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Etoposide (VP-16) Uptake by Tumour Spheroids and Activity in the Presence of Brij 30, Formulation Additives and Sodium Salicylate

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Abstract—*A number of additives typically used in the formulation of poorly soluble drugs can be shown to influence drug transport across various physiological barriers. Multicellular spheroids from a human neuroblastoma cell line (NB1-G) were used to investigate the effect of etoposide in solution, as its commercial formulation, Vepesid, in the presence of a nonionic surfactant, Brij* 30, *and a hydrotropic agent, sodium salicylate. Enhanced growth delay, apparently related to increased drug uptake, was observed both with the Vepesid and the sodium salicylate formulations. Brij* 30, *however, showed no enhancement of growth delay or drug uptake at a concentration at which it was not in itself cytotoxic. Significant morphological changes in the spheroid were observed at higher concentrations of additives, particularly with Brij* 30, *emphasizing the fact that many formulation additives cannot be used with impunity in tissue culture systems. The enhanced uptake of drug into tumour cells and potential synergy between additive and drug is worthy of further investigation.*

INTRODUCTION

MANY poorly soluble drugs such as etoposide are formulated in surfactant and co-solvent systems. The potential effect of such formulation additives on the penetration and activity of drugs used in *in vitro* cell studies and in animals is often neglected. Yet surfactants have been shown to reduce the barrier properties of many biological membranes, thereby enhancing drug penetration [1]. Multicellular tumour spheroids have been used for some time [2–7] as models for tumours particularly to study the penetration and activity of anticancer drugs. In this work we have used spheroids to investigate the influence of a variety of formulation additives (pharmaceutical adjuvants) on the activity of etoposide (VP16).

It has been suggested that resistance to some cancer chemotherapeutic agents is in some cases due to inadequate diffusion of the drug to sensitive cells in the interior of tumours. Few systematic studies have been carried out on the diffusion of a range of drugs into tumours but calculations by Levin *et al.* [8, 9] suggest that drug penetration can be limited and that this is a problem in the chemotherapy of tumours of detectable size. On the other hand, there have been reports of the rapid penetration into tumours of some molecules, both of high molecular weight (such as antibodies) as well as those of low molecular weight.

One approach to the problem of inadequate diffusion is to use penetration enhancers such as surfactants and to investigate the influence of agents which might be used in the formulation of drugs for clinical use. Materials such as ethanol, the polyoxyethylene glycols, nonionic surfactants and hydrotropic solubilizing agents are used as pharmaceutical adjuvants but are rarely biologically inert [10–12]. Spheroids present a useful model for studying the influence of formulation on anticancer drug penetration and their subsequent activity.

In this work we have studied the penetration of etoposide [13–15], a drug with low solubility (of approx. 100 μg/ml), available commercially (Vepesid, Bristol Meyers) as a solubilized preparation in polysorbate 80 (Tween 80), ethanol and polyoxyethylene glycol (PEG) 300 [13, 14]. We have investigated the effect of unformulated etoposide and as a solution in water up to its solubility limit and the commercial formulation on cultured NB1-G spheroids. The influence of a hydrotropic agent, sodium salicylate, and a nonionic surfactant, Brij 30, on spheroid behaviour has also been studied and we have considered the applicability of the spheroid system as a model to study additive effects. Uptake of etoposide into mouse leukaemia L1210

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cells has been reported to be by passive diffusion [15], so it is a useful model compound. Surfactants have potentially several effects whatever mode of etoposide penetration obtains: by increasing membrane fluidity they can increase transmembrane transport; by decreasing cell contact and increasing the distance between the cells, paracellular 'channel' widths are increased. Some surfactants used in this study cause the spheroids to swell and ultimately disaggregate. The increase in solubility of drugs brought about by micellar solubilization or hydrotropic activity might maintain also a higher concentration gradient and thus increase drug flux into the cells.

