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Review article

Impact of liposomes as delivery systems in veterinary medicine

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Abstract – The development of drug resistance and the inability of the drug to reach the location of the etiologic agents are major challenges for anti-infective and cancer therapies. As the development of new drugs with improved pharmacodynamic and pharmacokinetic properties is a slow and difficult process, drug delivery systems appear as promising alternatives. Liposomes are lipid vesicles formed when phospholipids are exposed to an aqueous environment. They arrange themselves in bilayers and close up, forming a vesicle. During this process they capture the aqueous phase of the dispersion, and any substance dissolved in it, within the vesicle. Liposomes have remarkable features that make them an almost ideal delivery system. They are biodegradable, with few side effects, can deliver drugs with different physico-chemical properties together and can be formulated for different routes of administration. The potential to modify the pharmacokinetic behaviour of encapsulated drugs to deliver them selectively to the site of action is the most important feature of liposomes as drug delivery systems. Liposomes are already used in human medicine for the treatment of bacterial, viral and parasitic diseases, and cancer. They have also been proven useful as immunoadjuvants and vaccines. Liposomes are used in certain avian vaccines. The possible uses of liposomes and their impact in veterinary medicine in the treatment of infectious diseases and cancer as well as in the prevention of diseases are discussed in the present article. © Inra/Elsevier, Paris

liposome / pharmacokinetics / delivery system / therapy

Résumé – Impact des liposomes en tant que systèmes fournisseurs en médecine vétérinaire. L'augmentation de la résistance aux médicaments et la difficulté à atteindre le site d'action sont des problèmes très importants pour la thérapie contre les maladies infectieuses et tumorales. Le développement de nouvelles drogues avec des propriétés pharmacodynamiques et pharmacocinétiques améliorées est un processus lent et difficile. Alors, les systèmes de transport de drogues sont une alternative prometteuse. Les liposomes sont des vésicules lipidiques qui apparaissent lorsque des phospholipides sont exposés à un environnement aqueux. Ils se disposent en couches superposées qui finissent par se fermer en formant une vésicule. Lors de ce processus, ils captent

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la phase aqueuse du milieu et toute substance qui s'y trouverait dissoute. Les liposomes possèdent certaines caractéristiques qui en font un système fournisseur presque idéal. Ils sont biodégradables avec peu d'effets secondaires. Ils peuvent transporter des drogues possédant différentes propriétés physico-chimiques et ils peuvent aussi être utilisés par différentes voies d'administration. La modification du comportement pharmacocinétique des drogues encapsulées dans les liposomes et la capacité de ceux-ci à transporter les drogues au site d'action sont les caractéristiques les plus importantes de ce système fournisseur. Actuellement, on emploie les liposomes en médecine humaine pour la thérapie des maladies bactériennes, virales, parasitaires et tumorales. Ils sont également utiles comme substances immunomodulatrices et comme vaccins. Les liposomes sont déjà employés dans quelques vaccins aviaires. L'utilisation thérapeutique des liposomes, leur impact dans la thérapie des maladies infectieuses et du cancer ainsi que la prévention des maladies, sont discutés dans cet article. © Inra/Elsevier, Paris

liposome / pharmacocinétique / système fournisseur / thérapie

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1. INTRODUCTION

The use of drug delivery systems has been proposed to improve selectivity by altering the pharmacokinetic behaviour of drugs (clearance, metabolism, excretion). This would favour the therapeutic as opposed to the toxic effects of the drug, and would deliver the drug or biologically active compound specifically to the target organ or tissue. Although liposomes have a great range of possible applications, their use as drug carriers offers the greatest potential, not only because they are biodegradable and easy to prepare, but also because of their remarkable versatility in terms of composition, size and other structural characteristics.

The pathogenesis of a number of bacterial and protozoan diseases is due to the ability of the pathogenic agent to avoid being killed by macrophages and polymorphonuclear leukocytes [64, 101]. Organisms such as Brucella spp. and Mycobacterium spp. are found almost exclusively within cells, while others, such as Salmonella spp. and Staphylococcus aureus, exist both intracellularly [101].

Most of the presently available antibiotics are ineffective against intracellular
infections, mainly due to poor penetration and poor retention of the drugs inside the macrophages, and due to their decreased activity within the intracellular milieu [64, 101]. Moreover, one of the challenges in the development of effective antiviral drugs is to deliver the agents to the sites of virus replication [101]. The capacity of liposomes to encapsulate a wide range of hydrophilic and/or lipophilic biologically active compounds and their natural distribution to the mononuclear phagocytic system (MPS) cells make them extremely attractive as potential vehicles for drug delivery in anti-infective therapy.

Many delivery systems have the ability to sustain almost invariable concentrations of a drug in plasma and tissues. Even though it is not always desirable to maintain sustained concentrations of antibiotics for therapeutic efficacy, controversy exists over the benefits of sustained release delivery systems for antimicrobial therapy. In some veterinary applications, sustained release preparations may be the only feasible approach to improving anti-microbial therapy [101] and liposomes could play a major role in this process.

The successful evolution of liposomes from experimental tools to industrially manufactured products for human and veterinary use depends on efficient drug entrapment in vesicles of a narrow size distribution using simple and reproducible methods. There has been considerable progress in this area and well-defined formulations containing a variety of active agents can now be produced in a stable form. A number of these formulations are currently undergoing clinical trials and a few (including liposome-based veterinary vaccines) are already licensed [39, 72, 111].

