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Colloidal anticancer drugs bioavailabilities in oral administration models

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Abstract

Liposomes have been prepared with a polymerised core. Drug release and gastrointestinal (GI) degradation of liposomes with this polymerised core was slightly less important than those of classical liposomes. Vincristine, 5-fluorouracil (5-FU), and methotrexate (MTX) have been incorporated into the liposomes, and studies carried out using the differentiated cell lines Caco-2 and TC7, and with 150 histologically normal sections of human colon. Encapsulation of the drugs in liposomes had variable effects, depending on the test system and the drug used. For 5-FU and MTX calculated to be in a therapeutic range, liposomal formulation enhanced drug permeation, but not for the other drugs tested. In the excised human colon model, the treatment history of the patients can affect bioavailability: pre-operative radiation increased the drug tissue uptake. Transmucosal transport of ions was modified by prior chemotherapy. These results should be taken into account in the design of oral anticancer treatments both at the level of nutritional and pharmacological considerations.

Keywords: Oral route model; Chemotherapy; Mucosal permeability

1. Introduction

An understanding of the oral absorption of drugs in man is essential for the development of new products destined for oral use. The intestinal permeabilities measured after oral administration normally reflect the absorption in the small intestine, rather than in the colon (Nejdfors et al., 1998). The use of cell monolayers such as Caco-2 (Artursson, 1990) or the sub-clone TC7 (Caro et al., 1995) is now recognised as an efficient method for estimating the oral absorption of drugs in man (Rubas et al., 1993). Nevertheless, the results are often contradictory when compared with those obtained in vitro/ex vivo using animal tissue sections in Ussing diffusion chambers (Grass and Sweetana, 1988).

Colorectal cancers are the second cause of death by cancer in the Western world, and despite curative surgical resection, the 5-year survival rate remains around 50% (Boring et al., 1991). In France, colorectal cancer occupies the top position for incidence and the second for mortality, with 33,000 new cases a year and 16,000

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deaths (Atlan et al., 2000). Since 1990, intravenous adjuvant chemotherapy has been shown to be effective in patients who have been operated on for colon cancer and who have ganglionic metastasis. New approaches including utilisation of guanylyl cyclase C agonists are very promising (Pitari et al., 2001). But the use of anticancer drugs administered by the oral route is a very attractive prospect and has been the subject of a number of international trials (Fabre et al., 2000). Despite the obvious comfort for the patient and the decreased cost, the oral route has been little used in practice, although some studies are reported using oral 5-fluorouracil (5-FU) after curative resection (Sakamoto et al., 1999). To render anticancer drugs amenable to oral administration, a number of different chemical modifications have been described, including the fluorinated pyrimidines (capacitabine, tegafur-uracil, BMS247616) (Pazdur et al., 1998) or the glycosylated nucleoside analogues (Rohlff et al., 1999). One promising development has been the oral administration of mixed-backbone antisense nucleotides (MBO) to inhibit the expression of the PKA RIα sub-unit, in synergy with the use of cisplatin administered intraperitoneally (Wang et al., 1999). When immunomodulators such as JBT-3002 are administered by the oral route they stimulate the endogenous production of interleukin-15 by intestinal macrophages and thus protect the mucosa from the damage caused by intraperitoneal irinotecan. During these studies, the investigators showed that the beneficial effect of preventing irinotecan-induced ulceration could be optimised by encapsulating the JBT-3002 in liposomes or better still in micelles (Shinohara et al., 1999). Even if it is the case that with irinotecan and doxorubicin, it is the metabolites that are responsible for the ulceration (Morelli et al., 1996), it has been established that most cytostatic drugs themselves induce modifications in the intestinal mucosa. The co-administration of drugs to reduce these secondary effects of orally administered cytostatics can be envisaged, for example, by the administration of amifostine which has been demonstrated to reduce the toxicity of platinates and alkylating agents (Kurbacher and Mallmann, 1998).

With the aim of optimising the oral bioavailability of anticancer drugs, and at the same time reducing their cytotoxicty towards healthy cells in the gastrointestinal (GI) tract, we have encapsulated various anticancer drugs into polymeric particles, which are themselves the core of a lipidic vesicles, that is, liposomes with polymeric cores (LSP). The objective is to reduce the free drug concentration in the GI tract, and thus reducing toxicity, while increasing the drug concentration at the absorption sites.

Initially we investigated the stability of double radiolabelled LSP liposomes in vitro and then established their site of degradation in the GI tract by oral administration to rats. Several radiolabelled anticancer drugs were then entrapped in the liposomes. The permeability and tissue accumulation of the free and entrapped drugs were measured using the differentiated cell layer systems Caco-2 and TC7 and with sections of human colon, obtained from cancer patients during bowel resections, and mounted in Grass transport chambers. The pre-operative effects of chemo- and radiotherapy on the drug transport and tissue accumulation, as well as transport in different lower gut sites were also examined.

2. Materials and methods

2.1. Production of radiolabelled liposomes

The nanoparticle structure consists of a liposome that has a polymeric internal phase, which radiates out to the external surface. To determine the stability of this particle a double radiolabelling was carried out. The phospholipids used were pharmaceutical grade from Lipoid (Ludwigschafen, Germany), and all other chemicals were from Sigma-Aldrich (St. Quentin Fallavier, France).

Two hundred milligrams of phospholipid mixture (PC:PI:C, 2:1:1) was added to 2 ml of acetone–ethanol v/v 1:1 and 10 μCi of ethanolic [^{14}C]-dioleoyl-phosphatidylcholine ([^{14}C]-DOPC) added. The radiolabel was situated on the fatty acid ([^{14}C]-1-dioleoyl; NEN, Zaventem, Belgium) of the [^{14}C]-DOPC. The solvents were dried off at 60 °C under nitrogen. One hundred and twenty milligrams of collagen were added to 26 mg of a 1:1 w/w mixture of iota and kappa carrageenans (SBI, L'isle sur Sorgue, France), 150 mg of sucrose and 10 μCi of [^{3}H]-collagen (NEN, Zaventem, Belgique). In this case the radiolabel ([^{3}H]-2,3-propionate) is covalently bound to the collagen. This mixture, corresponding to the internal polymeric phase, was added to the lipidic film and

then rehydrated with 2.5 ml of NaCl (0.9% w/v) at 75 °C. The preparation was sonicated for 1 min at a frequency of 20,000 Hz (Sonoreactor, Undatim ultrasonic, Nivelle, Belgium) and filtered by tangential diafiltration (miniultrasette; Pall-Filtron, St. Germain en Laye, France). At the end of the process, preparation was sterilised by heat with positive pressure on a 0.22 μm membrane (type rezist; Labo-moderne, Paris, France). A suspension of liposomes was obtained that were double labelled ([^{14}C]-DOPC)/([^{3}H]-collagen).

The same method was carried out to label liposomes on the choline moiety of the phospholipid, by replacing the 10 μCi of [^{14}C]-DOPC with an ethanolic solution of [^{14}C]-1-methyl choline (NEN, Zaventem, Belgium). Liposomes containing 10 μCi of [^{14}C]-PEG 4000 or [^{14}C]-D-glucose (NEN, Zaventem, Belgium) were produced by the same method with the marker being added during the hydration of the lipidic film. Classic liposomes (LS) were prepared in the same way but without the addition of the polymers. The lipidic film was simply hydrated with 2 ml of 0.9% NaCl.

