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## **S29434, a Quinone Reductase 2 Inhibitor: Main Biochemical and Cellular Characterization**

Jean A. Boutin, Frédéric Bouillaud, Elzbieta Janda, István Gacsalyi, Gerald Guillaumet, Etienne C. Hirsch, Daniel A Kane, Françoise Nepveu, Karine Reybier, Philippe Dupuis, et al.

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1           **S29434, a quinone reductase 2 reference inhibitor: main biochemical and cellular**  
2   **characterization.**

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35

36 **Running title:** Reference quinone reductase 2 inhibitor: S29434.

37

38 **Abbreviations:** BNAH: *N*-benzylidihydronicotinamide; CLQ: chloroquine; DIPEA: *N,N*-  
39 diisopropylethylamine; DMEM: Dulbecco's modified essential medium ; DMF:  
40 dimethylformamide; FBS: fetal bovine serum; HATU: hexafluorophosphate azabenzotriazole  
41 tetramethyl uronium or 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium  
42 3-oxid hexafluorophosphate; ITC: isothermal titration calorimetry; MPP+: 1-methyl-4-  
43 phenylpyridinium; MPTP: 1-methyl-4-phényl-1,2,3,6-tetrahydropyridine; NaH: sodium  
44 hydride; NRH: *N*-ribosylidihydronicotinamide; QR1: NAD(P)H dehydrogenase (quinone 1),  
45 EC 1.6.5.2; QR2: quinone reductase 2 or ribosylidihydronicotinamide dehydrogenase  
46 (quinone), formerly E.C. 1.10.99.2 now E.C. 1.10.5.1 ; ROS: reactive oxygen species;  
47 S29434: [2-(2-methoxy-5*H*-1,4*b*,9-triaza(indeno[2,1-*a*]inden-10-yl)ethyl)-2-furamide; TCEP:  
48 tris(2-carboxyethyl)phosphine; TES: 2-[tris(hydroxymethyl)-methylamino]-ethanesulfonic  
49 acid; THF: tetrahydrofuran.

50

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- 52 • Number of tables: 1,
- 53 • Number of figures: 13,
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- 56 • Words in Introduction: 738
- 57 • Words in Discussion: 1897

58

59 **Abstract:** Quinone reductase 2 (QR2, E.C. 1.10.5.1) is an enzyme with a feature that has  
60 attracted attention for several decades: in standard conditions, it does not recognize NAD(P)H  
61 as an electron donor but instead recognizes some putative metabolites of NADH such as *N*-  
62 methyl- or *N*-ribosyl-dihydronicotinamide. Several publications have showed in recent years  
63 its implication in various systems, particularly related to reactive oxygen species (ROS) on  
64 one hand and memory on another, thus strongly suggesting a link between Quinone reductase  
65 2 (as a possible key element in pro-oxidation), autophagy and neurodegeneration in various  
66 pathological conditions. In molecular and cellular pharmacology, a lack of specific and  
67 powerful tools often is an obstacle to understanding physiopathological associations. Here we  
68 present a thorough description of the potent, nanomolar inhibitor S29434 from chemical,  
69 molecular, cellular and stability perspectives, including its actions (and lack thereof) in  
70 various ROS-producing systems. The inhibitor is fairly stable *in vivo*, penetrates cells,  
71 specifically inhibits quinone reductase 2, and has various activities that suggest a key role for  
72 this enzyme in different types of pathological conditions including neurodegenerative  
73 diseases.

74

75

76

## Introduction

77 Quinone reductases are pyridine nucleotide–dependent enzymes involved in the detoxification  
78 of natural quinones, described as early as 1954 in peas (Wosilait and Nason, 1954). In  
79 mammals, the reference quinone reductase enzyme, initially designated DT-diaphorase, was  
80 first described by Conover and Ernster (1960). In the 1990s, another quinone reductase was  
81 cloned: quinone reductase 2 (QR2 or NQO2) with an unusual history (Zhao et al., 1997).  
82 Indeed, the initial description pointed at this enzyme to be a strict analogue of quinone  
83 reductase 1 (a.k.a., DT-diaphorase, or NAD(P)H dehydrogenase (quinone 1), QR1, NQO1,  
84 E.C. 1.6.5.2) with similar properties, but later research revealed that it was quite different  
85 from its closest relative. Early research in the 1960s identified QR2 as the only reductase  
86 using non-canonical co-substrates such as *N*-ribosyl- and *N*-methyl-dihydronicotinamide and  
87 para-quinones as substrates (Liao and Williams-Ashman, 1961; Liao et al., 1962), but not  
88 NADH or NADPH (Zhao et al., 1997; Ferry et al., 2010). Later, Talalay and colleagues  
89 cloned and crystallized QR2 (Zhao et al., 1997). However, the physiologic role of the enzyme  
90 and its specificity were poorly documented and understood, except for the fact that QR2 could  
91 recognize non-quinone compounds such as CB1954 as a substrate (Wu et al., 1997). This  
92 finding was the basis for attempts to develop CB1954 as a prodrug with interesting cytotoxic  
93 properties (Knox et al., 2003). QR2 has been implicated in various situations, distantly related  
94 to quinone metabolism, such as antimalarial actions (Kwiek et al., 2004; Cassagnes et al.,  
95 2017), AMPA-signaling pathway (Rappaport et al., 2015), paraquat-mediated toxicity (Janda  
96 et al., 2013), oxidative stress sensing (Leung and Shilton, 2013) and oocytes maturation (Chen  
97 et al., 2017)

98 While searching for the elusive melatonin MT3 binding site, described by Dubocovich and  
99 colleagues (Duncan et al., 1988) and our team (Paul et al., 1999), we found that this binding  
100 site was QR2 (Nosjean et al., 2000). A screen of many compounds from our library

101 [comprising natural products such as flavonoids (Boutin et al., 2005)] added further  
102 compounds to the list of QR2 inhibitors, together with the already described resveratrol  
103 (Buryanovsky et al., 2004), tacrine (den Braver-Sewradj et al., 2017) or chloroquine (CLQ)  
104 (Kwiek et al., 2004), dabigatran, a prescribed thrombin inhibitor (Michaelis et al., 2012) and  
105 tetracyclic compounds (Boussard et al., 2006) such as S29434. Since some of the products of  
106 the reduction of quinones are fairly unstable, especially in the absence of conjugating  
107 system(s), the products of the reaction, quinols, may spontaneously yield back the original  
108 quinones in the presence of oxygen. Similar quinone cycling was described for QR1 (Bindoli  
109 et al., 1990; Nutter et al., 1992; Bian et al., 2017). Of note, in the absence of cellular  
110 conjugating capacities that neutralize the process by conjugating the quinol (*e.g.* menadiol)  
111 with glucuronic acid (Kappus and Sies, 1981; Bolton et al., 2000; Nishiyama et al., 2010), the  
112 by-product of this futile cycle is the production of reactive oxygen species (ROS). The  
113 generation of ROS can be detected by electron paramagnetic resonance spectroscopy (EPR)  
114 (Reybier et al., 2011). Thus, we used that way to show that under some conditions, the pro-  
115 oxidant property of QR2 can be confirmed and measured.

116 QR2 is expressed in many different organs and cells (Nosjean et al., 2001) and might have a  
117 key role in several pathological conditions. This makes QR2 an interesting study case. For  
118 this reason, we sought to generate new data on the specificity of S29434, its actions in several  
119 situations and put them in perspective with the already available data on this inhibitor to  
120 consolidate its use as an established QR2 inhibitor tool. The present paper deals with the full  
121 description of the characteristics of the inhibitor S29434. Despite previous uses in several  
122 publications, no report exists on its synthesis, on the specificity toward alternative enzymes or  
123 receptors, or on its characteristics in term of metabolism. Thus, we brought as much  
124 information as possible on this molecule, in order to make its use 'safer' in term of  
125 pharmacology of QR2. Here we cover the most important characteristics of the compound,

126 from chemistry to molecular and cellular pharmacology, hints of its specificity towards other  
127 ROS-generating systems as well as some activities *in vivo*. These data illustrate that S29434  
128 or one of its unknown metabolite(s) reaches QR2 and inhibits this enzyme in situ.  
129 Furthermore, its specificity seems to be limited to QR2, making this compound a valuable  
130 tool for understanding the role of this enzyme in multiple scenarios, even in vivo.

131

## Materials and Methods

132

133 **Chemical syntheses.** S29434 synthesis has never been published. Two different paths were  
134 explored for its synthesis. Both are summarized in **Fig. 1 & 2**. The synthetic routes are given  
135 in full details in the supplementary materials, as well as the various steps permitting to obtain  
136 the compounds are detailed in the **Supplementary data 1**. The final analyses of the product  
137 obtained with the second route are available as **Supplemental Data 1** (spectral analyses of  
138 compounds 3, 4 and S29434 obtained by the first synthetic route). S29434 was obtained with  
139 purity above 99%. Analytical details of the second route **Supplementary Figure S2** is also  
140 given as **Supplementary Data 2, 3, 4, 5, 6 and 7**.

141 **QR2 inhibition measurements.** The potency of S29434 was measured in standard kinetic  
142 assays. Briefly, QR2 enzymatic activity was measured under FAD saturation using substrate  
143 (menadione) and 100  $\mu$ M co-substrate BNAH or NRH. An oxidoreduction reaction was  
144 performed at 25 °C in a Tris–HCl 50 mM, pH 8.5, FAD 500 nM, octyl-glucopyranoside 1  
145 mM, dimethylsulfoxide (DMSO) 5% buffer. Enzymatic kinetics measured the decrease in co-  
146 substrate absorbance corresponding to its oxidation. This reaction was followed either at 350  
147 nm (BNAH) or 340 nm (NRH) on a FLUOstar 384-well plate reader (BMG, Offenburg,  
148 Germany). The slope of absorbance decrease was determined using FLUOstar Optima  
149 software (BMG) and expressed in units of optic density (UDO).s-1 then in M.s-1 using the  
150 Beer–Lambert law. This measurement was next corrected from its corresponding non-  
151 enzymatic (spontaneous) oxidation rate measured in the absence of enzyme, which  
152 corresponds to the specific maximum-activity oxidoreduction reaction. For the inhibitory  
153 hQR2 enzymatic activity assay, S29434 was used in the range of 50 pM to 5  $\mu$ M. The  
154 inhibitory concentration 50 % ( $IC_{50}$ ) and the inhibition percentage of co-substrate oxidation  
155 were determined using the PRISM program (GraphPad Software Inc., San Diego, CA, USA).



156 These experiments were conducted over 50 times (inhibition, **Fig. 3A**) or in triplicate (**Fig.**  
157 **3B**).

158 **Binding kinetics of S29434 and resveratrol to QR2 FADox (oxidized FAD).** Transient  
159 kinetics were measured at 25 °C (S29434) or 4 °C (resveratrol) with a SFM-300 stopped-flow  
160 mixer fitted with a FC-15 cell and coupled to a MOS-200 M rapid spectrophotometer  
161 equipped with a 150 W Xe–Hg lamp (Bio-Logic Science Instruments, Seyssinet-Pariset,  
162 France). Typical mixing dead time was 2.6 ms. QR2 tryptophans were excited at 295 nm to  
163 minimize photobleaching and excitation of tyrosines. Fluorescence emission was recorded  
164 using a 320-nm cutoff filter combined with a UG11 band-pass filter to block S29434  
165 fluorescence. Binding experiments were performed under pseudo-first-order conditions by  
166 mixing an equal volume (75 µl) of QR2 and ligand in Tris–HCl 50 mM, β-octyl-D-  
167 glucopyranoside 1 mM, DMSO 5%, pH 8.5. QR2 monomer concentration after mixing was 1  
168 µM. Fluorescence traces were first analyzed individually with the BioKine software (Bio-  
169 Logic, v4.80) as the sum of up to two exponential terms, as described by equation 1, with n  
170 being the number of exponentials, ai the amplitude of the ith exponential, kobs i the rate  
171 constant of the ith exponential, c the trace end point, and bt accounting for the slow linear  
172 drift caused by photobleaching. **Fig. 4** was generated with Prism (GraphPad, v7.03).

173

$$174 \quad y = \left( \sum_{i=1}^n a_i e^{(-k_{obs_i} t)} \right) + bt + c \quad (1)$$

175

176 The kobs values were plotted against ligand concentration. When a linear increase of kobs  
177 was observed, data were fitted to equation 2 describing a one-step binding mechanism, using  
178 Prism (GraphPad, v7.03).

179

$$180 \quad k_{obs} = k_f[\text{ligand}] + k_{-1} \quad (2)$$

181 **Specificity tests. General specificity.** Most of the tests were subcontracted to Eurofins. All  
182 experimental details can be found online on their site ([www.eurofinsdiscoveryservices.com](http://www.eurofinsdiscoveryservices.com)).

183 **Specificity tests. Kinases.** The panel of kinases for screening is available from Eurofins  
184 (ExpressDiversityKinase). It comprises 46 different kinases chosen to cover a maximal  
185 structural diversity among this large family of over 500 different enzymes. S29434 was tested  
186 at 100 nM on the activity of all the kinases using a standard radiometric assay.

187 **Specificity tests Sirtuin experiments.** The use of this original technology has been reported in  
188 a technical note by Agilent (see [https://www.agilent.com/cs/library/applications/5990-9345en\\_lo.pdf](https://www.agilent.com/cs/library/applications/5990-9345en_lo.pdf)). In brief, recombinant sirtuin 1 (SIRT1) was produced in E. coli and purified.  
190 The non-acetylated and acetylated Foxo-3 substrates (hFoxo-3a-290-K(Ac): Ac-DSPSMLS-  
191 K(Ac)-WPGSPTS-NH<sub>2</sub>) were synthesized by GENEPEP SA (Saint-Jean-de-Védas, France).  
192 Deacetylase reactions were carried out in reaction buffer (Tris-HCl 50 mM, NaCl 137 mM,  
193 KCl 2.7 mM, MgCl<sub>2</sub> 1 mM, bovine serum albumin 0.05%, TCEp 1 mM, NAD<sup>+</sup> 0.8 mM) and  
194 20 nM SIRT1 for 30 min at room temperature. The reaction was initiated by the addition of  
195 peptide substrate and quenched by the addition of 2% formic acid. The transitions of peptides  
196 detection: substrate: (excitation at 829.1 nm; emission at 544.6 nm) and product (excitation at  
197 808.4 nm; emission at 544.5 nm) were detected.

