APRIL 1982
VOLUME 71 NUMBER 4

JOURNAL OF PHARMACEUTICAL SCIENCES

LITERATURE SURVEY

Experimental Methods in Cancer Therapeutics

KENNETH J. WIDDER *¶x, **ANDREW E. SENYEI** ‡¶, and **BARRY SEARS** §

Received from the *Department of Pathology, Duke University Medical Center, Durham, N.C.*, the ‡*Department of Obstetrics and Gynecology, University of California at Irvine, Orange, Calif.*, and the §*National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Mass.* ¶Present address: Kendrew Biosystems, Inc., San Diego, Calif.

Keyphrases □ Liposomes—as drug carriers, cancer therapeutics, literature survey □ Albumin—magnetically responsive microspheres, cancer therapeutics, literature survey □ Cancer therapeutics—experimental methods, literature survey

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During the last 10 years, the search for methods of selectively targeting antineoplastic agents has increased significantly. Because of the lack of tumor specificity of systemically administered antitumor drugs, toxic side effects are common, often resulting in greater harm to the host tissue than to the neoplastic process. Currently, the therapeutic index of most antineoplastic agents used in systemic therapy remains marginal, despite efforts to achieve tumor specificity. Despite various chemical modifications of chemotherapeutic agents (e.g., altering their partition coefficient, attaching immunologic ligands, or altering charge density), only variable success has been achieved in increasing specificity. An alternative approach for increasing the effectiveness of antitumor drugs is to alter their distribution in the body by incorporating them into particulate carriers, which theoretically can be concentrated at the tumor site. In this way, the toxicity of the drug for the tumor might be enhanced while the systemic toxicity is minimized.

Two major approaches for achieving this goal are discussed in this article. The first consists of new types of liposomes which impart an element of selectivity to desired target sites; the second consists of magnetically responsive drug-carrying microspheres which are capable of being targeted to tissue sites by use of an external magnetic field. A detailed comparison of all the various methods of drug delivery has been reviewed elsewhere (1).

BACKGROUND

The goal of any drug delivery system involves the altering of the pharmacokinetics and physiological disposition of the drug in question in order to obtain a higher therapeutic index. This can be accomplished either by decreasing the toxicity of the drug or by increasing its efficacy. In treating neoplastic disease, these concentrations become essential. Since the biochemical differences between normal and transformed cells are minimal and are difficult to exploit (2), the chemical synthesis of more efficacious drugs has not been entirely fruitful. Cancer therapy currently involves the administration of toxic drugs with a relatively small safety margin. Any drug delivery system that could significantly increase the therapeutic index of an antitumor drug would have a major impact in cancer treatment.

Many approaches have been tried in attempts to design a "magic bullet" for cancer. Implanted drug reservoirs give a more uniform dosage rate of the entrapped drug. The pharmacokinetics of the drug and its eventual disposition, however, remain identical to those of the free drug administered *via* normal methods. Covalently attaching antitumor agents to antibodies specific for the tumor has been suggested as a method of achieving tumor specificity. The results so far are only suggestive of potential applicability (3, 4). This approach is limited further by the extent that the attachment of the drug to the antibody de-

0022-3549/ 82/ 0400-0379$01.00/ 0
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creases the immunological specificity of the antibody. The most encouraging prospect of increasing the therapeutic index of antitumor drugs is to encapsulate them inside macromolecular carriers that can circulate within the body. In theory, macromolecular carriers can be localized at the tumor site with high specificity, thereby increasing the therapeutic index of the encapsulated drug. Two such macromolecular carriers have been exploited recently in the targeting of drugs. The first, the liposome, is an assembly of phospholipids held together by noncovalent forces. The other carrier is the magnetically responsive albumin microsphere which can be targeted to tissue sites *via* external magnetic fields. We will critically evaluate the current experimental data for both of these systems and then speculate on their future clinical use in treating cancer.