MATERIALS AND METHODS

Materials

Etoposide (VP16) was obtained from Bristol Myers (U.K.) and used as received. VP16 formulated as Vepesid (Bristol Myers) was also obtained from the manufacturers and used as received. The formulation contains: etoposide (100 mg), citric acid 10 mg, benzyl alcohol, polysorbate 80 (400 mg), PEG 300 (3.25 g) and ethanol to 5 ml. Sodium salicylate was obtained from BDH Ltd. (Poole, U.K.) and used as received.

Brij 30, a nonionic surfactant with the structure $C_{12}H_{25}(OCH_2CH_2)_4OH$ was obtained from ICI (Leatherhead, U.K.) and used without further purification.

Spheroid culture

The cell line used was NB1-G, established in a monolayer culture originating from a xenograft in athymic nude mice of tumour fragments from a stage IV human abdominal neuroblastoma [16]. Monolayers and spheroids were cultured in Eagles minimum essential medium supplemented with 10% foetal calf serum (FCS) and incubated at 37°C in a humid atmosphere containing 5% CO_2. Cell suspensions were obtained from trypsinized monolayer cultures, 10^6 cells being seeded into stirrer culture vessels (500 ml, Techne, Cambridge, U.K.) for spheroid initiation at a spinner speed of 30 rpm. Spheroids of around 250 μm could then be harvested by Pasteur pipette after 5–7 days.

Experiments with etoposide and additives

Five millilitre aliquots of spheroid suspensions were distributed between six 20 ml sterile plastic universal containers. The spheroids were allowed to settle and the medium drained off and replaced with 5 ml of the treatment solution. All drug solutions were prepared in foetal calf serum-free medium to eliminate protein binding of etoposide. Even distribution of spheroids within the bathing solution was ensured before incubation at 37°C for 1 h with a single agitation after 30 min. Control spheroids were treated in an identical fashion.

Unformulated etoposide was dissolved up to its solubility limit directly in the tissue culture medium. In the case of systems containing surfactants or sodium salicylate, drug was dissolved in medium containing the final concentration of these adjuvants. The solutions were filtered through a 0.2 μm filter. The Vepesid dilutions were prepared aseptically from the commercial injection formulation.

Spheroid growth and growth delay

Post-treatment spheroids were rinsed three times with FCS-free medium and 24 individual spheroids of chosen diameter were selected under the microscope by Pasteur pipette and each placed in a 1% agar base coated well [24 well test plates (Corning)]. Experiments were carried out in duplicate. Plates were incubated as above with weekly additions of 0.5 ml of fresh medium, containing 10% FCS as before.

Three times weekly, spheroid cross-sectional areas were determined by image analysis and the median log volume at 97.7–99.1% confidence limits was calculated. The growth curves obtained from a plot of log volume against time were used to calculate growth delay which is defined as the time in days for the median spheroid volume to reach 10 times the original volume, V_0 [16].

Drug uptake

Spheroids were treated as before (three spheroids of similar size in each universal flask) using [^{3}H]etoposide (Amersham, U.K.) at 5 μCi/100 μg drug. Individual spheroids were then digested overnight using 33% KOH, neutralized with a 1.5 M HCl and duplicate samples of 200 μl counted in 10 ml 'ecoscint' for ^{3}H activity by liquid scintillation. The total amount of drug per spheroid was calculated.

Histology

Spheroids of 500–100 μm diameter were treated for 1 h as before, washed three times immediately transferred, and fixed in neutral buffered formalin. Fixed spheroids were paraffin-embedded and 5 μm sections cut through the centre of each. Sections were stained with haemotoxolin and photographed at ×20 or ×80 under a Polyvar (Reichert-Jung) microscope.

