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Synthesis and Radioprotective Effects of Adamantyl Substituted 1,4-Dihydropyridine Derivatives

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Abstract—A series of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid diesters substituted at the N-1 and/or C-4 positions of the dihydropyridine ring was synthesized. The in vitro cytotoxicity and in vitro and in vivo radioprotective efficacy of these agents were evaluated in Chinese hamster (V-79) cells and CD2F1 male mice, respectively. Compounds with at least one adamantyl substituent afforded better radioprotection than those without this substituent. Substitution of an aromatic ring at the C-4 position of the dihydropyridine ring did not enhance the radioprotectant action of the compounds.

Introduction

Radiation accidents such as the one that occurred at Chernobyl and increasing use of nuclear techniques in military and medical settings continue to fuel the need for safe and effective radioprotective agents. Phosphorothioate derivatives, such as S-[2-(3-aminopropyl)-aminoethyl]phosphorothioic acid ester (WR 2721), have demonstrated significant radioprotective effects.[1–3] Although these sulfhydryl-containing compounds are effective radioprotectors, they have a narrow therapeutic index and are plagued by significant side effects even at low doses.[4] The search for more effective and less toxic radioprotectants has spurred interest in the development of nonsulfur-based compounds. Recently, diadamantyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid diester (**1**) was reported as a nonsulfur-based synthetic radioprotective agent.[5] The compound demonstrated low toxicity and high radioprotective activity against ionizing irradiation in mice and rats. The adamantyl group has also been shown to enhance the activity of other radioprotectors, notably the mercaptoacetamidine phosphorothioates and disulfides.[6]

We have synthesized and examined the structure–activity relationships for introduction of substituents at the N-1 and C-4 positions of the dihydropyridine ring of **1** (see Chart 1). A 2-chlorophenyl moiety was selected as the C-4 substituent because Lehuede and coworkers[7] have recently demonstrated that incorporation of aromatic rings into the dihydropyridine ring increases the free radical scavenging ability of the compounds. Thus, the 2-chlorophenyl group could potentially increase the radioprotective effect of **1** by enhancing its free radical scavenging ability.[7] Additionally, the 2-chlorophenyl moiety has been shown to stabilize dihydropyridines towards hepatic metabolism.[8]

Results and Discussion

Chemistry

Synthesis of the key intermediate, adamantyl acetoacetate (**11**), is shown in Scheme 1. Adamantyl acetate (**10**) was prepared from 1-adamantanol (**8**) and acetic anhydride (**9**) with zinc chloride as the catalyst. Compound **10** was transformed to **11** in the presence of sodium and acetic acid. Hantzsch's procedure was adopted to synthesize the 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid diesters 1–7 (Chart 1).[9]

Key words: Radioprotectors; adamantyl; dihydropyridine; radiation; ionizing.
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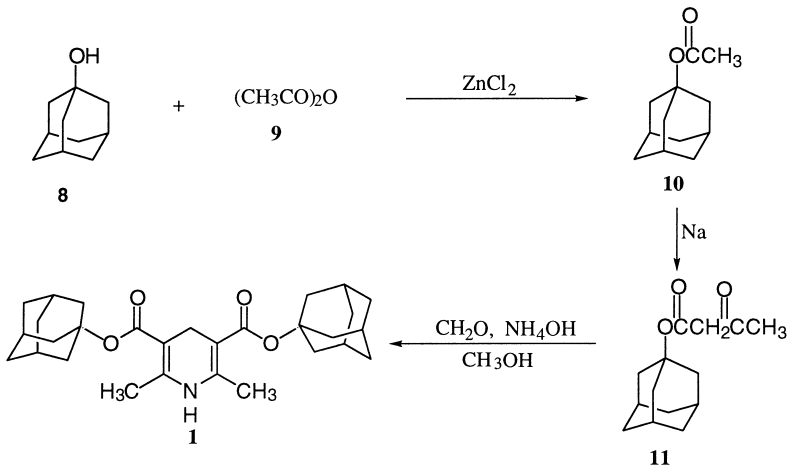


Scheme 1.

Briefly, two equivalents of the appropriate alkyl acetoacetate were reacted with one equivalent of the appropriate aldehyde and one equivalent of ammonium hydroxide or the appropriate alkylamine in methanol (Scheme 2). The reaction mixtures were refluxed for 6 h to obtain the corresponding 1,4-dihydropyridine derivative. Final products were purified by column chromatography and/or recrystallization.