LIPOSOMES

Liposomes form spontaneously when dry phospholipids are hydrated with an aqueous solution. These structures, which are held together by noncovalent forces, consist of concentric bilayers of phospholipids that are separated by an aqueous space (5, 6). Within this aqueous space, water soluble compounds such as antitumor drugs can be stored. Drug-containing lipsosomes can be separated from nonsequestered drug by centrifugation of the liposomes or by gel filtration. However, all phospholipids do not form liposomes; in fact only very few naturally occurring phospholipids form liposomes that are stable under physiological conditions. Phosphatidylcholine, the most abundant natural phospholipid, readily forms stable liposomes, whereas phosphatidylethanolamine, the second most abundant natural phospholipid, does not. Furthermore, phospholipid liposomes only form if they are hydrated above a temperature that corresponds to the physical transition of the phospholipid from a crystal-like (*i.e.*, gel) state to a liquid crystalline state. This temperature, which is dependent on the fatty acid composition of the phospholipid, is known as the transition temperature. Liposomes formed under these conditions are multilamellar bilayers that range from 1 to 5 μm in diameter. Upon sonication, these multilamellar bilayers are reduced in size to 0.02 μm in diameter. Other size ranges between these two extremes are possible *via* polycarbonate filter extrusion techniques (5).

The potential use of liposomes as drug delivery systems for antineoplastic agents has been summarized in many articles (6–10). This review will attempt to update and critically evaluate recent experimental results using liposomes in treating neoplastic disease and to anticipate potential new approaches in this area that may be useful in the future.

Physiological Fate of Liposomes—It is now appreciated that the simple encapsulation of an antitumor drug in unsonicated dispersions of phosphatidylcholine is a relatively naive approach to greater *in vivo* drug specificity. This is partly due to the complexity of the interaction of both the plasma and the reticuloendothelial system with liposomes. In the former case, the drug is released from the liposome so that its fate becomes that of the free drug. In the latter, the rapid removal of liposomes and their associated antitumor drug by fixed macrophages of the liver and spleen limits the number of liposomes capable of reaching a target site.

In the rush to demonstrate the ability of liposomes to treat neoplastic disease, few controlled studies have been conducted into the physiological fate of systemically injected liposomes. Such studies would require having liposomes of defined size and composition. Without this definition, relatively simple experiments such as *in vitro* exchange of cholesterol between sonicated phosphatidylcholine vesicles, can be misinterpreted (11). Rigorous studies on the physiological fate of liposomes require the use of radioactive phospholipids rather than easily obtained commercial radioactive markers such as cholesterol or cholesteryl esters. Such markers exchange readily with blood components (12) or are metabolized in the plasma to components that can exchange easily (13) resulting in an erroneous interpretation of carrier distribution. It is also essential to determine whether actual mass transfer of the phospholipid from the liposome is observed or simply the exchange of radioactive phospholipid equivalents with other blood components. Finally, the structural integrity of the liposome and the associated antitumor drug in the systemic circulation should be verified. Due to the complexity of such experiments, few studies exist that fulfill these criteria. The most complete work is that of Tall (14) in which all of these experimental parameters are examined. This particular study is important in that it gives insight into the molecular modification of liposomes by the lipoprotein apoproteins.

A wide variety of lipoproteins circulate in the plasma. Lipoprotein particles are responsible for transporting highly insoluble lipids such as triglycerides and cholesteryl esters to their site of utilization. The protein moieties of the lipoproteins, known as apoproteins, appear to play a role in the structural integrity of the lipoprotein particle as well as functioning as a recognition signal for various receptors in the body.

Apoproteins, especially apo A, can leave their respective lipoprotein particles and associate with phospholipid liposomes. With apo A, this association can eventually lead to the disruption of liposomal integrity (15–17) and the release of the associated water soluble antitumor agent. It has become clear that the inclusion of cholesterol in the liposome bilayer minimizes the interaction of apo A with phospholipid liposomes *in vitro* (18). However, the inclusion of cholesterol may not significantly alter the physiological fate of most of the liposomes *in vivo* (14).

