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Synthesis and Anticonvulsant Activity of *N*-Benzyloxycarbonyl-Amino Acid Prodrugs of Phenytoin

GERHARD K. E. SCRIBA AND DIDIER M. LAMBERT*

*Department of Pharmaceutical Chemistry, University of Münster, Hittorfstrasse 58-62, 48149 Münster, Germany and *Laboratory of Medicinal Chemistry and Radiopharmacy, Catholic University of Louvain, Avenue E. Mounier 73, CMFA 73.40, 1200 Brussels, Belgium*

Abstract

Glycine, which has weak anticonvulsant properties, has been shown to potentiate the activity of several antiepileptic drugs but not phenytoin. Recently, studies have shown that *N*-(benzyloxycarbonyl)glycine (Z-glycine) antagonized seizures more than glycine in addition to possessing activity in the maximal electroshock test, a convulsive model in which glycine is inactive. In the present study esters of 3-hydroxymethylphenytoin, a phenytoin prodrug, and Z-glycine as well as the homologous *N*-(benzyloxycarbonyl)-ω-amino acids, Z-β-alanine and Z-γ-aminobutyric acid (Z-GABA), were prepared and tested for their anticonvulsant and acute neurotoxic activities.

The phenytoin prodrugs were obtained by esterification of bis(2-oxo-3-oxazolidinyl)-phosphinic acid chloride-mediated esterification of 3-hydroxymethylphenytoin with the respective *N*-benzyloxycarbonyl-protected amino acid. The Z-glycine-phenytoin ester was the most active anticonvulsant derivative. Compared with phenytoin the compound exhibited a decreased median effective dose (ED50) in the MES test and an increased median toxic dose (TD50), resulting in an significantly improved protective index expressed as the ratio between TD50 and ED50.

The present data suggest that covalent binding of phenytoin to Z-glycine results in an improved pharmacological profile of the drug.

Glycine is one of the major inhibitory amino acids in the central nervous system (CNS) acting via the strychnine-sensitive glycine receptor localized mainly in the brain stem and the spinal cord (Betz 1992). Simultaneously, glycine acts as a co-agonist of the excitatory neurotransmitter glutamate by interaction with the strychnine-insensitive glycine binding site of the *N*-methyl-D-aspartate (NMDA) receptor complex (Leeson & Iversen 1994).

In-vivo, glycine displays weak anticonvulsant activity in several seizure models including the 3-mercaptopropionic acid test and the strychnine test, while it is inactive in the maximal electroshock test and the pentylenetetrazole test (Lapin 1981; Toth et al 1983). Recently, it was shown that *N*-(benzyloxycarbonyl)glycine (Z-glycine) exhibits a higher anticonvulsant activity than glycine after intraperitoneal administration, especially in the maximal electroshock test (Lambert et al 1994). Amide and ester derivatives of Z-glycine further increased the seizure-antagonizing action (Lambert et al 1995; Geurts et al 1998).

Co-administration of glycine potentiates the activity of several anticonvulsant drugs such as phenobarbital, carbamazepine, diazepam and valproate (Seiler & Sarhan 1984a, b; Toth & Lajtha 1984; Seiler et al 1985; Liu et al 1990; Peterson et al 1990; Peterson 1991). However, co-administration of glycine does not alter the anti-convulsant activity of phenytoin (Peterson et al 1990), one of the most frequently used antiepileptic drugs for the treatment of generalized tonic-clonic seizures (Rall & Schleifer 1990). Moreover, large doses of glycine ranging between 3 and 11 $g\,kg^{-1}$ (40–150 $mmol\,kg^{-1}$) administered either subcutaneously or orally were necessary to exert potentiation. Structural homologues of ω-amino acids such as β-alanine and γ-aminobutyric acid (GABA) were less effective than glycine (Seiler & Sarhan 1984a). GABA is an inhibitory

Correspondence: G. K. E. Scriba, Department of Pharmaceutical Chemistry, University of Münster, Hittorfstrasse 58-62, 48149 Münster, Germany.
E-Mail: scriba@uni-muenster.de

neurotransmitter in the CNS while β-alanine acts as an agonist at the strychnine-sensitive glycine receptor (Marvizon et al 1986).

The present study was conducted to evaluate the potential of prodrugs combining phenytoin and Z-amino acids with regard to a synergism of the compounds. The derivatives were synthesized by esterification of 3-hydroxymethylphenytoin, a phenytoin prodrug (Varia et al 1984), with Z-glycine, the homologous Z-β-alanine and Z-GABA and with Z-phenylalanine as an amino acid devoid of CNS activity.

