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Synthesis of six mexiletine derivatives with isoindolines attached as potential antioxidants and their evaluation as cardioprotective agents†

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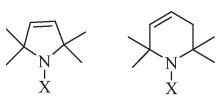
Six derivatives containing mexiletine and its 2,6-dichloro analogue to which the potential antioxidant moiety isoindoline, its N-oxide and the *N*-hydroxy derivatives have been attached, have been evaluated as cardioprotective agents. Four of the derivatives at different oxidation states, NH, NO and NOH had significant cardioprotective properties in an *in vitro* ischaemia model. In contrast to previous reports the NH compounds were not the most active.

Introduction

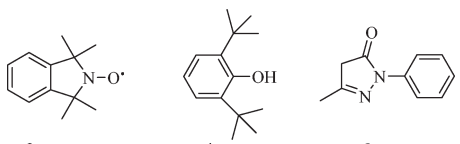
Mexiletine has been used clinically as a class Ib antiarrhythmic agent for more than 40 years.[1] Mexiletine has also been shown to have cardioprotective activity in animal models, an effect mediated by the opening of cardiac K^+ channels.[2,3] Inuo and colleagues found that mexiletine administration decreased infarct size and increased left ventricular functional recovery in a rabbit closed chest ischaemia model, and that glibenclamide reversed these protective effects.[2]

More recently, the modification of mexiletine by attachment of antioxidant moieties has been reported to lead to improved activity in both cardioprotective and antiarrhythmic activity. Most of the studies have used 2,2,5,5-tetramethylpyrroline based compounds (**1**) as the potential antioxidant[4–7] and more recently closely related six membered ring compounds based on 2,2,6,6-tetramethyltetrahydropyridine (**2**) have been used.[8]



1 **2**

A related nitroxide (**3**) has been attached to edaravone[9] and a conventional antioxidant 2,6-di-*tert*-butylphenol (**4**) attached to mexiletine.[10] These have been reported to give improved cardioprotection and improved potency in decreasing neuropathic pain respectively.



3 **4** **edaravone**

In most cases the parent amine was used[6–8] but it was shown that conversion to the nitroxide (NO•) and hydroxylamine (NOH) readily occurred during the testing. Halmosi *et al.*[7] showed that the amine substituted mexiletine series of compounds and a related series of phthalimide compounds had similar effects on both thiobarbituric acid reactive substances (TBARS) formation and the % protein oxidation. Thus the N-oxides and *N*-hydroxyl amines of both series of compounds were shown to have no antiarrhythmic effect but to be less toxic than the active amines. Similarly, Hankovsky *et al.*[4] showed that the nitroxides and hydroxylamines prepared from different compounds chemically close to class I antiarrhythmic agents, showed no or decreased antiarrhythmic effects relative to the amino compounds.

Walker *et al.*[9] showed that an edaravone derivative with an isoindoline N-oxide substituent (**3**) significantly attenuated cell death relative to the parent edaravone but a compound modified by attachment of an *N*-OMe containing substituent that cannot function effectively as an antioxidant was inactive.

Attachment of a conventional 2,6-di-*tert*-butylphenol group (**4**) to mexiletine gave a compound which was shown to be approximately 80 times more potent than mexiletine in

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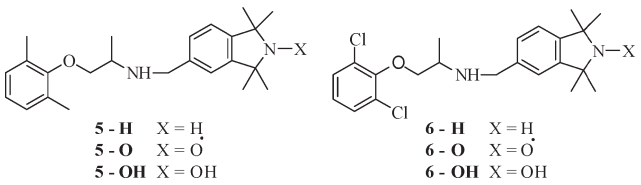
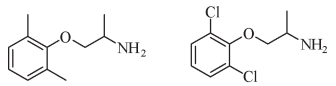
competing with the radioligand ^{3}H-batrachotoxinin for binding to Na^{+} channels in rat brain membrane and to be more effective than mexiletine in attenuating pain behaviour in three different rat models of neuropathic pain.[10]

