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Reduction of pain on intravenous infusion with bile salt formulations for a macrolide antibiotic

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Abstract

Macrolide antibiotics are well-known to cause pain on intravenous injection. The studies reported here indicate the feasibility of using bile salts, especially bile salt-phospholipid mixed micelle systems, to reduce the pain on injection of clarithromycin (6-*O*-methyl erythromycin). Sodium glycodeoxycholate was identified as the bile salt which was most effective in forming physically stable formulations of clarithromycin and in reducing the pain on injection, as shown by two animal models, the mouse scratch test and the rat paw lick test. Clarithromycin-bile salt formulations generally caused hemolysis, but this could be reduced by HSA and, especially, phospholipids. A formulation composed of clarithromycin lactobionate/glycodeoxycholate/egg phosphatides (1 : 3 : 2 molar ratio) was identified as a formulation which is chemically and physically stable, reduces the pain by at least 50% according to the animal models, has comparable efficacy to clarithromycin lactobionate in a mouse protection test, and had toxicity in rats which was less than that of clarithromycin lactobionate. The results suggest the potential of using mixed micelle systems in the formulation of macrolides and other drugs which are painful on intravenous administration.

Keywords: Clarithromycin; Macrolide; Pain on injection; Bile salt; Mixed micelle

1. Introduction

Intravenous infusion of drugs is known to cause venous irritation in a number of cases (Brown, 1970; Langdon et al., 1973; Hecker et al., 1984; Falchuk et al., 1985; Lewis and Hecker, 1985; Hilleman et al., 1987). The irritation may take one of many forms including phlebitis, thrombosis, thrombophlebitis, pruritus and erythema, and may, in some cases cause substantial pain (Stampfl and Franson, 1986; Klement and Arndt, 1991). Although some of the symptoms cited above may occur without pain, it appears that when pain is felt, it is usually accompanied by one or more of these symptoms. 'Vein irritation' is a general term which may be used to describe one or all of the above symptoms. However, in this paper, we

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will use the term to describe gross, observable changes in morphology, e.g., discoloration at or near the injection site. We use the term 'pain' to refer to the sensation felt whether or not it is simultaneously accompanied by morphological changes.

There are several possible reasons why any given drug solution may cause pain on infusion. For example, the drug may precipitate at the site of the injection or the solution may be contaminated with foreign particles. A significantly hypo- or hyper-osmolar solution may also cause irritation with accompanying pain. A fourth possibility is that the pain sensation is intrinsic to the drug molecule, i.e., it occurs as a result of the interaction of the drug with nerve endings in the venous wall. Erythromycin, a macrolide antibiotic, has been reported to cause pain on infusion (Marlin et al., 1983; Stampfl and Franson, 1986). Although the severity of the pain was reduced by installing an in-line filter between the solution and the patient, the pain was still present (Marlin et al., 1983). Erythromycin also is reported to cause venous irritation, which is reduced somewhat, but not eliminated, by decreasing the infusion rate and/or diluting the drug in a large volume (Marlin et al., 1983; Stampfl and Franson, 1986).

Clarithromycin (Cm), the 6-*O*-methyl derivative of erythromycin (see Fig. 1) is an antibiotic which is highly active against gram positive organisms and which, compared to erythromycin, exhibits superior pharmacokinetics (Fernandes et al., 1986). The tablet was recently approved for indications such as pneumonia, sinusitis and bronchitis, and is now marketed by Abbott Laboratories as Biaxin®. Because of the potential for intravenous infusions of macrolides such as Cm to produce pain, a project was initiated to formulate the drug to reduce pain on infusion. The objectives of this work were to develop a formula which: (i) was less painful than the current formulation, Cm lactobionate, in the animal model(s) used to evaluate pain on injection; (ii) was physically and chemically stable; (iii) was not more toxic than the neat parent drug; (iv) was visually clear before infusion; and (v) could be infused at a drug concentration of 5 mg/ml.

