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Original article

Polycyclic cage structures as carrier molecules for neuroprotective non-steroidal anti-inflammatory drugs

Louis H.A. Prins [a], Jan L. du Preez [b], Sandra van Dyk [a], Sarel F. Malan [a,*]

[a] Pharmaceutical Chemistry, School of Pharmacy, North-West University, Potchefstroom 2520, South Africa
[b] Central Analytical Laboratory, School of Pharmacy, North-West University, Potchefstroom, 2520, South Africa

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ABSTRACT

The blood–brain barrier is formed by the brain capillary endothelium and plays the predominant role in controlling the passage of substances between the blood and the brain. Recent studies on polycyclic structures, i.e. pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine, indicated favourable distribution thereof to the brain and it was concluded that these polycyclic structures and their derivatives penetrate the blood–brain barrier readily. A series of novel polycyclic prodrugs incorporating the well known non-steroidal anti-inflammatory drugs (NSAIDs), acetylsalicylic acid and ibuprofen, were synthesised and screened for blood–brain barrier permeability and antioxidant activity. Increased levels of both NSAIDs were detected in the brain tissue of C57BL/6 mice after administration of the synthesised prodrugs, indicating favourable blood–brain barrier permeation. Results from a lipid peroxidation assay indicated that the ester and amide prodrugs significantly increased the ability of the drugs to attenuate lipid peroxidation. These novel prodrugs thus readily penetrate the blood–brain barrier and exhibit increased antioxidant activity when compared to the free NSAIDs.

1. Introduction

Neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases are detrimental to the health and well being of millions of people around the globe. In the quest to successfully prevent and treat these diseases, the blood–brain barrier presents as a major obstacle by preventing the entrance of certain substances into the central nervous system (CNS). Drugs with limited transport over the blood–brain barrier have been conjugated with lipophilic carriers to vastly improve their blood–brain barrier permeability [1]. It was recently described that the polycyclic structures, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine, exhibit favourable distribution to the brain and therefore penetrate the blood–brain barrier readily [2,3]. This property makes these structures very useful as carrier molecules for CNS delivery of drugs. The polycyclic cage thus appears to be a useful scaffold to yield drugs with a wide scope of applications, and can also be used to modify and improve the pharmacokinetic and pharmacodynamic properties of drugs in current use [4].

Mandel et al. [5] demonstrated that, at the time of death, patients suffering from Parkinson's disease presented with a cascade of lethal events. These events may contribute to the demise of the melanin-containing nigro-striatal dopamine neurons and include increased levels of iron and monoamine oxidase B (MAO-B) activity, oxidative stress, inflammatory processes, glutamatergic excitotoxicity, aberrant nitric oxide synthesis, abnormal protein folding and aggregation, reduced expression of trophic factors, depletion of endogenous antioxidants such as reduced glutathione, and altered calcium homeostasis. Applying the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) animal models of Parkinson's disease, their studies indicated that neuroprotection could be afforded with iron chelators, radical scavenger antioxidants, MAO-B inhibitors, glutamate antagonists, nitric oxide synthase inhibitors, calcium channel antagonists and trophic factors.

Several mechanisms have been proposed for the neuroprotective activity of NSAIDs, including the inhibition of oxidative stress [6,7] and it is postulated that their neuroprotective effect might be independent of the ability to inhibit the cyclooxygenase (COX) enzyme isoforms [8]. This notion is supported by Guerrero et al. [9] who indicated that salicylic acid, the main acetylsalicylic acid metabolite, does not inhibit COX in whole blood samples while still exhibiting antioxidant capacity. The neuroprotective properties

* Corresponding author. Tel.: +27 18 2992266; fax: +27 18 2994243.
E-mail address: sarel.malan@nwu.ac.za (S.F. Malan).

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of acetylsalicylic acid and acetaminophen were recently investigated and it was demonstrated that these two drugs alone and in combination significantly attenuated cyanide-induced superoxide anion ($O_2^{-\bullet}$) generation in vitro [10,11]. In vivo, acetylsalicylic acid and acetaminophen were shown to alleviate the quinolinic acid-induced rise in $O_2^{-\bullet}$ generation in the hippocampus and were also effective in reducing the MPP^+-induced rise in $O_2^{-\bullet}$ levels. A further in vivo observation was that these drugs, alone and in combination, prevented the MPP^+-induced inhibition of the electron transport chain and complex I activity. The potent activity exhibited by acetylsalicylic acid was attributed to possible roles in the enhancement of cellular respiration and superoxide dismutase activity in the presence of MPP^+. Asanuma et al. [12] also reported that ibuprofen, amongst other drugs, afforded protective effects against apoptosis mainly due to its direct nitric oxide radical ($NO^{\bullet}$) scavenging activities in neuronal cells.

