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Lipidic peptides. VIII. Cellular uptake studies of lipidic amino acid, its oligomers and highly lipophilic drug conjugates

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Summary

Cellular uptake of two series of lipidic amino acid and peptides **1a–1c**, benzoquinolizine **2a** and conjugates **2b–2d** was studied. The lipidic amino acid methyl ester **1a** and benzoquinolizine monomer conjugate **2b** were taken up by erythrocytes, *E. coli* and hepatocytes more readily and to a greater extent than the fully protected dipeptide **1b**, tripeptide **1c** and conjugates **2c** and **2d**. It is believed that the dimer **1b**, trimer **1c** and the conjugates **2c** and **2d**, due to their increased lipophilic structures are retained in the plasma membrane. Metabolism of the conjugate **2b** in red blood cells was slow; after 30 min incubation no significant metabolites were detected.

Introduction

The lipidic amino acids and oligomers (Gibbons et al., 1990) can be covalently conjugated with or incorporated into poorly absorbed pharmacologically active peptides and drugs, in order to enhance their passage of the compounds across biological membranes (Toth et al., 1991). Studies on the cellular uptake of lipidic compounds have been reported (Cooper et al., 1987; Burns et al., 1988; Yung et al., 1989; Borlakoglu et al., 1990). Investigations on nucleosides showed that the liposome of the phospholipid conjugate of AZT has greater antiviral activity than that of the parent compound (Hosteler et al., 1989; Shea et al., 1990). If lipidic amino acids and their oligomers are to be effective as a drug or peptide delivery system, they should confer improved adhesion to and/or passage across cell membranes on the compounds to which they are conjugated. To investigate this, the behaviour of lipidic amino acids and their oligomers towards cell membranes was examined in a series of experiments using the ^{3}H-labelled amino acid methyl ester **1a**, the fully protected dimer **1b** and trimer **1c** in single-cell suspensions.

Uptake studies were carried out in red blood cells, to compare the cellular uptake of the ben-

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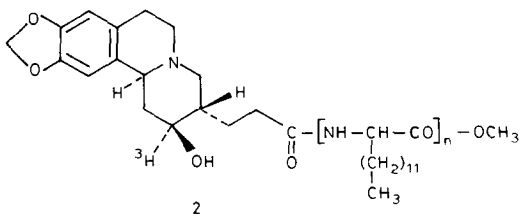
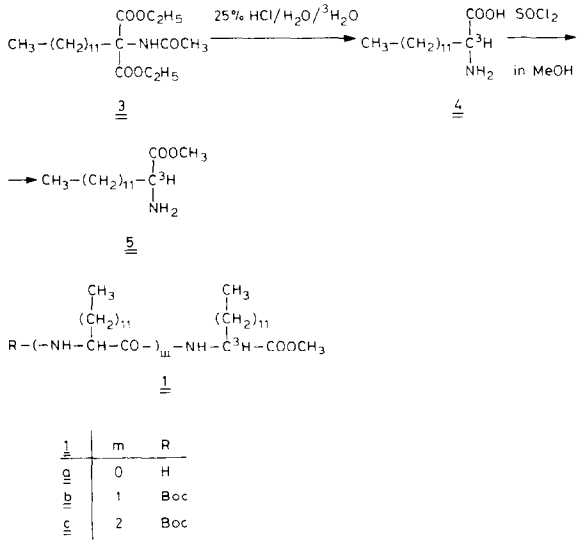


2

2	n
a	0
b	1
c	2
d	3

zoquinolizine conjugates **2a–2d** with that of the underivatised lipidic amino acid **1a** and oligomers **1b** and **1c**.



1	m	R
a	0	H
b	1	Boc
c	2	Boc

Scheme 1. Procedures followed for the synthesis of ^{3}H-labelled lipidic peptides.

Materials and Methods

Infrared spectra were recorded with a Perkin Elmer 841 spectrophotometer. ^{1}H-NMR spectra were obtained in Varian XL-300 and Bruker AM500 instruments operating at fields of 300 and 500 MHz, respectively; chemical shifts are reported in ppm downfield from internal TMS. Mass spectra were run on a VG Analytical ZAB-SE instrument, using fast atom bombardment (FAB) techniques. Reaction progress was monitored by thin-layer chromatography (TLC) on Kieselgel PF_{254} using dichloromethane : methanol 10 : 1 as the mobile phase. Purification was achieved by TLC using Kieselgel $PF_{254+366}$ (Merck) on 20 × 20 cm plates of 1.5 mm thickness, or column or flash chromatography through Kieselgel G. Solvents were evaporated under reduced pressure with a rotary evaporator.

