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Original Article

Development of a neuroprotective antioxidant by a "mix-and-match" strategy

Vijaya Kenche[1], Carolin Tumpach[1] Kevin J. Barnham[1,2], Steven J. Collins[3], Cathryn L. Haigh[3*], Simon C. Drew[1*]

[1]Mental Health Research Institute, [2]Department of Pharmacology, [3]Department of Pathology; The University of Melbourne, Parkville, Victoria; ***Australia***

*CLH and SCD contributed equally to this work.

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Corresponding Author
Simon C. Drew
Mental Health Research Institute,
The University of Melbourne,
Parkville, VIC 3010, Australia.
sdrew@unimelb.edu.au

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Mix-and-match; Multi-target drug discovery; Neuroprotection; Radical scavenger; Prostaglandin

Abstract

The compound 4-{[(4-hydroxyphenyl)amino]methyl}-2,6-di-tert-butylphenol (TRS1) was developed using a "mix-and-match" drug design approach for antioxidant-based neuroprotection and therapy. The commonly-used antipyretic drug, paracetamol, which is also found to act as a mild anti-inflammatory, was further modified by attaching a radical scavenger moiety to design a bifunctional bioactive molecule. Using a cell-free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reduction assay, and a 2',7'-dichlorofluorescein (DCFH) oxidation assay in cultured N2a mouse neuroblastoma and SH-SY5Y human neuroblastoma cells exposed to oxidative insult (serum deprivation), the radical scavenging ability of TRS1 was found to be comparable with other well-studied antioxidants such as Trolox. The maintenance of antioxidant action of this compound in cells suggests this avenue and other multi-targeted drug discovery paradigms may provide potential therapeutic strategies for targeting complicated neurodegenerative conditions.

INTRODUCTION

Excessive production of reactive oxygen and nitrogen species (ROS and RNS) and inflammation are observed in many neurological disorders, cardiovascular disease and cancer [1-3]. In such diseases, natural antioxidants such as vitamin C, vitamin E and glutathione reach insufficient levels *in vivo* to effectively mitigate the ROS and/or RNS, with consequent oxidative and/or nitrosative stress; the process wherein ROS/RNS levels exceed the cellular capacity to detoxify them, resulting in damage to major cellular components, leading to impaired cell function and/or cell death [4-6]. The processes catalysed by ROS/RNS often involve free radicals that can trigger chain reactions leading to oligomerisation of various proteins, with consequent disruption of normal cell signalling [7]. To counter such metabolic derangements, the use of dietary antioxidant supplements has become commonplace and antioxidants such as α-tocopherol (vitamin E) have been evaluated in both Alzheimer's disease (AD) and Parkinson's disease (PD) therapies, albeit with negative therapeutic outcomes [8-10].

The levels of pro-inflammatory proteins such as cytokines and chemokines produced in both microglia and astrocytes of the central nervous system (CNS) have been shown to be elevated in the AD brain [3]. In addition, activated microglia and reactive astrocytes are found in the proximity of amyloid deposits [11]. Prostaglandin E2 (PGE2), a pro-inflammatory lipid signalling molecule derived from arachidonic acid metabolism, controls various facets of inflammation [12] and its levels in the cerebrospinal fluid are elevated in neurodegenerative diseases, including AD [13] and amyolateral sclerosis [14]. These and other data [15] have therefore led some researchers to classify AD a primarily neuroinflammatory disease and various non-steroidal antiinflammatory drugs (NSAID) have been investigated for their preventative [16] as well as therapeutic efficacy in AD [17, 18].

The pathogenic mechanisms of the above diseases are multifaceted. Drug candidates directed against a single biological target frequently show promising results in pre-clinical studies but fail as therapeutics in formal clinical trials [19]. For example, therapeutics aimed at depleting β-amyloid (Aβ) peptides, believed to be responsible for impairment of synaptic plasticity and cognitive dysfunction associated with AD, have to date not conclusively demonstrated clinical efficacy [20]. In keeping with multifaceted disease mechanisms, defining an appropriate drug discovery strategy is also increasingly challenging for medicinal chemists seeking to design a single chemical entity with multiple functions [21]. Over the past decade, the 'multi-target drug discovery' (MTDD) strategy has gained increasing interest among the drug discovery community and the outcomes of this strategy are already showing promising results [22-24]. The MTDD strategy consists of constructing a single molecule comprising two or more appropriate fragments with distinct pharmacological properties [21]. For example, hybrid molecules that target inhibition of acetylcholinesterase (AChE) and simultaneously scavenge radicals have been investigated as therapeutic agents for AD [25]. Based on this MTDD paradigm, we investigated a hybrid molecule 4-{[(4-hydroxyphenyl) amino]methyl}-2,6-bis(2-methyl-2-propanyl)phenol (TRS1), which can be seen as a "mix and match" of two fragments, one being the weak anti-inflammatory and antioxidant acetaminophen (paracetamol) and the other being derived from the common antioxidant di-*tert*-butylphenol (Fig.1).

