International Journal of Pharmaceutics, 12 (1982) 323–330
Elsevier Biomedical Press

The uptake of liposomal and free [^{14}C]chloramphenicol by *Tetrahymena pyriformis*

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(Received March 1st, 1982)
(Accepted May 6th, 1982)

Summary

The uptake of free and liposomal [^{14}C]chloramphenicol by the protozoan, *Tetrahymena pyriformis*, is compared. The results obtained are consistent with a passive mode of entry into the cell, i.e. diffusion, for the free label and an active mode of entry for the liposomal label, the latter form of the label being accumulated within the cell against the apparent transmembranal concentration gradient. Uptake studies on liposomes double-labelled with [^{14}C]chloramphenicol and [^{3}H]cholesterol indicate that the anionic dipalmitoyl phosphatidylcholine liposomes used here are taken up intact by *Tetrahymena pyriformis*.

Introduction

In a previous communication (Onaga and Baillie, 1980) it was shown that the acute toxicity of chloramphenicol (CAP) for the protozoan, *Tetrahymena pyriformis*, could be markedly enhanced by entrapment of the drug in liposomes. It was proposed that the observed enhancement was due to a liposome-mediated alteration in the micro- or cellular pharmacokinetics of the drug in this single-celled organism. In this present paper we present evidence, based on uptake studies of labelled free and liposomal CAP, that at least one aspect, absorption, of CAP cellular pharmacokinetics is significantly increased by the use of the drug in carrier form.

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0378-5173/82/0000–0000/$02.75

Materials and methods

Materials

D,L-α-phosphatidylcholine, dipalmitoyl (DPPC), crystalline; cholesterol (CHOL); dicetyl phosphate (DCP); 2,5-diphenyloxazole (PPO), crystalline scintillation grade, (Sigma). Triton X-100; Hyamine 10-X hydroxide solution; toluene, all scintillation grade (BDH Chemicals). [7(n)-^{3}H]cholesterol (9.5 Ci mmol^{-1} in toluene); D-threo[dichloroacetyl-1-^{14}C]chloramphenicol (14.9 mCi mmol^{-1}) (The Radiochemical Centre, Amersham). Sepharose 6B (Pharmacia). Dimethylsulphoxide (Fisons Scientific). *Tetrahymena pyriformis*, strain GL (The Culture Centre, Cambridge).

Methods

Preparation of vesicles. 75 mg of lipid mixture, comprising DPPC–CHOL–DCP in a 7 : 2 : 1 molar ratio was trace-labelled with [^{3}H]CHOL so that 1 μCi [^{3}H]CHOL ≡ 40 μmol DPPC; this was then formed into a thin film by vacuum rotary evaporation from chloroform at 37°C. The dry film was hydrated at 50°C for 2 h under N_2 with 4 ml 3.3×10^{-3} M, pH 7.2 phosphate-buffered 0.3% w/v saline (PBS) containing 1.25 μCi [^{14}C]CAP ml^{-1}. Hydrated lipid suspensions were sonicated at 50°C under N_2 in sealed 10 ml vials in a bath sonicator (Walker Ultrasonics) for 20 min to near optical clarity then fractionated over Sepharose 6B (40 × 2.3 cm column) with PBS eluant. The 3 ml fractions containing liposomes (absorbance 400 nm) and those containing free [^{14}C]CAP label were pooled.

Exposure of cells to labels. Standardized *T. pyriformis* cell suspension prepared as before (Onaga and Baillie, 1980) was added to a range of dilutions of labelled liposome suspension to give a final cell density of 5×10^{4} ml^{-1}. After 60, 120 and 180 min incubation at room temperature, 1 ml samples were withdrawn into 9 ml ice-cold PBS and thrice centrifuged (400 *g* for 10 min at 4°C) and washed in 9 ml ice-cold PBS. 1 ml Hyamine hydroxide was added to the final pellet which was dissolved in the sealed centrifuge tube by incubation at 60°C with occasional shaking. After cooling, duplicate 0.5 ml samples were transferred each to 5 ml counting cocktail (0.5% w/v PPO in toluene : Triton X-100 2 : 1 v/v) and duplicate counts made on each vial on a Tri-Carb 460C Automatic scintillation counter (Packard) of ^{3}H and ^{14}C activities using separate external standards at a counting efficiency of 38% and 90%, respectively.