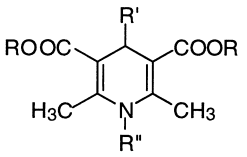
Radioprotection evaluation

The cytotoxicity and radioprotective effects of the dihydropyridine derivatives were initially determined in vitro against Chinese hamster (V-97) cells. The results of these studies are summarized in Table 1. All of the compounds were essentially nontoxic at the concentrations employed in these experiments against Chinese hamster (V-79) cells except compound **2** which caused approximately 28% inhibition in the colony forming ability of the cells at 0.1 mM. The data indicate that the substitution of a 2-chlorophenyl group at the C-4 position of the dihydropyridine ring and the presence of adamantyl esters at the 3 and 5 positions (as in compound **2**) appear to enhance the cytotoxicity of the resulting derivative. All the other derivatives which were either methyl esters (compounds **3**–**6**) or lacked 2-chlorophenyl group at the C-4 position were comparatively less toxic to the cells at the highest concentration of 0.1 mM than was compound **2**.

In vitro radioprotective effects of the compounds were studied with Chinese hamster (V-79) cells at a nontoxic dose of 0.05 mM. The dose modifying factor (DMF) was calculated from the radiation survival curve generated for each compound tested. The higher the DMF, the greater the radioprotective effect. The results of this study are shown in Table 1. Compound **1** with a DMF of 1.71 exhibited the highest radioprotective effect. None of the analogs was as effective as **1**. Compounds **2**–**4** demonstrated moderate DMF (1.32 to 1.45) while **5** and **6** had little effect. It should be noted that compounds **1**–**4** have 1 or 2 adamantyl substituents while **5** and **6** lack the same. This observation corroborates previous reports that adamantyl groups may be important in the radioprotective action of 1,4-dihydropyridines.[5] Since the position of the adamantyl moiety is significantly different from each other in compounds **1** and **3**, it appears that the lipophilic effects of the adamantyl group may be more important than its spatial location.

The compounds were evaluated in vivo for their radioprotective effects using male CD2F1 mice. Thirty day survival and mean survival times (MST) are reported in Table 1. In comparison to vehicle treated mice which had a MST of 7.7 days, all of the compounds except **6** showed a significant increase in MST (from 12.8 days for **7** to 18.3 days for **2**). In comparison to **1**, however, none of the new compounds were significantly more active. In agreement with the results of the in vitro studies, the adamantyl group afforded greater radioprotective effects to the compounds.

The objective of this study was to determine if structural analogs of **1** would afford similar or better radioprotection to mice. The in vitro and in vivo results indicate that the structural modifications reported in this study resulted in no significant improvement in the radioprotective effect. The results, however, reveal that while the position of substitution of the adamantyl group on the 1,4-dihydropyridine ring is not critical, its presence does enhance radioprotection in comparison to



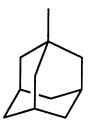
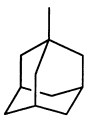
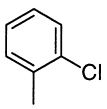
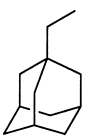
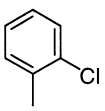
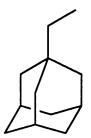
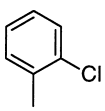
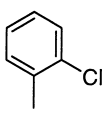
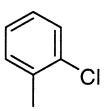
#	R	R'	R"
1.		H	H
2.			H
3.	CH_3	H	
4.	CH_3		
5.	CH_3		H
6.	CH_3		
7.	CH_2CH_3		

Chart 1.

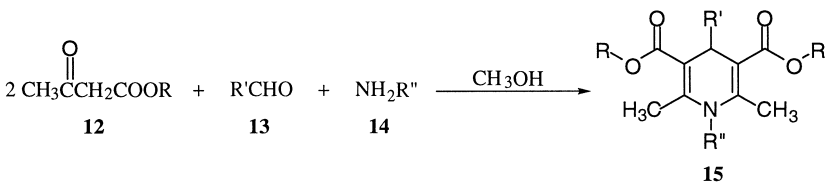
similar compounds lacking this group. This finding is in agreement with earlier reports that the adamantyl moiety enhances the radioprotective effects of potential radioprotectants.[5,6] Our results are however, at odds with an earlier report[7] which indicated that substitution of aromatic rings in dihydropyridines increases their free radical scavenging ability. Based on the earlier report,[7] we introduced a 2-chlorophenyl moiety at the 4-position of the dihydropyridine ring of **1** (as in compound **2**) but did not observe enhancement in the radioprotective effect of **1** with respect to 30-day survival.

