Predicting Injection Site Muscle Damage III: Evaluation of Intramuscular Formulations in the L6 Cell Model

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INTRODUCTION

A variety of *in vivo* methods for assessing IM toleration exist and have been discussed in previous reports and papers (1). The most commonly used predictor of muscle damage has been the rabbit lesion model. However, this model is time and labor intensive requiring visual, mechanical, and histological evaluation of tissue. Other *in vivo* models such as rabbit serum creatine phosphokinase (creatine kinase, CK) are fairly good predictors of muscle tolerance for immediate release formulations, but either require surgical implantation of vascular access ports, or if not cannulated, require excessive handling and restraint during bleeding. Rat CK models are less labor intensive but not as sensitive as the rabbit models. Brazeau and Fung (2) developed an isolated rat muscle model to screen for muscle damage following IM injections. However this method also required animals as a tissue-source. Svensen *et al.* (3) developed a cell culture line of MRC-5 fibroblasts from human fetal tissue. This method was also time and labor intensive, since it involved microscopic evaluation of morphological changes and histological measurements. A method developed by Williams *et al.* (4) used a rat muscle cell line (L6) to evaluate the cellular damage and cellular CK loss after exposure to eight parenteral antibiotics. The results illustrated a significant relationship between the irritation of the antibiotics and the extent of L6 cell damage, as manifested by CK depletion. Hoover *et al.* (5) expanded on this study by demonstrating the direct correlation between CK release from the L6 cells and microscopic changes. Finally, a collaborative project among 10 pharmaceutical companies clearly showed the usefulness of the L6 model as a reliable screen for evaluating the muscle irritation of parenteral formulations (6).

This report is an extension of our earlier work, which evaluated animal models for predicting muscle damage from IM immediate release (1) and extended release (7) formulations. Using the L6 cell model, we report a correlation between CK depletion and muscle damage from formulations which had been tested in the rabbit lesion volume model, rabbit and rat CK models, and the rat foot edema models.

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MATERIALS AND METHODS

Test Formulations

The positive control digoxin 0.25 mg/ml (lot 301443, Lanoxin® Burrough Wellcome) was purchased at a local pharmacy. Digoxin vehicle, azithromycin immediate release formulation (0, 100 mg/ml), danofloxacin immediate release formulation (0, 60 mg/ml), and danofloxacin oily suspension extended release formulation (0, 60 mg/ml), and danofloxacin aqueous suspension extended release formulation (0, 60 mg/ml) were obtained from Pfizer Central Research (1,7).

Cell Culture and Irritation Assay

The method according to Laska (8) was used. Briefly, rat skeletal muscle myoblast, L6 cells (American Type Culture Collection, CRL 1456) were plated between passage 2 and 5, at a density of about 1.4×10^6 cells/ml in 10% FBS (fetal bovine serum)/medium 199 in a 6 well tissue culture plate at a volume of 2 ml cell suspension per well. The plates were then allowed to incubate for 5 hours, then the medium was aspirated and the plates fed with 2.0 ml/well of 10% FBS/medium-199. Approximately 24 hours later, the medium was aspirated and the cells were exposed to serum-free medium-199 drug treatment and allowed to incubate for one hour. As determined in controls, very little cell loss was attributed to sample handling. The monolayers were then washed with 3.0 ml of Hank's salt solution (HSS). Any remaining intact cells were then dissolved by adding 200 µl 1.25% Triton-X100 and rotating for 20 minutes. Samples (500 µl) were then collected into tubes, snap-frozen in liquid nitrogen and CK activity was determined.

Creatine Phosphokinase (CK) Activity Determination

A commercially available kit (47-UV, Sigma, St. Louis, MO), was used to determine plasma CK activity (1).

Calculation of CK Depletion

As demonstrated by others (3), formulation irritation could be related to cell lysis. If the formulation was irritating, most of the cells would be lysed during the incubation, and the number of intact cells (and total CK) remaining in the monolayer would be quite low. When CK depletion was significant, the formulation was further diluted with serum-free medium-199 and run through the test again. The CK depletion caused by exposure to the formulation was expressed as a percent of the concurrently run control (serum-free medium-199 only, sample CK content: 30–50 U/L). In each test, a 6-well tissue culture plate was used for a control, and for each dilution of the formulation (n = 6). Differences between means were considered significant when $p < 0.05$ (ANOVA). The dilution of formulation required to deplete 50% of the monolayer CK (EC_{50}) was determined using a procedure essentially analogous to ref. 6. The natural logarithm of dose vs. logit of percent response was linearly regressed using the equation $\ln (P/100 - P) = \alpha +$

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$\beta \ln D$, where P was the percent of response, dose (D) expressed as % original concentration, α and β were the intercept and slope, and $EC_{50} = e^{(-\alpha/\beta)}$.