2. STRUCTURE AND PREPARATION

Liposomes are closed lamellar structures. They are lipid vesicles that have

![Figure 1. Structure of a multi-lamellar vesicle (liposome) showing the arrangement of the lipid bilayers and the localization of entrapped compound or drug.](image-url)
many or one (sometimes only a few) lipid bilayers or lamellae, deployed in a concentric way, surrounding a central aqueous core (figure 1). The bilayers are separated by aqueous spaces. Liposomes have the ability to encapsulate various solutes present in the aqueous phase during their formation. Technically, liposomes are simple to prepare. There are various methods for their preparation. The details of the different methods have been well reviewed elsewhere [23, 102]. The selection of a particular method for the preparation of liposomes depends on the lipids to be used, the vesicle type to be obtained and, especially, the compound to be encapsulated; some compounds are sensitive to the formation conditions, e.g. exposure to organic solvents and short periods of sonication that may possibly result in the denaturation of sensitive proteins. The most well-known method is based on the exposure of certain amphipathic lipids (i.e. phospholipids) to an aqueous environment. The lipids are solubilized in an organic solvent and by rotary evaporation under reduced pressure of a nitrogen atmosphere, they are deposited, forming a thin film on the walls of the flask. Then the lipids are hydrated adding an aqueous buffer at a temperature determined by the lipids selected and with gentle shaking [23, 102]. The lipids orient themselves with their hydrophilic head groups facing the water molecules, thereby isolating the hydrophobic hydrocarbon tails from the water. Depending on the lipids, the temperature and other conditions, amphipathic lipids can form a number of macromolecular configurations, including micelles, hexagonal phases and bilayers. In order to obtain a more homogeneous group of bilayer-forming lipids with a smaller size, the mixture can be either extruded through polycarbonate membranes with different pore diameters [23, 102] or vigorously vortexed so that the bilayers will break-up into small closed vesicles or liposomes [42, 61, 62, 101].

**Figure 2.** Liposomes classified according to the number of bilayers and the aqueous space volumes. a) Multi-lamellar vesicle (MLV); b) large uni-lamellar vesicle (LUV); c) small uni-lamellar vesicle (SUV).
Two methods are described that are simple and easy to scale up. The first one is based on the dehydration–rehydration of lipids [38] and the second one is based on microfluidization of the lipid emulsion [107]. Other methods that avoid the use of organic solvents, which are pharmaceutically unacceptable have been described [82, 103].

3. TYPES OF LIPOSOMES

Different types of liposomes can be prepared according to their internal aqueous space volume or their bilayer composition. Multilamellar vesicles (MLVs) have many bilayers or lamellae surrounding a central aqueous core and are generally heterogeneous. They have an encapsulation efficiency of 5 to 15% for small water-soluble substances. Small unilamellar vesicles (SUVs) can be prepared by many methods [23, 102]. One of them is the sonication of MLVs, which will produce a fairly homogeneous population of SUVs, that has an encapsulation efficiency of about 1%. These low entrapment efficiencies led researchers to develop methods for producing large unilamellar vesicles (LUVs) that can capture more aqueous volume with a smaller amount of lipids. LUVs have only one compartment and a 40% encapsulation efficiency [101, 102] (figure 2).

In terms of composition, liposomes are composed of either natural, synthetic lipids or a lipid mixture. Phospholipids are the most commonly used lipids. Those with more complex bilayers may have proteins within or upon their bilayers (proteoliposomes, immunolipo-

Figure 3. Types of liposomes with different substances attached to their surfaces. a) S-liposomes (sterically stabilized or long-circulating liposomes); b) S-immunoliposomes (S-liposomes + antibodies).
somes), gangliosides or polyethylene glycol polymers (sterically stabilized liposomes) [3, 5, 39, 61, 111] (figure 3). These sterically stabilized liposomes (also termed as S-liposomes, stealth or long circulating liposomes) have different pharmacokinetic properties with long half-lives. This is due to the incorporation of GM, gangliosides or polyethylene glycol to the liposome bilayer surface, which reduces the degree of recognition and the rate of uptake by the mononuclear phagocyte system (MPS) [3, 5, 20, 111] (figure 3).

4. ENTRAPMENT OF DRUGS IN LIPOSOMES

Liposomes have the advantage that drugs are entrapped without chemical modifications, but there are many factors affecting drug entrapment and retention in liposomes. These factors should be considered carefully because the optimal pharmacological effects will be only achieved if the amount of drug contained in a liposome is maximized [25, 101]. Factors relating to both the entrapped drug and the liposome (membrane stability and size) must be considered [102].

Water-soluble drugs or biologically active compounds are entrapped within the aqueous core and the different compartments for multilamellar vesicles or only within the aqueous compartment in the cases of large and small unilamellar vesicles. Lipid-soluble substances are retained within the bilayers. Therefore, the greater the solubility of the substance in the non-polar solvent, the larger the amount that can be retained in the hydrophobic regions of liposomes (figure 1). In addition to the solubility of the different drugs or compounds, the size of the liposome may influence the amount entrapped, because the larger the liposome, the greater the size of the aqueous compartment and the number of hydrophobic regions present [24, 55, 74]. High loading efficiency (i.e. high drug to lipid ratio) is necessary to achieve the maximum pharmacological effect. However, other conditions may be required to obtain an optimal response. Adequate drug retention within liposomes must be achieved, especially in the case of long-circulating liposomes, to avoid side-effects [54]. Many drugs are able to cross or destabilize membranes and may leak out of the liposome [7, 78, 79, 101, 109]. The liposomal membrane, composed of lipid bilayers, is, in general, impermeable to ions and large hydrophilic ions, but permeation of neutral or weakly hydrophobic molecules can be controlled by concentration gradients. However, some weak acids or bases can be transported through the membrane due to various gradients, including electrical, pH (ionic) or chemical potential gradients [54]. The permeability and stability of liposome formulations are important both over time and in the presence of physiologic fluids [40, 77, 85]. When stored, liposomes leak their contents after 1 to 2 days for different reasons, one of which is the oxidation of the unsaturated fatty acids. The oxidation can be prevented by either incorporating antioxidants in the membranes (e.g. vitamin E) or including EDTA in the suspension medium and storing the product at 4 °C. These conditions assure long-term stability [15]. The presence of physiological fluids may have either beneficial or detrimental actions [15, 39, 115] depending on the composition of the fluid [7, 15, 33, 77, 79, 85, 95, 115]. By modifying the composition and thickness of the bilayers, these disadvantages can be minimized [7, 33, 79, 85, 95]. Cholesterol plays an important role in membrane permeability and stability. Large amounts of cholesterol (33 % of the lipids) increase liposome stability in the serum and decrease its uptake by the liver and spleen [76]. Cholesterol-induced changes in mem-
brane permeability are dependent on an intact sterol side chain, the planar sterol nucleus, and the 3β-hydroxy group [102]. This structure–function relationship is due to:

