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Synthesis and neuroprotective activity of dictyoquinazol A and analogues

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ABSTRACT

A flexible and efficient synthesis of the neuroprotective alkaloid, dictyoquinazol A, is reported. Several structural analogues of the target molecule were produced, and the neuroprotective activity of this series of compounds was investigated using three different cell-based models of stroke. Several of the new compounds were found to have superior activity compared to the natural product. This work has established a new molecular scaffold that holds promise for a novel pharmaceutical treatment for stroke.

1. Introduction

Stroke is a leading cause of mortality and morbidity in the Western world, resulting in more deaths than coronary heart disease and causing approximately two-thirds of survivors to suffer a permanent disability.[1] Stroke is defined as a disruption in the blood flow to the brain. This can be caused by a clot (ischaemic stroke) or a bleed (haemorrhagic stroke).[2] In both cases, the normal delivery of nutrients and oxygen to neurons in the affected area is prevented, and this triggers a cascade of adverse biochemical responses. Excitotoxicity (i.e., the flooding of synapses by excitatory neurotransmitters), oxidative stress, and apoptosis have all been identified as major biochemical processes that contribute to the pathology of stroke.[3]

Despite the prevalence and severity of the disease, current treatments for acute stroke are very limited. The only pharmacological treatment used regularly is rtPA (recombinant tissue plasminogen activator), a thrombolytic protein-based drug that can break up blood clots and restore blood flow to brain areas affected by an ischaemic stroke.[4] However, this medication must be administered within four hours of stroke onset to be effective, and it is actively harmful to patients who have suffered a haemorrhagic stroke. Therefore there is clearly a need for new, more effective treatments for stroke.

Dictyoquinazol A (**1**, Fig. 1) is a natural alkaloid derived from the mushroom *Dictyophora indusiata*.[5] This natural product has been shown to have neuroprotective activity: specifically it reduces the damage caused by excitotoxicity and oxidative stress in mouse cortical neurons by blocking over-activity of the *N*-methyl-D-aspartic acid subtype of NMDA glutamatergic receptors and the monoamine oxidase enzyme respectively.[5] Dictyoquinazol A (**1**) contains a quinazolinone moiety, which has been identified as a privileged structure in medicinal chemistry[6] and has a wide presence in pharmaceutical agents such as the clinically used sedative, methaqualone.[7] Dictyoquinazol A (**1**) is 'Lipinski-compliant'[8] in terms of its low molecular weight (312 g mol^{-1}), as well as its number of H-bond donor and acceptor groups, and its *c*Log*P* value of 1.95 which is typical for CNS-active drugs.[9] All of these attributes suggest that compound **1** may be a promising candidate for development into a novel treatment for stroke.

The medicinal development of **1** would rely upon two initial milestones. First, an efficient and flexible synthetic approach would need to be available. Second, the biological activity of **1** (and its analogues) would need to be investigated in a variety of cell-based models of stroke, so that a preliminary structure–activity relationship profile could be developed. Realising these twin milestones became the aim of the current study.

One total synthesis and one formal synthesis of **1** have previously been published by the groups of Oh[10] and Ma.[11] These syntheses required only six and five steps, respectively. However, it could be argued that these syntheses are not ideally suited to the

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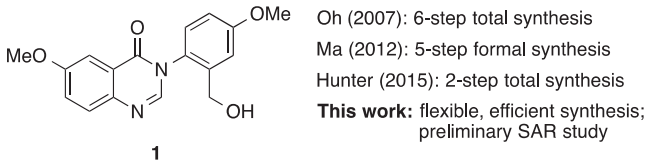


Figure 1. Structure and syntheses of dictyoquinazol A (**1**).

rapid production of structural analogues of **1**, because both syntheses featured a linear stepwise design,[10,11] and both also required a radical bromination reaction which called for toxic reagents and precise experimental conditions. Recently, we reported a two-step synthesis of **1** that avoided the need for this radical bromination reaction.[12] The brevity of our synthesis was enabled by the insight that the target molecule possesses hidden symmetry, and hence could be constructed through a dimerisation approach. However, our concise synthesis still suffered from some limitations in terms of overall yield and the ability to produce non-symmetrically substituted analogues.[12] Therefore, new synthetic methods are still desirable.

The second requirement for the medicinal development of **1** would be to further investigate its biological activity, in comparison with analogues, in order to build a preliminary structure–activity relationship profile. This is non-trivial because of the variety of adverse biochemical processes that are caused by stroke (e.g., excitotoxicity, oxidative stress and apoptosis amongst others).[3] An ideal pharmaceutical agent would protect nerve cells against several of these different types of injury mechanisms. Therefore it would be important to perform a variety of assays using several different models of stroke,[13] in order to ensure that protective activity can be optimised against one particular injury stimulus, without compromising activity towards another form of injury.

2. Results and discussion

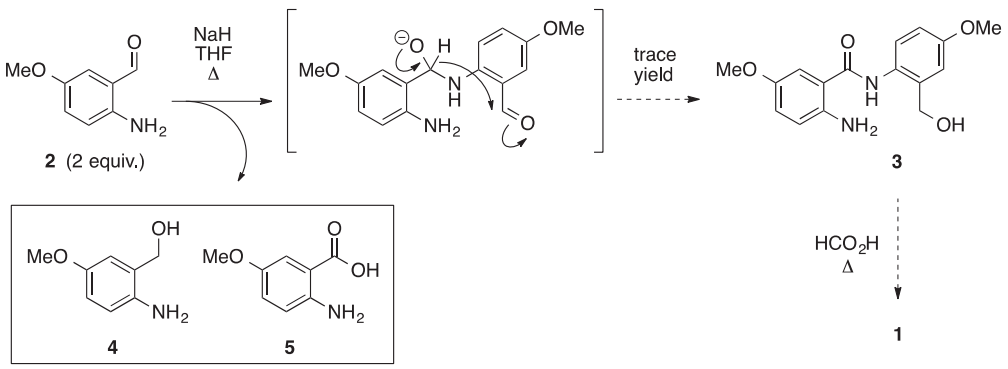
2.1. Synthesis

In order to produce analogues of **1** for biological screening, it was decided to investigate several different synthetic routes and to assess them in terms of step count, yield and flexibility.