Materials and Methods

General methods

Melting points were determined on a Kofler melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian Gemini 200 (Varian AG, Bremen, Germany). The chemical shifts are reported as δ (ppm) values relative to tetramethylsilane. Mass spectra were obtained on a Varian MAT 44S (Varian AG, Bremen, Germany) at 70 eV. Z-glycine, Z-phenylalanine and bis(2-oxo-3-oxazolidinyl)phosphinic acid chloride were obtained from Fluka (Deisenhofen, Germany). 3-Hydroxymethylphenytoin (Varia et al 1984), Z-β-alanine (Fiedler et al 1993) and Z-GABA (Blankespoor et al 1984) were synthesized as described. Methylene chloride was distilled over P_2O_5, triethylamine was distilled over KOH.

Prodrug synthesis

Two grams (7·1 mmol) of 3-hydroxmethylphenytoin, 7·1 mmol of the respective Z-amino acid, 1·5 g (15 mmol) of triethylamine and 1·9 g (7·5 mmol) of bis(2-oxo-3-oxazolidinyl)phosphinic acid chloride were stirred in 50 mL methylene chloride under nitrogen at room temperature for 2 h. The mixture was poured into ice-cold 0·05 M HCl and extracted twice with methylene chloride. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Column chromatography on silica gel (Merck, Darmstadt, Germany) using methylene chloride–methanol (100 : 1, v/v) as eluent yielded amorphous solids.

N-*Benzyloxycarbonyl-[(2,4-dioxo-5,5-diphenyl-3-imidazolidinyl)methoxy]acetic acid ester (Z-glycine-phenytoin).* Yield 2·2 g, 65%; mp 62–63°C, ^{1}H NMR (200 MHz) $CDCl_3$ δ: 7·66 (1H, s), 7·38–7·30 (15H, m), 5·58 (2H, s), 5·38 (1H, t, J = 5·6 Hz), 5·07 (2H, s), 3·93 (2H, d, J = 5·6 Hz); ^{13}C NMR (50 MHz) $CDCl_3$ δ: 172·2, 169·1, 156·3, 154·6, 138·5, 136·6, 128·9, 128·8, 128·6, 128·2, 128·1, 126·8, 70·4, 67·2, 62·4, 42·5; MS m/z: 473 (M^+), 266, 237, 194, 180, 165, 107, 91.

*3-(*N-*Benzyloxycarbonylamino)-[(2,4-dioxo-5,5-diphenyl-3-imidazolidinyl)methoxy]propionic acid ester (Z-β-alanine-phenytoin).* Yield 2·0 g, 58%; mp 49–50°C, ^{1}H NMR (200 MHz) $CDCl_3$ δ: 7·65 (1H, s), 7·38–7·30 (15H, m), 5·55 (2H, s), 5·37 (1H, t, J = 6 Hz), 3·34 (2H, dt, J = 6, 5·8 Hz), 2·48 (2H, t, J = 5·8 Hz); ^{13}C NMR (50 MHz) $CDCl_3$ δ: 172·3, 171·1, 156·3, 154·6, 138·6, 136·5, 129·3, 128·9, 128·8, 128·5, 128·1, 126·8, 70·4, 66·8, 62·1, 36·4, 34·2; MS m/z: 487 (M^+), 266, 237, 194, 180, 120, 91.

*4-(*N-*Benzyloxycarbonylamino)-[(2,4-dioxo-5,5-diphenyl-3-imidazolidinyl)methoxy]butanoic acid ester (Z-GABA-phenytoin).* Yield 2·7 g, 76%; mp 54–55°C, ^{1}H NMR (200 MHz) $CDCl_3$ δ: 8·04 (1H), 7·40–7·30 (15H, m), 5·51 (2H, s), 5·08 (1H, s), 5·03 (2H, s), 3·12 (2H, t, J = 7·2 Hz), 2·28 (2H, t, J = 7·2 Hz), 1·73 (2H, m); ^{13}C NMR (50 MHz) $CDCl_3$ δ: 172·4, 171·9, 156·6, 154·8, 138·7, 136·6, 128·8, 128·7, 128·5, 128·2, 127·5, 126·6, 70·3, 66·7, 62·0, 40·2, 31·0, 24·9; MS m/z: 501 (M^+), 394, 266, 237, 194, 180, 91.

*2-(*N-*Benzyloxycarbonylamino)-3-phenyl-[(2,4-dioxo-5,5-diphenyl-3-imidazolidinyl)methoxy]propionic acid ester (Z-phenylalanine-phenytoin).* Yield 2·0 g, 49%; mp 67–68°C, ^{1}H NMR (200 MHz) $CDCl_3$ δ: 8·01 (1H, s), 7·40–7·02 (20H, m), 5·63 (1H, d, J = 10·3 Hz), 5·53 (1H, d, J = 10·3 Hz), 5·32 (1H, d, J = 8·3 Hz), 5·06 (1H, d, J = 12 Hz), 4·98 (1H, d, J = 12 Hz), 4·64 (1H, m), 3·00 (2H, m); ^{13}C NMR (50 MHz) $CDCl_3$ δ: 172·1, 170·4, 155·6, 154·6, 138·6, 138·5, 135·3, 129·3, 128·9, 128·7, 128·6, 128·5, 128·1, 128·0, 127·9, 127·1, 126·8, 70·4, 67·0, 62·6, 54·7, 37·9; MS m/z: 563 (M^+), 413, 266, 237, 208, 194, 180, 160, 131, 91.

