Chemicals Possessing a Neurotrophin-Like Activity on Dopaminergic Neurons in Primary Culture

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Abstract

**Background:** Neurotrophic factors have been shown to possess strong neuroprotective and neurorestaurative properties in Parkinson’s disease patients. However, the issues to control their delivery into the interest areas of the brain and their surgical administration linked to their inability to cross the blood brain barrier are many drawbacks responsible of undesirable side effects limiting their clinical use. A strategy implying the use of neurotrophic small molecules could provide an interesting alternative avoiding neurotrophin administration and side effects. In an attempt to develop drugs mimicking neurotrophic factors, we have designed and synthesized low molecular weight molecules that exhibit neuroprotective and neurotrophic potential for dopaminergic neurons.

**Principal Findings:** A cell-based screening of an in-house quinoline-derived compound collection led to the characterization of compounds exhibiting both activities in the nanomolar range on mesencephalic dopaminergic neurons in spontaneous or 1-methyl-4-phenylpyridinium (MPP+)-induced neurodegeneration. This study provides evidence that rescued neurons possess a functional dopamine transporter and underlines the involvement of the extracellular signal-regulated kinase 1/2 signaling pathway in these processes.

**Conclusion:** Cell-based screening led to the discovery of a potent neurotrophic compound possessing expected physicochemical properties for blood brain barrier penetration as a serious candidate for therapeutic use in Parkinson disease.

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Introduction

Loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc) is a consistent feature of Parkinson’s disease (PD). This is mainly clinically characterized by motor impairments [1]. Except for some cases linked to specific gene defects (<10%), PD is a sporadic combination of unknown factors [2]. Mitochondrial dysfunction, oxidative stress, and proteasome failure are among the several hypotheses put forward to explain the molecular basis of neuronal damage [3,4]. The symptoms of PD can be improved by drugs that replace neurotransmitters, but these treatments are unable to slow down the disease progression and often induce undesirable side effects [5]. New therapies are required to preserve DA neurons and stimulate their DA activity and to limit or halt the progression of the disease [6]. Several neurotrophins implicated in the development and maintenance of different neuronal populations have been shown to provide protection against cell death in *in vitro* and *in vivo* models of PD through diverse signaling pathways including activation of phosphatidylinositol–3-kinase (PI3-kinase)/Akt, ras-dependent mitogen-activated protein kinase (MAPK), and phospholipase C (PLC) [7]. These signaling cascades lead to the prevention of apoptotic cell death, promotion of cellular differentiation and neuritogenesis. Glial-cell derived neurotrophic factor (GDNF) has been proposed as a therapeutic agent to delay the development of PD [8]. Despite the therapeutic potential of GDNF, clinical trials have been disappointing [9], probably due to inherent drawbacks associated with the use of polypeptides applied as drugs [10], including pleiotropic effects, short half-life and inability to cross the blood-brain barrier (BBB), thus imposing repeated transcranial injections, with dramatic side effects. To obviate these issues, substantial efforts have been made to design non-peptidic small molecules with neurotrophin-like activities.

Lembheyne A (LBA), a natural polyacetylene isolated from *Haliclona sp*., marine sponges, was previously described for its neurotrophic properties for the mouse neuroblastoma cell line Neuro2a [11]. Structure–activity relationship studies determined the minimal structure required for activity [12]. Inspired by this natural biologically active product, we designed and synthesized...
an in-house collection of quinoline-derived compounds by linking the neuritogenic part of LBA to a putative neuroprotective quinoline ring largely described for its high biological potential [13–17] with the aim of producing chemicals possessing a neurotrophin-like activity. Herein, we report the design, synthesis and cell-based screening of small molecules exhibiting both neuroprotective and neuritogenic activities on rat mesencephalic DA neurons against spontaneous [18] or MPP+ -induced [19] degeneration. Preliminary studies of the mechanism of action revealed that the most active compound exerts its activity through ERK1/2 signaling pathway activation, especially on DA neurons.

