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Research paper

Chemical and enzymatic stability evaluation of lipoamino acid esters of idebenone

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Abstract

Lipophilic conjugates of idebenone (IDE) with short-chain alkylamino acids were previously synthesized and evaluated in vitro for their antioxidant properties. In this study, their susceptibility to chemical and enzymatic hydrolysis was evaluated. Results indicated that these derivatives release the parent drug quantitatively via enzymatic hydrolysis by serum and liver esterases, with a cleavage rate related to the length of the alkyl side chain. Consequently, the present lipoamino acid conjugates of IDE are prodrugs and their in vivo effects are mediated through the parent compound released in the body.

Keywords: Idebenone; Lipoamino acids; Prodrugs; Hydrolysis; Stability; Lipophilicity

1. Introduction

Lipophilic prodrugs of CNS-active agents can represent a useful strategy to overcome the resistance created by the blood–brain barrier (BBB) against many drugs, which would need to reach their target site of action inside the brain.

In our previous studies, the conjugation of drugs with lipoamino acids (LAAs) has been proposed as a means of enhancing the lipophilicity of drugs, while giving them *membrane-like character* which can increase the uptake by/penetration through biological membranes and barriers [1–3]. LAAs are α-amino acids bearing an alkyl side chain, the structure and length of which can be varied to obtain the desired physico-chemical properties [4,5].

In a recent study [6], LAAs with short alkyl side chains (4–7 carbon atoms) have been conjugated through an ester bond to idebenone (IDE), to give conjugates **1–4** (Fig. 1). IDE [2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone], structurally related to ubiquinone, is a benzoquinone compound with antioxidant properties, whose potential in ameliorating the symptoms of cognitive decline frequently associated with dementia is known [7]. Thus, it is used in the treatment of vascular and degenerative diseases of the central nervous system, such as Friedreich's ataxia and Alzheimer's disease. IDE has recently also deserved attention for the treatment of oxidative diseases of the eye [8,9]. Ubiquinone is an essential component of the mitochondrial respiratory chain and, as a consequence, IDE can be expected to improve bioenergetic production in the respiratory chain in the presence of a mitochondrial complex I defect by acting as an electron-trapper at the mitochondrial membrane [10,11].

In a preliminary biological evaluation, the lower homologues **1** and **2** (Fig. 1) displayed an antioxidant activity comparable to or higher than IDE as scavengers of superoxide anion and as inhibitors of lipoperoxidation [6]. As is obvious for ester-structures, IDE–LAA conjugates are possible substrates for various esterases when administered in vivo. Therefore, the in vivo effects of the IDE–LAA conjugates may be mediated through the conjugate itself or its parent compound, IDE. Thus, for interpretation of the future experiments in vivo the susceptibility of compounds **1–4** to enzymatic bioconversion in serum and liver homogenate was studied.

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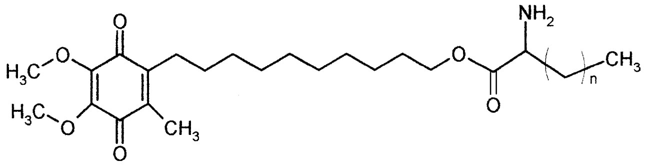

Conjugate	n
1	1
2	2
3	3
4	4

Fig. 1. Structure of IDE–LAA conjugates.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol was purchased from Labscan (Dublin, Ireland) and acetonitrile from Rathburn Chemical (Walkerburn, Scotland). Bovine serum albumin (BSA, >96%) was from Sigma Chemical Co. (St Louis, USA). All other solvents and reagents were of high-purity reagent-grade and used without further purification. IDE–LAA conjugates **1–4** were synthesized according to the methods previously described [6]. Stock solutions of **1–4** ($2\ \mathrm{mg\ ml^{-1}}$) were prepared in methanol and stored at − 20 °C.

2.2. Instrumentation

HPLC determinations were performed with a Merck LaChrom HPLC system, consisting of a Model L-7250 programmable autosampler, a Model L-7100 HPLC pump, a Model D-7000 interface module and a Model D-7000 HPLC system manager (Hitachi, Tokyo, Japan). An Orion SA 520 pH-meter (Orion Research Inc., Boston, MA, USA) equipped with a combination pH electrode was used for pH determination. The centrifuge used was an Eppendorf 5804 R (Eppendorf AG, Hamburg, Germany).