Uptake of free [^{14}C]CAP was investigated by incubating mixtures of equal volumes of cell suspension and pooled free [^{14}C]CAP fractions at room temperature then sampling as described above.

The ^{3}H and ^{14}C activities of liposome suspensions and the ^{14}C activity of free label solution was determined after addition of 0.2 ml of the suspension/solution to 5 ml counting cocktail.

Results and discussion

Entrapment efficiency

For the different batches of double-labelled liposomes used in the experiments

TABLE 1

[^{14}C]CAP:[^{3}H]CHOL RATIOS FOR LIPOSOME SUSPENSIONS (EXTRACELLULAR) AND FOR LABELS FOUND ASSOCIATED WITH 5×10^4 *T. Pyriformis* CELLS AFTER 120 MIN INCUBATION AT ROOM TEMPERATURE

Extracellular label concentration (nCi · ml^{-1})		Extracellular labels' ratio [^{14}C]CAP:[^{3}H]CHOL	Cell-associated label (nCi)		Cell-associated labels' ratio [^{14}C]CAP:[^{3}H]CHOL
[^{14}C]CAP	[^{3}H]CHOL		[^{14}C]CAP	[^{3}H]CHOL	
0.33	4.13	0.080	0.17	2.42	0.070
0.55	6.88	0.080	0.22	2.92	0.075
0.64	10.29	0.062	0.19	3.51	0.054
0.69	9.56	0.072	0.20	3.32	0.060
0.83	10.33	0.080	0.25	3.39	0.074
0.98	15.66	0.063	0.24	4.39	0.055
1.04	14.56	0.072	0.25	4.19	0.060
1.14	14.15	0.081	0.31	3.75	0.083
1.40	22.38	0.063	0.29	5.35	0.054
1.49	20.78	0.072	0.32	5.08	0.063
1.66	20.66	0.080	0.41	4.75	0.086
1.96	31.33	0.063	0.32	6.96	0.046
2.09	29.1	0.072	0.36	6.68	0.054

The mean extracellular and cell-associated labels' ratios are 0.072 and 0.064, respectively, so that the cell-associated ratio is some 89% of the extracellular ratio.

described here values for the ratio [^{14}C]CAP/[^{3}H]CHOL lay between 0.062 and 0.081 (Table 1) which, using the ratio 40 μmol DPPC : 1 μCi [^{3}H]CHOL in the original lipid mixture and a concentration of 1.25 μCi [^{14}C]CAP ml^{-1} in the hydrating solution, may be calculated to represent respective entrapped volumes of 1.25 and 1.62 l mol^{-1} DPPC. These values are in good agreement with the entrapment figure of 4.2 μg CAP mg^{-1} DPPC, equivalent to 1.62 l mol^{-1} DPPC, reported previously (Onaga and Baillie, 1980) for anionic vesicles of identical composition to those described here but which were assayed chemically for CAP and DPPC content. Although no measurements were made on the labelled liposome suspensions described here, photon correlation spectroscopy (Baillie et al., 1979) gives a mean radius of 50 nm for vesicles prepared according to the present method and this size is assumed in this communication. The vesicles may thus presumably be described as small multilamellar vesicles and we estimate that each comprises some 5×10^4 DPPC molecules and since in the original lipid mixture 1 μCi [^{3}H]CHOL ≡ 40 μmol DPPC we calculate that 1 nCi [^{3}H]CHOL ≡ 4.88×10^{11} vesicles.

Drug uptake

It was found for both free (Fig. 1A) and liposomal (Fig. 1B) forms of the [^{14}C]CAP label that as the extracellular concentration of label increased, the quantity of cell-associated label increased. However, although an obviously linear relationship could be established between these parameters for the free label, this was not the case for the liposomal label data for which a curve is required if the fitted line was to pass through the origin. This hyperbolic relationship between cell-associated label and extracellular label concentration suggests an uptake mechanism for the liposomal label which is saturable at high concentration. The linear relationship for free drug is compatible with a diffusional mechanism of entry. It is also apparent that at any particular extracellular label concentration the quantity of cell-associated label increased with incubation time for free and liposomal forms.