As glutamate excitotoxicity is implicated in Alzheimer's, Parkinson's and other neurodegenerative diseases, Casper et al. [13] evaluated whether acetylsalicylic acid, acetaminophen and ibuprofen protect dopaminergic neurons against excitotoxicity. All three NSAIDs significantly attenuated the decrease in dopamine uptake caused by glutamate, indicating preservation of neuronal integrity. Ibuprofen increased the relative number of dopaminergic neurons by 47%. These effects were most likely due to the ability of NSAIDs to inhibit oxidative damage.

The observation that regular NSAID use is associated with a decreased risk of developing Parkinson's disease [14] and that these drugs possess antioxidant activity [10–12], served as rationale for selecting acetylsalicylic acid and ibuprofen for our study. From the above discussion the need for an effective CNS delivery mechanism for these drugs is also clear, and prodrug synthesis of the selected NSAIDs, acetylsalicylic acid and ibuprofen, were pursued by conjugation with pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine.

2. Chemistry

The well-described cage compound, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione, served as primary basis for the synthesis of the proposed compounds. In order to obtain the ester prodrugs (**2**, **3**), a tetradecane cage structure (**1**) was synthesised [15] and conjugated to acetylsalicylic acid and ibuprofen, respectively. Amantadine.HCl (**4**), a well known anti-Parkinsonian agent, was used to synthesise the amide prodrugs (**5**, **6**) of the selected NSAIDs.

3. Results and discussion

3.1. Blood–brain barrier permeability

The average blood and brain concentration as well as percentage recovery from the blood and brain were determined using the LC-MS/MS results. Percentage recovery from blood and brain were approximately equal for all the tested compounds, except for **5**, which had a percentage recovery from blood of nearly twice the value from the brain. The LC-MS/MS results were also applied to determine the brain to blood concentration ratios (Table 1).

From the results obtained the blood–brain barrier permeability of the test compounds and more importantly, the extent to which the prodrug conjugates improved the CNS delivery of the free drugs, were calculated. It was confirmed that acetylsalicylic acid, does not readily penetrate the blood–brain barrier, presenting with a brain to blood concentration ratio of 0.49. Most of the drug is observed as salicylic acid, the primary acetylsalicylic acid metabolite, presenting with a brain to blood concentration ratio of 1.06. Although salicylic acid is ionised at physiological pH and does not cross the blood–brain barrier readily, the reason for the relatively high brain concentration thereof is probably that once it is produced in the brain as a result of the hydrolysis of acetylsalicylic acid, it is too hydrophilic to permeate back to the systemic circulation from the CNS.

From the calculation of the total salicylates it is however clear that the distribution of the drug between the blood and brain does not favour brain delivery of the free drug.

After administration of compound **2**, the acetylsalicylate ester prodrug, the free drug was observed at about twice the concentration in the brain when compared to free drug administration. This indicated a more favourable blood–brain barrier permeation for compound **2**. HPLC results indicated that salicylic acid was also observed in brain tissue after administration of compound **2**, but to a lesser extent than that observed for acetylsalicylic acid. This may be attributed to drug latentiation, where the acetyl group on acetylsalicylic acid is hydrolysed at a slower rate than normally because of the polycyclic molecule conjugated to it. With administration of compound **5** (acetylsalicylamide prodrug), free acetylsalicylic acid was observed in both the brain and the blood at roughly one third of the concentration of that observed after the free drug was administered. This observation is attributed to the fact that the amide bond of the prodrug is less prone to hydrolysis than the ester bond. Less acetylsalicylic acid is thus observed at 1 h after drug administration than compared to that for compound **2**, because at this specific time point less of the amide prodrug is hydrolysed to release acetylsalicylic acid. Compound **5** presented with a brain to blood concentration ratio of 0.49 The percentage recovery from the blood was however nearly twice that of the value from the brain, for this specific compound, which indicated that at 1 h after administration there was approximately equal amounts of the compound in the blood and in the brain.

Free ibuprofen presented with a brain to blood concentration ratio of 0.90, which indicated more favourable blood–brain barrier permeation than observed for acetylsalicylic acid. Once conjugated to the tetradecane structure (**1**) to produce compound **3**, the brain to blood concentration ratio for free ibuprofen increased to 1.39, indicating a higher ibuprofen concentration in the brain than that observed in the blood. Once again the amide prodrug, compound **6**, exhibited lower blood and especially brain concentrations than observed for the free drug and a ratio of 0.39 was observed. This ratio is even lower than that observed for free ibuprofen, and may indicate a much lower rate of hydrolysis for the amide compound, **6**, especially in the brain.

