Synthesis, antioxidant properties and neuroprotection of α-phenyl-tert-butyl nitronate derived HomoBisNitrones in in vitro and in vivo ischemia models

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We herein report the synthesis, antioxidant power and neuroprotective properties of nine homo-bis-nitrones HBNs 1–9 as alpha-phenyl-N-tert-butyl nitronate (PBN) analogues for stroke therapy. In vitro neuroprotection studies of HBNs 1–9 against Oligomycin A/Rotenone and in an oxygen-glucose-deprivation model of ischemia in human neuroblastoma cell cultures, indicate that (1Z,1′Z)-1,1′-(1,3-phenylene)bis(N-benzylmethanimine oxide) (HBN6) is a potent neuroprotective agent that prevents the decrease in neuronal metabolic activity (EC50 = 1.24 ± 0.39 μM) as well as necrotic and apoptotic cell death. HBN6 shows strong hydroxyl radical scavenger power (81%), and capacity to decrease superoxide production in human neuroblastoma cell cultures (maximal activity = 95.8 ± 3.6%), values significantly superior to the neuroprotective and antioxidant properties of the parent PBN. The higher neuroprotective ability of HBN6 has been rationalized by means of Density Functional Theory calculations. Calculated physicochemical and ADME properties confirmed HBN6 as a hit-agent showing suitable drug-like properties. Finally, the contribution of HBN6 to brain damage prevention was confirmed in a permanent MCAO setting by assessing infarct volume outcome 48 h after stroke in drug administered experimental animals, which provides evidence of a significant reduction of the brain lesion size and strongly suggests that HBN6 is a potential neuroprotective agent against stroke.

Bis-nitrones are well-known antioxidant and neuroprotective agents showing high clinical potential. For instance, bis-nitronate W-AZN (Fig. 1), an azulenyl spin trap possessing neuroprotective effects in an animal model of

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cerebral ischemia, is able to attenuate the in vivo MPTP neurotoxicity, suggesting a possible application in the treatment of Parkinson’s disease. Similarly, the neuroprotective capacity of bis-nitrone STAZN, a second-generation of potent antioxidant azulenyl nitrone, has been confirmed in focal ischemia models. Bis-nitrone TN-2 also exhibits a high neuroprotective effect in either in vitro or in vivo models of stroke, very likely as a consequence of its ability to trap HO· and O2−, two of the most reactive oxygen species (ROS) to brain tissues.

Despite ROS play key roles in physiological functions at low concentrations, they can also be very toxic in highly oxidative stress dysregulated conditions. For instance, hydroperoxides formed in the reaction of ROS with unsaturated fatty acids are very reactive, and constitute the origin of extensive cell death. Nowadays, it is widely accepted that the formation of ROS is one of the main biological events involved in the etiology of stroke. For this reason, there exists a growing interest in the search for new and more efficient ROS scavengers as potential therapeutic agents for stroke.

In our current program targeted to identify new nitrones for the therapy of stroke, we have already investigated nitrones derived from (hetero)aromatic aldehydes, quinolinynitrones, and cholesteronitrones. More recently, we have designed bis-nitrones derived from alpha-phenyl-N-tert-butylnitrone (PBN) (Fig. 1), a well-known radical scavenger that prevented and reversed traumatic shock injury in rats, and the starting point of several new nitrones, such as NXY-059, the first nitrone to reach clinical trials. As shown in Fig. 1, homo-bis-nitrones (HBNs) result from the incorporation of a second identical nitrone moiety at the para (p-HBNs 1–3), meta (m-HBNs 4–6) and ortho (o-HBNs 7–9) positions and bearing methyl, tert-butyl or benzyl substituents, respectively, as the N-alkyl groups attached to the nitrone motif. Among these nitrones, only HBNs 1, 2, 3, 4, and 5 have been previously described in the literature, but in studies not related to their antioxidant properties and/or potential use for stroke therapy. In fact, the present work is the first study aimed at exploring the neuroprotective and antioxidant properties of bis-nitrones, analogues of the parent PBN. The hypothesis behind the present design is that two “nitrone” scavenging motifs in the same scaffold should afford a higher antioxidant power than only one. Indeed, as it will be shown later on, we have identified (1Z,1′Z)-1,1′-(1,3-phenylene)bis(N-benzylmethanimine oxide) (HBN6) as a potent neuroprotective ligand (EC50 = 1.24 ± 0.39 µM), whose neuroprotective and antioxidant capacities are higher than those of PBN.

Results and discussion

Chemistry. The synthesis of HBNs 1–9 (Scheme 1) was achieved from commercial and readily available carbaldehyde precursors (terephthalaldehyde, isophthalaldehyde, and phthalaldehyde) and the appropriate N-methyl(--butyl, benzyl)hydroxylamine hydrochlorides (Methods and Supplementary Information). All compounds were isolated as pure Z isomers at the double PhC=N(O)R bond, and exhibited analytical and spectroscopic data in agreement to those previously described.

Neuroprotection studies

Oligomycin A/rotenone. One of the first events taking place in the initial stages of stroke is the collapse of the mitochondrial electron transport chain (ETC), which leads to extended cell death and brain damage due to the formation of ROS. In order to mimic this event into suitable experiments, we tested the effect of the bis-nitrones on cell death induced by Oligomycin A and Rotenone (O/R), inhibitors of mitochondrial complexes V and I, respectively. To this end, we used the XTT cell viability test, a colorimetric assay that detects the cellular metabolic activities. Based on a previous work from our laboratory, we selected the appropriate experimen-
tal conditions and tested the neuroprotective effect of HBNs 1–9 at different concentrations (0.1–1,000 μM), added 10 min before the administration of O10 μM/R30 μM (O/R), and using PBN, at the same concentrations (0.1–1,000 μM), as a reference compound28.

As shown in Fig. 2, a 42.31 ± 4.43% (mean ± SEM) inhibition of neuroblastoma cells viability was observed upon treatment with O10/R30 for 24 h. This effect was reverted after incubation with PBN and HBNs 1–9 for 24 h in a concentration-dependent manner (Fig. 2). The neuroprotection study, considering the 100% neuroprotection as the difference between C24 h viability (100 ± 4.75%; mean ± SEM; n = 20) and OR (57.69 ± 10.46; mean ± SEM; n = 16) revealed that the most potent nitrones were HBNs 4–6. Table 1 gathers the analyses of concentration–response curves for HBNs 1–9 and PBN, in the range of 0.1 μM to 1 mM, the corresponding EC\textsubscript{50} values, and the highest neuroprotective activities. EC\textsubscript{50} values, from the lowest to the highest, follows the order: HBN5 ≤ NAC ≤ HBN6 ≤ HBN4 ≤ HBN3 ≤ HBN2 << HBN9 < HBN8 ≤ HBN1 ≤ PBN << HBN7.