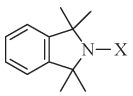
In this paper we report on the effectiveness of the isoindoline-amine (**5-H**), N-oxide (**5-O**) and hydroxylamine (**5-OH**) derivatives of mexiletine and similarly substituted 2,6-dichloro analogues (**6-H**, **6-O** and **6-OH**) as cardioprotective agents. The dichloroanalogues were used as it has been shown that mexiletine is oxidised *in vivo* leading to a hydroxymethyl mexiletine.[11] It has been shown that the hydroxymethyl mexiletine is a much weaker sodium channel blocker than mexiletine[12] and also can be conjugated in the liver to form a glucuronide. The compounds in which the 2,6-methyl groups have been replaced by chloro groups cannot be oxidised, have a similar steric demand and were thus investigated as possible analogues of the mexiletine based compounds.

The results are compared with those obtained using the parent compounds (**7** and **8**). Experiments to assess the potential antioxidant activity were also performed using three commonly used methods, red blood cell (RBC) lysis, lucigenin-enhanced chemiluminescence and L-012-enhanced chemiluminescence.





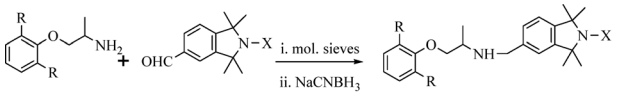
mexiletine (**7**) 2,6-dichlorophenoxy-2-propanamine (**8**) (dichloromexiletine analogue)



The isoindoline derivatives used previously by Walker *et al.*[9] were chosen as they are proposed to be more thermally stable, less reactive, detectable by UV and relatively inert to radical attack at other than the N-containing functions.[13] Thus, it was considered worth evaluating the possible increase in effectiveness of these mexiletine derivatives.

2. Results and discussion

The six modified mexiletine (**5**) and 2,6-dichloroanalogues (**6**) were synthesised using standard methods[14,15] as summarised in Scheme 1 and evaluated as described below.



7 (R = CH_3) Isoindoline (X =H) **5-H, 5-O, 5-OH** (R = CH_3, X = H, O$^{\cdot}$, OH)
8 (R = Cl) Isoindolin-2-yloxyl (X = O$^{\cdot}$) **6-H, 6-O, 6-OH** (R = Cl, X = H, O$^{\cdot}$, OH)

Scheme 1 Synthesis of mexiletine and 2,6-dichlorophenoxy-2-propane derivatives.

2.1 Cardioprotective activity

An *in vitro* ischaemia assay was carried out on cells from a H9c2(2-1) embryonic rat atrial cell line that were incubated at 37 °C for 12 h under 100% N_2 in a hypoxic simulated media as described in the Experimental section. Then propidium iodide was used to stain dead cells (~25–40%) and the concentration of compounds to reduce % cell death was calculated. The H9c2 cell line is a very common experimental tool that provides a reliable and consistent model of cardiac ischaemia, and cardioprotective effects in this cell line correspond very closely to whole heart experiments and also to experiments carried out in ventricular myocytes.[16]

This model showed that four of the mexiletine derivatives had significant cardioprotective properties, as evidenced by a decrease in cardiomyoblast cell death after 12 h. The maximal protective effect of 55.0 ± 5.5% reduction in cell death was observed with compound **5-H** at 5 μM. Compounds **6-O** and **5-OH** were both also active at 5 μM. Mexiletine, had no significant effect at this concentration, indicating that **5-H**, **6-O** and **5-OH** all have greater cardioprotective activity than the parent compound. It should be noted however, that increasing the concentration to 10 μM led to a decrease in effectiveness for these three and most of the other compounds tested and some evidence of toxicity was observed.