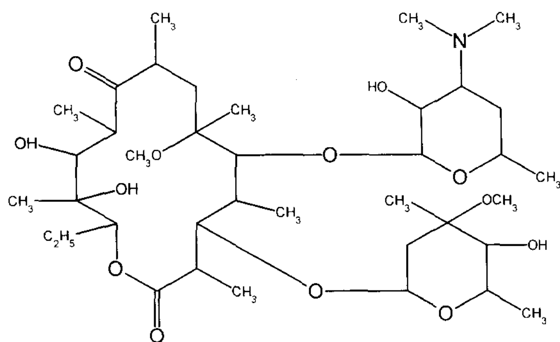

Fig. 1. Structure of clarithromycin (6-*O*-methyl erythromycin).

To accomplish these ends, Cm may be incorporated into a surfactant micelle, shielding the portions of the drug molecule which are responsible for pain. One possibility is to incorporate drug into an oil-in-water emulsion such as Liposyn™ or Intralipid™. To achieve visually clear infusions, however, other strategies are necessary using surfactants of high hydrophilic-lipophilic balance (HLB) number. However, few of these are approved for parenteral use. Bile salts represent one class of surfactants which may allow preparation of suitable formulations. Bile salts are naturally occurring, with serum levels of 3–30 mg/l for total bile salts (Tietz, 1976), 0.7 mg/l for glycocholate and 0.4 mg/l for glycodeoxycholate (Neale et al. 1971). Sodium deoxycholate is currently used in an intravenous formulation of amphotericin B (Fungizone™) at a level of up to 1.2 mg/kg per day (Physicians Desk Reference, 1993). Mixed micelle formulations, composed of phospholipids and bile salts, have also been examined as pharmaceutical formulations. For example, phosphatidylcholine-glycocholate mixed micelles were used to solubilize diazepam for parenteral administration (Steffen and Schmidt, 1979), and lecithin-glycocholate formulations were used to solubilize and improve the local tolerance of non-steroidal anti-inflammatory agents, e.g., carprofen, ibuprofen, indomethacin, and naproxen (Ferro and Steffen, 1989). In the work reported here, bile salt formulations, especially mixed micelles, appear promising in decreasing the pain on injection of macrolides such as Cm.

2. Materials and methods

2.1. Materials

Freeze-dried Cm lactobionate (CmL), 500 mg/vial, was obtained from Abbott Laboratories. Egg lecithin (EL), human serum albumin (HSA), and the sodium salts of cholic acid (Cho), glycodeoxycholic acid (GDC), and taurodeoxycholic acid (TDC) were obtained from Sigma Chemical Co. (St. Louis, MO). The sodium salts of deoxycholic acid (DC) and ursodeoxycholic acid (UDC) were purchased from Aldrich Chemical Co. (Milwaukee, WI); sodium chenodeoxycholate (CDC) was obtained from Calbiochem Corp. (San Diego, CA). Egg phosphatides (EP) were obtained from Pfanstiehl Laboratories Inc. (Waukegan, IL). Dextrose and sodium phosphates were USP grade. For calculating mole ratios, a molecular weight of 785 g/mol was used for EL and EP (which is a mixture of about 65% phosphatidylcholine and 35% other phospholipids). Animals were obtained from Charles River Inc. (Portage, MI).

2.2. Analytical

The HPLC system consisted of a Waters (Milford MA) WISP automatic sample injector, an Applied Biosystems (Foster City CA) Spectraflow 400 pump, an Applied Biosystems 783 UV-visible detector and a Spectra-Physics (San Jose CA) 4270 integrator. Samples were assayed using a reverse-phase method with the following conditions: mobile phase, 51:49 acetonitrile/0.05 M potassium phosphate buffer pH 4.0; flow rate, 1.0 ml/min; column, 5 cm × 4.6 mm ODS2 3 mm 'Little Champ' (Regis, Morton Grove, IL); injection volume, 25–75 μl; detection wavelength, 225 nm. Working standards and samples were diluted to concentrations of 15–175 μg/ml in 51:49 acetonitrile/0.05 M potassium phosphate buffer pH 7.0. Physical stability was monitored by observation of physical appearance, clarity, and visible presence or absence of particulate matter. Lack of change in these observations after 30 days was taken as the criterion for acceptable physical stability.

