Regional Anesthesia and Pain

Liposomal formulations of prilocaine, lidocaine and mepivacaine prolong analgesic duration

[Des préparations liposomiques de prilocaïne, de lidocaïne et de mépivacaïne prolongent la durée de l'analgésie]

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Purpose: A laboratory investigation was undertaken to compare the *in vivo* antinociceptive effects of 2% liposomal formulations of prilocaine (PLC), lidocaine (LDC) and mepivacaine (MVC) compared to plain solutions of each of these three local anesthetics.

Methods: Large unilamellar vesicles were prepared by extrusion (400 nm), at pH 7.4. The membrane/water partition coefficients were obtained from encapsulation efficiency values, after incorporation of each local anesthetic to the vesicles. The anesthetic effect of each liposomal formulation was compared to the respective local anesthetic solution in water, using the infraorbital nerve-blockade test, in rats.

Results: The partition coefficients were: 57 for PLC, 114 for LDC and 93 for MVC. *In vivo* results showed that local anesthetic-free liposomes, used as control, had no analgesic effect. In contrast, the encapsulated formulations induced increased intensities of total anesthetic effect (35.3%, 26.1% and 57.1%) and time for recovery (percentage increases of 30%, 23.1% and 56%), respectively, for PLC, LDC and MVC when compared to the plain solutions ($P < 0.01$).

Conclusions: These results indicate that liposomes provide effective drug-delivery systems for intermediate-duration local anesthetics. Mepivacaine was affected to the greatest extent, while LDC benefited least from liposome encapsulation, possibly due to greater vasodilatory properties of LDC.

***Objectif** : Une recherche en laboratoire a été entreprise pour comparer les effets antinociceptifs in vivo de préparations liposomiques de prilocaïne (PLC), de lidocaïne (LDC) et de mépivacaïne (MVC) à 2 %, à des solutions simples de chacun de ces anesthésiques locaux.*

***Méthode** : De grandes vésicules unilamellaires ont été préparées par extrusion (400 nm), à un pH de 7,4. Les coefficients de partage membrane/eau ont été obtenus des valeurs d'efficacité de l'encapsulation, après l'introduction de chaque anesthésique local dans les vésicules. L'effet anesthésique de chaque préparation liposomique a été comparé à la solution respective d'anesthésique local dans l'eau par le test de blocage du nerf infra-orbitaire chez des rats.*

***Résultats** : Les coefficients de partage ont été de : 57 pour la PLC, 114 pour la LDC et 93 pour la MVC. Les résultats in vivo ont montré que les liposomes témoins sans anesthésique local n'avaient pas d'effet analgésique. Par contre, les préparations en capsules ont augmenté l'intensité anesthésique totale (35,3 %, 26,1 % et 57,1 %) et le temps de récupération (30 %, 23,1 % et 56 %) respectivement pour la PLC, la LDC et la MVC comparées aux solutions simples ($P < 0,01$).*

***Conclusion** : Ces résultats indiquent que les liposomes sont des systèmes de vecteurs de médicaments efficaces pour les anesthésiques locaux de durée moyenne. La MVC a surtout bénéficié, et la LDC le moins, de l'encapsulation liposomique, peut-être à cause de ses plus importantes propriétés vasodilatatrices.*

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PRILOCAINE (PLC), lidocaine (LDC), and mepivacaine (MVC) are structurally related local anesthetics (LA), commonly used for regional anesthesia, with fast onset and intermediate durations of action (90–240 min in clinical studies).[1] The structure of these aminoamide LA comprises two major components: a lipophilic fraction (an aromatic group) and a polar region, connected by an intermediate carboxyl group in an amide bond (Figure 1).[2]

The structure and physicochemical features of each LA molecule determines drug potency, onset of action, duration of sensory block, and toxicity.[3] Water solubility is an important property influencing transportation of the anesthetic molecule to the nerve fibres, as well as the ionization equilibrium that guarantees the presence of charged and uncharged LA species in the axoplasm, at physiologic pH. In contrast, hydrophobicity is also crucial for drug partitioning into the axon[1] so that a sufficient amount of LA molecules remain within that membrane in order to maintain the voltage-gated sodium channel protein in the inactive, non-conducting state.[4]

Amongst the desirable properties of an ideal LA molecule are long duration of action, low toxicity and adequate solubility in water and lipids.[5] While the search for ideal molecules continues, we speculated that it may be possible to enhance the effects of currently-available LA by their encapsulation into liposome delivery systems.[6] Liposomes are lipid vesicles that have been extensively described in the literature as effective drug-carriers, since they are able to enhance drug bioavailability, reduce systemic toxicity, and increase the half-lives of LA *in vivo*.[7–12]

The present study was undertaken to compare the *in vivo* antinociceptive effects of intermediate-duration LA, when encapsulated in large unilamellar liposomes (LUV). Prilocaine, LDC and MVC (Figure 1) were used at the same (2%) concentration, and each drug was administered to rats either in both plain solutions and encapsulated liposomal formulation. To better understand the interactions of LA molecules with liposomes, the results are related to the physicochemical properties of these molecules.

