Biochemical Studies**
Cells were seeded onto 6-well plates (0.4 x 106 cells/well) and cultured overnight. Cells were serum-starved for 2 hr, and treated with vehicle, compounds or growth factor (GF) as indicated in the text (generally for 20 minutes). Cell lysates were prepared (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 containing a protease inhibitor cocktail). Protein was quantified in cleared lysates using the Bradford assay (BioRad). After SDS-PAGE, Western blotting was evaluated with the primary antibodies pAkt, pErk1,2 (Cell Signaling, catalogue #4060, #4370), or Actin control for loading (Sigma Aldrich, catalogue A2066). Signals were developed using Western Lightning Plus ECL (PerkinElmer), films were scanned and quantified using ImageJ software.

**Phosphatase Inhibition Screens**
PTP activity/inhibition assays are as we reported previously (4). Tagged catalytic domain or full length of the following phosphatases were used for the screen. GST-PTP1B aa 1-321, GST-LAR-D1D2, GST-Sigma-D1D2, GST-MKPx aa 1-184 and His-SHP-1 aa 1-595. Enzyme reactions were performed in assay buffer 50mM HEPES pH7.0 for PTP1B, LAR, Sigma and MKPx and buffer 50 mM Bis-Tris pH 6.3, 2 mM EDTA for SHP-1. DiFMUP (Invitrogen) was used as substrate for all assays, in black 96-well plates (Corning) in a final volume of 100 µL at 25°C. The reaction was monitored by measuring the fluorescence (excitation wavelength 358 nm/emission 455 nm) with the Varioskan plate reader (Thermo electron). For the kinetic assays, fluorescence was monitored over 10 minutes in 30 seconds intervals and rates were calculated in FU/minutes. Km was determined from rates at various substrate concentrations using Michaelis-Menten equation from GraphPad Prism software. A substrate concentration equivalent to the Km value for each PTP was used for the screening of the compound. Inhibitors were diluted in DMSO, then a dilution to 10 µM or 40 µM was made in assay buffer. Controls contains 1-2% DMSO final. Phosphatas and compounds were pre-incubated 2-5 minutes prior to addition of

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DiFMUP for enzyme inhibition quantification.

**Cell Survival Assays**

Cell survival was measured by the MTT assay (Sigma Aldrich) using optical density readings as the endpoint. 2000-5000 cells were plated in 96-well format in serum-free media (SFM) containing 0.03% FBS (HCell-100, Wisent). The indicated test agents, or DMSO vehicle were added to the wells. The respective growth factors (GF, 30 ng/ml GDNF for MG87 RET/GFRa1, 30 ng/ml NGF for MG87 TrkA) at optimal concentrations were added as control. Assay length was typically 72 hr since this time point yields an optimal signal-to-noise ratio between vehicle and growth factor controls. Assays were repeated at least 3 times (each assay n=4-6 wells per condition). MTT optical density data were standardized to growth factor = 100%, and SFM = 0%, using the formula \[(\text{ODtest} – \text{OD}_{\text{SFM}})*100 / (\text{OD}_{\text{GF}} – \text{OD}_{\text{SFM}})\].

**Animal models**

All animal procedures respected the IACUC guidelines for use of animals in research, and to protocols approved by McGill University Animal Welfare Committees. All animals were housed 12 hr dark-light cycle with food and water ad libitum. We used the “RHOP347S” transgenic mouse (expressing the human Rhodopsin mutated at amino acid position 347) in a C57BL/6J (B6) background (kindly donated by Dr. T. Li). This model of RP faithfully replicates the features of disease progression in humans.

**Retinal Organotypic Cultures**

Whole eyes were enucleated and whole retinas dissected from wild-type and RHOP347S mice at post-natal day 18 were used for organotypic culture experiments. Following enucleation, eyes were placed in a petri dish with PBS. The cornea was perforated and cut away along the ora serrata, leaving room to remove the lens. Whole intact retinas were then freely dissected away from the sclera and immediately transferred into 24 well plates containing 500 µl of culture medium (DME/F12 supplemented with 10 mM NaHCO₃, 100 µg/ml Transferrin, 100 µM Putrescine, 20 nM Progesterone, 30 nM Na2SeO3, 0.05 mg/ml Gentamicin, 2 mM L-Glutamine, and 1 mM Sodium Pyruvate). Under sterile conditions, the media was gently removed and replaced with fresh media containing the treatments or controls, and incubated at 37°C and 5% CO₂ for 24 hr. Compounds were tested at a concentration of 20 µM. Cell grade DMSO was used for vehicle treatments and was 0.5% by volume. Retinas were then used for TUNEL staining.