The effect of another apoprotein, apo E, on phospholipid liposomes is often unappreciated. Apo E is associated with the uptake of chylomicron and very low density lipoprotein (VLDL) remnants in the liver (19, 20). Its association with phospholipids either as liposomes (21) or as phospholipid–triglyceride emulsions (22) is well documented. One hypothesis is that apo E may have a higher affinity for cholesterol-rich liposomes than for cholesterol-poor liposomes. If so, the cholesterol rich liposomes *in vivo* would be taken up by the hepatic receptor that recognizes apo E. Tall indicates that this may be the case (14). Therefore, the physiological fate of most cholesterol rich liposomes *in vivo* may be the liver, even though the fractional amount of the liposomes that remains in the plasma may appear to be structurally intact (18).

It is also well documented that the inclusion of choles-

terol in a liposome will reduce the rate of leakage of entrapped drugs. The reduction in the rate of loss of an antitumor agent from such cholesterol rich liposomes can be beneficial to the efficacy of cell cycle sensitive drugs such as cytarabine (23–25). Cytarabine entrapped within liposomes is sequestered from the plasma and is not subject to rapid metabolism; hence, the liposome acts as a long-lived depot for the drug. For other noncell cycle-dependent drugs, however, large increases in the efficacy of encapsulated drug regardless of the composition of the liposome, has not been observed (26), probably because most of the drug is removed to distant sites before the tumor can be reached. Thus, the inclusion of cholesterol in the liposome only decreases the permeability of the drug from a liposome and does not appear to significantly alter liposome tissue distribution.

The unresolved basic problem is how to gain greater target specificity for the drug. The most exploited way is to reduce the size of the liposome to that of a small unilamellar vesicle with a diameter of ~200 Å. This size reduction increases the plasma lifetime of the liposome compared to unsonicated dispersions (27), thereby increasing the possibility that greater tumor localization can be achieved compared to larger liposomes. The reduction in size of the liposome, however, also decreases the amount of interior aqueous space, thereby limiting the amount of water soluble antitumor drug that can be encapsulated. Moreover, the *in vitro* stability of small unilamellar vesicles is less than that of larger liposomes, either as large unilamellar vesicles or as unsonicated dispersions. The reduction in the size of the liposome while increasing plasma circulation time does not appear to alter extensively the physiological fate of the liposome (*i.e.*, localization primarily in the liver and the spleen).

Incorporation of Glycolipids—One new approach to alter the tissue distribution of liposomes is the inclusion of natural and synthetic glycolipids into the liposomes. Taking advantage of the asialo galactose receptor in the liver, vesicles containing an exposed galactose moiety accumulated to an even greater extent in the liver than liposomes without such glycolipids (28). On the other hand, the presence of a sialic acid group on the glycolipid slightly retards the accumulation of the such liposomes in the liver (29). Using a variety of synthetic glycolipids, such as amino mannose derivatives, Baldeschwieler and coworkers have shown that enhancement of vesicle stability can occur along with a highly altered tissue distribution, especially to the lung (30, 31). More recent studies have indicated that the same amino mannose derivatives enhance the uptake of liposomes by macrophages (32). Whether or not such altered stability and different tissue location have any beneficial effects for cancer chemotherapy remains to be tested. These results indicate that suitable modifications of the liposomal surface can dramatically alter *in vivo* characteristics.

Incorporation of Immunoglobulins—Another new methodology that has received much attention is the use of immunoglobulins to direct liposomes and the associated antitumor drug to a specific site. In theory, suitable tumor-specific antibodies can be bound to the liposomal surface and then can impart the necessary tissue specificity so that targeting can be achieved. An early study indicated that specificity could be achieved *in vitro* (33). Although internalization of the liposome and its associated antitumor drug was demonstrated, the studies were done with highly phagocytic cells. As attractive as this potential may be, there are factors that must be critically assessed. First, as shown by Weinstein and coworkers, the increased binding of a liposome containing the appropriate hapten to a cell, mediated by the $F(ab)_2$ fragment of an IgG specific antibody, does not ensure internalization of the liposome and its associated antitumor drug into nonphagocytic cells (34). Even the localization of liposomes containing methotrexate near tumor cells *in vitro* does not enhance the cytotoxic behavior of methotrexate (34). Recent studies have indicated that sonicated vesicles containing methotrexate and a surface-oriented hapten can be internalized into highly phagocytic cells having an F_c receptor by prior reaction of the lysosomes with antihapten IgG antibody (35). However, the therapeutic uses of this technique may be limited to highly phagocytic cells. Moreover, many normal cells also have F_c receptors that appear to be needed for carrier internalization (35).