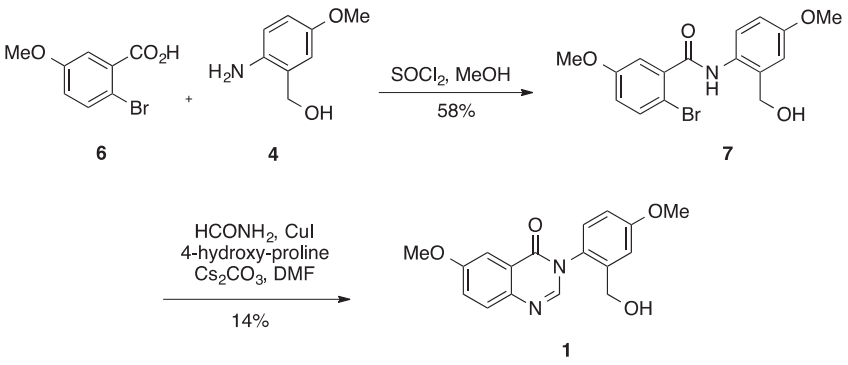
The first synthetic route to be investigated had the potential to deliver the target (**1**) in just two synthetic operations. It was recognised that compound **1** could conceptually be derived from the aldehyde **2** (Scheme 1). Two molecules of **2** could potentially be united through an amidation-Cannizzaro sequence[14] to deliver the amide **3**, which could then undergo cyclocondensation[15] with a C1 reagent such as formic acid to deliver the target **1**. In addition to the brevity of this strategy, an additional attraction was the fact that no stoichiometric redox transformations would be required. Thus, the aldehyde **2**[16] was treated with base in an attempt to effect the desired amidation-Cannizzaro reaction (Scheme 1). Several experimental parameters were varied, including the identity of the base (sodium hydride; potassium bis(trimethylsilyl)amide; *sec*-butyllithium), the stoichiometry (0.3–1.5 equiv of base relative to the aldehyde), the reaction concentration (13–80 mM in aldehyde), the temperature (0–100 °C with conventional or microwave irradiation), and solvent (THF; toluene). Unfortunately however, after all these attempts only a trace quantity of the desired product **3** was tentatively identified in one instance by HRMS and TLC (by comparison with an authentic sample, vide infra). It was interesting to note that the benzylic alcohol **4**[17] and the benzoic acid **5**[18] were sometimes detected in the crude product mixtures (Scheme 1), hinting that an intermolecular Cannizzaro process might have been dominating. Given that the desired intermediate **3** was not isolable in synthetically useful quantities, this approach to **1** was deemed to be unsuitable for our purposes.

The second new route to be investigated was a streamlined version of Ma's synthesis:[11] with the novelty in the current work being that the primary alcohol group of **1** was included from the start of the synthesis (Scheme 2). Accordingly, the first step was the coupling of starting materials **4** and **6** to give amide **7** (Scheme 2). A key copper-catalysed cyclisation reaction[11] was then investigated. This reaction did afford some of the desired target **1**, thereby completing a concise total synthesis, but unfortunately the target compound was only obtained in 14% yield due to the concomitant formation of two unidentifiable side-products which had very similar polarity to that of **1**. Efforts to optimise this process failed to increase the yield of **1**.

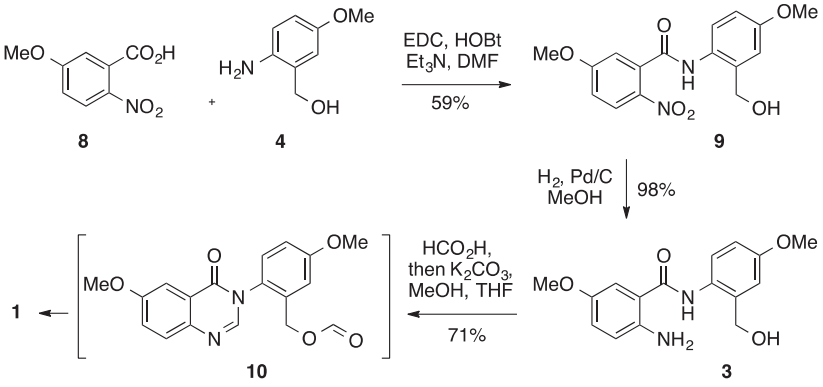
The third synthetic route to be investigated was a streamlined version of Oh's synthesis:[10] again, the novelty in the current work was that the primary alcohol group of **1** was included from the start of the synthesis (Scheme 3). The first step was the coupling of acid **8** and aniline **4** (Scheme 3). This reaction delivered the amide **9** in moderate yield, along with a smaller quantity of a side-product (see ESI) in which the primary alcohol group of **4** had become esterified by reaction with another equivalent of the acid **8**. Nevertheless, the desired product **9** was readily purified by flash chromatography and the yield (59%) was deemed sufficient to proceed with the synthesis. The next step in the sequence was reduction of the nitro group of **9**, which was achieved using catalytic hydrogenation conditions and smoothly delivered the aniline **3** in excellent yield (Scheme 3). Finally, the aniline **3** was treated with formic acid at reflux to deliver the natural product **1**. It was found that some of the ester **10** was also formed under these reaction conditions, but it could readily be converted to the desired target **1** simply by treating the crude product mixture with



Scheme 1. Attempted two-step synthesis of **1** via an amidation-Cannizzaro sequence.[14]



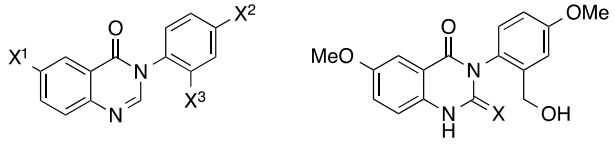
Scheme 2. A new, streamlined variant of Ma's synthesis.[11]



Scheme 3. A new, streamlined variant of Oh's synthesis.[10]

mild base in a one-pot operation. Overall, this 'streamlined' synthesis delivered the natural product **1** in just three discrete chemical operations, and in high overall yield (41%).

With these preliminary synthetic investigations completed, the next objective was to produce several analogues of **1** in order to commence a structure–activity relationship study. The various synthetic approaches to **1** (Schemes 1–3) were compared in terms of their step count, overall yield and flexibility. It was decided that the route illustrated in Scheme 3 offered the best approach. Accordingly, this synthetic strategy was utilised in the preparation of several structural analogues of **1** (e.g., **11** and **13**, Fig. 2; also see Supporting information). Additional analogues were also available through separate work (e.g., **12**, **14**–**16**).[12] From the total collection of analogues, only the small subset that is illustrated in Figure 2 was selected for biological investigation in this study, in order to collect preliminary structure–activity data in a focused manner. Analogue **11** was designed to probe the importance of the methoxy groups of **1**; analogues **12**–**14** probed the significance of the benzylic alcohol group of **1**; and analogues **15**–**16** probed the importance of the heterocyclic core of **1**.