2.3. Formulation preparation

In general, formulations containing GDC or TDC were prepared by reconstituting vials containing 500 mg of freeze-dried CmL (all CmL quantities refer to base equivalent) to 15–20 mg/ml with water. The appropriate bile salt(s) was then added to the reconstituted solution and stirred with a magnetic stirrer until dissolved. HSA or phospholipids were then added as indicated and stirred until a visually clear solution was obtained. A sufficient volume of 0.25 M sodium phosphate buffer at pH 7.5 was added to give a final phosphate molarity of 0.05 M and final pH as indicated in Table 1. Where indicated, sufficient dextrose was then added for isotonicity and the solution was made up to volume with water to a final Cm concentration of 5 mg/ml. The formulations containing Cho or CDC were prepared by dissolving the bile salt and phospholipid together in water, adding this mixture to a 50 mg/ml CmL solution, and diluting to volume with 5% dextrose to make an isotonic 5 mg/ml CmL formulation.

2.4. In vitro hemolysis

The in vitro hemolysis of the formulations was evaluated according to two methods: the method

Table 1
Composition of physically stable clarithromycin-bile salt formulations

Formula	Composition (molar ratio)	pH
A	CmL/TDC (1:5)	7.4
B	CmL/GDC (1:3)	7–7.5
C	CmL/GDC (1:3)+5%HSA	7.2
D	CmL/GDC/EP (1:3:1)	7.4
E	CmL/GDC/EP (1:3:2)	7.4
F	CmL/GDC/EP (1:3:3)	7.4
G	CmL/CDC/EP (1:5:0.9)	8.1
H	CmL/Cho/EL (1:11:1.6)	7.5
I	CmL/GDC/GC/EP (1:1.5:1.5:2)	7.4

CDC, chenodeoxycholate; Cho, sodium cholate; Cm, clarithromycin; CmL, clarithromycin lactobionate; EL, egg lecithin; EP, egg phosphatides; GDC, glycodeoxycholate; GC, glycocholate; TDC, taurodeoxycholate.

of Husa and Adams (1944) and the modified Husa method developed by Reed and Yalkowsky (1985). The Husa method involves incubation of red blood cells (RBC) with the formulation, wherein 0.5 ml of formulation was mixed with 4.5 ml of heparinized fresh human whole blood. The mixture was equilibrated for 10 min and centrifuged at $1600 \times g$ for 10 min. The absorbance of the supernatant was read at 540 nm and used to evaluate relative hemolysis.

In the method of Reed and Yalkowsky (1985), the test solution is mixed with blood at various volume ratios and compared to the hemolysis from a negative control of normal saline or 5% dextrose (D5W). The test solution-blood mixture was agitated and incubated at 25°C for 2 min, and 5 ml of normal saline or D5W was added to stop the hemolysis by making the solution nearly isotonic. The solution was centrifuged for 5 min at 3500 rpm which caused precipitation of intact RBCs. The supernatant was then discarded by vacuum suction. The residual test solution and hemoglobin from the lysed RBCs were removed by washing the RBCs with 5.0 ml aliquots of normal saline or D5W three successive times. After each wash, the suspension was centrifuged and the supernatant discarded. Water for injection (5.0 ml) was added to the final precipitate and the mixture was agitated and centrifuged for 5 min at 3500 rpm. The supernatant was removed, filtered through a 0.45 μm filter and the absorbance measured at 540 nm.

2.5. *In vivo hemolysis*

For each formulation tested, two beagle dogs were given Cm (100 mg in 20 ml) by intravenous infusions into the femoral vein at a rate of 0.25 ml/min. Blood samples were withdrawn at 0, 0.33, 0.67, 1, 2, 4, 6, 8 and 24 h after the start of the infusion. The samples were centrifuged at 3500 rpm for about 5 min and the supernatant checked visually for redness which would indicate hemolysis, and given a grading of zero to three for no and severe hemolysis, respectively by an experienced unbiased observer. Centrifugation by itself did not cause lysis of unlysed RBCs.