2.2. Preparation of liposomal anticancer drugs

The same procedure as above was repeated, but the radiolabel was exclusively on the entrapped anticancer drugs, with 30 μCi of each drug added at the hydration step: [^{3}H]-methotrexate ([^{3}H]-MTX), [^{3}H]-vincristine ([^{3}H]-VIN), [^{14}C]-5-FU (NEN, Zaventem, Belgium). The different radiolabelled drugs, MTX, VIN, or 5-FU were dissolved in the appropriate solvents: methanol for the lipophilic ones and 150 mM NaCl for the hydrophilic ones. In the case of methotrexate at therapeutic concentration (MTX-TC), the preparation was carried out as above, but with the labelled drug diluted with a solution of cold drug, in order to obtain a liposomal suspension containing a similar amount of drug as might be used in the therapeutic situation. It is possible that the transmucosal passage of MTX is modified due to cytotoxicity leading to cytolysis and consequent erroneous results. During the tangential diafiltration step, the membrane cut-off was 300 kDa, so that any drug not encapsulated or absorbed to the liposomes was eliminated. To obtain size reduction of the liposomes, 2 mM chenodeoxycholate (i.e. less than the critical micellar concentration) was added during homogenisation.

2.3. Drug release from liposomes or LSP

For the evaluation of drug release from the two liposomal forms (LS and LSP), radiolabelled drugs were encapsulated in liposomes according the aforesaid method. Then encapsulated drug amount in LS or LSP were checked by dialysis. Sample of external medium were collected after 24 h incubation, and total radioactivity label release was analysed as it is described below (Section 2.4).

2.4. Stability of liposomes in isotonic medium

In order to measure the basic stability of the liposomes, release experiments were carried out by dialysis at 37 °C in an isotonic medium. Two liposome preparations were compared: liposomes with or without a polymerised core, in which the lipidic phase is the same in both cases. These formulations were incubated inside a dialysis membrane (cut-off 500 kDa) that retained the particles but allowed the passage of degradation products. Incubation was at 37 °C for 24 h with shaking in an isotonic medium Optimem:Ham F12 1:1 (Gibco, Cergy-Pontoise France) containing 20% foetal calf serum (FDA grade, Panbiotech, Brumath, France), 1% L-glutamine (Gibco, Cergy-Pontoise, France). The liposomal lipids were radiolabelled with [^{14}C]-DOPC (1 mCi, 80–120 mCi mmol^{-1}), and the polymer core with [^{3}H]-collagen. Aliquots of the external medium were removed at time intervals and replaced with cold medium. The radioactivity, representing the passage of [^{14}C]-DOPC or [^{3}H]-collagen across the membrane, was determined by scintillation counting with internal chemiluminescence correction (Counter: 1600TR, Packard, Meriden, USA).

2.5. Stability of LSP in the presence of intestinal enzymes

Liposomes were incubated in the presence of enzymes, that is, collagenase type IV and a mixture of phospholipase A_2 and C (Sigma-Aldrich, St.Quentin Fallavier, France) which have the ability to hydrolyse the liposome components. The liposomes were radiolabelled at the level of fatty acids with [^{14}C]-DOPC (80–120 mCi mmol^{-1}) and the polymeric core with [^{3}H]-collagen (0.1–1 mCi mg^{-1}). The liposomes (LS

or LSP) were included in dialysis tubing with a cut-off of 3500 Da (Spectra/Por, Spectrum, Houston, USA), with shaking at 37 °C and placed in 50 ml of a buffer containing $CaCl_2$ (0.735 g ml^{-1}), Tris (2.42 g ml^{-1}) NaCl 9% w/v, pH 7.65, to optimise enzyme activity. The collagenase was added (0.25 mg ml^{-1}) to the liposome suspension at time 0 and the phospholipases (0.1 mg ml^{-1}) were added after 60 min. Samples of the external medium were taken at time intervals up to 24 h and the volume replaced with fresh medium. The samples were analysed by double counting to determine the passage of the degradation products of the lipids and of the polymeric cores across the dialysis membrane.

2.6. Morphological characteristics of liposomal formulations

The mean diameters and number of liposomes was determined by quasi-elastic light scattering (QELS). Briefly, the measurements were carried out with liposome preparations at a concentration of 0.3 mg ml^{-1} with respect to phospholipids. The samples were analysed at 37 °C at an angle of 90° using a Sematech RTG3 analyser (Sematech, Nice, France) with a 5 mW helium/neon laser source giving a laser beam of 632.8 nm.

Atomic force microscopy (AFM) was used to visualise liposomes and their interactions with the polymeric network. The technique was performed in air with a Nanoscope IIIa scanning probe microscope, equipped with an "E head" scanner (Digital Instruments, Santa Barbara, USA). Images were acquired in the tapping mode using cantilevers with sharpened silicon nitride tips (TESP, Digital Instruments, Santa Barbara, USA). Topographic images were processed by mean plane subtraction and colour-coded from black to white (bottom to top) in the *Z* direction by image analysis software. AFM samples were elaborated by the deposit of 10 μl onto a freshly cleaved mica surface (PROVAC, Balzers, Liechtenstein) and dried at room temperature before analysis.

2.7. Body distribution of LSP after oral administration to rats

Two groups of 6 male rats (250 g weight) of the Wistar strain (Iffa Credo SA, L'abresle, France) were placed in individual cages at random. Forty-eight hours before the experiment they were given a glucose diet (50 g l^{-1}), and 24 h before were put on starvation. The rats were administered 2 ml of liposome suspension by gastric intubation. Liposomes were radiolabelled with [^{14}C] (10 μCi) on the fatty acids or the choline of the phospholipids and in all cases with [^{3}H] (10 μCi) on the internal polymer core. Four hours after administration, that is after gastric emptying, the animals were anaesthetised. The whole blood was recovered and the following organs: kidneys, spleen, liver, stomach, lungs, skin, bone, intestine, and heart. The intestine was separated into three parts: caecum and colon, jejunum and ileum, and duodenum. The organs were kept at 4 °C until processing. The whole blood was centrifuged at 4 °C to separate the plasma from the erythrocytes. The organs were washed in isotonic phosphate-buffered saline, weighed, and a sample of each taken and stored at −20 °C. The tissue samples were incubated at 50 °C for 16 h in 1 ml of soluene (Packard, Meridien, USA) before the addition of 10 ml of Picofluor 40 scintillant (Packard, Meridien, USA), and counted for radioactivity.

2.8. Differentiated cell lines Caco-2 and TC7

The cell line Caco-2 (ECACC, Sophia Antipolis, France) at a high passage number (103) or their sub-clone TC7 (passage 63, generously provided by Dr. C. Alquier, INSERM U-476, Marseille, France) were maintained in 75 cm^2 flasks in medium DMEM/Ham F12 1:1 containing 20% foetal calf serum, 4.5 g l^{-1} glucose, 1% L-glutamine et 1% non-essential amino acids, in the absence of antibiotics. The cells were stripped off when they were 90% confluent. Twenty-four hours before seeding, transwell clear filters (pore size 3 μm, 4.7 cm^2 surface area, Costar, Brumath, France) were coated with a solution of Type IV collagen (Becton Dickinson, Le Pont de Claix, France) to minimise non-specific binding. On the day of seeding, the filters were washed three times with phosphate-buffered saline without Ca^{2+} or Mg^{2+}, and the cells seeded at a density of 5×10^5 per filter. The cells were cultured for 24 days and the impermeability of the monolayer verified by the measure of transepithelial electrical resistance (TEER) using a millicell ERS (Millipore, St. Quentin

en Yvelines, France), as well as by measuring the transport of the non-absorbable marker [^{14}C]-PEG 4000 (NEN, Dupont de Nemours) and analysing the lactate dehydrogenase (LDH) in the basolateral compartment (Promega cytotoxicity assay, Technical bulletin No. 163). The results are a mean of 6–12 independent experiments.