The compound TRS1 has previously been used as a stabilizing agent in polymer chemistry and the manufacture of synthetic rubber [26]. However, to the best of our knowledge, applications of this molecule in biological systems have yet to be explored. Based upon an MTDD strategy, we therefore selected TRS1 for further investigation as a novel neuroprotective agent and demonstrate that TRS1 promotes neuronal survival under conditions of oxidative stress.

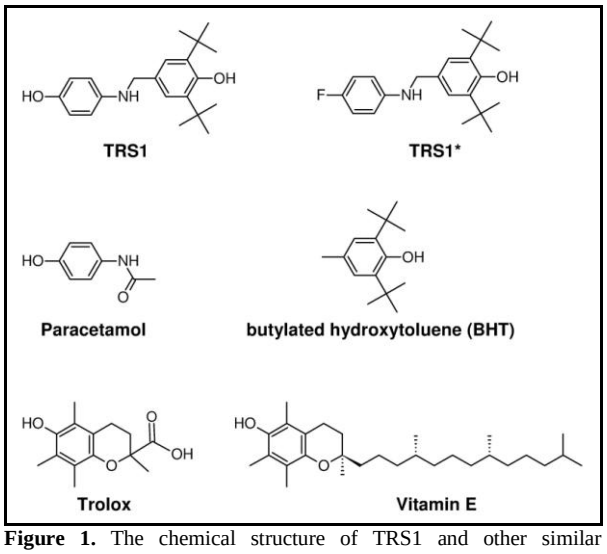

Figure 1. The chemical structure of TRS1 and other similar compounds.

MATERIALS AND METHODS

Synthesis

Trolox, vitamin E and paracetamol were purchased from Sigma-Aldrich. The reported synthetic procedure for TRS1 [26] was modified to a more viable approach and was synthesized in a single step from commercially available reagents with good yields (*vide infra*). A mixture of 4-aminophenol (2 mmol), 3,5-di-tert-butyl-4-hydroxybenzaldehyde (2 mmol) and methanol (MeOH, 6 ml) was stirred at room temperature overnight, before cooling on ice. To the ice-cold mixture, sodium borohydride ($NaBH_4$, 1 mmol) was added with continued stirring for further 1 h before quenching the reaction with acetone, followed by addition of 1 M HCl. The reaction mixture was concentrated *in vacuo*, the residue suspended in water, then filtered and dried. The crude product was recrystallized with ethanol/water to give TRS1 as a crystalline solid. ^{1}H NMR (400 MHz, DMSO): δ 8.52 (s, 1H), 6.87 (s, 2H), 6.68 (s, 1H), 6.62 (d, J= 9.2Hz, 2H), 6.52 (d, J = 9.2Hz, 2H), 4.15 (s, 2H), 1.25 (s, 18H) ESI-MS: m/z 328.2276 [M+H].

A similar procedure was followed for the synthesis of 4-{[(4-fluorophenyl)amino]methyl}-2,6-bis(2-methyl-2-propanyl)phenol (TRS1*) using 4-fluorophenol and 3,5-ditert-butyl-4-hydroxybenzaldehyde as raw materials. ^{1}H NMR (400 MHz, DMSO): δ 6.93 (bs, 1H), 6.9 (m, 2H), 6.73 (m, 2H), 4.37 (s, 2H), 1.28 (s, 18H).

DPPH antioxidant assay

A 100 μM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) was prepared in MeOH or in Tris/MeOH (50% 10 mM Tris HCl pH 7.5, 50% MeOH). For consistency with subsequent cell culture assays, stock solutions of all test compounds were first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, before serial dilution in either MeOH or in Tris/MeOH to concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 μM. To a 96-well clear microplate, 100 μL of each test compound was added in duplicate wells, then 100 μL of 100 μM DPPH solution was rapidly added to all wells using a multichannel pipette to initiate the reaction. The absorbance of DPPH• at 517 nm (A_{517}) was read using a spectrophotometer (SPECTROstar Nano; BMG Labtech) every 2 min for 1 h. Absorbance measurements were corrected for any intrinsic absorbance of each test compound at 517 nm (negligible in most

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instances) and normalised by A_{517} (DPPH) at each time point to correct for the slow inherent degradation of DPPH• alone or any change in well volume due to solvent volatility. The efficiency of the radical scavenging was evaluated as follows:

Efficiency (%) = 100 x [1 – A_{517} (*t* = 60 min)/A_{517} (*t* = 0)]

The compound concentration that reduces the initial DPPH• concentration by 50% (EC_{50}) was computed from the dose response curve by non-linear least squares fitting to the 4-parameter sigmoidal equation:

$$y = bottom + (top - bottom) / (1 + [EC_{50} / x]^{slope})$$

-x, the compound concentration after mixing with DPPH,

-slope, the Hill slope (corresponding to the steepness of the curve)

-y, the DPPH• concentration

-top, the maximum response (allowed to vary)

-bottom, the baseline response (in this instance restricted to zero)

The mean and standard error in the parameters were calculated from the variation in the fits to the individual assay repeats. Corresponding continuous-wave (CW) electron paramagnetic resonance (EPR) spectra were acquired using an X-band spectrometer (E500; Bruker) fitted with a Bruker super-high sensitivity probe-head and a flat solution cell (WG-808-Q; Wilmad-LabGlass).