1) a condensation of the area of phospholipid molecules in monolayers;
2) an inhibition of motion within the outer segment of the phospholipid acyl chain bilayers;
3) an increase in width of bilayers composed of short chain phospholipids; and
4) an increased perpendicular orientation of the acyl chains [98, 102].

Thus, cholesterol decreases the permeability of phospholipid bilayers to ions and small polar molecules and reduces the ability of many proteins to penetrate [102]. It should be pointed out that lipids that increase the bilayer rigidity, such as sphingomyelin and cholesterol, decrease both the in vitro and in vivo uptake of liposomes into the MPS cells [4]. This fact plays an important role in the in vivo kinetics of liposomes designed to reach organs or tissues other than the MPS.

One particular case of entrapping biologically active compounds concerns protein molecules. These can be bound to the surface of the liposome. This has great advantages in terms of modifying the distribution and selecting the target for the lipid vesicles (e.g. antibodies) [3, 11, 25, 40, 97].

Another advantage of liposomal entrapment occurs when two synergistic drugs have different solubilities, i.e. one is water-soluble and the other one is lipid-soluble. Thus, both drugs can be formulated together in a single liposome formulation. The hydrophilic compound will be entrapped in the aqueous spaces, while the lipophilic one will be present within the bilayers.

5. IN VIVO KINETICS OF LIPOSOMES

A very attractive prospect for the use of liposomes is the idea of targeted liposomes for site-specific drug delivery. However, when the first formulations with conventional liposomes (e.g. phosphatidylcholine and cholesterol) were injected intravenously, they did not behave as predicted. These liposomes immediately interact with plasma high density lipoproteins (HDL) which remove the phospholipid molecules from the bilayers, disrupt the membrane and could result in their water-soluble contents leaking out into the systemic circulation [39, 101, 115]. In addition to HDLs, opsonins are another factor that markedly affect the distribution of liposomes in the organism. They bind to the surface of the liposomes and make them detectable by the MPS [3], resulting in their uptake and retention. The result is a short plasma half-life and a low distribution, mainly to liver, spleen and bone marrow, which are the major MPS organs. The interaction between opsonins and liposomes depends on various factors, the surface charge is one factor determining if opsonins are a necessary component in the uptake process. Thus, the uptake of neutral and negatively charged liposomes does not involve serum components; however, liver uptake of positively charged liposomes is most likely due to liposome aggregation caused by opsonins [28, 58]. Differences among species in the necessity of opsonins for recognition and uptake of liposomes by the MPS and the serum opsonic activity were reported both in vitro and in vivo [59, 60]. In mice, for example, liposome uptake by the liver was independent of serum proteins, while in rats this uptake was strongly dependent on the activity of opsonins. Also, the affinity of opsonins for liposomes of a given composition differed among animal species. In vitro,
human serum exhibited higher activities for phosphatidylcholine/cholesterol (PC/Cho) liposomes than bovine sera, while rat serum had no activity for PC/Cho liposomes but had a high opsonic activity for monosialoganglioside (GM\_1) liposomes [60]. Serum components appeared to enhance the liver uptake of GM\_1 liposomes in rats. Such activity was also found with human and bovine sera, but not with mouse serum [59]. The health status of the animal may also influence the opsonic activity of the serum; tumour-bearing rats showed changes in the opsonic capacity with a decreased liver uptake of liposomes compared to healthy rats [67]. The activity of opsonins is a crucial factor to be considered when designing long-circulating liposomes. Other plasma components, such as haemoglobin, methaemoglobin, phospholipase A\_2, may affect liposome stability [85, 115].

Although most of the intravenous administered conventional liposomes follow this main pattern of distribution, some of them can reach other organs. These exceptions are the lungs and the skin. This can be explained by the second carrier theory. In the blood, some liposomes are taken up by circulating monocytes, which can migrate to the tissues resulting in the liposomal load being removed from the circulation. Included among these tissues are the pulmonary interstice and skin. This process is particularly prevalent when an inflammatory process is present. Also, for the lungs, mechanical factors may be responsible, since the lungs are the first capillary bed encountered and LUVs may be retained in this site [83, 85, 94, 101]. On the other hand, liposomes injected in the peritoneal cavity (i.p.) behave like other particles. A certain proportion of intact liposomes enters the lymphatics and eventually reaches the circulation. Once in the bloodstream, they are taken up by phagocytes of the major MPS organs. Subcutaneous or intramuscular injections are slowly cleared from the site of injection; then they follow the same route as those administered i.p. Topical administration may provide a sustained release of drug by this route. This natural pattern of distribution has been proven to be useful for drug administration in certain conditions (e.g. cutaneous leishmaniasis, pulmonary metastases, immunomodulators) [83, 86, 94, 101]. Other routes of administration include intra-tracheal, intra-articular, per os [8, 98] and by aerosols [16, 68].

