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HEPATOTOXIC AND CELLULAR UPTAKE INTERACTIONS AMONG SURFACE ACTIVE COMPONENTS OF ERYTHROMYCIN PREPARATIONS*

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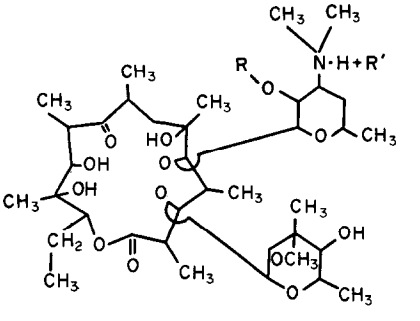
Abstract—Rat hepatoma cell cultures and Chang cell cultures derived from human liver were exposed to erythromycin estolate, erythromycin cetyl sulfate or to the individual components of these preparations, erythromycin propionate and sodium lauryl sulfate or erythromycin base and sodium cetyl sulfate respectively. Cytotoxicity was evaluated by leakage of cytoplasmic and lysosomal enzymes into the media. The uptake of the drugs by the cells was measured with radiolabeled compounds. The surface activity of the drugs was determined *in vitro* with a surface tension balance. Those drugs with the greatest surface activity were found to have the greatest cytotoxic effects on both cell culture lines. The cytotoxic effects of erythromycin cetyl sulfate were exclusively from the cetyl sulfate. With the erythromycin estolate combination, both lauryl sulfate and erythromycin propionate were cytotoxic. The presence of the latter increased the uptake of lauryl sulfate by the cells and resulted in a potentiation of the cytotoxic effect. Thus, the detergent ingredients used to enhance the gastrointestinal absorption of the drugs may also enhance their cell penetration and determine their hepatotoxicity *in vitro*; this form of cytotoxic drug interaction mechanism at the cellular level may explain the hepatotoxicity of some of these drugs in man.

We have reported the cytotoxic effects of various structurally related phenothiazines, a hepatotoxic laxative preparation, and several general anesthetics on Chang liver, rat hepatoma or mice liver explant cells in culture. The toxicity produced *in vitro* correlated with the known or suspected differences in clinical hepatotoxic potential of the various drugs and their congeners *in vivo* [1–4]. We have also found a positive correlation between the surfactant potency of the drugs and their cytotoxic effects *in vitro*.

Erythromycin estolate, a combination of the propionate salt of erythromycin with a detergent, lauryl sulfate (Fig. 1), is the only erythromycin derivative reported to produce hepatotoxicity in man [5]. The investigational drug, erythromycin cetyl sulfate, is the combination of erythromycin base and the detergent, cetyl sulfate. The detergents are added to enhance gastrointestinal absorption of the antibiotics. We have previously reported that the cetyl sulfate combination damages liver cells *in vitro* [3]. When clinical evidence of hepatotoxicity developed in 10–15 per cent of human volunteers, erythromycin cetyl sulfate was withdrawn from further trials in man. Since erythromycin base has never been shown to be hepatotoxic *in vivo* or *in vitro*, cetyl sulfate appeared to be the most likely moiety responsible for this effect. Consequently, we wondered whether lauryl sulfate, which is chemically similar to cetyl sulfate (Fig. 2), could be responsible for the toxicity of the estolate preparation.

We report here the role of the surfactant ingredients of these preparations in producing cytotoxic effects *in vitro*.



Structural formula of erythromycin.

Fig. 1. Chemical structure of the erythromycins.

	R	R¹
Erythromycin base	H	
Erythromycin propionate	CH_3CH_2CO	
Erythromycin estolate	CH_3CH_2CO	$C_{12}H_{26}OSO_3$
Erythromycin cetyl sulfate	H	$C_{16}H_{34}OSO_3$

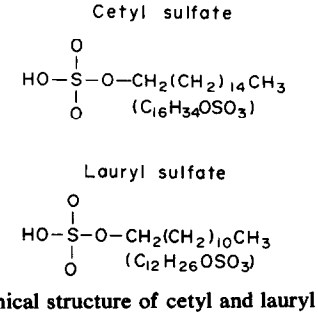


Fig. 2. Chemical structure of cetyl and lauryl sulfate.