3.2. Lipid peroxidation

This assay was applied as it is quick and relatively easy to use and is performed in an isolated biological system. The results are expressed as nmoles malondialdehyde (MDA)/mg tissue, and give an indication of the concentration of MDA produced in the presence (and absence) of the test compounds. From the results it is clearly evident that especially compound **2** exhibits substantial antioxidant capacity in the presence of the toxin ($H_2O_2 + FeCl_3 +$ ascorbic acid) (Fig. 1).

From the results obtained it is clear that the novel synthesised prodrugs as well as the free drugs, exhibit a significant attenuation of lipid peroxidation in the presence of the toxin (all presenting with $p < 0.001$). Acetylsalicylic acid and ibuprofen's abilities to attenuate lipid peroxidation are significantly increased by both their amide and ester prodrug forms (all having p values of <0.001). Compound **2** (acetylsalicylate ester prodrug) exhibited a significantly higher inhibition of lipid peroxidation than either

Table 1
LC-MS/MS results.

	Average [Blood] (ng/mL)	Average [Brain] (ng/mL)	[Brain]/ [Blood]		Average [Blood] (ng/mL)	Average [Brain] (ng/mL)	[Brain]/ [Blood]
Acetylsalicylic acid	45.50	22.47	0.49	*Acetylsalicylic acid from compound* **5**	13.84	6.81	0.49
Salicylic acid from acetylsalicylic acid	6.95	7.37	1.06	*Salicylic acid from compound* **5**	LOQ[a]	LOQ[a]	NC[b]
*Total salicylates from acetylsalicylic acid**	41.45	24.60	0.59	*Ibuprofen*	61.37	52.51	0.90
Compound **2**	LOQ[a]	LOQ[a]	NC[b]	*Compound* **3**	LOQ[a]	LOQ[a]	NC[b]
Acetylsalicylic acid from compound **2**	44.11	59.03	1.34	*Ibuprofen from compound* **3**	40.28	55.80	1.39
Salicylic acid from compound **2**	LOQ[a]	LOQ[a]	NC[b]	*Compound* **6**	LOQ[a]	LOQ[a]	NC[b]
Compound **5**	17.38	8.58	0.49[#]	*Ibuprofen from compound* **6**	30.45	11.83	0.39

*To calculate the total salicylate values the molecular mass ratio of salicylic acid to acetylsalicylic acid was used. [#]Recovery from blood was double that from brain and effective ratio is thus ±1.
[a] Obtained values were below the limit of quantification.
[b] Brain to blood concentration ratios were not calculable.

acetylsalicylic acid or compound **1**, the cage structure used in its synthesis ($p < 0.001$). This observation can be explained by a possible synergistic effect or the much improved lipophilicity profile of the conjugate. This is also true for compound **6** (ibuprofen amide prodrug), which attenuates lipid peroxidation more effectively than amantadine.HCl ($p < 0.001$). Compound **5** (acetylsalicylamide prodrug) also attenuated lipid peroxidation to a significantly greater extent than observed for amantadine.HCl ($p < 0.05$).

From the results of the study the notion that both acetylsalicylic acid and ibuprofen possess certain antioxidant abilities [10–12], was confirmed. It was also observed that amantadine.HCl exhibited a certain amount of antioxidant activity in the presence of the toxin used. A further conclusion drawn from the study is that the synthesis of acetylsalicylic acid and ibuprofen prodrugs through esterification and amide formation with the tetradecane cage structure and amantadine respectively, significantly increased the free drug's abilities to attenuate lipid peroxidation.

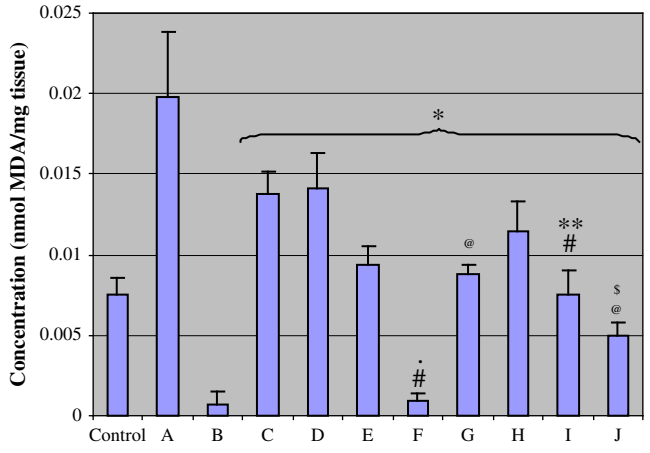


Fig. 1. Antioxidant activity of the different compounds in vitro. *X* axis labels are as described in Table 2 and each bar represents the mean ± SEM; $n = 5$ ($^{*}p < 0.001$ vs. toxin ($H_2O_2 + FeCl_3 +$ ascorbic acid); $^{\#}p < 0.001$ vs. acetylsalicylic acid; $^{@}p < 0.001$ vs. ibuprofen; $^{\bullet}p < 0.001$ vs. compound 1; $^{**}p < 0.05$ vs. amantadine.HCl; $^{\$}p < 0.001$ vs. amantadine.HCl).