The administration of liposome-encapsulated drugs by aerosols could be a feasible, non-invasive way to achieve high concentrations of a drug in the lungs [8, 68]. After inhalation, aerosolized liposomes have been shown to be uniformly distributed to all the lung lobes [8]. Although the alveolar macrophages are the desired target cells, liposomes can remain intact for a period of time [68] after which they are taken up by both types of pneumocytes (I and II) [8]. In addition to this pattern of distribution, chronic exposure to liposome aerosols had no detrimental effects on either the survival of the animals or on their macrophage functioning [68]. These characteristics are particularly useful when delivering antibiotics in the treatment of infections of the lower respiratory tract. Liposome-encapsulated ciprofloxacin administered by aerosols using commercially available nebulizers had high therapeutic efficacy in the treatment of Francisella tularensis infection in mice [16].

In order to achieve a targeted drug delivery, liposomes must be altered by coupling certain molecules (monoclonal antibodies, GM\_1 ganglioside, sugars) to their surfaces [3, 5, 11, 40, 70, 97]. The first difficulty to overcome in this process was increasing the length of their half-lives to increase their chances of reaching
the desired target. This is achieved by coating the liposome surface with polyethylene glycol (PEG) by covalent attachments (sterically stabilized liposomes). They then have reduced affinity for the MPS cells and can avoid detection. They also have an enhanced lubrication property (vascular permeability coefficient), which enables them to travel through the capillaries more easily [3, 111]. A more precise targeting is achieved by attaching specific monoclonal antibodies to sterically stabilized liposomes (S-immunoliposomes) [11, 40, 66, 94, 97].

As a sustained or controlled drug delivery system, liposomes have the following advantages:

1) slower drug clearance from the bloodstream;

2) ability to protect the drug from enzymatic or hydrolytic degradation;

3) retain the drug at the site of injection (intra-ocular, intra-articular) for an extended period of time; and

4) slow release of the drug from the site of injection (i.m., s.c., subconjunctival) or from the site of liposome localization after i.v. administration (liver, spleen) [14, 98, 101].

6. CELLULAR UPTAKE OF LIPOSOMES

Two general mechanisms have been described to explain how liposomes are taken up by cells [108]. In the first one, regardless of the way they attach to the cells and the ligand involved, liposomes are taken up by endocytosis and they end up in lysosomes with their bilayers disrupted. Depending on the drug stability in the presence of enzymes and different pH conditions, the released drugs can then act either within the organelles (as seen in a number of intracellular microbial and storage diseases) or, following their diffusion through the lysosomal membrane, in other cell compartments (e.g. in the nucleus during cancer chemotherapy) [39, 108]. Recently, liposomes have been designed to evade lysosomal localization and the resulting inactivation of their labile contents. When these pH-sensitive liposomes are in the acidic environment of endosomes, they are believed to fuse with the vacuole membrane and to release their contents in the cytoplasm [51]. However, it remains to be seen whether the encouraging results that have been obtained in vitro can be reproduced in vivo, where the blood proteins that are adsorbed in the liposomal surface may interfere with its fusogenic properties [39]. An enhanced penetration of liposomes may occur into cells infected with certain viruses, such as rheoviruses [90, 92]. This enhancement could be explained by an increased membrane fluidity, which could be a consequence of the mechanisms of the virus exit process. This preferential uptake was not specific for liposomes because a similar uptake was also observed with others nanoparticles [92]. Regardless of the mechanisms involved, the fact that a viral infection stimulates the cellular uptake of liposomes facilitates the targeting of liposome-entrapped antiviral drugs [90]. In addition, this strategy only works well with target cells with relatively high endocytic activity such as tumour cells. This activity may be smaller in virus-infected cells which have been induced to shut down their metabolic activity, particularly the endocytic activity [64].

In a second mechanism, unilamellar liposomes appear to fuse with the plasmatic membranes of the target cells and to introduce their contents directly into the cytosol [64, 98].
7. THERAPEUTIC APPLICATIONS OF LIPOSOMES

The treatment and prevention of diseases is a main research goal in human and veterinary medicine. While finding or creating new drugs is one approach, there are now interesting alternative approaches, among them we can emphasise: a) the modification of the drug formulation to obtain a controlled release or targeting to specific sites, thereby enhancing the efficacy of existing drugs, and b) the stimulation of the natural defences of the host using immunomodulating substances. These alternatives are not mutually exclusive. In both cases, as pointed out above, liposomal formulations have been investigated and have shown some usefulness.

Tailoring the structural characteristics of the liposome to take advantage of certain properties of the biological environment has led to the production of a variety of types of vesicles that are optimal for drug transport and, ultimately, therapy. There are many proposed therapeutic applications that are based on successful results with experimental animal models of disease. These include: therapy of viral, bacterial and protozoan infections; treatment of a variety of cancer cells; gene and antisense therapy; enzyme-replacement therapy of inherited metabolic disorders, where substrates accumulate in tissues as a result of enzyme deficiencies; hormone-replacement therapy; detoxifying tissues that contain intracellularly deposited metals; arthritis treatment; the administration of haemoglobin-loaded liposomes as a blood surrogate (haemosomes) in emergencies where appropriate donor blood is not readily available; and for vaccinations [39]. There is a sizeable reduction in the adverse effects that different drugs may have when they are administered as a liposomal formulation [81, 101]. This may have a positive impact on the clinical use of liposomes. A list of some liposomal formulations that are already on the market or under study for approvals is given in table 1.

7.1. Bacterial diseases

In many microbial diseases the bacteria have an intracellular location and are therefore able to escape destruction within phagocytic cells (e.g. preventing phagolysosomal fusion). This represents a therapeutic challenge. Most of the antibiotics presently available are ineffective against intracellular infections, mainly because of poor penetration, poor retention of these drugs inside the macrophages, and decreased activity within the intracellular milieu. The ability of liposomes to target antibiotics to the site of a facultative intracellular bacterial infection has been examined in several in vitro cultures and experimental animal models. Liposomal formulations have been compared with free drug formulations against bacteria such as Staphylococcus aureus, Salmonella spp., Brucella spp. and Mycobacterium spp. [101].