Methods

Materials and animal model

Prilocaine, LDC and MVC hydrochloride formulations, and thiopental, were obtained from Cristália – Produtos Químicos e Farmacêuticos Ltda (SP, Brazil). Egg phosphatidylcholine (EPC), cholesterol (Ch) and α-tocopherol (α-TC) were purchased from Sigma Chemical Co. (MO, USA). All other reagents were of analytical grade.

Male Wistar rats, 250–350 g, were obtained from CEMIB – UNICAMP (Centro de Bioterismo - State University of Campinas – UNICAMP, SP, Brazil) and were given free access to water and food throughout the study. The experiment was approved by the Institutional Committee for Ethics in Animal Research of UNICAMP (Protocols 824-1 and 559-1), which follows the recommendations of the Guide for the Care and Use of Laboratory Animals.

Liposomal LA and plain solution preparations

A dry lipid film, containing EPC, Ch and α-TC at a 4:3:0.07 molar ratio was prepared by solvent evaporation under nitrogen flow.[8] Multilamellar liposomes were obtained by adding 20 mM HEPES buffer, pH 7.4 (containing 154 mM NaCl) to the dry lipid film and vortexing the mixture. Unilamellar liposomes were prepared by extrusion (12 cycles through 400 nm polycarbonate membrane, at 25°C) of the multilamellar vesicles. The total lipid concentration in the LUV was 5 mM.[7,8] Since LA exhibit a fast equilibrium between EPC membranes and the aqueous phase,[13] LA molecules were added directly to the liposomes after extrusion, up to a concentration of 2% (corresponding to 77.9 mM of PLC, 73.8 mM of LDC and 70.7mM of MVC). Plain LA solutions with the same therapeutic LA concentrations[1] were prepared in 0.9 % saline (154 mM NaCl). Liposome LA formulations were incubated for 12 hr and stored at 4°C until further use.

The selection of LA concentration, 2%, was determined by the clinical efficacy of LDC and MVC. Comparisons among the drugs were directed by determination of their partition coefficient and the relationship between these values and enhanced analgesic effect provided by encapsulation into the liposomes is discussed.

Partition coefficient determination

The partition coefficient (P) between liposome/water was obtained from the encapsulation efficiency values, according to equation 1:[13]

$$P = \frac{n_m / V_m}{n_w / V_w} \qquad (1)$$

where: n corresponds to the number of moles of the anesthetic and V, to membrane volume, and m and w refer to the liposome and aqueous phase, respectively.

The encapsulation efficiency was determined by centrifugation (120.000 × g, two hours, 10°C) of liposome suspensions (4 mM lipid concentration), in the presence of an appropriate LA concentration

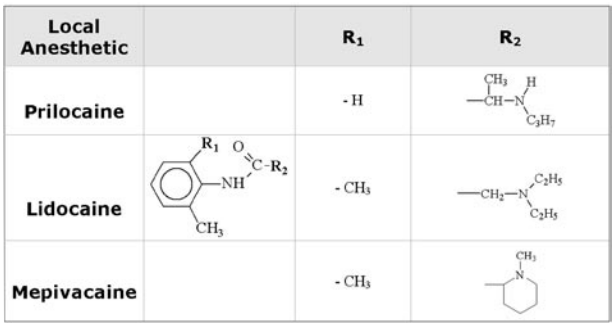

FIGURE 1 Chemical structure of the local anesthetics (prilocaine, lidocaine and mepivacaine).

TABLE I Physicochemical properties of prilocaine, lidocaine and mepivacaine

Local anesthetic	*Molecular weight*[a]	*pK*[b]	ε *($M^{-1}.cm^{-1}$)*	*P (pH 7.4)*
Prilocaine	256.8	7.9	5000[c] at 224nm	57 ± 6[c]
Lidocaine	270.8	7.8	382 at 263 nm	114 ± 16
Mepivacaine	282.8	7.6	429 at 263 nm	93 ± 7

pK = ionization constant; ε = molar extinction coefficient; P = partition coefficient. [a]Local anesthetic – hydrochloride form; [b]According to de Paula and Schreier;[13] [c]According to Cereda *et al.*[8]

(2 mM) for UV light absorption detection,[13] LA and liposomes were incubated for 12 hr at 4°C before phase separation. The amount of LA remaining in the supernatant was determined at 224 nm for PLC and 263 nm for LDC and MVC. To determine the amount of LA bound to the lipid phase and the encapsulation efficiency, LA concentration in the supernatant was subtracted from the initial LA concentration.

