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The Effect of the Non-ionic Surfactant Brij 30 on the Cytotoxicity of Adriamycin in Monolayer, Spheroid and Clonogenic Culture Systems

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Abstract—*The effects of a non-ionic polyoxyethylated lauryl ether surfactant (Brij 30) on monolayer uptake and spheroid penetration of adriamycin have been studied. Co-incubation of adriamycin with Brij 30 increases intracellular adriamycin levels by 2–3-fold. Although, in the concentrations used, Brij 30 alone is not cytotoxic, adriamycin and Brij 30 mixtures are significantly more cytotoxic (monolayer* ID_{90} = 0.6 μg/ml; *disaggregated spheroid* ID_{50} = 1.9 μg/ml) *and induce significantly longer spheroid growth delay than adriamycin alone (monolayer* ID_{90} = 2.1 μg/ml; *disaggregated spheroid* ID_{50} = 3.3 μg/ml). *Adriamycin is equally cytotoxic to mouse normal granulocytes and chronic myeloid leukaemic (M1 cell line) cells in agar clonogenic cultures. The addition of Brij 30 appears to enhance preferentially the activity of adriamycin against these tumour cells relative to the normal granulocytes.*

INTRODUCTION

STUDIES in several mammalian cell culture systems have shown that experimentally developed resistance to adriamycin is associated with reduced membrane permeability of the drug and reduced cellular retention in cells expressing the multidrug resistant phenotype [1–3]. Levin *et al.*'s calculations [4] showed that the slow diffusion of drug into tumours could limit the effectiveness of chemotherapy, and have encouraged the search for penetration enhancers [5]. Many non-ionic surfactants particularly of the poly(oxyethylene) alkyl and aryl ether class interact with biological membranes increasing their permeability and allowing increased trans-membrane solute transport [6]. While the mechanisms are not fully understood, interaction of the surfactant molecules with the membrane occurs following adsorption and the disruption of membrane integrity and function is at the centre of many of their observed biological effects [7].

Not all surfactants are capable of inducing increased membrane fluidity and drug transport. However the non-ionic detergent polysorbate 80 has been reported to increase the absorption of methotrexate [5] and to potentiate the antineoplastic effect of adriamycin *in vivo* and *in vitro* [8–10]. These effects were attributed to an increase in cellular drug uptake in the presence of the detergent, presumably mediated by altered membrane fluidity [9]. There is evidence, also, that surfactants might alter cellular retention of anthracyclines in cells expressing the multidrug resistant phenotype. Klohs *et al.* have shown that Tween 80 potentiates the activity of adriamycin in P388 leukaemia cells by altering drug accumulation and efflux [11].

The multicellular spheroid model was developed as a system of intermediate complexity between solid tumours and monolayers in which 3-dimensional growth simulated development of micrometastases [12]. Resistance of cells in intact spheroids to drug treatment has been reported for adriamycin and the existence of drug penetration barriers has been postulated [12, 13].

In this study we have assessed the effects of an alkyl ether polyoxyethylated surfactant, one of a series of surfactants studied to determine a relationship between structure and influence on membrane permeability [15] on the cellular accumulation and cytotoxicity of adriamycin in monolayer and spheroid culture systems. A lauryl ether, Brij 30, was chosen because of the finding that this alkyl chain

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length was optimum in enhancing permeability in other test systems.

MATERIALS AND METHODS

Monolayer culture

The L-DAN cell line was derived from our own patient with squamous lung cancer. The cells were maintained as a monolayer in exponential growth in Ham's F10/DMEM (50 : 50) with 8 mM $NaHCO_3$ supplemented with 15% calf and 20% horse serum, respectively.

Conditions of drug exposure and determination of cell survival

The monolayers were exposed to adriamycin (Farmitalia Carlo Erba), Brij 30 (Atlas Chemical Industries, U.K.) and a combination of the two over a range of concentrations for 1 h. The clonogenic dose response curve for Brij 30 was determined over a concentration range of 0.003–3 μl/ml culture medium, whereas the adriamycin concentration ranged from 0.1–10 μg/ml. During combined treatment, the concentration of Brij 30 was fixed and the concentration of adriamycin varied from 0.1–10 μg/ml.