RESULTS

Growth delay

Typical spheroid growth curves are shown in Fig. 1 plotted as log (spheroid volume) versus time. Characteristic growth curves were obtained for untreated spheroids and those treated with the adjuvants alone, these exhibiting an exponential

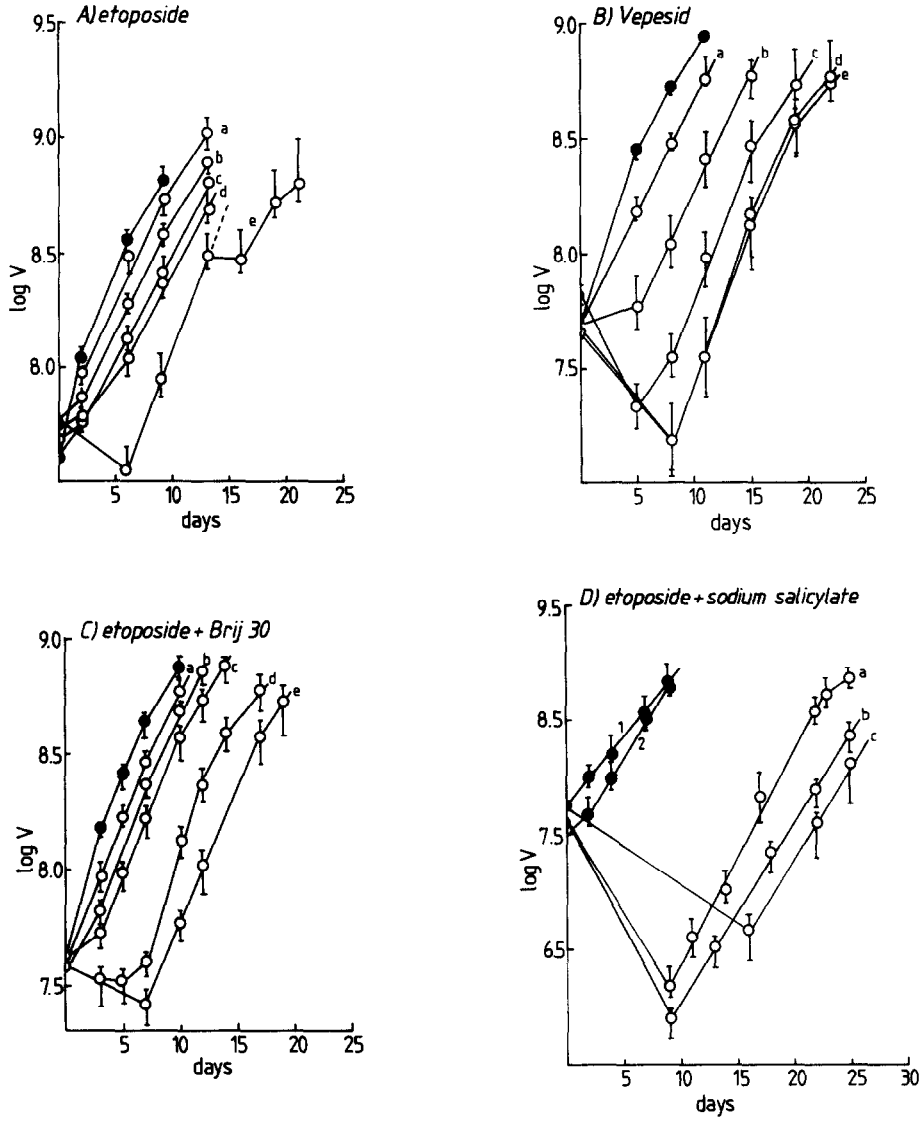

Fig. 1. Representative growth curves for NB1-G spheroids in the presence of various concentrations of etoposide with and without formulation additives: (A) etoposide (VP-16) in aqueous solution: ● = control, ○ a = 20 μg/ml, b = 40 μg/ml, c = 60 μg/ml, d = 80 μg/ml, e = 100 μg/ml of drug; (B) Vepesid injection formulation: ● = control ○, a,b,c,d,e, as before; (C) etoposide with Brij 30 (0.1 μg/ml) control and a–e as before; (D) etoposide with sodium salicylate (= 0.1 M) ● = control (1 = sodium salicylate alone; 2 = no drug or additive); a = 40 μg/ml, b = 60 μg/ml, c = 80 μg/ml etoposide.

phase with a spheroid volume doubling time of around 2 days and a retardation phase once the spheroids reach a diameter of around 1000 μm, illustrating that at the concentrations used the surfactants and co-solvents had in themselves no effect on spheroid growth.