Other workers have shown that IgG or $F(ab)_2$ fragments can be covalently linked to liposomes *via* oxidized glycolipids present in the bilayer of the liposome (36). Using $F(ab)_2$ fragments specific for red cells, a high degree of binding to red cells has been observed (37). Likewise, monoclonal antibodies can be rendered hydrophobic and linked to the liposome with subsequent *in vitro* specificity (38) or can be covalently linked *via* phosphatidylethanolamine in the liposome (39). Nonetheless, the mere increased binding to the target cell does not ensure greater efficacy of the associated antitumor drug, even *in vitro* (34). Therapeutic uses of antibody-directed liposomes must also be considered based on work demonstrating the physiological fate of purified ^{131}I-labeled anticarcinoembryonic antigen administered to patients with colorectal carcinomas. It has been shown that although diagnostic specificity (*i.e.*, higher radioactivity in tumor tissue compared to normal adjacent tissue) is observed, only 0.1% of the injected antibody localizes in the resected tumor (40). Such low absolute specificity raises a question for the potential use of antibodies as a way of targeting liposomes and their associated antitumor drugs to a tumor, since the carcinoembryonic antigen is one of the most characterized tumor markers. Also, since solid tumors are known to shed their antigens into the plasma, much of the liposome–antibody complex would most likely bind with circulating antigens, severely limiting the number of liposomes capable of binding to antigens on the tumor surface (40). Finally, there remains the problem of whether the liposome–antibody complex can leave the plasma compartment and enter into the extravascular compartment. If the liposome complex is being simply removed from the circulation by the fixed macrophages of the liver and spleen, then there is little liklihood that the liposome will encounter the bulk of the tumor. On the other hand, if the liposome–antibody complex can leave the vascular system at the tumor site, or enter the lymphatic circulation, then the possibility exists that the complex can interact with the tumor. The problem of the cellular uptake of the liposome and its associated antitumor drug *in vivo* remains the same as discussed for the *in vitro* studies.

Use of Biological Factors—A more novel approach to the problem of tissue specificity is the use of biological

factors to stimulate the body's immune system to attack tumor sites. The use of interferon as an immuno-modulating agent has received much attention, but its use has failed to produce uniformly positive results (41). Attempts have been made to restrict biological response modifiers selectively at the site of tumors. One example of this approach is the encapsulation of lymphokines within phospholipid liposomes (42). Liposomes, composed of phosphatidylcholine and phosphatidylserine, were shown to localize in the lung after intravenous injection. Such localization allowed the alveolar macrophages to phagocytose the liposomes and their associated macrophage activating factor. Thus, by activating the alveolar macrophages, metastases, which have migrated to the lung from a primary tumor, can be attacked with high efficiency, but only if the primary tumor had been excised from the animal (43). Further experiments indicated that macrophage activating factor can be replaced by the synthetic immuno-potentiating compound muramyl dipeptide with similar results (44). This approach indicates that if one can localize biological response modifiers such as macrophage activating factor and muramyl dipeptide to macrophages in the lung, substantial efficacy can be observed under certain conditions (*i.e.*, removal of the primary tumor). In a similar approach, immune RNA has been encapsulated in liposomes coated with antilymphocyte antibody studies. The phagocytosis of these liposomes by lymphocytes *in vitro* resulted in enhanced cytotoxicity to tumor cells (45). The *in vivo* utility of a system using antibodies to target liposomes to lymphocytes to enhance chemotherapy, has the same limitations as outlined previously.