Prodrug hydrolysis

Hydrolysis was performed at 20 ± 0·5°C in 5% rat plasma diluted with phosphate-buffered saline and 50 mM phosphate buffer, pH 7·4 containing 30% acetonitrile. Stock solutions of the compounds in acetonitrile were added to the prewarmed incubation mixtures to give a final concentration of 1 mM. The reaction mixtures were vigorously stirred during the kinetic run. Samples (100 μL) were

quenched by addition to 50 μL ice-cold 0·5 M perchloric acid followed by dilution with 850 μL water–acetonitrile (70 : 30, v/v) and assayed by HPLC using a LiChrospher RP Select B column (125 × 4·6 mm, 5 μm) (Merck, Darmstadt, FRG) equipped with a guard column (25 × 4·6 mm, 5 μm) filled with the same adsorbent. The mobile phase consisted of 0·05 M phosphate buffer, pH 5·0, containing 40% acetonitrile (v/v). The flow rate was 1·0 mL min^{-1}. Phenytoin and prodrug concentrations were calculated from calibration curves obtained by analysis of the pure compounds under identical chromatographic conditions.

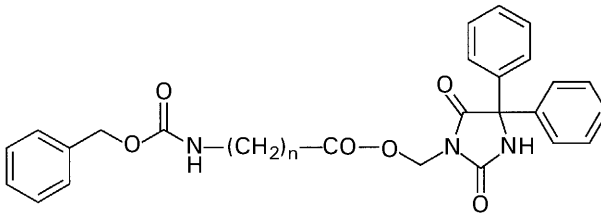
Pharmacology

Anticonvulsant testing was provided by the Antiepileptic Drug Development Program, Epilepsy Branch, Division of Convulsive, Developmental and Neuromuscular Disorders, National Institutes of Health, according to standard procedures (Porter et al 1984) and included the maximal electroshock test and the seizure threshold test with subcutaneous pentylenetetrazole. The acute neurological toxicity was determined in the rotorod test. For all these evaluations the compounds were dissolved or suspended in 0·5% aqueous methyl cellulose.

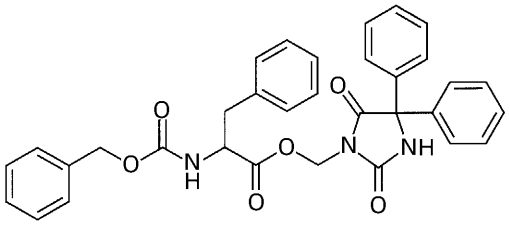
Results and Discussion

The structures of the phenytoin Z-amino acid esters are given in Figure 1. They were readily obtained by bis(2-oxo-3-oxazolidinyl)phosphinic acid chloride-mediated esterification of 3-hydroxymethylphenytoin with the respective N-benzyloxycarbonyl-protected amino acid. The compounds degraded slowly in aqueous buffer, pH 7·4, with half-lives between 208 h (Z-glycine-phenytoin) and 313 h (Z-GABA-phenytoin). In contrast, phenytoin was quickly liberated from all esters by rat plasma esterases (Table 1). Phenytoin was the only detectable degradation product in the incubations. The primary product of the hydrolysis, 3-hydroxymethylphenytoin, has been shown to liberate phenytoin at physiological pH with a half-life of 2–4 s (Varia et al 1984).

Pharmacological testing included the maximal electroshock test and the subcutaneous pentylenetetrazole test as well as the determination of the acute neurotoxicity using the rotorod test. For comparison, an equimolar mixture of 3-hydroxymethylphenytoin and Z-glycine was also investigated. The median effective doses (ED50) in the maximal electroshock test and the median toxic



n = 1 Z-glycine-phenytoin
n = 2 Z-β-alanine-phenytoin
n = 3 Z-GABA-phenytoin



Z-phenylalanine-phenytoin

Figure 1. Structures of the Z-amino acid phenytoin esters.

doses (TD50) in the rotorod test, upon intraperitoneal administration of the compounds to mice, are summarized in Table 1. All esters, as well as the 1 : 1 mixture, exhibited anticonvulsant activity in the maximal electroshock test. Z-Glycine-phenytoin was the most effective derivative. Compared with the administration of phenytoin on a molar basis, the ED50 was reduced by about 10% while the TD50 increased significantly (approx. 2·6-fold). As a net result the protective index (the ratio between TD50 and ED50) increased by a factor of 1·7. Based on these data, Z-glycine-phenytoin is a safer drug than the parent compound phenytoin. Interestingly, co-administration of equimolar amounts of Z-glycine and 3-hydroxymethylphenytoin also increased the protective index due to a slight increase of the TD50, while the ED50 in the MES test remained unaltered. However, neither increase seen with the mixture was significantly different from phenytoin itself.