4. Conclusion

Conjugation of acetylsalicylic acid and ibuprofen to the tetradecane structure through esterification (**2**, **3**) substantially improved their blood–brain barrier permeability and thus attainable brain concentrations. Nielsen and Bundgaard [16] however stated that esterification renders the acetyl ester of acetylsalicylic acid extremely susceptible to enzymatic hydrolysis, with the half-life for deacetylation of acetylsalicylic acid esters being 1–3 min compared to a half-life of 2 h for the free drug in human plasma. In this study acetylsalicylic acid was detected in the CNS at far higher concentrations than salicylic acid after administration of the acetylsalicylate ester prodrug (**2**), indicating that this novel synthesised ester prodrug did not enhance the hydrolysis of the acetyl ester of the free drug. The levels of the two ester prodrugs were below the level of quantification after the 1 h time period indicating that, hydrolysis of the polycyclic ester took place at a high rate. This seems to be especially true for the brain as was confirmed by the higher brain to blood concentration ratio observed for the free drugs after administration of the ester prodrugs (**2**, **3**). The lower free drug concentrations observed with the amide prodrugs (**5**, **6**) could be attributed to incomplete hydrolysis of the amide bond, especially in the brain. This observation was in accordance with literature [17] and could probably also explain the much lower brain concentration of the free drug observed for both prodrugs. The acetylsalicylic acid brain to blood ratio observed for the acetylsalicylamide prodrug corresponds to that of the free drug (Table 1) and is a probable indication that hydrolysis of this compound, though slow, mostly occurs in the periphery.

The modified thiobarbituric acid (TBA) assay applied in this study measured the amount of MDA as an index of lipid peroxidation. From the results it is clear that all of the test compounds possess antioxidant properties in the presence of the H_2O_2, $FeCl_3$, ascorbic acid system. The study results also indicate that the novel synthesised prodrugs positively influenced the antioxidant activity of the drugs. Decades of research have highlighted the profound role oxidative stress plays in the pathogenesis of neurodegenerative disorders [18–20]. These novel prodrugs may therefore be neuroprotective because of their increased antioxidant activity, either through COX inhibition and anti-inflammatory action and/or by providing protection against oxidative damage through other mechanisms.

The novel synthesised prodrugs thus present enhanced blood–brain barrier permeability and increased antioxidant activity. In other words, more of the drugs were delivered to the site of action and its

neuroprotective ability was also increased. Furthermore, the amide prodrugs (**5**, **6**), though showing lower distribution to the brain, may be dual acting drugs in that they deliver free acetylsalicylic acid and ibuprofen into the CNS, with amantadine, which has widely described neuroprotective activity [21–23]. The kinetics and distribution of these prodrugs are currently being investigated further.

5. Experimental

5.1. Materials

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione was prepared according to the method of Cookson et al. [24] and amantadine.HCl was obtained from Sigma–Aldrich (South Africa). Analytical grade acetonitrile (Multisolvent) and methanol were purchased from Sharlau (Spain). Formic (99%) and hydrochloric acids (32%) were obtained from Saarchem (South Africa). Chloroform (Hipersolv for HPLC) was purchased from BDH (England) and 25% ammonia solution for analysis, was procured from Merck (Germany). Throughout analyte preparation double-distilled, de-ionised water was used.

5.2. Synthesis

A rearranged cage alcohol (**1**) was synthesised in order to effectively esterificate the relevant NSAIDs to the polycyclic cage (Scheme 1) [15]. Activation chemistry using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), yielded the ester prodrugs of acetylsalicylic acid (**2**) and ibuprofen (**3**). The corresponding amide prodrugs were synthesised employing amantadine (**4**) as the polycyclic moiety. *o*-Acetylsalicyloyl chloride was applied for the synthesis of the acetylsalicylamide prodrug (**5**) and activation chemistry, using N,N′-dicyclohexylcarbodiimide (DCC), was employed to obtain the amide prodrug of ibuprofen (**6**).

Characteristic MS and NMR signals served as confirmation for each structure.