As the highest neuroprotective activity (maximal activities) was similar in all cases, we can conclude, by regarding the EC\textsubscript{50} values, that HBNs 4–6 bearing the nitrene motifs in meta position gave the best neuroprotection, followed by HBNs 2–3 bearing the nitrene motifs in para position, and HBNs 7–9 bearing the nitrene motifs in ortho position. The high neuroprotection observed for HBNs 4–6 exceeds that of the parent PBN and is very similar to that of N-acetyl-L-cysteine (NAC) (EC\textsubscript{50} = 5.16 ± 1.60 μM). From the structure–activity

**Scheme 1.** Synthesis of HBNs 1–9.

**Figure 2.** Neuroprotective effect of HBNs 1–9 on SH-SYSY human neuroblastoma cells viability after treatment with O/R. Bars show % cell viability after treatment with O10/R30, with, or without, HBNs 1–9 and PBN, at the indicated concentrations. Values are the mean ± SEM of three experiments, each one performed in triplicate. The statistics compare the effect of OR on its control (C) (red ***), or the effect of the different compounds after O/R (24 h) with O/R (24 h) alone, in the absence of these compounds (black **). Data were statistically analyzed by one-way ANOVA, followed by Holm-Sidak as test post hoc. *P < 0.05; **P < 0.01; ***P < 0.001.
relationship (SAR) point of view, note that, among the bis-nitrones of the same group, HBNs having a benzyl or tert-butyl group at the nitrogen atom of the nitrone motif systematically afforded a higher neuroprotection.

Neuroprotection analysis in an OGD model. Next, the neuroprotective effect of HBNs 1–9 was evaluated in an in vitro oxygen glucose deprivation (OGD) model, followed by ischemic reperfusion (IR)\(^9\). Tested compound concentrations ranged from 0.01 to 1,000 \(\mu M\), after IR. After OGD (I) (4 h), a loss of metabolic activity between 50–80% was observed, showing a small cell recovery after 24 h reperfusion (IR) of 38% to 61% (49.29 ± 3.26; mean ± SEM; \(n = 16\)).

HBNs 1–9 (Fig. 3) were able to partially or even totally reverse the cell loss of metabolic activity induced by IR, in a concentration-dependent manner. These data revealed that HBN5 and HBN6 were the most potent bis-nitrones. Among HBNs 1–3, HBNs 2 and 3 provided 60% neuroprotection, regardless of the dose. Furthermore, HBNs 7–9 afforded a concentration–response curve, with the best neuroprotection reached in the 25–100 \(\mu M\) range. Strikingly, HBN9 showed high neuroprotection, in the same range than HBN5 and HBN6. To sum up, in the OGD experiment, HBN5 and HBN6 showed the best neuroprotective profile, in good agreement with the results observed with the inhibitors of the mitochondrial ETC.

Based on these encouraging results, we have determined the \(EC_{50}\) and the highest neuroprotective activities for HBNs 1–9 comparing them with that of PBN and NAC. As shown in Table 2, the \(EC_{50}\) values, from the lowest to the highest neuroprotective nitrone, follows the order: HBN3 ≤ HBN6 ≤ HBN5 < HBN9. However,
and based on the observed highest neuroprotective activity (maximal activities), this order was as follows: HBN3 < HBN6 ≤ HBN 5 ≤ HBN 9. Then, given that HBN3, despite having the lowest EC50, has a low maximal activity, we could conclude that the neuroprotective capacity of HBN3 is similar to that of HBN9, a compound that shows a high maximal neuroprotective activity at a higher EC50 and that both HBNs have lower overall neuroprotective capacity than HBN6 and HBN5, both with a low EC50 and a high maximal neuroprotective capacity.

From the SAR point of view, note that: (1) the best neuroprotective HBNs 3, 6 and 9 bear a benzyl group at the nitrogen atom of the nitrone motif, (2) HBN9 bears the two nitrone motifs in an ortho arrangement at the aromatic ring, and (3) the meta relative position of nitrones, present in HBN5 and HBN6, is the preferred arrangement to provide an effective neuroprotection. Moreover, the neuroprotection afforded by HBN5 and HBN6 is very similar to that of NAC (EC50 = 2.58 ± 0.91 μM).

Table 2. Neuroprotective effect of HBNs 1–9, PBN and NAC after OGD-IR in human neuroblastoma SH-SY5Y cells. The estimation of EC50 (in μM) and maximal activities (in % neuroprotection) values were performed by a weighted nonlinear regression of minimum squares using logistic curves, as is described in the "Statistical Analysis" section of "Neuroprotection Assessment Assays". Values are the mean ± S.E.M. Data analysis was carried out with SigmaPlot v.12., and ANOVA one-way to get the significant statistics of HBNs respect to PBN, or to HBN6. Differences are statistically significant when P ≤ 0.05. EC50 and maximal activities were calculated from the data obtained from three experiments, each one in triplicate. The statistics compares differences with PBN or HBN6 at *P < 0.05, **P < 0.01 and ***P < 0.001 (one-way ANOVA, followed by Holm–Sidak analysis as a post hoc test.

| HBN | R       | Neuroprotection (EC50 ± SEM), μM | P < (PBN) | P < (HBN6) | Maximal Activity (Mean ± SEM), % | P < (PBN) | P < (HBN6) |
|-----|---------|---------------------------------|-----------|------------|----------------------------------|-----------|------------|
| 1   | Me      | 227.07 ± 15.92                  | ***       | ***        | 68.55 ± 3.96                     | *         | ***        |
| 2   | tert-Bu | 45.09 ± 4.73                    | ns        | ***        | 77.82 ± 2.46                     | ns        | ***        |
| 3   | Bn      | 0.78 ± 0.09                      | **        | ns         | 69.01 ± 0.87                     | *         | ***        |
| 4   | Me      | 38.85 ± 4.05                    | ns        | **         | 78.21 ± 2.23                     | *         | ***        |
| 5   | tert-Bu | 1.70 ± 0.18                     | **        | ns         | 113.31 ± 4.69                    | ***        | ns         |
| 6   | Bn      | 1.24 ± 0.23                     | **        | ns         | 104.07 ± 3.06                    | **        | **         |
| 7   | Me      | 20.36 ± 1.21                    | ns        | *          | 68.28 ± 2.13                     | **        | ***        |
| 8   | tert-Bu | 20.58 ± 4.66                    | ns        | *          | 55.43 ± 3.49                     | ***        | ***        |
| 9   | Bn      | 10.14 ± 0.66                    | *         | ns         | 120.41 ± 6.63                    | ***        | ns         |
| PBN | –       | 42.01 ± 5.41                    | –         | **         | 82.54 ± 6.23                     | –         | **         |
| NAC | –       | 2.58 ± 0.91                     | **        | ns         | 110.30 ± 2.81                    | **        | ns         |

Figure 4. Effect of HBNs 1–9 on the LDH release in SH-SY5Y cells after IR. Bars show % LDH release after OGD (4 h) and IR (24 h), without treatment (IR 24 h) or treated with HBNs1–9, PBN and NAC, at the indicated concentrations. Values are the mean ± SEM of three experiments, each one performed in triplicate, and compare the effect of OGD and IR on respective controls, C4h and C24h, respectively (red *** or the effect of the different compounds after IR (24 h) with IR (24 h) in the absence of these compounds (black ***). Data were statistically analyzed by one-way ANOVA, followed by Holm–Sidak as test post hoc. *P < 0.05; **P < 0.01; and ***P < 0.001.

and based on the observed highest neuroprotective activity (maximal activities), this order was as follows: HBN3 < HBN6 ≤ HBN 5 ≤ HBN 9. Then, given that HBN3, despite having the lowest EC50, has a low maximal activity, we could conclude that the neuroprotective capacity of HBN3 is similar to that of HBN9, a compound that shows a high maximal neuroprotective activity at a higher EC50 and that both HBNs have lower overall neuroprotective capacity than HBN6 and HBN5, both with a low EC50 and a high maximal neuroprotective capacity.