**TUNEL staining**

Staining was performed using the DeadEnd Fluorometric TUNEL system (Promega) as per manufacturer’s instructions and as described by us (16,17). RHOP347S retinas in culture were first fixed in 4% PFA in PBS and kept at 4°C overnight, followed by permeabilization using 2% Triton-X in PBS and re-fixed in PFA for 30 minutes. Retinas were incubated with 50 µl of equilibration buffer for 20 minutes, then 25 µl of TdT reaction mixture for 2.5 hr at 37°C, and the reaction was then terminated. The retinas were washed and mounted using Vectashield with DAPI. For image acquisition (17,18), the retinas were divided into 4 quadrants, and 3 pictures with a 20X objective were taken in each area (central, mid, peripheral) for a total of 12 images of the outer-nuclear layer (ONL) per retina. Total TUNEL-positive cells were counted in each image semi-automatically (ImageJ). Counts were verified by at least one other person blinded to the experimental conditions. Retinal flat mounts from wild-type mice (where there is no mutation-driven photoreceptor death) were used as negative controls.

**Immunohistochemistry**
Staining was performed as described by us (17). After enucleation, the cryoprotected eyes were mounted in Tissue-TEK (Sakura) and cryostat sections were cut and mounted onto gelatin-coated glass slides. Sections (14 µm thick) were incubated in PBS containing 3% normal goat serum, 0.2% Triton X-100 and 3% bovine serum albumin (BSA) for 2 hr. Retinal sections were incubated overnight at 4°C with primary antibody (1:250 p-MAPK, Cell Signaling #4370; 1:250 p-Akt, Cell Signaling #4060; 1:400 CRALBP, Abcam ab183728). The sections were rinsed and incubated with secondary antibody for 1 hr at room temperature. Then, sections were washed and mounted (Vectashield mounting media with DAPI).

Image acquisition (fluorescence microscopy) and data analysis

Pictures were taken as Z-stacks of confocal optical sections using a Leica confocal microscope at a 20X objective. Images were equally adjusted using Adobe Photoshop CS 8.0 to remove background signals. For each experimental condition, a minimum of 6 images were acquired from 3 sections cut from different areas of the retina (n=3 retinas per group). The area of the profiles of the cells expressing pErk and pAkt was measured using ImageJ software.

Intravitreal injections

Briefly, mice were anesthetized with isoflurane, delivered through a gas anesthetic mask. The treatments were delivered using a Hamilton syringe. Injections were done using a surgical microscope to visualize the Hamilton entry into the vitreous chamber and confirm delivery of the injected solution (3 µl of a 2 mM stock solution). After the injection, the syringe was left in place for 30 seconds and slowly withdrawn from the eye to prevent reflux. In each animal the right eyes were injected with the test agents (experimental eye) and the left eyes were untreated (internal control).

Statistical analyses

The quantitative data were subjected to statistical analyses using GraphPad Prism 5 software, and are presented as mean ± SD for all studies. The differences between groups were determined by ANOVA (multiple groups) followed by Dunnett’s or Bonferroni-corrections as indicated in Legends. Student t-tests were performed to compare two groups. p-values below 0.05 were considered to indicate statistically significant differences between groups.

Data availability.

All data are contained within the article. The complete NMR resonances are available on request to the corresponding author.
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Patent applications have been filed by McGill University (SJ, YS, MS, and HUS, authors).

Authorship Contributions
Participated in research design: Jmaeff, Saragovi
Conducted experiments: Jmaeff, Sidorova, Nedev
Contributed new reagents or analytic tools: Nedev
Performed data analysis: Jmaeff, Sidorova, Saragovi
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List of Abbreviations:
BSA: Bovine Serum Albumin
CRALBP: Cellular Retinaldehyde Binding-Protein
DAPI: 4’,6-diamidino-2-phenylindole
DMEM: Dulbecco’s Modified Eagle’s Medium
DMSO: Dimethyl sulfoxide
FBS: Fetal Bovine Serum
GDNF: Glial cell line-derived neurotrophic factor
GFRα1: GDNF Family Receptor alpha 1
INL: Inner Nuclear Layer
NGF: Nerve Growth Factor
ONL: Outer Nuclear Layer
PBS: Phosphate Buffered Saline
PTP: Protein Tyrosine Phosphatase
PFA: Paraformaldehyde
pTyr: phospho-tyrosine
RGC: Retinal Ganglion Cell Layer
RP: Retinitis Pigmentosa
RTK: Receptor Tyrosine Kinase
SAR: Structure-Activity Relationship
SFM: Serum-free Media
TdT: Terminal Deoxyxynucleotidyl Transferase
TrkA: Tropomyosin Receptor Kinase A
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Table Legends