In vivo experiments

The rat infraorbital nerve blockade technique[14] was used to evaluate the analgesic effect. This method has been used previously,[8,15–17] and provides a simple and very reproducible experimental method for the animal model. As the rat infraorbital nerve is homologous with human infraorbital nerve, the rat infraorbital nerve block technique can be considered an important research tool. The antinociceptive effect was assessed by observation of the aversive response to the rat upper lip pinching, according to the scores: 0 (aversive response) or 1 (no aversive response). The animals had been lightly anesthetized with thiopental (25 $mg \cdot kg^{-1}$) by the *ip* route, before the plain or liposomal formulations were injected into the infraorbital notch, situated above a gap between the posterior four molars and the anterior incisor. The degree of sedation did not interfere with the generalized aversive response to the upper lip artery forceps pinching. For each LA studied a group of animals (n = 7–8) received 0.1 mL of the following formulations: Group I – prilocaine solution (PLC_{PLAIN}); Group II – liposomal prilocaine (PLC_{LUV}); Group III – lidocaine solution (LDC_{PLAIN}); Group IV – liposomal lidocaine (LDC_{LUV}); Group V – mepivacaine solution (MVC_{PLAIN}); Group VI – liposomal mepivacaine (MVC_{LUV}); Group VII (control) – local anesthetic-free liposomes ($LUV_{LA\text{-}FREE}$). Each formulation was injected unilaterally, into the rat's right side and the intact left side served as an internal control for each animal. The same investigator performed all experiments. The animals were tested every five minutes up to the time when the first aversive sign in the injected side was detected. The efficacy of infraorbital nerve block was analyzed by the time for sensory function recovery and the total LA effect. Local anesthetic effect was estimated by the area under the time curve (AUC) expressed as score/hour[18] and calculated using Origin 6.0 (Microcal™ Software, Inc., Northampton, MA, USA) program.

Statistical analysis

Infraorbital nerve blockade data (time for recovery and AUC) were analyzed by the Mann-Whitney test and expressed as medians (minimum and maximum limits). Statistical significance was defined as $P < 0.05$. Sample size calculation (n = 7–8 animals/group) was performed according to the equation for a finite population[19] and also considering previous literature reports.[8,15–17]

Results

Select physicochemical properties of PLC, LDC and MVC – taken from the literature – as well as the LA - liposome affinity inside the liposomes at pH 7.4, expressed as the partition coefficient, are presented in Table I. The partition coefficient values were: 57, 114 and 93 for PLC, LDC and MVC, respectively.

The antinociceptive effects induced by the three LA - plain and liposomal formulations – as tested by infraorbital nerve block, are presented in Figure 2 (panels A, B and C). These results are expressed as a percent of animals with analgesia.[12] Table II summarizes the times for recovery, and the total anesthetic effect (expressed as AUC) on nerve block, obtained with the different formulations. $LUV_{LA\text{-}free}$, used as control, presented no effect, whereas the encapsulated formulations induced an improvement on intensity of total anesthetic effect (35.3%, 26.1% and 7.1%) associ-