198 **Specificity tests. NAD(P)H oxidase experiments.** This enzyme activity was measured using  
199 the lucigenin assay as described by Sasaki et al. (2013) and by Kuribayashi et al. (2008). This  
200 assay was adapted to the U937 cell line as biological source.

201 **ROS production in acellular and cellular experiments using electron paramagnetic**  
202 **resonance (EPR).** 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from  
203 Interchim (Montluçon, France). Menadione, adrenochrome, 3,3'-methylene-bis(4-  
204 hydroxycoumarin) (dicoumarol), NADH, and PBS were purchased from Sigma-Aldrich-Fluka  
205 Co. (Saint Quentin Fallavier, France). BNAH was purchased from TCI Europe (Zwijndrecht,

206 Belgium). NRH was custom synthesized by O2h (Ahmedabad, India). DMSO was purchased  
207 from Fisher (UK, Loughborough). The DMPO stock solution (1 M) was prepared in water  
208 and stored at -80 °C until required. The inhibitors (S29434 and dicoumarol) were prepared as  
209 1 mM solutions in DMSO and diluted in PBS to yield 0.2 mM. The substrates and co-  
210 substrates stock solutions were prepared in DMSO. Experiments on pure enzyme were  
211 performed with demineralized (18 M $\Omega$ ) and deaerated water containing  
212 tris(hydroxymethyl)amino-methane 50 mM and  $\beta$ -octyl-glucoside 1 mM, final pH 8.5. A  
213 Chinese hamster ovary cell line overexpressing QR2 (CHO-k1-QR2) was described  
214 previously (Cassagnes et al., 2015). The experiments were performed on  $5 \cdot 10^6$  cells  
215 suspended in a final volume of 400  $\mu$ l. EPR spectra were obtained at X-band and at room  
216 temperature on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity  
217 (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). The analyses were  
218 performed with a flat quartz cell FZKI160-5 X 0.3 mm (Magnettech, Berlin, Germany).  
219 WINEPR and SIMFONIA software programs (Bruker) were used for EPR data processing  
220 and spectrum computer simulation. Typical scanning parameters were as follows: scan  
221 number, 5; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 1  
222 mW; sweep width, 100 G; sweep time, 41.94 s; time constant, 20.48 ms; and magnetic field  
223 3465–3560 G. The intensities of the EPR signals were evaluated by measuring the amplitude  
224 peak to peak of the second line.

225 **Liver and brain mitochondrial respiration.** The animal experimentation component of the  
226 studies was conducted with the approval of the local Animal Care and Use Committee and  
227 according to the applicable guidelines of the CNRS  
228 (<http://www.cnrs.fr/infoslabos/reglementation/euthanasie2.htm>). Rats (male, 150 g) were  
229 sacrificed by cervical dislocation followed by decapitation (fast bleeding). The liver and  
230 brain were quickly removed and placed in ice-cold mitochondria isolation buffer (sucrose 300

231 mM, Tris 10 mM, EGTA 1 mM, pH 7.4). Mitochondria were prepared by differential  
232 centrifugation, and the final mitochondrial pellet was resuspended in isolation buffer (20–80  
233 mg/mL). Mitochondria were resuspended in 5 mL of mitochondrial respiration buffer (KCl  
234 100 mM, sucrose 40 mM, TES 10 mM, MgCl<sub>2</sub> 5 mM, EGTA 1 mM, 5 mM phosphate, fatty  
235 acid-free bovine serum albumin 0.4 %, pH 7.2), and this suspension was distributed in each  
236 of the two chambers (2 mL final volume) of the Oxygraph 2K (Oroboros Instruments,  
237 Innsbrück, Austria). Temperature was set to 25 °C. Respiration was initiated with addition of  
238 glutamate and malate (5 mM each), and a fully stimulated phosphorylating state 3 was  
239 obtained by addition of 1.25 mM ADP. Increasing amounts of the S29434 were added to one  
240 chamber using 1,000 × concentrated working solutions in DMSO, and simultaneously the  
241 same volume (2 µl) of DMSO was added in the other chamber.

242 **Muscle mitochondrial respiration.** With the exception of Amplex Red Ultra (Life  
243 Technologies) and BNAH (see above), all chemicals and reagents were purchased from  
244 Sigma-Aldrich. Male Wistar rats were purchased from Charles River Laboratories, Inc.  
245 (Montréal, QC) and acclimated for approximately 2 weeks in the Animal Care Facility at St.  
246 Francis Xavier University (Canada). During the acclimation period, the rats were housed in  
247 pairs in standard cages with a plastic tunnel to provide environmental enrichment. All rats had  
248 access to standard rat chow and water, ad libitum. The room in which the rats were housed  
249 was maintained at 20 °C/22 °C and 40%/60% relative humidity and on a reversed 12-h  
250 light/dark cycle. Approval was granted by the institutional Animal Care Committee at St.  
251 Francis Xavier University prior to commencing the study, in accordance with guidelines of  
252 the Canadian Council on Animal Care guidelines. Variations of the permeabilized myofiber  
253 preparation are published elsewhere (Kuznetsov et al., 2008; Perry et al., 2011; Pesta and  
254 Gnaiger, 2012; Perry et al., 2013). In brief, immediately following euthanasia with sodium  
255 pentobarbital, two cuts were made on the right gastrocnemius muscle of each animal. Red

256 (i.e., oxidative) portions of the gastrocnemius were extracted and placed in ice-cold buffer X,  
257 consisting of (in mM): 50 MES, 7.23 K<sub>2</sub>EGTA, 2.77 CaK<sub>2</sub>EGTA, 20 imidazole, 0.5 DTT, 20  
258 taurine, 5.7 ATP, 14.3 phosphocreatine, and 6.56 MgCl<sub>2</sub>·6H<sub>2</sub>O (pH 7.1). Four fiber bundles  
259 from each gastrocnemius section were separated along the longitudinal axis, using needle  
260 tipped forceps (Fine Science Tools, Inc., Vancouver, CB, Canada) in ice-cold buffer X under  
261 magnification (Discovery V8, Carl Zeiss, Oberkochen, Germany). Following dissection, fiber  
262 bundles were placed in vials containing saponin (50 µg/mL) dissolved in 2.0 mL of buffer X.  
263 These vials were then placed on a nutating mixer (VWR International, Radnor, PA, USA) and  
264 kept at 4 °C for 30 min. After permeabilization, fiber bundles were transferred to a wash  
265 buffer solution, MiR05, consisting of (in mM): 20 taurine, 0.5 EGTA, 3 MgCl<sub>2</sub>, 60 K-  
266 lactobionate, 10 KH<sub>2</sub>PO<sub>4</sub>, 20 HEPES, 110 sucrose, 20 creatine, 1 g/L fatty acid-free bovine  
267 serum albumin, pH 7.1. Fiber bundles remained in MiR05 for approximately 15 min with  
268 regular inversion on the nutating mixer until experimentation, after which the samples were  
269 placed into the Oxygraph-2k (Oroboros). Blebbistatin was included in the permeabilization  
270 buffer, wash buffer, and assay to inhibit spontaneous contraction of muscle fibers (Perry et al.,  
271 2011; Perry et al., 2013). Substrate-dependent respiratory oxygen consumption was measured  
272 with the Oxygraph 2K (Oroboros). H<sub>2</sub>O<sub>2</sub> production was monitored in permeabilized rat red  
273 gastrocnemius muscle fibers simultaneously with respirometry using an amperometric add-on  
274 LED module to the Oroboros Oxygraph-2k. Prior to the first substrate addition of the  
275 respirometric protocol, 10 µM Amplex Red Ultra, 5 U/mL superoxide dismutase, and 1 U/mL  
276 horseradish peroxidase were added to the oxygraph chambers containing the fibers. H<sub>2</sub>O<sub>2</sub>  
277 calibration experiments were performed under the same parameters used for experimental  
278 data collection. The relationship between H<sub>2</sub>O<sub>2</sub> and fluorescence intensity was linear to at  
279 least 0.7 µM. Next the following were added, in order: 4 mM malate, 0.2 mM  
280 octanoylcarnitine, 5 mM ADP, 20 mM L-lactate, 5 mM NAD<sup>+</sup>, 5 mM pyruvate, and 10 µM

281 UK-5099, and 10 mM glutamate was then added to test glutamate oxidation. Then, 10 mM  
282 succinate, 1  $\mu$ M auranofin (thioredoxin reductase inhibitor), and 100  $\mu$ M carmustine (BCNU;  
283 inhibitor of thioredoxin and glutathione reductases) were added to the chambers consecutively  
284 to inhibit the H<sub>2</sub>O<sub>2</sub> scavenging systems mitochondria. Finally, H<sub>2</sub>O<sub>2</sub> was titrated to internally  
285 calibrate the resorufin signal for H<sub>2</sub>O<sub>2</sub>. Following conclusion of the respirometry experiments,  
286 fibers were placed directly into water to clear out any remaining substrates in the cytosol.  
287 After 5 min, fiber bundles were then placed in a clean dry Eppendorf tube and freeze-dried  
288 using Labconco® FreeZone1. Following freeze-drying for 5 h, fiber bundles were weighed on  
289 a Mettler Toledo® Excellence Plus XP6 Ultra-Microbalance.

290 Data are expressed as mean  $\pm$  SEM. A two-way, repeated measures ANOVA was completed  
291 for each oxygen flux and hydrogen peroxide production to identify any interaction among the  
292 three groups of exercise and histamine receptor antagonists, exercise only, and control. This  
293 step was followed by a one-way ANOVA with repeated measures (Bonferroni post hoc  
294 analysis) to examine differences between different substrate additions. Data analysis was  
295 completed using Prism 7 (GraphPad). The  $\alpha$ -level for statistical significance was set at 0.05.

296 **Measurement of mitochondrial ROS levels by flow cytometry in cells.** MitoSox (Thermo  
297 Fisher ScientificLife Tech., Molecular probes, Waltham, MA, USA) was used to measure  
298 mitochondrial superoxide levels in cells by flow cytometry. HepG2 were a kind gift from Dr.  
299 Eugenio Arcidiacono ( University Magna Graecia, Catanzaro, Italy), were cultured in high-  
300 glucose (4.5 g/L) Dulbecco's modified essential medium (DMEM), as described (Lascalea et  
301 al., 2018). U373 cells were cultures in low-glucose DMEM. Both types of DMEM were  
302 supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin and streptomycin solution  
303 (Pen-Strep) and 1 % glutamine, all from Carlo Erba srl (Milan, Italy). HepG2 and U373 cells  
304 seeded on 24-well plates at the density 80 x 10<sup>3</sup> and 60 x 10<sup>3</sup> per well, respectively, 2 days  
305 before starting treatments. Cells were exposed to S29434 or vehicle (DMSO) for 6, 18 and 24

306 h before the end of the experiment. Next, the cells were washed with pre-warmed serum-free  
307 DMEM (high and low-glucose, depending on the cell type) and then incubated with MitoSox  
308 (2.5  $\mu$ M, 15 min) diluted in DMEM as above. The experiment was finished by washing the  
309 cells twice with pre-warmed phosphate-buffer saline followed by trypsin-mediated  
310 detachment of cells and flow cytometry analysis according to a previously published  
311 protocols (Janda et al., 2013)

312 **Transient silencing of QR2 expression and autophagy assay in hepatic cells.** HepG2 cells,  
313 were cultured as described above. Cells were seeded on 12-well plates one day before  
314 transfection at a density of 30,000  $\text{cm}^{-2}$ . Non-silencing or control (Ctrl) siRNA (All-stars) and  
315 a set of human QR2-targeting siRNA were purchased from Qiagen (Hilden, Germany).  
316 Transfection of HepG2 cells was performed using Lipofectamine 2000 (L2000, Life  
317 Technologies, Invitrogen, Monza MB, Italy) and Optimem (Life Technologies, Invitrogen)  
318 according to the manufacturer's instructions. Briefly, 360 pmol siRNA QR2 or control siRNA  
319 and 18  $\mu$ l of L2000 were mixed and incubated in 600  $\mu$ l of Optimem for 15–20 min at room  
320 temperature. Each well containing HepG2 cells in 500  $\mu$ l of serum-free medium was overlaid  
321 with 100  $\mu$ l of siRNA-Lipofectamine complexes and incubated in a cell-culture incubator.  
322 After 5 h, the medium was changed for DMEM supplemented with FBS without Pen-Strep.  
323 At 24 h later, the cells were treated with S29434 or DMSO for 24 h in regular medium with  
324 Pen-Strep. CLQ 25  $\mu$ M was added 3 h before the end of the experiment to half of the samples.  
325 The cells were lysed, and the lysates were run on 12% SDS-PAGE gels and assayed for LC3  
326 and QR2 expression by western blotting, as previously described (Janda et al., 2015). The  
327 antibodies were polyclonal rabbit anti-LC3A/B (MBL, Enzo Life Sciences, Farmingdale, NY,  
328 USA) used at 1:2000, anti-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotech,  
329 Dallas, TX, USA) used at 1:500 and anti-QR2 (Sigma-Aldrich) used at 1:1000).