The recognized mechanism of action of dihydropyridine calcium channel blockers is the inhibition of the entry of calcium ions across the cell membrane. The mechanism of action of these compounds as radioprotective agents has not been fully investigated. However, it has been suggested that this class of agents may interfere with the damaging effects of intracellular influx of calcium after membrane injury by radiation-induced free radicals.[10] Alternatively, the calcium antagonists may also directly inactivate free radicals.[11]

Experimental

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI, USA.). Solvents were dried prior to use when necessary. Tetrahydrofuran was freshly distilled from sodium and benzophenone. Melting points were determined on a HAAKE micromelting point apparatus and are uncorrected. Proton NMR spectra were recorded on a 500 MHz GE NMR Spectrometer, and values are reported in parts per million (ppm) from Me_4Si. Thin layer chromatography (TLC) was performed on precoated silica gel plates (Sigma Chemical Company, St. Louis, MO, USA.). The compounds were visualized at 254 nm under an UV lamp. Column chromatography was performed with silica gel 60 Å (230–400 mesh, Aldrich, Milwaukee, WI). Elemental analyses (C,H,N) were performed at M-H-W Laboratories (Phoenix, AZ, USA.) and are within 0.4% of the theoretical values.

Adamantyl acetate (10). A mixture of 1-adamantanol (7.6 g, 50 mmol), acetic anhydride (50 mL) and zinc



Scheme 2.

Table 1. Cytotoxicity and radioprotective effects of dihydropyridine derivatives

Compound	Cytotoxicity							
	Conc. (mM)	Survival (%)	In vitro DMF[a]	Dose[b]	In vivo survivors[c]	MST[c]	T/C%[d]	p[e]
Control	NA[f]		NA		0/10	7.7 ± 0.9	NA	—
WR2721	0.1	100.0	1.40	200	8/10	27.4 ± 1.8	263	< 0.001
	0.5	2.0						
	1.0	0.0						
1	0.01	91.6						
	0.05	83.5	1.71	23.3	3/10	19.1 ± 2.6	248	0.002
	0.1	90.0						
2	0.01	86.1						
	0.05	79.3	1.35	28.9	2/10	18.3 ± 3.1	238	0.005
	0.1	72.2						
3	0.1	91.8						
	0.05	93.3	1.45	18.6	3/10	17.9 ± 2.6	286	0.004
	0.1	90.0						
4	0.01	87.8						
	0.05	86.9	1.32	24.2	1/10	14.0 ± 1.9	182	0.012
	0.1	85.4						
5	0.01	95.4						
	0.05	85.0	1.13	16.8	1/10	13.3 ± 2.0	173	0.039
	0.1	83.0						
6	0.01	96.1						
	0.05	91.9	1.02	17.5	0/10	9.1 ± 1.1	118	> 0.1
	0.1	90.5						
7	0.01	ND[f]						
	0.05	ND	ND	18.9	0/10	12.8 ± 2.4	166	0.070
	0.1	ND						

[a]Dose modifying factor (DMF) was determined by dividing the D_o value (the radiation dose required to reduce the survival by a factor of 0.37 in the exponential region of the curve) obtained from the radiation survival curve in the presence of a radioprotective agent by the D_o value obtained from the control radiation survival curve. The drug concentration used in radioprotective activity study on Chinese hamster (V-79) cells was 0.05 mM.

[b]The dose is in mg/kg and was based on 0.05 mmol/kg for all the compounds except for WR2721, which was based on 1 mmol/kg.

[c]30-Day survival and mean survival time (MST) ± S.E.M. were evaluated at 10 Gy. Mice surviving more than 30 days were counted as 30 day survivors in the calculation of MST.

[d]T/C% = MST of treated mice (T) divided by MST of control mice (C) multiplied by 100.

[e]p value for a two-sample t-test with unequal variances.

[f]nd = not determined; na = not applicable.

chloride (0.1 g) was refluxed with stirring for 4 h. The reaction mixture was allowed to cool to room temperature and the pH was adjusted to 10 with 6 N potassium carbonate followed by extraction with ethyl acetate (3×30 mL). The combined organic extracts were dried over sodium sulfate and evaporated to dryness. The resulting residue was purified by column chromatography with methylene chloride as the eluant to give 6.6 g (68%) of **10** as a colorless liquid. ^{1}H NMR ($CDCl_3$) δ 1.65 (m, 6H, adamantyl), 1.95 (s, 3H, CH_3), 2.08 (d, 6H, adamantyl), 2.14, (br. s, 3H, adamantyl).