The activity of these three compounds shows that there appears to be no effect in replacing the Me groups with Cl in the mexiletine aromatic ring and that the enhanced activity observed with the attached potential antioxidising group was not associated with any specific oxidation level of the amine, amine oxide and hydroxyl amine sequence. The cardioprotective efficacy of some phthalimide derivatives with 2,2,5,5-tetramethyl-3-pyrroline derivatives has been shown to be the most effective when the parent pyrroline derivative was used.[6] Thus the amine was shown to be metabolically oxidised to its nitroxide using *ex vivo* ESR studies and samples of the *N*-O$^{\cdot}$ and *N*-OH compounds were shown to give a significant increase in protection but less than that of the parent amino derivative.[6] Halmosi *et al.*[7] also showed that the parent pyrroline compounds were more effective than their N-oxides in protection against ischaemia-reperfusion myocardial injuries for a wide range of compounds. The results reported by us show that the greater effectiveness of the amino compounds is not generally applicable (Fig. 1).

2.2 Effect on guinea-pig atrial rate and refractory period

The effect of four of the compounds that showed cardioprotective activity in the previous section, (**5-H**, **5-O**, **5-OH**

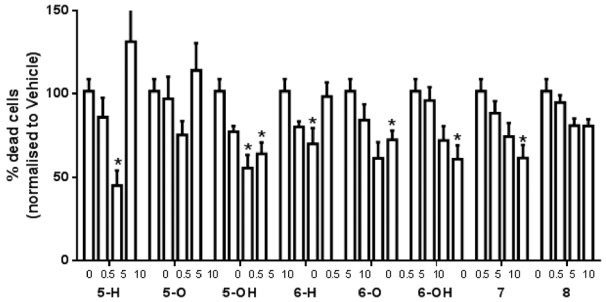


Fig. 1 The effect of mexiletine and its derivatives at concentrations 0–10 μM on cell death in a simulated ischaemia assay in H9c2 cells. Cells were incubated in 100% N_2 for 12 h prior to identification of dead cells using a propidium iodide exclusion assay. Mean ± S.E.M., n = 3–4, * indicates $P < 0.05$.

and **6-O**) on the rate and absolute refractory period (ARP) of guinea pig paced isolated right atria were compared with mexiletine. The resting atrial rate was between 175–200 bpm for the different treatment groups and the baseline refractory period in the absence of drugs was 70–75 ms. Mexiletine and dichloromexiletine analogue **8** had little effect on atrial rate (with only a 15–25 bpm decrease) at 100 μM. In contrast, the corresponding **5-OH**, **5-O**, **5-H** and **6-O** analogues all stopped the spontaneous beating of the atria at 100 μM (Fig. 2a). As shown in Fig. 2b, with a threshold between 1–10 μM, mexiletine and dichloromexiletine analogue (**8**) increased the ARP. The activity of the **5-OH**, **5-O** and **5-H** analogues was not distinguishable from mexiletine. The **6-O** analogue showed less effect on the ARP than the parent analogue **8**. This suggests that mexiletine (**7**) and dichloro compound (**8**), in contrast to their

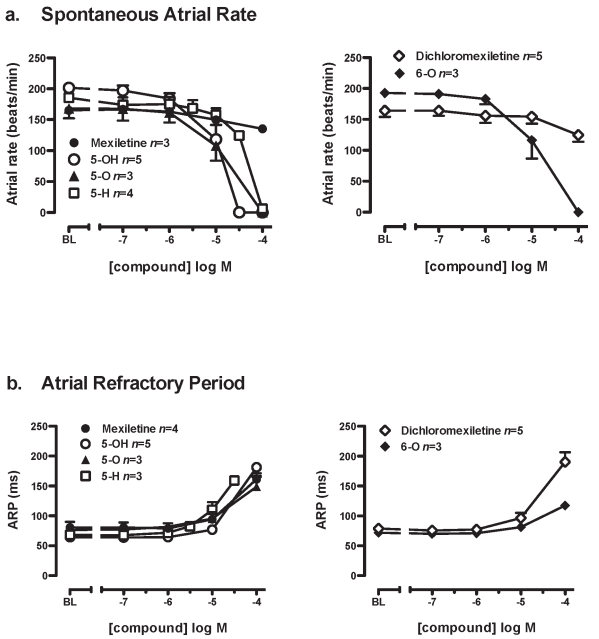


Fig. 2 The effects of mexiletine derivatives on a. spontaneous atrial rate and b. the atrial refractory period (ARP) of guinea pig isolated right atria. Error bars are ± S.E.M.

derivatives, show a selectivity for affecting ARP without affecting sinus rate. None of the analogues were more potent than the parent compounds on papillary muscle refractory period determined in a similar manner (data not shown).