2.6. *Mouse scratch test*

Pain on injection of Cm formulations was evaluated by subcutaneous administration to mice (CD-1 mice, 19–27 g. each). Each formulation (5 mg/ml) was administered at a dose of 5 ml/kg to 10 mice. The number of times that each mouse scratched the injection site was then counted for exactly 5 min. A control of CmL at 5 mg/ml was run with each group of formulations, which were counted by the same observer. Statistical analyses, comparing each formulation with the corresponding CmL control, were performed using Student's *t*-test.

2.7. *Rat paw-lick test*

The other model used to evaluate pain on injection was the rat paw-lick test (Comereski et al., 1986; Gupta et al., 1994). 10 weanling rats (Sprague-Dawley, 70–120 g each) were tested with each formulation. Each rat was given a single injection of 0.1 ml of one of the test formulations into the foot pad of the right hind paw. The number of times the paw was licked was counted over a 15 min time period (five separate 3 min intervals for each rat); the total amount of time each rat licked was also recorded.

2.8. *Rabbit ear vein irritation test*

Rabbits were divided into test groups of three rabbits (New Zealand white, 2–3 kg). Each animal was given a single infusion of 8 ml/kg at a rate of 1 ml/min into the marginal ear vein. Visual observations of the appearance of the veins were made at time points until 24 h after the injection by an experienced unbiased observer.

2.9. *Rat tail vein irritation test*

Rats were divided into test groups of six rats each (three male and three female, Sprague-Dawley, 150–250 g each). The tail vein of each animal was infused once daily for 3 consecutive days with 0.3 ml/min, taking care that no extravasation occurs from the vein into the surrounding tissue. The daily dose given was 20 ml/kg (100 mg/kg).

Visual observations of the appearance of the tails were made at time points until 24 h after the last infusion by an experienced unbiased observer.

2.10. Acute toxicity

To determine the LD_{50} of each formulation, rats (Sprague-Dawley, 10 rats/dose) were given i.v. infusions at 2 ml/min of each formulation. LD_{50} results were reported in ml/kg with 95% confidence intervals. For cases in which no deaths were reported, the LD_{50} was recorded as above 20 ml/kg (100 mg/kg) since doses greater than this are considered excessive.

2.11. Mouse protection

The antibacterial activity of 4, 1, 0.25, and 0.0625 mg/ml Cm concentrations of the CmL/GDC/EP (1:3:2) formulation was compared to the activity of CmL in mouse protection tests (Fernandes et al., 1986). Female CF-1 mice were infected intraperitoneally with *Staphylococcus aureus* (strain 10649) and treated 1 h after infection by injecting the formulation intravenously via the tail vein. Drug efficacy was determined by enhanced survival of infected mice and was expressed as an ED_{50} (i.e., the dose required to keep half the mice alive) in mg/kg.

3. Results and discussion

3.1. Physical stability of clarithromycin-bile salt formulations at room temperature

In preliminary experiments, a number of surfactants were examined for their ability to form stable micelles of Cm at neutral pH. As a class, bile salts were found to be the most promising in this regard, apparently because of the opposite charges of the drug and bile salt near neutral pH, which would contribute to the stability of the micelles. Formulations were prepared by dissolving all ingredients at low pH and adjusting to neutral pH. The choice of particular bile salt was found in preliminary experiments to have a marked influence on the physical stability of Cm bile salt mixtures. For many formulations, it was found that metastable or supersaturated solutions could be prepared which initially appeared clear, but which precipitated drug within minutes or hours, or in some cases, several days. Table 1 shows the composition of the Cm-bile salt formulations which appeared physically stable for at least 1 month. Most formulations were not monitored beyond this period, except for formulations B, C, and E, which were monitored for over 60 days with no sign of physical instability. Stable formulations could not be prepared using taurocholate (TC), deoxycholate (DC), or ursodeoxycholate (UDC) as surfactants; formulations prepared using these bile salts generally precipitated within 1 h.