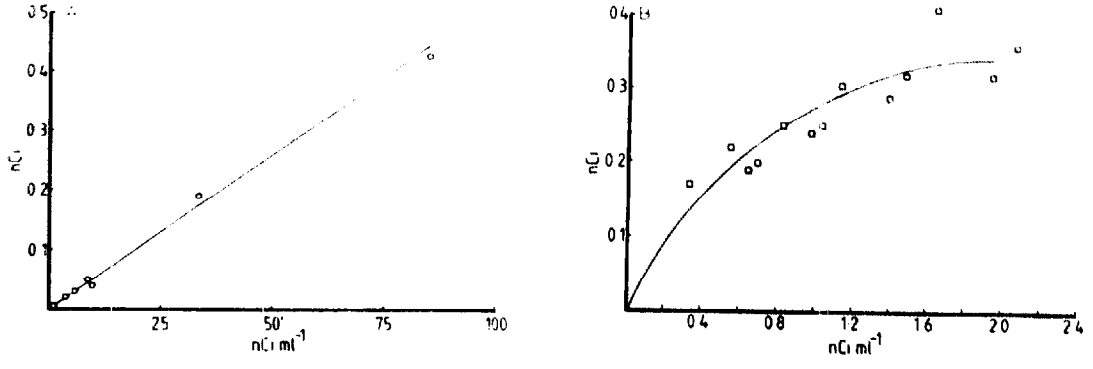

Fig. 1. The influence of the extracellular concentration of (A) free and (B) liposomal [^{14}C]CAP label on the quantity of cell-associated label after 120 min incubation at room temperature. Abscissae: extracellular [^{14}C]CAP label concentration (nCi·ml^{-1}); ordinates: quantity of label (nCi) associated with 5×10^4 *T. pyriformis* cells after 120 min.

TABLE 2

CONCENTRATION FACTORS C FOR FREE [^{14}C]CAP LABEL INCUBATED WITH 5×10^4 *T. pyriformis* CELLS FOR 120 MIN AT ROOM TEMPERATURE

Extracellular [^{14}C]CAP concentration, C_e(nCi · ml^{-1})	Cell-associated [^{14}C]CAP (nCi)	Cell-associated [^{14}C]CAP concentration C_i (nCi · ml^{-1})	$C = C_i/C_e$
0.63	0.003	0.92	1.46
0.95	0.005	1.54	1.62
3.73	0.02	6.15	1.65
5.71	0.03	9.23	1.62
8.59	0.05	15.38	1.79
9.47	0.04	12.31	1.30
33.65	0.19	58.46	1.74
86.10	0.43	132.31	1.54

Cell-associated label concentrations are calculated on the basis of a total volume of 3.25×10^{-3} ml for 5×10^4 cells.

The quantity of cell associated label may be converted to an intracellular concentration using a value of 3.25×10^{-3} ml for the total volume of 5×10^4 cells which assumes each cell to be a sphere of 25 μm radius. This allows calculation of C, the concentrative effect of the cells on the free and liposomal [^{14}C]CAP label as shown in Table 2. For free drug, all C values determined were greater than unity which probably reflects the lipophilicity of CAP which has an octanol–water partition coefficient (log P) of 1.14. (Leo et al., 1971). The highest C value for free label was 2.48, obtained with cells incubated in 5.71 nCi · ml^{-1} [^{14}C]CAP for 150 min. The C values for liposomal [^{14}C]CAP after 120 min incubation (Table 3) lay in the range 53–155 at respective extracellular label concentrations of 2.09 and 0.33 nCi · ml^{-1} demonstrating the ability of *T. pyriformis* to concentrate this form of the label within the cell. Although the present data do not allow direct comparison of C values for the same concentration of free and liposomal label it is apparent that intracellular accumulation of the latter is some 30 times greater than that of the former, a value which is in accord with the apparently greater toxicity of liposomal over free CAP for this cell (Onaga and Baillie, 1980).