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METHODS

The following substances singly or in various combinations were studied: erythromycin base,* erythromycin propionate, erythromycin estolate,† sodium lauryl sulfate, erythromycin cetyl sulfate,‡ and sodium cetyl sulfate. Their chemical structures are depicted in Figs. 1 and 2.

Cytotoxicity experiments. Liver cell cultures (rat hepatoma CCl 144, The American Type Culture Collection) were purchased from Gibco Laboratories (Grand Island, NY). Cells were grown in monolayers by standard techniques in HAMS F-12 medium supplemented with glutamine and 10 per cent fetal bovine serum on the glass surfaces of 16 × 125 mm screwcap tubes incubated at 37° in a horizontal position.

Chang cell cultures, originally derived from human liver and now an established line in The American Type Culture Collection (CCL 13), were purchased from Flow Laboratories (Rockville, MD). Chang cells were grown in identical fashion to rat hepatoma cells except that minimum essential medium§ was used.

Monolayers were grown until total confluency was reached, at which time each culture tube contained approximately 10^6 cells. Cells in cultures were counted in a Coulter counter B after suspension by exposure to 0.25 per cent trypsin for 10 min.

Drugs were dissolved in propylene glycol (PG) and then diluted in Parker 199 medium containing Hanks' salts and L-glutamine (TCM) without animal serum or antibiotics. This solution was added to the cell cultures to attain final concentrations from 10^{-6} to 10^{-2} M. Cultures containing the same final concentrations of PG (1%) in TCM were used as controls. The pH of the medium in the cultures was maintained at 7.2 ± 0.2. Three or more cultures were treated simultaneously with each drug or drug combination, or served as controls. The same stock of cultures was used for each experiment. All procedures were performed using aseptic techniques.

To initiate a study, the growth medium of each tube was discarded and replaced with 1.5 ml of drug or control solution. After incubations of 1–4 hr, the medium was withdrawn, centrifuged for 15 min at 600 *g*, and the supernatant fraction immediately assayed for lactate dehydrogenase (LDH) and beta-glucuronidase (BG) [3]. Alterations in cell membrane permeability and/or integrity were quantitated by the activities of the cytoplasmic enzyme (lactate dehydrogenase) and the lysosomal enzyme (beta-glucuronidase) which leaked from the cells into the culture media after exposure to the drugs for 4 hr. As described in previous reports [3], additional experiments were performed to exclude the possibility that factors (e.g. interference of the drugs with the assay) other than cell damage were responsible for changes in enzyme activity.

The cell cultures were evaluated for cytopathic changes by their appearance under phase light microscopy after exposure to control or drug containing medium. Vital staining was performed in some parallel experiments by replacing the medium with 2 ml of 0.15% trypan blue for 10 min. Dead cells acquire a blue color whereas viable cells exclude the dye.

Surface tension measurements. The measurements of surface tension of the single drug or combinations were done at 25° in a Cahn electrobalance, as described previously [3]. Drugs were dissolved in the same balanced salt solution (Hanks) which was contained in the tissue culture medium used for the cytotoxicity experiments. The surface tension of the salt solution without drug was 71 dynes/cm. The surface pressure of a given drug solution was calculated by subtracting its measured surface tension from 71. Therefore, surface pressure refers to surface activity of a drug solution compared to the solute without drug. The greater the surface pressure given by a drug the greater its surface activity.

Cellular uptake of drugs. Radiolabeled drug¶ was introduced with the respective "cold" drug dissolved in the culture medium. After exposure of the cultures to the drugs for various periods of time, the medium was withdrawn, centrifuged to recover possible detached cells and assayed for LDH and BG. The monolayers were dispersed by treatment with 0.25% trypsin for 5 min and centrifuged at 600 *g* for 45 min. The cell pellets were rinsed twice with 0.9% saline and dissolved in Protosol (New England Nuclear); the radioactivity was measured in a liquid scintillation counter by standard techniques. Disintegrations per minute, after correction for quenching (using an external standard), were converted to nmoles of drug per 10^6 cells.

