Binding of temoporfin to the lipoprotein fractions of human serum

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The binding of a new photosensitizer, temoporfin, to human serum lipoproteins was investigated. [^{14}C]-Temoporfin ($0.1\text{--}10\text{ }\mu\text{g ml}^{-1}$) was incubated with human serum for 30 min at room temperature or for 20 h at 4°C , prior to stepwise density flotation to separate the lipoprotein fractions. The distribution of the drug was independent of the initial concentration or time and temperature of the incubation. The proportion of temoporfin in each fraction was: very low density lipoprotein 6%, low density lipoprotein 22%, lipoprotein(a) 17%, high density lipoprotein 39% and lipoprotein deficient serum 16%. Autoradiography of agarose gels showed that the drug was associated with the lipoprotein in the fractions. Fractionation of plasma samples collected from a patient after an intravenous infusion of temoporfin revealed a binding profile similar to that obtained in the *in vitro* study.

Keywords temoporfin (*m*-THPC) photodynamic therapy human serum lipoproteins protein binding

Introduction

Photodynamic therapy of cancer is based on the use of photosensitizing agents which concentrate relatively selectively in tumours. Exposure of the target tissue to light of the appropriate wavelength results in the production of reactive oxygen species which are cytotoxic. Of major importance in the use of photosensitizers is their selectivity for tumour cells. This selectivity may be due to: (i) the chemical properties of the photosensitizer and/or (ii) the binding of the photosensitizer to carriers which have increased affinity for tumours. As low density lipoprotein (LDL) uptake may be increased in tumours as compared with normal cells [1–3], it has been suggested that the LDL receptor pathway is an important mechanism by which a drug-lipoprotein complex concentrates in tumour cells. The role of the LDL receptor pathway in the delivery of lipophilic photosensitizers has been reviewed by Mazière *et al.* [4].

Temoporfin (5,10,15,20-tetra[*m*-hydroxyphenyl]chlorin, *m*-THPC) is a new photosensitizer [5] which is undergoing clinical trials [6]. It is normally administered as a single i.v. dose and the tumour irradiated 2 or 3 days later. Temoporfin has some of the desired properties of a good photosensitizer: it is a single compound, it has a high absorption in the red region of the spectrum and data from *in vitro* and *in vivo* studies indicate that

it has a high tumoricidal potential [6–10]. This study reports the first data on the distribution of temoporfin between the lipoprotein fractions of human serum.

Methods

Materials

Temoporfin (MW 677) and [^{14}C]-temoporfin were gifts from Scotia Pharmaceuticals (Guildford, Surrey, UK). The radiolabelled drug, custom synthesized by American Radiolabeled Chemicals (St Louis, MO, USA), was supplied as being 93% pure by h.p.l.c. with a specific activity of 36 mCi mmol^{-1} . Lipoprotein standards were purchased from Sigma Chemical Company Ltd (Poole, UK) or Calbiochem (Nottingham, UK). H.p.l.c. grade acetonitrile was from Fisons Scientific (Loughborough, UK). Kits for measuring total cholesterol and albumin were from Sigma.

Sample collection

Venous blood, from five healthy volunteers (23–46 years, 59–83 kg; four male, one female) who gave informed

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consent, was collected in plain glass tubes and allowed to clot at room temperature (23–25°C) for 30 min. Serum was separated by centrifugation (1000 g, 15 min). Blood samples from a patient who had received 0.3 mg kg⁻¹ of temoporfin as an intravenous infusion over 15 min, were collected into EDTA tubes at 20, 44 and 69 h after the dose. The study was approved by the ethics committee of the Royal London Hospital.

Incubation of serum and separation of lipoprotein fractions

[¹⁴C]-Temoporfin was added to freshly separated serum to give concentrations of 10, 1 or 0.1 µg ml⁻¹. Tubes were allowed to stand for 30 min at room temperature (23–25°C) or placed in the refrigerator overnight (20 h at 4°C).