330 **Testing S29434 against neuronal degeneration in vitro.** Animals were housed, handled,  
331 and taken care of in accordance with recommendations of the Guide for the Care and Use of  
332 Laboratory Animals of the National Institutes of Health (NIH Publication no. 85-23, revised  
333 1996) and the European Union Council Directives (2010/63/EU). Experimental procedures  
334 were authorized by the ethical committee on animal experiments (Comité Charles Darwin #5).  
335 Cultures were prepared from the ventral midbrain of Wistar rat embryos at gestational age  
336 15.5 days (Janvier LABS, Le Genest-St.-Isle, France). Dissociated cells in suspension  
337 obtained by mechanical trituration of midbrain tissue pieces were seeded at a density of 1.2–  
338  $1.5 \times 10^5$  cells/cm<sup>2</sup> onto Nunc 48-well multi-dish plates precoated with 1 mg/mL  
339 polyethylenimine diluted in borate buffer, pH 8.3, as previously described (Toulorge et al.,  
340 2011). In some experiments, the cultures were maintained in N5 medium supplemented with 5  
341 mM glucose, 5 % horse serum, and 0.5 % fetal calf serum, except for the first 3 days *in vitro*,  
342 when the concentration of fetal calf serum was set at 2.5 % to favor initial culture maturation  
343 (Guerreiro et al., 2008). In the other experiments, we used a chemically defined serum-free  
344 medium consisting of equal volumes of Dulbecco's minimal essential medium and Ham's  
345 F12 nutrient mixture (DMEM/F12, Life Technologies, Invitrogen), supplemented with 10  
346 µg/mL insulin, 30 mM glucose, and 100 IU/mL Pen-Strep (Troadek et al., 2001). Dopamine  
347 neurons were detected by tyrosine hydroxylase immunofluorescence staining using  
348 procedures previously described (Toulorge et al., 2011). These neurons represented  
349 approximately 2-3% of the total number of neuronal cells present in these cultures after  
350 plating. The cultures, fixed for 12 min using 4% formaldehyde in Dulbecco's phosphate-  
351 buffered saline (PBS), were washed twice with PBS before an incubation step at 4 °C for 24 -  
352 72 h with primary antibodies. A monoclonal anti- tyrosine hydroxylase antibody diluted  
353 1/5000 (ImmunoStar, Inc., Hudson, WI, USA) or a polyclonal anti- tyrosine hydroxylase  
354 antibody diluted 1/1000 (US Biologicals, Salem, MA, USA) was used to assess survival of



355 dopamine neurons. Cell counting was performed with a Nikon TE 2000 inverted microscope  
356 (Nikon, Champigny-sur-Marne, France) at 200 time magnification, using a 20 × objective  
357 matched with a 10 × ocular. The number of tyrosine hydroxylase<sup>+</sup> neurons in each culture  
358 well was estimated after counting visual fields distributed along the x- and y-axes. The  
359 functional integrity and synaptic function of dopamine neurons were evaluated by their ability  
360 to accumulate [<sup>3</sup>H]-dopamine (50 nM; 40 Ci/mmol; PerkinElmer, Courtaboeuf, France), as  
361 previously described (Guerreiro et al., 2008). When using N5 medium supplemented with  
362 serum, dopamine neurons in culture degenerate spontaneously and progressively during the  
363 maturation process of these cultures (Toulorge et al., 2011). Thus, in this setting,  
364 pharmacological treatments were initiated immediately after plating and were then renewed  
365 daily after replacing a fraction (two thirds) of culture medium. Dopamine cell survival was  
366 assessed in 9 days in vitro cultures, i.e., at a stage where about 70 % of dopamine neurons  
367 have already died (Toulorge et al., 2011). Some sets of cultures were treated with veratridine  
368 (0.8 μM), a depolarizing agent used as a positive control for neuroprotection in this setting  
369 (Salthun-Lassalle et al., 2004). Treatments with the mitochondrial toxin MPP<sup>+</sup> (1-methyl-4-  
370 phenylpyridinium) were performed in cultures in which the spontaneous death process was  
371 prevented by supplementing the N5 medium with a depolarizing concentration of K<sup>+</sup> (30 mM)  
372 in the presence of 1 μM of the *N*-methyl-D-aspartate receptor antagonist dizocilpine  
373 ([5R,10S]-[1]-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine, a.k.a. MK-  
374 801), as previously described (Salthun-Lassalle et al., 2004). Note that this treatment did not  
375 interfere with the neurotoxic effects of MPP<sup>+</sup>. MPP<sup>+</sup> was applied to midbrain cultures between  
376 4 and 6 days in culture, whereas protective treatments were added after 2 days in vitro culture  
377 and renewed thereafter until fixation of the cultures at 6 days in culture. Finally, oxidative  
378 stress-mediated dopamine cell death was achieved by placing the cultures in a defined serum-  
379 free DMEM/F12 medium containing 1.5 μM of ferrous iron (Troadek et al., 2001). The

380 antimitotic compound ARA-c (1.5  $\mu$ M) was added to the cultures after plating to prevent glial  
381 cell proliferation. Dopamine cell survival was assessed at 5 days in culture in these  
382 conditions. Experimental values expressed as mean  $\pm$  SEM were derived from triplicates of  
383 three independent experiments. Data were analyzed using the SigmaPlot 12.5 software (Systat  
384 Software Inc, San Jose, CA, USA) with one-way analysis of variance (ANOVA) followed the  
385 Student–Newman–Keuls post hoc test for all pairwise comparisons.

386 **Object recognition in mice.** Experiments were performed in a quiet, dimly lit room in a dark  
387 Plexiglas chamber (L  $\times$  W  $\times$  H: 25  $\times$  35  $\times$  25 cm). On the day before the test day (day 0,  
388 familiarization phase), the animals were allowed to explore the test apparatus for 2.5 min.  
389 Twenty-four hours later (day 1, acquisition phase), after a 30-min pretreatment time after drug  
390 treatment, each test animal was placed in the middle of the test box from the previous day,  
391 and the 5-min acquisition trial was begun. In the first trial, mice were allowed to explore two  
392 identical objects (10 sec per object within a total 5-min period). During test time, the  
393 exploration time (ET) was measured with a stopwatch. ET is defined as direct, active  
394 olfactory exploration of objects 1 and 2. In general, it consists of nosing and sniffing of the  
395 edge and the top, as well as approaching and crossing the line approximately 1 cm around the  
396 objects. Posturing and mounting are not included in measures of investigation. On day 2 (24 h  
397 later, retention phase), a new object and a familiar object were placed in the test box, and the  
398 animals were replaced in the box for 4 min and ET measured again. Discrimination index =  
399 [Exploration time of new object in seconds (N) – Exploration time of familiar object in  
400 seconds (F)]/[Exploration time of new object in seconds (N) + Familiar object exploration  
401 time in seconds (F)] = [N-F]/[N+F]. A thorough description of these methods can be found in  
402 previous publications (Ennaceur and Delacour, 1988; Bartolini et al., 1996; Bevins and  
403 Besheer, 2006). For in vivo data, one-way ANOVA was performed (followed by Dunnett’s  
404 multiple comparisons test) for the DI data.

## Results

405  
406 **Chemistry.** S29434 was synthesized by Guillaumet and colleagues in the late 1990s as a  
407 compound to explore the melatonergic system(s). We introduced the notion that it could be  
408 an interesting tool for QR2 studies after we discovered that MT3, the elusive and  
409 unconventional melatonin binding site we and others described (Duncan et al., 1988; Paul et  
410 al., 1999) was indeed QR2 (Nosjean et al., 2000). The first synthesis, as usual, aimed at  
411 producing several examples of tetracyclic chemicals that could be assimilated into two indolic  
412 structures fused together. The goal at that stage was to document the chemical series. The  
413 synthetic route is summarized in **Fig. 1**. The second synthesis scheme was intended to  
414 rationalize the yields of each step, even if alternative synthetic steps had to be substituted in  
415 the original protocols, as summarized in **Fig. 2**. Full details of the synthetic routes are given as  
416 supplementary data. Of key note, the final product, used in most of the experiments presented  
417 herein, is 99% pure, as can be seen from the analytical details given in the **supplementary**  
418 **data section**.

419 **Inhibition of quinone reductase 2.** Various reports from the literature show that, depending  
420 on the substrate/co-substrate couples used to measure the catalytic activity of QR2, the  
421 recorded IC<sub>50</sub> values range from 1 to 40 nM as summarized in **Table 1**. We stabilized our  
422 system in a standard configuration (see materials and methods section) for all the internal  
423 needs for screening on this enzyme (during which S29434 was used as a reference compound)  
424 and ended up with an IC<sub>50</sub> of  $16 \pm 3.2$  nM (n = 51). The considerable amount of data  
425 accumulated on this material during several years render this information quite robust, at least  
426 using the human cloned enzyme, BNAH as co-substrate and menadione as substrate. A typical  
427 sigmoid curve of concentration/activity relationship is presented in **Fig. 3A**. We also present  
428 the comparison of the inhibition by S29434 of the enzyme catalytic activity while using  
429 BNAH, a synthetic co-substrate, or NRH, a natural candidate co-substrate of QR2 (**Fig. 3B**).

430 One can see that the inhibition using the natural co-substrate is slightly moved leftward,  
431 towards a more potent  $IC_{50}$  in the low 5 nM range. Nevertheless, the use of BNAH is  
432 recommended because the compound is commercially available and slightly more robust than  
433 its ribosylated counterpart (Boutin et al., 2005).

434 **Inhibitor specificity:** *Standard panel of molecular targets* (Eurofins-CEREP profiles). It was  
435 important to start as early as possible on checking the specificity of the inhibitor S29434. Our  
436 classical way of doing this was to check its activity in a series of standard assays designed to  
437 evaluate its behavior against a selection of receptors and enzymes [see, for example, Millan et  
438 al. (2012)]. All the tests were done in duplicate, independently, at two concentrations: 100 nM  
439 and 10  $\mu$ M. None of these assays revealed a capacity of the compound to inhibit the binding  
440 at or the activity of those selected targets, even at fairly high concentrations ( $>10 \mu$ M) (data  
441 not shown). One notable exception is the capacity of S29434 to displace the binding of 2-  
442 iodomelatonin at the melatonin receptor MT<sub>2</sub>, for which the compound has an affinity of 0.14  
443  $\mu$ M, which is not surprising because the compound was initially synthesized as part of a  
444 melatonin receptor agonist discovery process. Finally, S29434 has no cytotoxic activities  
445 towards the following standard cancer cell lines Caco-2, HCT116, HT-29, and L1210 up to 10  
446 mM.

447 **Inhibitor specificity:** *Kinases*. Duncan et al. (2008) reported the toxicity of two casein kinase  
448 2 inhibitors: TBBz (4,5,6,7-*H*-tetrabromobenzimidazole) and DMAT (2-dimethylamino-  
449 4,5,6,7-tetrabromo-1*H*-benzimidazole), presumably because of their inhibition of QR2  
450 catalytic activity. Similarly, Rix et al, reported that the only non-kinase target of the BCR-  
451 ABL inhibitors, imatinib and nilotinib, was QR2 (Rix et al., 2007). Thus, we wanted to know  
452 if this QR2 inhibitor was able to inhibit one or several catalytic activities of a panel of  
453 kinases. Of the 46 different kinases of the panel, none was significantly (more than 20 %)

454 inhibited by S29434 at 100 nM (data not shown). Because of the high potency of S29434, no  
455 higher concentrations were tested.

456 **Inhibitor specificity: *Quinone reductase 1*.** The main component of a possible specificity for  
457 S29434 would be its activity with the closest relative of QR2, namely, QR1. Inversely, we  
458 wanted to confirm that the standard QR1 inhibitor (Ernster et al., 1962), dicoumarol, which  
459 has been widely used over the last decades, was not an inhibitor of QR2. Using the catalytic  
460 activity measurement of the enzymes, the comparison has been made in the past (Ferry et al.,  
461 2010), showing a good selectivity on pure enzymes of S29434 on QR2 over QR1 and vice-  
462 versa for dicoumarol. In a new set of experiments, (see **Fig. 4**), we show the absence of  
463 inhibition of QR1 by 10  $\mu$ M of S29434 compared to the full inhibition by dicoumarol.

464 **Inhibitor specificity: *sirtuin*.** Because of the link between sirtuin-mediated NAD degradation  
465 (Jiang et al., 2017) and the use by QR2 of a possible catabolite of NADH, we wanted to make  
466 sure that the activity of the inhibitor did not lead to upstream inhibition of this enzyme,  
467 diminishing the availability of the co-substrate of QR2. Even at 10  $\mu$ M, S29434 does not  
468 inhibit this enzyme while SOD or diphenyleneiodonium chloride are potently inhibiting  
469 sirtuin activity (not shown).

470 **Inhibitor specificity: *NADPH oxidase*.** In addition to mitochondria, a main cellular source of  
471 ROS production are NADPH oxidase(s) (Sorce et al., 2017; Teixeira et al., 2017). Thus we  
472 checked the capacity of S29434 to inhibit this family of enzymes. The test was assessed with  
473 the NOX inhibitor diphenyleneiodonium. While this reference compound led to a complete  
474 inhibition, S29434 remained poorly active up to 10  $\mu$ M, at which point it began to inhibit  
475 about 20% of the ROS production from those cells (see **Supplemental Figure 3**).

476 **S29434/quinone reductase 2 characteristics: *Binding kinetics*.** Resveratrol has been  
477 described as a potent inhibitor of QR2 (Buryanovskyy et al., 2004) and thus might serve as a  
478 reference compound in some conditions. The binding of both compounds (S29434 and

479 resveratrol) leads to the quenching of the intrinsic fluorescence of QR2 (FADox). Pre-  
480 equilibrium stopped-flow spectroscopy was used to measure the real-time binding kinetics of  
481 the two compounds and compare their binding mechanism (Fig. 5). On one hand, resveratrol  
482 binding kinetics being too fast at 25°C, they were slowed-down by decreasing the temperature  
483 to 4 °C. Data were consistent with a single-step binding mechanism, governed by an  
484 association rate constant  $k_1$  of  $19 \cdot 10^6 \text{ s}^{-1} \text{ M}^{-1}$  and a dissociation rate constant  $k_{-1}$  of  $27 \text{ s}^{-1}$ . A  
485  $K_D$  estimate of  $1.4 \mu\text{M}$  was calculated from the ratio  $k_{-1}/k_1$ , which is in fairly good agreement  
486 with isothermal titration calorimetry (ITC) data obtained on the same enzyme batch, yielding  
487 a  $K_D$  of  $0.6 \mu\text{M}$  at  $25 \text{ °C}$  (data not shown). On the other hand, S29434 displayed slower but  
488 biphasic binding transients, consistent with a two-step binding mechanism involving a  
489 conformational change. Re-plot of the  $k_{\text{obs}}$  rate constants for the fast phase yielded a linear  
490 plot, diagnostic of a bimolecular association step, governed by a  $k_1$  of  $14 \cdot 10^6 \text{ s}^{-1} \text{ M}^{-1}$  and a  
491 dissociation rate constant  $k_{-1}$  of  $61 \text{ s}^{-1} \cdot \text{M}^{-1}$ . A  $K_D$  estimate for this encounter complex of  $4.4$   
492  $\mu\text{M}$  can be calculated from the ratio  $k_{-1}/k_1$ . The observed rate of the slow phase was  
493 independent of the S29434 concentration in the investigated range, staying close to  $30 \text{ s}^{-1}$ .  
494 This step is therefore likely reflecting a conformational rearrangement, either following  
495 binding (induced-fit) or preceding it (conformational selection). While preliminary, these  
496 kinetic data strongly suggest the evolution of the binding mechanism from the low-affinity  
497 single-step binder resveratrol to the high-affinity two-step binder S29434. They also are in  
498 agreement with results from our mass spectrometry studies of the inhibitor/enzyme binding  
499 (Antoine et al., 2012).