Table 1. Overview and compound structures, listed as they appear to show SAR progression. Letters A-D denote the position and order in which the modifications took place throughout the paper, and the compounds that resulted. The adjacent text on the core structure describes the parameters that the modification influenced. Starting from Q121 and Q128, side chain substitutions at position A generated compounds that activated RET without GFRa1 expression. Modification at position B through removal of the Cl atom generated compounds that were much less potent, although toxicity was improved and they potentiated GDNF survival. However, A and B modified compounds exhibited poor selectivity. Modification at position C by N-substitution yielded compounds with improved selectivity for RET and were pursued in further studies. Finally, compounds tested with N-substitutions at both positions C and D were found to be generally inactive.

Table 2. Select compounds were screened for their ability to inhibit the phosphatases LAR, Sigma, PTP1B, MKPX, and SHP-1 and data are shown as the percent inhibition relative to DMSO control. Sodium Orthovanadate (Na3VO4) was used as positive control as a broad spectrum PTP inhibitor.

Table 3. Summary of pharmacological profiles. Comparison of signaling (maximal efficacy of activation of Akt/Erk in RET-expressing cells) by the compounds relative to each other and to GDNF. Potency is not indicated (e.g. GDNF is nM and compounds are µM). RET selectivity was gauged by quantification of signaling in cells not expressing RET but expressing other RTKs (negative Akt/Erk activation is wanted). Culture of various cell types with the compounds in serum-containing media gauged general toxicity, and culture in serum-free conditions gauged accelerated or delayed cell death. The use of various cell types in such assays evaluated RET-dependence. Q525 is highlighted as a potential lead with the best balance overall. + low/poor ++ moderate +++ high – not tested/not applicable. The summary illustrates the interplay that exists between the different parameters and profiles, and the challenges of choosing a lead agent.
Figure Legends

Figure 1. Screening and SAR of naphthoquinone RET activators

**A.** Compounds Q121 and Q128 activity in MG87 RET/GFRa1 cells. Following serum starvation, cells were exposed for 20 min to either vehicle, 10 ng/ml GDNF, or compounds at the indicated μM range. Lysates were collected and probed for pAkt, pErk, and total-phosphotyrosine (4G10). Representative Western blot data are shown. Actin was probed as internal loading standard. **B.** Representative total RET immunoprecipitation from MG87 RET cells transfected with either GFRa1 or GFP control. Q121 treatment results in RET phosphorylation at concentrations of 50 μM and 100 μM in the cells expressing GFRa1. Total RET was probed as internal loading standard. For MG76 RET/GFRa1 cells, G represents GDNF as positive control. For MG87 RET/GFP, G+a1 represents GDNF+GFRa1 co-treatment which is needed to induce RET phosphorylation in these cells lacking GFRa. **C.** Activities of naphthoquinone derivatives. Side chain modifications generated compounds active in MG87 RET/GFRa1 as demonstrated by the representative Western blot. **D.** The naphthoquinone derivatives are also active in MG87 TrkA cells as demonstrated by the representative Western blot. **E.** Compound Q151 induces RET phosphorylation in both MG87 RET/GFRa1 and in RET/GFP cells lacking GFRa1. **F.** MG87 RET/GFRa1 cells in SFM were treated with Q2003 and Q2004 alone or in combination with sub-optimal GDNF (GDNF SO, 5 ng/ml). Survival was assessed by MTT after 72 hours. Both compounds had low but significantly trophic activity at a concentration of 1 μM. Compounds significantly potentiate GDNF SO. **G.** MG87 TrkA cells in SFM were treated with Q2003 and Q2004 alone or in combination with sub-optimal NGF (NGF SO, 60 pg/ml). Survival was assessed by MTT after 72 hours. Both compounds had low but significantly trophic activity at a concentration of 1 μM, but did not potentiate NGF SO.