11: X^1=H, X^2=H, X^3=CH_2OH
12: X^1=OMe, X^2=OMe, X^3=H
13: X^1=OMe, X^2=OMe, X^3=CH_2F
14: X^1=OMe, X^2=OMe, X^3=CO_2Me

15: X=O
16: X=S

Figure 2. Structural analogues of **1** that were selected for biological investigation.

2.2. Bioassays

SH-SY5Y cells, which are a human-derived neuroblastoma cell line, were grown in culture then exposed to one of three different injury stimuli that were designed to mimic various mechanisms involved in brain injury that occurs after a stroke: L-glutamate (to mimic excitotoxicity); hydrogen peroxide (to mimic oxidative stress); or staurosporine (STS, to induce apoptosis). Each injury stimulus was administered at a concentration that caused approximately 50% of the cells to die within 20 h (i.e., L-glutamate = 13.3 mM; H_2O_2 = 44 μM; STS = 125 nM). The experiments were then repeated with co-administration of varying concentrations of the dictyoquinazol A analogues (Fig. 2).[19] Any improvements in cell viability were attributed to the compounds' neuroprotective activity. It was of particular interest to establish whether protective activity could be optimised towards one injury stimulus without compromising activity towards a different injury stimulus.

The first injury stimulus to be investigated was L-glutamate (Fig. 3). The natural product (**1**) exhibited modest protective activity: it caused a maximum of 10% improvement in cell survival (i.e., from 50% to 60%) at a drug concentration of 0.3 μM. Analogue **11**, which lacks both methoxy groups of **1**, showed greater protective activity against L-glutamate (Fig. 3a): it caused a 35% increase in cell survival at a concentration of 0.6 μM. The analogues **12**–**14** were next tested (Fig. 3b), to reveal the significance of the benzylic alcohol group of **1**. Analogue **12**, which lacks the benzylic alcohol group entirely, showed equal protective activity against L-glutamate compared to **1** (Fig. 3b). In contrast, analogues **13** and **14**, which contain a benzylic fluoride or methyl carboxylate group at this position, exhibited considerably greater protective activity than **1** across all of the concentrations tested (Fig. 3b). Finally,

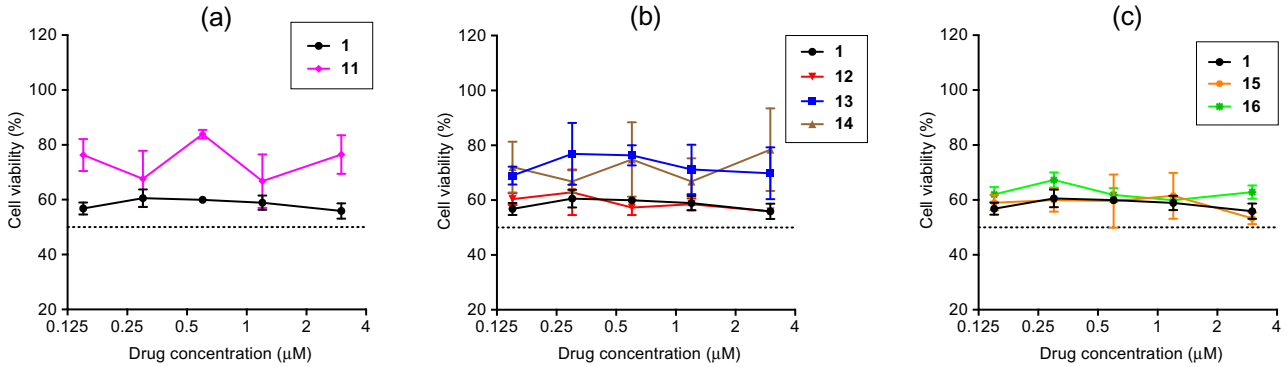


Figure 3. Neuroprotective activity of **1** and analogues **11**–**16** against L-glutamate-induced toxicity. The dashed line indicates cell viability (%) in the presence of 13.3 mM L-glutamate alone. For clarity, the results are split between three graphs to illustrate the importance of (a) the methoxy groups of **1**; (b) the benzylic alcohol group of **1**; (c) the heterocyclic core of **1**.

the analogues **15** and **16** were tested (Fig. 3c), to reveal the importance of the heterocyclic core of **1**. Both of these analogues were found to have approximately equal protective activity against L-glutamate-induced injury compared to **1**.

The next injury stimulus to be investigated was H_2O_2 (Fig. 4). The natural product (**1**) was found to exhibit a robust, concentration-dependent protective action against H_2O_2, causing a maximum of 22% improvement in cell viability at 0.6–1.2 μM (Fig. 4a). In contrast, the analogue **11**, which lacks both methoxy groups, showed essentially no protective activity (Fig. 4a). Turning attention to the analogues with variation at the benzylic alcohol position (Fig. 4b), the results were mixed. Analogue **12**, which contains no substituent at this position, was considerably more potent than **1** particularly at low drug concentrations (Fig. 4b); analogue **13**, which contains a benzylic fluoride, was less potent; while analogue **14**, containing a methyl carboxylate group, exhibited approximately equal protective activity compared to **1**. Finally, analogues **15** and **16**, which contained variations on the heterocyclic core, were both more potent than **1** particularly at low drug concentrations (Fig. 4c).