500 **S29434/quinone reductase 2 characteristics:** *Pause of S29434 in QR2.* S29434 has been co-  
501 crystallized in QR2 on several occasions (Pegan et al., 2011; Gerard et al., 2018), but its pause  
502 inside the crystal was poorly discussed. In brief, as shown in Fig. 6, the ligand S29434 fits  
503 deeply into a QR2 hydrophobic cavity (PDB: 3OX3), interacting with both subunits A and B

504 with its amido-furan side chains pointing toward the solvent. It makes  $\pi$ -edge interactions  
505 with Trp105 and Phe126  $\pi$ - $\pi$  interactions with Phe178 and a water-mediated H-bond with  
506 Asn161. Additional  $\pi$ - $\pi$  interactions exist with co-factor FAD. Although the furan moiety  
507 makes an additional hydrophobic interaction with Ile194 from QR2, it also seems that this  
508 part of the molecule somehow hangs out of the catalytic site, potentially offering the  
509 possibility to modify/decorate this part of the molecule with associated properties, as for  
510 instance a fluorophore moiety.

511 **Notions of S29434 drug metabolism.** As often happens, at this stage of the research  
512 programs, some experiments were conducted to better understand the properties of the  
513 compound in terms of pharmacokinetics or more generally from the drug's cellular  
514 penetration. The first experiments were those involving measurement of the behavior of  
515 S29434 in an *in cellulo* blood-brain barrier model (Cechelli et al., 1999), based on co-cultures  
516 of bovine brain micro-vessel endothelial cells and rat astrocytes (Booher and Sensenbrenner,  
517 1972). We measured the permeability surface total (PSt) (filter + collagen + cells) and the  
518 permeability surface filter (PSf) (filter + collagen without cells) expressed in milliliters per  
519 minute. These values were 4.58 cm/min and 3.78 cm/min, respectively. Therefore, the ratio  
520 PSt/PSf (permeability class) for S29434 was determined to be 121%, classifying it into the  
521 high category of permeable compounds.

522 **Reactive Oxygen Species and quinone reductase 2: QR2-mediated ROS production.** QR2  
523 activity can be followed by EPR (Reybier et al., 2011; Cassagnes et al., 2015). As stated  
524 above, this functional assay is indirectly linked to the enzyme. Indeed, the product of the  
525 reaction, a quinol, has a poor stability in standard conditions. Thus, in the presence of oxygen,  
526 the quinol is oxidized back in the original quinone which in turn serves as substrate for the  
527 enzyme, starting a futile cycle between the two chemical species with the concomitant  
528 production of reactive oxygen species [see Reybier et al (2011) for complete discussion]. This

529 process seems to occur in a-cellular as well as in cellular contexts (Cassagnes et al., 2017).  
530 Here, we completed and reinforced these data by showing by EPR spectroscopy that pure  
531 QR2, in the presence of its co-substrate (BNAH), produces ROS during the catalytic activity  
532 (**Fig. 7**) that is inhibited by S29434. Furthermore, under those experimental conditions,  
533 neither NADH nor NAD(P)H can substitute for BNAH. Note that in basal conditions (no  
534 substrate and co-substrate of QR2), very low levels of ROS were recorded.

535 **Reactive Oxygen Species and quinone reductase 2: Mitochondrial respiration in liver and**  
536 *brain*. Because S29434 diminishes the amount of ROS produced during the futile cycle  
537 between quinol and quinone in aerobic conditions (Reybier et al., 2011), we wanted to make  
538 sure that this effect was not the result of some direct interference of S29434 with ROS even  
539 though the compound is inactive in other ROS-generating systems (such as mitochondria).  
540 Thus, it was confirmed that S29434 cannot trap hydroxyl radicals ( $\bullet\text{OH}$ ) produced via the  
541 Fenton reaction or superoxide radicals ( $\text{O}_2^{\bullet-}$ ) produced from crown ether and  $\text{KO}_2$  (not  
542 shown). Furthermore, another source of cellular ROS production was tested: the  
543 mitochondria-mediated production of radical species. To evaluate the mitochondrial effect of  
544 drugs, mitochondrial respiration was settled with complex I substrates (glutamate/malate) and  
545 in phosphorylating conditions (1.25 mM ADP). The relative effect of increasing amounts of  
546 S29434 is indicated by the ratio of respiratory rates compound/solvent, with a value of 1  
547 indicating a lack of effect. With this approach, S29434 did not produce a significant effect at  
548 concentrations up to 10  $\mu\text{M}$  and revealed no statistically significant changes in respiratory rate  
549 with rat liver or rat brain mitochondria (**Fig. 8**). The system was validated with experiments  
550 with rotenone or oligomycin, two known inhibitors of the mitochondrial respiratory chain,  
551 leading to results similar to those reported in the literature (data not shown).

552 **Reactive oxygen species and quinone reductase 2: Mitochondrial respiration in muscle.**  
553 Thorough studies were then independently conducted on mitochondria in saponin-



554 permeabilized rat gastrocnemius muscle fibers. As illustrated in **Fig. 9 A**, no differences in  
555 respiration were observed between conditions, confirming that neither BNAH nor S29434  
556 affected respiration in these samples. Likewise, BNAH and S29434 did not affect H<sub>2</sub>O<sub>2</sub>  
557 production (**Fig. 9 B**). When H<sub>2</sub>O<sub>2</sub> was expressed as a percentage of O<sub>2</sub> flux, a significant  
558 difference was observed between the BNAH and BNAH + S29434 (**Fig. 9 C**) only in the  
559 absence of ADP, suggesting that BNAH may increase mitochondrial H<sub>2</sub>O<sub>2</sub> in non-  
560 phosphorylating (i.e., State 4, or LEAK) conditions. However, because neither respiration nor  
561 H<sub>2</sub>O<sub>2</sub> alone was affected, a more plausible explanation is that the difference in proportional  
562 H<sub>2</sub>O<sub>2</sub> production is the result of non-mitochondrial sources of H<sub>2</sub>O<sub>2</sub> in the samples and not of  
563 bulk O<sub>2</sub> flux.

564 **Effects of S29434 on cells. Nervous system cells.** S29434 was described to have a beneficial  
565 effect against paraquat-induced toxicity *in cellulo* and *in vivo* (Janda et al., 2013; Janda et al.,  
566 2015), suggesting a potential role of QR2 in Parkinson's disease. This prompted us to analyze  
567 its effect in models of neuronal degeneration *in cellulo*. To this aim, primary midbrain  
568 dopamine neurons in culture have been established from fetal rat ventral midbrains. S29434  
569 (up 100 μM) could not reduce the spontaneous loss of dopamine neurons in culture (**Fig. 10**  
570 **A**), while the reference neuroprotective molecule, veratridine, efficiently rescued these cells.  
571 S29434 also failed to protect dopaminergic cells from iron-induced oxidative stress, (in  
572 presence of iron in the culture medium) caused by Fenton-type reaction occurring  
573 spontaneously (**Fig. 10 B**). At 100 μM, there was even a tendency for S29434 to become  
574 slightly toxic. However, as expected, the chelation of iron with deferoxamine (10 μM)  
575 afforded a robust protection under these conditions. In contrast to what we observed in the  
576 two previous settings, S29434 showed concentration-dependent protective effects against the  
577 mitochondrial complex I inhibitor MPP<sup>+</sup> (**Fig. 10 C**). The effect of S29434 was already  
578 significant at 0.1 μM, peaked at 1 μM, and declined progressively between 10 and 100 μM.

579 Dopamine cell rescue was also observed with the positive control, nicotine, used at 10  $\mu$ M.  
580 Because MPP<sup>+</sup> is selectively taken up by the dopamine transporter to exert its selective  
581 toxicity towards dopamine neurons, we tested whether S29434 was operating as an inhibitor  
582 of dopamine uptake. This was not the case because S29434 does not bind to the ad hoc  
583 transporter (not shown) strongly suggesting that S29434 was blocking the intrinsic  
584 mechanism of toxicity of MPP<sup>+</sup>.

585 **Effects of S29434 on cells. Autophagy.** Autophagy is a vesicle-mediated pathway that ensures  
586 the recognition and transport of defective organelles and protein aggregates to lysosomes,  
587 where they are digested. We tested if S29434 could induce autophagy, in the human HepG2  
588 cells, derived from liver, the organ with the highest level of QR2 expression (Nosjean et al.,  
589 2000). S29434 dose-dependently induced LC3-II, a marker of autophagy vesicles (**Fig. 11A**).  
590 Silencing of QR2 by more than 50% increased basal LC3-II levels and suppressed LC3-II  
591 induction by S29434, indicating that the autophagy response to this compound does not  
592 depend on its off-target effects, but it is directly mediated by QR2 inhibition (second panel in  
593 **Fig. 11 A & B**). Of importance, LC3-II was further accumulated in S29434-treated cells,  
594 when autophagy flux was blocked by the lysosomal inhibitor CIQ, indicating a true induction  
595 of autophagy by the QR2 inhibitor (**Fig. 11 B**). Further details are given, including the  
596 comments on the whole blots as **Supplementary Data 8**. These data confirm that QR2 plays  
597 a role in regulation of autophagy. Oxidative stress and redox reactions have a strong impact  
598 on autophagy machinery (Janda et al., 2012; Janda et al., 2015). However, since QR2  
599 inhibition does not reduce basal ROS levels neither in HepG2 cells (**Fig. 12A**), nor in U373  
600 astrocytes (**Fig. 12B**), the mechanism by which S29434 triggers autophagy should be  
601 mitochondrial ROS-independent and QR2-dependent as suggested by the data presented  
602 above (**Fig. 11A & B** and **Supplementary Data 8**).

603 **Effects of S29434 *in vivo*.** *Object recognition tests in CH3 wild-type mice.* The interplay  
604 between QR2 and “memory” has been observed in several occasions (Brouillette and Quirion,  
605 2008; Benoit et al., 2010; Rappaport et al., 2015). Thus, we wanted to document further this  
606 relationship, using the inhibitor S29434 in the object recognition test. The test trial with the  
607 new object was carried out 24 h after the acquisition trial with two identical objects. S29434  
608 was given intraperitoneally, 30 min before the acquisition trial. The discrimination index is  
609 shown, where N is the time spent inspecting the new object, and F is the time spent exploring  
610 the familiar object during the test trial (**Fig. 13A**). Both concentrations of S29434 led to an  
611 enhanced time of exploration of the unfamiliar object. Under these circumstances and  
612 experimental conditions, the object recognition memory of wild-type mice is fairly enhanced  
613 by the treatment. As other authors working with other models have reported (Rappaport et al.,  
614 2015), this finding suggests that the compound, by inhibiting QR2, has a positive effect on at  
615 least this particular aspect of memory.

616 ***In vivo* considerations on S29434.** *Object recognition memory in the KO CH3QR2<sup>-/-</sup> mice.*  
617 The same experiments were carried out on KO CH3QR2<sup>-/-</sup> mice. The corresponding results  
618 are presented in **Fig. 13 B**. For QR2 KO animals, the results strongly reinforce the previous  
619 experiments. Indeed, the treated (1 or 15 mg/kg) and control animals did not differ, indicating  
620 that the product has no further effect on this parameter. Because the QR2 KO animals were  
621 genetically deficient only in this enzyme (Mailliet et al., 2004), this lack of activity of S29434  
622 strongly suggests that the compound is specific to QR2. Furthermore, we know these animals  
623 did not lose their learning capacity compared to the wild-type mice as demonstrated in several  
624 tests including the Morris water maze, object recognition, and rotarod performance test. All  
625 their performances in those tests were superior to those by their wild-type counterparts  
626 (Benoit et al., 2010).

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## Discussion

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*General.* Molecular pharmacology is based on either the manipulation (including the mutagenesis) of targets or their inhibition/activation by synthetic or natural chemicals. Hundreds of compounds have been described to serve such tasks. Using those tools, it is possible to better understand the mechanisms by which targets (receptors, enzymes) regulate physiological systems, up to a point where pathology replaces physiology and drugs replace chemicals. This naïve way of describing what is the basis of Pharmacology and thus of Therapeutics permits to better understand the limits of those approaches. Indeed, finding a compound is not usually complicated, and the modern use of high-throughput screening campaigns together with the power of the virtual analyses led very often to compounds that interact with a target in a given system. Obviously, going from chemicals to drug involves many aspects that are often complicated obstacles to overcome. A way to clarify the future use of a new compound is to deliver as many information as possible on it. In an area not distant from QR2 pharmacology, a good counterexample is certainly dicoumarol, the QR1 reference inhibitor. This compound has been widely used in the literature (over 2700 references) and still, many of its off-targets effects biases the interpretation of the data obtained with this compound [see discussion in Scott *et al* (2011)].

For many reasons, the project to which S29434 was originally attached (melatonergic ligands) led us to quinone reductase 2 and a completely different avenue of research, as described elsewhere (Boutin, 2016). By distributing the compound to colleagues looking for the role of this curious enzyme in various systems, we did not follow the best path. Indeed, no information was available at that time on the various situations in which the compound was tested: *in cellulo* or *in vivo*. The stability, permeability and overall specificity of S29434 were among the information that we did not know. Furthermore, no clear synthesis method was

653 published so far. We thus decided to complete the already available information on S29434 by  
654 bringing either completely new or by reinforcing previously but minimally documented  
655 findings.