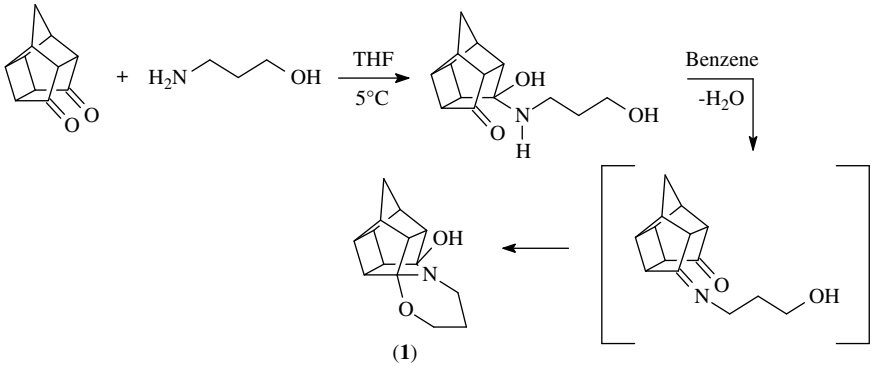
5.2.1. Physical data

*5.2.1.1. 3-Hydroxy-4-aza-8-oxo-heptacyclo[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradecane (**1**).* $C_{14}H_{17}NO_2$; **mp**: 170 °C; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H: 4.97–3.94 (bs, 1H, H-17), 3.85–3.74 (m, 2H, H-7a, 7b), 3.73–3.67 (m, 2H, H-5a, 5b), 3.02–2.53 (3 × m, 8H, H-1, 2, 10, 11, 12, 13, 14, 15), 1.80:1.52 (AB-q, 2H, **J** = 10.58 Hz, H-16a, 16b), 1.75–1.55 (m, 2H, H-6a, 6b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C: 101.45 (2 × s, C-3, 9), 62.69 (t, C-7), 54.97 (d, C-13), 53.14 (d, C-14), 45.73 (d, 1C), 44.63 (d, 1C), 44.00 (d, 1C), 42.92 (d, 1C), 42.41 (t, C-5), 41.67 (t, C-16), 41.48 (d, 1C), 41.01 (d, 1C), 24.33 (t, C-6); **HR-MS**: calc. 231.1259, exp. 231.1258; **MS** (EI, 70 eV) *m*/*z*: 231 (M^+), 174, 151, 139, 91, 41, 28; **IR** (KBr) ν_{max}: 3452, 1484, 1347, 1321, 1165 cm^{-1}.

*5.2.1.2. 3-{4-Aza-8-oxo-heptacyclo[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradec-yl}-2-(acetyloxy)benzoate (**2**).* $C_{23}H_{23}NO_5$; **mp**: 115 °C; **^{1}H NMR** (300 MHz, CD_3OD) δ_H: 7.96 (dd, 1H, **J** = 7.83, 1.51 Hz, H-24), 7.70–7.60 (m, 1H, H-22), 7.40–7.30 (m, 1H, H-23), 7.16 (dd, 1H, **J** = 8.10, 0.89 Hz, H-21), 4.82–3.67 (2 × m, 4H, H-5a, 5b, 7a, 7b), 3.30–2.61 (3 × m, 8H, H-1, 2, 10, 11, 12, 13, 14, 15), 2.31 (s, 3H, H-27a, 27b, 27c), 1.87–1.57 (m, 2H, H-6a, 6b), 1.85:1.57 (AB-q, 2H, **J** = 11.05 Hz, H-16a, 16b); **^{13}C NMR** (75 MHz, CD_3OD) δ_C: 171.26 (s, C-18), 163.72 (s, C-26), 152.00 (s, C-20), 135.28 (d, C-22), 132.31 (d, C-24), 127.19 (d, C-23), 124.90 (d, C-21), 124.66 (s, C-19), 63.91 (t, C-7), 58.23 (d, C-14), 56.50 (d, C-13), 47.04 (d, 1C), 45.10 (t, 2C), 44.97 (d, 1C), 44.36 (d, 1C), 43.03 (t, C-5), 41.37 (t, C-16), 30.91 (d, 1C), 24.68 (t, C-6), 21.04 (q, C-27); **HR-MS**: calc. 393.1576, exp. 393.1598; **MS** (EI, 70 eV) *m*/*z*: 393 (M^+), 365, 351, 270, 231, 230, 163, 121, 91; **IR** (KBr) ν_{max}: 1762, 1710, 1603, 1480, 1454, 1198, 1046, 912, 753, 701, 649 cm^{-1}.