The third injury stimulus to be investigated was staurosporine (STS), which causes cell death by apoptosis (Fig. 5). The natural product (**1**) was found to exhibit strong protective activity against STS toxicity, causing a 41% increase in cell survival at a drug

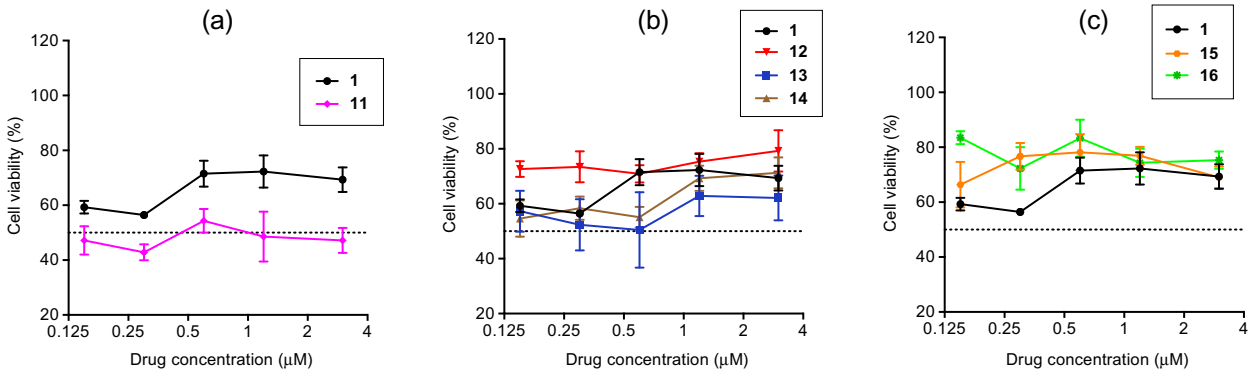


Figure 4. Neuroprotective activity of **1** and analogues **11**–**16** against H_2O_2-induced toxicity. The dashed line indicates cell viability (%) in the presence of 44 μM H_2O_2 alone. For clarity, the results are split between three graphs to illustrate the importance of (a) the methoxy groups of **1**; (b) the benzylic alcohol group of **1**; (c) the heterocyclic core of **1**.

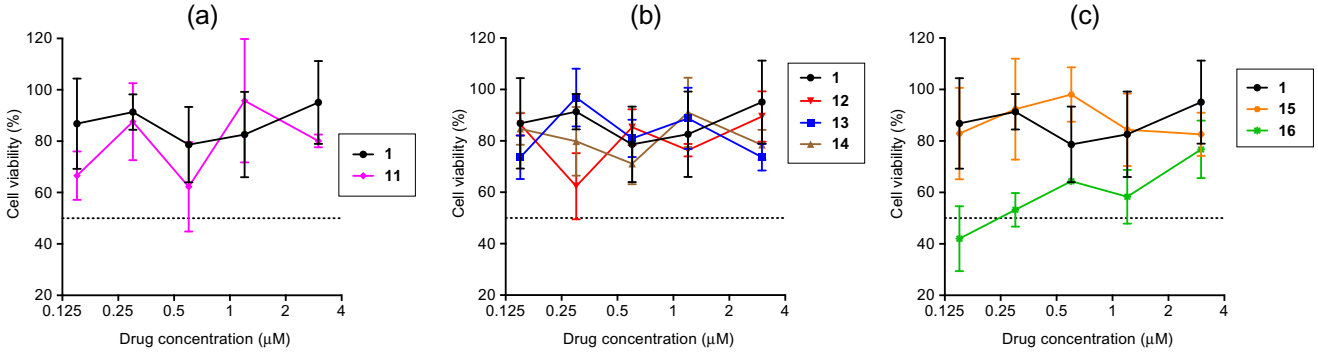


Figure 5. Neuroprotective activity of **1** and analogues **11**–**16** against staurosporine-induced toxicity. The dashed line indicates cell viability (%) in the presence of 125 nM STS alone. For clarity, the results are split between three graphs to illustrate the importance of (a) the methoxy groups of **1**; (b) the benzylic alcohol group of **1**; (c) the heterocyclic core of **1**.

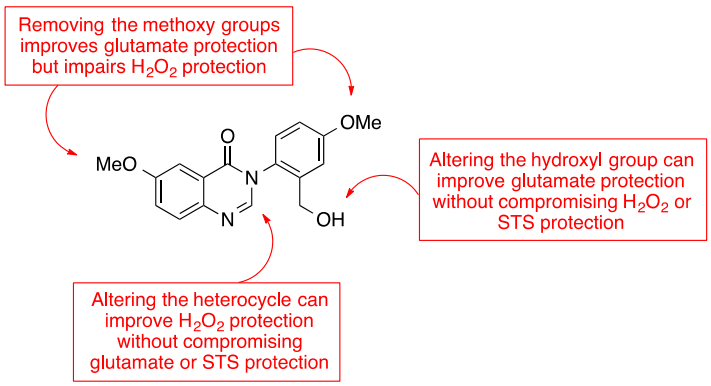


Figure 6. Preliminary structure–activity data for compound **1** against the three injury stimuli.

concentration of 0.3 μM (Fig. 5a). Analogue **11**, which lacks both methoxy groups, exhibited approximately equal protective activity (Fig. 5a). In a similar vein, the analogues **12**–**14**, which contained variation at the benzylic alcohol position, also exhibited approximately equal protective activity against STS compared to **1** (Fig. 5b). Finally, the results with analogues **15** and **16**, which contained variations on the heterocyclic core, were mixed (Fig. 5c). Analogue **15**, containing a C=O substituent, had approximately equal protective activity compared to **1**, while analogue **16**, containing a C=S substituent, was much less potent against STS toxicity.

2.3. Structure–activity relationships

The preliminary structure–activity relationship data for compound **1** is summarised in Figure 6. The importance of the methoxy groups of **1** is somewhat complex to interpret: the methoxy groups were essential for protective activity against H_2O_2, but unnecessary for protective activity against STS and actually detrimental for protective activity against L-glutamate. The benzylic alcohol group of **1** was found to be non-essential (Fig. 6), as its alteration could improve the protection against L-glutamate without compromising activity against H_2O_2 or STS. Finally, it was found that the heterocyclic core of **1** could also safely be modified without loss of activity against any of the injury stimuli (Fig. 6). Overall, perhaps the most important conclusion is that the activity of compound **1** can indeed be improved against one particular injury stimulus without impairing activity against all of the other different types of injury. For example, analogue **14** had better protective activity against L-glutamate (Fig. 3b) without loss of activity against H_2O_2 or STS (Figs. 4b and 5b); and analogue **15** had better activity against both H_2O_2 and STS (Figs. 4c and 5c) without loss of activity against L-glutamate (Fig. 3c).