656 *Chemistry.* S29434 belongs to a family of flat tetracyclic compounds some of which were  
657 described by us (Boussard et al., 2006) or by others: cryptolepines (Onyeibor et al., 2005;  
658 Lavrado et al., 2008; Whittell et al., 2011) or other types of indolizino-indolones  
659 (Bhattacharya et al., 2001) for different purposes. Compounds known to inhibit QR2 activity  
660 were also reported in the literature, such as pyrroloquinoline ammosamides (Reddy et al.,  
661 2011), or furan-amidines (Alnabulsi et al., 2018). A few, if any, were tested and documented  
662 outside their enzymatic test activities, thus we believe that the present report is remarkable  
663 because it described S29434 in different *in cellulo* as well as *in vivo* situations.

664 *Acellular behavior, specificity:* In an a-cellular system, the compound is a potent inhibitor of  
665 the activity of the QR2 2. It does not or only marginally inhibit QR1. Furthermore, among  
666 more than a hundred other targets, it does not potently interfere with any, with the sole  
667 exception of the melatonin receptor 2 for which it has a binding affinity in the micromolar  
668 range. Of course, the notion of specificity is limited to the targets the measures of which are  
669 available. Indeed, it is impossible to know if the compound will uniquely recognize QR2  
670 among the protein products of the 26 000 genes of the human genome. The mechanism by  
671 which S29434 binds to and interacts with QR2 has been scrutinized at several occasions  
672 (Pegan et al., 2011; Antoine et al., 2012). Here, we bring more information on the way the  
673 compound fills up the catalytic site of the enzyme, with trends on how this can be further  
674 accomplished, as well as a way to measure the affinity of the compound for the enzyme, in  
675 complement to its capacity to inhibit it. These two notions (affinity for an enzyme and  
676 inhibition potency) are too often mixed up. It is important to be able to segregate between the  
677 affinity with which the compound recognizes the enzyme and its capacity to impair its

678 activity. Furthermore, the first of those biophysical measures permits to evaluate the time of  
679 residency during which the compound stays inside the catalytic site. This time can widely  
680 vary inside a series of analogue compounds for a given enzyme ranging from seconds to  
681 hours. The implication of this measure is the time during which the compound stays in the  
682 enzyme.

683 *Stability in vivo, membrane crossing.* S29434 seems to be fairly stable in animal experiments,  
684 based on previously published data on S29434 (Benoit et al., 2010; Janda et al., 2013; Janda  
685 et al., 2015) as well as the study on its blood-brain barrier crossing provided here. Based on  
686 several published observations, it was already clear that the compound was penetrating very  
687 different types of primary or cultivated cells (Benoit et al., 2010; Janda et al., 2013; Chen et  
688 al., 2017). It was also clear that because the compound was active in rodent models,  
689 administered by intraperitoneal (Janda et al., 2015; Rappaport et al., 2015) or by intra-  
690 cerebroventricular (Janda et al., 2013) routes, it should be fairly stable in mammals and that  
691 sufficient quantities were reaching the brain. Indeed, in each case, the effects seen *in vivo*  
692 could have been interpreted as an inhibition of QR2, strongly suggesting stability and  
693 penetration.

694 *Cellular aspects, reactive oxygen species.* As explained, there is a crucial link between QR2  
695 and ROS production. Thus, our main goal was to ensure that the compound was not a  
696 scavenger *per se*, and that its capacity to diminish ROS bursts in cellular experiments was due  
697 only to its interference with QR2. Indeed, experiments conducted on cells show that the  
698 compound is capable to inhibit QR2-dependent production of ROS in cellular experiments, on  
699 various types of cells such as hepatoma HepG2 cells, the astroglial cell line U373, (Janda et  
700 al., 2013) as well as on primary dopaminergic neurons (Cassagnes et al., 2017). However, the  
701 compound does not alter basal ROS levels in cells when applied in the absence of exogenous  
702 QR2 activators. For example, we treated HepG2 and U373 cells with S29434 at 6, 18, and 24

703 h before measuring the intracellular ROS levels with MitoSox, a fluorescent compound  
704 recognizing preferentially mitochondrial superoxide anion (Polster et al., 2014). MitoSox  
705 fluorescence was unaltered by S29434 at all experimental times in HepG2, while an  
706 unspecific antioxidant, *N*-acetyl cysteine (NAC), significantly reduced basal ROS levels (Fig.  
707 12). Mitochondria are the main source of cellular ROS in physiological conditions. It was thus  
708 important to conduct detailed studies on mitochondria from various organs and to validate that  
709 in all conditions, S29434 was not able to inhibit this ROS production. Furthermore, it does not  
710 inhibit Nox, nor it impairs ROS produced from a Fenton-type reaction to be toxic to neuronal  
711 cells. These data strongly suggest that S29434 is specific of QR2.

712 *Cellular aspects, autophagy.* The autophagy pathway mitigates the cellular damage that  
713 occurs in response to stress and toxic agents and plays an important role in ensuring basal  
714 protein and organelle turnover (Janda et al., 2012; Dagda et al., 2013). While studying the  
715 antioxidant effects of S29434 on paraquat-induced toxicity, we discovered that this compound  
716 has yet another property, which is the ability to restore basal autophagy in paraquat-treated  
717 astrocytes. In addition, we found that S29434 induces autophagy in the absence of paraquat  
718 and that this response was mediated by a QR2-dependent mechanism in astrocytes (Janda et  
719 al., 2015). The data presented in the present work indicate that QR2-dependent mechanism of  
720 autophagy induction can be generalized to other cell types such as liver cells. Furthermore, we  
721 show here that this effect does not correlate with any detectable modulation of ROS levels in  
722 liver cells, thus excluding possible ROS-dependent basis of the pro-autophagic activity of  
723 S29434. The accumulation of LC3-II in the presence of CIQ is believed to result from a  
724 blockade of lysosome activity, but it might partially depend on the ability of the anti-malaria  
725 drug CIQ to bind and inhibit QR2 directly, although with a poor affinity when compared to  
726 S29434 (Kwiek et al., 2004; Leung and Shilton, 2015). Indeed, a direct effect of CIQ on QR2

727 is much weaker than its inhibitory effect on lysosomal digestion because LC3-II still  
728 accumulates when QR2 expression is reduced by siRNA (Fig. 11 B, compare lines 1 and 4).

729 *Cellular aspects, neuronal cells.* Another key result is presented here. MPP<sup>+</sup>, the active  
730 metabolite of MTPT, is an inhibitor of the mitochondrial complex I. This compound once  
731 delivered to mice, leads to a Parkinson-like syndrome. We know that QR2 does not recognize  
732 MPTP as a substrate; nevertheless, S29434 provides a concentration-dependent protection in  
733 this model. Of interest, paraquat and MPP<sup>+</sup> are both Parkinsonian toxins and share common  
734 toxicity mechanisms, including the inhibition of complex 1 activity in the mitochondria and  
735 dysregulation of autophagy (Dagda et al., 2013).

736 *In vivo activity, ultimate specificity.* Of course, one of the possible ultimate proofs of  
737 specificity would be in a case where an enzyme activity is linked to a clear observation (for  
738 instance, memory or certain type(s) of memory) in vivo. In that case, assuming or proving a  
739 reasonable penetration, distribution and stability of a compound, if this compound is active –  
740 inhibiting the activity – in wild type mice and inactive on the same parameter in knock-out  
741 mice for this particular protein, one can assume, once again, a higher degree of specificity of  
742 the molecule. It is exactly the situation we described herein: wild type mice in object  
743 recognition memory test are sensible to S29434: after treatment, mice are spending an  
744 enhanced period of time exploring the unfamiliar object compared to the untreated mice,  
745 suggesting also a role of QR2 in memory after others (Harada et al., 2001; Benoit et al., 2011;  
746 Rappaport et al., 2015). Finally, the same experiments in engineered mice in which QR2 has  
747 been deleted, the compound is not active anymore. Of note, since the first observation  
748 (Brouillette and Quirion, 2008), it seems that the QR2 catalytic activity aims at suppressing  
749 several types of memory, potentially linked to dopaminergic neurons (McNab et al., 2009;  
750 Rossato et al., 2009; Nobili et al., 2017), see also review by D’Amelio et al (2018).



751 The present paper does not cover the actions of S29434 in various models on inflammation,  
752 that are often linked to ROS production, but S29434 does not influence Toll-like receptors or  
753 inflammasome signaling (Groß et al., 2016) as an activator or inhibitor (O. Gross, personal  
754 communication). Further work is needed to better understand a possible role of QR2 in  
755 inflammation responses.

756 In summary, S29434 is a powerful inhibitor of QR2. It is specific over QR1 and over a range  
757 of standard pharmacological targets. It is fairly stable *in vivo*, and penetrate cells.  
758 Nevertheless, the body of results gathered in the present work does not guarantee the  
759 specificity of S29434 but clearly delineates some of its actions, and particularly in various  
760 models that help confirming that its actions are largely the result of its capacity to powerfully  
761 inhibit QR2. Several other molecules have been described as inhibiting QR2, such as  
762 resveratrol, melatonin, and some flavonoids, but they are non-specific and inhibit many  
763 different enzymes in many different pathways; thus, using them to dissect a given pathway is  
764 difficult. As such, we believe that the present work lays a strong foundation for the  
765 pharmacological use of S29434 in many situations to evaluate the role of QR2.

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788 Thierry Le Diguarher developed and ran the drug metabolism experiments and wrote the  
789 related materials and methods and the results. Frederic Bouillaud, Hala Guedouari, Karen  
790 Brebner, and Daniel A. Kane designed and ran the mitochondria experiments, wrote the  
791 related materials and methods and the results, and designed the corresponding figures. Marie-

792 Claude Viaud-Massuard, Hervé Da Costa, and Gérald Guillaumet conceived the molecule,  
793 synthesized it, wrote the related chemical materials and methods section, and developed the  
794 related figures. Vishalgori Goswami and Aakash Patel conceived the alternate synthetic route,  
795 applied it to the synthesis of the molecule, wrote the related chemical materials and methods  
796 section, and developed the related figures. István Gacsalyi ran the in vivo experiments, wrote  
797 the related materials and methods section and the results, and designed the corresponding  
798 figures. Adeline Giganti and Jérôme Paysant designed and ran the specificity experiments,  
799 wrote the related materials and methods section and the results, and designed the  
800 corresponding figure. Pierre Ducrot ran the molecular modeling experiments, wrote the  
801 related results, and designed the corresponding figure. Etienne C. Hirsch and Patrick P.  
802 Michel designed and ran the neuron experiments, wrote the related materials and methods  
803 section and the results, and designed the corresponding figure. Elzbieta Janda designed and  
804 ran the autophagy experiments and flow cytometry ROS measurements, wrote the related  
805 results and materials & methods sections, and designed the corresponding figures. Monivan  
806 Chhour, Françoise Nepveu and Karine Reybier designed and ran the EPR and ROS  
807 experiments, wrote the related materials and methods section and the results, and designed the  
808 corresponding figures.

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## Figure Legends

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813 **Fig. 1: Schematic representation of the synthesis of S29434.** Note that the yield presented  
814 here is not to be taken for the purity of the final compound(s) that were usually above 95 %.

815 **Fig. 2: Schematic representation of an alternate synthesis of S29434.** Note that the yield  
816 presented here is not to be taken for the purity of the final compound(s) that were usually  
817 above 95 %.

818 **Fig. 3: Quinone reductase 2 inhibition by S29434. A)** Inhibition profile of S29434 on pure  
819 quinone reductase 2. The pure enzyme (2 nM) was incubated with 100  $\mu$ M of BNAH and 100  
820  $\mu$ M of menadione in a buffer consisting of 1 mM n-octyl-beta D-glucopyranoside in 50 mM  
821 Tris-HCl, pH 8.5 and 500 nM FAD. In the absence of enzyme, the reduction does not occur.

822 **B)** Inhibition profile of the compound S29434 as a function of hQR2 co-substrate. IC<sub>50</sub>  
823 values were here assessed in a standard enzymatic assay (Tris-HCl 50mM pH 8.5, FAD  
824 500nM, n-octyl-B-d-glucopyranoside 1mM, DMSO 5%) consisting in 0.5nM E.coli expressed  
825 human QR2 (PDB: 2QX4) preincubed with S29434 [50pM - 5 $\mu$ M], either BNAH or NRH  
826 100 $\mu$ M and triggered by addition of menadione 100 $\mu$ M. Results are expressed as % inhibition  
827 of the control activities deduced from BNAH (350nm) or NRH (340nm) absorbance  
828 measurements, and are the mean of 3 independent experiments.

829 **Fig. 4: Specificity of S29434 towards quinone reductase 1. Comparison with dicoumarol.**  
830 Reactive oxygen species production recorded with purified QR1 (5  $\mu$ g/mL) in the presence of  
831 NADH (100  $\mu$ M) and menadione (100  $\mu$ M) once pre-incubated with buffer (control), S29434  
832 (10  $\mu$ M) or dicoumarol (10  $\mu$ M). The corresponding EPR spectra are presented on the right  
833 panels. The production of ROS was evaluated by the double integration of the EPR spectra  
834 obtained with DMPO (50 mM) as spin-trap.

835 **Fig. 5: Binding kinetics of resveratrol and S29434 to QR2 (FADox).** **A, B, C)** Resveratrol.  
836 **D, E, F)** S29434. **A)** Fluorescence transients measured after mixing QR2 and resveratrol at  
837 4°C, final concentrations were 1  $\mu$ M and 8  $\mu$ M, respectively. **B)** Residuals of the mono-  
838 exponential fit. **C)** Re-plot of the  $k_{obs}$  values overlaid with the corresponding linear fit. **D)**  
839 Fluorescence transients measured after mixing QR2 and S29434 at 25°C; final concentrations  
840 were 1  $\mu$ M and 8  $\mu$ M, respectively. **E)** Residuals of the bi-exponential fit. **F)** Re-plot of the  
841  $k_{obs}$  values for the fast (upper plot) and slow (lower plot) phases. The fast-phase plot is  
842 overlaid with the corresponding linear fit.