2.3. Chromatography

The analytical HPLC separations were performed on a Zorbax SB-Phenyl column (4.6 × 150 mm, 5 μm; Agilent, USA). The mobile phase for isocratic elution consisted of 20% (v/v) 20 mM phosphate buffer solution (pH 6.0) and 80% methanol, delivered at a flow-rate of $1.5\ \mathrm{ml\ min^{-1}}$. The detection was carried out by a UV detector at a wavelength of 280 nm. The injection loop used was a 100 μl stainless steel loop and injection volumes of 50 μl were typically used.

2.4. Chemical hydrolysis in aqueous solution

The chemical hydrolysis of conjugates **1–4** (initial concentration $25\ \mathrm{\mu g\ ml^{-1}}$) was studied in phosphate buffer (50 mM, $\mu = 0.15$, pH 7.4, 2.5% methanol, $30\ \mathrm{mg\ ml^{-1}}$ BSA). As this experiment serves as a control for serum hydrolysis studies, addition of BSA at a concentration close to human serum albumin concentration (35–50 mg/ml) was considered a practical means to increase the solubility of the starting material and hydrolysis products. The solution was placed in a thermostated water-bath at 37 ± 1 °C and, at suitable intervals, 200-μl samples were withdrawn and added to 400 μl of acetonitrile. After centrifugation (10 min, 10 000 rev./min, 5 °C), the supernatant was analysed by HPLC to determine the rate of disappearance of the compound. Pseudo-first-order half-lives ($t_{1/2}$; Table 2) were calculated from the linear slope of the logarithmic plot of remaining compound over time [12].

2.5. Enzymatic hydrolysis in human serum

Stock solution (100 μl) of **1–4** was added into 0.7 ml of phosphate buffer (50 mM, $\mu = 0.15$, pH 7.4). Pre-heated human serum (3.2 ml) was added and the solution was kept in a water bath at 37 ± 1 °C. At suitable intervals, 200-μl aliquots were taken and added to 400 μl of acetonitrile, in order to precipitate serum proteins. After mixing and centrifugation (10 min at 10 000 × *g* and 5 °C), 200 μl of the supernatant was taken for the HPLC analysis.

2.6. Enzyme hydrolysis in rabbit liver homogenate

The hydrolysis of compounds **1–4** was also studied in rabbit liver homogenate at 37 °C. Rabbit liver was homogenized with approximately four weight-equivalents of isotonic phosphate buffer (pH 7.4) using an Ika Ultra Turrax homogenizer (Janke and Kunkel, Staufen, Germany). This 20% homogenate was stored at − 80 °C until used. A volume of 100 μl of test compound stock solution was added to 3.88 ml of isotonic phosphate buffer (pH 7.4), followed by addition of 20 μl of pre-heated 20% liver homogenate. BSA ($30\ \mathrm{mg\ ml^{-1}}$) was added to increase the aqueous solubility of the test compounds and hydrolysis products. The solution was then incubated at 37 ± 1 °C. At appropriate intervals, 200 μl samples were withdrawn and added to 400 μl of acetonitrile. After mixing and centrifugation (10 min, 11 000 rev./min at 5 °C) the supernatant was analysed by HPLC.

2.7. Calculation of physicochemical properties

The partition coefficients and water solubilities for compounds **1–4** and IDE were calculated using two different software: the ACD LogP software, ver. 5.15 (Advanced Chemistry Development Inc., Toronto, Canada),

Table 1
Calculated LogP and water solubility values for IDE and its conjugates **1–4**

Compound	cLog *P*[a]	cLog *P*[b]	Water solubility[b] (Log *S*)[c]
IDE	3.49	4.21	−3.16
1	4.52	4.08	−3.79
2	5.06	4.54	−4.06
3	5.59	5.00	−4.33
4	6.12	5.47	−4.60

[a] Calculated by ACD LogP software.

[b] Calculated by Osiris Property Explorer.

[c] Unit stripped logarithm (base 10) of solubility in mol/l.

and the Osiris Property Explorer [13]. The results are presented in Table 1.

3. Results and discussion

IDE–LAA conjugates **1–4** (Fig. 1) showed an increased lipophilicity compared to the parent drug, judging by the calculated partition coefficients (Table 1) and a better ability to interact with biomembrane models, as assessed with calorimetric (DSC) experiments on phospholipid liposomes (unpublished data). The estimated aqueous solubility of the conjugates fall around 10^{-4} M, a value considered compatible with a drug molecule [13]. Additionally, the conjugates should have higher solubility in low pH due to their basic amine functionality, and this opens further formulation opportunities.

The susceptibility of conjugates **1–4** to enzymatic hydrolysis was assessed in two enzymatic systems (human serum and rabbit liver homogenate) using a corresponding enzyme-free chemical environment (pH 7.4 phosphate buffer solution) as a control.