*5.2.1.3. 3-{4-Aza-8-oxo-heptacyclo[9.4.1.0^{2,10}.0^{3,14}.0^{4,9}.0^{9,13}.0^{12,15}]tetradec-yl}-2-(4-isobutylphenyl)propanoate (**3**).* $C_{27}H_{33}NO_3$; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H: 7.15 (d, 2H, **J** = 8.10 Hz, H-22, 26), 7.05 (d, 2H, **J** = 8.24 Hz, H-23, 25), 3.98–3.06 (3 × m, 5H, H-5a, 5b, 7a, 7b, 19), 3.04–2.48 (3 × m, 8H, H-1, 2, 10, 11, 12, 13, 14, 15), 2.46–2.33 (d, 2H, H-27a, 27b), 1.98–1.29 (3 × m, 8H, H-6a, 6b, 16a, 16b, 20a, 20b, 20c, 28), 1.00–0.73 (d, 6H, H-29a, 29b, 29c, 30a, 30b, 30c); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C: 172.59 (s, C-18), 140.45 (s, C-21), 140.41 (s, C-24), 129.27 (d, C-22/26), 127.13 (d,.C-25), 127.10 (d, C-23), 102.80 (s, C-3), 102.30 (s, C-9), 62.96 (t, C-7), 55.44 (d, C-13), 55.19 (d, C-14), 45.30 (d, 1C), 45.23 (d, 1C), 45.00 (t, C-5), 44.98 (t, C-16), 43.77 (d, 1C), 41.97 (d, 1C), 40.21 (d, 1C), 38.89 (d, 1C), 30.13 (d, C-28), 23.85 (t, C-6), 22.31 (q, C-20), 22.27 (q, C-29/30), 22.26 (q, C-29/30); **HR-MS**: calc. 419.2460, exp. 419.2447; **MS** (EI, 70 eV) *m*/*z*: 419 (M^+), 231, 230, 214, 188, 161, 57, 43; **IR** (KBr) ν_{max}: 2867, 1740, 1510, 1458, 1339, 1269, 1124, 849 cm^{-1}.

*5.2.1.4. 1-Adamantan-yl-2-[(adamantylamino)carbonyl]phenyl-acetate (**5**).* $C_{19}H_{23}NO_3$; **mp**: 173 °C; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H: 7.65 (dd, 1H, **J** = 7.69, 1.65 Hz, H-18), 7.44–7.34 (m, 1H, H-16), 7.30–7.20 (m, 1H, H-17), 7.03 (dd, 1H, **J** = 8.10, 1.09 Hz, H-15), 6.06–5.74 (s, 1H, H-11), 2.41–2.27 (s, 3H, H-21a, 21b, 21c), 2.15–2.00 (2 × m, 9H, H-2a, 2b, 3, 5, 7, 8a, 8b, 9a, 9b), 1.76–1.60 (m, 6H, H-4a, 4b, 6a, 6b, 10a, 10b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C: 169.06 (s, C-20), 164.68 (s, C-12), 147.44 (s, C-14), 131.11 (d, C-16), 130.12 (d, C-18), 129.43 (d, C-17), 126.22 (d, C-15), 122.84 (s, C-13), 52.35 (s, C-1), 41.63 (3 × t, C-2, 8, 9), 36.31 (3 × t, C-4, 6, 10), 29.42 (3 × d, C-3, 5, 7), 21.06 (q, C-21); **HR-MS**: calc. 313.1678, exp. 313.1668; **MS** (EI, 70 eV) *m*/*z*: 313 (M^+),



Scheme 1. Synthesis of the tetradecane cage compound (**1**).

271, 150, 135, 121, 94, 43; **IR** (KBr) ν_{max}: 3422, 1759, 1640, 1194, 909, 805, 753 cm^{-1}.