C values calculated for the [^{3}H]CHOL label also indicate that the lipid liposomal label is accumulated within the cell to an extent similar to the [^{14}C]CAP marker. However, it is more useful to compare the extra- and intracellular labels ratio (Table 1) which indicates that the association mechanism of liposomes with *T. pyriformis* involves uptake of essentially entire intact vesicles.

A replot of the data shown in Fig. 1A and B in double-reciprocal form (Fig. 2A and B) also reveals the apparent difference between cell uptake of free and liposomal [^{14}C]CAP label. This technique has been used in the analysis of the interaction between PMN cells and liposomes (Stendahl and Tagesson, 1977) and between macrophages and protein-coated oil droplets (Ueda et al., 1981). It is presupposed

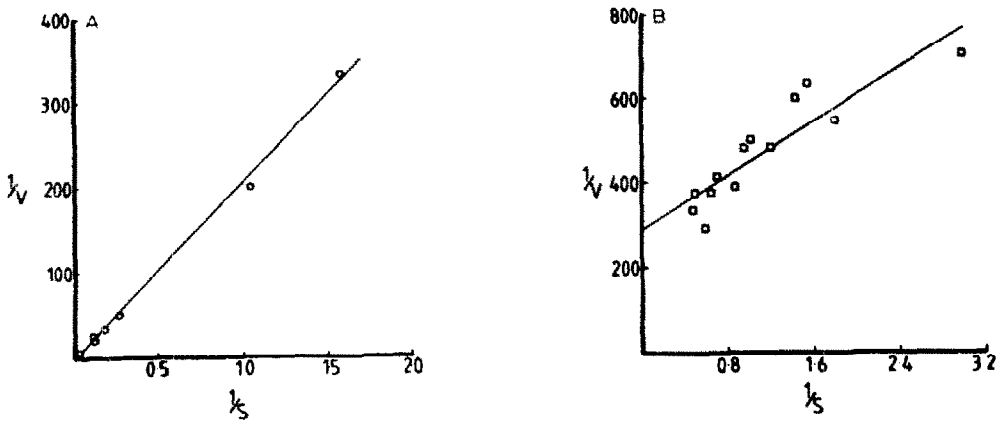

Fig. 2. A: double-reciprocal plot of the free [^{14}C]CAP data shown in Fig. 1A. Abscissa: 1/s, where s is the extracellular concentration (nCi·ml^{-1}) of free [^{14}C]CAP; ordinate, 1/V, where V is the rate (nCi·min^{-1}) of association of free [^{14}C]CAP with 5×10^4 *T. pyriformis* cells during room temperature incubation. B: double-reciprocal plot of the liposomal [^{14}C]CAP data shown in Fig. 1B. Abscissa, 1/s, where s is the extracellular concentration (nCi·ml^{-1}) of liposomal [^{14}C]CAP; ordinate, 1/V where V is the rate (nCi·min^{-1}) of association of liposomal [^{14}C]CAP with 5×10^4 *T. pyriformis* cells during room temperature incubation. The intercept on the ordinates, 1/V = 291, yields a value for V_{max} of 3.44×10^{-3} nCi [^{14}C]CAP min^{-1} for 5×10^4 cells. Line fitted by least-squares method, *r* the correlation coefficient = 0.896.

that the interaction between cell and liposome is of the Michaelis-Menten type:

$$C + L_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CL \xrightarrow{k_2} C + L_2$$

where C is the cell, L_1 and L_2 are liposomes located extracellularly and intracellularly, respectively, and CL is an intermediate unstable complex of liposome and cell, arising as a result of, e.g. adsorption, which mediates the transfer of liposomes into the cell. When the intermediate state CL is fully occupied in the transfer of liposomes into the cell, the uptake rate approaches it maximum V.

The linear plot for free drug which passes through the origin (Fig. 2A) would be expected for a diffusional uptake mechanism which does not conform to Michaelis-Menten kinetics; i.e. there is no V. This implies that the entry of free label increases indefinitely with extracellular concentration as the principal driving force for its entry into the cell is the transmembranal concentration gradient.