Lipoprotein fractions, corresponding to very low density lipoprotein (VLDL), low density lipoprotein (LDL), lipoprotein(a) (Lp(a)) and high density lipoprotein (HDL) were obtained by stepwise differential flotation [11]. Sodium bromide solutions (0.71, 14.9, 13.4 and 36.3% (w/v) containing 0.1% (w/v) Na₂EDTA) were used to obtain the required final densities of 1.006, 1.063, 1.087 and 1.21 g ml⁻¹, respectively.

The radioactive content of the lipoprotein layers was determined by liquid scintillation counting of 0.1 ml samples. The temoporfin in patient samples was quantified by h.p.l.c as described previously [12]. The intra-assay coefficients of variation were 2.7% and 5.1% at 1 and 0.1 µg ml⁻¹, respectively.

Electrophoresis

Serum lipoproteins were separated on an agarose gel in barbitone buffer, pH 8.6 (Ciba–Corning Diagnostics, Halstead, UK). Samples (1 µl) of serum, lipoprotein fractions or lipoprotein standards were applied to the wells and electrophoresed at 90 V for 35 min. After drying (56°C, 20 min), the gels were subjected to autoradiography for up to 6 weeks at 4°C and then stained with Fat Red 7B.

Determination of protein and cholesterol concentrations

The protein content of the fractions was determined by Lowry's method [13] and total cholesterol and albumin were determined using commercially available kits. The precisions of the methods, assessed by conducting replicate (6) assays at 100 and 50 mg dl⁻¹ (cholesterol) or 5 and 2.5 g dl⁻¹ (albumin), were <1% and <2%, respectively.

Statistical analyses

Data were expressed as mean ± s.d. Differences between treatments were tested by one-way ANOVA.

Results

The proportions of drug in each fraction were unaffected by the drug concentration ($P > 0.2$) or incubation conditions ($P > 0.9$) (Table 1). The mean percentages (± s.d.) in the fractions were: VLDL 6.0 ± 3.1, LDL 22.3 ± 5.6, Lp(a) 17.3 ± 3.6, HDL 38.6 ± 3.6 and lipoprotein deficient serum 16.0 ± 3.1%. The recovery of added radioactivity was always >98%. The distribution of drug between the lipoprotein fractions from patient plasma gave the same pattern as that obtained from the serum of healthy volunteers. Temoporfin plasma concentrations were 0.26, 0.15 and 0.10 µg ml⁻¹ at 20, 44 and 69 h, respectively. The proportion (mean % ± s.d.) of temoporfin in each fraction was: VLDL 7.8 ± 2.2, LDL 18.3 ± 2.8, Lp(a) 11.5 ± 0.8 and HDL 34.8 ± 2.5.

Autoradiography of agarose gels confirmed that temoporfin was associated with the lipoproteins of each fraction (Figure 1). The migration of the lipoproteins was as expected [14], HDL > VLDL > LDL, and generally the LDL band was the most intensely stained with Fat Red 7B. However, the intensity of the HDL band on the autoradiograph was of similar or greater intensity than that for LDL.

Albumin could not be detected in the VLDL, LDL and Lp(a) layers but the HDL layer contained measurable amounts (6.8 ± 1.6 g l⁻¹). The albumin concentration in the residual lipoprotein deficient serum was 30.3 ± 2.4 g l⁻¹. The total protein concentration in each fraction was 0.27 ± 0.09, 1.27 ± 0.51, 1.09 ± 0.22, 7.55 ± 3.2 and 65.7 ± 3.5 g l⁻¹ for VLDL, LDL, Lp(a), HDL and lipoprotein deficient serum, respectively. The cholesterol values in the corresponding fractions were 14 ± 10, 95 ± 25, 31 ± 9, 30 ± 8 and 7 ± 4 mg dl⁻¹, respectively.

Discussion

In common with other photosensitizers, over 80% of the temoporfin was associated with the lipoprotein fractions. Generally, the fraction collected in the density range 1.063–1.21 g ml⁻¹ is taken as being HDL [15]. This fraction corresponds to the sum of our Lp(a) and HDL fractions, i.e. 56%. Thus, the distribution of temoporfin between the fractions was similar to that reported for benzoporphyrin derivative monoacid: VLDL 3%, LDL 15% and HDL 55% [16].