In order to rule out the possibility that the trypsin treatment affected our evaluation of drug uptake, a number of parallel experiments were performed as follows. After the incubation with drugs, the culture tubes were centrifuged for 15 min at 600 *g* to sediment possible free cells, the medium was discarded and the cells were dissolved by shaking in 2 ml Protosol. This aliquot of lysed cells was then mixed with a scintillation counting solution and the radioactivity measured as before. The comparative differences in uptake among the drugs were similar using the two procedures described, thus demonstrating that treatment with trypsin did not result in a loss of radioactivity large enough to alter our interpretation of the previous results.

RESULTS

Surface pressure determinations. Surface pressure changes of solutions containing single drugs or combinations are shown in Fig. 3. The higher

* Marketed as E-Mycin, Upjohn, Kalamazoo, MI; Erythrocyn, Abbott Lab., Chicago, IL; and Ilotycin, Eli Lilly Co., Indianapolis, IN.

† Ilosone (propionyl erythromycin lauryl sulfate), Eli Lilly Co., Indianapolis, IN.

‡ A37753, Abbott Lab., Chicago, IL.

§ All tissue culture media were purchased from GIBCO, Grand Island, NY. Compositions of the media are shown in their catalog (1976).

¶ [^{35}S]sodium cetyl sulfate (Abbott Lab.), [^{35}S]sodium lauryl sulfate (New England Nuclear), [^{14}C]erythromycin and [^{14}C]erythromycin propionate (Eli Lilly Co.).

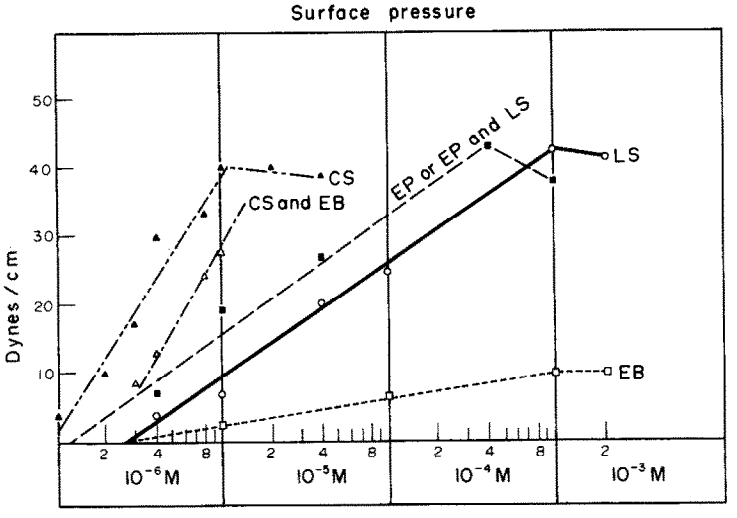

Fig. 3. Surface pressure in dynes/cm of solutions of various concentrations of erythromycin drugs and their components. Abbreviations: CS, cetyl sulfate; EB, erythromycin base; EP, erythromycin propionate; and LS, lauryl sulfate.

the surface pressure shown, the greater the surface activity of the drug. The drug concentration at which the concentration-surface pressure relationship becomes nonlinear represents the critical micellar concentration of the drug. In general, the greater the surface activity of a drug, the smaller the concentration required to lower the surface tension of the solute. Cetyl sulfate was the most surface-active drug, followed by erythromycin propionate, lauryl sulfate and erythromycin base. The latter had relatively little surface activity and its addition to a solution of cetyl sulfate at any concentration resulted in reduction of the surface activity of the solution. The addition of lauryl sulfate did not change the surface activity of the solution of erythromycin propionate.

In vitro *hepatotoxicity and drug–cell uptake experiments.* The cytotoxic effect of the drugs was most evident at 4 hr. These times were then selected for all of the cytotoxicity experiments shown. Determinations of drug uptake were done after incubations of 5 min to 4 hr. The optimum time to determine drug uptake by the cell cultures was 60 min because the peak concentrations of drugs found in the cells were reached at this time; furthermore longer incubations with some of the cytotoxic drugs resulted in an underestimation of drug uptake due to leakage of the drugs from the cells secondary to plasma membrane damage.