2.3 Estimation of antioxidant activity

The results reported in this paper show no evidence that the parent amines were more effective than the corresponding N-oxides or *N*-hydroxylamines. As discussed above this is in contrast to reports in the literature dealing with related compounds where the parent amine was the most active.[4,6,7] The antioxidant activity of some of the mexiletine derivatives was studied using three independent assays, a L-012 enhanced chemiluminescence assay,[17] a RBC lysis (AAPH-induced haemolysis)[18,19] and a lucigenin-enhanced chemiluminescence assay.[20]

L-012-enhanced chemiluminescence is a well established technique for the detection of reactive oxygen species (ROS) released from tissues and cells. Hence, in the present study, the antioxidant activities of the isoindoline compounds (**9-H**, **O**, **OH**),[21] mexiletine derivatives (**5-H**, **O**, **OH**) and dichloromexiletine derivatives (**6-H**, **O**, **OH**) together with mexiletine (**7**) and dichloromexiletine analogue (**8**) were assessed by examining their ability to quench the L-012 enhanced chemiluminescence signal detected in RAW246.7 cells following stimulation with the NADPH oxidase-activating compound, phorbol 12,13-dibutyrate. Stimulation with phorbol 12,13-dibutyrate caused a marked increase in the L-012-enhanced chemiluminescence signal measured in monolayers of RAW264.7 cells. Importantly, whereas neither the vehicle (1% DMSO), nor the parent compounds mexiletine and dichloromexiletine analogue, had any effect on the L-012-enhanced chemiluminescence signal, the three isoindoline compounds (**9-H**, **O**, **OH**) and the dichloromexiletine derivatives (**6-H**, **O**, **OH**), decreased the signal by 15–35%, suggesting that they were acting as antioxidants in this cell system. The parent amino compounds were more active antioxidants than their oxidised derivatives in agreement with the reported greater activity of amino compounds as cardioprotective agents[4–6] but in contrast to the results reported in our study.

To ensure that the inhibitory effects of the isoindoline (**9-H**, **O**, **OH**) and dichloromexiletine derivatives (**6-H**, **O**, **OH**) on ROS levels were not merely due to cytotoxic effects of the compounds, their impact on cell viability was assessed *via* MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. None of the compounds led to a significant decrease on the ability of RAW264.7 cells to metabolise MTS into its formazan derivative,[22] indicating that they had no effect on cell viability.

None of the isoindoline derivatives (**9-H**, **9-O** and **9-OH**) or mexiletine showed antioxidant activity in the NADPH-stimulated lucigenin assay using rat aorta despite effective activity of the control antioxidant diphenylene iodonium (DPI). Similarly in the AAPH driven red blood cell lysis assay, in which antioxidants provide protection by increasing

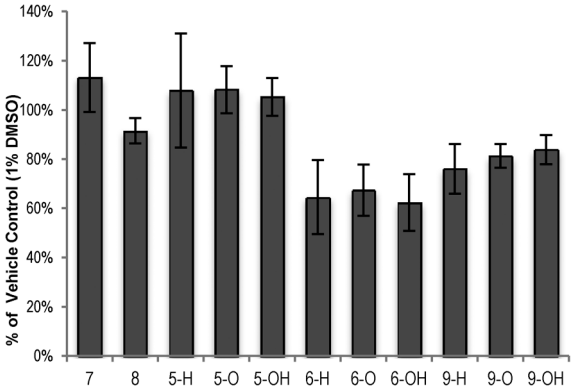


Fig. 3 Effects of mexiletine derivatives on ROS levels in phorbol 12-13-dibutyrate-stimulated RAW264.7 cells measured by L-012-enhanced chemiluminescence (n = 3). Values are expressed as a percent of ROS levels in vehicle (1% DMSO) treated RAW264.7 cells.