From the SAR point of view, note that: (1) the best neuroprotective HBNs 3, 6 and 9 bear a benzyl group at the nitrogen atom of the nitrone motif, (2) HBN9 bears the two nitrone motifs in an ortho arrangement at the aromatic ring, and (3) the meta relative position of nitrones, present in HBN5 and HBN6, is the preferred arrangement to provide an effective neuroprotection. Moreover, the neuroprotection afforded by HBN5 and HBN6 is very similar to that of NAC (EC50 = 2.58 ± 0.91 μM).

Effect of HBNs on necrotic and apoptotic cell death induced by OGD. During an ischemic stroke, there is massive cell death due to necrosis, and, as a consequence, the plasma membrane is broken or significantly permeabilized[25]. Under these circumstances, lactate dehydrogenase (LDH), a soluble cytosolic enzyme, easily crosses the damaged membrane, and for this reason, it is possible to determine the extent of the cell necro-
sis taking place in the OGD experiment by comparing its extracellular to its intracellular activity. As shown in Fig. 4, from the values obtained from the measurement of the LDH release after OGD for 4 h, followed by 24 h reperfusion (IR) on neuroblastoma cells, by adding HBNs 1–9 at 1–500 µM concentrations (PBN and NAC as the reference compounds), we concluded that all HBNs, with the exception of HBN3, PBN and NAC, significantly decreased the release of LDH, reaching 100% of the LDH activity inhibition (Fig. 4).

HBNs 1–3 were, in general, less potent than HBNs 4–6, whereas HBN8 and HBN9 were the most efficient bis-nitrones (Fig. 4). Despite that, HBNs 1–9 exhibited a rather similar inhibitory potency of LDH activity than PBN and NAC.

Next, and in order to evaluate the extent of cell death by apoptosis, we determined the caspase-3 activity, by using DEVD-AMC as a substrate, which affords fluorescent AMC upon hydrolysis. So, after OGD (4 h), and adding HBNs 1–9, PBN and NAC, at 1–250 µM concentration doses, followed by IR (24 h), the cells were lysated, DEVD-AMC was added, and the fluorescence measured. As shown in Fig. 5, it can be concluded that, in general, the tested compounds protect less efficiently from the apoptotic than from necrotic cell death. Among the para-HBNs, HBN3 was the best agent, as the caspase-3 activity was reduced at 10 µM dose. HBN5, HBN6, HBN8 and HBN9 showed also potent antiapoptotic activity, being ortho-HBN8 and HBN9, which bear tert-Bu and Bn substituents, respectively, more potent than the corresponding meta-HBN5 and HBN6. Both, the antiapoptotic and antinecrotic effects of the most potent HBNs (HBN8, HBN9, HBN5 and HBN6), were very similar to those found for NAC. However, the antiapoptotic effect of HBNs 1–9 was greater than that of PBN, despite the fact that they have a similar anti-necrotic effect to this base nitrone.

**Basal neurotoxicity of HBNs.** Due to the observed decrease of the neuroprotective effect by HBNs 4–6 (Fig. 3) or the effect on the LDH release by HBN3 (Fig. 4) at the highest concentrations tested (250–1,000 µM), the possible neurotoxicity of HBNs was investigated. The experiments were carried out by measuring the cell viability with XTT, but without adding any toxic insult. As shown in Fig. 6, none of the HBNs, at 250–1,000 µM doses, with the exception of HBN3, (62.76 ± 10.57% cell viability at 1 mM; P<0.001 versus 100% C), HBN4...
Antioxidant capacity of HBNs 1–9: production and scavenging of radical superoxide in human neuroblastoma SH-SY5Y cells. The results shown in the previous sections prompted us to investigate whether the observed neuroprotection was a consequence of their capacity to act as antioxidants and ROS scavengers, particularly of superoxide radical anion (O$_2^-$). O$_2^-$ detection was carried out by using dihydroethidium (DHE), after OGD (3 h) and IR (3 h), with or without HBNs 1–9, including PBN and NAC as standards. Compound concentrations from 0.1 to 1,000 μM were tested, after IR. As shown in Fig. 7A, ROS level production after IR (1.46 ± 0.19 UAF/min/150,000 cells; mean ± SEM; n = 16) was higher (P < 0.05, one way Anova test) than ROS production after OGD alone (1.14 ± 0.07 UAF/min/150,000 cells mean ± SEM; n = 16). As expected, HBNs 1–9 were able to partially or totally reverse the increase in ROS levels induced by IR, in a concentration-dependent manner (Fig. 7A). A concentration–response curves and calculations of EC$_{50}$ and the highest antioxidant activities for HBNs 1–9 and PBN (a graphic example is presented for HBN6 in Fig. 7C), is shown in Table 3. The EC$_{50}$ values, from the lowest to the highest, follows the order: NAC ≤ HBN 4 ≤ PBN ≤ HBN6 ≤ HBN 5 ≤ HBN2 ≤ HBN1 < HBN 9 < HBN8 < HBN7. As the highest neuroprotective activity (maximal activities) was lower for HBNs 1–4 and higher for HBNs 5–6 and HBN8–9, we conclude that, regarding the antioxidant capacity against IR-induced superoxide production, m-HBNs 4–6 exhibit the best antioxidant properties followed by p-HBNs 1–3, whose effect is very similar to that of PBN. However, the fact that o-HBN8 and o-HBN9 have higher maximal activity than the other HBNs, despite their higher EC$_{50}$ makes them highly antioxidant nitrones with a similar activity to those of HBNs 1–3, which, despite their lower EC$_{50}$ exhibit the lowest maximal activity.

In summary, and from the SAR point of view, once again HBN5 and HBN6, bearing tert-Bu and Bn, substituents, respectively, were confirmed to be the most potent bis-nitrones of the entire series. Finally, although the antioxidant effects of HBN5 and HBN6 were very similar to that of PBN, the fact that PBN has a lower maximum antioxidant activity, led us to conclude that both nitrones exceed PBN as ROS scavengers.
Finally, to examine whether the antioxidant effect of HBNs could be responsible for their neuroprotective effect, we performed a linear correlation analysis of neuroprotective power (Fig. 7B) versus the antioxidant capacity (Fig. 7C), as shown in Fig. 7D for HBN6. In all cases, there was a very significant correlation between both effects, with Pearson correlation coefficients (r) ranging from 0.807 to 0.983, and with a statistical significance > 0.001 in the case of nitrones with tert-Bu and Bn substituents and < 0.01 or < 0.05 for HBNs with Me substituent and PBN (data not shown).

To sum up, it becomes clear that the results of the ROS trapping experiment are in good agreement with the neuroprotection analyses and firmly confirm that HBN5 and HBN6 are the best and most balanced bis-nitrones of the studied series in terms of neuroprotection (OGD plus IR, and O/R) and antioxidant power. In addition, the antioxidant power of HBN5 and HBN6 is very similar to the antioxidant power of NAC (EC50 = 3.23 ± 0.35 μM).

Based on the neuroprotection results, we have also investigated the antioxidant power analysis of HBNs 5–9 on diverse antioxidant tests, using PBN, NDGA and Trolox as standards for comparative purposes.