The cytotoxic effects in Chang cell cultures exposed to the components of erythromycin cetyl sulfate singly and in combination are shown in Table 1. Erythromycin base at 8×10^{-5} M concentration had no effect, whereas cetyl sulfate was cytotoxic. Simultaneous exposure to both drugs, each at 8×10^{-5} M, reduced the toxicity from cetyl sulfate. Uptake of cetyl sulfate by cells, measured at 60 min, was decreased by the presence of erythromycin base in the medium, but the concentration of erythromycin base in cells was the same in the presence or absence of cetyl sulfate in the medium.

The effect of the components of erythromycin estolate and the relationship between cytotoxicity and cellular uptake are shown in Table 2. At 4 hr both erythromycin propionate and lauryl sulfate were cytotoxic at 8×10^{-5} M. This effect was markedly enhanced when they were placed together at the same concentration. The presence of both drugs in the medium did not modify the total erythromycin propionate content of cells at 60 min but increased the lauryl sulfate content by about 70 per cent. In additional experiments not shown in the tables, the drug uptake was measured at 4 hr; the increment in lauryl sulfate content by the addition of erythromycin propionate was only 24 per cent. This was most likely the result of leakage of drug from cells, consequent to cellular damage by the drugs after the longer period of incubation.

The same drug interactions were evaluated on another cell line, rat liver hepatoma cell cultures. Although the results were quantitatively different,

Table 1. Erythromycin base and cetyl sulfate interactions in Chang cell cultures

Drugs* (8×10^{-5} M)	Enzyme leakage (units/ml)		Drug uptake (nmoles/10^6 cells)	
	LDH†	BG‡	EB	CS
Control	25 ± 5	3 ± 1		
Erythromycin base (EB)	27 ± 4	3 ± 1	3.7 ± 0.1	
Cetyl sulfate (CS)	465 ± 67§	25 ± 4§		14 ± 1
EB + CS	350 ± 46	16 ± 3‖	3.6 ± 0.1	10 ± 0.7‖

* Four to six cultures in each group.

† LDH = lactate dehydrogenase leaked from cells into surrounding medium.

‡ BG = beta glucuronidase leaked from cells into surrounding medium.

§ $P < 0.01$ difference from control and each of the single drugs.

‖ $P < 0.01$ difference from control and each of the single drugs.

Table 2. Erythromycin propionate and lauryl sulfate interactions in Chang cell cultures

Drugs* (8×10^{-5} M)	Enzyme leakage (units/ml)		Drug uptake (nmoles/10^6 cells)	
	LDH†	BG‡	EP	LS
Control	19 ± 3	4 ± 0.5		
Erythromycin propionate (EP)	47 ± 3§	18 ± 1§	8 ± 0.1	
Lauryl sulfate (LS)	46 ± 2§	12 ± 1§		7 ± 0.2
EP + LS	300 ± 30‖	30 ± 2‖	7 ± 0.4	12 ± 1.2¶

* Four to six cultures in each group.
† LDH = lactate dehydrogenase leaked from cells into surrounding medium.
‡ BG = beta glucuronidase activity leaked from cells into surrounding medium.
§ $P < 0.01$ difference from control.
‖ $P < 0.01$ difference from control and each of the single drugs.
¶ $P < 0.01$ difference from cultures exposed to LS singly.

the direction and type of interactions between the drugs were identical. A typical experiment is shown in Table 3. Erythromycin propionate added to lauryl sulfate resulted in approximately a 10-fold increase in enzyme leakage from the cells and almost a 3-fold increase in the concentration of lauryl sulfate in the cells.