the time taken for hemolysis[23] neither the three isoindoles or mexiletine demonstrated any protective effect. These findings were in contrast to the L-012 assay and surprising in that the nitroxide (**9-O**) has been shown to be an effective antioxidant on the basis of its redox potential obtained from cyclic voltammetry.[24] These data indicate the care that needs to be taken in determining antioxidant activity in biological systems (Fig. 3).

Conclusions

In conclusion, some analogues tested showed cardioprotective effect in a cell-based assay but they also showed an effect of slowing atrial rate that was not evident in the parent mexiletine and its dichloroanalogue. Moreover, the results reported, using a variety of assays, demonstrate the difficulties associated with the measurement of antioxidant activity in biological systems. In addition it has been shown that there is no reproducible ranking of activity between amines, their N-oxides and their hydroxylamine derivatives, although previous works have suggested that the amines are usually the most active.[4–6]

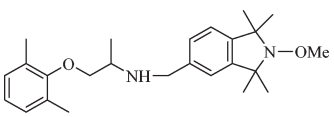
Experimental section

All the animal experiments were performed in compliance with institutional guidelines. The University of Melbourne Animal Ethics Committee approved the experiments requiring the use of animal tissue.

Synthesis of mexiletine derivatives

1-(2,6-Dimethylphenoxy)-*N*-((1,1,3,3-tetramethylisoindolin-5-yl)methyl)propan-2-amine (5-H). 5-Formyl-1,1,3,3-tetramethylisoindoline[14] (245 mg, 1.20 mmol) and 4 Å molecular sieves (300 mg) were added to a solution of (±) mexiletine (216 mg, 1.20 mmol) in dichloromethane (7 mL). The reaction mixture was stirred overnight at ambient temperature under N_2 atmosphere. The molecular sieves were removed and dichloromethane was evaporated. The residue was dissolved in methanol (7 mL) and sodium cyanoborohydride (250 mg, 3.97 mmol) was added. After stirring overnight at ambient temperature under N_2 atmosphere, the reaction mixture was evaporated to dryness. Distilled water (25 mL) was added and extracted with dichloromethane. The organic layer was washed with saturated sodium chloride solution and dried over anhydrous magnesium sulphate. The solvent was evaporated and the residue was purified by chromatography (silica gel, 9 : 1 dichloromethane/methanol) to give **5-H** (340 mg, 77%) as a clear liquid. H^1 NMR ($CDCl_3$, 400 MHz) δ = 7.21 (d, 1H, J = 7.7 Hz, 7″-H), 7.09 (s, 1H, 4″-H), 7.03 (d, 1H, J = 7.7 Hz, 6″-H), 6.94 (d, 2H, J = 7.4 Hz, 3′-H & 5′-H), 6.85 (t, 1H, J = 7.4 Hz, 4′-H), 3.93 (d, 1H, J = 13.0 Hz, $NHCH_2$), 3.81 (d, 1H, J = 13.0 Hz, $NHCH_2$), 3.72–3.69 (m, 2H, OCH_2), 3.18 (m, 1H, CH), 3.15 (bs, 2H, NH), 2.22 (s, 6H, 2 × $PhCH_3$), 1.45 (s, 6H, $C(CH_3)_2$), 1.44 (s, 6H, $C(CH_3)_2$), 1.15 (d, 3H, J = 6.4 Hz, CH_3). C^{13} NMR ($CDCl_3$, 100 MHz) δ = 155.31, 148.16, 148.09, 146.69, 139.35, 130.49, 128.73, 127.34, 123.71, 121.26, 120.86, 75.43, 63.16, 63.05, 52.72, 51.04, 49.66, 31.24, 31.21, 16.70, 16.12, 16.08. MS (ESI): m/z: 367 $(M + H)^+$, 389 $(M + Na)^+$. HRMS (ESI): m/z calc. for $C_{24}H_{35}N_2O^+$ $(M + H)^+$ 367.2749; found 367.2731. (Found: C, 76.98; H, 9.79; N, 7.54. Calc. for $C_{24}H_{34}N_2O{\cdot}1/2H_2O$: C, 76.76; H, 9.39; N, 7.46%).