Results

Chemical syntheses

Terminal alkynols 3a–c are the first intermediates of our multistep synthesis leading to the lateral chain of our products. First, propargylic alcohol 1 was coupled to bromoalkanes through a previously described cross-coupling reaction [20] involving an iron catalyst and lithium amide to give compounds 2a–c. In a second step, a zipper reaction [21] led to terminal alkynols 3a–c. This was followed by a Sonogashira cross-coupling reaction [22] between 2-chloroquinoline or 3-bromoquinoline, both commercially available, and terminal alkynols 3a–c giving alcohol intermediates 4a–f. Best yields were obtained by using PdCl2(PPh3)2 and CuI as catalysts and Et3N as base. Alcohol intermediates 4a–f were then oxidized into the corresponding aldehydes 5a–f. This oxidation was followed by a final coupling reaction between aldehydes 5a–f and commercially available trimethylsilylacetylene after metallation with n-butyllithium to give a silylated intermediate that was immediately deprotected with tetrabutylammonium fluoride leading to compounds 6a–f. In order to study the influence of the benzyl triple bond on the biological activity, another series was synthesized from compounds 4a–f after catalytic hydrogenation of the triple bond, leading to saturated alcohol intermediates 7a–f. Compounds 7a–f were then oxidized into the corresponding aldehydes 8a–f and coupled to trimethylsilylacetylene before deprotection through the same steps as for compounds 4a–f. With the aim of studying the influence of terminal propargylic alcohol on the biological activity,
other compounds were synthesized in a similar manner from aldehyde 5e by adding vinyl or cyclopropyl magnesium bromide to give the corresponding compounds 6g and 6h. Compound quinoline-free 9g was prepared in the same way from the corresponding aldehyde 8g of commercially available alcohol 7g. The different steps are presented in Fig. 1. Spectroscopic data are available in the Supporting Information S1 (see Supplementary Information - Spectroscopic analysis).

Screening for both Neuroprotective and Neuritogenic Activities on DA Neurons

Synthesized compounds were then assessed for their ability to protect DA neurons from degeneration and activate neurotogenesis. The screening was performed in primary mesencephalic cultures displaying a progressive degeneration of DA neurons [18] (see Supplementary Information - Mesencephalic cultures). Cultures were maintained for 8 days in the presence or absence of the different tested compounds, and then tyrosine hydroxylase (TH) was immunolabeled to allow the analysis of DA neurons (see Supplementary Information - Immunocytochemistry). Neuroprotection was assessed by TH immunopositive (TH+) neuron counting (see Supplementary Information - Survival quantification). Neurotogenesis, expressed as total neurite length per DA neuron, was quantified using image analysis software on at least 100 neurons randomly photographed per condition (see Supplementary Information - Neuritogenesis quantification). Results are given in Table 1 and give rise to several interesting observations. First, compounds such as 6a–c, 9a–c, 6b and 6g,h exhibited both protective and neuritogenic activities in the nanomolar range. Second, a strong selectivity for both activities was observed between the 2- and 3-substituted quinolines (6a–c vs. 6d–f, P<0.001, Table 1), as represented in Fig. 2B–C. Third, the activities were significantly increased with the length of the lateral chain (6a vs. 6b vs. 6c, P<0.05, Table 1) and the best dual activities were observed for compound 6c (Table 1, Fig. 2A–C). Furthermore, saturation of the intrachain triple bond significantly decreases the activities (6a–c vs. 6a, P<0.001, Table 1). Fourth, the removal (6b vs. 4b) or replacement (6c vs. 6e, 6c vs. 6h) of the terminal triple bond significantly decreases (P<0.001, Table 1) the protective effect but not the neuritogenic effect. The dose–effect relationship (data not shown) revealed that maximal effect was observed at a 10 nM concentration for compounds 6g,h (data not shown).

Table 1. Neuroprotective and neuritogenic activities of LBA-derived compounds on mesencephalic DA neurons.