After treatment, L-DAN monolayers were harvested with 0.25% trypsin in PBS, centrifuged and washed with ice-cold medium. The cells were then diluted in medium and seeded at 200 cells/ml in 5 ml in 5 cm Petri dishes. The plates were incubated for 12 days in a humid 2% CO_2 atmosphere. The colonies were then fixed and stained with crystal violet and colonies of ≥ 40 cells were counted. Clonogenic cell survival for the mouse granulocytes from bone marrow and M1 myeloid leukaemic cells was determined from an agar capillary method [16]. Following the usual convention, the cloning efficiency of the untreated cells was normalized to 100% and the cloning efficiency of the treated cells was expressed as a percentage of control survival.

Spheroid culture

L-DAN monolayers were disaggregated enzymatically with 0.25% trypsin in PBS and the resultant cell suspension used to provide cells for initiation of tumour spheroids, using the "agar underlay" static method [17].

Cytotoxic drug efficacy was assessed by measuring spheroid growth delay and disaggregated spheroid clonogenic survival. Spheroids from two flasks were pooled and a number of glass universal tubes were prepared each containing 200–300 spheroids with a mean diameter of approximately 350 μm. The spheroids were treated with similar drug concentrations as used in monolayer for 1 h at 37°C with intermittent agitation. At the end of this period the spheroids were allowed to sediment, the drug containing medium was removed and they were washed with fresh, ice-cold medium. The spheroids were then resuspended in medium and subdivided for assays of response.

For clonogenic assay approximately half of the spheroids were incubated with 0.125% trypsin in PBS for 15 min at 37°C, after which the trypsin was removed and replaced with fresh medium. The spheroids were then mechanically disaggregated to a single cell suspension by repeated pipetting. The clonogenic assay was performed as previously described.

For spheroid growth delay measurement spheroids from the other group were transferred to agar coated wells in a plastic tissue culture multidish with one spheroid per well. Twenty-four spheroids were taken from each treatment group and measurements of cross-sectional area of individual spheroids were made twice weekly using a Micromeasurements image analysis system coupled via a television camera to an inverted optical microscope. The area measurements were subsequently converted to volumes, assuming spherical geometry. Sequential estimates of median spheroid volume were made and growth delay could be calculated from the time taken for median spheroid volume to increase by a factor of 10 above initial size [18].

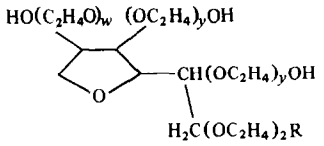
Determination of intracellular drug levels: L-DAN monolayers were exposed to adriamycin (5 μg/ml) and Brij 30 (0.01 μl/ml) for varying times (0.5–2 h). The cells were then washed twice with ice-cold PBS, harvested by brief trypsinization, counted (Coulter Counter Ltd, U.K.) and resuspended in distilled water. Adriamycin was then extracted and measured by reversed phase high pressure liquid chromatography with fluorescence detection, as previously described [19]. Adriamycin levels were expressed as ng/10^5 cells. Drug uptake curves were fitted by the method of least squares.

Fluorescent and electron microscopy

Intact spheroids approx. 500 μm in diameter were exposed to adriamycin (5 μg/ml) ± Brij 30 (0.01 μl/ml) for varying times. The spheroids were then washed to remove loosely bound drug, placed in gelatin capsules filled with OCT embedding gel (Lurker Labs Ltd) and frozen in liquid nitrogen. Thin cryotome sections (6 μm) were mounted in uvinert and examined under a Polyvar fluorescent microscope (λ excitation = 468 nm; λ emission = 550 nm).

The effect of Brij 30 on the morphology and ultrastructure of L-DAN cells was assessed by electron microscopy. Monolayers of intact cells were exposed to Brij 30 (0.01–1 μl/ml) for 1 h and the cells subsequently processed for routine electron microscopy.

Tween 80 = sum of w, x, y, z is 20; R is $(C_{17}H_{33})COO$



Brij 30 = $CH_3(CH_2)_{10}CH_2\text{-}[OCH_2CH_2]_4OH$
Brij 36T = $CH_3(CH_2)_{10}CH_2\text{-}[OCH_2CH_2]_{10}OH$