To perform the first experiment correctly, due to the low solubility of IDE and its conjugates in the buffer solution, BSA was added to the solution. The selected BSA concentration (30 mg ml^{-1}) is comparable to serum albumin concentration in humans.

The stability of conjugates **1–4** towards chemical hydrolysis was related to the length of the alkyl side chain, with $t_{1/2}$ values ranging from 7.1 h for the butyl derivative **1** to 17 h for the longer heptyl derivative **4** (Table 2). HPLC analysis of the incubation media showed in all cases that the hydrolysis of the conjugates led quantitatively to the parent drug IDE, with no sign of further degradation of the latter into other degradation products.

The susceptibility of IDE–LAA conjugates to undergo enzymatic hydrolysis was first studied in 80% human serum. The observed degradation rate was much higher than in the buffer solution, with $t_{1/2}$ values ranging from 28 to 71 min. This result strongly suggests that these conjugates are susceptible to serum esterases. Also, in this case we observed that the longer the alkyl chain the higher the stability of the conjugate towards the hydrolytic cleavage of the ester bond, even if we actually detected a little discrepancy with compounds **3** and **4**, whose $t_{1/2}$ values were close. IDE–LAA conjugates released parent compound quantitatively (Fig. 2 gives a representative example of the serum hydrolysis experiments), and as discussed above for the chemical hydrolysis, no further degradation of released IDE was observed (data not shown).

Table 2
Hydrolysis half-lives (min) after incubation at 37 °C in human serum, rabbit liver homogenate and 50 mM phosphate buffer (pH 7.4) for lipoamino acid esters of IDE

Compound	Phosphate buffer (pH 7.4)	80% human serum	0.1% rabbit liver homogenate
1	426 ± 6	27.7 ± 0.2	0.72 ± 0.03
2	396 ± 6	41.2 ± 0.4	0.47 ± 0.04
3	696 ± 48	87.1 ± 1.0	0.49 ± 0.07
4	1044 ± 0	71.1 ± 1.6	0.80 ± 0.10

Values indicate the mean ± SD of two replicates.

The hydrolysis experiments performed in 0.1% rabbit liver homogenate (Table 2) further support the enzymatic lability of IDE–LAA conjugates. In fact, the hydrolytic sensitivity of conjugates **1–4** in liver homogenate was so high that the determined half-lives were below 1 min for all the compounds.

4. Conclusions

The present study suggests that IDE–LAA conjugates behave as prodrugs when administered in vivo, as they are able to release the parent IDE rapidly via enzymatic hydrolysis in vitro in serum and liver homogenate. However, high enzymatic degradation rates may account for poor availability of intact IDE–LAA conjugates for uptake at the BBB. For example, first-pass metabolism by liver esterases

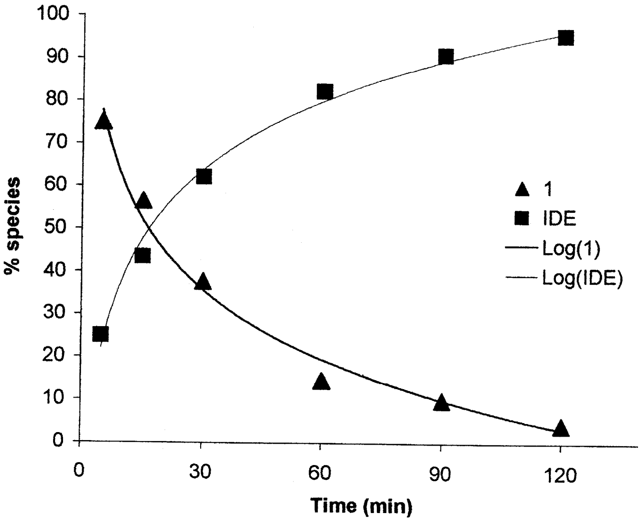

Fig. 2. Degradation profile of compound **1** to IDE in 80% human serum (pH 7.4, 37 °C).

may be anticipated to occur extensively after oral absorption. In addition to the latter route of administration, which however is not of primary interest to us, also topical routes like dermal, transdermal and ophthalmic applications may be desirable for IDE administration in the future.

As reported above, IDE has been recently proposed for treating oxidative eye diseases [8,9]. In this case, the rapid conversion of IDE–LAA conjugates into the active drug would be advantageous after absorption through the cornea into the anterior chamber of the eye. At the present time, pharmacological studies in the rabbit are in course to verify this potential application of IDE–LAA lipophilic conjugates.

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