| Compound* | Functional group (Fg) | R¹ | R² | n | % relative to control ± SEM | TH⁺ neuronsᵇ | Length/TH⁺ neuronˢ |
|-----------|----------------------|----|----|---|-----------------------------|-------------|------------------|
| Control   |                      |    |    |   |                             |             |                  |
| dbc-AMP   |                      |    |    |   | 100±2.7                     | 100±2.9     |                  |
| 6a        | C==C                | 2-quinolyl | C==CH | 1 | 127.2±7.4                  | 150.6±7.6   |                  |
| 6b        | C==C                | 2-quinolyl | C==CH | 3 | 141.4±4.6                  | 155.2±6.3   |                  |
| 6c        | C==C                | 2-quinolyl | C==CH | 5 | 154.5±4.1                  | 186.0±7.7   |                  |
| 6d        | C==C                | 3-quinolyl | C==CH | 1 | 86.8±9.5                   | 51.3±3.7    |                  |
| 6e        | C==C                | 3-quinolyl | C==CH | 3 | 89.6±2.8                   | 55.2±2.0    |                  |
| 6f        | C==C                | 3-quinolyl | C==CH | 5 | 98.2±6.3                   | 78.5±2.2    |                  |
| 9a        | CH₂=CH₂            | 2-quinolyl | C==CH | 1 | 115.8±3.9                  | 126.5±10.8  |                  |
| 9b        | CH₂=CH₂            | 2-quinolyl | C==CH | 3 | 129.6±5.3                  | 122.3±8.7   |                  |
| 9c        | CH₂=CH₂            | 2-quinolyl | C==CH | 5 | 142.6±2.6                  | 128.8±1.0   |                  |
| 9d        | CH₂=CH₂            | 3-quinolyl | C==CH | 1 | NRᵈ                       | NRᵈ         |                  |
| 9e        | CH₂=CH₂            | 3-quinolyl | C==CH | 3 | NRᵈ                       | NRᵈ         |                  |
| 9f        | CH₂=CH₂            | 3-quinolyl | C==CH | 5 | 117.6±6.1                  | 95.8±2.9    |                  |
| 4b        | C==C                | 2-quinolyl | H    | 3 | 121.4±3.1                  | 157.9±13.9  |                  |
| 4e        | C==C                | 3-quinolyl | H    | 3 | 103.9±4.2                  | 114.3±10.7  |                  |
| 6g        | C==C                | 2-quinolyl | <CH=CH₂ | 5 | 135.5±2.7                  | 160.6±11.4  |                  |
| 6h        | C==C                | 2-quinolyl | CH=CH₂ | 5 | 139.1±4.2                  | 163.5±14.2² |                  |
| 9g        | CH₂=CH₂            | H      | C==CH | 5 | 95.8±5.9                   | 163.8±11.8  |                  |

*Compounds used at 100 nM concentration.
¹Number of TH⁺ neurons per well as a percentage of untreated cultures expressed as the mean±SEM of at least three independent experiments performed in triplicate.
²Neurite length per neuron as a percentage of untreated neurons measured on at least 100 neurons per condition using an image analyser (Neurite Outgrowth, Explora Nova, France) and expressed as the mean±SEM of three independent experiments performed in triplicate.
³Non-determined.
⁴Related values obtained at 10 nM because of a weak toxicity observed at 100 nM.
⁵1= cyclopropyl. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test (P<0.001, reported in text).

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shown). Dbc-AMP at 200 μM was used as a positive control. Values are normalized to the non-treated control (CTL) and represent the average of at least three assays realized in triplicate and are expressed as mean±SEM. *P<0.001. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post hoc test. (C) Compound 6c (b) increased the number and the length of DA neuron processes compared to non-treated cultures (a). In contrast, neuritogenesis of DA neurons in compound 6f-treated cultures (c) was slowed down, showing the crucial role of the substitution position on the combined activity. (b) and (c) represent (tyrosine hydroxylase) TH immunolabeled neurons in cultures treated with 100 nM of compounds 6c and 6f. A dose-dependent neurotrophic effect was also observed for compound 6c (data not shown), while compound 6f was still inactive at higher concentrations (data not shown). Dbc-AMP at 200 μM (d) was used as a positive control. Images were acquired with an inverted fluorescent microscope coupled to a digital camera.