5-(((1-(2,6-Dimethylphenoxy)propan-2-yl)amino)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl (5-O). Using the same procedure the **5-O** compound was prepared from 5-formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl[14] and mexiletine. **5-O** was obtained as a thick liquid (320 mg, 94%). MS (ESI): m/z: 382.3 $(M + H)^+$. HRMS (ESI): m/z calc. for $C_{24}H_{34}N_2O_2^+$ $(M + H)^+$ 382.2620; found 382.2636. (Found: C, 75.47; H, 8.67; N, 7.50. Calc. for $C_{24}H_{33}N_2O_2$ C, 75.55; H, 8.72; N, 7.34%). The nitroxide was converted to methoxy amine **10** according to Bottle[25] for NMR studies.



10

5-(((1-(2,6-Dimethylphenoxy)propan-2-yl)amino)methyl)-2-methoxy-1,1,3,3-tetramethylisoindoline (10). H^1 NMR ($CDCl_3$, 200 MHz) δ = 7.18–7.00 (m, 3H, Ph), 6.96–6.82 (m, 3H, Ph), 3.82 (q AB, 2H, J = 13.3 Hz, $NHCH_2$), 3.70 (s, 3H, OCH_3), 3.75–3.64 (m, 2H, OCH_2), 3.42–3.10 (m, 1H, CH), 2.32 (bs, 1H, NH), 2.19 (s, 6H, 2 × $PhCH_3$), 1.35 (s, 12H, 2 × $C(CH_3)_2$), 1.16 (d, 3H, J = 6.42 Hz, CH_3). MS (ESI): m/z 397.3 $(M + H)^+$.

5-(((1-(2,6-Dimethylphenoxy)propan-2-yl)amino)methyl)-1,1,3,3-tetramethylisoindolin-2-ol (5-OH). **5-O** was dissolved in absolute ethanol and dry HCl gas was bubbled for 5 min. The resulted solution was refluxed until the colour of the nitroxide disappeared (30 min) and evaporated to give a white solid. MS (ESI): m/z: 383.4 $(M + H)^+$. HRMS (ESI): m/z calc. for $C_{24}H_{35}N_2O_2^+$ $(M + H)^+$ 383.2699; found 383.2691.

Synthesis of 1-(2,6-dichlorophenoxy)propane-2-amine (8)

1-(2,6-Dichlorophenoxy)propane-2-amine (**8**)[15] was prepared from 2,6-dichlorophenol following a reaction scheme similar to preparation of mexiletine. H^1 NMR ($CDCl_3$, 300 MHz) δ = 7.13 (d, 2H, J = 8.0 Hz, 3-H & 5-H), 6.82 (t, 1H, J = 8.0 Hz, 4-H), 3.81 (m, 1H, CH_2), 3.61 (m, 1H, CH_2), 3.28 (m, 1H, CH), 1.54 (bs, 2H, NH_2), 1.03 (d, 3H, J = 6.6 Hz, CH_3). C^{13} NMR ($CDCl_3$, 75 MHz) δ 151.07, 129.07, 128.66, 124.68, 79.48, 46.81, 19.20. MS (ESI): m/z 220.1, 222.1.