Scheme 1. *Structural formulae of non-ionic surfactants.*

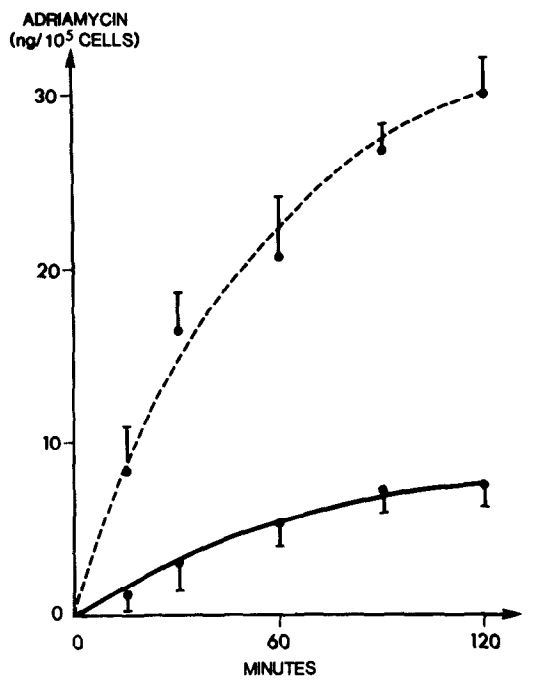


Fig. 1. *The time course of cellular accumulation of adriamycin (fixed drug concentration of* 5 μg/ml). ●——● *Adriamycin alone;* ●----● *adriamycin + Brij* 30 (0.01 μl/ml). *The vertical bars denote standard deviation (mean of* 5 *experiments).*

RESULTS

Intracellular drug levels

The relationship between intracellular adriamycin levels and time, for a fixed external drug concentration, is curvilinear. The intracellular adriamycin levels are significantly higher for the L-DAN cell line, at all time points following combined treatment with the surfactant (Fig. 1).

Monolayer survival

Clonogenic cell survival after treatment with adriamycin ± Brij 30 is summarized in Fig. 2 for the L-DAN cell line, M1 cell line and mouse granulocytes. The adriamycin concentration found to inhibit 90% clonogenic cell survival (ID_{90}) for each cell line is summarized in Table 1.

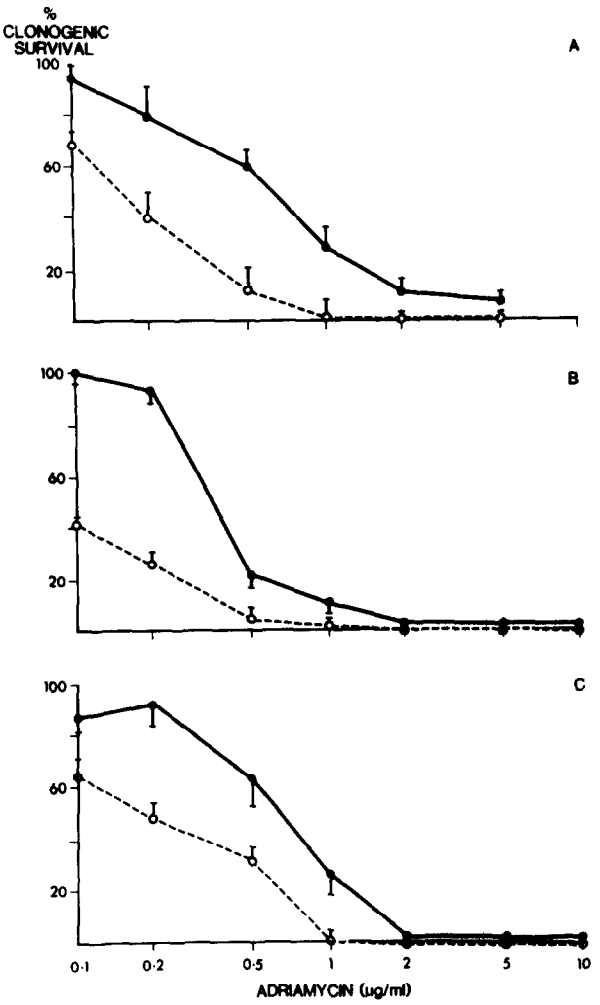


Fig. 2. *Clonogenic cell survival curves for: A, L-DAN cell line; B, M1 cell line–mouse myeloid leukaemic cells; C, normal mouse granulocytes.* ●——● *Adriamycin alone;* O-----O *adriamycin + Brij* 30. *The vertical bars denote standard deviation.*

Table 1. *Adriamycin* ID_{90} (± *Brij* 30) *in monolayers*

Cell line	Adriamycin ID_{90} (μg/ml) + Brij 30	− Brij 30
L-DAN	0.6	2.1*
M1	0.4	1.9*
Mouse granulocytes	0.8	1.5*†

Statistical differences by Student's *t*-test: $*P < 0.01$, significant reduction in ID_{90} in Brij 30 treated group, $†P < 0.05$, significant reduction in ID_{90} in M1 cell line relative to normal granulocytes treated with adriamycin + Brij.