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**Figure 2. Screening for both neuroprotective and neuritogenic activity on DA neurons.** (A) Two-dimensional representation of Table 1 showing four main groups of compounds exhibiting or not both neuroprotective and neuritogenic activity in different ratios. (B) The substitution position of the lateral chain is crucial for both activities (6c vs. 6f). 2-substituted compounds induced an increase in both TH neuron survival and neuritogenesis in a dose-dependent manner, while 3-substituted compounds inhibited both activities. Dbc-AMP at 200 μM was used as a positive control. Values are normalized to the non-treated control (CTL) and represent the average of at least three assays realized in triplicate and are expressed as mean±SEM. *P<0.001. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post hoc test. (C) Compound 6c (b) increased the number and the length of DA neuron processes compared to non-treated cultures (a). In contrast, neuritogenesis of DA neurons in compound 6f-treated cultures (c) was slowed down, showing the crucial role of the substitution position on the combined activity. (b) and (c) represent (tyrosine hydroxylase) TH immunolabeled neurons in cultures treated with 100 nM of compounds 6c and 6f. A dose-dependent neurotrophic effect was also observed for compound 6c (data not shown), while compound 6f was still inactive at higher concentrations (data not shown). Dbc-AMP at 200 μM (d) was used as a positive control. Images were acquired with an inverted fluorescent microscope coupled to a digital camera.

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**Specificity of Compound 6c-induced Neurotrophic Effect on DA Neurons**

DA neurons represent only a few percent among the total neuronal population in ventral mesencephalon culture, which is mainly constituted by GABA (gamma-aminobutyric acid)-ergic neurons [24]. GABA neurons are not affected under these conditions so their number, assessed by counting microtubule-associated protein 2 (MAP2) immunolabeled-neurons, is only representative of total neuronal viability [Fig. 3A] [25]. To explore the phenotypic specificity of compound 6c, uptakes of [7,8-3H]-DA ([3H]-DA) and 4-Amino-α-[2,3-3H]-butyric acid ([3H]-GABA) were measured under the same conditions (see Supplementary Information – Uptake of neurotransmitters). Uptake measurements were carried out at DIV12 to allow neuronal maturation. Fig. 3B provides evidence that DA uptake was increased in compound 6c-treated cultures in a dose-dependent manner, while the latter has no influence on [3H]-GABA uptake. These data suggest that compound 6c-induced neurotrophic activity is specific to DA neurons under these conditions.

**Protective Effect of Compound 6c Against Neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) Toxicity**

Since toxins could be involved in PD onset [26–29], there is growing interest finding the search for compounds able to protect DA neurons from toxin-induced death. To examine more closely the protective potential of compound 6c, MPP⁺ was added to mesencephalic cultures (see Supplementary Information – MPP⁺ intoxication). This inhibitor of mitochondrial complex I is specifically toxic for DA neurons in a dose-dependent manner [19]. In the present study, addition of 2, 3, or 4 μM of MPP⁺ to mesencephalic cultures between DIV5 and DIV7 decreased the number of TH⁺ neurons and induced neurite degeneration, as shown in Fig. 4A-B. Addition of compound 6c significantly increased the survival of TH⁺ neurons at 10 nM on 2 μM MPP⁺-treated cultures (P<0.05). The effect decreased with MPP⁺ concentration but was still significant at 1 μM in 4 μM MPP⁺-treated cultures (P<0.05). In addition to rescuing TH⁺ neurons from MPP⁺-induced toxicity, compound 6c protected neurites from MPP⁺-induced degeneration, as illustrated in Fig. 4B.

**Compound 6c-induced Neurotrophic Effect is Independent of Glial Proliferation**

In vitro neuroprotective processes were previously linked to an inhibition or an activation of glial proliferation [30,31]. To explore the influence of compound 6c on proliferating cells in mesencephalic cultures, [methyl-3H]-thymidine, a marker of DNA synthesis used to label proliferating cells, was incorporated in the cultures for 18 hours at 37°C then washed before fixation at DIV8, as previously described [32]. An autoradiographic revelation allowed spotting and counting of radioactive nuclei. As outlined in Fig. 5, no effect of compound 6c was observed on proliferating cells under these conditions in comparison with the mitogenic
performed by one-way ANOVA followed by Bonferroni's test.

We report here the discovery of quinoline-derived compounds as neurotrophin mimetics. These compounds behave as neurotrophins with nanomolar activity unprecedented for low molecular weight neurotrophic compounds. Structure–activity relationship analysis revealed not only that the presence of the quinoline ring is crucial for dual activity but also that the substitution position plays a decisive role in the neurotrophic effect. The presence of intrachain triple bond increases the activity which is in contrast with previous reports showing that unsaturations have no influence on the LBA-induced differentiation [12]. These findings suggest that the electronic delocalization between the benzylic donor moiety and the heterocyclic nitrogen in 2-substituted quinolines is strongly implicated in the effect. Furthermore, the removal or replacement of the terminal triple bond decreased the protective effect, which correlates with studies related to rasagiline [42,43] demonstrating the potential of the propargyl group in rasagiline-induced neuroprotection. In these cases, the neurotrophic effect is not affected, which is in accordance with previous reports of compounds possessing lateral hydroxylated long chain as differentiation inducers led to compounds combining dual protective and neuritogenic activities in the nanomolar range.