2.9. Transport studies with human colonic mucosa in vitro

The removal of human tissue for these experiments was carried out according to the regulations in force (articles L.672-1 and 665-11 of the Public Health code), and each patient gave informed consent. Within 30 min of the ligation of the vasculature preceding the operation, a segment of non-cancerous colon was obtained. Histological examination was used to confirm the absence of tumour nodules in the segments used for study. Six mucosal sections of around 1 cm^2 were obtained from each segment. The sections were dissected longitudinally to remove the serosal muscle in order to retain the mucosa, sub-mucosa and muscularis mucosa. The dissection lasted a maximum of 10 min. The sections were mounted in Grass diffusion chambers (Costar, Brumath, France) and maintained in a state of cold ischemia in Wisconsin University medium (Belzer's fluid, Belzer and Southard, 1988). The sections were then bathed in Krebs bicarbonate ringer (NaCl: 6.9 g l^{-1}; KCl: 0.35 g l^{-1}; KH_2PO_4: 0.163 g l^{-1}; $MgSO_4$: 0.1446 g l^{-1}; $CaCl_2$: 0.2778 g l^{-1}; $NaHCO_3$: 2.1 g l^{-1}; D-glucose: 2 g l^{-1}; EDTA: 0.0097 g l^{-1}) at pH 7.4 at 4 °C and oxygenated with 95:5% O_2:CO_2. Two separate compartments are obtained: the intestinal lumen surface and the plasmatic (serosal) surface, each containing isotonic medium. The whole system was kept at 4 °C, before being gradually warmed to 37 °C to reactivate the tissue after the cold ischemia. The sections in the apparatus were washed five times with 2.5 ml of Krebs bicarbonate ringer supplemented with mannitol (3.64 g l^{-1}) on the mucosal side (pH 7.8) and D-glucose (3.6 g l^{-1}) on the serosal side (pH 7.45). After stabilisation, the system was verified by assaying LDH in the two compartments, measuring the passage of [^{14}C]-PEG 4000, and by recording the variation of TEER. In total, experiments were carried out on 150 samples.

2.10. Transport experiments

For each type of system (Caco-2 and TC7 monolayers, Grass chambers with human intestinal sections), the same procedure was followed. The mucosal compartment was filled with Krebs bicarbonate ringer containing 3.64 g l^{-1} mannitol at pH 7.8–8.0, and the serosal (basolateral) compartment with DMEM/Ham F12 1:1 medium containing 20% foetal calf serum and 4.5 g l^{-1} glucose, at pH 7.45. The different preparations of drugs, either free or encapsulated were added to the apical compartment at $t = 0$, and a 10 μl sample taken. The concentrations of drugs either in the free form or encapsulated were: MTX: 374 nM; MTX-TC: 286 μM; VIN: 848 nM; 5-FU: 89.2 nM. Every 15 min, 1 ml of the medium in the serosal compartment was sampled for counting and replaced with fresh medium. In each model, the TEER was measured every 60 min during the total incubation of 180 min. At the end of the incubation, the cells or the tissue sections were rinsed four times with phosphate-buffered saline, and frozen at −80 °C for subsequent analysis of the tissue accumulation of the drugs following dissolution in soluene (50 °C, 16 h) and for the counting of radioactivity as described earlier.

2.11. Calculations and statistics

For comparison with other studies and for clarity, the drug transport data obtained are presented in two different forms: as a calculation of the apparent permeability (P_{app}) and as the area under the curve (AUC).

The P_{app}, expressed as cm s^{-1} were calculated using the following equation, as described by Artursson et al. (1993).

$$P_{app} = \frac{FV_D}{S_A M_D}$$

where F is the flux rate (dQ/dt) (g s^{-1}), V_D is the apical volume (cm^3), S_A is the area of tissue or cells exposed to the apical compartment (cm^2) and M_D is the mass (g) of the test drug in the apical compartment. AUC (mg min^{-1}) corresponds to the AUC of the kinetics of the drug appearance in the serosal medium, related to the initial dose at $t = 0$ in the apical medium. AUC $= \int_0^{180} tC_p dt$ (C_p is the basolateral concentration of the test molecule during the time course of the experiment). AUC values were calculated using the

Table 1
Treatment received by patients prior to resection

Transport experiment	Pre-operative radiotherapy (Y/N)	Pre-operative chemotherapy (Y/N)	Metastasis (Y/N)	Carcinosis (Y/N)	Sample localization (L/T/I)	Sex (F/M)
5-FU	6/9	0/15	6/9	3/12	12/3/0	12/3
LSP-5-FU	6/9	0/15	6/9	3/12	12/3/0	12/3
VIN	15/12	6/21	9/18	0/27	18/0/9	15/12
LSP-VIN	15/12	6/21	9/18	0/27	18/0/9	15/12
MTX	3/9	0/12	3/9	3/9	6/6/0	6/6
LSP-MTX	3/9	0/12	3/9	3/9	6/6/0	6/6
MTX-TC	9/12	9/12	6/15	9/12	12/3/6	9/12
LSP-MTX-TC	9/12	9/12	6/15	9/12	12/3/6	9/12

Abbreviations: Y, yes; N, no; L, left colon; T, transversal colon; I, ileum; F, female; and M, male.

trapezoidal rule. The calculation of M_D or the AUC was not just a mean extrapolation between $t = 0$ and $t = 180$, but each value at time t was calculated based on the previous value $(t - 1)$, according to the equation: $X_t = (5x_t + x_{t-1}) + (5X_{t-1}/6)$ for the colon sections, and $X_t = (20x_t + x_{t-1}) + (X_{t-1}/2.4)$ for the monolayers Caco-2 et TC7; where x_t corresponds to the amount (mg) of the drug recovered at time t, and X_t corresponds to the amount of drug linked to the loss and dilution due the previous sampling. Results are expressed as means ± S.E.M., and analysed by ANOVA for repeated values. The differences were determined by Fisher's test protected least significant difference (PLSD) or the more discriminative Scheffe's test at a confidence level of 95%, and when appropriate by the Student's t-test.

2.12. Patient characteristics and medical history

The colonic resections were classified according to the pathology and treatment of the patients and this is summarised in Table 1, along with details of the drug transport tests carried out on the sections obtained.

3. Results

3.1. Stability of liposomes in isotonic medium

Using the dialysis technique, it was shown that at the end of the experiment the dialysis membrane absorbed a negligible amount of the radioactivity (less than 0.01% of the total), and therefore did not effect the results.

The effect of the polymer on the stability of the LSP was tested following incubation in medium containing 20% serum at 37 °C with shaking. The stability was followed by measuring the radioactivity outside the dialysis tubing released from the labelled phospholipids or collagen by their degradation. The results are shown in Fig. 1. It can be seen that the percentage of radioactivity released from the phospholipids ([^{14}C]-DOPC) was always higher from the LS than from the LSP. The release pattern between the two types of liposomes, LS and LSP, showed significant differences at 30 min, 10 h, 12 h and between 18 and 24 h. The LSP contain the [^{3}H]-collagen in their core and its release (insert in Fig. 1) increased progressively with time ($r^2 = 0.874$, $P < 0.0001$). But, after 24 h, 82% of the collagen was still entrapped in the LSP. In the case of the release of the radiolabelled phospholipids, there was a clear difference between the LS and the LSP. LSP only released 0.35% of their radioactivity, compared with 1.2% for the LS over 24 h of incubation. Thus, the presence of the collagen core stabilises the lipidic bilayer when the liposomes are incubated at 37 °C in the presence of 20% serum.