Staphylococcus aureus produces chronic bovine mastitis which is difficult to treat and therapy rarely results in bacteriological cure. MacLeod and Prescott [64] used an intramammary infusion (IMI) of liposomally entrapped gentamicin to treat this type of mastitis, but failed to achieve a bacteriological cure. Other workers obtained the same result when using liposomal cloxacillin. These failures were attributed to the inhibitory activity of the cellular milieu (i.e. the acid environment of the phagolysosome) as well as to the metabolic inactivity of intracellular S. aureus. These results do not correlate with other reports where aminoglycosides greatly enhanced the destruction of S. aureus in mouse peritoneal macrophages and canine mono-
Table I. Liposomal formulations available or for which approval is under way for some drugs of interest to veterinary medicine.

<table>
<thead>
<tr>
<th>Trademark</th>
<th>Drug</th>
<th>Status</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-microbial agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomal Nystatin</td>
<td>Nystatin</td>
<td>Not approved in USA</td>
<td>Aronex Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>(no marked yet)</td>
<td>Amphotericin B</td>
<td>Approved in Europe and USA</td>
<td>NeXstar Pharmaceuticals (former Vestar) (USA)</td>
</tr>
<tr>
<td>MiKasome</td>
<td>Amikacin</td>
<td>Not approved in USA</td>
<td>Aronex Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>Anticancer drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomal Annamycin</td>
<td>Annamycin</td>
<td>Not approved</td>
<td>Aronex Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>(no marked yet)</td>
<td>Daunorubicin</td>
<td>Approved in UK, Sweden and USA</td>
<td>NeXstar Pharmaceuticals (former Vestar) (USA)</td>
</tr>
<tr>
<td>DaunoXome</td>
<td>Daunorubicin</td>
<td>Approved in USA</td>
<td>NeXstar Pharmaceuticals (former Vestar) (USA)</td>
</tr>
<tr>
<td>VincaXome</td>
<td>Vincristine</td>
<td>Not approved</td>
<td>NeXstar Pharmaceuticals (former Vestar) (USA)</td>
</tr>
<tr>
<td>Doxil</td>
<td>Doxorubicin</td>
<td>Approved in USA</td>
<td>SEQUUS Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>D99</td>
<td>Doxorubicin</td>
<td>Not approved</td>
<td>The Liposome Company (USA)</td>
</tr>
<tr>
<td>Vaccines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>Newcastle disease</td>
<td>Licensed by USDA</td>
<td>IGI, Vineland Laboratories (USA)</td>
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<tr>
<td>disease vaccine</td>
<td>virus (killed)</td>
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<tr>
<td>Avian Rheovirus</td>
<td>Avian Rheovirus (killed)</td>
<td>Licensed by USDA</td>
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<tr>
<td>vaccine</td>
<td>virus (killed)</td>
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<tr>
<td>Epaxal-Berna Vaccine</td>
<td>Inactivated hepatitis A</td>
<td>Approved in Switzerland</td>
<td>Swiss Serum and Vaccine Institute (Switzerland)</td>
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<td>virions</td>
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cytes [64]. Grayson et al. [37] found that the administration of liposomal gentamicin in mice provides sustained drug concentrations in the regional tissues with a protective effect against a massive challenge of *S. aureus* for at least four subsequent days. These contradictory results may be explained due to the relatively poor bactericidal capacity of the mammary macrophages [64]. Nevertheless, the study of MacLeod and Prescott [64] suggests that intraphagocytic activity of antimicrobial agents is as important as phagocytic penetration. Liposomal entrapment may be useful in the treatment of bovine mastitis for those drugs which penetrate phagocytic cells poorly and have high intraphagocytic antimicrobial activity.

In the case of *Brucella* spp., a vaccine is available and eradication programmes in food animals are being undertaken in many countries, involving the destruction of animals with positive serological reactions. Treatment has only been suggested for valuable breeding animals. The co-administration of a long acting oxytetracycline and free streptomycin is effective in the elimination of *B. abortus* in udder secretions or in achieving an apparent cure. However, this only occurs when repeated doses are used [72]. The search for a short duration therapeutic regimen with no or few side effects continues to be a main goal. Domingo et al. [22] compared a newer macrolide antibiotic, azithromycin, with the combination of doxycycline and streptomycin. A short oral treatment with azithromycin was only able to reduce the infection; it was not able to cure the animals as effectively as the doxycycline–streptomycin regimen administered for a long period of time. A similar effect can be obtained with fewer doses of each preparation using liposomal instead of free streptomycin in the combination with long acting tetracycline. Liposomal streptomycin has to be administered both IV and by IMI; otherwise, the flushing of the compound by milking would render IMI alone ineffective [73]. Vitas et al. [106] found that the administration of liposomal gentamicin can result in an enhancement of the therapeutic activity of gentamicin in a murine model of systemic acute brucellosis. The treatment with liposomal gentamicin protected 70% of the animals when administered 1 day after a lethal challenge [106]. Further studies are required to evaluate this potential treatment of bovine brucellosis and, more importantly, for human brucellosis.