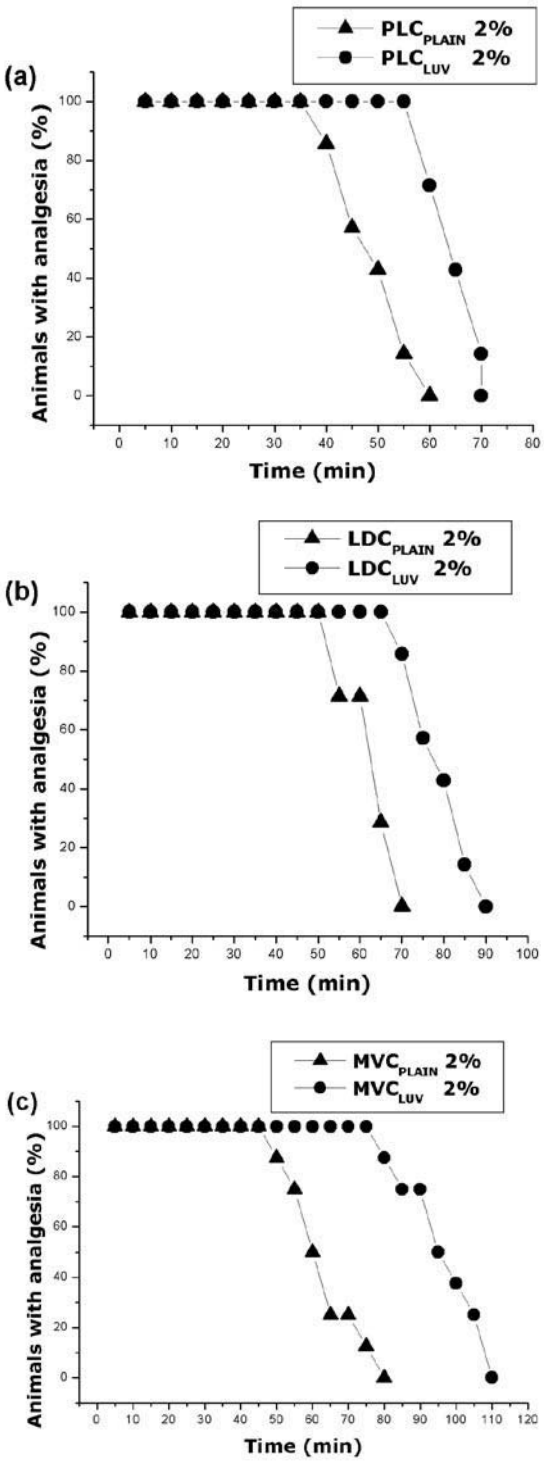

FIGURE 2 Time-course (min) showing the per cent of animals with analgesia evaluated by the infraorbital nerve blockade test in rats (n = 7–8 per group). Prilocaine solution *vs* liposomal prilocaine (panel A); lidocaine solution *vs* liposomal lidocaine (panel B); mepivacaine solution *vs* liposomal mepivacaine (panel C).

ated with prolonged times for recovery (percentage increases of 30%, 23.1% and 56%), for PLC, LDC and MVC groups respectively, when compared to plain solutions of each of the three drugs.

No signs of sensory blockade were observed on the intact left side of animals in any of the three groups, punctuated with score 0, i.e., aversive response to pinch (data not shown).

Discussion

The aminoamide family LA have been preferentially used in clinical practice since theses drugs exhibit less allergenic properties than LA from other families, such as the aminoesters.[20] The intermediate durations of action and fast onset are pharmacological features shared by the three aminoamide LA studied here[2,21] that are beneficial for a variety of surgical procedures. These LA are also less toxic to the nervous and cardiovascular systems than long-acting LA, such as bupivacaine.[1] In previous work, we have shown that MVC and bupivacaine liposomal formulations are associated with a dose-dependent block in mice sciatic nerve fibres[7] that, only in the case of MVC, was significantly larger than the block induced by plain LA solutions of equivalent concentrations. In another study, the antinociceptive effects of a 3% liposomal PLC formulation on rat infraorbital nerves were shown to be larger than that of 3% plain PLC, and comparable to that elicited by 3% vasoconstrictor-associated PLC.[8] Here, for a preset LA concentration of 2% (using the rat infraorbital nerve blockade test) we report and compare an increase in the duration of anesthetic effect induced by PLC, LDC and MVC liposome formulations, relative to the plain solutions of each of these three drugs.

Lidocaine and PLC are linear aminoamide LA homologues with similar substitutions, although PLC is a very asymmetric molecule (Figure 1). Mepivacaine is a cyclic aminoamide, i.e., its amine group is part of a piperidine ring with a methyl substitution (Figure 1). Since the hydrophobic character of an LA molecule can be determined by its aromatic group substitutions,[21] the two ortho-methyl groups in the structure of LDC and MVC can be responsible for their higher P values (Table I) and Van der Waal's volumes,[22] in comparison to PLC. The nerve blocking effect of plain LA: LDC ≥ MVC > PLC (Figure 2, Table II) followed what was expected from their P values at pH 7.4. However, liposome encapsulation had affected in a different manner each of the three LA: MVC_{LUV} effect prevailed over those of LDC_{LUV} and PLC_{LUV}. It is easy to understand why PLC, the less hydrophobic molecule, benefited least from liposome encapsulation. We could also rationalize that the higher ratio

TABLE II Total effect of sensory blockade (AUC) and time for recovery for plain and liposome local anesthetic formulations