Synthesis of dichloromexiletine analogues

1-(2,6-Dichlorophenoxy)-*N*-((1,1,3,3-tetramethylisoindolin-5-yl)methyl)propan-2-amine (6-H). **6-H** compound was prepared from 5-formyl-1,1,3,3-tetramethylisoindoline[14] and 1-(2,6-dichlorophenoxy)propane-2-amine. **6-H** was obtained as a thick liquid (218 mg, 60%). H^1 NMR ($CDCl_3$, 300 MHz) δ = 7.28 (d, 2H, J = 8.0 Hz, 3′-H & 5′-H), 7.26 (d, 1H, J = 7.0 Hz, 7″-H), 7.15 (s, 1H, 4″-H), 7.07 (d, 1H, J = 7.0 Hz, 6″-H), 6.97 (t, 1H, J = 8.0 Hz, 4′-H), 4.06 (dd, 1H, J = 8.8, 4.0 Hz, $NHCH_2$), 3.95–3.78 (m, 3H, $NHCH_2$ & OCH_2), 3.24 (m, 1H, CH), 1.50 (s, 6H, 2 × CH_3), 1.49 (s, 6H, 2 × CH_3), 1.23 (d, J = 6.5 Hz, 3H, CH_3), 1.19 (bs, 2H, NH). C^{13} NMR ($CDCl_3$, 75 MHz) δ = 151.19, 148.24, 146.70, 139.73, 129.30, 128.90, 127.36, 124.98, 121.29, 120.91, 76.67, 63.29, 63.15, 52.63, 51.13, 31.53, 17.05. MS (ESI): m/z: 407.2 $(M + H)^+$. HRMS (ESI): m/z calc. for $C_{22}H_{29}N_2OCl_2^+$ $(M + H)^+$ 407.1657; found 407.1657 (found: C, 63.99; H, 6.99; N, 6.84; Cl, 18.58. Calc. for $C_{22}H_{28}N_2OCl_2$: C, 64.86; H, 6.93; N, 6.88; Cl, 17.41%).

5-(((1-(2,6-Dichlorophenoxy)propan-2-yl)amino)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl (6-O). **6-O** compound was prepared from 5-formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl[14] and 1-(2,6-dichlorophenoxy)propane-2-amine. **6-O** was obtained as a thick liquid. (471 mg, 86%) MS (ESI): m/z: 422.1 $(M + H)^+$. HRMS (ESI): m/z calc. for $C_{22}H_{28}N_2O_2Cl_2^+$ $(M + H)^+$ 422.1528; found 411.1531 (found: C, 62.33; H, 6.74; N, 6.51; Cl, 16.99. Calc. for $C_{22}H_{27}N_2O_2Cl_2$: C, 62.56; H, 6.44; N, 6.63; Cl, 16.79%).

Compound **6-OH** was prepared similar to 5-OH. HRMS (ESI): m/z calc. for $C_{22}H_{29}N_2O_2Cl_2^+$ $(M + H)^+$ 423.1606, 425.1577; found 423.1599.

Cell culture and *in vitro* ischaemia model

The H9c2(2-1) embryonic rat atrial cell line (American Type Culture Collection-ATCC, Manassas, VA, USA) was used for this study. The cell line was grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamate, 4.5 g L^{-1} glucose, 3.7 g L^{-1} sodium bicarbonate, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin supplemented with 10% fetal bovine serum (Invitrogen, Mount Waverley, VIC, Australia) in a 5% CO_2 incubator. Cells were used at 60–70% confluence and plated one day prior to assay at 40 000 cells per well of 96 well plate. Simulated ischaemia was induced using conditions developed in our laboratory and described previously.[9] In short, ischaemia was achieved by incubating the cells in hypoxic simulated ischaemia (SI) medium at pH 6.4 containing (in mM): 137 NaCl, 3.5 KCl, 0.88 $CaCl_2{\cdot}2H_2O$, 0.51 $MgSO_4{\cdot}7H_2O$, 5.55 D-glucose, 4 HEPES, 10 2-deoxy-D-glucose and 20 DL-lactic acid (Sigma, Castle Hill, NSW, Australia) plus 2% fetal bovine serum. Cells were incubated under nitrogen (100% N_2 gas atmosphere) for 12 h at 37 °C. Fresh simulated ischaemia medium was prepared for each experiment and sterile-filtered prior to experimentation. Mexiletine and derivatives were dissolved in SI buffer and added to the wells at concentrations between 0.5 and 20 μM. Each treatment was repeated in 4 independent assays performed in triplicate wells.