After 24 h incubation of LS or LSP in the same medium, end point sample of external dialysis medium show 82 ± 3% for [^{3}H]-MTX from LS samples and 65 ± 2% for LSP; 89 ± 4% for [^{3}H]-VIN from LS sample versus 64 ± 3% for LSP and 92 ± 6% for [^{14}C]-5-FU LS formulation and 54 ± 7% for the corresponding LSP sample. These results (only expressed in the text) show that presence of polymer in liposomal formulation lead to a slight decrease in the drug release from the liposomal formulations.

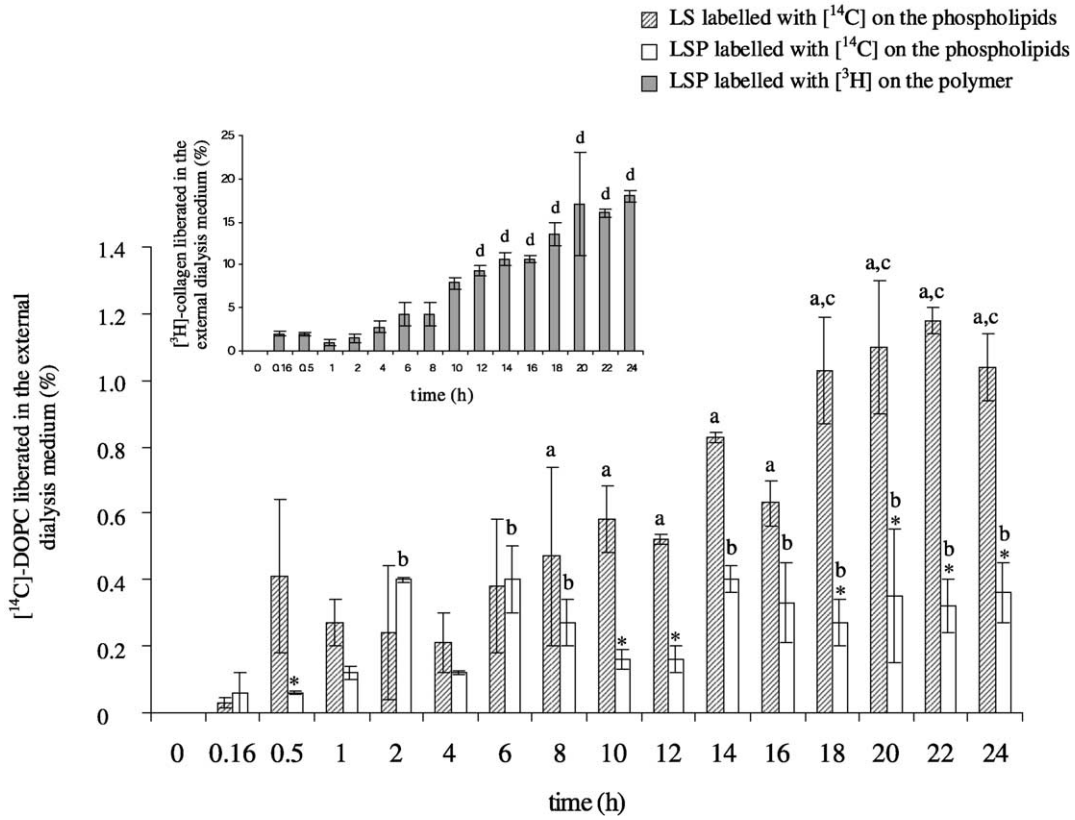

Fig. 1. The stability of LS and LSP in isotonic medium at 37 °C, shown as the percentage of the initial radioactivity recovered in the exterior dialysis medium compared with time. Results are expressed as means ± S.E.M. ($n = 3$). An asterisk (∗) indicates a significant difference between the LS and the LSP at given times (*t*-test, $P < 0.05$). ANOVA was used for the following comparisons: (a) LS time 0 vs. time 8–24 h; (b) LSP time 0 vs. time 2, 6, 8 h, and time 0 vs. 14–24 h; (c) LS time 8 h vs. time 18–24 h; and (d) LSP time 0 vs. time 12–24 h.

LSP formulation appear slightly more resistant than the polymer less liposomes formulations. These data are consistent with experiments using intestinal enzymes in the incubation medium.

3.2. Stability of LSP in the presence of intestinal enzymes

The size of the LSP as measured by QELS was 124 ± 21.2 nm ($n = 32$), slightly larger than the LS which measured 106±23.5 nm ($n = 12$). AFM (Fig. 2) suggested that the collagen polymeric core radiated out and formed a surface layer on the LSP. Pictures clearly show three kind of structures. First consists in a roughness globular shape with 144 nm of horizontal distance and 65 nm of vertical distance. Second shows a polymeric network radiated out the particle (thickness: 3 nm). Third (thickness: 7–8 nm) corresponds to a single bilayer structure due to a partial collapse of the liposomes. To test this hypothesis, the LSP were submitted to successive exposure to degradative enzymes, starting with collagenase, which would degrade any collagen surface layer, and subsequently (1 h later) with phospholipases which would hydrolyse the phospholipids of the bilayer with the release of fatty acids, once the surface layer had been degraded. The amounts of [^{3}H]-collagen and [^{14}C]-DOPC (lipidic bilayer marker) breakdown products, found in the external dialysis medium during the incubation with the enzymes are shown in Fig. 3. It can be seen that there was a release of collagen breakdown products after 5 min (insert in Fig. 3), and this steadily increased to reach a plateau of around 50% degradation after 21 h incubation. By contrast, the action of the

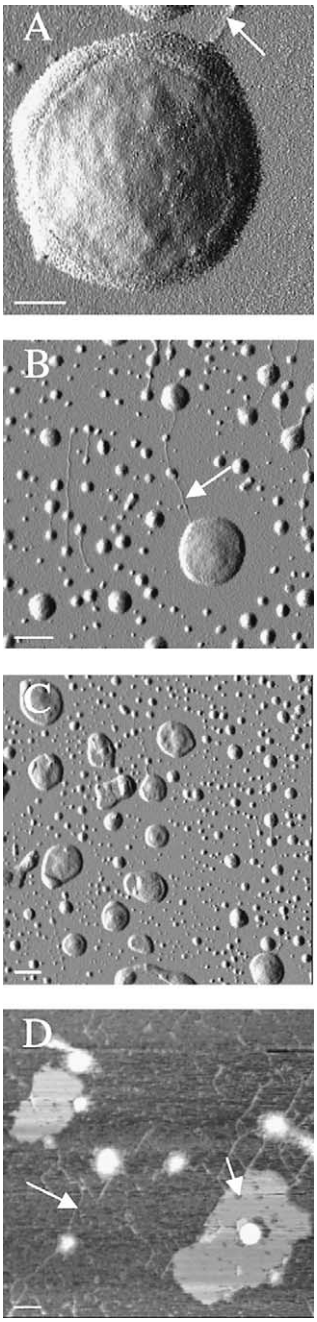

Fig. 2. (A) AFM pictures show the vesicular structure of a LSP (bar corresponds to 20 nm), the arrow indicates an extra-liposomal part of the polymer. (B) Arrow show polymeric network (bar corresponds to 100 nm). (C) Most of the LSP are not collapsed (bar corresponds to 100 nm). (D) There is no bilayer resulting from destroyed liposomes, but a polymeric network with a thickness of 3 nm (bar corresponds to 100 nm).

phospholipases resulting in the transfer of labelled breakdown products to the exterior dialysis compartment was much slower and did not manifest until at least an hour after the addition of the enzymes. The percent breakdown products finally reached only 1.2% after 24 h. The release of [^{3}H]-collagen fragments during the first few minutes of the incubation would be evidence that while the majority of the collagen forms the core of the LSP, there is a surface layer of collagen on LSP as suggested by the AFM observations. It is this internal and external presence of collagen which induces the changes in the biological behaviour of the liposomes.