843 **Fig. 6: Co-crystallization of S29434 with quinone reductase 2.** **A)** QR2 molecular surface  
844 colored by lipophilicity (blue: hydrophilic regions, green: hydrophobic regions). Ligand  
845 S29434 (coral, space filling model). **B)** Ligand S29434 binding cavity (orange: chain 1; pink:  
846 chain B; coral: ligand; lipophilic surface). **C)** Left: QR2/S29434 interactions; right:  
847 QR2/S29434 interactions with FAD (grey).

848 **Fig. 7: Effect of co-substrate and inhibitor on the QR2-dependent production of ROS.**  
849 *Left panel:* ROS production recorded with purified QR2 (25.6  $\mu$ g/mL) and different co-  
850 substrates: BNAH, NADH or NAD(P)H, in the presence or not of 20  $\mu$ M S29434. The  
851 production of ROS was evaluated by the double integration of the EPR spectra obtained with  
852 DMPO (50 mM) as spin-trap. *Right panel:* Crude EPR spectra recorded with BNAH or NADH  
853 and menadione.

854 **Fig. 8: Effect of S29434 on respiration of rat liver and brain mitochondria.** Rat liver  
855 mitochondria (**A, B**) and rat brain mitochondria (**C, D**) oxygen consumption was measured  
856 simultaneously in the two chambers of the respirometer O2k ([www.orooboros.at](http://www.orooboros.at)). Successive  
857 additions are indicated below the X-axis (time). Firstly, (left to the vertical dotted line) we  
858 settled a fast respiring phosphorylating state by addition of substrates glutamate/malate (GM)  
859 and ADP. Then, (right to the dotted line) four additions of increasing concentrations ( $10^{-7}$  to

860  $10^{-4}$  M) of S29434 were made (black line and empty dots) as a control same volumes of  
861 solvent (DMSO) were added (grey line). **A, B:** mean value of the oxygen consumption rate [in  
862 pmol/(s. mg protein)] \*  $p < 0.05$  with a paired t-test to compare the oxygen consumption rate  
863 with S29434 (100  $\mu$ M) or solvent for the same period of time. **C, D:** ratio between the rates  
864 S29434/Solvent.. \*\*  $p < 0.01$  with the Mann–Whitney rank test (<http://vassarstats.net/>) for the  
865 comparison of the ratio observed with S29434 at  $10^{-4}$  with each of the others comparable  
866 situations (ADP and no further addition, S29434  $10^{-7}$  to  $10^{-5}$  M).

867 **Fig. 9: Effects of S29434 on mitochondrial respiration or H<sub>2</sub>O<sub>2</sub> production in**  
868 **permeabilized skeletal muscle.** Saponin-permeabilized rat red gastrocnemius skeletal muscle  
869 fibers were incubated in the following prior to assay: 100  $\mu$ M BNAH, 30  $\mu$ M S29434, 100  
870  $\mu$ M BNAH + 30  $\mu$ M S29434, or control (vehicle: 18.75  $\mu$ l DMSO). Subsequently, respiration  
871 was measured in all conditions (**A**), and H<sub>2</sub>O<sub>2</sub> in S29434 and BNAH + S29434 conditions (**B**).  
872 H<sub>2</sub>O<sub>2</sub> as a percentage of O<sub>2</sub> consumed was also calculated. \* $p < 0.05$ , \*\* $p < 0.01$ ,  $n = 7$ .

873 **Fig. 10: S29434 effects on neuronal cell cultures.** **A)** S29434 does not protect DA neurons  
874 that degenerate spontaneously in midbrain cultures maintained with serum-supplemented N5  
875 medium. The effect of S29434 against cell death was tested at the concentrations indicated  
876 above. The reference compound is veratridine (VERA; 0.8  $\mu$ M). \* $p < 0.05$  vs. corresponding  
877 controls. **B)** S29434 does not protect DA neurons that degenerate from oxidative stress in  
878 midbrain cultures maintained with a chemically defined medium containing catalytic iron.  
879 S29434 was tested at concentrations indicated above. The reference compound was  
880 deferoxamine (DESF; 10  $\mu$ M). \* $p < 0.05$  vs. corresponding controls. **C)** S29434 protects DA  
881 neurons against MPP<sup>+</sup>-induced cell death. Survival of TH<sup>+</sup> neurons in midbrain cultures  
882 treated (open bars) or not with MPP<sup>+</sup> at a concentration of 3  $\mu$ M. The effect of S29434 against  
883 MPP<sup>+</sup>-induced cell death was tested at concentrations indicated above. Reference compound  
884 is nicotine (Nic; 10  $\mu$ M). \* $p < 0.05$  vs. corresponding controls.

885 **Fig. 11: Induction of autophagy by S29434 in HepG2 cells is QR2-dependent.** HepG2  
886 cells were transfected with 100  $\mu$ M siRNA against QR2 or non-targeting siRNA (negative  
887 control). The following day, cells were treated with S29434 (5 and 10  $\mu$ M) or vehicle  
888 (DMSO) for 24 h. **(B)** Lysosomal function inhibitor chloroquine (25  $\mu$ M) or vehicle (water)  
889 **(A)** was added to duplicate experimental points 2.5 h before the lysis and analysis of LC3  
890 levels by western blot (SDS-PAGE 12%). Optical density (OD) relative to loading control  
891 (glyceraldehyde-3-phosphate dehydrogenase) was analyzed for LC3-II and normalized to  
892 control DMSO-treated cells for chloroquine-treated and untreated samples, separately.

893 **Fig. 12: S29434 has no effect on basal mitochondrial ROS levels in cells.** Subconfluent **A)**  
894 human hepatoma (HepG2) and **B)** astrocytoma (U373) cell lines were treated with S29434 (10  
895  $\mu$ M), N-acetylcysteine (5 mM) or vehicle (DMSO) for 6, 18, and 24 h before analysis. ROS  
896 levels were determined by flow cytometry after staining with MitoSOX. The graphs show the  
897 mean  $\pm$  SEM from three independent experiments performed at least in triplicate. Statistical  
898 analysis two-way ANOVA, followed by Tukey post-test, time versus pharmacological  
899 treatment, 3 groups per 3 times, n = 3 or 4 samples each group, \*\*, p < 0.01 when compared  
900 to control at the same time point.

901 **Fig. 13: Effect of S29434 in vivo on object recognition memory.** **A)** Object recognition  
902 memory is enhanced by S29434 in wild-type mice. The test trial with the new object was  
903 carried out 24 h after the acquisition trial with two identical objects. S29434 was given  
904 intraperitoneally 30 min before the acquisition trial. The discrimination index (DI) is shown  
905 where N is the time spent inspecting the new object and F is the time spent exploring the  
906 familiar object during the test trial. Data are mean  $\pm$  SEM, n = 9–10/group. \*\*\* p < 0.001,  
907 one-way ANOVA followed by Dunnett's test vs. medium-treated control animals (0 mg/kg)  
908 group. **B)** Lack of effect of S29434 on object recognition memory in CH3QR2<sup>-/-</sup> mice. The  
909 test trial with the new object was carried out 24 h after the acquisition trial with two identical

910 objects. S29434 was given intraperitoneally 30 min before the acquisition trial. The  
911 discrimination index (DI) is shown where N is the time spent inspecting the new object and F  
912 is the time spent exploring the familiar object during the test trial. Data are mean  $\pm$  SEM, n =  
913 10/group. \*\* p < 0.01 one-way ANOVA followed by Dunnett's test *versus* medium-treated  
914 control animals (0 mg/kg) group.

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**Table 1: Summary of the IC<sub>50</sub> obtained in different laboratories and conditions with S29434**

Enzyme	Co-substrate	Substrate	Signal	IC <sub>50</sub> (nM)	References
h QR2 expressed in <i>E. coli</i>	BNAH (100 μM)	Menadione	Abs* 350 nm	20	Unpublished
h QR2 expressed in <i>E. coli</i>	NRH (100 μM)	Menadione	Abs* 340 nm	5	Unpublished
h QR2 expressed in CHO	BNAH (100 μM)	Menadione	Fluo** BNAH	14	(Pegan et al., 2011)
h QR2 expressed in <i>E. coli</i>	NMeH (100 μM)	Menadione	Abs* 360 nm	2.4	(Pegan et al., 2011)
h QR2 expressed in <i>E. coli</i>	NMeH (100 μM)	Formosan	Abs*** 612 nm	2.4	(Pegan et al., 2011)
h QR2 expressed in insect cells	BNAH (100 μM)	Menadione	Fluo** BNAH	7	(Ferry et al., 2010)
h QR2 expressed in insect cells	NRH (100 μM)	Menadione	Fluo** NRH	0.7	(Ferry et al., 2010)

Notes: \*Abs: absorbance assay; \*\* Fluo: fluorimetric assay; \*\*\* Abs of MTT.

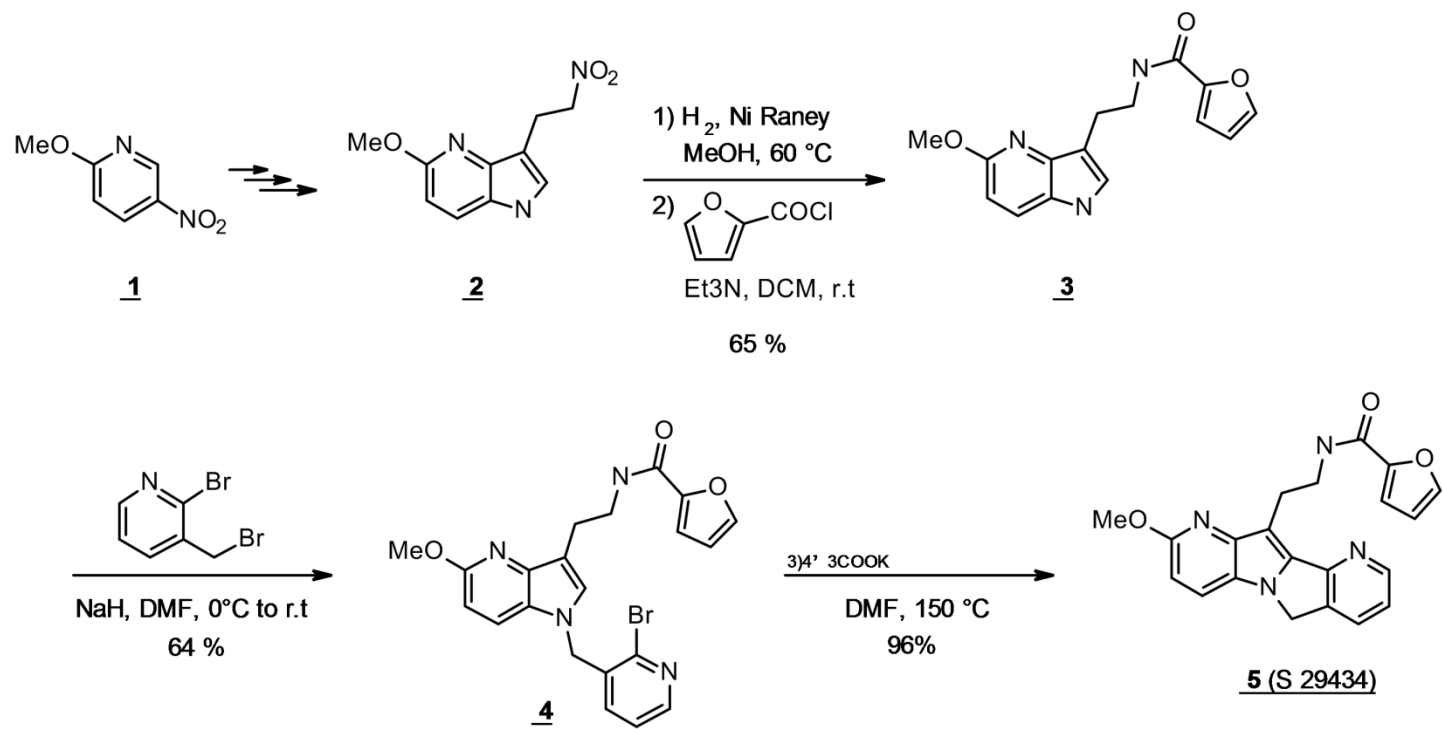


Figure 1

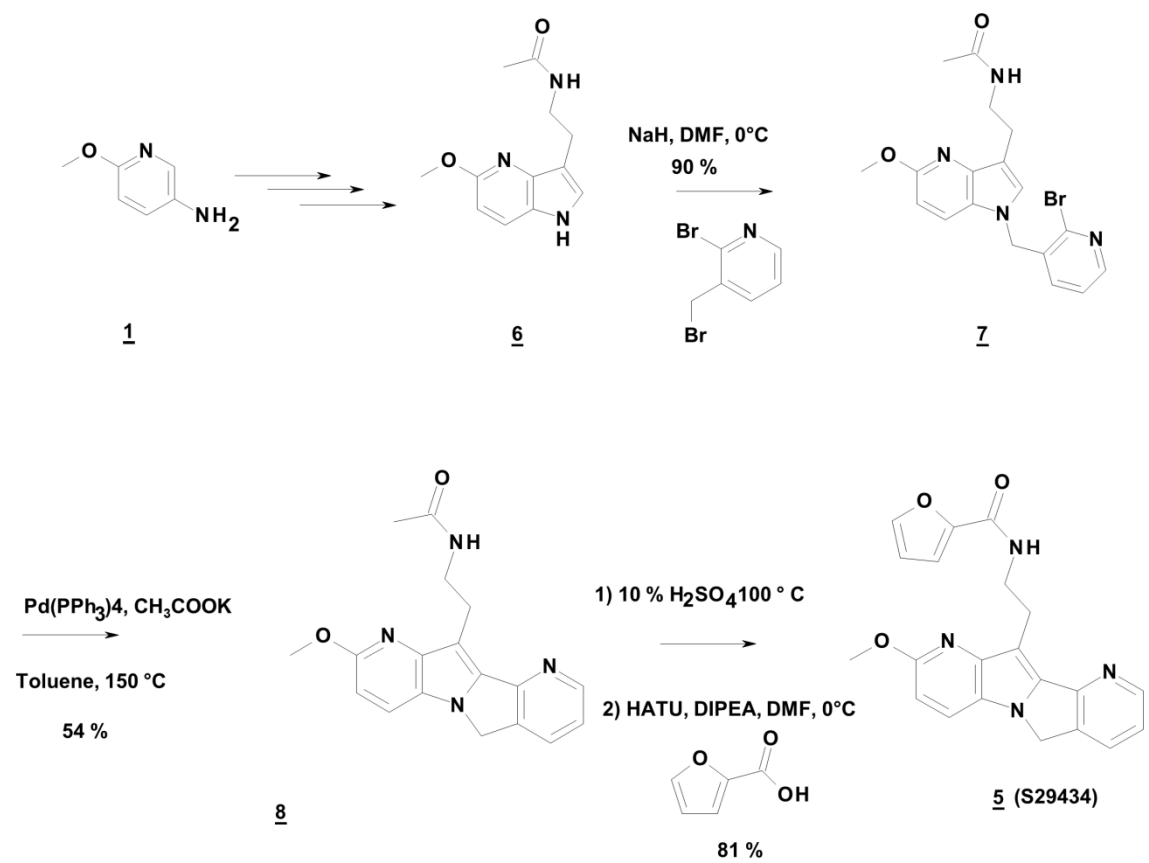
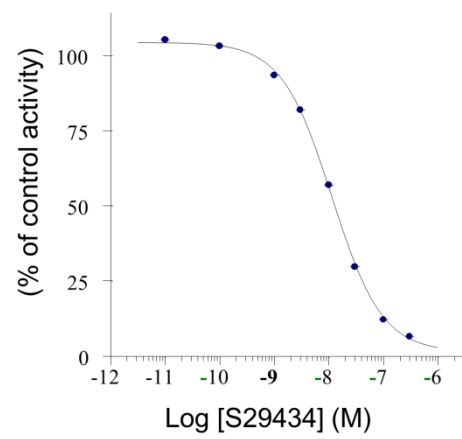