Growth curves for treated spheroids exhibited a regression phase at higher etoposide doses, this effect being particularly marked with the Vepesid and sodium salicylate formulations.

Spheroids of 250 μm initial diameter were treated with etoposide alone (in increasing concentrations up to its maximum aqueous solubility of 100 μg/ml) and with Vepesid injection over a range of 0 to 200 μg/ml etoposide. The resultant plot of growth delay versus concentration of etoposide (Fig. 2) shows that etoposide presented to the cells as Vepesid formulation exhibits an enhanced effect. In both cases a plateau in the growth delay plot appears to be reached close to the maximum aqueous solubility of the drug (growth delay = 19 days for the unformulated drug and 23 days when formulated as Vepesid).

Larger spheroids (with an average initial diameter 450 μm) were treated with four formulations, the results being shown in Fig. 3. The growth delay observed with etoposide alone was considerably less than with the 250 μm spheroids and slightly less in the case of the Vepesid formulation. Again there tended to be a levelling off of chemosensitivity near to the maximum drug solubility. Spheroids treated with 0.1 M sodium salicylate–etoposide solutions exhibited a greatly enhanced growth delay and at a concentration of 100 μg/ml of etoposide a 92%

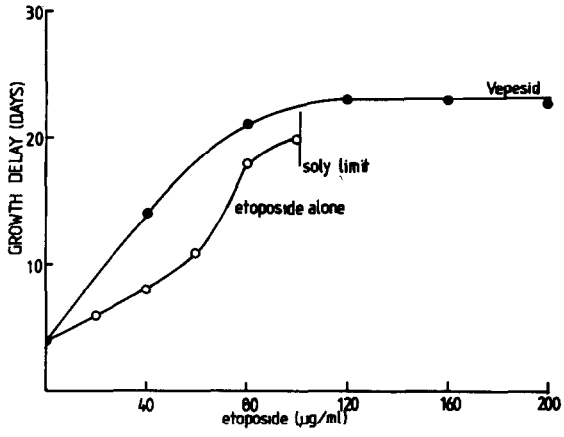

Fig. 2. Growth delay of spheroids of initial diameter ca 250 μm: ● *as a function of etoposide concentration up to the solubility limit* (100 μg/ml) *in unformulated aqueous solution; and* ○: *presented as the commercial Vepesid formulation containing polysorbate* 80, *polyoxyethylene glycol* 300, *and alcohols (see text).*

'cure' was observed, i.e. after 30 days there was no sign of regrowth in 92% of the wells. The growth delays of both untreated spheroids and those treated with sodium salicylate alone were slightly larger than the controls in the other experiments; growth rate in all spheroids observed in the sodium salicylate experiment appears to be slightly slower. The reason for this is unclear. However, the effect is small compared with the effect sodium salicylate has on etoposide activity. Further investigation is required into the effect of sodium salicylate on cell growth.

Spheroids treated with the Brij 30 surfactant (0.1 μg/ml) exhibit similar growth delay to those treated with unformulated drug. This concentration of Brij 30 was shown [17] to increase the chemosensitivity to and penetration of adriamycin into L-Dan spheroids. At higher concentrations Brij 30 is cytotoxic and produces disruptive effects on spheroids (see below).