3.3. Body distribution of the LSP after oral administration to the rat

In order to study the stability of the particles in the GI tract, we argue that if they remain intact then the ratio of the radioactivity in the surface lipids to radioactivity in the collagen core will remain the same. A large difference in this ratio would represent degradation. It could be possible, however, that in tissues the same ratio could be obtained from degradation products. The results (Fig. 4) show that only a small amount of the metabolites resulting from the degradation of the LSP actually cross the intestinal barrier. They follow the normal route of elimination by the reticuloendothelial system. It appears that the LSP are stable in the upper parts of the GI tract. Once they reach the caecum and colon they are rapidly degraded. In other tissues examined the LSP appear to be degraded. It was possible that when the radiolabel on the lipidic surface was in the form of the fatty acid, it could have been released by the action of pancreatic phospholipase A_2. To check this, the experiment was repeated with the phospholipid labelled in the choline moiety. No difference was observed in the labelling distribution obtained following oral administration. Whatever the localisation of the radiolabel (fatty acids or choline) on phospholipids, same results are found (data not shown).

3.4. Transport of free and liposome entrapped drugs by cell layers and tissue sections

The results of the transport experiments with the cell lines and the human colon sections are shown in Table 2. With the Caco-2 cell line, the most highly

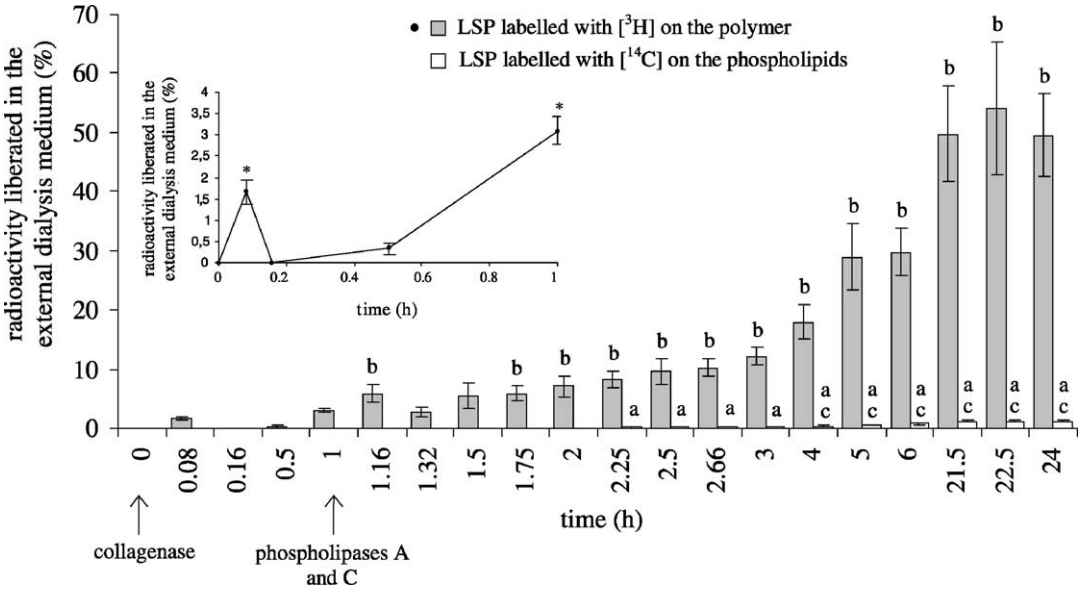

Fig. 3. The stability of the LSP in the presence of intestinal enzymes, shown as the percentage of the original radioactivity recovered in the external dialysis medium compared with time. Results are expressed as means ± S.E.M. ($n = 3$) and are analysed by ANOVA for the following comparisons: (a) [^{14}C]-phospholipids LSP time 0 vs. time 2.25 to time 24 h; (b) [^{3}H]-polymer LSP time 0 vs. time 1.16 and time 0 vs. time 1.75–24 h; and (c) [^{14}C]-phospholipids LSP time 2.25 vs. time 4–24 h. An asterisk (∗) indicates a significant difference between time 0 vs. time 0.08 and 1 h (*t*-test, $P < 0.05$).

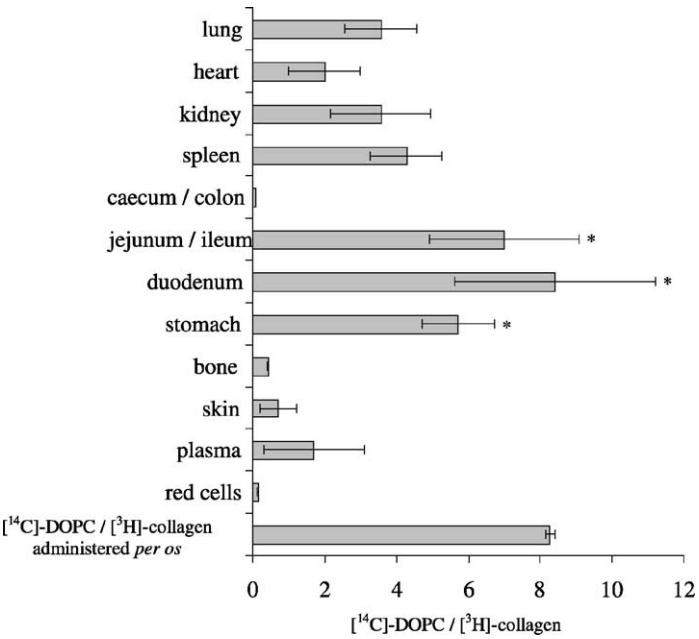

Fig. 4. Body distribution of LSP after oral administration to rats. Results are expressed as means ± S.E.M. ($n = 6$) and are analysed by ANOVA for the following comparisons: reference ratio: [^{14}C]-DOPC/[^{3}H]-collagen vs. same ratio in the whole body. An asterisk (∗) indicates no significant difference.