Antioxidant tests. As shown in Table 4, HBN9 was able to inhibit 80% lipid peroxidation (LP), in the same range as Trolox (88%), in the same experiment, as well as lipooxygenase (LOX) (85 μM) and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS+•) (23%), albeit in a poorer extent than NDGA (0.45 μM), and Trolox (91%), respectively. Note also that HBN6 was the most potent hydroxyl radical scavenger (81%), overcoming HBN5 and HBN9, and in the same range that Trolox (83%). Finally, compared to PBN, HBN5, HBN6 and

| Table 3. Antioxidant effect of HBNs 1–9, PBN and NAC after OGD-IR in human neuroblastoma SH-SY5Y cells. The estimation of EC50 (in μM) and maximal activities (in % neuroprotection) values were performed by a weighted nonlinear regression of minimum squares using logistic curves, as is described in the "Statistical Analysis" section of "Neuroprotection Assays." Values are the mean ± S.E.M. Data analysis was carried out with SigmaPlot v.12., and ANOVA one-way to get the significant statistics of HBNs 1–9 respect to PBN, or to HBN6. Differences are statistically significant when P ≤ 0.05. EC50 and Maximal Activities were calculated from the data obtained from three experiments, each one in triplicate. The statistics compares differences with PBN or HBN6 at *P < 0.05, **P < 0.01 and ***P < 0.001 (one-way ANOVA, followed by Holm–Sidak analysis as a post hoc test.

| HBNs/standards | ClogP | ILPO (%) | LOX inhibition (IC50 [μM]/%) | Scav. activity for OH (%) | ABTS+• (%) |
|----------------|-------|----------|-----------------------------|--------------------------|-------------|
| PBN            | 3.02  | 11       | 23                          | no                       | 5           |
| HBN5           | 4.51  | 55       | 6                           | 67                       | no          |
| HBN6           | 4.96  | 37       | 29                          | 81                       | no          |
| HBN7           | 2.56  | 64       | 60 μM                       | 29                       | 22          |
| HBN8           | 0.61  | 46       | 57.5 μM                     | 59                       | 4           |
| HBN9           | 4.95  | 80       | 85 μM                       | 16                       | 23          |
| NDGA           | nd    | nd       | 0.45 μM                     | nd                       | nd          |
| Trolox         | nd    | 88       | nd                          | 83                       | 91          |

| Table 4. Antioxidant activity of HBNs 5–9, PBN, Trolox and NDGA. Bold is for emphasis Nitrones tested at 100 μM; Values are means of three or four different determinations. No, no activity under the experimental conditions. Means within each column differ significantly (P < 0.05). nd not determined, no no activity.

| HBNs/standards | ClogP | ILPO (%) | LOX inhibition (IC50 [μM]/%) | Scav. activity for OH (%) | ABTS+• (%) |
|----------------|-------|----------|-----------------------------|--------------------------|-------------|
| PBN            | 3.02  | 11       | 23                          | no                       | 5           |
| HBN5           | 4.51  | 55       | 6                           | 67                       | no          |
| HBN6           | 4.96  | 37       | 29                          | 81                       | no          |
| HBN7           | 2.56  | 64       | 60 μM                       | 29                       | 22          |
| HBN8           | 0.61  | 46       | 57.5 μM                     | 59                       | 4           |
| HBN9           | 4.95  | 80       | 85 μM                       | 16                       | 23          |
| NDGA           | nd    | nd       | 0.45 μM                     | nd                       | nd          |
| Trolox         | nd    | 88       | nd                          | 83                       | 91          |
HBN9 showed more potent balanced antioxidant capacity, in good agreement with the higher calculated ClogP values, which are also consistent with the neuroprotection results (see above).

**Contribution of HBN6 to brain damage prevention.** Permanent ischemia models reflect the most frequent variants of stroke in patients who are outside of therapeutic windows, or are non-responders to recombinant tissue plasminogen activator, or surgical thrombectomy. In addition, permanent ischemia (no reperfusion)
has also been associated with substantial accumulation of ROS. Permanent middle cerebral artery occlusion (pMCAO) is a commonly used stroke model in mice. Using the pMCAO procedure, we analyzed the in vivo contribution of HBN6 to brain damage prevention. As expected, animals in the sham operated group showed no infarct (not shown). In striking contrast, groups subjected to pMCAO showed, 48 h after the occlusion procedure, infarcted regions which included exclusively the cerebral cortex (Fig. 8). The average size of the infarcted brain area was of 4.26 ± 0.2 mm³ (mean ± S.E.M.; n = 6) in vehicle treated animals, a value significantly greater than that for the HBN6 treated group, 0.37 ± 0.15 mm³ (media ± SEM) (n = 6; *P* < 0.001 two-tailed Student's t test assuming equal variances) (Fig. 8A,B). Considering that the total brain volume for the vehicle-treated mice was 458.42 ± 18.41 mm³ and for the mice treated with HBN6 458.26 ± 26.16 mm³ (mean ± SEM; n = 6; ns, two-tailed Student's t-test), this area represents 0.92 ± 0.05% and 0.088 ± 0.03% of the total brain volume, for vehicle and HBN6-treated animals, respectively (n = 6; *P* < 0.001 two-tailed Student's t test).

**Table 5.** Computed free reaction energies (ΔGₗ, at 298 K, in kcal/mol) for the reactions involving HBN6, PBN and HBN5 and radicals HO·. All data have been computed at the B3LYP-D3/def2-SVP level.

| Reaction path | HBN6 | PBN | HBN5 |
|---------------|------|-----|------|
| INT1          | −45.7 | −43.6 | −45.1 |
| INT2          | −30.7 | −22.7 | −30.4 |
| INT3 + H₂O    | −35.5 | −13.1 | −14.0 |

**Computational studies.** Density Functional Theory (DFT) calculations were carried out at the dispersion corrected B3LYP-D3/def2-SVP level (see computational details in the Supplementary Information) to gain more insight into the higher neuroprotective response of HBN6 as compared to the parent nitrone PBN. To this end, we first explored the reaction between the oxygen-centered radical HO· and HBN6. Three different pathways were envisaged (see Fig. 9), namely the addition of the radical to the carbon atom of the nitrone moiety leading to INT1, the addition to the aryl carbon atom placed in adjacent position to both nitrone moieties (leading to INT2), and the hydrogen abstraction reaction leading to INT3 which releases a water molecule.

From the data in Fig. 9, it becomes evident that the processes are thermodynamically controlled in view of the rather low activation barriers computed for the different possible pathways. Interestingly, the thermodynamically preferred pathway involves the addition of the hydroxyl radical to the electrophilic C=N bond of the nitrone therefore leading to the radical intermediate INT1. The higher stability of this species with respect to INT2 or...
INT3 may be in part ascribed to the occurrence of a stabilizing π–π non-covalent interaction involving the phenyl group of the benzyl group and the central aryl group, as easily visualized by means of the corresponding NCI plot (see green surface in the inset of Fig. 9).

Table 5 gathers the computed reaction energies of the different pathways involving HBN6 and PBN. Data for HBN5 are also included to enable a direct comparison (i.e. PBN and HBN5 possess a tert-Bu group as a nitrone substituent). As clearly seen from the computed energy values, all the possible pathways are less favored for the processes involving the parent PBN, which is fully consistent with the lower neuroprotective response observed for this nitrone. Except for the hydrogen abstraction reaction, the addition of OH- is only slightly favored for HBN6 as compared to its t-Bu substituted counterpart HBN5, which again is also consistent with the slightly higher response of the HBN6 as compared to HBN5 (see above). Therefore, it can be concluded that the neuroprotective ability of the considered bis-nitrones may be directly related to the exergonicity of their initial reaction (mainly addition reaction) with the corresponding ROS.