Cell culture

Neuro2a (N2a) mouse neuroblastoma and SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's Modified Eagles Media (DMEM; Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS, Lonza), 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. For microtitre plate assays, cells were plated to be 90-95% confluent at the start of the assay.

Dichlorofluorescein (DCFH) assay

Cells were incubated in Dulbecco's phosphate buffered saline (dPBS; Lonza) containing 5 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFHDA; Invitrogen) at 37°C for 20 min. Probe solution was then removed and replaced with pre-warmed Opti-MEM® I Reduced Serum Medium (Invitrogen) without phenol red with or without test reagent added. Fluorescence intensity was measured every 5 min for 12 h using a microplate reader (Fluostar Optima; BMG Labtech) fitted with 490 nm excitation and 520 nm emission filters and initial rates were calculated using tangents to the curve.

Cell viability assay

Five microliters of One Solution Cell Proliferation Assay (MTS) reagent (Promega) per 100 µl media was added to test and media only background control conditions, and incubated under normal culture conditions for 90 min. Reaction product was quantified using absorbance at 462 nm in the Fluostar Optima microplate reader.

Trypan blue staining

Cells were resuspended in 1 ml of dPBS by trituration then mixed 50:50 with trypan blue dye (Invitrogen). Sufficient cell suspension was pipetted into a counting chamber slide (Countess®; Invitrogen) to completely fill the chamber; cell counting was done using the Countess® and analyzed with the corresponding software.

Prostaglandin E2 enzyme immunoassay

Cultures were plated with equivalent numbers of cells per well and allowed to adhere to the plate under normal incubation conditions. Prior to the start of the assay, cells were washed twice with dPBS before transferral into serum-free media, either alone, with 10 µM TRS1 or with equivalent DMSO. Cells were returned to the incubator for 2 h then media was collected and briefly centrifuged (3000 rpm, 3 min) to remove any contaminating cells. PGE2 levels were measured with a commercially available enzyme immunoassay kit (Cayman Chemical, Item No. 514010) and the SPECTROstar Nano spectrophotometer. Media was assayed directly without dilution.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism 5 software. The relevant statistical test applied is indicated in the figure legends. Where significant differences were found, Dunnett, Bonferroni and Dunn tests were used for multiple comparisons of one-way, two-way and non-parametric ANOVA, respectively. Graphs show the mean and standard error of the mean (SEM) of 'n' independent experiments unless otherwise stated.

RESULTS AND DISCUSSION

We first characterized the ability of TRS1 to scavenge radicals in a cell-free environment using the DPPH radical assay. Reduction of DPPH• to its hydrazine form (DPPH-H) in the presence of TRS1 was monitored in both non-aqueous (MeOH) and semi-aqueous (1:1 Tris pH 7.5/MeOH) solutions by following the decrease of the characteristic absorbance of DPPH• at 517 nm (Fig.2). Using a log2 dilution series, the efficiency of the radical reduction by TRS1 was calculated and compared with that of a known antioxidant, vitamin E, and its more water soluble analogue, Trolox (Fig.3). In MeOH, the radical reduction of DPPH by TRS1 followed kinetics (Fig.2) and efficiency (Fig.3) comparable with Trolox and vitamin E. In semi-aqueous conditions, TRS1 followed slower kinetics (Fig.2), although it displayed slightly higher radical reduction efficiency (Fig.3)