Table 2
Drug transport and accumulation by cell culture models and human intestinal tissue

Drug treatment	Model	P_{app} ($cm\,s^{-1} \times 10^{-6}$)	AUC ($mg\,min^{-1} \times 10^{-5}$)	Tissue uptake (%)	n
5-FU	Human tissue	15.2 ± 0.8	34.8 ± 3.1[a*]	2.15 ± 0.3[a*,b**]	15
	Caco-2 cells	22.7 ± 1.5[c*]	363.1 ± 30.3[a*,c*,d*]	0.66 ± 0.02[c**]	6
	TC7 cells	6.52 ± 2.3[d*]	96 ± 4.1[a*,c**,d*]	0.44 ± 0.02[d**]	6
LSP-5-FU	Human tissue	19.6 ± 0.1[c**]	50.7 ± 5.4[c*]	2.66 ± 0.11[c**]	15
	Caco-2 cells	7.7 ± 0.3	114.2 ± 4.7	0.57 ± 0.03	6
	TC7 cells	4.3 ± 0.5	65.2 ± 7.7	0.43 ± 0.01	6
VIN	Human tissue	11.9 ± 2.1	1.4 ± 0.1	6.07 ± 0.42[e*,f**,g*]	27
	Caco-2 cells	22.5 ± 1.6	11.8 ± 1.3[f*]	0.64 ± 0.03[e*,f*]	6
	TC7 cells	3.6 ± 0.9[f**]	0.9 ± 0.1[f**]	0.71 ± 0.12[a*]	6
LSP-VIN	Human tissue	9.3 ± 1.2	1.6 ± 0.3	2.81 ± 0.24	27
	Caco-2 cells	3.2 ± 0.5	1.6 ± 0.2	0.50 ± 0.02	6
	TC7 cells	1.4 ± 0.3	0.4 ± 0.07	0.84 ± 0.05	6
MTX	Human tissue	23.2 ± 5.4[e**]	1.1 ± 0.5	1.76 ± 0.32	12
	Caco-2 cells	23.7 ± 1.6[h*,i*]	40.7 ± 3.4[i*]	0.47 ± 0.02	6
	TC7 cells	2.2 ± 0.2	3.5 ± 0.3	0.32 ± 0.01	6
LSP-MTX	Human tissue	15.1 ± 2.1	0.6 ± 0.1	1.57 ± 0.23[j*]	12
	Caco-2 cells	6.1 ± 0.7	9.8 ± 1.2	0.39 ± 0.02	6
	TC7 cells	2.1 ± 0.3	3.4 ± 0.7	0.31 ± 0.01	6
MTX-TC	Human tissue	14.9 ± 2.1[k**]	$4.4 \times 10^5 \pm 0.5 \times 10^5$[g*,k*,l**,m*]	1.2 ± 0.11	21
	Caco-2 cells	37.8 ± 0.9[l*]	$2.8 \times 10^5 \pm 0.1 \times 10^5$[l*]	1.93 ± 0.11[l*]	12
	TC7 cells	Toxic	Toxic	Toxic	12
LSP-MTX-TC	Human tissue	30.5 ± 1.1[l**]	$10.1 \times 10^5 \pm 3.3 \times 10^5$[n*,o*]	1.22 ± 0.1	21
	Caco-2 cells	6.5 ± 2.1	$0.8 \times 10^5 \pm 0.1 \times 10^5$	1.43 ± 0.12	12
	TC7 cells	Toxic	Toxic	Toxic	12
Controls					
PEG 4000	Human tissue	0.05 ± 0.001	0.01 ± 0.005	n.d.	3
LSP-PEG 4000	Human tissue	0.06 ± 0.002	0.01 ± 0.006	n.d.	3
G	Human tissue	7.1 ± 0.1	0.2 ± 0.001	n.d.	3
LSP-G	Human tissue	11.8 ± 2.2[p**]	0.42 ± 0.06[p*]	n.d.	3

Values are expressed as means ± S.E.M. Results were analysed by ANOVA for repeated values, and significant differences were determined by Scheffe's test (*) or Fisher's post hoc test (**) at a confidence interval of 95%.

[a] 5-FU vs. VIN.
[b] 5-FU vs. MTX-TC.
[c] 5-FU vs. LSP-5-FU.
[d] 5-FU vs. MTX.
[e] VIN vs. MTX.
[f] VIN vs. LSP-VIN.
[g] VIN vs. MTX-TC.
[h] MTX vs. LSP-VIN.
[i] MTX vs. LSP-MTX.
[j] LSP-MTX vs. VIN.
[k] MTX-TC vs. MTX.
[l] MTX-TC vs. LSP-MTX-TC.
[m] MTX-TC vs. LSP-MTX.
[n] LSP-MTX-TC vs. LSP-MTX.
[o] LSP-MTX-TC vs. MTX.
[p] LSP-G vs. G.

transported molecule was free MTX, which gave a P_{app} of 23.7×10^{-6} cm s^{-1}, whereas the encapsulation of this drug reduced the permeability by four-fold. By contrast, with the TC7 cell monolayer, the permeability of the free and encapsulated form of the drug was similar, but considerably lower than in the Caco-2. At low concentrations, a similar result was observed with the human tissue sections. At MTX-TC (i.e. compatible with concentrations used clinically), the free drug had a five-fold higher permeability than the encapsulated drug with Caco-2 cells. By contrast, with the human tissue sections, the encapsulation of MTX-TC increased its permeability by two-fold.

In the case of VIN, the same variation between the models is observed as for MTX. The encapsulation of the drug in liposomes reduced the permeability by six-fold with Caco-2 and 2.5-fold with the TC7 cells. By contrast, the transport of VIN across human colon sections was similar whether it was encapsulated in liposomes or not.

With 5-FU, encapsulation also reduced the permeability in the two cell monolayer models by factors of 3 and 1.5 in the cases of Caco-2 and TC7 cells, respectively. However the permeability across human colon sections was increased in 25% by encapsulation (Fisher's test).

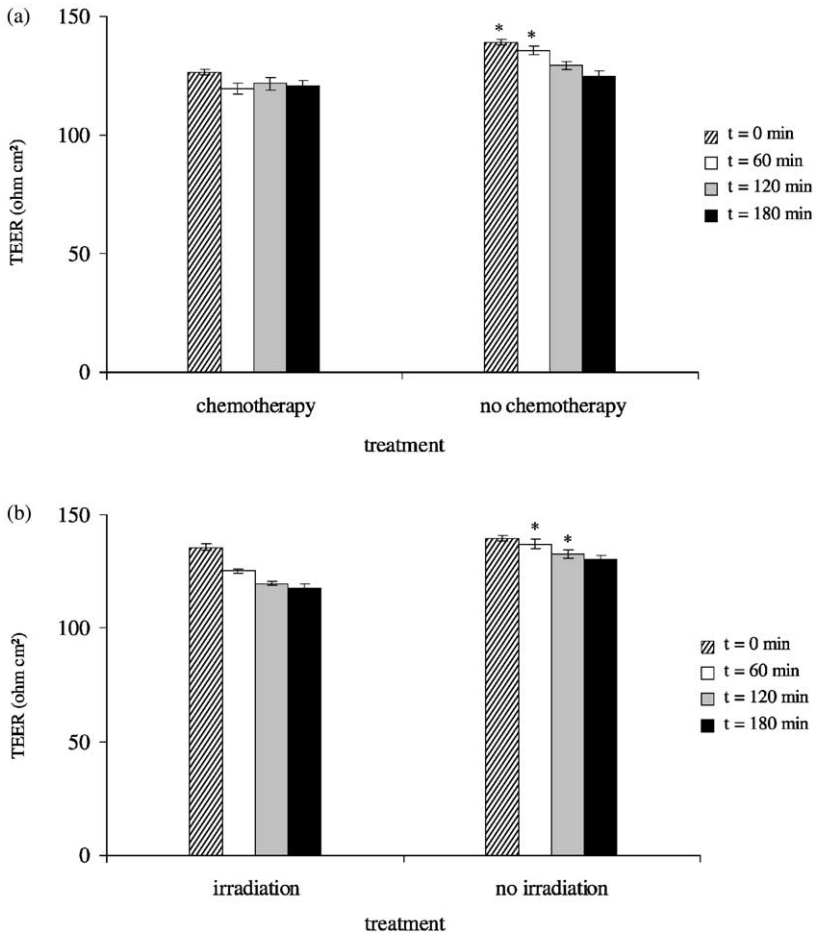

Fig. 5. The influence of (a) pre-operative chemotherapy and (b) pre-operative radiotherapy on the TEER of the human intestinal sections, regardless of the formulation studied. Values are expressed as means ± S.E.M. (chemotherapy, $n = 30$; no chemotherapy, $n = 120$; irradiation, $n = 66$; no irradiation, $n = 84$). Results were analysed by ANOVA for repeated values, and significant differences ($*$) were determined by Scheffe's test, at a confidence interval of 95%.