Data are expressed as % survival ± SD from 4 experiments, with the respective optimal trophic factor (GF O, 30 ng/ml) standardized to 100%, and vehicle to 0%. Symbol * vehicle versus all treatments. Symbol # sub-optimal GF versus optimal GF. Symbol + sub-optimal GF versus all treatments. One symbol, p<0.05, two symbols p<0.005, three symbols p<0.0005, Bonferroni-corrected t-test.
Figure 2. Chlorine substitution affects toxicity and directs lead compound development
MG87 RET/GFRα1 and TrkA expressing cells were exposed to 1 μM compounds in regular growth media and assessed by MTT. Q2003 and Q2004 bearing a hydrogen in place of the chlorine were not toxic, indistinguishable from vehicle control. However, Q1047, the chlorine-containing analogue of Q2003, showed significant signs of toxicity under the same conditions. Additionally, the lack of toxicity observed by Q525 demonstrated that the chlorine could be maintained, and directed the development of more potent, RET-selective leads. The comparable toxicity between both cell lines indicated that the origin of the toxicity is not due to RET-overactivation, but rather a generalized mechanism.

Figure 3. Development of Lead Quinoline derivatives
A-B. Biochemical screening of Q525 in MG87 RET/GFRα1 cells. The compound generated large increases in pAkt/pErk within the 5-20 μM range. Western blot data were quantified from 3 experiments and expressed as mean ± SD versus Vehicle, standardized to 100%. For pAkt *p<0.05, **p<0.005, ***p<0.0005. For pErk, #p<0.05, Dunnett’s test.
C-D. Counter screens with Q525 were performed in MG87 TrkA cells. Only slight increases in pAkt/pErk were observed within the same concentration range. For comparison, the poorly selective Q1047 bearing an identical side-chain is also shown. Results were quantified from 3 experiments and expressed as mean ± SD versus Vehicle, standardized to 100%. For pAkt **p<0.005. For pErk, #p<0.05, Dunnett’s test.
Figure 4. Differential signaling through pAkt/pErk is mediated by XIB4035-induced modulation of GFRa1.
A. Representative blots taken from separate experiments showing pAkt and pErk signaling of compounds Q1047, Q525, Q112, and Q508 with or without XIB4035 pretreatment. B. Quantification of pErk signals of compounds Q1047, Q525, Q112, and Q508 in combination with the GFRa1-modulator XIB4035. XIB4035 pretreatment did not affect pErk signals induced by Q112 and Q508. However, Q1047 and Q525 signals were significantly increased. Data are expressed as mean ± SD from 5 experiments, standardized to compound alone which was set at 100%. *<p<0.05, ***p<0.0005, Bonferroni-corrected t-test. C. Quantification of pAkt signals of compounds Q1047, Q525, Q112, and Q508 in combination with the GFRa1-modulator XIB4035. XIB4035 pretreatment did not affect pAkt signals induced by Q1047 and Q525. However, Q112 and Q508 signals were significantly decreased. Data are expressed as mean ± SD from 5 experiments standardized to compound alone, which was set at 100%. *<p<0.05, **p<0.005, Bonferroni-corrected t-test.

Figure 5. Q525 is neuroprotective ex vivo in a mouse model of retinal degeneration and activates trophic signals in Muller glial cells.
A. Representative confocal images of retinal flat mounts following treatment with Q525 and Q143. Freshly dissected retinas from 18-day old RHOP347S mice were incubated in culture media for 24 hours. One retina with the indicated compounds, and the contralateral retina of the same mouse with vehicle control (e.g. each individual had its own internal control). Tissues were processed for TUNEL staining and flat-mounted, 12 images were taken per retina and quantified semi-automatically. B. Quantification of TUNEL-positive cells after treatment. Cell counts from n=3 mice per group were standardized versus vehicle control. Q525 treatment affords a significant reduction in TUNEL staining, while Q143 treatment was comparable to vehicle. *<p<0.05, Student t-test. C. and D. Images of retinal sections stained for pErk and pAkt following intravitreal injection of compound Q525 in vivo. Eyecups were collected 1 hour after injection. Significant increases in staining for pErk and pAkt co-localized with the Muller cell marker CRALBP, suggesting activation within the glial population. RGC = retinal ganglion cell layer, INL= Inner nuclear layer, ONL = outer nuclear layer. Scale bar 25 µm. E. pErk quantification of Q525 and Q143 retinal sections (n=3 per group) expressed as the fold-change in pixel area over vehicle ± SD, *** p<0.0005, Student t-test. F. pAkt quantification of Q525 and Q143 retinal sections (n=3 per group) expressed as the fold-change in pixel area over vehicle ± SD, * p<0.05, Student t-test.
Table 1.