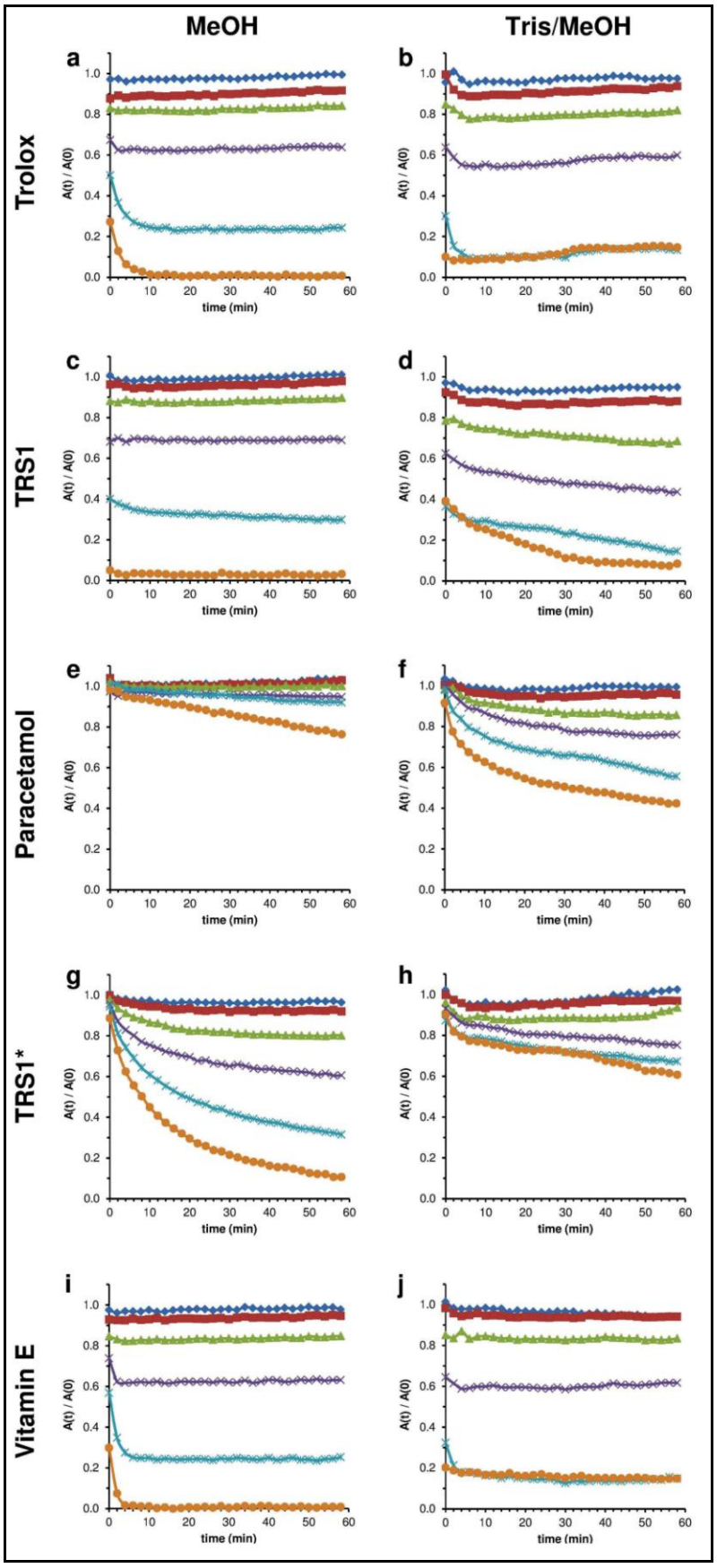

Figure 2.
Time-dependence of the reduction of DPPH• to DPPH-H as determined by visible absorption spectrophotometry in **(left)** MeOH and **(right)** Tris-buffered MeOH solutions in the presence of **(ab)** Trolox, **(cd)** TRS1, **(ef)** paracetamol, **(gh)** TRS1*, and **(ij)** vitamin E. Final concentrations: **(♦)** 1.56 μM, **(■)** 3.125 μM, **(▲)** 6.25 μM, **(◊)** 12.5 μM, **(×)** 25 μM, and **(●)** 50 μM. Data correspond to a single representative assay performed in duplicate.

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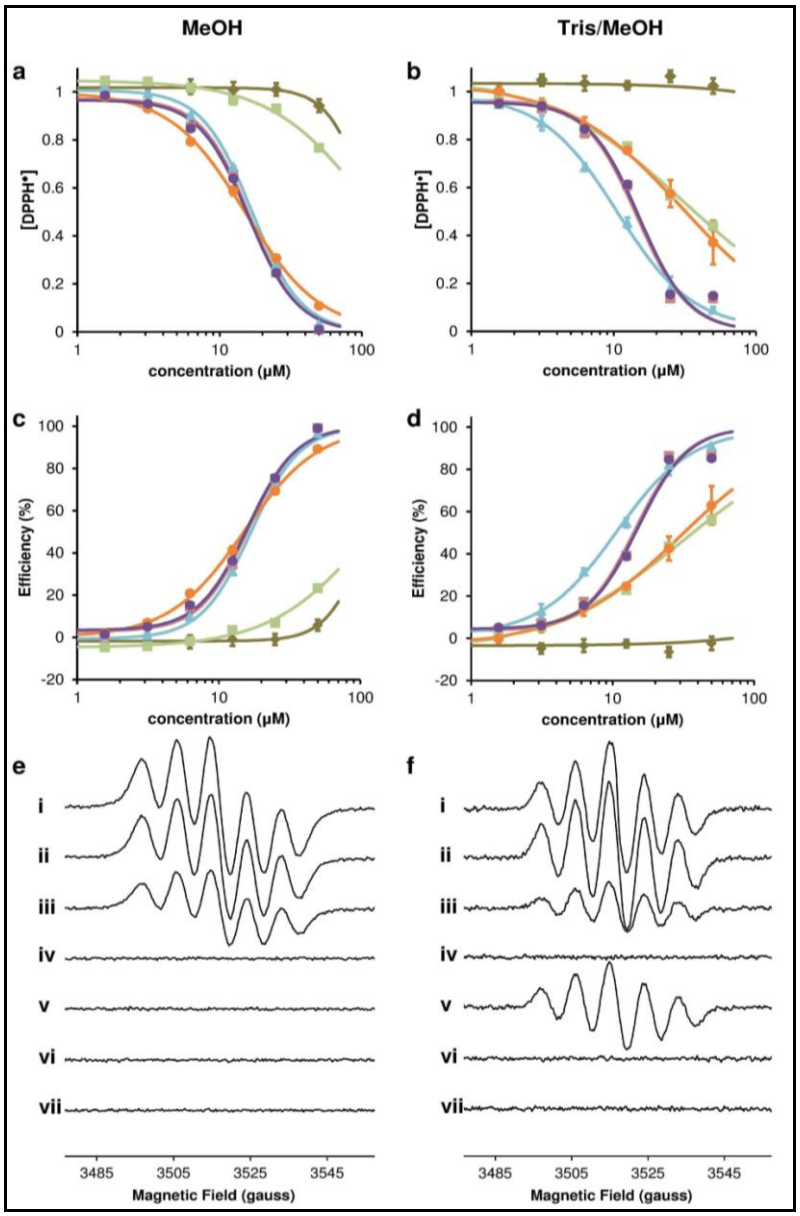