L-DAN

Brij 30 alone, at the concentration used in these experiments (0.01 μl/ml), reduced clonogenic survival by approx. 10% so this was subtracted from the figure attributed to combined treatment. Clearly, combined treatment significantly enhances the cytotoxicity of adriamycin.

Table 2. Spheroid growth delay in response to adriamycin (± Brij 30)

Adriamycin concentration (μg/ml)	Growth delay (days) + Brij 30	− Brij 30
Control	9	8
2	16.8	11.5
5	19	13.5
10	35	18
15	37	21

M1 myeloid leukaemic and normal mouse granulocytes

The concentration of Brij 30 (0.007 μl/ml) used in these experiments was not cytotoxic. There is no difference in the response of myeloid leukaemic cells or granulocytes to adriamycin over the concentration range used. The combination of adriamycin and Brij 30 is significantly more toxic to both cells than adriamycin alone, however the tumour cells are more adversely affected than the granulocytes. It would appear that Brij 30 selectively enhances the activity of adriamycin in the tumour cell line at lower drug concentrations (0.1–0.6 μg/ml) by approx. 20%. This effect is statistically significant ($P < 0.05$).

L-DAN spheroid survival

Growth delay over a range of adriamycin concentrations was determined in the presence and absence of Brij 30 (Table 2). Co-treatment with the surfactant induces significantly longer growth delays at each adriamycin concentration.

Disaggregated spheroid clonogenic cell survival is higher than for monolayer at identical adriamycin concentrations implying that adoption of the spheroid configuration confers a degree of resistance to adriamycin (Fig. 3). Treatment with Brij 30 significantly ($P < 0.05$ at drug concentrations > 2 μg/ml) improves the clonogenic cell kill of adriamycin, but it is still less than that found in monolayer (Fig. 3). The respective disaggregated spheroid ID_{50}s for adriamycin alone and the combination of adriamycin and Brij 30 are 3.3 and 1.9 μg/ml.

Fluorescence and electron microscopy

It was possible to determine qualitatively the degree of adriamycin penetration into the spheroids. The spheroids are approx. 16–20 cell layers in diameter (400–500 μm), and adriamycin had diffused into the outer 3–4 cell layers after incubation for 1 h. Brij 30 increased the penetration depth to approximately 6–7 cell layers (Fig. 4).

Electron microscopy revealed that Brij 30 alone caused significant ultrastructural alterations to L-DAN cells which were concentration dependent.

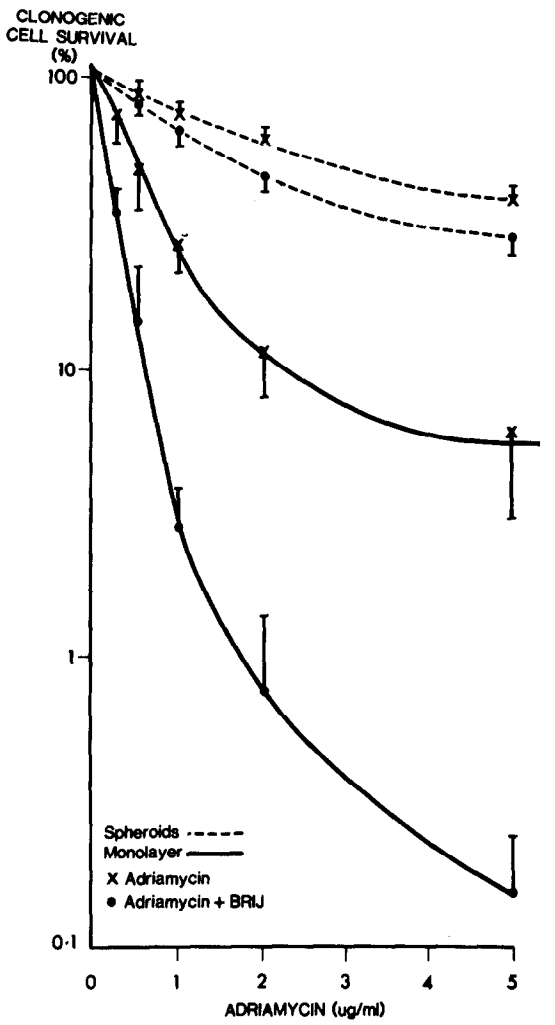


Fig. 3. Spheroid and monolayer clonogenic cell survival for L-DAN cell line.