A number of mixed micelle formulations (i.e., containing bile salts and phospholipids) are also listed in Table 1. Stable formulations containing cholate (Cho) could only be prepared at relatively high concentrations of bile salt and > 1 mol of phospholipid. Chenodeoxycholate (CDC) was capable of forming stable formulations at lower levels than cholate, but the presence of phospholipid was still required for stable micelle formation. Similarly, glycocholate (GC) yielded stable formulations only at high levels of bile salt, unless GDC was also present.

Glycodeoxycholate (GDC) and taurodeoxycholate (TDC), which have relatively low critical micelle concentrations, were found to be the bile salts most effective at solubilizing Cm, with 3 and 5 mol bile salt/mol drug, respectively, being the minimum amount of bile salt required for stable micelles. HSA (5% w/v) could be added to the CmL/GDC (1:3) formulation with no loss of physical stability, but higher levels (> 7.5%) of HSA led to precipitation of drug within several days, probably because of albumin's ability to bind bile salts (Hanson et al., 1977).

Egg phosphatides (EP), a phospholipid mixture used in intravenous lipid emulsions, and composed of phosphatidylcholine (65%), phosphatidylethanolamine (25%) and other lipids, could also be added to CmL/GDC (1:3) at 1–3 mol/mol drug. Above 3 mol EP/mol drug (i.e., > 1 mol EP/mol bile salt), formulations were not optically clear, apparently due to the formation of

liposomal structures in addition to the micelles. The formation of small amounts of liposomal material below this level of egg phosphatide cannot be ruled out. The chemical stability of drug in the mixed micelle formulation at pH 7.5 also appeared to be good; no degradation could be detected within 11 days at room temperature. Because GDC was found to be the bile salt which afforded stable formulations of Cm at the lowest bile salt/drug ratio, most work was concentrated on developing GDC formulations for future examination.

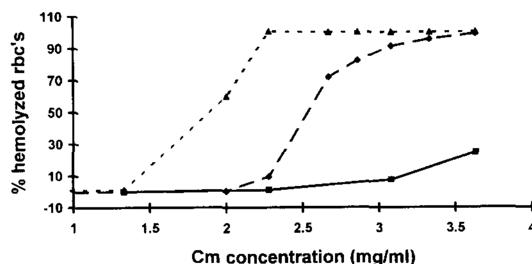

Fig. 2. In vitro hemolysis of clarithromycin-bile salt formulations (method of Reed and Yalkowsky (1985)). (■——■) CmL; (▲----▲) CmL/GDC; (♦---♦) CmL/GDC/EP.

3.2. *Hemolysis of clarithromycin bile salt formulations*

Bile salts are known to be hemolytic. In preliminary examinations of hemolysis of Cm-bile salt formulations, using the method of Husa and Adams (1944), the bile salts GDC and, especially, TDC were found to lead to significant levels of hemolysis. The following trend was observed in the relative degree of hemolysis arising from 5 mg/ml CmL formulations: CmL/TDC > CmL/GDC > CmL/GDC/5% HSA > CmL/GDC/7.5%HSA > CmL; the CmL/GDC formulation (formulation B of Table 1) was approx. 15 times as hemolytic as CmL. Addition of increasing amounts of HSA decreased the hemolytic potential as observed in previous studies of bile salts (Hanson et al., 1977). Several of the GDC formulations were tested in vivo in dogs for hemolytic tendency. Hemolysis, judged as being severe at 1 h after injection, was observed for formulations containing CmL/GDC (1:3) and 5 or 7.5% HSA. Slight hemolysis was observed even up to 24 h after administration, even though it was expected that the formulation components should be cleared by this time. Bile salt molecules, because of their surface activity, have been found to partition into phospholipid bilayer membranes and red blood cell membranes at bile salt/lipid mole ratios of less than 0.25 (Saito et al., 1983). Such partitioning invariably leads to membrane damage and leakage of cell contents. At higher concentrations, bile salts have been reported to solubilize membrane components (Billington et al., 1977; Coleman et al., 1979, 1980; Lowe and Coleman, 1981). It is possible that, in this study, the bile salt molecules were released back into the plasma from hemolyzed red blood cells or another compartment, and as such were free to penetrate other red blood cells and continue to release hemoglobin. Alternatively, the degree of hemolysis observed may have been sufficient to saturate or retard the normal mechanism for clearance of stroma-free hemoglobin.