3. Conclusions

A concise and efficient synthesis of the neuroprotective alkaloid, dictyoquinazol A (**1**) has been developed. Several structural analogues of this natural product were also produced. Compound **1** and its analogues were assayed in three different cell-based models of stroke, using cultured human neuroblastoma cells. This revealed that it is possible to improve the protective activity of compound **1** against a particular neural injury pathway, without impairing activity towards other injury mechanisms; this is a confirmation that compound **1** holds promise as a lead compound for the development of a novel treatment for stroke. Towards that goal, this work has also revealed which functional groups of **1** can safely be derivatised without impairing activity (e.g., the benzylic alcohol); this information will be used to facilitate the design of pull-down experiments to identify the protein target(s) of compound **1**.

4. Experimental

4.1. Synthetic reagents and instrumentation

Reactions were performed in oven-dried glassware at room temperature and under nitrogen atmosphere with magnetic stirring unless stated otherwise. All commercial reagents were of synthetic grade and were used as received. Reactions were monitored by TLC using Merck aluminium-backed silica gel 60 F254 (0.2 mm) TLC plates. TLC spots were visualised under short-wave UV light (254 nm) followed by staining with phosphomolybdic acid or potassium permanganate dip. Flash chromatography was performed using Davisil 40–63 mesh silica gel and eluents are stated as volume-to-volume ratios. Melting points were determined using an OptiMelt melting point apparatus MPA100. IR spectra were recorded using a Cary 360 Fourier Transform Infrared (FTIR) spectrometer equipped with attenuated total reflectance (ATR) with a diamond crystal inset. NMR spectra were obtained using Bruker Avance III 300, 400 and 600 MHz instruments at 300 K. Residual solvent peaks were used as an internal reference to calibrate ^{1}H and ^{13}C spectra. HMRS results were acquired at the UNSW Bioanalytical Mass Spectrometry Facility using an LCQ Deca XP Plus ion trap MS in positive ion mode using electrospray ionisation (ESI).

4.2. Dictyoquinazol A (1)

4.2.1. Method 1

A mixture of compound **3** (395 mg, 1.31 mmol), formic acid (500 μL, 13.3 mmol) and tetrahydrofuran (2 mL) was stirred at 66 °C for 8 h. The reaction mixture was cooled to room temperature and diluted with methanol (2 mL). Potassium carbonate (2.00 g, 14.5 mmol) was added, and the resulting mixture was stirred under N_2 atmosphere at room temperature for 12 h. The mixture was diluted with ethyl acetate (20 mL) and filtered through a silica plug with ethyl acetate washing. The filtrate was concentrated onto silica, and the crude product was subjected to flash chromatography eluting with 8:2 dichloromethane/ethyl acetate to yield the title compound as a pale yellow powder (292 mg, 71%); spectral data in accordance with literature values.[5,10,12]

4.2.2. Method 2

Cesium carbonate (0.110 g, 0.338 mmol) and purified[20] copper (I) iodide (0.008 g, 0.04 mmol) were added to an oven-dried Schlenk tube and dried at approximately 150 °C overnight. The vessel

was cooled, refilled with nitrogen and the lower compartment of the tube was sealed. Compound **7** (0.06 g, 0.2 mmol) and *trans*-4-hydroxy-L-proline (0.005 g, 0.04 mmol) were added to the upper compartment and dried under vacuum for 3 h. The vessel was refilled with nitrogen and the solids were allowed to fall into the main compartment. Dry formamide (0.02 mL, 0.5 mmol) and DMF freshly distilled from calcium hydride (0.7 mL) were added via syringe and the mixture was stirred at 80 °C for 24 h under a nitrogen atmosphere. The mixture was cooled, diluted with ethyl acetate and washed with aqueous ammonium chloride, water and brine. The organic layer was dried with anhydrous magnesium sulfate and the solvent was removed in vacuo. The residue was purified by column chromatography to afford the title compound (0.007 g, 14%).

4.3. 2-Amino-5-methoxybenzaldehyde (2)

Compound **4**[17] (216 mg, 1.41 mmol) and MnO_2 (900 mg, 10.4 mmol) were dissolved in dry THF (20 mL). The reaction mixture was stirred under nitrogen atmosphere at room temperature for 4 h. After that time another portion of MnO_2 (900 mg, 10.35 mmol) was added, followed by a third portion of MnO_2 (900 mg, 10.35 mmol) after the same time interval. The reaction mixture was stirred under N_2 atmosphere at room temperature for a total of 24 h. The mixture was filtered through a layer of celite and washed with small amount of THF. The filtrate was evaporated under reduced pressure to produce the title compound as a brown oil (225 mg, 99%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.85 (d, J = 0.3 Hz, CHO), 7.01 (dd, J = 8.8, 2.9 Hz, ArH4), 6.96 (d, J = 2.9 Hz, ArH6), 6.63 (d, J = 8.8 Hz, ArH3), 5.83 (s, 2H, NH_2), 3.79 (s, 3H, OCH_3), ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 193.7, 150.9, 144.9, 124.8, 118.6, 117.9, 116.9, 56.0. This compound is unstable at room temperature and must be kept in a vial filled with nitrogen gas at −20 °C; for this reason it was not fully characterised.

4.4. 2-Amino-*N*-(2-(hydroxymethyl)-4-methoxyphenyl)-5-methoxybenzamide (3)

Compound **9** (206 mg, 0.62 mmol) was dissolved in methanol (5 mL), and 10% Pd/C (60 mg, 0.06 mmol) was added to this solution. The reaction mixture was stirred under H_2 atmosphere at room temperature for 4 h. The mixture was filtered through a layer of Celite and washed with methanol. The filtrate was concentrated under reduced pressure to produce the title compound as a brown powder (184 mg, 98%); mp 132–137 °C; IR (neat) ν_{max} (cm^{-1}) 3375, 3248, 2830, 2723, 2377, 2098, 1640, 1574; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (s, 1H, NH), 7.90 (d, J = 8.8 Hz, ArH6′), 7.07 (d, J = 2.8 Hz, ArH6), 6.92 (dd, J = 8.8, 2.8 Hz, ArH4), 6.90 (dd, J = 8.8, 2.8 Hz, ArH5′), 6.83 (d, J = 2.8 Hz, ArH3′), 6.70 (d, J = 8.8 Hz, ArH3), 4.79 (s, 2H, CH_2), 3.81 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3); ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 167.7 (C═O), 156.8 (C4)′, 151.4 (C5), 143.2 (C2), 132.8 (C1′ or C2′), 130.3 (C2′ or C1′), 124.8 (C6′), 120.2 (C4), 119.3 (C3), 116.9 (C1), 115.0 (C3′), 113.8 (C5′), 111.7 (C6), 64.5 (CH_2), 56.1 (OCH_3), 55.7 (OCH_3); HRMS (ESI,+ve) $C_{16}H_{18}N_2O_4Na^+$ [MNa^+] requires m/z 325.1151, found 325.1159.