Figure 3.
TRS1 has a radical scavenging activity comparable with Trolox. Reduction of DPPH• (50 μM) to its hydrazine form (DPPH-H) as determined by visible absorption spectrophotometry in
(left) MeOH and
(right) Tris-buffered MeOH solutions.
(ab) DPPH• concentration (normalised to 50 μM DPPH• control), and
(cd) the corresponding efficiency curves 1 h after reaction initiation.
(■) Trolox,
(▲) TRS1,
(■) paracetamol,
(●) TRS1*),
(●) vitamin E, and
(♦) DMSO control (0-0.5% v/v).
Data points represent the mean and standard error of three independent assays, each performed in duplicate.
(ef) Room-temperature CW-EPR spectra of containing
(i) 50 μM DPPH alone or 50 μM DPPH plus,
(ii) 0.5% v/v DMSO,
(iii) 50 μM paracetamol (0.5% v/v DMSO),
(iv) 50 μM TRS1 (0.5% v/v DMSO), **(v)** 50 μM TRS1* (0.5% v/v DMSO),
(vi) 50 μM vitamin E (0.5% v/v DMSO), and
(vii) 50 μM Trolox (0.5% v/v DMSO).
The spectra represent a single independent end-point measurement acquired approximately 1 h after reaction initiation.
Experimental conditions: microwave frequency, 9.866 GHz; microwave power, 10 mW; magnetic field modulation amplitude, 1 G; field modulation frequency, 100 kHz; receiver time constant, 82 ms; receiver gain, 82 dB; sweep rate, 4 G s^{-1}; averages, 4.

compared with Trolox and vitamin E. The dependence of kinetics and efficiencies on solvent composition reflects the complex variation in ligand solubility, solvent acidity [27] and pK_a of ionizable groups of both ligands and DPPH [28, 29] with organic solvent fraction.

Since the reaction kinetics between DPPH and antioxidants are not linear with respect to DPPH concentration, the common practice of expressing antioxidant capacity using EC_{50} values is subjective [29]. Nonetheless, since the kinetics were rapid enough to produce a plateau in the absorbance (Fig.2), we also calculated EC_{50} parameter by least-squares fitting of the dose response curves of TRS1, Trolox and vitamin E (Fig.3). TRS1 possesses an EC_{50} comparable with Trolox and vitamin E in MeOH and slightly lower EC_{50} compared with these compounds in semi-aqueous conditions (Table 1).

Table 1. EC_{50} values (μM) calculated from the dose response curves in Fig.3ab. Uncertainties denote the standard error of the mean. Reliable EC_{50} estimates for paracetamol, TRS1* and DMSO could not be obtained because their slow kinetics did not lead to a plateau of the radical reduction curves within 60 min (Fig.2).

	MeOH	Tris/MeOH
TRS1	16.8 ± 0.3	10.6 ± 0.2
Trolox	16.2 ± 0.1	14.5 ± 0.2
Vitamin E	15.9 ± 0.1	15.0 ± 0.1

Since TRS1 is an amalgamation of two functional species, either moiety may contribute to its antioxidant activity. Therefore, we also compared the activity of TRS1 with paracetamol and with TRS1*, wherein the 4-hydroxyaniline moiety of TRS1 was replaced with 4-fluoroaniline (Fig.1). The reaction kinetics of DPPH• with paracetamol and butylated hydroxytoluene (BHT) have previously been characterized and observed to be slow [30, 31]. Consistent with this, paracetamol displayed slow kinetics (Fig.2e) and minimal efficiency (Fig.3c) in MeOH, whereas TRS1* exhibited intermediate kinetics (Fig.2g) and an efficiency comparable with TRS1 (Fig.3c). In semi-aqueous conditions, both paracetamol and TRS1* exhibited slow kinetics; with comparable efficiencies whose aggregate approximated that of TRS1 (Fig.3d).