Figure 2



A



B

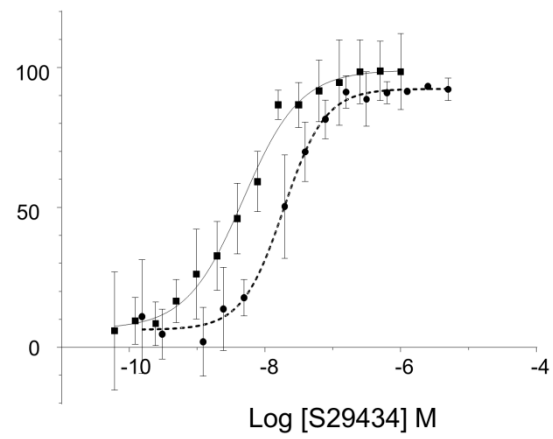


Figure 3

# QR1

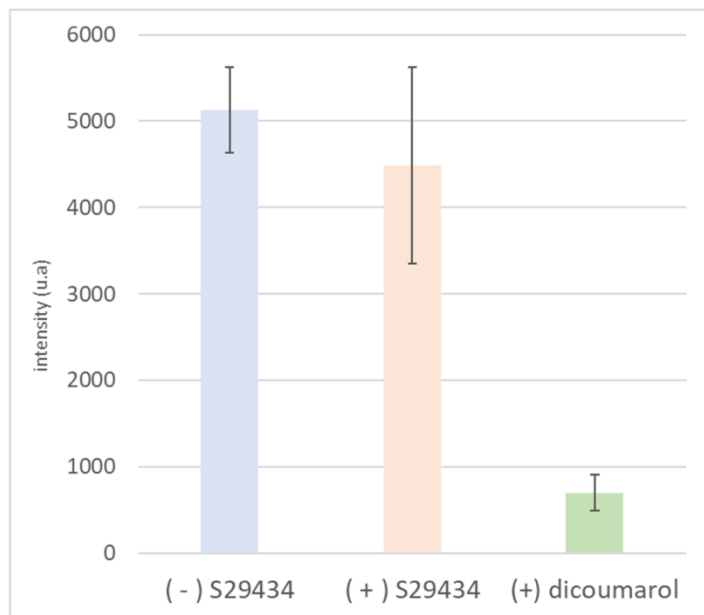
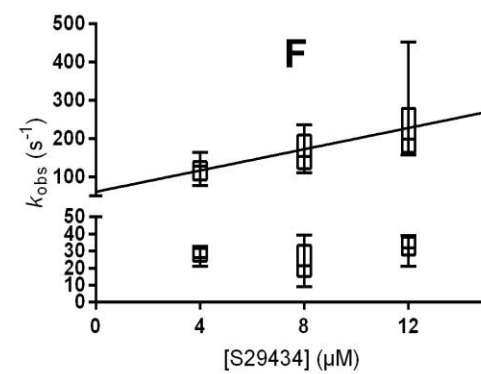
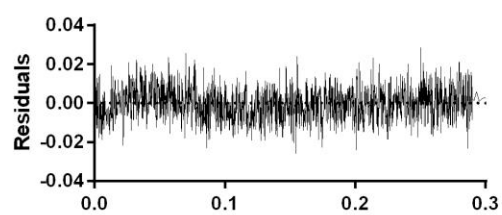
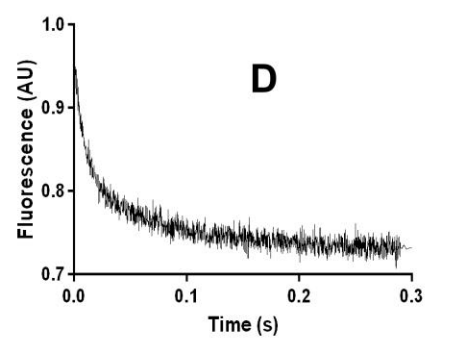
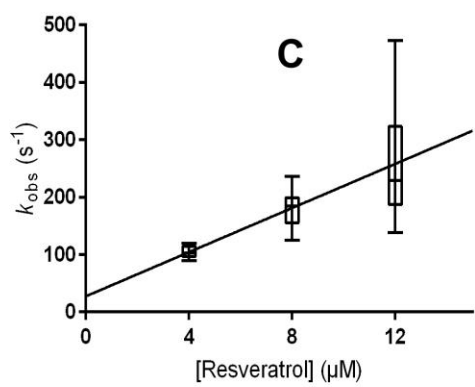
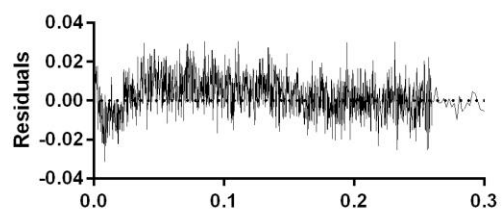
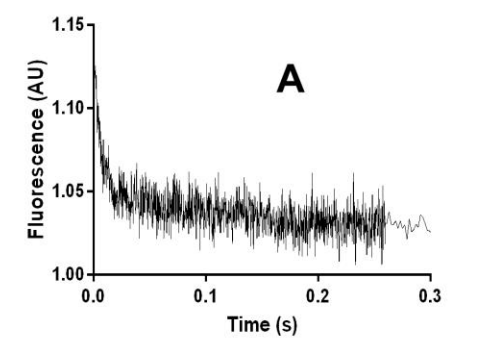
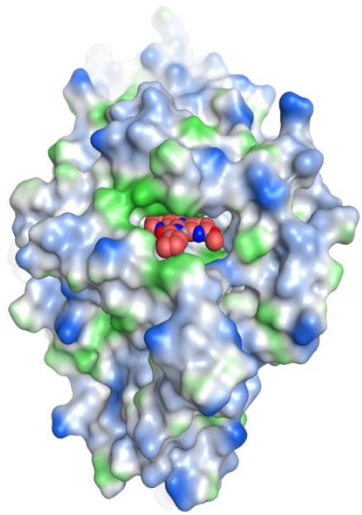


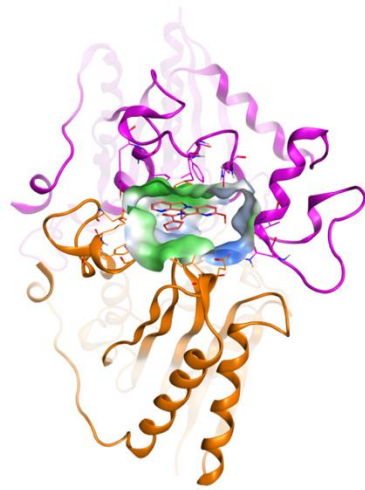
Figure now 4



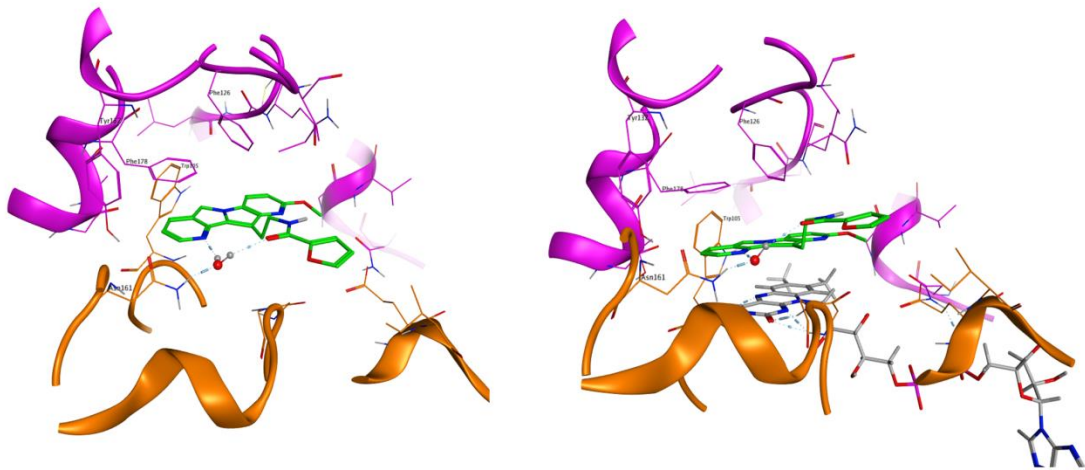
**Figure 5**



**A**



**B**



**C**

Figure 6

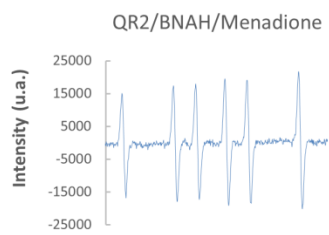
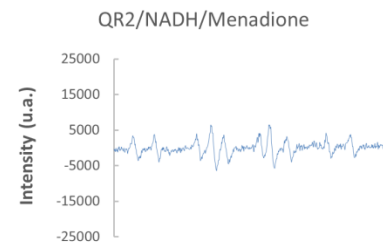
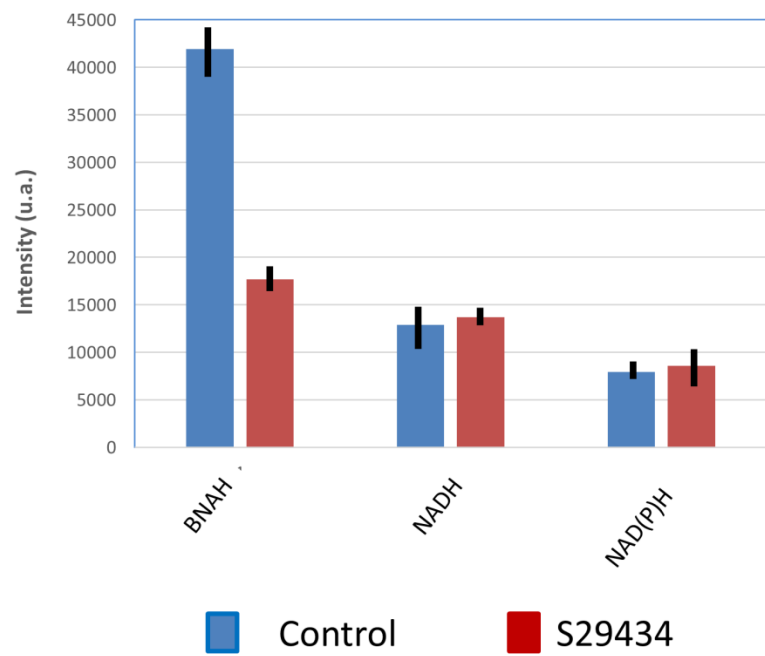