Adamantyl acetoacetate (11). A mixture of adamantyl acetate (**10**, 6 g, 31 mmol) and sodium metal (0.36 g, 15 mmol) cut into small pieces were gently heated and stirred under nitrogen until the mixture solidified. After cooling, 20 mL of 50% aqueous acetic acid was added and the mixture was extracted with ethyl acetate (3×20 mL). The combined extracts were dried over sodium sulfate and evaporated to dryness. The resulting residue was purified by column chromatography using methylene chloride as an eluant to give 0.78 g (22%) of **11** as a colorless liquid. ^{1}H NMR ($CDCl_3$) δ 1.64–2.15 (m, 15H, adamantyl), 2.22 (s, 3H, CH_3), 3.33 (s, 2H, CH_2).

General procedure for the preparation of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylates (1–7). A mixture of the appropriate alkyl acetoacetate (**12**, 2.2 mmol), the appropriate aldehyde (**13**, 1 mmol), and concentrated ammonium hydroxide or the appropriate amine (**14**, 1 mmol) in methanol (100 mL) was refluxed for 6 h. The mixture was allowed to cool to room temperature and the resulting solid was filtered and purified by either

recrystallization or column chromatography to yield the corresponding 1,4-dihydropyridine derivative (**15**).

Diadamantyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (1). Compound **1** was obtained in 45% yield after recrystallization from methylene chloride/methanol mixture. Melting point 216 °C (dec.). ^{1}H NMR ($CDCl_3$) δ 1.62–2.22 (m, 30H, adamantyl), 2.25 (s, 6H, 2,6-dimethyl), 2.78 (s, 2H, CH_2), 4.82 (br. s 1H, NH). Anal. calcd for $C_{29}H_{39}NO_4$: C, 74.80; H, 8.40; N, 3.01. Found: C, 74.60; H, 8.04; N, 2.94.

Diadamantyl-2,6-dimethyl-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (2). Compound **2** was obtained in 31% yield after purification by column chromatography with methylene chloride as the eluant. Melting point 135 °C (dec.). ^{1}H NMR ($CDCl_3$) δ 1.64–2.16 (m, 30H, adamantyl), 2.27 (s, 6H, 2,6-dimethyl), 5.36 (s, 1H, 4-CH), 5.53 (s, 1H, NH), 7.17–7.41 (m, 4H, aromatic). Anal. calcd for $C_{35}H_{42}ClNO_4$: C, 72.96; H, 7.35; N, 2.43. Found: C, 72.74; H, 7.16; N, 2.32.

Dimethyl-2,6-dimethyl-*N*-(1-adamantylmethyl)-1,4-dihydropyridine-3,5-dicarboxylate (3). Compound **3** was obtained in 69% yield after purification by column chromatography with ethyl acetate:hexane (1:4) as the eluant. Melting point 78–84 °C (dec.). ^{1}H NMR ($CDCl_3$) δ 1.52–1.98 (m, 15H, adamantyl), 1.88 (s, 6H, 2,6-dimethyl), 2.83 (s, 2H, 4-CH_2), 3.62 (s, 6H, 3,5-di$COOCH_3$), 4.41 (s, 2H, N-CH_2). Anal. calcd for $C_{22}H_{31}NO_4$: C, 70.75; H, 8.37; N, 3.75. Found: C, 70.46; H, 8.37; N, 3.86.

Dimethyl-2,6-dimethyl-*N*-(1-adamantylmethyl)-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (4). Compound **4** was obtained in 65% yield after recrystallization from methylene chloride/methanol mixture. Melting point 148–151 °C. ^{1}H NMR ($CDCl_3$) δ 1.55–2.03 (m, 15H, adamantyl), 1.80 (s, 6H, 2,6-dimethyl), 3.01 (m, 2H, N-CH_2), 3.52 (s, 3H, $COOCH_3$), 3.70 (s, 3H, $COOCH_3$), 6.23 (s, 1H, 4-CH), 7.09–7.28 (m, 4H, aromatic). Anal. calcd for $C_{28}H_{34}ClNO_4$: C, 69.47; H, 7.08; N, 2.89. Found: C, 69.62, H, 6.86, N, 2.82.

Dimethyl-2,6-dimethyl-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (5). Compound **5** was obtained in 60% yield after recrystallization from methylene chloride/methanol. Melting point 192–193 °C. ^{1}H NMR ($CDCl_3$) 2.31 (s, 6H, 2,6-dimethyl), 3.60 (s, 6H, 3,5-di$COOCH_3$), 5.29 (s, 1H, 4-CH), 5.64 (br. s, 1H, NH), 7.03–7.36 (m, 4H, aromatic). Anal. calcd for $C_{17}H_{18}ClNO_4$: C, 60.81; H 5.40; N, 4,17. Found: C, 61.00; H, 5.42; N, 4.56.