Synthesis of tritium-labelled lipidic amino acid oligomers

A series of tritiated lipidic peptides, **1a–1c** labelled in the α-position of the C-terminal residue, were synthesized (Scheme 1). Their synthesis was achieved by the hydrolysis of ethyl (2-acetamido-2-ethoxycarbonyl)tetradecanoate **3**, using 25% HCl in the presence of ^3H_2O (100 mCi, 200-fold excess). In this way, 1 mmol (500 μCi) of amino acid **4** was produced. The labelled amino acid **4** was esterified by refluxing overnight in thionyl chloride in methanol to yield the methyl ester **1a** (1 mmol, 500 μCi). One part of this compound was used to prepare each of the two oligomers **1b** and **1c**, using the procedures described in the literature (Gibbons et al., 1990). Thus, starting from 0.25 mmol of methyl ester **1a**, 0.25 mmol of oligomers **1b** and **1c** were synthesized, each oligomer having 125 μCi radioactivity. Compounds **2a–2d** were prepared by literature methods (Toth et al., 1991).

Cell preparation

Erythrocytes Blood (19 ml) was collected with a syringe from the aorta of two male Sprague Dawley rats (300–400 g/rat, anaesthetized with 600 mg/kg phenobarbitone) into 1.0 ml sodium EDTA (0.1 M, pH 7.4). The plasma and buffy layer were collected by centrifugation (2500 rpm, MSE Major centrifuge, 8 min) and discarded. The erythrocytes were resuspended in 50 ml Ringer's saline and washed three times in this volume and once with 10 mol BSA-Ringer's (4% bovine serum albumin [98–99% fraction V powder, Sigma A7906] in Ringer's saline; filtered through 0.45 μm Millipore filter before use). The buffy layer was removed at each wash. Cells (7

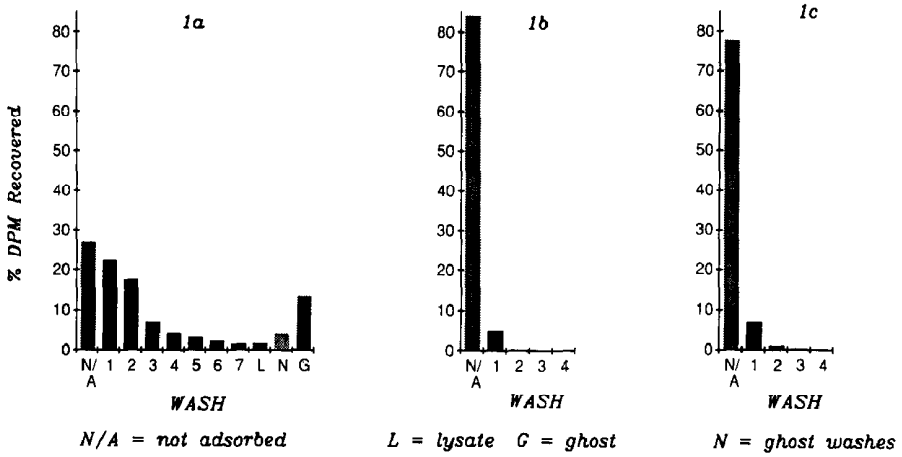


Fig. 1. Uptake of compounds **1a–1c** by rat erythrocytes.

ml) were concentrated by centrifugation at 3000 rpm (MSE Major centrifuge, 15 min) during the final wash. Of this concentrate, 1.2 ml centrifuged for 8 min at 13500 rpm in a Biofuge yielded a supernatant of 0.18 ml, indicating the erythrocyte concentrate was 85% packed cells. The concentrate was stored on ice until use. Rat hepatocytes were prepared as previously described (Moldeus et al., 1978). Gross cell aggregates were allowed to settle out (2 min, 0 °C) and discarded. Supernatant cells were collected by centrifugation (250 rpm, 1 min, 22 °C, MSE Major centrifuge) and washed twice with BSA-Ringers in which it was stored as a 50% suspension at 0 °C before use.

Escherichia coli strain KL16 *E. coli* cells were grown in Oxoid nutrient broth No. 2 (code: CM67) (Bachmann, 1972), and collected by centrifugation (0 °C, 7000 rpm, Sorvall GSA rotor, 5 min). Pelleted cells were resuspended in 500 ml glucose-salt solution (Hedgeman, 1966), held with shaking at 220 °C for 60 min, and collected by centrifugation. Pelleted cells were resuspended in 10 ml ice-cold 4% BSA-Ringers and stored at 0 °C until used.