*5.2.1.5. 2-(4-Isobutylphenyl)-N-(adamantyl)propanamide (**6**).*

$C_{23}H_{33}NO$; **mp**: 145 °C; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H: 7.14 (d, 2H, **J** = 8.10 Hz, H-16, 20), 7.07 (d, 2H, **J** = 8.34 Hz, H-17, 19), 5.11–4.81 (s, 1H, H-11), 4.04–3.80 (q, 1H, H-13), 2.54–2.33 (d, 1H, H-21), 2.06–1.96 (m, 1H, H-22), 1.95–1.71 (m, 7H, H-2a, 2b, 8a, 8b, 9a, 9b, 3/5/7), 1.71–1.49 (m, 6H, H-4a, 4b, 6a, 6b, 10a, 10b), 1.47–1.39 (d, 3H, H-14a, 14b, 14c), 0.94–0.76 (d, 6H, H-23a, 23b, 23c, 24a, 24b, 24c); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C: 173.53 (s, C-12), 140.44 (s, C-15), 140.36 (s, C-18), 129.60 (d, C-20), 129.47 (d, C-16), 127.18 (d, C-17), 126.88 (d, C-19), 51.64 (s, C-1), 47.50 (d, C-13), 45.01 (t, C-21), 41.46 (3 × t, C-2, 8, 9), 36.33 (3 × t, C-4, 6, 10), 30.12 (d, C-22), 29.41 (3 × d, C-3, 5, 7), 22.33 (q, C-14), 22.27 (q, C-23/24), 22.24 (q, C-23/24); **MS** (EI, 70 eV) *m/z*: 412 (M^+), 287, 236, 162, 135, 119, 83, 55, 43; **IR** (KBr) ν_{max}: 3296, 2852, 1655, 1447, 1376, 805 cm^{-1}.

5.3. Biological evaluation

Approval for this study was obtained from the Ethics Committee for Research on Experimental Animals of the North-West University (Potchefstroom campus).

5.3.1. Animals

All animals were maintained under controlled laboratory conditions at a temperature of 21 ± 0.5 °C, and relative humidity of 50 ± 5%. Free access to food and water was allowed at all times.

5.3.2. Blood–brain barrier permeability (in vivo)

In order to determine the blood–brain barrier permeability of the novel synthesised prodrugs (**2**, **3**, **5**, **6**) (Fig. 2), an in vivo test model previously used in our laboratory was applied [2]. This involved the intraperitoneal (i.p) injection of the test compounds and free NSAIDs to C57BL/6 laboratory mice followed by decapitation 1 h after administration. This arbitrary time-point (1 h) was chosen as the result of pilot studies done by the above authors, using 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01). The hydrochloric salts of the ester compounds (**2**, **3**) were prepared to facilitate aqueous solubility. The amide compounds (**5**, **6**) were used as such. The test compounds were administered in the following solvent systems: Acetylsalicylic acid and compounds **1**–**3** in 40% (v/v) ethanol in water for injection; Compounds **5**, **6** in methanol (100%); Ibuprofen in polyethylene glycol 300 and amantadine.HCl (**4**) in propylene glycol (all volumes administered were below the LD_{50} for any particular solvent). Freshly constituted test preparations (38 mg/mL) were administered intraperitoneally. To obtain similar molar ratios of free NSAID in relation to the relevant cage conjugate, the following doses were administered: 150 mg/kg for acetylsalicylic acid, ibuprofen, amantadine.HCl (**4**) and compound **1**; 327.58 mg/kg for compound **2**; 305.08 mg/kg for compound **3**; 260.935 mg/kg for compound **5** and 246.883 mg/kg for compound **6**. After administration of the relevant compound, the animals were allowed free movement and ad lib access to water and food.

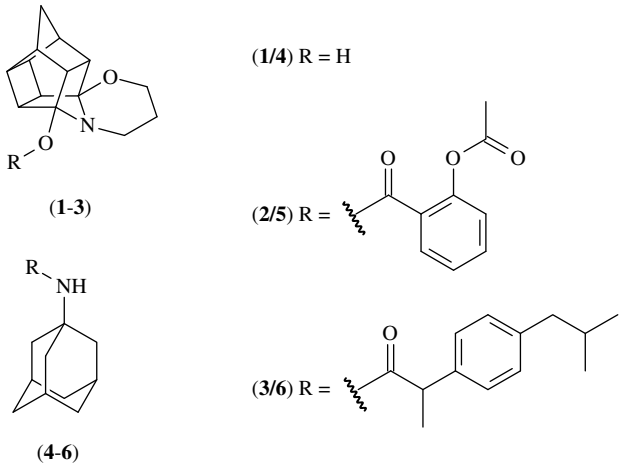


Fig. 2. Polycyclic structures synthesised in this study.

Animals were promptly sacrificed 1 h after administration by means of decapitation. Whole brain and blood samples were collected respectively in pre-weighed open-topped 15 mL Pyrex® vials and 4 mL BD Vacutainer™ vials containing 7.2 mg K3E anticoagulant. Samples were frozen with liquid nitrogen and kept at −77 °C until analysed. Experiments were repeated in triplicate. Naproxen (200 μg/mL) was used as internal standard throughout the experiments.

5.3.2.1. Instrumentation and analysis. After brain tissue and blood extractions were performed, the organic residues were re-dissolved in 250 μl methanol where after HPLC as well as, LC-MS/MS analyses were carried out.