To confirm the UV-vis results, we also directly detected radical reduction using EPR spectroscopy. The DPPH radical in solution is characterized by a 5-line EPR spectrum, while the one-electron oxidized phenoxyl-type radical intermediates formed by each test compound during the reaction with DPPH• are too short-lived to be detected by EPR under ordinary experimental conditions [28, 32]. Stoichiometric 50 μM mixtures of DPPH• and TRS1, Trolox and vitamin E confirmed the complete reduction of DPPH• to non-paramagnetic DPPH-H, the solvent-dependent partial reduction by paracetamol and TRS1* (Fig.3ef).

Neurons are amongst the most vulnerable cells within the body and heightened oxidative damage is known to be present in many neurodegenerative diseases [33]. To test the activity of TRS1 in a neuronal lineage cell system, N2a cells were subjected to serum deprivation (an inducer of intracellular oxidative stress) using a system characterized previously [34]. First, the toxicity of TRS1 to the N2a cells was determined by incubating the cells in serum-free medium with a log10 serial dilution of TRS1. Viability of the cells (MTS) was assessed after 24 h using formazan metabolism. TRS1 was well tolerated up to 10 μM but significant toxicity was seen at 100 μM (Fig.4). The same toxicity profile was observed for N2a cells cultured in serum-containing media, representing non-oxidative conditions (data not shown). The non-toxic concentration range (≤ 10 μM) was subsequently used to determine TRS1 antioxidant efficiency.

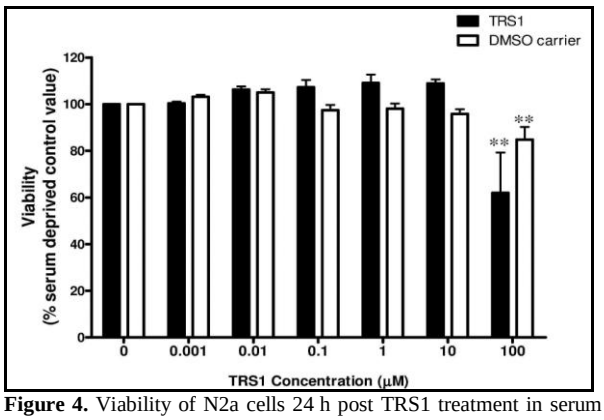

Figure 4. Viability of N2a cells 24 h post TRS1 treatment in serum free media, as determined using cellular metabolism of formazan (MTS). There was no significant difference between TRS1 and the DMSO control (two-way ANOVA, $F = 0.237$, $P = 0.63$, $n = 3$). A significant reduction in cellular viability was observed only at 100 μM TRS1 (two-way ANOVA, $F = 9.405$, $P < 0.001$, $n = 3$; Bonferroni test, $^{**}P < 0.01$).

The intracellular availability and ability of TRS1 to scavenge intracellular ROS was assessed using DCFHDA. This dye is cell permeable until cleaved by intracellular esterases into DCFH, after which it becomes trapped within the cell and is oxidized to a fluorescent DCF product by intracellular ROS [35]. Although the precise reaction mechanisms leading to DCF product formation remain complicated [36], the DCF assay provides a general measure of oxidative stress in biological systems [37]. Nutrient starvation caused a rapid and prolonged increase in intracellular ROS, which was detected by DCF fluorescence (Fig.5a). Serial dilution of TRS1 demonstrated a dose-dependent ability to reduce ROS detection by this dye, significant at 1 μM (~30% efficiency) and 10 μM (~50% efficiency) concentrations (Fig.5b). A full dose response curve could not be constructed from the TRS1 titration due to the onset of toxicity at high doses (Fig.4). Therefore, no attempt to calculate an EC_{50} value by curve fitting was made; however, 50% efficiency was achieved in the vicinity of 10 μM TRS1 (Fig.5).

To ensure the above observations were not a cell line-specific response, these assays were repeated using SH-SY5Y cells with identical results obtained (data not shown). Trypan blue staining also confirmed that the decrease in intracellular DCF fluorescence following TRS1 treatment was not due to loss of cell membrane integrity (data not shown). Trypan blue is a non-membrane-permeable stain that is excluded from cells with intact membranes but can permeate cells with leaky membranes and therefore increased staining of cells indicates loss of membrane integrity or cell death.

TRS1 was also compared with Trolox, vitamin E, TRS1* and paracetamol at concentrations where the greatest antioxidant efficiency was observed in serum-deprived cells, namely 1 μM and 10 μM. The efficiency of TRS1 was comparable with Trolox at 10 μM, whilst the efficiency of vitamin E was not significantly different from the DMSO control (Fig.6). The latter observation is consistent with the preferential localization of the lipophilic chain of vitamin E within cell membranes (logD = 11.9 [38]), making it