The human sections in Grass chambers were monitored by using the non-absorbable marker PEG 4000, and with glucose as a marker of transcellular/active transport. As expected (Uil et al., 1997) the PEG 4000 showed no significant passage whether formulated or not. Glucose permeability was similar to the cytotoxic drugs, and was increased in 40% by liposomal encapsulation.

Examination of the AUC values obtained, shows that whatever the drug studied, the values were always greater with the Caco-2 cells than those obtained with the TC7 cell line. The AUC values obtained with the human colon sections were generally lower than those obtained with the cell lines. Overall, the AUC values correlate reasonably with the P_{app} values.

The release of LDH into the basolateral (serosal) compartment was followed in all the systems. The formulation LSP-MTX-TC caused a significant (Fisher's test) decrease (15%) in the LDH released compared with the free drug. Compared with the control the free drug itself caused a significant release of LDH (12%) over the 3 h of incubation. None of the other formulations caused any significant variation in the LDH release whatever the systems used. The measure of the TEER showed that with the two cell culture systems, there was a 25% reduction in TEER over the 3 h incubation period regardless of the formulation, while for the human tissue sections, this decrease was around 10%. However, with the cell line models, the reduction was less of a drop in the TEER when the drugs were encapsulated in liposomes. It should be noted that the cell layers of TC7 were destroyed by the high concentration of MTX, even in its liposomal formulation.

Table 2 also shows the tissue levels of drugs at the end of the incubation period, obtained from the tissue radioactivity after the equivalent surface area of cultured cells or tissue sections had been rinsed five times with phosphate-buffered saline. Tissue levels of drugs are expressed as a percentage of the initial radioactivity added. In all cases the tissue accumulation was always higher in the human colon sections than in the cell lines. VIN showed the highest accumulation in the tissue sections, followed in descending order by 5-FU, MTX, and MTX-TC. Liposomal encapsulation of VIN reduced the tissue accumulation in the tissue sections, while encapsulation of low concentration of MTX had no effect. By contrast, the encapsulation of 5-FU and MTX-TC significantly increased tissue accumulation. In the Caco-2 system, the highest cell accumulation was with the MTX-TC, while VIN and 5-FU had similar values but one-third less. The lowest tissue uptake was seen with the low concentration of

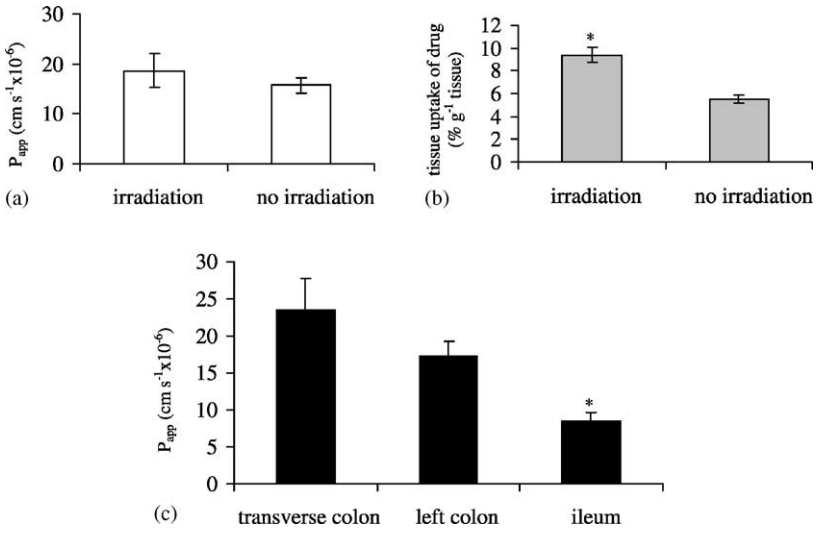

Fig. 6. (a) The influence of pre-operative radiotherapy on drug permeability (P_{app}) and tissue accumulation and (b) the effect of tissue site on drug permeability, regardless of formulation in each case. Values are expressed as means ± S.E.M. (irradiation, $n = 66$; no irradiation, $n = 84$; transverse colon, $n = 24$; left colon, $n = 96$; ileum, $n = 30$). Results were analysed by ANOVA for repeated values, and significant differences (∗) were determined by Scheffe's test, at a confidence interval of 95%.

MTX. In all cases the encapsulation of the drug in liposomes reduced the cell accumulation. With the TC7 cell line, VIN showed the highest cell accumulation, followed by 5-FU and finally MTX, but in no case the liposomal encapsulation modified the cell uptake.

It should be noted that the human tissue used was obtained adjacent to areas undergoing resection. The test tissue was checked histologically to verify that it was non-tumoral before use. However, the patients had received a number of different treatments before undergoing surgery (summarised in Table 1). The effect of pre-treatments on the TEER is shown in Fig. 5a and b. It can be seen that both radio- and chemotherapy had a significant effect. Fig. 6a shows the effects of these treatments on the permeability and tissue accumulation of the drugs. It can be seen that radiotherapy had no effect on permeability, but increases tissue accumulation by two-fold. The anatomical location of the tissue is also important and it can be seen that regardless of the formulations, certain gut regions showed higher permeabilities. Fig. 6b shows that the tissue from the transverse colon gave the highest permeabilities, and the ileum the lowest.

4. Discussion

Compared with classical liposomes, the structure of the LSP renders them less sensitive to factors such as bile salts, temperature, mobility and exposure to degradative enzymes in conditions similar to those found in vivo. Kokkona et al. (2000) have reported that the stability of liposomes in the GI tract depends partly on the phase transition temperature of the constitutive lipids. In our case, the presence of the polymeric internal phase, and polymer on the exterior surface, as shown by the AFM (Fig. 2) and enzyme degradation experiments, leads to enhanced resistance. Jass et al. (2000) have shown that conventional liposomes attached on a solid support during AFM analysis, spread and flattened from the outer edge to the centre. Some of these structures are shown on Fig. 2C. But most of the LSP seems to resist to the collapse imposed by the tapping mode of the analysis. We can not observe large bilayer resulting from liposome degradation. The thickness of the network (Fig. 2D) is different than the bilayer one. For this reason we can assume that it could correspond to the external polymer of the LSP.

LSP stability was confirmed by the experiments with the addition of the collagenase and phospholipase enzymes. The results showed that this form is stable to pancreatic enzymes in vitro. In addition, release rate of encapsulated substances (drugs or the internal polymer) shows the potential of this structure to limit the leakage and cytotoxic effects of entrapped molecules. We have noted that the pre-surgery treatments received by patients modify substantially the mucosal function (Fig. 6a and b). Thus, using the LSP formulation, a diminution of the toxicity for an equivalent quantity of drug will be beneficial where the mucosal function has been altered by radio- or chemotherapy.