4.5. 2-Bromo-*N*-(2-(hydroxymethyl)-4-methoxyphenyl)-5-methoxybenzamide (7)

A mixture of compound **6** (0.081 g, 0.35 mmol) and thionyl chloride (1.12 mL, 15.4 mmol) was stirred at reflux for 5 h. The excess thionyl chloride was removed and the residue was cooled to 0 °C. Aqueous saturated sodium bicarbonate solution (10 mL) and a solution of compound **4** (0.046 g, 0.30 mmol) in methanol (2 mL) were added slowly with rapid stirring. The mixture was stirred at room temperature overnight and the resultant pink-orange solid was collected and washed with aqueous HCl (1 M, 5 mL), water (5 mL), diethyl ether (5 mL) and hexane (5 mL). The solid was dried to obtain the title compound as a sticky orange powder (0.063 g, 58%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.81 (s, 3H), 3.82 (s, 3H), 4.71 (s, 2H), 6.82–6.93 (m, 3H), 7.17 (d, J = 3.1 Hz, 1H), 7.49 (d, J = 8.8 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H), 8.57 (br s, 1H); this material was carried forward to the next step without further purification.

4.6. *N*-(2-(Hydroxymethyl)-4-methoxyphenyl)-5-methoxy-2-nitrobenzamide (9)

A solution of compound **8** (240 mg, 1.22 mmol) and EDC·HCl (277 mg, 1.44 mmol) in DMF (2 mL) was stirred at room temperature for 5 min, then cooled to 0 °C. To this solution was added successively 1-hydroxybenzotriazole (195 mg, 1.44 mmol), a solution of compound **4** (207 mg, 1.35 mmol) in DMF (2 mL), and triethylamine (0.25 mL, 1.80 mmol). The mixture was warmed to room temperature and stirred under N_2 atmosphere for 6 h. The solvent was removed under reduced pressure and the crude product was partitioned between aqueous HCl (10 mL, 1 N) and ethyl acetate (20 mL). The aqueous layer was extracted with ethyl acetate (4 × 20 mL). The organic layers were combined and washed with water (10 × 20 mL), then dried with $MgSO_4$ and concentrated onto silica under reduced pressure. The residue was subjected to flash chromatography eluting with 1:9→3:7 ethyl acetate/dichloromethane, to afford the title compound as a pale yellow solid (238 mg, 59%); mp 168–170 °C; IR (neat) ν_{max} (cm^{-1}): 3375, 3257, 3067, 3021, 2946, 2840, 2656, 2321, 2085, 2008, 1926, 1790, 1647, 1578, 1538, 1505; ^{1}H NMR (400 MHz, CD_3CN) δ 8.64 (s, 1H, NH), 8.14 (d, J = 9.1 Hz, ArH3), 7.66 (d, J = 8.7 Hz, ArH6′), 7.20 (d, J = 2.8 Hz, ArH6), 7.15 (dd, J = 9.1, 2.8 Hz, ArH4), 6.98 (d, J = 3.0 Hz, ArH3′), 6.92 (dd, J = 8.7, 3.0 Hz, ArH5′), 4.63 (s, 2H, CH_2), 3.94 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.45 (s, 1H, OH); ^{13}C{^{1}H} NMR (101 MHz, CD_3CN) δ 165.8 (C═O), 164.9 (C5), 158.6 (C4′), 140.2 (C1), 137.1 (C1′), 136.6 (C2), 129.1 (C2′), 128.2 (C3), 126.7 (C6′), 115.9 (C4), 115.1 (C6), 114.4 (C3′), 113.7 (C5′), 62.5 (CH_2), 57.2 (OCH_3), 56.1 (OCH_3); HRMS (ESI,+ve) $C_{16}H_{16}N_2O_6Na^+$ [MNa^+] requires m/z 355.0901, found 355.0894.

4.7. 5-Methoxy-2-(6-methoxy-4-oxoquinazolin-3(4*H*)-yl)benzyl formate (10)

Data for **10**: mp 127–132 °C; IR (neat) ν_{max} (cm^{-1}) 3064, 3003, 2919, 2839, 2098, 1712, 1673, 1600, 1482; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (s, 1H, CHO), 7.96 (s, 1H, ArH2), 7.74–7.69 (m, 2H, ArH5, ArH8), 7.40 (dd, J = 9.0, 3.0 Hz, ArH7), 7.22 (d, J = 8.7 Hz, ArH3′), 7.12 (d, J = 2.9 Hz, ArH6′), 7.02 (d, J = 8.7, 2.9 Hz, ArH4′), 5.10 (d, J = 13.0 Hz, C*H*H), 5.00 (d, J = 13.0 Hz, CH*H*), 3.93 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3); ^{13}C{^{1}H} NMR (76 MHz, $CDCl_3$) δ 160.9 (C4), 160.6 (C5′), 160.3 (O—C═O), 159.3 (C6), 144.7 (C2), 142.4 (C9), 134.7 (C2′), 129.6 (C3′), 129.21 (C8 or C5), 129.20 (C1′), 125.0 (C7), 123.1 (C10), 115.8 (C6′), 115.3 (C4′), 106.8 (C5 or C8), 62.0 (CH_2), 56.1 (OCH_3), 55.8 (OCH_3); HRMS (ESI,+ve) $C_{18}H_{16}N_2O_5H^+$ [MH^+] requires m/z 341.1132, found 341.1129; $C_{18}H_{16}N_2O_5Na^+$ [MNa^+] requires m/z = 363.0951, found 363.0948.

4.8. 3-(2-(Hydroxymethyl)phenyl)quinazolin-4(3*H*)-one (11)

4.8.1. Step 1

2-Nitrobenzoic acid (0.34 g, 2.0 mmol) and EDC·HCl (466 mg, 2.40 mmol) were dissolved in DMF (4 mL). After stirring for 5 minutes, 1-hydroxybenzotriazole (301 mg, 2.40 mmol), a solution of 2-aminobenzyl alcohol (0.36 g, 3.0 mmol) in DMF (2 mL), and triethylamine (0.41 mL, 3.0 mmol) were added to this solution. The reaction mixture was stirred at room temperature for 36 h.