Drug uptake

An increase in drug uptake was observed with an increase in drug concentration, uptake being

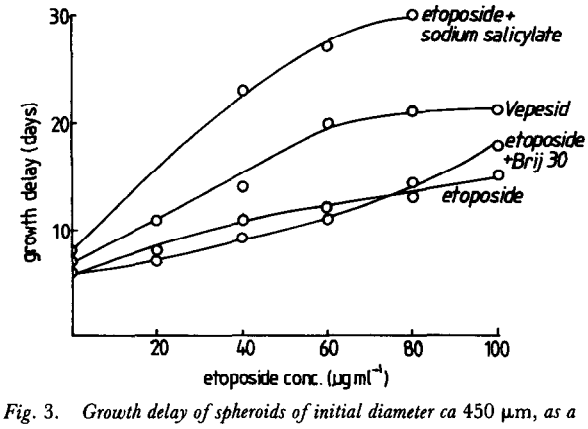

Fig. 3. Growth delay of spheroids of initial diameter ca 450 μm, *as a function of etoposide concentration up to the aqueous solubility limit of etoposide itself and in the presence of sodium salicylate* (0.1 M), *or Brij* 30 *or as the Vepesid formulation as indicated.*

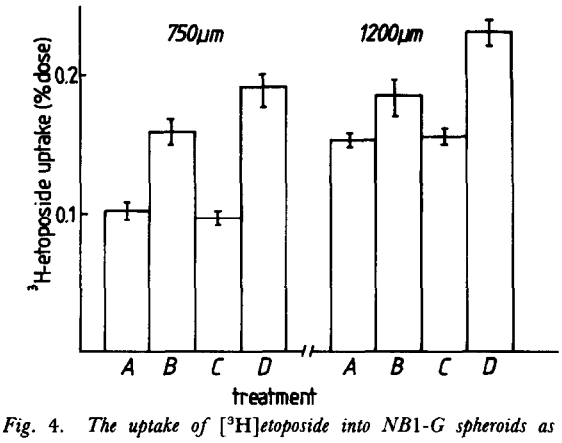

Fig. 4. The uptake of [^{3}H]*etoposide into NB1-G spheroids as a percentage of total applied radioactivity, into spheroids of* 750 *and* 1200 μm *diameter; A = etoposide, B = Vepesid, C = etoposide + Brij* 30 (0.1 μg/ml), *D = etoposide + sodium salicylate* (0.1 M).

directly proportional to dose in the range tested. Similarly uptake was linear with time over a 2 h period. Figure 4 shows the uptake of [^{3}H]etoposide from various formulations: it indicates increased uptake of drug in the presence of the formulation additives in the Vepesid injection, sodium salicylate (0.1 M) compared with etoposide alone and etoposide + Brij 30 in low concentration (0.01%). These data, however, do not distinguish between bound and unbound drug.

Assessment of spheroid disruption

Figure 5 shows some of the morphological effects of the adjuvants used on the NB1-G spheroids. The most marked effect is observed with Brij 30 (1 μg/ml) (Fig. 5B) showing virtual destruction of the viable rim (Fig. 5E) compared with the control (Fig. 5A). Even the structure of the necrotic area appears to have been disrupted. However, all formulations had some effect on this outer layer of cells, as can be seen from Figs. 5C and D. Vepesid injection (Fig. 5C) causes a less dramatic effect as cells remain intact but the outermost cells are necrotic in appearance (Fig. 5F). In Fig. 5D little change can be observed with the sodium salicylate even at the 1 M level.