Virtual ADME analysis. Drug-like properties were determined for HBNs 1–9 and PBN as reference compound. We have used the QikProp software [QikProp, version 5.1, Schrodinger, LLC, New York, NY, 2017-1], and results are summarized in Table 1S (Supplementary Information). Out of ten compounds, seven nitrones were found to have no Lipinski’s rule34 violation and three nitrones showed one violation (Table 1S). According to Lipinski’s rule34, the partition coefficient (QPlogPo/w) value should be ≤ 5. For nitrones bearing the N-benzyl moiety (HBN3, HBN6 and HBN9) QPlogPo/w values range from 6.048 to 6.247. The number of hydrogen bond donors (NDonorHB) and hydrogen bond acceptors (NaccPHB) for all the nitrones were in agreement with the drug-likeness requirements of the Lipinski’s rule of five34 (NDonorHB ≤ 5, NaccPHB ≤ 10). The predicted central nervous system (CNS) activity with a score range from −2 (inactive) to +2 (active) indicated that bis-nitrones HBNs 1–9 had lower activity in the CNS (predicted value = 0) than PBN (predicted value = 1). Molecular volume is another crucial factor for binding at the active site. It was found that all bis-nitrones have molecular volumes between 667.298 and 1,188.993 Å³ (the reference value of molecular volume is 500–2000 Å³). The aqueous solubility (QPlogS) of a compound significantly affects its absorption and distribution characteristics. Typically, a low solubility goes along with a bad absorption. Only HBN3 (QPlogS = −6.81) and HBN6 (QPlogS = −6.87) presented solubility values out of the limits (−6.5 to 0.5). This is why the reason these two nitrones showed one violation of the rule-of-three (ROT)35,36. QPPCaco, which predicts permeability of a molecule for the gut-blood barrier through passive transport, is also one important factor to be considered. Compounds with high Caco-2 permeability (QPPCaco) are easy to absorb. Caco-2 cell permeability prediction of the tested bis-nitrones indicated excellent results, predicting good intestinal absorption.

The prediction of Blood Brain Barrier (BBB) permeability, determined by the logBB was also assessed. Compounds with logBB below −1 are poorly distributed to the brain and are improbable to operate as effective CNS drugs. All bis-nitrones displayed logBB > −1, pointing towards potential BBB permeability. The number of likely metabolic reactions (metab) is necessary for determining the level of accessibility of compounds to their target sites after entering into the blood stream. The predicted average number of possible metabolic reactions indicated that all bis-nitrones possessed metabol values in the recommended range (1–8). All bis-nitrones also exhibited an excellent predicted percentage of oral absorption, 100%. The optimum value polar surface area (7–200 Å) holds a great importance on the oral bioavailability of the molecules; in the present study, bis-nitrones were exhibited 18.99–45.83 Å value of PSA, indicating good bioavailability by oral route.

In particular, for HBN6 and PBN, the more significant observed data were the following: (1) Total Solvent Accessible Surface Area (SASA), in square Å, using a probe with a 1.4 Å radius: 686.317 and 429.378, respectively (limits 300.0–1,000.0); (2) Estimated number of hydrogen bonds (donorHB) that would be accepted by the solute: 0 for both compounds (limits: 0.0–6.0); (3) Predicted octanol/water partition coefficient (QPlogPo/w): 6.244 and 3.450, respectively (limits 2.0 to 6.5); (4) Predicted aqueous solubility, S, in mol/dm³, is the concentration of the solute’s saturated solution that is in equilibrium with crystalline solid (QPlogS): −6.873 and −3.342, respectively (limits 6.5 to 0.5); (5) Predicted brain/blood partition coefficient (QPlogBB): −0.601 and 0.197, respectively (limits −3.0 to 1.2); (6) Van der Waals surface area of polar nitrogen and oxygen atoms (PSA): 43.368 and 18.991, respectively (limits 7.0–200.0); (7) Number of violations of Lipinski’s Rule Of Five (ROF): 1 and 0, respectively (limits −3.0 to 1.2); (8) Number of violations of Jorgensen’s rule of three (ROT): 1 and 0, respectively; (9) Number of violations of Lipinski’s Rule Of Five: 1 and 0, respectively (limits 7.0–200.0); (10) Number of violations of Jorgensen’s rule of three: 1 and 0, respectively.

Table 6 lists the calculated partition coefficients (logBB) of the tested nitrones, with respect to 1-octanol, and their aqueous solubility values (QPlogS). The lipophilicity of the nitrones (as measured by logBB) is an important parameter to be considered in vivo. Cerebral Blood Barrier (BBB) permeability plays a significant role in the oral administration of nitrones. BI-3 shows the lowest logBB (−2.85) and is therefore the most suitable for BBB permeation compared to the remaining nitrones. In line with this assumption, HBN5 and HBN6 show the highest values of logBB (−0.601 and 0.197, respectively, Table 1S). The computed logBB values are consistent with the observed low solubility of these nitrones (Table 1S). This is in agreement with a previous study, which showed that nitrones with logBB > −1, pointing towards potential BBB permeability. The number of likely metabolic reactions (metab) is necessary for determining the level of accessibility of compounds to their target sites after entering into the blood stream. The predicted average number of possible metabolic reactions indicated that all bis-nitrones possessed metabol values in the recommended range (1–8). All bis-nitrones also exhibited an excellent predicted percentage of oral absorption, 100%. The optimum value polar surface area (7–200 Å) holds a great importance on the oral bioavailability of the molecules; in the present study, bis-nitrones were exhibited 18.99–45.83 Å value of PSA, indicating good bioavailability by oral route.

Conclusions

In this work we have described the design, synthesis and biological evaluation of nine bis-nitrones HNBs 1–9 derived from PBN for the potential treatment of stroke. The biological evaluation of the bis-nitrones included neuroprotection against Oligomycin A/Rotenone, and in an ischemia in vitro model under OGD conditions in human neuroblastoma SH-SY5Y cells, and diverse antioxidant tests. Our design, supported on literature precedents, was based on the hypothesis that two nitrone motifs installed in the same scaffold should afford higher neuroprotective power than only one nitrone group. For our approach, we have used PBN (Fig. 1) as the nitrone standard to implement this strategy. The homo-bis-nitrones HNBs 1–9 are the result of the incorporation a second identical nitrone at para, meta and ortho positions leading to HNBs 1–3, HNBs 4–6 and HNBs 7–9, respectively, and bearing methyl, tert-butyl or benzyl substituents, as the N-alkyl groups at the nitrone motif (Fig. 1). The desired ligands were easily obtained by simple methods from readily available starting precursors. From all the neuroprotection results, we were able to identify bis-nitrone (1Z,1′Z)-1,1′-(1,3-phenylene)
bis(N-benzylmethanimine oxide) (HBN6), bearing the two nitrone motifs in meta position, and two N-benzyl groups at the nitrone scaffold, as a potent neuroprotective agent (EC_{50} = 1.24 ± 0.39 µM) with strong hydroxyl radical scavenger power (81%), in the same range as Trolox (83%), and a strong capacity to decrease superoxide production in human neuroblastoma cell cultures, which exceed the neuroprotective and antioxidant capacities of the parent PBN. This may be related to the exergonicity of the addition of the oxygen-centered radical to the carbon atom of the nitrone moiety. In silico results allowed us to conclude that HBN6 is predicted to be a potential drug candidate, via oral administration, due to its relevant drug-likeness profile, bioavailability, excellent liposolubility and adequate pharmacokinetics, including CNS permeability, although with low water solubility. To sum up, all these observations confirmed that our initial design hypothesis was correct (“two better than one”), and could be used as a guideline to design new and more efficient neuroprotective agents for the therapy of stroke.