The mixture was concentrated under reduced pressure, and the crude product was dissolved in aq HCl (15 mL, 1 N) and ethyl acetate (25 mL). After partitioning of the two layers, the aqueous layer was extracted with ethyl acetate (4 × 25 mL). All organic layers were combined and washed with water (10 × 20 mL). Finally the solvent was dried with $MgSO_4$ and evaporated under reduced pressure onto silica. The crude product was purified by flash chromatography eluting with 1:9→3:7 ethyl acetate/hexane (containing 0.5% acetic acid) to produce *N*-(2-(hydroxymethyl)phenyl)-2-nitrobenzamide (**17**) as a white solid (88 mg, 16%); mp 170–172 °C; IR (neat) ν_{max} (cm^{-1}) 3232, 3071, 2918, 2864, 2712, 2284, 2111, 2069, 1647, 1521; ^1H NMR (300 MHz, CD_3CN) δ 9.17 (s, 1H, NH), 8.09 (dd, J = 8.0, 0.7 Hz, ArH3), 7.95 (d, J = 8.4, 1.3 Hz, ArH6′), 7.83 (m, 2H, ArH5, ArH6), 7.74 (ddd, J = 8.0, 7.0, 2.1 Hz, ArH4), 7.40 (m, 2H, ArH5′, ArH3′), 7.24 (ddd, J = 8.4, 7.6, 1.1 Hz, ArH4′), 4.71 (d, J = 3.5, 2H, CH_2), 3.56 (s, 1H, OH); $^{13}C\{^1H\}$ NMR (76 MHz, CD_3CN) δ 165.4 (C═O), 148.1 (C2), 137.2 (C1′), 134.9 (C5), 133.7 (C1 or C2′), 133.6 (C2′ or C1), 132.1 (C4), 129.6 (C6), 129.4 (C3′), 129.1 (C5′), 126.3 (C4′), 125.5 (C3), 124.1 (C6′), 63.1 (CH_2); HRMS (ESI,+ve) $C_{14}H_{12}N_2O_4Na^+$ [MNa^+] requires m/z 295.0689, found 295.0672.

4.8.2. Step 2

Compound **17** that was synthesised above (68 mg, 0.25 mmol) was dissolved in methanol (2 mL), and 10% Pd/C (30 mg, 0.03 mmol) was added to this solution. The reaction mixture was stirred under H_2 atmosphere at room temperature for 4 h. The mixture was filtered through a layer of celite and washed with methanol. The filtrate was concentrated under reduced pressure onto silica, and the crude product was subjected to flash chromatography eluting with 3:1 hexane/ethyl acetate to yield 2-amino-*N*-(2-(hydroxymethyl)phenyl)benzamide (**18**) as a white crystalline solid (45 mg, 76%); mp 140–143 °C; IR (neat) ν_{max} (cm^{-1}) 3440, 3278, 3025, 2928, 2877, 2343, 2111, 1997, 1812, 1620, 1617, 1582, 1510; ^1H NMR (300 MHz, CD_3CN) δ 9.45 (s, 1H, NH), 8.04 (dd, J = 8.1, 1.2 Hz, ArH6′), 7.57 (dd, J = 8.0, 1.5 Hz, ArH6), 7.37−7.22 (m, 3H, ArH5′, ArH3′, ArH3), 7.13 (ddd, J = 14.9, 7.4, 1.2 Hz, ArH4′), 6.77 (dddd, J = 9.5, 8.2, 1.2, 0.4, Hz, ArH4), 6.67 (dddd, J = 8.2, 8.0, 7.2, 1.2 Hz, ArH5), 5.92 (s, 2H, NH_2) 4.69 (s, 2H, CH_2), 3.72 (s, 1H, OH); $^{13}C\{^1H\}$ NMR (76 MHz, $CDCl_3$) δ 168.6 (C═O), 151.0 (C2), 138.7 (C2′), 133.6 (C3), 132.5 (C1′), 129.4 (C3′), 129.0 (C5′), 128.4 (C6), 125.0(C4′), 123.5 (C6′), 118.0 (C4); 116.9 (C5), 116.1 (C1), 63.9 (CH_2); HRMS (ESI,+ve) $C_{14}H_{14}N_2O_2Na^+$ [MNa^+] requires m/z 265.0947, found 265.0944.

4.8.3. Step 3

A mixture of compound **18** that was synthesised above (16 mg, 0.06 mmol) and formic acid (100 μL, 1.86 mmol) was stirred at reflux (oil bath temperature 95 °C) for 12 h. The reaction mixture was cooled to room temperature and diluted with methanol (1 mL) and tetrahydrofuran (1 mL). Potassium carbonate (270 mg, 1.95 mmol) was added, and the mixture was stirred at room temperature under N_2 atmosphere for 12 h. The mixture was diluted with ethyl acetate (10 mL) and filtered through a silica plug with ethyl acetate washing. The filtrate was evaporated under reduced pressure onto silica, and the crude product was subjected to flash chromatography eluting with 1:3 ethyl acetate/hexane, to produce the title compound (**11**) as a light yellow powder (13 mg, 83%); mp 154–159 °C; IR (neat) ν_{max} (cm^{-1}) 3290, 3042, 2920, 2644, 2320, 2109, 1982, 1942, 1876, 1846, 1672, 1599, 1561; ^1H NMR (600 MHz, CD_3CN) δ 8.26 (dd, J = 7.9, 1.5 Hz, ArH5), 8.06 (s, 1H, ArH2), 7.86 (ddd, J = 15.3, 7.9, 1.5 Hz, ArH7), 7.77 (dd, J = 8.2, 1.2 Hz, ArH8), 7.64 (d, J = 7.6 Hz, ArH3′), 7.59 (m, 1H, ArH4), 7.56 (ddd, J = 15.2, 7.6, 1.4 Hz, ArH4′), 7.49 (ddd, J = 15.2, 7.6, 1.4 Hz, ArH5′), 7.37 (dd, J = 7.8, 1.4 Hz, ArH6′), 4.44 (d, J = 13.9 Hz, C*H*H), 4.42 (d, J = 13.9 Hz, CH*H*), 3.24 (s, 1H, OH); $^{13}C\{^1H\}$ NMR (150 MHz, CD_3CN) δ 161.8 (C4), 149.3 (C10), 148.1 (C2), 140.3 (C1′), 137.1 (C2′), 135.5 (C7), 130.7 (C4′), 129.9 (C3′), 129.6 (C5′), 129.3 (C6′), 128.5 (C8), 128.4 (C6), 127.5 (C5), 123.4 (C9), 61.2 (CH_2); HRMS (ESI,+ve) $C_{15}H_{12}N_2O_2H^+$ [MH^+] requires m/z 253.0972, found 253.0968.