The linear plot with a positive intercept on the ordinate obtained with the liposomal label data (Fig. 2B) is indicative of Michaelis-Menten kinetics, the intercept 1/V = 291 yielding a value for V of 3.44×10^{-3} nCi [^{14}C]CAP min^{-1}. Based on the mean intracellular CAP/CHOL ratio of 0.064 (Table 1) this is equivalent to 53.75×10^{-3} nCi [^{3}H]CHOL min^{-1} or 2.62×10^{10} vesicles min^{-1}. For a single cell this represents a figure of 8.73×10^3 vesicles s^{-1}. Assuming that adsorption, i.e. formation of CL, precedes vesicle uptake by either fusion or endocytosis this number of vesicles (n) of radius 50 nm represents an area of 1.37×10^{-6} cm^2 ($1/2\ 4\pi r^2 n$). The cell surface area of *T. pyriformis* is 7.9×10^{-5}

TABLE 3

CONCENTRATION FACTORS C FOR LIPOSOMAL [^{14}C]CAP LABEL INCUBATED WITH 5×10^4 *T. pyriformis* FOR 120 MIN AT ROOM TEMPERATURE

Extracellular [^{14}C]CAP concentration, C_e(nCi·ml^{-1})	Cell-associated [^{14}C]CAP (nCi)	Cell-associated [^{14}C]CAP concentration, C_i(nCi·ml^{-1})	$C = C_i/C_e$
0.33	0.17	52.31	158.52
0.55	0.22	67.69	123.07
0.64	0.19	58.46	91.34
0.69	0.20	61.54	89.19
0.83	0.25	76.92	92.67
0.98	0.24	73.85	75.36
1.04	0.25	76.92	73.96
1.14	0.31	95.38	83.67
1.40	0.29	89.23	63.74
1.49	0.32	98.46	66.08
1.66	0.41	126.15	75.99
1.96	0.32	98.46	50.23
2.09	0.36	110.77	53.00

Cell-associated label concentrations are calculated on the basis of a total volume of 3.25×10^{-3} ml for 5×10^4 cells.

cm^2 and the phagostome surface area 1.57×10^{-6} cm^2 which assuming an adsorption time scale of the order of 1 s indicates that the phagostome surface is the rate-limiting factor in vesicle uptake and that vesicle uptake is endocytic.

If the cell was stationary, vesicle supply to the cell surface is dependent on vesicle diffusion and is given by $4\pi rND$, where r is the cell radius, 25 μm, N the vesicle concentration which at the lowest extracellular [^{3}H]CHOL label concentration used of 4.13 nCi · ml^{-1} is 2.0×10^{12} ml^{-1}, and D the vesicle diffusion coefficient which for 50 nm radius vesicles has a value of 5×10^{-8} cm^2 · s^{-1} determined by photon correlation spectroscopy (Baillie et al., 1979). The vesicle supply based on these values is 3.14×10^3 vesicles s^{-1} which is of the same order of magnitude as the maximal vesicle uptake rate (3.73×10^3 vesicles s^{-1}) under the conditions of these experiments. *T. pyriformis* cell motility (speed 450 μm · s^{-1}) is thus not required for vesicle supply to the cell surface in view of the observed magnitude of vesicle uptake rate. However, a comparison of the contributions of cell motility and vesicle diffusion to vesicle supply at the cell surface lV/D, where l is the distance moved by the cell in 1 s, V the cell swimming speed and D the vesicle diffusion coefficient gives a value of 4.1×10^4 which shows that as a result of cell swimming there is an increase in vesicle supply and thus a vast excess of vesicles available at the cell surface for uptake.

Measurement of the uptake of the liposome internal marker, [^{14}C]CAP thus indicates that *Tetrahymena pyriformis* can accumulate liposomal contents within the cell. On the basis of the double label experiments such accumulation is the result of

the internalization of essentially intact vesicles so that the present findings support our earlier suggestion (Onaga and Baillie 1980) that phagocytosis of discrete packets of drug solution (i.e. liposomes) allows attainment of cytotoxic drug levels within the cell.

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