DISCUSSION

Surfactants and other excipients have been shown in a number of studies to affect the passage of drugs through physiological barriers such as the blood brain barrier [11], gastrointestinal tract, and the rectal and nasal [19] mucosa. Many surfactants because of their hydrophobic/hydrophilic nature can have a direct effect on the biological membrane causing an increase in permeability. At high concentrations they have been shown to disrupt membranes completely by solubilizing both the phospholipid and cholesterol components [1]. Even a small increase in membrane permeability could have a significant effect on the uptake of drugs to which the

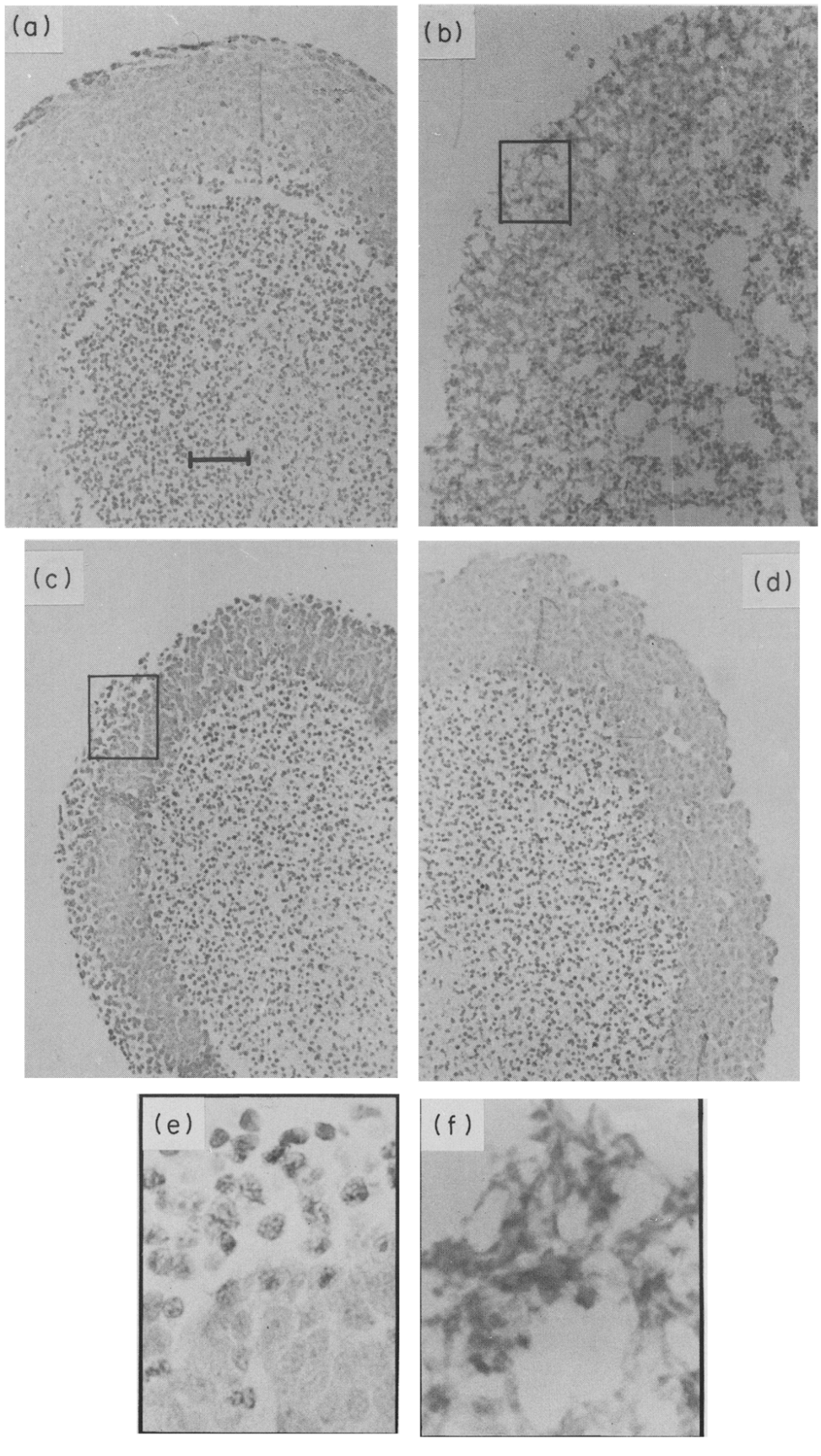

Fig. 5. Photomicrographs (×17) *of* 5 μm *sections of NB1-G spheroids in concentrations of additives approximately* 10 *times those used in growth delay experiments.* (*a*) = *control,* (*b*) = *after treatment with Brij* 30 (1 μg/ml) *for* 1 *h,* (*c*) = *after addition of Vepesid formulation equivalent to* 1 mg/ml *drug and* 4 mg/ml *polysorbate* 80, 32.5 mg/ml *PEG* 300 *for* 1 *h,* (*d*) = *after treatment with sodium salicylate* 1 M *for* 1 *h,* (*e*) = *portion of the outer rim of* (*b*) *above at* ×80 *magnification,* (*f*) = *portion of the outer rim of* (*c*) *at* ×68.

membrane barrier presents an important limiting factor to the intracellular action of the drug.

Although by *in vitro* methods it is impossible to mimic exactly the distribution and elimination of drugs, formulated or otherwise, it is reasonable to assume that excipients which can be absorbed from the bloodstream and distributed within the tissues can exert an effect at the cellular level and thus influence the action of drug.

In the data presented we have shown that compared to spheroids treated with etoposide alone, the formulated Vepesid injection at equimolar drug concentrations produces an increased cytotoxicity. The formulation excipients alone have no measurable effect. The surfactant, polysorbate (Tween) 80, present in the formulation will undoubtedly interact with the cell membrane in its native form or as its metabolite, oleic acid. Although at present we have no definitive evidence of increased permeability by such a mode of action, increased drug uptake leading to increased cytotoxicity could be due to such an effect. Such changes may be subtle in nature and not disruptive to the integrity of the cell. Indeed our morphological evidence would suggest that with Tween 80 at low concentrations this is the case. Brij 30, the other