The lack of any differences in binding due to the time for which the samples were incubated supports the view that the attainment of equilibrium for the distribution of photosensitizers between the lipoprotein classes is rapid [4]. However, Allison *et al.* [17] found that the distribution of benzoporphyrin derivative monoacid into lipoproteins increased from 49% after 1 h to 86.7% after 24 h of incubation at 4°C. The fact that the binding after storage for 20 h at 4°C was the same as that determined after the short incubation of fresh serum is of practical significance as it shows that sera from *in vivo* studies can be stored overnight at 4°C without affecting the distribution.

The concentrations of temoporfin used in the *in vitro*

Table 1 Distribution of [^{14}C]-temoporfin between lipoprotein fractions after incubation in serum at 23–25° C for 30 min or at 4° C for 20 h. Means $\pm$ (s.d.) from the data of five volunteers are presented.

Fraction*	% of initial radioactivity				
	30 min at 23–25° C			20 h at 4° C	
	0.1 $\mu\text{g ml}^{-1}$	1 $\mu\text{g ml}^{-1}$	10 $\mu\text{g ml}^{-1}$	1 $\mu\text{g ml}^{-1}$	10 $\mu\text{g ml}^{-1}$
VLDL	7.5 (2.3)	6.6 (3.1)	5.1 (2.8)	6.2 (2.7)	5.0 (2.7)
LDL	24.0 (5.8)	23.4 (6.7)	20.2 (4.8)	23.0 (6.2)	20.8 (6.2)
Lp(a)	16.5 (4.1)	17.2 (3.7)	15.8 (3.7)	19.2 (3.6)	17.6 (3.7)
HDL	38.7 (3.7)	38.9 (5.0)	38.3 (2.4)	38.8 (4.2)	38.2 (4.1)
LDS	15.0 (3.0)	15.3 (3.5)	18.5 (3.0)	14.7 (3.1)	16.4 (2.4)

*VLDL, very low density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein (a); HDL, high density lipoprotein; LDS, lipoprotein deficient serum.

†1 $\text{mg ml}^{-1} \equiv 1.5 \text{ mM}$

Figure 1 Agarose gel electrophoresis of serum and lipoprotein fractions from a volunteer. After autoradiography (upper) the gel was stained with Fat Red 7B to reveal the lipoproteins (lower). The initial concentration of temoporfin was 10 $\mu\text{g ml}^{-1}$ and 1 μl samples were applied to the wells. VLDL, very low density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein (a); HDL, high density lipoprotein; LDS, lipoprotein deficient serum.

study were in the range expected during therapeutic use [6]. As the proportion bound by any fraction was independent of the drug concentration over a 100-fold range, the biodistribution would not be expected to change as blood concentration falls with time. Similarly, the distribution pattern is unlikely to be affected by the size of the administered dose. The fractionation of patient plasma was carried out over a narrower range of total temoporfin concentrations than the *in vitro* study, but again there were no obvious differences in the proportion of drug in each fraction, supporting the view that the binding sites are not saturable at concentrations within the therapeutic range.

The evidence for LDL-mediated uptake of benzoporphyrin derivative monoacid into fibroblasts is compelling

[18] and it is likely that LDL bound temoporfin can be taken up via LDL receptor-mediated endocytosis. That less than 25% of the drug is found in the LDL fraction need not be clinically important as equilibrium between the classes is rapid and HDL bound drug could replenish the LDL bound pool. However, other mechanisms, including uptake by tumour associated macrophages [19] and high vascular permeability, cannot be ruled out [20]. Whatever the mechanism of uptake by tumour cells, it is clear that serum lipoproteins are important carriers of compounds of this type and they do have a role in solubilizing hydrophobic photosensitizers. In the absence of lipoprotein binding it is to be expected that the blood concentrations *in vivo* would be considerably lower, as such a hydrophobic drug would be taken up by tissues. Such uptake may not necessarily result in higher tumour concentrations. Further studies are required to determine how the knowledge of this distribution can be utilized to improve the clinical use of this drug.

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