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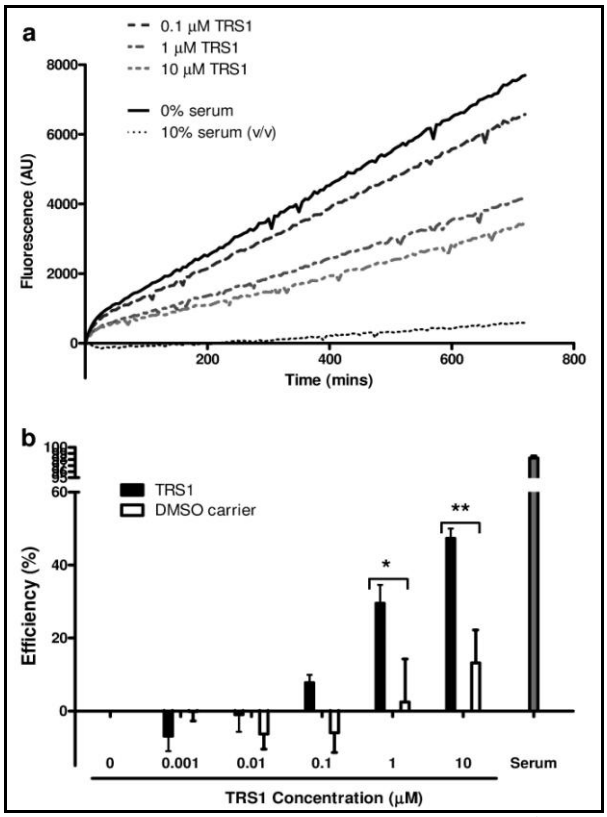

Figure 5. TRS1 reduces the ROS detected as a result of serum deprivation in N2a cells. **(a)** Raw rate curves of DCF fluorescence: ROS production was low in cells cultured under normal serum containing (10% v/v) conditions and was greatly increased in cells starved of the nutrients contained within the serum (0% serum); culturing the cells in serum-free media supplemented with TRS1 led to a significant decrease in intracellular radical production (two-way ANOVA, $F = 15.38$, $P < 0.001$, $n = 3$). **(b)** Data plotted as the efficiency of ROS reduction; the percentage by which the compound was able to reduce the intracellular ROS detection: Highest efficiency was measured for the 1 μM (*$P < 0.01$) and 10 μM (**$P < 0.001$) concentrations of TRS1 (Bonferroni test) and these conditions were significantly different from the DMSO controls (two-way ANOVA, $F = 12.72$, $P < 0.001$, $n = 3$).

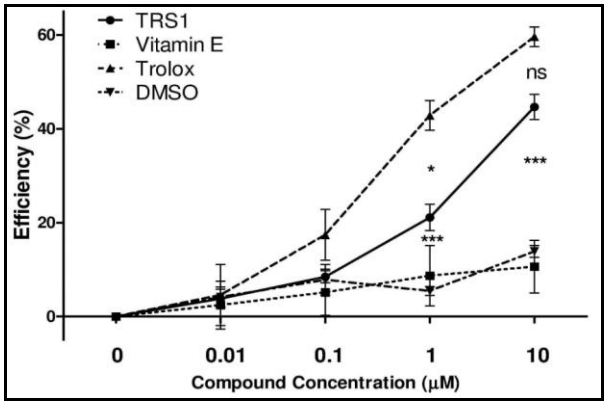

Figure 6. TRS1 has a cellular ROS reduction activity comparable with Trolox. The dose response curve of the serum-starved N2a cells with TRS1 was compared with responses to equivalent serial dilutions of Trolox and vitamin E. Both TRS1 and Trolox exhibit significantly greater radical reduction efficiency as compared with vitamin E and the DMSO control (two-way ANOVA, $F = 33.98$, $P < 0.001$, $n = 3$; Bonferroni test, *$P < 0.05$, ***$P < 0.001$).

ineffective against the intracellular ROS detected by the DCF assay. In line with other reports of ROS-attenuating effects [39, 40], the efficiency of paracetamol against intracellular ROS was also significantly different from the serum-deprived control, whilst that of TRS1* was not (Fig.7). The ineffectiveness of TRS1* alone, which still contains the radical-reducing BHT moiety (Fig.1), is consistent with the weaker activity of TRS1* observed in the cell-free assay in more aqueous conditions and may further be related to greater partitioning of paracetamol in the cytosol (where DCFH oxidation occurs) due to differences in lipophilicity (paracetamol: logD = 0.3 [41]; TRS1*: logD = 6.1 [42]). Interestingly, co-incubation of TRS1* with paracetamol exhibited no significant efficiency compared with the control (Fig.7), suggesting antagonistic interactions of these two compounds in the cellular environment. From these observations, we may conclude that both the hydroxyaniline and BHT functional groups contribute to the antioxidant activity of TRS1 in an intracellular environment and that the efficiency of TRS1 cannot be achieved by co-incubation of its separate functional moieties (TRS1* and paracetamol) due to antagonistic effects.