RESULTS AND DISCUSSION

Depletion of creatine kinase (CK) from monolayers incubated with immediate release formulations was summarized in Table I. Dilutions (1:1) of digoxin and digoxin vehicle, azithromycin and danofloxacin depleted significant amounts of CK from the monolayer, whereas azithromycin vehicle and danofloxacin vehicle did not. Further dilution of the active formulations eventually resulted in less then 50% CK depletion. EC_{50} values calculated from these data are also shown in Table I.

The % CK depleted from L6 cells exposed to 1:1 dilutions of immediate release formulations correlated well ($r^2 = 0.9$) with the rabbit lesion volume model (RbLV) (1). In fact, the EC_{50} generally agreed with the RbLV, rabbit CK (RbCK) and rat CK (RtCK) models.

The EC_{50} for digoxin, a known clinical irritant (9), digoxin vehicle, azithromycin and danofloxacin immediate release formulation were all below 50%. Williams and co-workers reported (2) in a PMA collaborative L6 study that an EC_{50} above 50% was required for clinical toleration of a formulation. As shown in Table I, these formulations were predicted to be as poorly tolerated as tetracycline and clindamycin.

Danofloxacin oily suspension vehicle and active formulation did not cause any depletion of CK. Danofloxacin aqueous suspension vehicle caused very little depletion of CK, while the active formulation caused extensive depletion of CK at a 1:1 and 1:9 dilution. As with whole animal CK models (7), chronic irritation of extended release formulations of danofloxacin was not well predicted by the L6 cell model. This was expected and likely due to the extended release nature of the formulation. Cell lysis and subsequent CK release after exposure to extended release formulations were probably related only to those muscle irritating components of the formulation that were immediately available at the time of initial exposure. The L6 model predicted that the acute irritation of the aqueous extended release danofloxacin formulation was apparently less than that of the immediate release formulation (EC_{50} 16.2% vs. 10.2%). This observation was qualitatively shown in the animal models (1,7).

No depletion of cellular CK was observed for the oily suspension extended release formulation, due in part to the phase separation, which minimized contact with the monolayer. Better contact with the monolayer might be possible if the

Table I. CK Depletion in *L6* Monolayers Following Exposure to Formulations, Comparison of Formulation EC_{50} Values, and Clinical Irritation

Formulations	Concentration (mg/ml)	Dilution[a]	CK Depleted (%)	EC_{50} (%)[b]	Clinical Irritant
Digoxin	0	1:1	93.0 ± 3.5*	31.0	
	0	1:4	11.4 ± 8.1*		
	0	1:9	0.1 ± 3.1		
	0.25	1:1	64.5 ± 6.4*	40.3	Yes[d]
	0.25	1:4	14.4 ± 7.4*		
	0.25	1:9	16.5 ± 3.6*		
Azithromycin	0	1:1	8.1 ± 7.6	>50	
	100	1:1	100 ± 0.0*	6.3	
	100	1:4	100 ± 0.0*		
	100	1:9	73.5 ± 12.8*		
	100	1:24	10.3 ± 6.0		
Danofloxacin solution	0	1:1	9.7 ± 7.2	>50	
	60	1:1	97.2 ± 3.6*	10.2	
	60	1:4	91.2 ± 3.1*		
	60	1:24	11.5 ± 7.6		
	60	1:49	0.8 ± 8.4		
Danofloxacin aqueous suspension	0	1:1	9.8 ± 7.0	>50	
	60	1:1	83.3 ± 15.1*	16.2	
	60	1:9	33.3 ± 11.9*		
	60	1:24	12.2 ± 20.4		
Danofloxacin Oily Suspension	60	1:1	0.0 ± 16.9	—	
Tetracycline[c]	125	—	—	<10	Yes/marked
Clindamycin[c]	150	—	—	24.8	Yes
Gentamicin[c]	40			54.9	No

Note: *different from concurrently run controls (serum-free medium-199 only) $p < 0.05$.
[a] Diluent was serum-free medium-199.
[b] The dilution (expressed as % of formulation concentration) required to deplete not more than 50% of the monolayer CK (creatine kinase).
[c] Toxicology Methods [4] 215–223, 1994.
[d] Clin Pharmacol Ther [16] 430–434, 1974.

suspension was homogenized with the diluting media. Although some water soluble muscle damaging components could also deplete monolayer CK, screening water immiscible IM formulations was not recommended with this model.

The authors recognized the complexity of CK release (10), and that additional studies are needed to further elucidate the relationship between CK release and muscle lesions following IM injection. While for immediate release formulations, animal CK models reportedly predicted the irritation observed in humans, they risk assay interference (11) from drug absorbed from the injected formulation. The potential of drug interference with CK assay was avoided in this model, since CK activity was determined in washed monolayers. For water miscible, immediate release formulations, the predictive value of the L6 cell monolayer model appeared to be similar to that of the animal CK models. It is not known whether this predictive value would apply to all compounds and all immediate release water miscible formulations. For example, formulation isotonicity and pH must be kept near physiological values, to minimize the potential of false positives (data not shown). With these precautions in mind, the model could serve as a useful, predictive initial formulation screen, since it was an animal-free test, lacked potential formulation interferences with the CK assay, and was resource and time sparing.

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