Because the in vitro hemolysis measurements used in these initial studies (Husa and Adams, 1944) are prone to interference from components of the test solution (Reed and Yalkowsky, 1985), and to improve the accuracy and predictive potential of the test, the CmL/GDC/EP (1:3:2) formulation was tested according to the method of Reed and Yalkowsky (1985). Results are shown in Fig. 2 and indicate that EP is effective in reducing, but not eliminating, the in vitro hemolysis due to the bile salt. In vivo testing of the same formulation in dogs showed no hemolysis up to 24 h after administration, in marked contrast to the formulation containing Cm, GDC and HSA. The observed effect of phospholipids on the hemolytic effects of bile salts is corroborated by literature data showing that phospholipids are very effective in reducing the hemolytic effects of bile salts (Okazaki et al. 1980; Sagawa et al., 1993). The fact that phospholipids and bile salts, in appropriate ratios, form optically clear mixed micellar solutions (Carey and Small, 1972; Zimmerer and Lindenbaum, 1979) may explain these observations. Presumably, the bile salts bind preferentially to the phospholipids in solution and

this prevents them from partitioning into or solubilizing the red blood cell membranes.

3.3. Animal models for pain on injection

3.3.1. Mouse scratch test results

Cm bile salt formulations were screened in two different animal models for pain on injection. Table 2 summarizes results from the mouse scratch test for screening the pain on injection of Cm bile salt formulations. CmL (5 mg/ml, pH 5.5), on average, elicited 21 scratches over a 5 min test period. Several formulations were tested in which Cm was solubilized at pH 7.5 without bile salts, by addition of 0.5% Pluronic F68 or 9% HSA. These formulations did not lead to reduction of pain, indicating that the low pH of the CmL formulation was not the cause of the pain on injection. Bile salt formulations containing GDC generally led to a significant reduction in pain by this model, giving 1/3–1/2 the pain on injection as CmL. Addition of HSA (5%) or EP (1–2 mol/mol drug) had little effect on the pain reduction by GDC. The most promising composition was CmL/GDC/EP 1:3:2 (molar ratios). The presence of dextrose (0 or 2.5%, to adjust to isotonicity) had no significant effect on the pain response from this formulation. The combined mean of all tests for this formulation (with or without dextrose) at pH 7.5 was 9.1 ± 0.6 scratches/5 min, compared to the CmL control at 20.7 ± 0.7 scratches/5 min, thereby decreasing the pain response by over 50%.

3.3.2. Rat paw lick test

The Cm/GDC/EP (1:3:2) formulation (pH 7.5, 2.5% dextrose) was also evaluated for pain by the rat paw lick test relative to 5% dextrose and CmL. When D5W was injected, only 1/10 animals licked for 2 s. When 5 mg/ml CmL was injected, all 10 animals licked; each rat licked its paw an average of 15 times out of the 15 min test period, for a total licking time of 115 ± 19 s (mean ± SE). When 5 mg/ml CmL/GDC/EP (1:3:2) was injected, 9/10 animals licked, averaging out to 6 times out of the test period, for a total licking time of 52 ± 12 s, which was significantly different from the CmL control ($p < 0.05$). Thus, CmL was the formulation most painful when injected, while the mixed micelle formulation appeared to be 50% as painful as CmL. This conclusion compares favorably with the results of the mouse scratch test.