4.9. 3-(2-(Fluoromethyl)-4-methoxyphenyl)-6-methoxyquinazolin-4(3*H*)-one (13)

Compound **1** (28 mg, 0.09 mmol) was dissolved in anhydrous dichloromethane (2 mL) and cooled to −78 °C. To this solution Deoxo-Fluor™ (25 μL, 1.4 mmol) was added. The reaction mixture was warmed to room temperature and stirred under N_2 atmosphere for 24 h. The mixture was diluted with dichloromethane (2 mL) and then quenched by dropwise addition of saturated $NaHCO_3$ solution (10 mL). After partitioning of the two layers, the aqueous layer was extracted with dichloromethane (4 × 3 mL). All organic layers were combined and the solvent was dried with $MgSO_4$ and evaporated onto silica under reduced pressure. The residue was purified by flash chromatography eluting with 1:9 ethyl acetate/dichloromethane to yield the title compound as a light yellow solid (12 mg, 43%); mp 164–170 °C; IR (neat) ν_{max} (cm^{-1}) 3751, 3328, 3077, 3021, 2922, 2844, 2100, 1983, 1912, 1740, 1667, 1612; ^1H NMR (300 MHz, $CDCl_3$) δ 7.94 (s, 1H, ArH7), 7.72 (m, 1H, ArH8); 7.70 (m, 1H, ArH5), 7.40 (dd, J = 8.9, 3.0 Hz, ArH7), 7.23 (dd, J = 8.6, 0.8 Hz, ArH6′), 7.13 (d, J = 2.7 Hz, ArH3′), 7.04 (dddd, J = 11.5, 8.6, 2.7, 1.2 Hz, ArH5′), 5.28 (dd, J = 47.5, 11.5 Hz, C*H*H), 5.21 (dd, J = 47.2, 11.5, Hz, CH*H*), 3.93 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3); $^{13}C\{^1H\}$ NMR (76 MHz, $CDCl_3$) δ 160.9 (C4), 160.6 (C4′), 159.3 (C6), 144.6 (C2), 142.5 (C9), 135.5 (d, J = 16.6 Hz, C2′), 129.5 (C6′), 129.3 (C8), 128.5 (d, J = 4.2 Hz, C1′)), 125.0 (C7), 123.0 (C10), 115.5 (d, J = 2.5 Hz, C5′)), 114.8 (d, J = 7.8 Hz, C3′)), 106.7 (C5), 81.2 (d, J = 168.0 Hz, CF), 56.0 (OCH_3), 55.8 (OCH_3); ^{19}F {^1H} NMR (76 MHz, $CDCl_3$) δ −212.5 (s, 1F); ^{19}F NMR (76 MHz, $CDCl_3$) δ −212.5 (t, J = 47.5 Hz); HRMS (ESI,+ve) $C_{17}H_{15}F_1N_2O_4H^+$ [MH^+] requires m/z 315.1139, found 315.1134.

4.10. Bioassay reagents and instrumentation

All bioassay chemicals were purchased from Gibco Life Technologies and used as received. All procedures were performed under aseptic conditions, unless stated otherwise. Optical density values were measured using a BMG FLUOstar OPTIMA microplate reader.

4.11. Cell culture model

SH-SY5Y neuroblastoma cells (ATCC® CRL-2266™) were maintained for between 10 and 18 passages in T-75 flasks in media (containing 5% FBS, 46% F12 growth media, 46% DMEM, 1% glutamax, 1% antibiotic/antimycotic and 1% sodium pyruvate) at 37 °C in a humidified atmosphere with 5% CO_2. Media was changed every four days. The cells were subsequently subcultured by 1:2 or 1:3 dilution into two or three T-75 flasks at 37 °C in a humidified atmosphere with 5% CO_2, until they were used for experimentation. When the cells achieved 85–95% confluency, 0.05% trypsin was used to detach the adherent cells from the flask. Once the cells detached, they were harvested and combined with media containing non-adherent cells. The cell suspension was then spun down at 700 rpm for 7 minutes at room temperature. Then, the supernatant was discarded and the cell pellet resuspended with 10 mL growth media. A small aliquot (10 μL) was taken to estimate cell number using a hemocytometer. Cells were plated into a Costar 96-well cell culture plate at a density 5×10^5 cells/mL, 100 μL/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 20 h prior to experimentation.

4.12. Cell treatment

A 3 mM stock solution of each compound of interest was prepared by dissolving 0.006 mmol pure synthetic compound in 2 mL sterile DMSO. A series of dilutions was performed to generate the desired concentration of the compound of interest. All dilutions of the compounds of interest were made such that the amount of DMSO was constant across the concentration range tested. In order to normalise data, 0%, 50% and 100% cell death controls were used in each culture plate. The 0% cell death control involved using Minimal Essential Media (MEM). The 50% cell death control was produced by treating cells with 13.33 mM L-glutamate (excitotoxicity) or 44 μM H_2O_2 (oxidative stress) or 125 nM STS (apoptosis). The 100% cell death control used 0.25% Triton X100 which killed all cells in the well (this was also verified by microscopic observation). All of the relevant controls and treatments with compounds of interest were performed in triplicate in each plate. Treated plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 20 h, then cell viability was measured.

4.13. Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[21] After 20 h of exposure to the injury stimulus, 10 μL MTT (5 mg/mL) was added to each well and incubated for 30 min. The assay was terminated by removing the MTT-media mixture, and adding 50 μL DMSO into each well to dissolve the formazan salt. Next, the plate was kept in the dark for 1 h. The absorbance was determined at wavelength 570 nm using a spectrophotometric plate reader. The obtained values were analysed by normalising experimental data to the average TX100 OD values as 0% cell viability, average MEM OD values as 100% cell viability. The OD density values of the treated cells were converted into percentage cell viability by comparison with these positive and negative controls.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.02.016.

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