These ranged from complete cytolysis (1 μl/ml) to minimal structural membrane changes in the plasma membrane and intracytoplasmic organelles at 0.01 μl/ml.

DISCUSSION

In this present study we have shown that combined treatment with Brij 30 enhances the cellular uptake of adriamycin and significantly improves its cytotoxicity in monolayer, clonogenic and spheroid culture systems. This effect is probably mediated by increased cellular uptake of adriamycin, which we found to be 2–3-fold higher in L-DAN monolayers in the presence of surfactant. It is apparent that the effect of Brij 30 is to shift the clonogenic survival curves (Fig. 2) to the left, which is compatible with its proposed mode of action in increasing membrane permeability to adriamycin.

It is interesting to note that despite virtually identical sensitivity to adriamycin alone, the combination of adriamycin and Brij 30 is slightly, but not significantly, more cytotoxic to the leukaemic cells. As can be seen in Fig. 2 this effect occurs at clinically

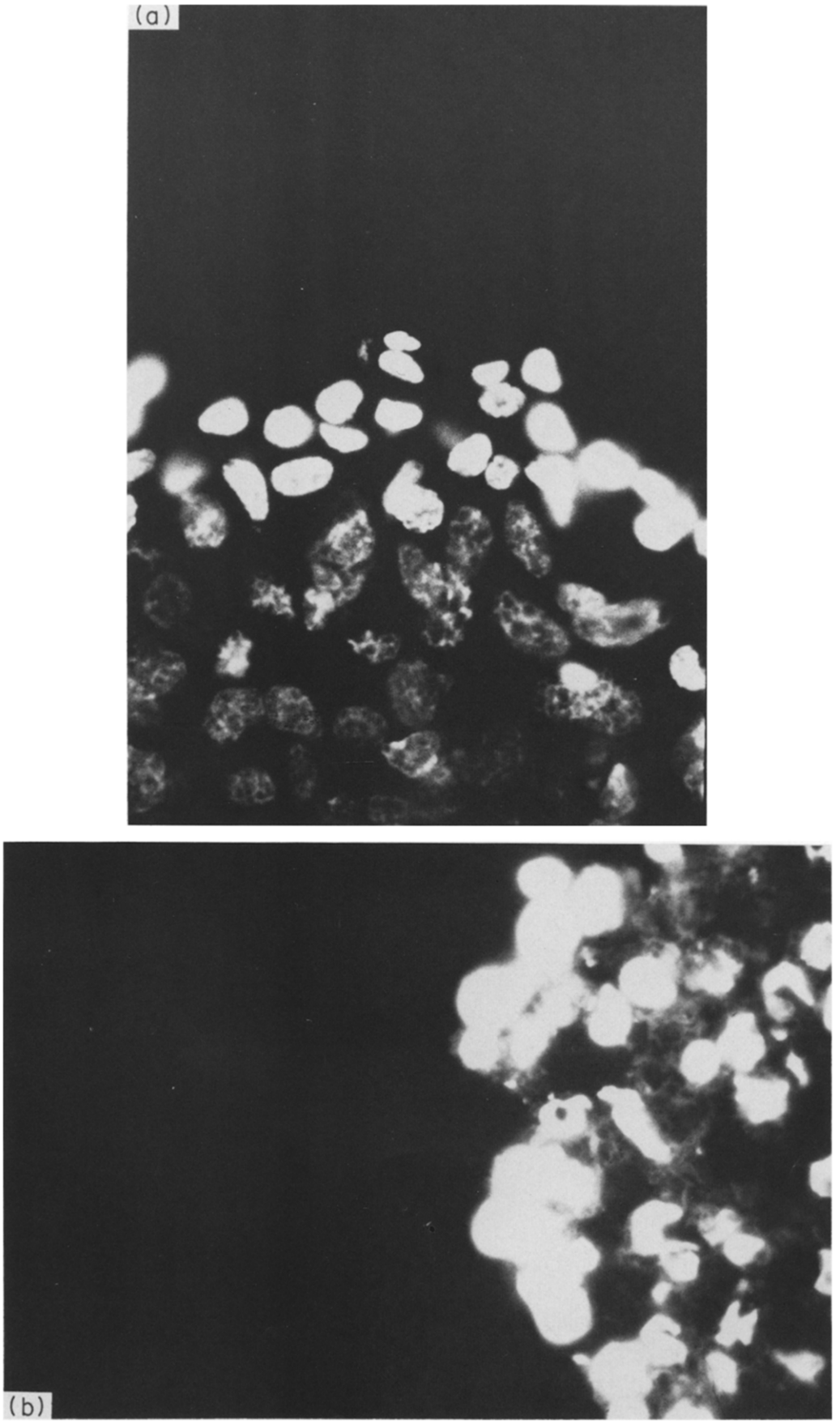


Fig. 4. *Fluorescent photomicrograph of external surface of spheroid. The labelled cells are seen mainly on the exterior of the spheroid. A, Adriamycin; B, adriamycin + Brij* 30. *Magnification* (× 80).

relevant concentrations of adriamycin [20].

The electron microscopic data suggest that the surfactant is altering membrane structure in some way, although the technique is too insensitive to give detailed information on the degree of membrane disruption. Spheroid fluorescent microscopy indicates that Brij 30 tends to increase the depth to which adriamycin can penetrate. We have not examined the penetration properties of the surfactant itself, however it presumably diffuses into the spheroid to a depth of at least 6–7 cell layers where it could increase intracellular uptake of adriamycin.

Previous studies [1–3] have indicated the importance of reduced membrane permeability to adriamycin relative to the development of experimentally induced drug resistance. Tween 80, a non-ionic surface active detergent (not structurally related to Brij 30), has been shown to potentiate the anti-neoplastic activity of a number of antitumour agents *in vitro*, including adriamycin, by increasing cellular drug uptake by resistant cells [8, 9] and decreasing drug efflux [11]. Dilution of adriamycin in a 10% water solution of Tween 80 produces a significant increase in the cytotoxic activity in a number of murine tumours (L1210 leukaemia, Gross leukaemia, MS-2 sarcoma) [10]. Toxicity was only slightly enhanced, therefore there was a significant increase in the therapeutic index. A recent study has shown a phamacokinetic interaction between adriamycin and Tween 80, resulting in elevated plasma adriamycin concentration by an apparent reduction in plasma volume [21], although this mechanism was found not to explain the enhanced absorption of methotrexate [5].