3.4. Acute toxicity

A number of bile salt-containing formulations were examined for toxicity in rats, giving the results in Table 3. Table 3 shows that formulation H actually had a lower toxicity than the neat drug, an unexpected result. The other significant feature of Table 3 is the effectiveness of HSA

Table 2
Mouse scratch test results

Formulation	pH	Dextrose	Mean ± SE [a]	Control ± SE [a]	% of control	n	P
CmL + 0.5% PF68	7.6	0	21.4 ± 2.8	21.6 ± 1.9	99%	10	0.5
CmL + 9% HSA	7–7.5	0	19.6 ± 3.1	25.9 ± 2.4	76%	10	0.065
CmL/TDC (1:5)	7.4	5%	8.8 ± 0.8	22.9 ± 2.1	38%	10	< 0.0001 [c]
CmL/GDC (1:3)	7.3	5%	5.9 ± 0.7	18.7 ± 1.0	32%	30 [b]	< 0.0001 [c]
CmL/GDC (1:3) + 5% HSA	7.2	4%	10.7 ± 1.6	25.9 ± 2.4	41%	10	< 0.0001 [c]
CmL/GDC/EP 1:3:1	7.6	0	7.0 ± 1.3	14.8 ± 2.8	47%	10	0.010 [c]
CmL/GDC/EP 1:3:2	7.5	0	9.2 ± 0.6	21.3 ± 0.8	43%	70 [b]	< 0.0001 [c]
CmL/GDC/EP 1:3:2	7.5	2.5%	9.0 ± 0.9	19.4 ± 1.1	46%	30 [b]	< 0.0001 [c]
CmL (control, weighted mean)	5.0	4.5%	20.8 ± 0.5		100%	180 [b]	

All formulations were tested at 5 mg/ml clarithromycin (base equivalent). n refers to total number of mice tested with given formulation.

[a] Number of scratches/5 min. Historical values for 0.9% saline are 3–7 scratches/5 min.

[b] These numbers represent more than one set of experiments.

[c] Significantly different from the control at the 95% level.

Table 3
Summary: LD_{50} of clarithromycin bile salt formulations

Formulation	Rat LD_{50} (mg/kg) [a]
(A) Clarithromycin lactobionate (CmL)	70 (55–80)
(B) CmL/GDC (1:3)	34 (28–41)
(C) GDC; vehicle for B	57 [b] (50–62)
(D) CmL/GDC (1:3)+5% HSA	52 (46–61)
(E) GDC+5% HSA; vehicle for D	72 [b] (68–78)
(F) CmL/GDC (1:3)+7.5% HSA	60 (53–70)
(G) GDC+7.5%HSA; vehicle for F	112 [b] (92–150)
(H) CmL/GDC/EP (1:3:2)	149 (132–173)
(I) GDC/EP; vehicle for H	172 (143–195) [b]

[a] Values in parentheses are 95% confidence limits.
[b] Based on 5 mg/ml clarithromycin.

and EP in reducing the toxic effects of bile salts, in this case GDC. For example, as seen in Table 3, formulation F containing 7.5% HSA had an LD_{50} of 60 mg/kg while formulation B with no HSA had an LD_{50} of 34 mg/kg, i.e., it was roughly twice as toxic. The effect of EP in reducing toxicity (which is even more pronounced than that of HSA at the concentrations used) is also evident when formulations B and H are compared, the reduction in this case being roughly 5-fold. When the toxicity of only the vehicles, i.e., formulations C, E, G and I are compared, it is also clear that the presence of HSA or EP reduces toxicity due to GDC and that EP is more effective, at the concentrations used, than HSA. As was mentioned earlier, it is known that bile salts cause hemolysis and that phospholipids reduce such effects (Okazaki et al. 1980). The results described above show clearly that phospholipids reduce bile salt toxicity (compare formulations C and I in Table 3). These findings suggest a correlation between the hemolytic property and the acute toxicity of these formulations, and it is reasonable to expect that the hemolytic property of a bile salt should at least partially contribute to the toxicity of the formulation. This is borne out by the fact that the acute toxicity of the CmL formulations follows the trend B > D > F > H (Table 3), and the corresponding vehicles show the same trend in toxicity: C > E > G > I. These were the same trends shown by relative hemolysis (vide supra).