non-ionic surfactant which we have studied, is much more toxic to cells in tissue culture and in experimental animals alike. It does not however appear to have any permeability enhancing action at a concentration at which it itself does not exert a cytotoxic effect, although in other studies we have observed increased penetration of Adriamycin into L-Dan spheroids [17]. At higher concentrations, as the photomicrograph (Fig. 5B) demonstrates, almost complete disruption of the outer rim of the spheroid occurs, the vast majority of the cells throughout the whole spheroid being necrotic in appearance. The above results suggest a direct effect by non-ionic surfactants on the cell membrane. The swelling of the spheroids in the presence of surfactants will allow drug to diffuse more freely through the extracellular space and thus reach the inner cells. The decreased cohesiveness of the viable cells in the outer rim of the spheroid accords with the reduced and often total loss of cell adhesion to flasks in monolayer culture seen in the presence of various surfactants [20]. This phenomenon of cell deaggregation raises the question of promotion of metastasis. There would be little point in increasing cell kill if those cells which do survive are likely to be released from the tumour site and become the source of metastatic growth elsewhere. However, in our growth delay experiments only one spheroid was seen to emerge from the debris and regrow. The concept, although difficult to assess, does merit some consideration. What can be concluded, however, from our results is that some surfactants do influence and indeed enhance the effects of etoposide at least within the confines of *in vitro* spheroid culture.

The mechanism of action of the salicylate is unclear. Very slight morphological changes in the outer rim of the spheroid are observed (Fig. 5D). Sodium salicylate achieves the greatest effect of the adjuvants studied, albeit at high concentrations. It seems to possess a permeability enhancing property without cytotoxicity. It has been shown [18] that sodium salicylate and other hydrotropic agents can enhance the absorption of various drugs across the rectal mucosa.

Our study illustrates that formulation excipients are not biologically inert and can influence drug uptake in culture. Further investigation is required to establish the exact mechanisms whereby the excipients affect etoposide action, including investigation of their effect on drug efflux as well as uptake.

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