Dimethyl-2,6-dimethyl-*N*-methyl-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (6). Compound **6** was obtained in 34% yield after recrystallization from methylene chloride:methanol (1:1). Melting point 134 °C (dec.). ^{1}H NMR ($CDCI_3$) δ 1.56 (s, 3H, CH_3), 1.80 (s, 3H, CH_3), 3.00 (s, 3H, N-CH_3), 3.50 (s, 3H, $COOCH_3$), 3.73 (s, 3H, $COOCH_3$), 6.26 (s, 1H, 4-CH), 7.03–7.34 (m, 4H, aromatic). Anal. calcd for $C_{18}H_{20}ClNO_4$: C, 61.80; H, 5.76; N, 4.00. Found: C, 62.08; H, 5.79; N, 3.99.

Diethyl-2,6-dimethyl-*N*-methyl-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (7). Compound **7** was obtained in 40% yield after recrystallization from methylene chloride:methanol (1:1). Melting point 128–129 °C. ^{1}H NMR ($CDCI_3$) δ 1.20 (t, 6H, CH_3 of 3,5-diCOOCH2CH_3), 2.41 (s, 6H, 2,6-dimethyl), 3.21 (s, 3H, N-CH_3), 4.05–4.14 (m, 4H, CH_2 of 3,5-di-$COOCH_2CH_3$), 5.56 (s, 1H, 4-CH), 7.06–7.25 (m, 4H, aromatic). Anal. calcd for $C_{20}H_{24}ClNO_4$: C, 63.57; H, 6.40; N, 3.71. Found: C, 63.70; H, 6.39; N, 3.71.

Evaluation of in vitro radioprotective effects

The cytotoxicity and radioprotective efficacy of the dihydropyridine derivatives were determined using asynchronous exponentially growing monolayer cultures of Chinese hamster (V-79) cells. The methods of culturing and measuring cell survival by colony formation were reported previously by Agrawal et al.[12] V-79 cells were grown in Eagle's minimum essential medium (MEM) with 15% fetal bovine serum. For cytotoxicity studies, approximately 250 cells were plated in petri dishes (60×15 mm) containing 3 mL of medium and were allowed to attach for 2 h. The medium was then removed by aspiration and replaced with 3 mL of medium without drug (controls) or containing the various concentrations of the drugs. The plates were incubated for 2 h at 37 °C. At the end of a 2 h period, the medium containing the drug was removed and replaced with 3 mL of fresh medium. The cultures were then incubated for 6 days at 37 °C in an atmosphere of 95% air and 5% CO_2. The resulting colonies were fixed in absolute ethanol, stained with methylene blue, and counted.

To determine in vitro radioprotective activity, varying numbers of cells were plated to provide approximately 200 colonies after exposure to various doses of irradiation (Gammacell 40 irradiator containing Cs-137 source, Nordion International, Ontario, Canada). The cell survival curves were generated for each compound after exposing the cells to 0.05 mM concentration of each drug under aerobic conditions at the radiation doses of 2 to 14 Gy. The D_o value (the radiation dose required to reduce the survival by a factor of 0.37 in the exponential region of the curve) was calculated for each compound, and the ratio of the D_o value of drug treated cells to the D_o value of the control cells provided the dose modifying factor (DMF) for each compound. The

D_o value for the control cells under these conditions was 2.3 Gy.

Evaluation of in vivo radioprotective effects

Male CD2F1 mice weighing between 22–24 g (Charles River Laboratories, Wilmington, MA, U.S.A.) were divided in groups of ten. Test compounds were dissolved in a mixture of normal saline and alcohol (9:1) and injected ip 30 min pre-irradiation at the doses indicated in Table 1. Whole body irradiation was carried out in a Gammacell 40 irradiator. The dose rate was equivalent to 1.16 Gy/min. The total irradiation dose was 10 Gy. Five mice held in a 5 in× 5 in ×1.2 in plastic box were placed in the middle of the chamber for irradiation. The animals after irradiation were housed in a vivarium and were observed for 30 days, keeping a daily record of deaths and survivors.[13] The radioprotective effects of the compounds were evaluated by comparing the mean survival time (MST) of treated mice with that of control mice (i.e. T/C, where "T" represents the MST of the treated group and "C" the MST of the control group). The per cent T/C values were calculated by multiplying the ratio of T/C by 100.

Acknowledgement

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