Cellular uptake studies

All experiments were performed in duplicate. Cell cultures in DMEM-10% FCS (5 ml) were centrifuged (23000 rpm) and the supernatant carefully removed. A 1 mM compound solution was prepared (0.5% DMSO-DMEM-10% FCS), filtered and divided into 1 ml vials. Compounds **1a–1c** were incubated in the presence of three cell types, namely, rat erythrocytes, rat hepatocytes and *E. coli*. BSA (4%) was added to the incubation medium to solubilise compounds **1a–1c**. After various time intervals, the radioactivity remaining in the supernatant was counted, giving the amount of radiolabelled compound associated with the cells. The uptake of compounds **2a–2d** was examined using erythrocytes.

Metabolic stability

The red blood cells after the cellular uptake studies were washed and vortexed in organic sol-

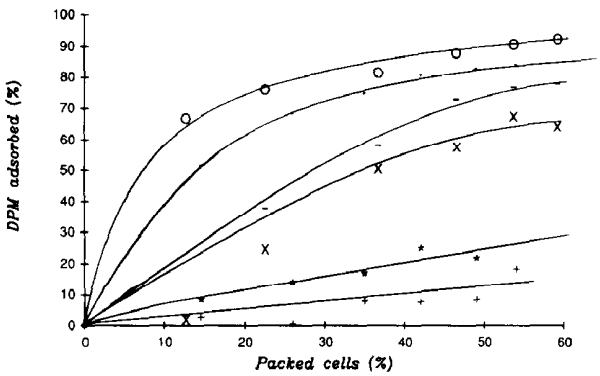


Fig. 2. Effect of cell concentration on uptake of compounds **1a–1c**; (·) **1a**, erythrocytes; (+) **1b**, erythrocytes; (*) **1c**, erythrocytes; (○) **1a**, *E. coli*; (×) **1b**, *E. coli*; (–) **1c**, *E. coli*.

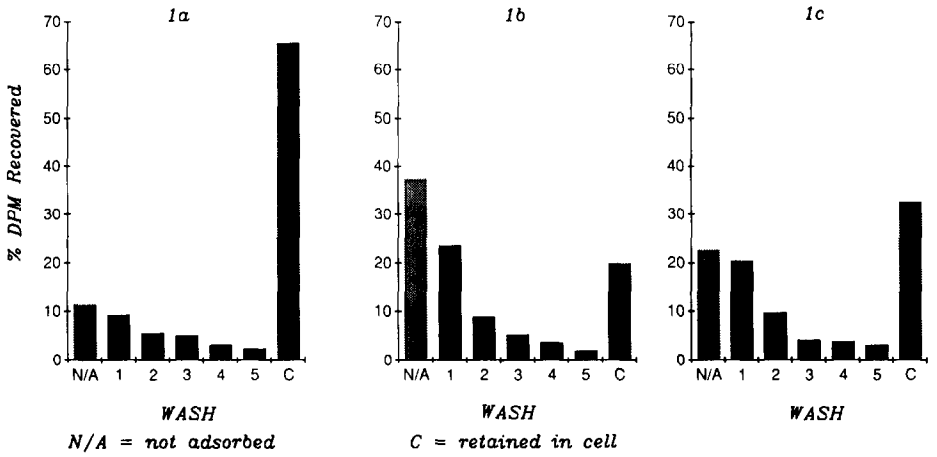


Fig. 3. Uptake of compounds **1a–1c** by *E. coli*.

vents (ethanol/chloroform mixture). The extracted compounds were analysed by TLC methods, using dichloromethane : methanol 10 : 1 as a mobile phase.

Results and Discussion

Affinity of lipidic amino acids and their oligomers for cells in suspension

After 0.5 h incubation with rat erythrocytes, most (74%) of the monomeric methyl ester **1a** disappeared from the supernatant to become associated with the cells. Washing with BSA in Ringer's saline solution (BSA/R) removed about 80% of **1a** associated with the cells. However, the remaining 20% of **1a** was not removed from the erythrocytes, even after seven BSA/R washes (Fig. 1). Three explanations can be given for this observation: (i) a proportion of **1a** was strongly bound to the surface of the cell membrane; (ii) compound **1a** was incorporated into the membrane; or (iii) compound **1a** was internalised by erythrocytes.