Cell viability (PI) assay and imaging of H9C2(2-1) cells

Detection of non-viable cells resulted from ischaemia was achieved by propidium iodide (PI) assay. Following 12 h simulated ischaemia, cells were first washed with PBS and stained with 5 μM PI (Sigma) for 5 min, followed by a PBS rinse twice prior to imaging. Images were taken using a confocal microscope (Nikon A1; Nikon Instruments, Tokyo, Japan) using a 561 nm excitation laser. The simulated ischaemia assay was repeated in at least 3 different passages. PI-positive cells were quantified using ImageJ (NIH Image; National Institute of Health, USA). The normalised dead cell percentage was calculated by dividing the number of PI-positive cells per well by the average number of PI-positive cells in the simulated ischaemia-treated wells for that experiment.

Statistical analysis

The effects of mexiletine and analogues on cardiomyoblast cell death during hypoxia were determined using a two-way analysis of variance (ANOVA), with one factor being antioxidant type and one factor being concentration. Identification of individual group-to-group differences was performed using Bonferroni *post hoc* analysis.

Effect on spontaneous atrial rate and atrial refractory period

Guinea-pigs of either sex were humanely killed in accordance with the *Australian code for the care and use of animals for scientific purposes* (8th edition, 2013, National Health and Medical Research Council, Canberra). The heart was rapidly removed and the right atrium dissected free from the ventricles and surrounding tissues. Atria were set up between two field electrodes to measure the electrogram and a punctate electrode was used to pace each atrium when required for determination of atrial refractory period as described by Angus & Harvey.[26] Atrial rate and ECG were monitored using LabChart 7.0 Pro (AD Instruments, Sydney, Australia) and allowed to stabilise during a 60 min equilibration period. Atrial rate was derived from the phasic force signal. The refractory period was determined by using a Grass S88 stimulator to deliver a single pulse at varying time intervals following a train of 7 pulses (0.2 ms duration at 150% threshold voltage) at 5 Hz. The average of three intervals required to deliver an incomplete beat was taken as the atrial refractory period. These tests were repeated at 30 min intervals in the presence of increasing concentration of drugs. Atria were

allowed to contract spontaneously when not being paced to determine the atrial refractory period.

L012-enhanced chemiluminescence

RAW264.7 cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% foetal bovine serum in clear-bottomed, white-walled, 96 well viewplates. At the time of assay, the culture medium was replaced with HEPES-buffered (pH 7.4) Krebs solution ± the compounds (**5-H**, **O**, **OH**), (**6-H**, **O**, **OH**), (**9-H**, **O**, **OH**), **7** and **8** (all at a concentration of 100 μM) or their vehicle (1% DMSO). The 96 well plate was then loaded into a Hidex Chameleon Luminescence Microplate Reader (Hidex, Denmark) and background photon counts in each well were monitored over a 30 min period (*i.e.* count time of 1 s per well, 10 count cycles, 3 min interval between each cycle). Next, L-012 was added directly to each well at final concentrations of 100 μM and photon counting was resumed for a further 30 min to monitor basal superoxide production by the cells. Finally, NADPH oxidase activity was stimulated in RAW264.7 cells by the direct addition of phorbol-12-13-dibutyrate (final concentration 10 μM). Photon emission, as a measure of ROS levels, was then monitored over a 60 min period. Upon completion of ROS measurements, RAW264.7 cells were washed with Krebs–HEPES and incubated for 3 h in 20% CellTiter 96R AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA) dissolved in Krebs–HEPES. Cell viability was assessed with this MTS-based assay by measuring the absorbance of the supernatant at 490 nm.

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