Methods

Chemistry. Compound purification was performed by column chromatography with Merck Silica Gel (40–63 µm) or by flash chromatography (Biotage Isola One equipment) and the adequate eluent for each case. Reaction course was monitored by thin layer chromatography (TLC), revealing with UV light (λ = 254 nm) and ethanolic solution of vanillin or ninhydrin. Melting points were determined using a Reichert Thermo Galen Koffer block and are uncorrected. Samples were dissolved in CDCl3 or DMSO-d6 using TMS as internal standard for 1H NMR spectra. In 13C NMR spectra, CDCl3 central signal (77.0 ppm) and DMSO-d6 (39.5 ppm) were used as references. 1H-NMR and 13C-NMR spectra were obtained in Bruker Avance 300 (300 MHz) and Bruker Avance 400 III HD (400 Hz) spectrometers. Chemical shifts (δ) are given in ppm. Coupling constants (J) are given in Hz. Signal multiplicity is abbreviated as: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), or multiplet (m). IR spectra were recorded on a Perkin-Elmer Spectrum One B spectrometer. Units are cm⁻¹. Low resolution mass spectra were recorded on an Agilent HP 1,100 LC/MS Spectrometer, whereas High Resolution mass spectrum (Exact Mass) was performed in an AGILENT 6,520 Accurate-Mass QTOF LC/MS Spectrometer. Elemental analyses were performed in an Elementary Chemical Analyser LECO CHNS-932.

General methods for the synthesis of nitrone. Method A: To a suspension of the bis(tris)carbaldelyde (1 mmol) in dry EtOH (20 mL), anhydrous NaHCO3 (3 equiv), Na2SO4 (4 equiv) and the corresponding N-alkylhydroxylamine hydrochloride (3 equiv) were added. The mixture was irradiated at 90 °C, and 15 bar, for the time indicated in each case. Then, the mixture was cooled, the solvent removed, and the crude purified by column chromatography. Method B: As in Method A, but at room temperature (rt). Method C: As in Method A, but in dry THF as solvent.

(IZ.1′Z)-1,1′-(1,4-Phenylene)bis(N-methylmethanimine oxide) (HBN1). Following the general Method A, the reaction of terephthalaldehyde (134 mg, 1 mmol) with NaHCO3 (252 mg, 3 mmol), Na2SO4 (568 mg, 4 mmol) and N-methylhydroxylamine hydrochloride (250.6 mg, 3 mmol), in EtOH (20 mL), for 1 h, after work-up and purification by column chromatography eluting with MeOH:CH2Cl2 at 3%, gave HBN1 (146.6 mg, 76%); mp > 220 °C; IR (KBr) ν 3,423, 1569, 1,361, 1,125 cm⁻¹; 1H NMR (500 MHz, DMSO-d6) δ 8.22 (s, 4 H, H-2, H-3, H-5, H-6), 7.86 (s, 2 H, H-7, H-9), 3.77 (s, 6 H, CH3); 13C NMR (126 MHz, DMSO-d6) δ 133.9 (2 C, C7, C-9), 132.3 (2 C, C-1, C-4), 127.9 (4 C, C-2, C-3, C-5, C-6), 54.6 (2 C, CH3); MS (ESI) m/z: 193 [M + 1]⁺, 215 [M + Na]⁺, 404 [2 M + Na]⁺. Anal. Calcd for C9H14N2O2: C, 69.53; H, 8.75; N, 10.14. Found: C, 69.31; H, 8.69; N, 10.13.

(IZ.1′Z)-1,1′-(1,4-Phenylene)bis(N-tert-butylmethanimine oxide) (HBN2). Following the general Method A, the reaction of terephthalaldehyde (134 mg, 1 mmol) with NaHCO3 (252 mg, 3 mmol), Na2SO4 (568 mg, 4 mmol) and N-tert-butylhydroxylamine hydrochloride (375 mg, 3 mmol), in EtOH (20 mL), for 3.5 h, after work-up and purification by column chromatography eluting with MeOH:CH2Cl2, at 3%, gave HBN2 (83.4 mg, 30%): mp > 220 °C; IR (KBr) ν 3,434, 1569, 1,361, 1,125 cm⁻¹; 1H NMR (500 MHz, DMSO-d6) δ 8.34 (s, 4 H, H-2, H-3, H-5, H-6), 7.86 (s, 2 H, H-7, H-9), 3.31 [s, 18 H, C(CH3)3]; 13C NMR (126 MHz, DMSO-d6) δ 132.6 (2 C, C-1, C-4), 128.9 (2 C, C-7, C-9), 128.3 (4 C, C-2, C-3, C-5, C-6), 71.0 (2 C, CH3); MS (ESI) m/z: 277 [M + 1]⁺, 299 [M + Na]⁺. Anal. Calcd for C16H24N2O2: C, 69.53; H, 8.75; N, 10.14. Found: C, 69.31; H, 8.69; N, 10.13.

(IZ.1′Z)-1,1′-(1,4-Phenylene)bis(N-benzylmethanimine oxide) (HBN3). Following the general Method A, the reaction of terephthalaldehyde (134 mg, 1 mmol) with NaHCO3 (252 mg, 3 mmol), Na2SO4 (568 mg, 4 mmol) and N-benzylhydroxylamine hydrochloride (477 mg, 3 mmol), in EtOH (20 mL), for 2.5 h, after work-up and purification by column chromatography eluting with AcOEt:hexane (2:3), gave HBN3 (110 mg, 32%): mp > 220 °C; IR (KBr) ν 3,435, 1,570, 1,459, 1,152 cm⁻¹; 1H NMR (400 MHz, DMSO-d6) δ 8.42 (s, 4 H, H-2, H-3, H-5, H-6), 8.12 (s, 2 H, H-7, H-9), 7.54–7.31 (m, 12 H, C6H5), 5.08 (s, 4 H, H-8, H-10); 13C NMR (101 MHz, DMSO-d6) δ 133.4 (2 C, C7, C-9), 132.3 (2 C, C1-C-4), 129.4, 128.9, 128.8 (C6H5), 128.2 (4 C, C-2-C-3-C-5-C-6), 70.6 (C-8-C-10); MS (ESI) m/z (%): 350 [M + 1]⁺, 372 [M + Na]⁺. Anal. Calcd for C23H20N2O2: C, 75.79; H, 5.93; N, 8.01. Found: C, 75.68; H, 6.22; N, 7.88.

(IZ.1′Z)-1,1′-(1,3-Phenylene)bis(N-methylmethanimine oxide) (HBN4). Following the general Method A, the reaction of isophthalaldehyde (134 mg, 1 mmol) with NaHCO3 (252 mg, 3 mmol), Na2SO4 (568 mg, 4 mmol) and N-methylhydroxylamine hydrochloride (250.6 mg, 3 mmol), in EtOH (20 mL), for 1 h, after work-up and purification by column chromatography eluting with MeOH:CH2Cl2 al 4%, gave HBN4 (185.9 mg, 97%): mp 277 [M + 1]⁺, 299 [M + Na]⁺. Anal. Calcd for C9H14N2O2: C, 69.53; H, 8.75; N, 10.14. Found: C, 69.31; H, 8.69; N, 10.13.
148–150 °C; IR (KBr) ν 3,419, 1584, 1,415, 1,172, 1,157 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 8.92 (s, 1 H, H-6), 8.34 (dd, J = 7.9, 1.7 Hz, 2 H, H-4, H-2), 7.90 (s, 2 H, H-9, H-7), 7.49 (t, J = 7.9 Hz, 1 H, H-3), 3.80 (s, 6 H, H-8, H-10); ¹³C NMR (101 MHz, DMSO-d₆) δ 134.0 (2 C, C-1, C-2), 132.1 (2 C, C-3, C-7, C-9), 129.1 (2 C, C-4, C-5), 128.9 (C-3), 127.6 (C-6), 54.6 (2 C, C-8); MS (ESI) m/z: 193 [M + 1]⁺, 215 [M + Na]⁺, 404 [2M + Na]⁺. Anal. Calcd for C₁₀H₁₂N₂O₂: C, 69.53; H, 8.75; N, 10.14. Found: C, 69.51; H, 8.75; N, 10.21.