The Z-β-alanine and Z-GABA derivatives also had an increased protective index, although to a lesser extent than Z-glycine-phenytoin. On a molar basis, the compounds were about half as active as phenytoin, but also less toxic than phenytoin itself. Both β-alanine and GABA were found to be less effective at potentiating the seizure-antagonizing activity of the GABA uptake inhibitor γ-vinyl-GABA in the 3-mercaptopropionic acid test when compared with glycine (Seiler & Sarhan 1984a). Esterification with Z-phenylalanine, an amino acid

Table 1. Median effective dose (ED50), median toxic dose (TD50) and protective index of phenytoin and the Z-amino acid phenytoin esters after intraperitoneal administration to mice and half-lives of the plasma-esterase-catalysed in-vitro hydrolysis of the ester derivatives.

Compound	MW	ED50 ($mg\,kg^{-1}$)[a]	TD50 ($mg\,kg^{-1}$)[a]	Protective index (TD50/ED50)	Plasma half-life (min)[b]
Phenytoin[c]	252·3	6·5 (5·6–7·2) [2 h]	42·8 (36·4–47·5) [2 h]	6·6	–
Phenytoin + Z-glycine[d]	491·5	12·4 (10·1–15·1) [2 h]	105 (90·3–167) [2 h]	8·5	–
Z-Glycine-phenytoin	473·5	10·6 (9·2–12·1) [2 h]	118 (100–138) [4 h]	11·2	1·0 ± 0·1
Z-β-Alanine-phenytoin	487·5	29·0 (22·5–35·0) [8 h]	256 (213–306) [8 h]	8·8	2·3 ± 0·4
Z-GABA-phenytoin	501·5	26·2 (18·8–35·0) [2 h]	245 (220–262) [2 h]	9·3	1·1 ± 0·1
Z-Phenylalanine-phenytoin	563·6	32·6 (26·1–42·0) [2 h]	193 (142–271) [4 h]	5·9	1·6 ± 0·3

[a]The data were calculated from 5–6 doses (n = 8–16 animals per dose). 95% confidence intervals are given in parentheses. The time of testing is listed in square brackets. [b]5% heparinized rat plasma in phosphate-buffered saline, 20°C, mean ± s.d., n = 3–4. [c]Data from Dimmock & Baker (1994). [d]Equimolar mixture of 3-hydroxymethylphenytoin and Z-glycine.

devoid of CNS activity, did not increase the protective index. Thus, combination of phenytoin with an inhibitory amino acid appears to be necessary for an improvement of the drug. Z-β-alanine, Z-GABA and Z-phenylalanine did not display any effect in the maximal electroshock test nor any toxicity up to a dose of $300\,mg\,kg^{-1}$.

As with phenytoin itself (Porter et al 1984; Dimmock & Baker 1994), none of the investigated compounds or the equimolar mixture of Z-glycine and 3-hydroxymethylphenytoin, exhibited any significant activity in the subcutaneous pentylenetetrazole test up to a dose of $300\,mg\,kg^{-1}$. Thus, combinations of phenytoin and Z-amino acids did not alter the pharmacological profile of the drug, as has been reported for dihydropyridine esters of 3-hydroxymethylphenytoin (Shek et al 1989). The dihydropyridine derivatives only slightly increased the protective index after intravenous administration to rats (Shek et al 1989).

It is interesting to note that the time of the peak effect of the maximal electroshock activity of Z-β-alanine-phenytoin is 8 h compared with 2 h for the other esters and phenytoin itself. The peak of the neurotoxicity of all amino acid esters, as well as the equimolar mixture of 3-hydroxymethylphenytoin and Z-glycine, is also retarded compared with phenytoin, although plasma esterase-catalysed hydrolysis proceeded extremely quickly for all esters. The rapid hydrolysis suggests a prodrug mechanism for the Z-amino acid phenytoin esters. However, the retardation of the peak effects suggests that factors other than just hydrolysis into the individual components of the prodrugs might contribute to the pharmacological activity.

In conclusion, esterification of phenytoin with *N*-benzyloxycarbonyl derivatives of inhibitory amino acids resulted in an increase of the protective index compared with the parent compound, phenytoin, while the pharmacological profile remained unaltered. Z-Glycine-phenytoin was the most effective compound. Co-administration of equimolar amounts of Z-glycine and 3-hydroxymethylphenytoin also increased the protective index although the synergism was not as effective as by covalent binding.

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