Microscopic evaluation by phase microscopy and vital staining after exposure to the drugs revealed that the drug or drug combination producing the greatest enzyme leakage also resulted in the greatest degree of morphologic alterations of the cell membrane and in the largest number of dead cells. Trypan blue staining is not as sensitive an index of membrane injury as intracellular enzyme leakage and was utilized only to determine the fact that there was cell damage from the drugs sufficient to result in a larger number of dead cells in cultures exposed to the drug which, in turn, resulted in the greater enzyme leakage. The morphological changes seen with phase microscopy consisted of granulations of the cytoplasm, shrinking and loss of adherence of the whole cell to the monolayer and ultimately death of many of the cells. The differences in appearance between cultures exposed to erythromycin base and erythromycin cetyl sulfate are depicted in Fig. 4.

DISCUSSION

Although the effects *in vitro* observed with the drugs studied parallel their hepatotoxic potential in man, the connection between our results and the experience *in vivo* should be interpreted with due reservation. The true identity of Chang cells as hepatocytes has been questioned despite their origin from cultures of human liver parenchymae. There is no evidence that these cells handle drugs similarly to liver cells *in vivo*, but recent studies revealed that Chang cells and human liver cell plasma membranes have similar immunochemically reactive properties [6]. Nevertheless, studies with these cells do appear useful to predict the relative cytotoxicity of a series of related drugs. Chang cell cultures have constant biological characteristics and allow studies in a controlled environment with predetermined concentrations of drugs. Furthermore, the results in Chang cells were similar to those obtained with rat liver hepatoma cells, and we have performed preliminary studies showing the same effects on isolated rat hepatocytes. The latter two cell lines have been shown to preserve their liver characteristics, *in vitro* [7].

The drug concentrations used in these *in vitro* studies are within the range of concentrations of some of these drugs which were found in human bile in individuals receiving therapeutic doses [8].

The results described suggest that the sulfated surfactant is the ingredient responsible for the cytotoxic potential *in vitro* of erythromycin cetyl sulfate and that a similar type of detergent, lauryl sulfate, is at least equally or more toxic than the erythromycin moiety in the estolate derivative.

The greater the surface activity of a drug the greater its lipid–water partition coefficient and the greater its adsorption onto the membrane interphase; these properties might result in differences

Table 3. Erythromycin propionate and lauryl sulfate interactions in rat hepatoma cell cultures

Drugs*	Concentration(s) (M)	Enzyme leakage (units/ml)		LS uptake (nmoles/10^6 cells)
		LDH†	BG‡	
Controls		25 ± 5	13 ± 1	—
Lauryl sulfate (LS)	8×10^{-5}	29 ± 4	14 ± 1	5 ± 0.2
Erythromycin propionate (EP)	8×10^{-4}	62 ± 9	18 ± 3	—
LS + EP	8×10^{-5} + 8×10^{-4}	616 ± 25§	161 ± 12§	13 ± 1.2§

* Four to six cultures in each group.
† LDH = lactate dehydrogenase leaked from cells into surrounding medium.
‡ BG = beta glucuronidase leaked from cells into surrounding medium.
§ $P < 0.01$ difference from control or each of the single drugs.