In a series of similar experiments with Tween 80 and the surfactant Brij 36T, we found neither enhancement of cell uptake, nor improved cytotoxicity in combination with adriamycin in the L-DAN lung tumour line (Kerr, unpublished data). It is possible that we saw no effects with these surfactants due to an intrinsic property of the cell membrane of the lung tumour line under study. However Tween 80 and the Brij surfactants have different molecular structures (Materials and Methods) and markedly different physicochemical properties.

Surfactants are amphipathic molecules and their relative hydrophobicity is described by a hydrophile–lipophile balance (HLB) number; the higher the HLB number, the more hydrophilic the molecule. Tween 80 (HLB = 14.9) and Brij 36T (HLB = 15.2) are evidently more hydrophilic than Brij 30 (HLB = 9.7). The optimum HLB for surfactant-mediated increases in permeability depends on the nature of the membrane as well as the surfactant series and drug under study [6]. The lower the HLB the greater the affinity of the surfactant for a non-aqueous phase; at the lowest HLB, however, surfactant activity is limited by aqueous insolubility. Steric factors also intrude. The areas/molecule of Brij 30 and 36T are 0.38 and 1.09 nm^2, respectively. The latter was most active on the isolated rabbit gastric mucosa [15] and on adsorption through rat nasal mucosa [22].

It is possible that this increased cellular permeability accounts for the apparent synergy of Brij 30 with adriamycin in the cell lines studied. There is a small but significant enhancement of the activity of adriamycin + Brij 30 against tumour cells relative to normal granulocytes. This could reflect a differential membrane response to the surfactant by normal and neoplastic cells and may impart a degree of selectivity to combination treatment with surfactant and cytotoxic.

In summary, we have demonstrated that the non-ionic polyoxyethylated surfactant Brij 30 enhances cellular accumulation of adriamycin and its antineoplastic activity *in vitro*.

There are no toxicity data regarding intravenous administration of Brij 30, however, we plan to extend these studies to include an animal model as combined treatment may provide a means of enhancing the therapeutic index of adriamycin. In addition, it would be interesting to examine the effects of this surfactant on adriamycin accumulation in tumour cells which express the multidrug resistant phenotype.

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