All our results suggest that both the ring and the terminal propargyl alcohol contribute to the protective effect, while the neuritogenic activity seems to be mainly due to the presence of the lateral hydroxylated chain. However, this is complicated by the fact that the length of the lateral chain and the presence of the benzylic triple bond also have an influence on the protective effect. In addition, our study demonstrates that the compound 6c-induced neurotrophic effect on DA neurons is mediated via the activation of the ERK1/2 signaling pathway. This result is particularly interesting since the MAPK signaling cascade is involved in neurotrophin-induced neuronal survival and neurito-
The prerequisite activation of the ERK1/2 signaling pathway has also been shown to provide neuroprotection in stress-induced conditions [34], which is in line with the 6c-induced protection observed against the oxidative stress caused by MPP+. These results are exciting since targeting MAPK signaling pathways represents an interesting way to slow down neurodegeneration in PD [48].

Quantitative structure–activity relationship (QSAR) studies [49], allow us to predict the potency of compound 6c to cross the BBB by passive diffusion. Indeed, comparisons of determining factors for BBB penetration are given in Table 2. Excluding any currently unknown pharmacological parameters, the physicochemical properties of compound 6c are close to those expected of an orally available CNS drug, suggesting that compound 6c is a serious candidate for in vivo studies and therapeutic use. Furthermore, preliminary toxicology studies performed on mice show that compound 6c does not present any toxic effect in a chronic treatment (300 mg/kg/day in a 15 days treatment) when orally administrated (results not shown).

In summary, we have designed and synthesized a class of quinoline-derived small molecules, some of which show potent neurotrophic activity, expressed as dual protective and trophic activities. These compounds are synthetic small molecules derived from LBA that exhibit potency in the nanomolar range. Lead compound 6c promotes specific survival and neuritogenesis of DA neurons, preserving DA transporter activity in two relevant in vitro models of PD, and does not influence cellular proliferation, thus representing a potential candidate for therapeutic applications in PD.

Materials and Methods

More details are available in supplementary informations S1, S2, S3, S4, S5, S6, S7, S8, S9, S10.

Preparation and Spectroscopic Data of Compounds 2a–c-9a–g

see Supplementary Information - S1.

Primary Mesencephalic Cultures

The embryos were removed at embryonic day 15.5 from pregnant Sprague-Dawley rats (Elevage Janvier, Le Genest St. Isle, France) that had been anesthetized, then decerebrated. Ventral mesencephalon were dissected and collected. Cell suspensions prepared by mechanical trituration precoated overnight with 1 mg/ml polyethyleneimine in borate buffer, pH 8.3. The cells were then maintained for maturation and differentiation in B27-supplemented Neurobasal culture medium. Cultures were treated at DIV1 and every four days 300 μL of culture medium were replaced by medium supplemented with treatments. For more details see Supplementary Information - S2.
Tyrosine Hydroxylase (TH) Immunolabeling

After 12 min fixation with a 4% formaldehyde solution in Dulbecco's phosphate-buffered saline (PBS), cells were washed three times with PBS and then incubated in PBS (PBS containing 0.2% Triton X-100, 10% fetal bovine serum (Sigma, Saint Louis, MO) and 0.01% thimerosal (Sigma, Saint Quentin Fallavier, France) for 1 hour. The cells were further incubated overnight at 4°C with a rabbit anti-TH polyclonal antibody or a mouse anti-MAP-2 monoclonal antibody. Subsequent incubations were performed, at room temperature, with a secondary anti-rabbit IgG cyanin 3 conjugate or an Alexa Fluor 488 F(ab')2 fragment of goat anti-rabbit IgG or an anti-mouse IgG cyanin 3 conjugate. Concerning phospho-Erk1/2 (pp42/pp44) immunofluorescence staining, the cultures were incubated overnight at 4°C with a mouse monoclonal anti-phospho-ERK1/2 antibody diluted at 1:100 in PBS then washed and incubated with an anti-mouse IgG cyanin 3 conjugate. For more details See Supplementary Information - S3.