Following the general Method A, the reaction of isophthaldehyde (134 mg, 1 mmol) with NaHCO₃ (252 mg, 3 mmol), Na₂SO₄ (568 mg, 4 mmol), and N-tert-butyldimethylsilylhydrochloride (375 mg, 3 mmol), in EtOH (20 mL), for 3 d, after work-up and purification by column chromatography eluting with AcOEt: hexane (3:2), gave HBN₅ (248 mg, 90%): mp 147–9 °C; IR (KBr) ν 3,435, 2,976, 1573, 1,361, 1,180 cm⁻¹; ¹H NMR (500 MHz, DMSO-d₆) δ 8.92 (s, 1 H, H-2), 8.39 (dd, J = 7.9, 1.7 Hz, 2 H, H-4, H-6), 7.82 (s, 2 H, H-7, H-9), 7.44 (t, J = 7.9 Hz, 1 H, H-3), 1.50 [s, 18 H, C(CH₃)₃]; ¹³C NMR (126 MHz, DMSO-d₆) δ 131.88 (2 C, C-1, C-3), 129.59 (2 C, C-4, C-5), 129.82 (3 C, C-2, 7, 9), 128.50 (C-5), 70.95 (2C, 8, 10), 28.28 (6 C, C(CH₃)₃); MS (ESI) m/z: 277 [M + Na]⁺, 299 [M + Na]⁺. Anal. Calcd for C₁₆H₂₄N₂O₂: C, 69.53; H, 8.75; N, 14.57. Found: C, 69.26; H, 8.75; N, 9.87.

Following the general Method A, the reaction of isophthaldehyde (134 mg, 1 mmol) with NaHCO₃ (252 mg, 3 mmol), Na₂SO₄ (568 mg, 4 mmol), and N-benzylhydroxylamine hydrochloride (477 mg, 3 mmol), in EtOH (20 mL), for 2.5 h, after work-up and purification by column chromatography eluting with AcOEt: hexane (3:2), gave HBN₆ (165 mg, 48%): mp 185–187 °C; IR (KBr) ν 3,435,1582, 1,458, 1,175 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 8.99 (s, 1 H, H-2), 8.29 (dd, J = 7.9, 1.7 Hz, 2 H, H-4, H-6), 8.12 (s, 2 H, H-7, H-9), 7.51–7.32 (m, 11 H, H-5, C₆H₅), 5.06 (s, 4 H, H-8, H-10); ¹³C NMR (126 MHz, DMSO-d₆) δ 131.88 (2 C, C-1, C-3), 131.51 (2 C, C-4, C-5), 129.14 (2 C, C-2, C-7, C-9), 128.22 (2 C, C-4, C-6), 129.4 (C-5), 129.0, 128.8, 128.7 (C₆H₅), 128.0 (C-2), 70.5 (C-8, C-10); MS (ESI) m/z (%): 350 [M + 1]⁺, 372 [M + Na]⁺. Anal. Calcd for C₁₂H₁₄N₂O₂: C, 69.72; H, 5.85; N, 8.14. Found: C, 69.59; H, 6.02; N, 8.28.
medium was replaced with fresh medium containing 0.01–1,000 μM compound concentrations or PBS in the controls, as indicated in each assay.

**Neuroblastoma cell cultures exposure to Oxygen–Glucose deprivation (OGD).** Neuroblastoma cell cultures were exposed to OGD to induce cellular damage (experimental ischemia). Cultured cells were washed and placed in glucose-free Dulbecco’s medium (bubbled with 95% N₂/5% CO₂ for 30 min) and maintained in an anaerobic chamber containing a gas mixture of 95% N₂/5% CO₂ and humidified at 37 °C at a constant pressure of 0.15 bar. Cells were exposed to OGD for a period of 4 h (OGD 4h), as indicated. At the end of the OGD period, culture medium was replaced with oxygenated serum-free medium, and cells were placed and maintained in the normoxic incubator for 24 h to recovery (R24h). In the neuroprotection experiments, HBNs 1–9 and PBN (0.01 μM – 1 mM) were added at the beginning of the recovery period (see below). Control cultures in Dulbecco’s medium containing glucose were kept in the normoxic incubator for the same period of time as the OGD (C4h), and then culture medium was replaced with fresh medium and cells were returned to the normoxic incubator until the end of the recovery period (C24h). In each experiment a series of different controls were performed containing the same final % of dimethyl sulfoxide (DMSO) as the samples with the tested compounds (between 0.00001% and 1% of DMSO for the samples with compound concentrations between 0.01 μM and 1,000 μM. In them, cell viability ranged from 100% to 93.5%. This small decrease in cell viability induced by DMSO was taken into account when performing viability and neuroprotection calculations. The control represented in figures is the control of 24 h of incubation with normal culture medium, that is, in the absence of DMSO.

The experimental procedures were blindly performed, assigning a random order to each assayed nitrone. Nitrones were analyzed independently three-five times with different batches of cultures, and each experiment was run in triplicate.

**Assessment of cell viability.** Measurements of cell viability in human SH-SY5Y neuroblastoma cells were carried out into 96-well culture plates as described. Briefly, control and treated SH-SY5Y neuroblastoma cells (about 0.75–1 × 10⁵ cells/well) were incubated with the XTT solution (Cell Proliferation Kit II (XTT), Sigma, Aldrich, Madrid) at 0.3 mg/ml final concentration for 2 h in a humidified incubator at 37 °C with 5% CO₂ and 95% air (v/v) and the soluble orange formazan dye formed was spectrophotometrically quantified, using a Biotek Power-Wave XS spectrophotometer microplate-reader at 450 nm (reference 650 nm). All XTT assays were performed in triplicate in cells of at least three different cell batches. Control cells treated with DME alone were regarded as 100% viability. Controls containing different DMSO concentrations (0.001–1% DMSO) were performed in all assays.

**Measurement of LDH activity.** For these assays, cultured neuroblastoma cells grown in 96-well culture dishes at a density of 1.5 × 10⁵ cells/well were used. LDH activity was measured as the rate of decrease of the absorbance at 340 nm, resulting from the oxidation of NADH to NAD + as described. Data are given as the percentage of LDH release with respect to the total LDH content (LDH in the culture medium and LDH inside the cells).

**Analysis of caspase-3 activity.** For these assays, cultured neuroblastoma cells grown in 48-well culture dishes, at a density of 2.5 × 10⁵ cells/well, were used. After OGD treatment, cells were treated with different nitrones or indicated positive controls at 1 – 500 μM concentrations and subjected to 24 h reperfusion. Attached cells were lysed at 4 °C in a lysis medium containing 5 mM Tris/ HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid, and 0.5% Triton X-100 and centrifuged at 13.000 g for 10 min. The activity of caspase-3 was measured using the fluorogenic substrate peptide DEVD-amc (66081; BD Biosciences PharmMingen), as described. Proteins were measured by the Bradford assay. Results were expressed as arbitrary fluorescence units [(AFU)/μg protein/h].