As with brucellosis, infections caused by *Mycobacterium* spp. in animals are not treated because of the risk of human infections. However, animal models of the diseases could be useful to extrapolate experimental treatments to human beings. *Mycobacterium avium* is usually associated with chronic pulmonary disease and exhibits an intrinsic resistance to conventional chemotherapeutic agents. Various chemotherapeutic regimens have been studied in animal models, but most drugs show little activity [18]. Therapy with aminoglycosides (amikacin, capreomycin) may be effective, but only at high doses and for prolonged periods of time [18, 56]. Unfortunately, nephrotoxicity is likely to occur under such therapeutic regimen. Liposomal entrapment of amikacin and capreomycin has resulted in long half-lives for both drugs, enhancing their distribution and resulting in higher active concentrations in the spleen, liver and lungs compared to the treatment with the free form of the same drugs. Thus, smaller and fewer doses were used to significantly reduce the number of bacteria in those tissues. However, complete elimination of the infection did not occur. Nephrotoxicity evaluated by blood urea nitrogen levels was not observed using these liposomal formulations [18, 56].

As noted above, none of the experimental treatments with liposomal formu-
lations have been able to completely eliminate the infections. This could be explained by the fact that some bacteria (e.g. *Staphylococcus aureus*) are able to escape destruction within cells and, in addition, they can remain in a non-replicative state being insensitive to the action of the antibiotics. Another factor is the stability of the drug in the intracellular milieu where the enzymes and pH present can inactivate it. In conclusion, liposomal encapsulation did provide a therapeutic benefit. It reduced the bacterial load which may reduce morbidity and is therefore a worthwhile goal [18]. This partial success might be explained by the fact that it is not clear whether targeting a particular organ is sufficient to account for the improved efficacy or whether specific cellular and subcellular targeting (which has been shown in the case of liposomal meglumine antimoniate in the treatment of *Leishmania donovani* infection) is necessary for these diseases. Another conclusion that can be drawn from these reports is the capacity of liposomes to provide sustained drug delivery. Streptomycin had active concentrations in the serum for up to 72 h after IV or IMI administration [73]. Active levels in the milk for gentamicin and streptomycin have been detected for up to 72 h, which represents nine and six milkings, respectively [64, 73]. Also, an IM injection of liposome-encapsulated enrofloxacin, a fluoroquinolone, provided a sustained drug release and prolonged therapeutic concentrations [14]. It is not always desirable to maintain constant or prolonged levels of drug for therapeutic efficacy, and controversy exists over the benefits of sustained release delivery systems for anti-microbial therapy. However, in some applications in veterinary medicine, sustained release preparations may be the only feasible approach to antimicrobial therapy [101].

### 7.2. Fungal diseases

Despite the advent of the newer, less-toxic azole antifungal drugs, amphotericin B (AmB) remains the preferred drug for the treatment of most serious systemic fungal infections. This is, in part, due to the increase in fungal diseases world-wide [1, 39, 41, 45]. Among these diseases are candidiasis, aspergillosis, cryptococcosis and histoplasmosis. Candidiasis is a common complication in foals with Gram-negative infections which receive treatment with a number of antibiotics as well as parenteral nutritional support. Both of these treatments have been identified as factors that increase the risk of systemic candidiasis. In addition, many of these foals present neonatal maladjustment syndrome that increases the chances of a systemic candidiasis [88]. Canine blastomycosis is another fungal disease that can result in a systemic infection for which AmB is the drug of choice [12, 52]. However, in both cases, because of its toxic effects, clinicians prefer the use of other antifungal drugs such as ketoconazole, itraconazole or fluconazole, alone or in combination with AmB in order to reduce its dose, hence, reducing possibilities of nephrotoxicity [12, 52, 84, 88]. Thus, the toxicity of AmB, either acute or chronic, has limited its clinical use [45, 84, 101]. It has been established that liposomal AmB (LAmB) is more effective and less toxic than conventional preparation for the treatment of critically ill patients [39, 101]. Most of the increase in therapeutic effect is thought to be the result of a decrease in toxicity, which allows larger dosages of AmB to be used safely [10, 43, 45, 104]. The safer use and the higher efficacy of LAmb were demonstrated in different animal models [10, 36, 45, 53, 57] and human beings [13], Gondal et al. [35] and Hospenthal et al. [45] demonstrated that LAmb could be used at high doses administered at rapid infusion rates without significant side-effects. This is espe-
cially important in those cases where high activity levels have to be reached rapidly in the plasma. Krawiec et al. [52] used high doses of LAmB to treat dogs with blastomycosis with different degrees of severity (localized and disseminated) achieving successful outcomes and none of the patients developed renal failure. Fungal pneumonias are a particular case of fungal diseases that are difficult to treat because it is difficult to achieve and maintain high levels of the drug when it is parenterally administered. LAmB was proven to be more efficient in reducing dissemination of pulmonary aspergillosis from its localization to other areas of the lungs and to the liver and spleen [57], and therefore, its lethality in human beings [13]. The administration of LAmB by aerosols appears to be a more effective alternative in localized fungal pneumonias [53]. After the inhalation of aerosolized LAmB, AmB was not detected in the serum and other organs such as liver, kidneys and brain. This indicates that the toxic side-effects in other tissues are minimal. In addition, LAmB showed prolonged phases of elimination (half-lives) from the lungs depending on the surface charge of the liposome. Negatively charged liposomes had the shortest half-life (4.5 days), while positively charged and neutral liposomes had longer half-lives (15 and 22 days, respectively) [53]. These results may suggest a potential for a long-term protection.

7.3. Viral diseases

Although viral diseases in man and animals are major cause of morbidity, mortality and economical loss, early hopes for an antiviral panacea have not been fulfilled. The lack of success in the treatment of viral diseases has been due in part to the fact that viral replication is intimately associated with the host cell biosynthetic machinery. The major thrust of current antiviral research is directed toward the development of drugs that selectively block virus replication without causing toxic effects to the host cell. Amantadine and acyclovir are good examples of this. However, these drugs must be delivered to the sites of virus replication in order to be effective. It has been demonstrated that some groups of viruses replicate within macrophages (e.g. canine distemper, equine infectious anaemia, caprine arthritis encephalitis, Rift Valley fever) [50]. In addition, the major antiviral drug-related toxicity occurs outside phagocytic cells. Hence, it is reasonable to expect that liposomes could increase drug efficacy without inducing toxicity, delivering antiviral drugs where they have to act without inducing any side-effects [48]. Even though there is no liposomal formulation of antiviral drugs available on the market [39], liposomally-entrapped ribavirin [48] and 2',3'-dideoxycytidine [65] have been proven to be promising formulations for the treatment of viral diseases.