Neuroprotection was Assessed by TH Immunopositive (TH+) Neuron counting

For more details See Supplementary Information - S4.

Neuritogenesis, Quantification

For more details See Supplementary Information - S5.

MPP+ Intoxication of Mesencephalic Cultures

MPP+ intoxication was performed according to a method previously described [19]. For more details See Supplementary Information – S6.

Uptakes of [7,8-3H]-DA ([3H]-DA) and 4-Amino-n-[2,3-3H]butyric acid ([3H]-GABA)

High-affinity uptake of [3H]-DA and [3H]-GABA was determined according to a method previously described [25]. For more details See Supplementary Information – S7.

Figure 6. Involvement of extracellular signal-regulated kinase (ERK1/2) activation in compound 6c-induced neurotrophic effect. (A) Addition of PD98059 disrupted the compound 6c-induced neuroprotective effect. Neuronal survival was assessed by counting TH+ neurons at DIV12 in 100 or 1000 nM compound 6c-treated cultures in the presence or absence of the MEK inhibitor PD98059 at 10 μM. Dbc-AMP at 200 μM was used as a positive control. Values are normalized to the non-treated control and represent the average of three assays performed in triplicate and expressed as mean±SEM. Statistical analysis was performed by two-way ANOVA followed by Duncan’s post hoc test. *P<0.05. (B) Addition of PD98059 reduced the neurotrophic effect of compound 6c. Images were acquired with an inverted fluorescent microscope coupled to a digital camera. (C) Treatment with compound 6c resulted in an increase of ERK1/2 phosphorylation in a dose-dependent manner. Western blot analyses were performed at DIV12 with proteic lysates from cultures maintained in the presence of compound 6c. Quantification of protein levels was performed with Image J software in at least three independent experiments and is expressed as mean±SEM. Statistical analysis was performed by two-way ANOVA followed by Duncan’s post hoc test. *P<0.05. (D) Visualization of the ERK1/2 phosphorylated forms (Pp44/Pp42) in DA neurons by dual immunolabeling of TH (green) and Pp44/Pp42 (ERK1/2) (red) [48]. Treatment with compound 6c resulted in an increase of ERK1/2 phosphorylated forms in DA neurons. This effect was disrupted by the addition of PD98059 at 10 μM. Images were acquired with unchanged exposure using an inverted fluorescent microscope coupled to a digital camera. Merge were performed using image analysis software.

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Table 2. Prediction of the ability to cross the BBB of CNS drugs using QSARs.

| Attributes                     | Successful CNS Drug* | Compound 6c |
|--------------------------------|----------------------|-------------|
| Potent activity                | low to subnanomolar  | 10 nM       |
| Selectivity                    | High                 | unknown     |
| Molecular weight               | <450 g mol⁻¹         | 375 g mol⁻¹|
| logP                           | <5                   | 2.04³       |
| H-bond donor                   | <3                   | 1           |
| H-bond acceptor                | <7                   | 1           |
| Rotatable bonds                | <8                   | 12          |
| PKa                            | 7.5–10.5 (avoid acid)| 4–5 (estimated)⁶ |
| Polar surface area (PSA)       | <60–70 Å             | 33 Å        |
| Aqueous solubility             | >60 µg mL⁻¹          | >385 µg mL⁻¹⁶ |

*see ref [49].
⁳see ref [50].
⁶in water with 1% ethanol.

ERK1/2 Activation

ERK1/2 activation was assessed according to previously described methods [47]. For more details See Supplementary Information – S9.

Statistical Analysis

Comparisons between two groups were performed with Student’s t test. Multiple comparisons against a single reference group were made by one-way analysis of variance (ANOVA) followed by Dunnett’s or Bonferroni’s post-hoc tests. When all pairwise comparisons were made, two-way ANOVA was used followed by Duncan’s test. S.E.M. values were derived from at least three values per condition of three independent experiments.

Supporting Information

Supporting Information S1 Found at: doi:10.1371/journal.pone.0006215.s001 (15 MB PDF)

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