The affinity of the fully protected compounds **1b** and **1c** in the same surroundings was decidedly less than that of **1a**, with only 16 and 22% adsorbed, respectively (Fig. 1). Furthermore, the

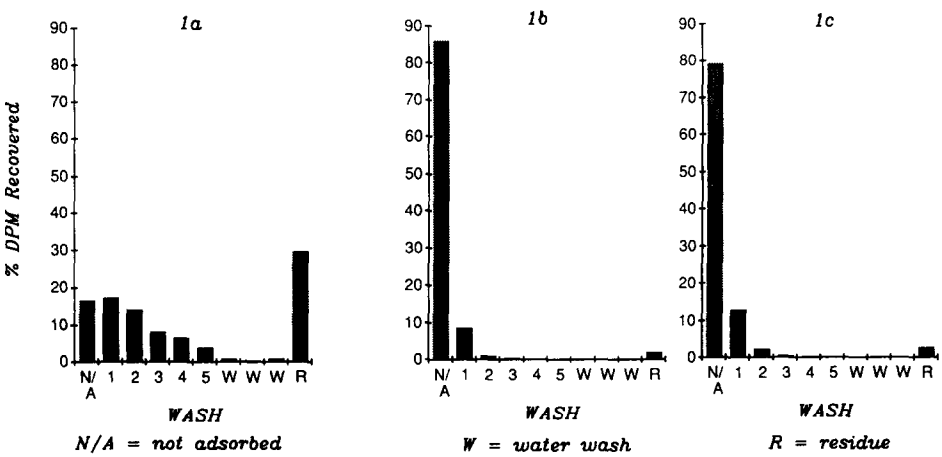


Fig. 4. Uptake of compounds **1a–1c** by hepatocytes.

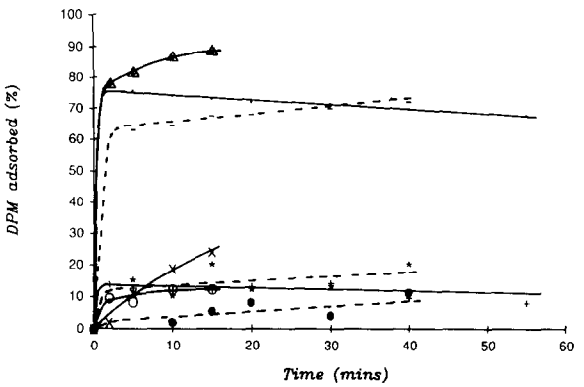


Fig. 5. Rate of uptake of compounds **1a–1c** as a function of cell type. (·) **1a**, erythrocytes; (+) **1b**, erythrocytes; (△) **1a**, hepatocytes; (○) **1b**, hepatocytes; (×) **1c**, hepatocytes; (–) **1a**, *E. coli*; (*) **1b**, *E. coli*; (●) **1c**, *E. coli*.

adsorbed quantities of **1b** and **1c** were removed from the cells after only three to four washes with BSA/R.

The effects of adsorption of **1a** were dependent upon cell concentration (Fig. 2). At a packed erythrocyte concentration of 10%, monomer **1a** was only 41% bound, while at a cell concentration of 50%, **1a** is 83% bound. The dimer **1b** and trimer **1c** were less avidly absorbed than **1a** at all cell concentrations.

In the case of *E. coli*, compounds **1a** (89%), **1b** (38%) and **1c** (22%) all showed affinity for the cells after 30 min incubation (Fig. 3). Furthermore, a proportion of **1b** and **1c** (20 and 33%, respectively) and the bulk of **1a** (66%) were tightly bound to the cells and could not be removed by BSA/R washes. However, all bound compound could be extracted from the cells with ethanol. The results suggest that a proportion of each compound **1a–1c** was bound tightly to the *E. coli* surface, but was probably not internalized.

Cell concentration had marked effects on the behaviour of **1a–1c** toward *E. coli* cell suspensions (Fig. 2). Compound **1a** showed the greatest affinity for *E. coli* at low cell concentrations. The percentage of **1a** adsorbed appeared to vary biphasically, with more than 60% adsorbed at a packed cell concentration of 10%, and 86% at a cell concentration of 60%. On the other hand, the adsorption of compounds **1b** and **1c** was found to increase steadily with increasing *E. coli* concentrations, before reaching a plateau at a cell concentration of 50%.