For the HPLC analysis the mobile phase consisted of 75% Milli-Q water (containing 0.2% triethylamine, pH 7) and 25% acetonitrile at a flow rate of 1 mL/min. After 10 min of each HPLC run, a solvent gradient program was initiated by decreasing the aqueous mobile phase from 75% to 10% with a concomitant increase of acetonitrile from 25% to 90%. Analyses were performed using an Agilent® 1100 series HPLC equipped with a quaternary gradient pump, autosampler, diode array UV detector and Chemstation® Rev. A. 08.03 data acquisition and analysis software.

The mobile phase for the LC-MS/MS analysis comprised of 70% Milli-Q water (containing 0.1% formic acid) and 30% acetonitrile at a flow rate of 200 μl/min. Here a solvent gradient program was also employed by decreasing the aqueous mobile phase from 70% to 20% with a resultant increase of acetonitrile from 30% to 80% after 10 min. Analyses were carried out using an Agilent® 1100 series HPLC with a binary gradient pump, autosampler and vacuum degasser coupled to an Applied Biosystems® API 2000 triple quadrupole mass spectrometer and Analyst® 1.4 data acquisition and analysis software. Standard solution samples of each of the eight test compounds were prepared in analytical grade methanol and analysed using the described mobile phases. LC-MS/MS analyses were optimised by means of direct infusion of a standard solution using a Harvard syringe pump and selection of the two most prominent parent/daughter ion pairs for each component. The most prominent Multiple Reaction Monitoring (MRM) pair was used as quantifier and the other as qualifier. The same procedure was followed for the internal standard.

5.3.3. Attenuation of lipid peroxidation (in vitro)

The TBA test is the most prominent and currently used assay as an index for lipid peroxidation products [25] but it has been widely criticised because of the low efficiency of fatty-acid hydroperoxide breakdown to MDA [26]. Keeping this in mind, the TBA test was performed with a few modifications to improve its efficacy: Male Sprague–Dawley rats, weighing approximately 180–220 g, were decapitated and the brains rapidly excised and homogenised in 0.1 M phosphate-buffered saline (PBS), pH 7.4, so as to give a final concentration of 10% w/v. A system containing H_2O_2, $FeCl_3$ and ascorbic acid (vitamin C) was used to produce the necessary hydroxyl radicals through the Fenton reaction. This system allowed the constant regeneration of the ferrous (Fe^{2+}) ions in a reaction in which the ferric (Fe^{3+}) ions are reduced by ascorbic acid. The test

Table 2
Sample preparation for antioxidant assay.

Test tubes	10% Homogenate	PBS	H_2O_2 (5 mM)	$FeCl_3$ (4.88 mM)	Ascorbic acid (1.4 mM)	Test Compound (10 mM)[a]
Control	0.8 mL	0.2 mL	–	–	–	–
A (Toxin: H_2O_2 + $FeCl_3$ + asc. acid)	0.8 mL	0.1 mL	0.05 mL	0.025 mL	0.025 mL	–
B (Trolox + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
C (Acetylsalicylic acid + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
D (Ibuprofen + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
E (Compound 1 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
F (Compound 2 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
G (Compound 3 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
H (Amantadine 4 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
I (Compound 5 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL
J (Compound 6 + toxin)	0.8 mL	–	0.05 mL	0.025 mL	0.025 mL	0.1 mL

[a] Resulting H_2O_2 and test compound concentrations were 1 mM respectively.

tubes were prepared as described in Table 2 (A = toxin and B = positive control (Trolox + toxin)), and vortexed for approximately 60 s. Incubation was carried out at 37 °C for 1 h in an oscillating water bath. The test tubes were centrifuged for 20 min at 2000 × g and the supernatants decanted into new tubes. 0.5 mL butylated hydroxytoluene (BHT), 1 mL trichloroacetic acid (TCA) and 0.5 mL TBA were then added to every test tube and vortexed for 60 s. The test tubes were incubated for 1 h at 60 °C in an oscillating water bath and were then cooled down using an ice bath. Butanol (2 mL) was added to each test tube and it was vortexed for 60 s followed by centrifugation for 10 min at 2000 × g. The absorbencies of the supernatants were subsequently measured at 532 nm and butanol was used as blank.

All test compounds were dissolved in DMSO to give an end concentration of 10 mM. Earlier lipid peroxidation studies done in our laboratory indicated that DMSO, at this concentration, had no significant anti-oxidant activity. In this study Trolox, a model antioxidant, was used as positive control.

5.3.3.1. Statistical analysis. All data are presented as means ± SEM, indicated by error bars on the graph. Data analysis was carried out using a one-way analysis of variance (ANOVA) followed by the Student–Newman-Keuls multiple range test. The level of significance was accepted at $p < 0.05$.

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