Antisense oligonucleotides are an alternative to antiviral drugs. They are short chains of complementary deoxyribonucleotides to the region of the initiation codon of the viral RNA. They bind to the RNA and inhibit the translation of viral proteins and, hence, inhibit viral replication. These oligomers cannot be administered in their free form because they are degraded by lysosomes [89]. Liposomes are a suitable method for the transfection of the oligomers to infected cells. pH-sensitive liposomes have shown a higher activity compared with non-pH-sensitive liposomes. This could be explained because the former makes it possible to avoid the degradation of the oligomers by lysosome enzymes [89], resulting in an increased intracellular stability and higher efficiency [91]. In contrast to the lack of liposomal antiviral compounds, there are liposomal vaccines which are licensed for veterinary use (Newcastle disease virus; avian
rheovirus) and which are in different experimental phases of their evaluation for human use (Influenza, Hepatitis A and B viruses) [39].

7.4. Parasitic diseases

The development of a novel anthelmintic molecule from its discovery to its sale on the market generally requires a large investment and many years of research and development. Even though these two factors could be hampered, it is unlikely that an ideal anthelmintic molecule with a broad spectrum and an acceptable activity against all classes of parasites such as nematodes, trematodes, cestodes and protozoa could be discovered or developed. Some alternative strategies have been developed. Novel delivery systems and administration of drug combinations have been evaluated. While the latter strategy may have limitations based on possible pharmacodynamic and pharmacokinetic interactions between drugs, delivery systems can improve certain pharmacokinetic features (e.g. absorption, distribution) of the currently available compounds making them more effective.

Liposomes represent a great advance in the treatment of those parasitic diseases which have their etiologic agent located within the MPS cells. Unfortunately, this cannot be said for parasites sequestered in other tissues such as skin and muscle (toxoplasmosis, Chagas' disease, trichinosis), thus effectively excluding liposomes for use in treating these other parasitic diseases [9, 17]. Although liposomal formulations result in an improved efficacy of the antiparasitic drugs, this is due to increased drug half-lives and a modified pattern of distribution [17]. Another advantage of liposomal drug formulations is the reduction of the toxic effects, which is an important factor when these drugs have a narrow therapeutic index (e.g. pentavalent antimonials, amphotericin B) [17, 32, 80, 112].

The cutaneous and visceral forms of leishmaniasis may be the parasitic diseases for which the use of liposomes has been most studied [9, 17, 31, 32, 80, 87, 101, 112]. Many compounds have been examined, such as pentavalent antimonial drugs and pentamidine [9, 17, 27]. However, most of the studies have been carried out using AmB. As mentioned above, the high toxicity of these compounds and the occasional failures in the treatment have made drug carriers that deliver anti-leishmania agents to the MPS an alternative to conventional therapy [27]. Currently, no drug carrier is available for antimonials and pentamidine has been linked experimentally to methacrylate polymer nanoparticles. Thus, LAmB is the only delivery system available which has already been used in human therapy [27]. Even though in vitro free AmB is 3 to 6 times more active than LAmB, in vivo free AmB has to be used at non-toxic doses which are not effective. In contrast, LAmB can be used at higher doses which have the same activity as free AmB but are without the side-effects and the drug is also distributed to the MPS (mainly the liver and spleen) [80, 112]. In addition, the use of LAmB reduces the course of treatment [21] and has efficacy in patients with antimony-unresponsive leishmaniasis [99].

Despite these successes, Oliva et al. [75] were unable to achieve a parasitological cure in dogs treated with LAmB, but the viscero-cutaneous signs of the disease showed improvement with regression of the splenomegaly and lymphadenomegaly and the healing of the skin lesions. These researchers concluded that the failure to eradicate the parasite may be due to inadequate drug delivery to parasitized cells, or to immune depres-
sion that is characteristic in canine leishmaniasis, or to both [75].

Drugs for the treatment of bovine trypanosomosis such as diminazene, homidium and isometamidium were evaluated as MLV-formulations for their prophylactic activity in cattle in the field. While a small increase in prophylactic activity was observed with liposomal formulations of diminazene and homidium [81, 114], a similar effect was not apparent with isometamidium [81]. Nevertheless, these formulations did reduce the local toxicity of these drugs. Further evaluation of liposomal formulations appears to have been hampered by problems with the standardization of the formulations [81]. Another example of improved efficacy for a liposomal formulation is primaquine for the treatment of malaria [17].

Despite the fact that helminths are not intracellular parasites, liposomal drugs have been studied against them, especially for their stages in the liver. Praziquantel (PZQ) is used in the therapy against liver flukes such as Schistosoma spp. and Opisthorchis spp. The liposomal formulation of PZQ (LPZQ) aimed to prevent its uptake by non-diseased tissue, reduce its metabolism, facilitate its absorption by parasite and extend parasite exposure to the drug [2, 17]. Thus, an increased efficacy in the treatment of opisthorchiasis [93, 100] and schistosomiasis [2, 17] was demonstrated. The persistence of PZQ concentrations in the liver after the administration of LPZQ compared with free PZQ (10 days and 1 h, respectively) led Akbarieh et al. [2] to suggest the use of LPZQ as a chemoprophylactic treatment of schistosomiasis.