Observations regarding the behaviour of compounds **1a–1c** towards rat hepatocytes (Fig. 4) were similar to those with erythrocytes. Thus, most of **1a** (83%) became associated with the hepatocytes, and 30% of this could not be removed by BSA/R washing. This 30% was found to be strongly associated with the cell residue (membranes, nuclei and microsomes) following lysis of the cells with water, and thus may represent internalised lipidic amino acid. Most of **1b** and **1c** (86 and 79%, respectively) failed to be

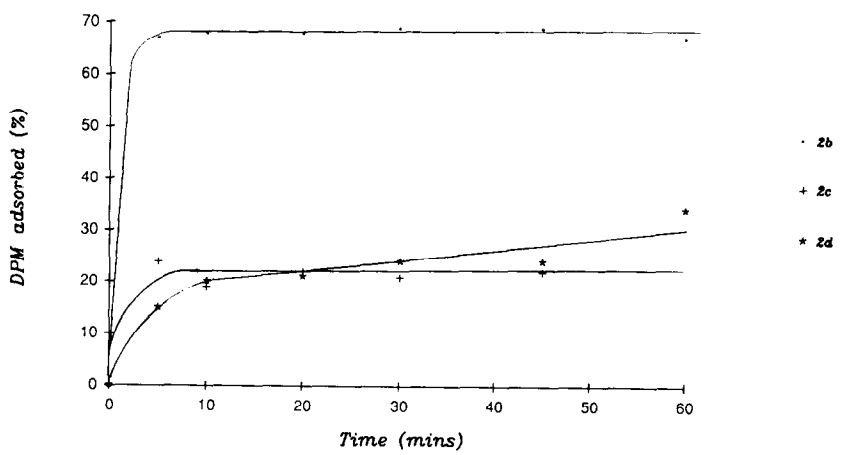


Fig. 6. Cellular uptake of conjugates **2b–2d**.

adsorbed, although small amounts were tightly bound.

Time course of association

The time course of association/uptake of compounds **1a–1c** for the three cell types was studied by taking samples from the incubation mixtures at various time intervals.

For rat erythrocytes, both **1a** and **1b** were rapidly taken up by the cell suspension in less than 5 min (Fig. 5). Thereafter, no further uptake could be detected. Indeed, there was evidence of a gradual decline in the percentage of compound absorbed over time. The rate of uptake of **1a–1c** by *E. coli* showed an initial rapid phase, such that adsorption/absorption had reached completion in under 10 min (Fig. 5). There was no evidence of the gradual desorption observed with erythrocytes.

The behaviour of **1a** and **1b** in hepatocytes mirrored the effects seen in the other two cell types, in that both compounds showed rapid association initially, followed by the attainment of steady-state levels (Fig. 5). These results are consistent with passive partitioning, association or absorption of compounds **1a** and **1b** into, with or by the hepatocyte membrane. In contrast, the fully protected trimer **1c** was taken up linearly by hepatocytes over the time scale of the experiments (15 min), an observation suggestive of an active mechanism of uptake.

The cellular uptake data on red blood cells of conjugates **2b–2d** (Fig. 6) confirmed the previous results obtained on **1a–1c**: the uptake of **2b** is greater than that of the dimeric and trimeric conjugates **2c** and **2d**.

Metabolic stability

After 30 min incubation of **2b** with red blood cells, the cells were washed with a 1:1 chloroform:ethanol mixture. The initial radioactive count of this organic solution was low, indicating a strong drug-cell association. After 30 min sonication, the radioactive **2b** taken up by the cells was dissolved by the organic solvent mixture. The long period of sonication (30 min) in chloroform/ethanol was necessary to disrupt the cells and liberate their contents. The compounds liberated were analysed by TLC. The preliminary studies showed that no significant amount of metabolite was formed after 30 min incubation; only the intact molecule was detected. The amide bond between the system and drug has been shown to be stable, and the methyl ester is not hydrolysed by esterases.

Conclusions

The lipidic amino acid methyl ester **1a** and benzoquinolizine monomer conjugate **2b** were more readily taken up by erythrocytes, *E. coli* and hepatocytes than the fully protected dipeptide **1b** and tripeptide **1c**, and were less readily recovered from the cells by washing. Compound **1c** tended to associate more avidly with all cell types than compound **1b**, although neither bound well to erythrocytes. It should be noted that compounds **1a–1c** do not form a homologous series, in that the methyl ester **1a** has a protonated amino function, while the peptides **1b** and **1c** are uncharged. The data on cellular uptake of benzoquinolizine conjugates **2b–2d** by erythrocytes were fully in agreement with those obtained on different cells with the lipidic system **1a–1c**. Thus, the monomer conjugate **2b** was taken up more rapidly and to a greater extent than were the dimer and trimer conjugates **2c** and **2d**. The preliminary studies showed that the metabolism of conjugate **2b** was slow; after 30 min of incubation with red blood cells, no significant amount of metabolite was produced.

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