| Q#  | D  | C  | B               | A               |
|-----|----|----|-----------------|-----------------|
| 121 | C  | C  | Cl              | OCH₃            |
| 128 | C  | C  | Cl              | OH              |
| 101¹ | C | C | Cl | NH-CH₂-Ph-COOCH₃ (p) |
| 105¹ | C | C | Cl | NH-Ph-CH₂-COOCH₃ (p) |
| 112¹ | C | C | Cl | NH-C(CH₂)₂-2-Me-Pyrrolidine |
| 141¹ | C | C | Br | NH-Ph-CH₂-COOCH₃ (p) |
| 143¹ | C | C | Cl | NH-Ph-N=C=S (p) |
| 151¹ | C | C | Cl | NH-Ph-COOCH₃ (p) |
| 1047¹ | C | C | Cl | NH-Ph-CH₂OH (p) |
| 2003² | C | C | H  | NH-Ph-CH₂OH (o) |
| 2004² | C | C | H  | NH-C(CH₂)₂-2-Me-Pyrrolidine |
| 508³ | C | N | NH-C(CH₂)₂-2-Me-Pyrrolidine | Cl |
| 525³ | C | N | NH-Ph-CH₂OH (p) | Cl |
| 1041⁴ | N | N | H  | NH-Ph-CH₂OH (p) |
| 1048⁴ | N | N | Cl | NH-Ph-CH₂OH (p) |

Diagram: Structure with labels for Potency, Toxicity, GDNF-modulation, and Selectivity.
Table 2.

| Q# | LARD1D2 | SigmaD1D2 | PTP1B | MKPX  | SHP-1 |
|----|---------|-----------|-------|-------|-------|
| 105 | 97      | 98        | 77    | 99.5  | 87    |
| 112 | 91.5    | 96.5      | 121.5 | 97    | 80    |
| 121 | 96.5    | 97.5      | 93    | 98    | 90    |
| 143 | 96      | 99        | 101   | 96.5  | 103.5 |
| 508 | 72      | 71        | 92.5  | 96.5  | 91    |
| 525 | 97.5    | 99.5      | 96    | 99    | 94    |
| Na3VO4 | 16.5  | 24        | 4     | 5     |       |
| DMSO | 99      | 97.5      | 99.5  | 99    | 93    |

Table 3.

| test | Potency | RET Selectivity | GFRa1 dependent | Toxicity |
|------|---------|-----------------|-----------------|----------|
| GDNF | ++++    | +++             | yes             | –        |
| 121  | ++      | +               | yes             | ++       |
| 128  | +       | +               | –               | ++       |
| 101  | +       | +               | –               | +++      |
| 105  | ++      | +               | no              | +++      |
| 112  | +++     | +               | no              | +++      |
| 141  | ++      | +               | no              | +++      |
| 143  | +       | –               | –               | +++      |
| 151  | ++      | +               | no              | +++      |
| 1047 | +++     | +               | no              | +++      |
| 2003 | +       | +               | –               | +        |
| 2004 | +       | +               | –               | +        |
| 508  | ++      | +++             | no              | +        |
| 525  | +++     | +++             | no              | +        |
| 1041 | +       | –               | –               | –        |
| 1048 | +       | –               | –               | –        |
Figure 1.
Figure 2.
Figure 4.

A. 

|       | V | G | 1047 | 525 | 112 | 508 |
|-------|---|---|------|-----|-----|-----|
| XIB4035 | - | - | +    |    | +   | -   |
| pAkt   |   |   |      |     |     |     |
| pErk   |   |   |      |     |     |     |
| Actin  |   |   |      |     |     |     |

B. 

![Graph showing percent relative to compound alone for pErk](image)

C. 

![Graph showing percent relative to compound alone for pAkt](image)
Small-molecule agonists of the RET receptor tyrosine kinase activate biased trophic signals that are influenced by the presence of GFRα1 co-receptors
Sean Jmaeff, Yulia Sidorova, Hinyu Nede, Mart Saarma and H. Uri Saragovi

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