Neurodegenerative conditions are characterized by both oxidative stress and neuro-inflammation [3, 11]. Paracetamol has been found to possess mild anti-inflammatory properties [43], which are connected to its role in decreasing levels of PGE2 [44-47]. Since TRS1 contains a similar hydroxyaniline moiety (Fig.1), we therefore tested whether TRS1 treatment altered PGE2 levels in our serum-deprived N2a cell culture model using a dosage associated with significant antioxidant activity. Whilst serum-deprivation for 24 h or more has been shown to reduce basal concentrations of PGE2 to very low levels in some cell lines [48], measurable levels of PGE2 have been observed in other cell types [49] and our relatively short TRS1 treatment time (2 h) was evidently short enough to permit detectable PGE2 levels in response to TRS1 treatment ($\sim 10^{-10}$ g/ml) under conditions of serum deprivation in N2a cells. Fig.8 shows that although the average measured level of PGE2 was reduced in comparison with the DMSO control, the results were too variable to conclude that TRS1 causes a significant reduction in PGE2 at a concentration of 10 μM.

Paracetamol has been hypothesized to reduce PGE2 levels by acting as a reducing co-substrate of the heme center present at the peroxidase active site of prostaglandin H2 synthase (PGHS). Thus, its inhibitory action will be abrogated by intracellular hydroperoxide levels, explaining why cells of different lineage are known to exhibit variable susceptibilities to paracetamol treatment [38]. If TRS1 is postulated to act via a similar mechanism, the therapeutic dose necessary

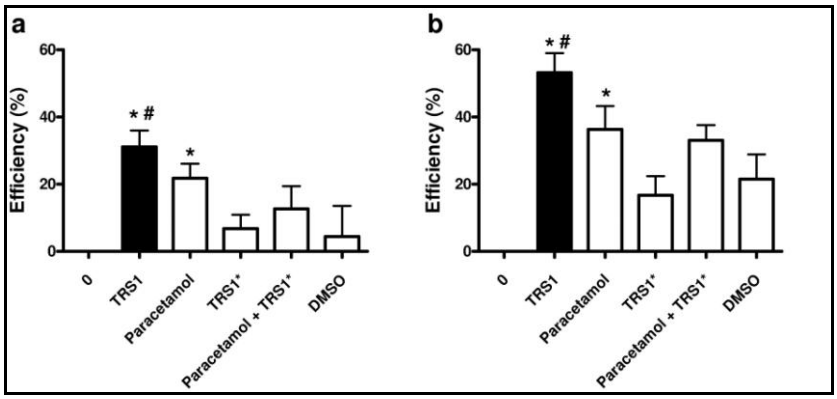

Figure 7.
The hydroxyl group on the aniline moiety of TRS1 is required for maximum efficiency. At concentrations where a significant reduction in ROS were observed (Fig.6), namely **(a)** 1 μM and **(b)** 10 μM, the activity of TRS1 cannot be obtained by co-incubation with separate molecules that mimic the sum of its parts. (*a*, One-way ANOVA, $F = 3.798$, $p = 0.0107$, $n = 3$; *b*, One-way ANOVA, $F = 6.643$, $p = 0.0005$, $n = 3$; Dunnett test, * significantly different from serum deprived control $p < 0.05$, # significantly different from DMSO control $p < 0.05$).

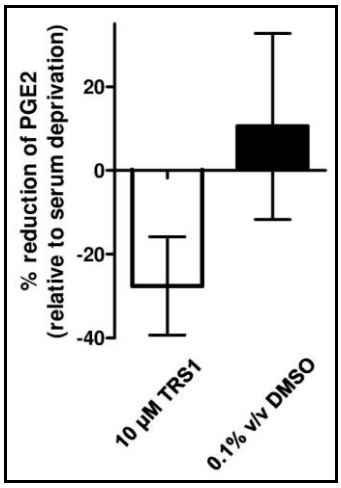

Figure 8.
In serum-deprived N2a cells, TRS1 treatment does not significantly reduce levels of PGE2 as compared with the DMSO control (one-tailed t-test, $t = 1.89$, $P = 0.066$, $n = 5$). Levels of PGE2 are expressed as a percentage change from untreated serum-deprived cells.

for effective anti-inflammatory action in the context of neurodegenerative conditions will depend critically upon the severity of oxidative stress, since lipid hydroperoxides are formed under these conditions. The ability of the hydroxyaniline moiety of TRS1 to directly reduce ROS may therefore deplete TRS1 levels necessary for effective inhibition of PGE2 production. We conclude that it remains possible that TRS1 may simultaneously act as both an antioxidant and anti-inflammatory, but not under the conditions for which we observed significant antioxidant activity.

In summary, the paradigm of "one-molecule, one-target" drug discovery has yielded several potential therapeutics for neurodegenerative disease, although the efficacy of such compounds *in vivo* has often been disappointing, which is at least partly due to their limited action in diseases characterized by multiple pathogenic pathways. Beginning with the commercially available paracetamol molecule, which possesses mild anti-inflammatory and antioxidant properties, we added an antioxidant moiety to produce a novel multi-target-directed compound TRS1. This hybrid compound maintained its radical scavenging capacity, demonstrating both antioxidant and neuroprotective capacity in cells suffering increased intracellular oxidative stress due to starvation. The effectiveness of the antioxidant action of this compound in cells suggests MTDD is a promising avenue for development of therapeutics for targeting complicated multifaceted neuro-degenerative conditions.

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