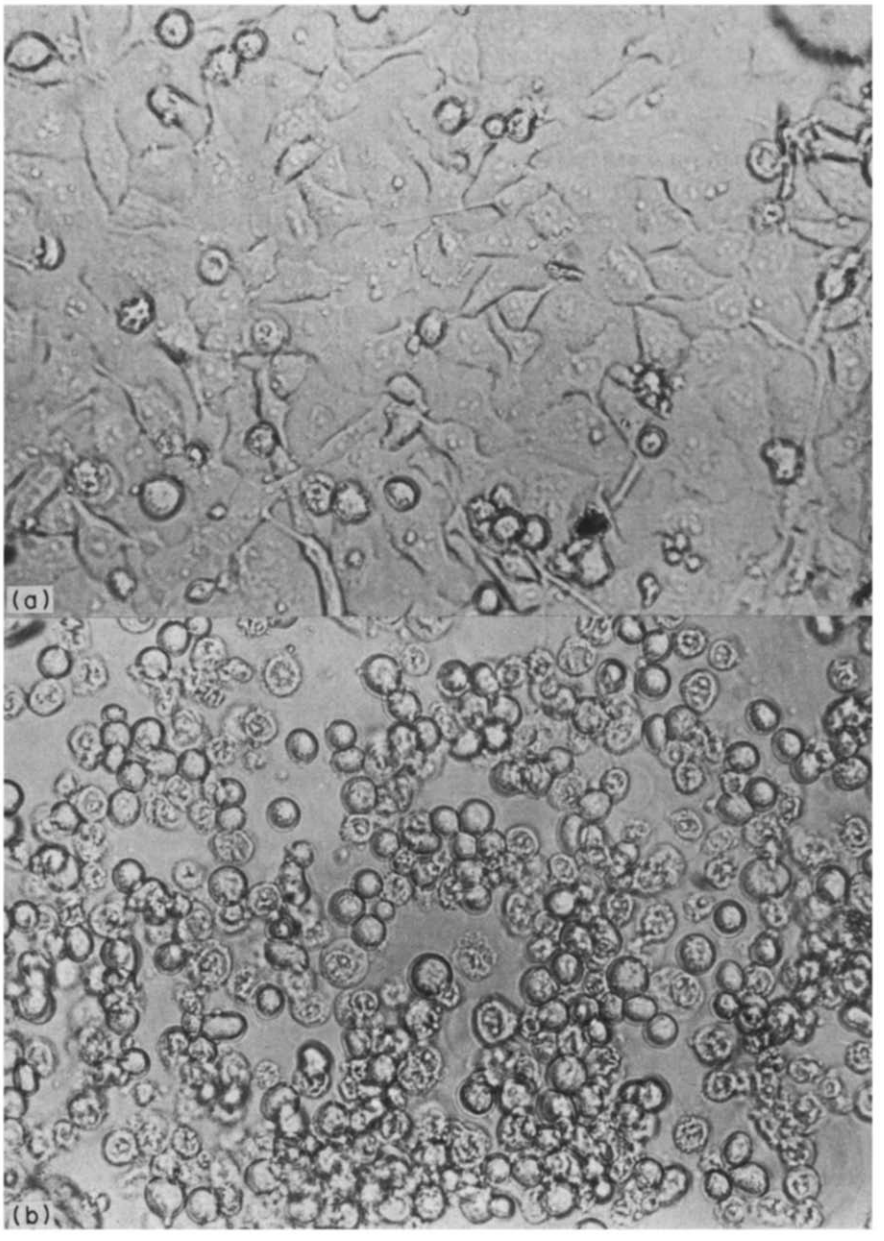

Fig. 4. Chang liver cell monolayers after exposure to erythromycin base (a) or erythromycin cetyl sulfate (b) for 4 hr. Phase contrast: ×200.

in uptake by, and consequent injury to, cell membranes. The type of interaction just described, whereby one drug inhibits or enhances the toxicity and uptake by liver cells of another drug by a surface activity effect, has been previously described *in vitro* between anionic detergents and antitumoral drugs [9]. To my knowledge this mechanism has not been described or postulated as playing a role in the hepatotoxic effects of a single drug or combinations of drugs.

These results demonstrate that when a drug (e.g. erythromycin base) lowers the surface activity of a medium containing a surfactant drug (e.g. cetyl sulfate) the former decreases the adsorption of the latter onto the cell membranes. Conversely, when a drug (e.g. erythromycin propionate) increases the surface activity of the medium, it may increase the adsorption of another drug (e.g. lauryl sulfate) in the combination. When the adsorption of drugs with potential cytotoxic effects is enhanced or inhibited, the magnitude of that effect is modified accordingly.

Individual drugs may have lesser or greater adsorption onto cell membranes depending on their surface activity and this may render them more likely to produce cytotoxicity. An endogenous surfactant, such as chenodeoxycholic acid, has been observed to produce hepatotoxicity in patients receiving it for the treatment of gallstones [10]. Another surfactant, dioctyl sulfosuccinate, has been associated with hepatotoxic effects in man when used as a laxative singly or in combina-

tion with oxiphenisatin [11, 12]. To date, based on our studies *in vitro* there appears to be a direct correlation between surface activity of drugs and their capacity to produce hepatocellular damage.

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