3.5. Vein irritation tests

Ear vein irritation tests have also been used to evaluate the irritation of formulations of drugs on intravenous injection. They have the advantage of

Table 4
Vein irritation results

Formulation	Results	
	Rabbit ear vein	Rat tail vein
5% dextrose	normal	5/6 [a]: slight purple/red/pink spots (mostly near injection sites)
CL/GDC/EP (1:3:2)	during infusion: 2/3 [a]: purple discoloration along marginal ear vein; 1/3: normal. 1–4 h after infusion: 1/3: swollen ear and purple discoloration along the entire marginal ear vein. 2/3: normal. 24 h post-dosing: 2/3: pinkish discoloration pinkish discoloration proximal to the end of the needle.	2/6: severe purple discoloration and scabbing of the tails; dosing discontinued after 2 days. 4/6: normal after first day of dosing but by the last observation time (24 h after the last dose) showed moderate to severe purple discoloration over 50–75% of the tail area.
Cm lactobionate	during infusion: 3/3: ears slightly flushed with blood during dosing. 1–4h after infusion: 3/3: bruises or reddish discoloration was observed at the injection site. 24 h post-dosing: 3/3: slight bruises around the vein at the injection site.	4/6: Slight to moderate pink/reddish purple areas observed at most time points over 50–75% of the tail. 2/6: slight to moderate discoloration near the injection site.

[a] Numbers refer to number of animals, e.g., 2/3 means two out of three animals.

being an intravenous injection; however, they do not model pain but only erythema, discoloration or visible damage of the vein used for the infusion. The Cm/GDC/EP (1:3:2) formulation was evaluated in the ear vein test relative to CmL as the positive control and 5% dextrose as the negative control in rabbits (ear vein) and rats (tail vein). The results of these tests are listed in Table 4, and indicate that in the rabbit, the mixed micelle formulation was more irritating than CmL, causing discoloration of the entire ear vein and swelling of the infused ear. Similarly, the mixed micelle formulation was more irritating than CmL in the rat tail vein test. It has been shown that, for certain other drugs, there is a correlation between in vitro hemolysis and in vivo intramuscular irritation (Brown et al., 1989). A comparison of the in vitro hemolysis and in vivo vein irritation observations for CmL and CmL/GDC/EP (1:3:2) appears to show the same trend, i.e., the more hemolytic mixed-micelle formulation (in the in vitro test) is also the more irritating to the vein (in the in vivo test).

3.6. Efficacy testing: mouse protection test

The CmL/GDC/EP 1:3:2 formulation was tested for efficacy against *S. aureus* in a mouse protection test (Fernandes et al., 1986), and compared to CmL. The ED_{50} for the mixed micelle formulation was 13.3 mg/kg per day, compared to 10.4 mg/kg per day for CmL. This difference was judged to be not significant ($p > 0.1$), indicating that there was no difference in efficacy between the CmL/GDC/EP 1:3:2 formulation and CmL.

4. Conclusions

Bile salt formulations, particularly the CmL/GDC/EP (1:3:2) formulation described here, appear promising as a means of decreasing the pain on injection of Cm and perhaps other drugs. However, GDC is not used currently in any parenteral formulation. While it is present in serum normally, the levels found to be effective for use here (i.e., up to 950 mg/day for a 500 mg dose of Cm) are much higher than levels seen in normal subjects, although they may approach GDC levels seen in patients with liver disease (Neale et al., 1971). While the higher toxicity originally seen from the bile salts appears to be eliminated by the phospholipid in the formulation, vein irritation may still occur, which, depending on the severity, may be unacceptable in patients. Furthermore, the animal tests used for pain on injection (mouse scratch and rat paw lick tests) may not adequately model intravenous infusion. All of this notwithstanding, this work has shown that the concept of reducing pain on injection by using a mixed micelle (or similar) approach is a viable one. Recently, we have reported that injectable oil-in-water emulsions of Cm also appear to be less than 50% as painful in animal models as CmL (Lovell et al., 1994). Both of these approaches warrant further investigation – ultimately leading, it is hoped, to a test in humans.

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