Figure 7

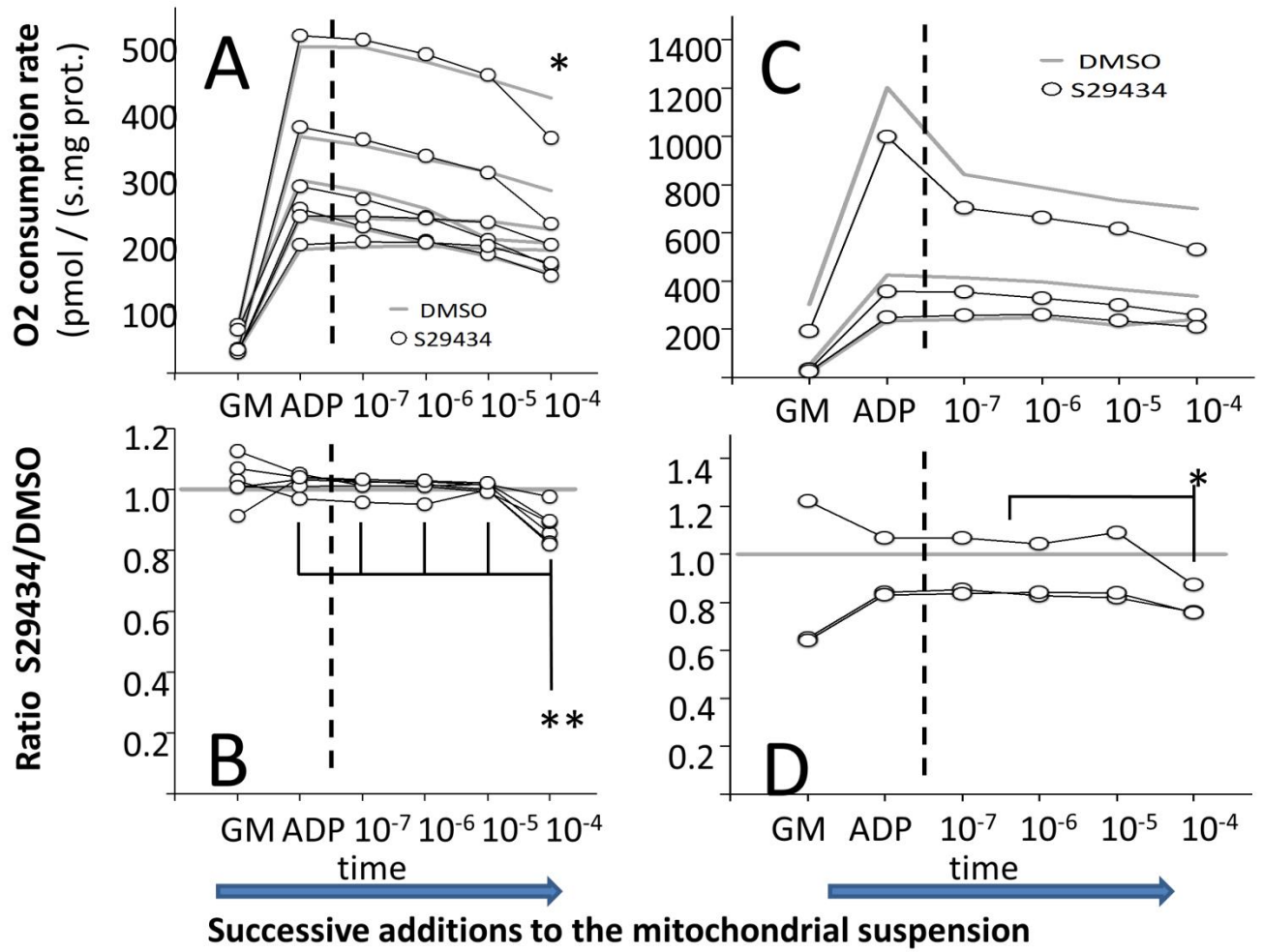


Figure 8

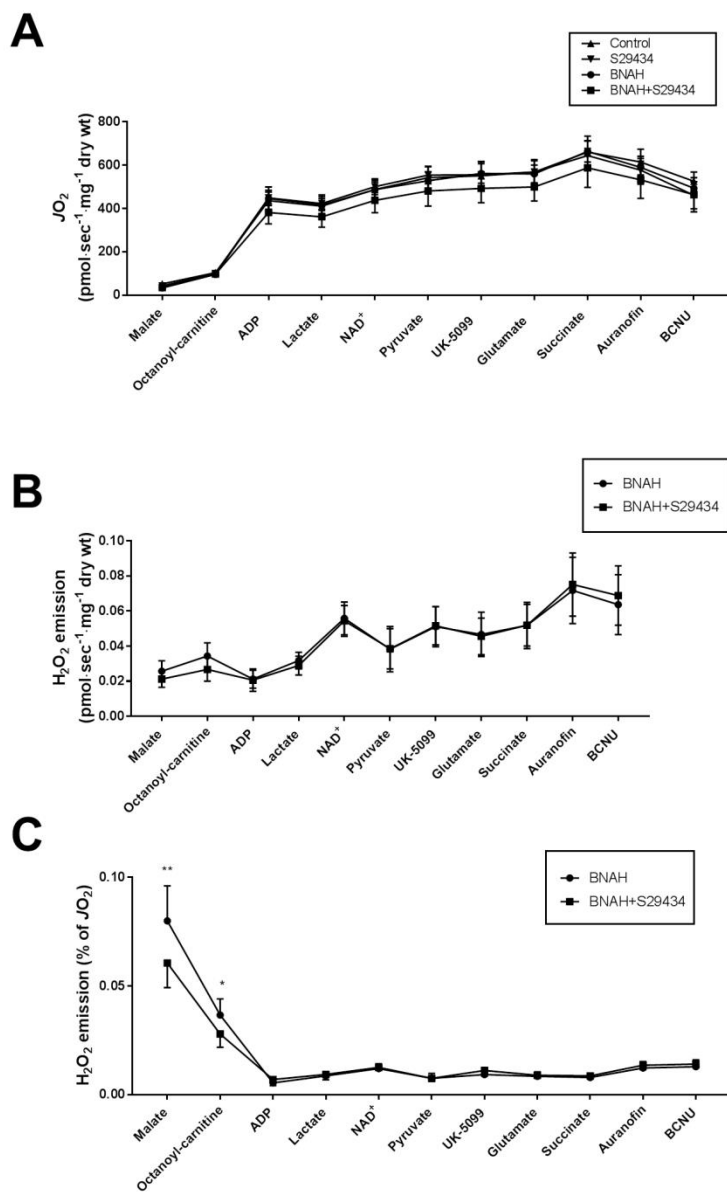


Figure 9

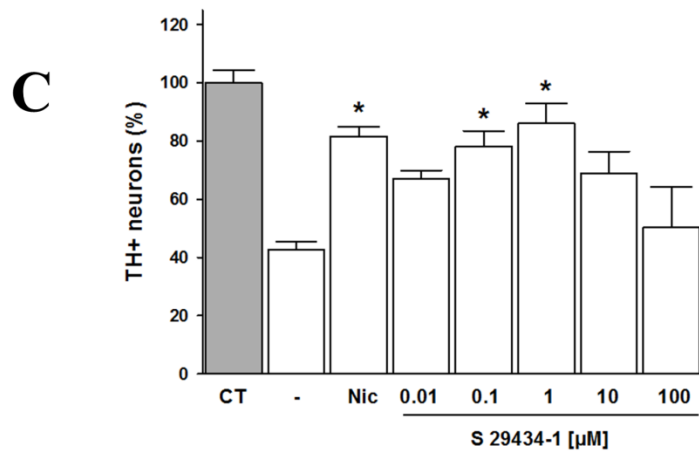
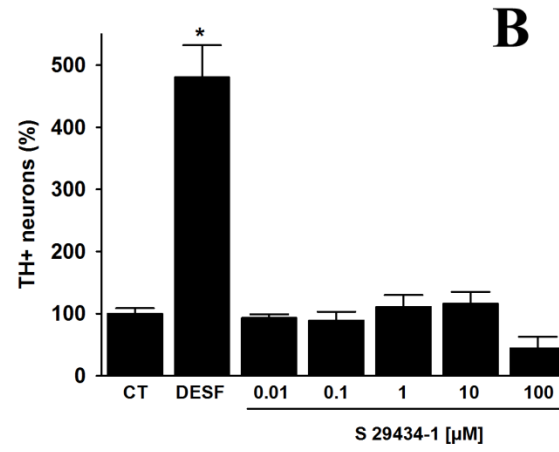
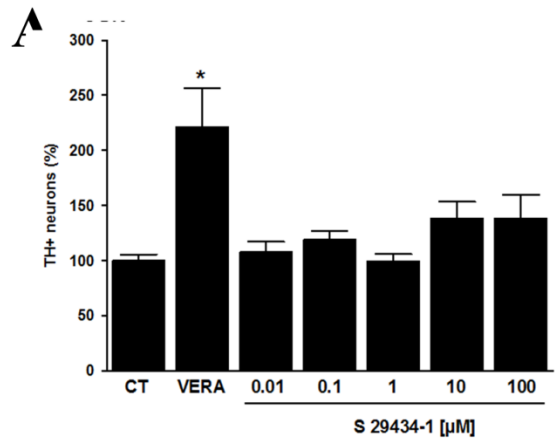


Figure 10



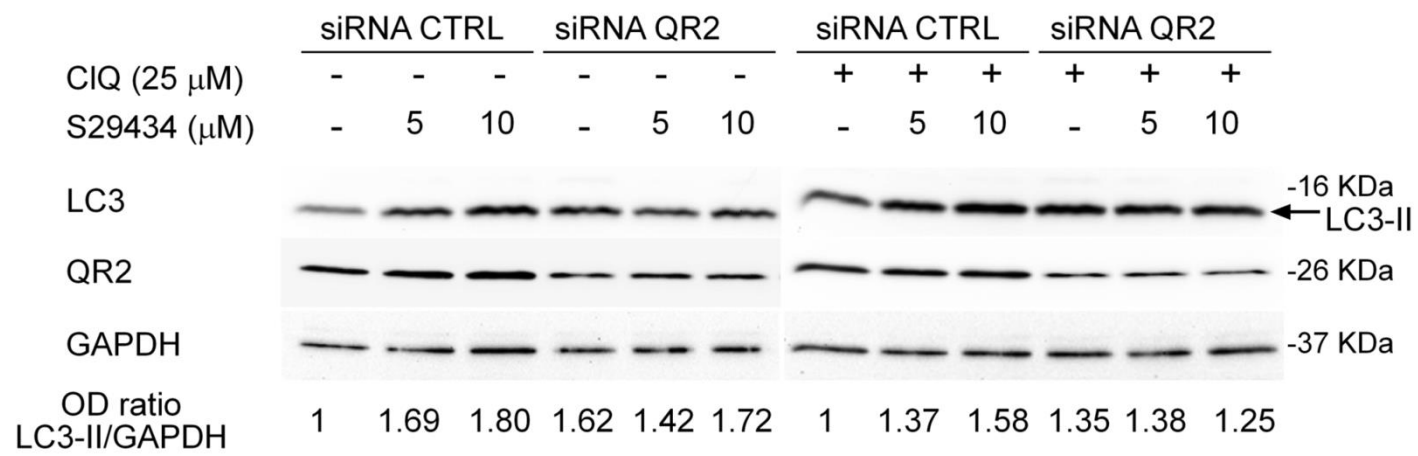


Fig. 11

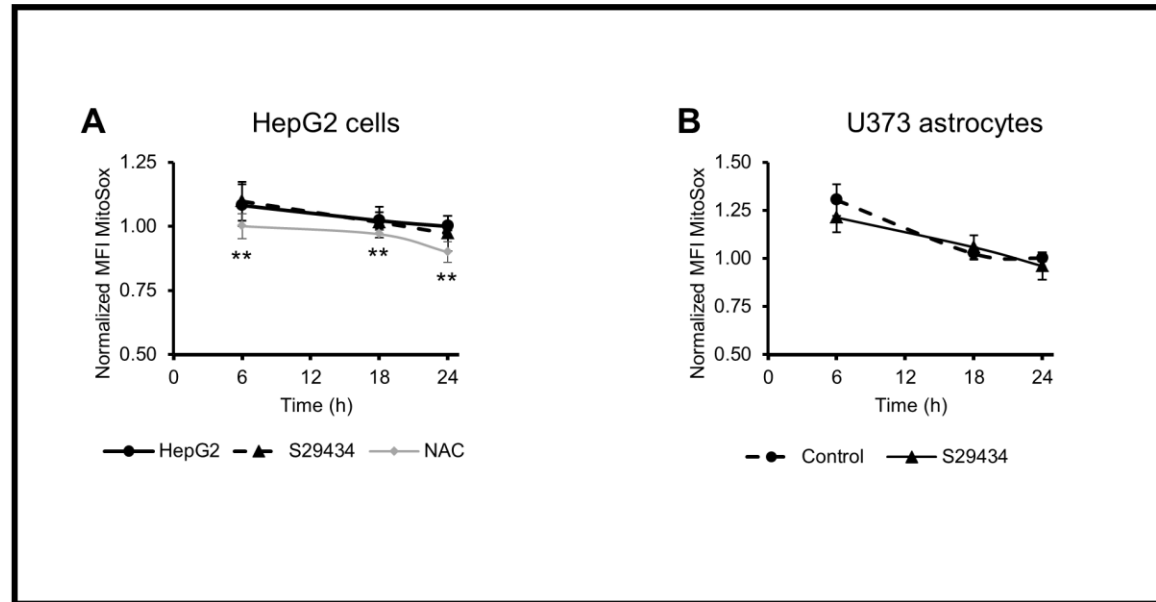


Figure 12

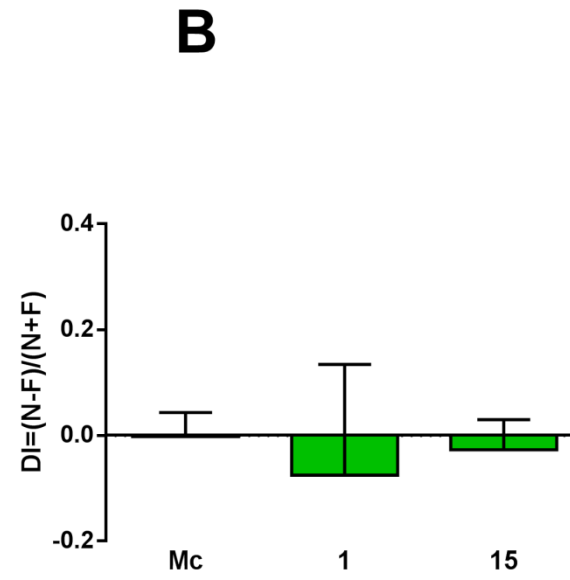
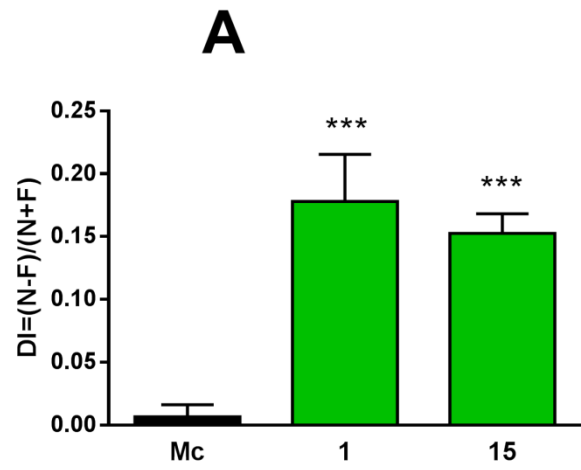


Figure 13