Albendazole (ABZ) is a well-known anthelmintic used in veterinary medicine for the treatment of helminth parasites. It also has efficacy in human hydatidosis (Echinococcus spp.) and fascioliasis (Fasciola spp.). As in the case of PZQ, the incorporation of ABZ into liposomes increased its efficacy in fascioliasis [113] and in cystic hydatidosis located in the liver [71, 110]. Liposomal mebendazole has also shown increased activity and reduced side effects in the treatment of echinococcosis [17].

It should be pointed out that the liposomal formulations were administered orally in monogastric animals [17, 71] and i.p. in ruminants [113]. The possible effects of the rumen environment should be taken into account if the liposomal formulations are going to be administered orally in ruminants. Such preparations have potential in both human and veterinary medicine.

7.5. Cancer therapy

Liposomal formulations containing cytostatic drugs have been developed to reduce side-effects and improve selective access to diseased areas in the body. Liposomal formulations prepared with anthracyclines daunorubicin and doxorubicin, tretinoin and annamycin have been assayed. Anthracycline antibiotics, for example, are widely used in the treatment of a variety of cancers. However, as with other cytostatics, their usefulness is limited by severe side-effects, including cumulative, non-reversible cardiac damage. Pre-clinical studies in animals and clinical work have revealed that when liposomal preparations are used for anthracycline compounds, there is a reduction in drug uptake by heart tissue and, therefore, a reduction in associated cardiotoxicity [20, 49]. The cholesterol-rich lipid composition of the anthracycline products assures minimal leakage of the drug from the circulating liposomes, and could explain the reduced toxicity [29, 39, 44]. Decreased rate and extent of uptake into the MPS diminish the chance of adverse effects on this important part.
of the host's defence system [3, 19, 20]. The combination of an adequate size, bilayer stability and/or a hydrophobic surface, as polymer-coated formulations, results in extended circulation times for vesicles, effective access to tumours and therapeutic efficacy [39]. The enhanced distribution also applies to an improved blood–brain barrier translocation of drugs [47, 66]. The location of tumours in the central nervous system (CNS) is a reason behind chemotherapy failure. Control of the passage through that barrier is critical to the treatment of CNS tumours [47]. Effectiveness against drug-resistant cancer cells appears to be other advantage of liposomal formulations, even using lower doses [96]. Currently, there are some liposomal formulations of anti-cancer drugs already approved (daunorubicin in the UK and Sweden; daunorubicin and doxorubicin declared approvable by the Food and Drug Administration, USA) or under study for approval (annamycin, tretinoin, vincristine, doxorubicin) for human use in different cancer types [39] (table 1).

7.6. Vaccines and immunomodulation

New-generation vaccines that are based on recombinantly made subunit and synthetic-peptide antigens are usually non-immunogenic, and the need for immunopotentiation is well recognized. Although many structurally unrelated agents (immunological adjuvants) are capable of inducing immune responses to vaccine antigens, most of them are toxic. Liposomes may be useful for this purpose. The way in which they induce immune responses to antigens is not clear, but has been attributed to a depot (slow release of antigen) mechanism, and to the ability of vesicles and antigen contents to migrate to regional lymph nodes following local injection (or, when given orally, to be endocytosed by M cells, which then direct the antigen to the lymph cells in the Peyer patches) [26, 111]. The enzyme glutathione S-transferase from Schistosoma mansoni has been shown to be the antigen that can induce a protective immune response against this parasite in both mice and humans. Ivanoff et al. [46], using an animal model demonstrated that incorporating this antigen into liposomes and administering it orally can be used for the immunization against schistosomiasis. After its administration, the liposome-entrapped parasite antigen is presented to the gut-associated lymphoid tissue. This results in both a mucosal and systemic immune response, which is demonstrated by the detection of specific IgA in the gut and specific IgG in serum. Thus, liposomal formulations may be a possible mucosal vaccination against schistosomiasis [46]. In the case of liposomes, the adjuvanticity differs from that of aluminium hydroxide [6, 69]. Liposomes induce IgG but not IgE antibody production, which indicates that they have potential for the development of vaccines with a lower allergic reaction and also for the application of immunotherapy [69]. There are some liposomal vaccines that are only licensed for use in veterinary medicine (Newcastle disease virus and avian rheovirus), and some human vaccines are on their way to being approved (Hepatitis A and B, influenza, tetanus, diphtheria) [39].

Immunomodulation with compounds such as lymphokines and muramyl dipeptide and its analogues can activate the host's defence. They can be toxic, however, if not used as a liposomal formulation [30, 34]. Liposome-entrapped muramyl tripeptide can increase the cytotoxicity of the mononuclear phagocyte system against tumours either alone [63] or used with interferon-gamma [30, 34]. This can be synergistic to traditional chemotherapy [30, 34].
8. CONCLUSION

The development of liposomes as drug- and vaccine-delivery systems appears promising. Clearly, the work thus far is only a starting point and there are important challenges to overcome. The prolonged presence of liposomes in the circulatory system, which is essential for most uses involving targets other than the MPS, has only been achieved for small vesicles. This must be extended to include larger vesicles if, for example, haemosomes are going to be used as a blood surrogate [39].

There is no doubt that liposomes will have an outstanding impact both on cancer and anti-infective therapies. In veterinary medicine, where economic aspects must be taken into account, particularly in food production animals, the main area affected will be the prevention of diseases either with liposomal vaccines or prophylactic liposomal drugs. Treatment of diseases of both small and large animals will have therapeutic benefits. However, these therapeutic benefits are mainly due to the altered kinetics of drugs with long half-lives and thus, prolonged periods where drug residues are present in the edible tissues (liver, kidneys, fat). This is of great concern as a hazard for public health. Ultimately, the cost of liposomal formulations has to be evaluated and compared with the improved effectiveness of therapy, especially when treating food animals.

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