Groups	T_{rec} *(min)*	*AUC (score/hour)*	Δ T_{rec} *(%)*	Δ *AUC (%)*
PLC_{PLAIN} (2%)	50 (40 - 60)	42 (32-42)	30%	35.3%
PLC_{LUV} (2%)	65 (60 - 75)*a***	58 (52-68)*a***		
LDC_{PLAIN} (2%)	65 (55 70)*d***	58 (48-62)*d***	23.1%	26.1%
LDC_{LUV} (2%)	80 (75 - 90)*b*** g***	72 (62-82)*b*** g***		
MVC_{PLAIN} (2%)	62 (50 - 80)*e** f*^{ns}	52 (42-68)*e** f*^{ns}	56%	57.1%
MVC_{LUV} (2%)	98 (80-110)*c**** h**** i**	82 (68-98)*c**** h**** i**		
$LUV_{L A -FREE}$	0	0	-	-

T_{rec} = Time for recovery; AUC = area under curve; Δ T_{rec} and Δ AUC = (liposomal – plain) / plain x 100; Statistical analysis (Mann-Whitney test): **P* <0.05 ; ***P* < 0.01 ;****P* < 0.001 a*** = PLC_{PLAIN} *vs* PLC_{LUV} ; b**** = LDC_{PLAIN} *vs* LDC_{LUV} ; c**** = MVC_{PLAIN} *vs* MVC_{LUV}; d*** = PLC_{PLAIN} *vs* LDC_{PLAIN}; e** = PLC_{PLAIN} *vs* MVC_{PLAIN}; f^{ns} (= not significant) = LDC_{PLAIN} *vs* MVC_{PLAIN}; g*** = PLC_{LUV} *vs* LDC_{LUV}; h**** = PLC_{LUV} *vs* $MVC_{PLAIN;}$ i** = LDC_{LUV} *vs* MVC_{LUV}. Data presented as seen by the infraorbital nerve blockade model. Data are expressed as median (minimum-maximum); (*n* = 7–8 / group). Please refer to text for definitions.

of uncharged/charged species of MVC (pKa = 7.6) at pH 7.4, accompanied by the fact that MVC is a cyclic aminoamide LA,[2,21] favoured its higher performance, in relation to the linear homologues LDC and PLC (pKa = 7.8 and 7.9, respectively). Therefore, we can infer that MVC_{LUV} and PLC_{LUV} showed an increase in their anesthetic effect as should be expected from their physicochemical properties.

Nevertheless, LDC_{LUV} did not benefit from encapsulation as might have been anticipated from its partition coefficient value. Moreover, the profile of the antinociceptive effect improvement obtained upon encapsulation was MVC > LDC ≥ PLC (Table II). Experimental studies performed with homologous linear and cyclic aminoamide LA in isolated rabbit nerve fibres[23,24] provide clues to help explain these results. It is well known that there is a correlation between the partition coefficients of LA and their conduction blockade, *in vitro*.[1,21,23,24] However, this was not the case observed *in vivo*, where MVC and LDC displayed similar potencies, what has been attributed to the fast vascular absorption of LDC.[1] This can be explained by the drug's notable vasodilatory effect[21] that favours drug clearance, and leaves fewer LDC molecules available for neural blockade. Accordingly, it is possible that LDC, having greater vasodilatory properties than the other two LA,[1] did not benefit from its higher liposome affinity, in relation to MVC and PLC, because its clearance was rapid and counterbalanced the increased antinociceptive effect attained with liposome encapsulation. This may have limited LDC_{LUV} effectiveness, *in vivo*. Another possible explanation could be a larger *in situ* resident time of LA, when carried in liposomal formulation. The controlled release of bupivacaine, for instance, has been well demonstrated *in vivo* with liposomes prepared with a pH gradient[10,25] or with lipids in the gel phase, such as hydrogenated soya lecithin[12] and could last for many hours. Nevertheless, this was not the case with the intermediate-duration LA and the liposome systems studied here.

In conclusion, this study shows that liposome encapsulation increases analgesic duration and intensity for three intermediate-duration aminoamide LA: LDC, MVC and PLC. The effects of encapsulation were greater with MVC compared to LDC or PLC. This may be due to the higher partition coefficient of MVC and the amount of uncharged drug at pH 7.4, in comparison to PLC. The greater partition of LDC in liposomes did not result in a proportional increase in duration of clinical effect, possibly due to the vasodilatory action of LDC that, *in vivo,* counterbalances the controlled release of anesthetic molecules from liposomes.

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