**Measurement of ROS formation.** SH-SY5Y human neuroblastoma cells (2 × 10⁵ cells/well) were exposed to OGD for a period of 4 h (OGD4h). At the end of the OGD period, the culture medium was replaced with oxygenated Dulbecco’s modified Eagle’s medium containing glucose and 10% fetal calf serum. Cells were treated in the absence (controls) or presence of indicated concentrations of nitrones or different known neuroprotective agents and maintained at 37 °C in a normoxic incubator for 3 h before and for 4 h after recovery. At the end of this period, 20 μM DHE (HEt; Molecular Probes) was added and fluorescence was recorded every 15 – 30 s during a 15 min period, using an excitation filter of 535 nm and an emission filter of 635 nm in a spectrofluorimeter (Bio-Tek FL 600) as previously described. Linear regression of fluorescence data [expressed as arbitrary fluorescence units (AFU)] was calculated for each condition, and the slopes (a) of the best fitting lines (γ = ax) were considered as an index of O₂⁻ production. SNP was used as a positive control of superoxide production.

**Statistical analysis.** Data were expressed as mean±SEM of results obtained from at least three independent experiments from different cultures, each of which was performed in triplicate. Statistical comparisons between the different experimental conditions were performed using one-way analysis of variance (ANOVA), followed by Holm–Sidak’s post–test when the analysis of variance was significant. A P value < 0.05 was considered statistically significant. Concentration–response curves for the estimation of EC₅₀ and maximal activities values were calculated by a weighted nonlinear regression of minimum squares using four parameters logistic curves (f1 = min + (max−min)/(1 + (x/EC₅₀)^a) (Hillslope), by using the program SigmaPlot v.11 (Systat Software INC., 2012). Lineal correlation analysis (straight line equations, correlation coefficients (r) and statistical significances of regression line equations) were performed by the Pearson Product Moment Correlation Test, carried out with SigmaPlot v.11.
pMCAO stoke model. To study of the effects of HBN6 on stroke recovery, administration of the vehicle and HBN6 was performed intraperitoneally, to 8weekold male C57BL/6 J mice (Harlan) weighing 25–30 g. All procedures with animals were carried out under a protocol approved by the Ethical Committee of the Spanish National Research Council (CSIC), and recommendations of the European Council. A special effort was made to keep to a minimum necessary the number of animals to achieve adequate significance. For surgery, anesthesia induction was carried out with 3% isoflurane (in 70% N2O, 30% O2), followed by 2% isoflurane for maintenance during stroke procedure. Rectal temperature was maintained at 36.5 °C with the use of a heating pad. The frontal branch of the MCA was, after craniotomy, exposed and occluded permanently by suture ligation as previously reported, with modifications. The permanent occlusion involved exclusively the frontal branch of the middle cerebral artery, the stem of this artery remaining untied. This procedure yielded a smaller infarct size than that determined by ligation of the arterial stem, allowing a better assessment of final infarct volume among vehicle control and HBN6 treated groups, and also reducing the sample (n) size. To ensure a complete artery during surgery, cortical blood flow was monitored by non-invasive laser Doppler flowmetry, as a quality control, with the aid of a Perimed equipment (PeriFlux System 5,000 Stroke Model Monitor, Perimed, Järfälla, Sweden). The study was exclusively performed in animals that showed post-ligation a drop of blood flow of at least 65%. Animals subjected to surgery for longer than 15 min were excluded of the study. Physiological parameters were maintained as previously reported. Experiments were performed in each of the following groups: (a) sham operated (n=6 animals); (b) pMCAO vehicle control group (saline buffer containing 29% dimethyl sulfoxide, DMSO) (n=6), and (c) pMCAO HBN6 treated group (100 mg/kg HBN6 dissolved in vehicle) (n=6). Drug administration was 15 min after arterial ligation. Determination of infarct size in vehicle and HBN6 treated mice was performed by means of the 2,3,5-triphenyltetrazolium chloride (TTC) staining procedure of sequential coronal 1 mm-thick brain slices obtained from the operated animals with the aid of a Brain Matrix (WPI, UK) as reported previously. The experiments compared infarct volume outcome between group’s b and c. Sham operated control group (a) showed with certainty that stroke was not due to the surgical pre-occlusive procedure. Infarct volumes, shown in mm³, were obtained integrating infarcted areas by counting pixels contained within the regions of interest. Each side of the coronal sections was sampled. Images were taken with the aid of a digital camera (Pentax Optio S7) that provided good resolution of infarct boundaries. With the use of the free software ImageJ 1.33a software (National Institutes of Health, Bethesda, MD), acquired images were analyzed. Student two-sample t-test was carried out to determine the statistical significance of differences of infarct values between the vehicle and the HBN6 treated mice. P value < 0.05 was considered significant.

Antioxidant activity tests of HBNs 5, 6, 9, and PBN. Estimation of Lipophilicity as Clog P. Biooom of Biobyte Corp was used for the theoretical calculation of lipophilicity as Clog P values (BioByte Home Page. Available online: https://www.biobyte.com).

Materials and methods. Nordihydroguaiaretic acid (NDGA), Trolox, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)Soybean LOX linoleic acid sodium salt were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Phosphate buffer (0.1 M and pH 7.4) was prepared mixing an aqueous KH2PO4 solution (50 mL, 0.2 M), and an aqueous of NaOH solution (78 mL, 0.1 M); the pH (7.4) was adjusted by adding a solution of KH2PO4 or NaOH). For the in vitro tests a Lambda 20 (Perkin-Elmer-PharmaSpectra 1,700) UV–Vis double beam spectrophotometer was used.

Inhibition of linoleic acid peroxidation. For initiating the free radical, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is used. The final solution in the UV cuvette consisted of ten microliters of the 16 mM linoleate sodium dispersion 0.93 mL of 0.05 M phosphate buffer, pH 7.4, thermostatted at 37 °C. 50 μL of 40 mM AAPH solution was added as a free radical initiator at 37 °C under air and 10 μL of the tested compounds.

Inhibition of soybean lipoxygenase. The oxidation of linoleic acid sodium salt results in a conjugated diene hydroperoxide. The reaction is monitored at 234 nm. Soybean lipoxygenase inhibition study in vitro. In vitro study was evaluated as reported previously. The tested compounds (several concentrations 1–100 μM, from the stock solution 10 mM were used for the determination of IC50) dissolved in DMSO were incubated at room temperature with sodium linolate (0.1 mM) and 0.2 mL of enzyme solution (1/9 x 10−4 w/v in saline). The conversion of sodium linolate to 13-hydroxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor NDGA (IC50 0.45 μM and 93% at 100 μM).

Hydroxyl radicals scavenging activity. The hydroxyl radicals were produced by the Fe3+/ascorbic acid system. EDTA (0.1 mM), Fe 3+ (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (0.1 mM) and ascorbic acid (10 mM) were mixed in test tubes. The solutions were incubated at 37 °C for 30 min. The reaction was stopped by CCl3COOH (17% w/v) and the % scavenging activity of the tested compounds for hydroxyl radicals was given.

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Author contributions
J.M.C. and M.J.O.G. coordinated the project and wrote the manuscript; D.D.I., B.S.M. and D.G.V. carried out the synthesis of the HBNs; M.C. supervised the synthesis and NMR analysis; D.H.L. performed the antioxidant tests; B.C. performed all the neuroprotection analysis; M.J.O.G. supervised and coordinated the neuroprotection studies; R.M.M. and D.G.N. coordinated the focal ischemia experiment; I.F. carried out the computational studies; I.I. carried out the ADME analysis; F.L.M., R.M.M., I.F. and I.I. corrected the manuscript.

Competing interests
The authors declare no competing interests.

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