With the Caco-2 cell line model, the encapsulation of MTX-TC reduced the passage of the molecule across the cell layer, compared with the free drug. However, it is likely that permeability of the free drug was a false high value caused by toxicity destroying the cell layer. Observation of this toxicity to the cells is backed up by the release of LDH measured, and in fact the level of this marker of cell cytolysis is lower with the MTX-TC entrapped in liposomes. In addition, the TEER values are higher in the presence of the encapsulated drug than with the free drug. Thus, the free form of the drug at therapeutic concentration would appear to damage the cell layer and distort permeability data. This was confirmed in the experiments with the TC7 cell, whereby the drug destroyed the cell layer and no transport data were obtained. It is important to be careful in interpreting transport data with the cytotoxic drugs when they are used in only "tracer" amounts and not at therapeutically equivalent concentrations, as in our MTX experiments. This view is supported by the results obtained with the transport of the MTX-TC across the human gut segments. The permeability of the liposomal drug was two-fold higher than that of the free drug, without any noticeable change in the toxicity markers.

The validity of the human mucosal sections in the Grass chambers was verified using the non-absorbable marker PEG 4000. Neither the free nor encapsulated form crossed the epithelial barrier confirming the viability of the system. That also suggested that the passage of the LSP formulation was not paracellular. On the other hand, the P_{app} of the active transport marker glucose was $7 \times 10^{-6}\,cm\,s^{-1}$ and the encapsulation significantly increased this value (by two-fold) by a mechanism which remains to be elucidated.

The membrane efflux transporter P-glycoprotein (P-gp) is known to cause the efflux of anticancer drugs from cancerous cells, where it is more active than in healthy cells. This transporter is a member of the ATP-cassette family of proteins and is overexpressed in colon cancer Robert (1999) as well as in cancer derived cell lines like Caco-2 cells (Van Hille et al., 1996). Among the strategies envisaged to reduce cell resistance to chemotherapy is the coupling of active cytotoxics to polymers (Kaye, 1999) or to encapsulate them in liposomes, with a view to avoid exposure of the drugs to P-gp transporting domains on the inner leaflet of the lipidic bilayer (Ueda et al., 1999). In our system using the human colon sections, which were non-cancerous, two main parameters were studied: the apical to basolateral permeability and the tissue accumulation of the drugs (Table 2). These values represent the balance between the tissue uptake, the transport out of the tissue towards the apical side consequent on the P-gp activity (possibly also linked to the function of the cytochrome P450 enzymes), and the passage across the epithelium. In the human tissue sections, VIN was the drug which showed the highest tissue accumulation and the lowest transepithelial passage. The same tendency was not seen with the Caco-2, where the tissue accumulation and permeability were very similar to 5-FU. Such differences between cell models and human models have been seen in previous studies, for example, within the liver (Clerc et al., 1996).

When the VIN was encapsulated the transepithelial flux was the same as for the free drug in the human colon sections, but was reduced by seven-fold in the Caco-2 system. When we examine the same comparisons with 5-FU, we can conclude that the encapsulation enhances the transepithelial passage and the tissue accumulation. In the Caco-2 system, the encapsulation of this drug reduced the transcellular passage by three-fold, and slightly reduced the cell accumulation. The variation of this reduction of permeation from one drug to another shows that the activity of the efflux. It also appears that, depending on the nature of the drug, the efflux transporters affecting transport to the lumenal surface, are not necessarily the same. The increase in 25% of the transepithelial transport of the liposomal form of 5-FU was not linked to an increase in the tissue accumulation of the drug, whereas a drug which accumulated more in the tissue, like VIN, crossed the epithelium to a lesser degree.

One can draw the same conclusions for MTX as for VIN. The transepithelial transport and tissue accumulation were both reduced by liposomal encapsulation in the human colon sections. In the Caco-2 model, the same variations were observed, but the reduction in permeation was by four-fold in this case. It can be concluded that the liposomal formulations of the drugs tested reduced the influence of the proteins responsible for their efflux. There is another important parameter to consider, which is the concentration of the tested drugs. In the case of MTX, we have tested a high concentration (286 μM) both free and in the liposomally entrapped form. Liposomal encapsulation increases the transepithelial passage by two-fold, while the tissue uptake was unchanged. The results obtained with the Caco-2 for MTX were the same as for 5-FU: the encapsulation decreased the passage by a factor of 5, as well as the tissue uptake. Thus the optimisation of the transepithelial passage of the drugs by the liposomal formulations depends on the nature of the drug and the concentration, but does not appear to be linked to the tissue accumulation.

In general terms, the values of permeability and tissue uptake by the TC7 cells were lower than in the Caco-2 system, and the reduction of transepithelial passage produced by the encapsulation of the drugs was less pronounced. In the case of MTX there was no change produced by the encapsulation, in contrast to the Caco-2 data. Quantitatively the values are far from those obtained with the human colon sections. Thus, it would appear that the efflux transporters are more active in the TC7 cells than in the other models. In this model, the reduction of the influence of the efflux transporters induced by encapsulation was only evident in the case of MTX. The modification of the P_{app} by encapsulated drugs was not a generalised phenomenon as in the case of VIN and MTX at tracer concentrations. Encapsulation of these molecules in the LSP had little effect on their transepithelial passage. Concerning 5-FU, the results showed that the encapsulation of this drug in the ex vivo model increased its passage.

Thus in summary, it appears that the formulation of 5-FU and MTX-TC in LSP should result in an slight increase of their bioavailability by the oral route. These results are confirmed by an analysis of the

AUC for these drugs. The mucoadhesion and tissue uptake of these drugs was not apparently correlated with their transmucosal passage, since VIN had high tissue accumulation but was relatively poorly transported, whereas 5-FU had lower tissue accumulation but higher permeability with both its encapsulated and free form.

In the case of the excised human colon sections, the treatment history of the patients can play a role in the tissue function. The results show that pre-operative radiation treatment had little effect on the transepithelial passage of the drugs, but increased the tissue uptake by a factor of 2. With systemic chemotherapy prior to operation, a significant drop in the TEER was observed, showing that the integrity, or more precisely, the transmucosal transport of ions was modified by prior chemotherapy. These results should be taken into account in the design of oral anticancer treatments both at the level of nutritional and pharmacological considerations.

Other parameters such as the localisation of the drug efflux mechanisms have been studied. It appears that the activity of the membrane transporters responsible for drug efflux into the lumen of the GI tract is decreased going from the ileum towards the colon [27] (Stephens et al., 2001). Thus, it is more interesting to deliver drugs at the level of the colon, where the lumenal efflux is less. While it is often stated that the ileum is the major site of xenobiotic absorption, our results would suggest that, at least for the anticancer drugs tested, this is not the case, as the transport of the drugs across the human tissue sections was highest with tissue from the transverse colon. This suggests that for the administration of anticancer drugs by the oral route, formulations should be designed so that the drugs are absorbed at the level of the transverse colon. The goal is to limit the dose administered orally in order to reduce the toxicity for the epithelial cells, already fragile as a result of treatments with adjuvants, and to reduce drug efflux by the transport proteins like P-gp. Thus the development of slow release formulations targeting to the colon would seem a judicious course to take.

In conclusion, the model of choice for evaluating the transmucosal passage of anticancer drugs is the human colon sections mounted in Grass chambers, where the tissue architecture is maintained, unlike the cell layer models Caco-2 and TC7. In addition, the results obtained with this model show weak correlation with those obtained with the Caco-2 and TC7 cell lines, probably as a result of the different expression of the efflux transporters. Because of their easy use, cellular models remains more available, but need to be functionally checked before using.

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