Thus far, most attempts at targeting liposomes to selected tissues *in vivo* has relied on size, glycolipids, or antibodies. Even if successful, unless the tumor cells in the target tissue are highly endocytic, the contents of the liposome are unlikely to enter the target cell unless they leak from the liposome. That is to say, the free drug must still enter the target cell. Another novel approach is the localization of the free antitumor drug at a desired site by using the physical parameters of the liposome phospholipid composition to increase the permeability of the drug.

Use of Transition Temperature—It has been shown that at the transition temperature, when the phospholipids of a liposome undergo a physical transition from the gel to liquid crystalline state, there is a dramatic increase in the permeability of the liposomal membrane (46). An encapsulated drug will leak out more rapidly at this transition temperature than at temperatures slightly higher or lower. Liposomes with entrapped methotrexate have been designed to have a transition temperature near 42° (47). Localized heating of the target tissue can raise the internal tissue temperature high enough to reach 42°, causing a large efflux of the encapsulated methotrexate from the liposome as the carrier enters the heated tissue. As the liposome is removed from the target site *via* the circulatory system, the permeability of the drug from the liposomes decreases rapidly due to the decrease in local temperature. Recent experimental results indicated that high density lipoproteins are necessary to destabilize the liposomes at the phase transition temperature (48). Theoretically this process can be repeated during every circulatory pass of the liposome thereby delivering the drug at high localized concentrations at the target site. A variation of this approach is to incorporate *N*-palmitoyl homocysteine into liposomes. By adjusting the composition of the liposome, one can increase the permeability of an encapsulated drug with decreasing external pH (49). This approach assumes that the pH near the tumor site will be sufficiently lower than that of normal tissue, hence giving a selective release of the drug at that site. Such a theoretical concept remains to be demonstrated *in vivo*. In both approaches, this type of targeting is a transient phenomenon, and while it does not localize the drug in high concentrations at the desired site, it does give greater concentration of the free drug at the target site than can be achieved with conventional administration. With the heat sensitive liposomes, some therapeutic benefit has been observed (47).

Current experimental evidence indicates there is much to accomplish in order to demonstrate the necessary increased efficacy of antitumor agents in liposomes before contemplating potential clinical use. Nonetheless, there are a number of theoretical factors that should be part of an ideal lipid-based drug delivery system. It must be stable *in vivo*; *i.e.*, it should be resistant to the interaction of apoproteins of the plasma lipoproteins on the vesicle surface, resulting in structural destabilization or hepatic localization (especially *via* apo E). The carrier should have a small size (less than 300 Å) in order to penetrate into the extravascular space or lymphatic circulation, thereby giving the drug and carrier maximum opportunity to interact with tumor cells. Furthermore, this small size will enhance the possibility of endocytosis of the lipid-based carrier in a similar fashion to low-density lipoproteins, especially by cells that are not highly phagocytic (50). Finally, the carrier should be able to be localized with high specificity to any desired part of the organism *in vivo*. It is unlikely that immunological means will be able to accomplish this final goal.

MAGNETICALLY RESPONSIVE ALBUMIN MICROSPHERES

By using magnetically responsive albumin microspheres as a drug carrier, an entirely different approach to drug targeting can be considered (51). Microspheres composed of a denatured human serum albumin matrix serve as the vehicle in which a chemotherapeutic agent and ultrafine particles of magnetite (Fe_3O_4), are entrapped. Because of the magnetic material within the microspheres, they are susceptible to the effects of a magnetic field.

Albumin microspheres alone have some of the major problems discussed previously regarding liposomes; namely, clearance by the fixed macrophages of the liver and spleen and the lack of target site specificity. However, magnetically responsive drug-carrying microspheres infused into an artery supplying a tumor can be retained within the capillaries of tumor by an external magnet over the tumor site. Retention of the microspheres in the microvasculature can be achieved by taking advantage of the difference in the linear flow velocity of blood in a large artery (15–30 cm/sec) *versus* that in capillaries (0.05 cm/sec). Since the linear flow velocity of the microspheres is much slower within the capillaries, a much smaller magnetic field is sufficient to retain them within these vessels. By targeting the microspheres in this manner, they can be focused to a desired site with high specificity with

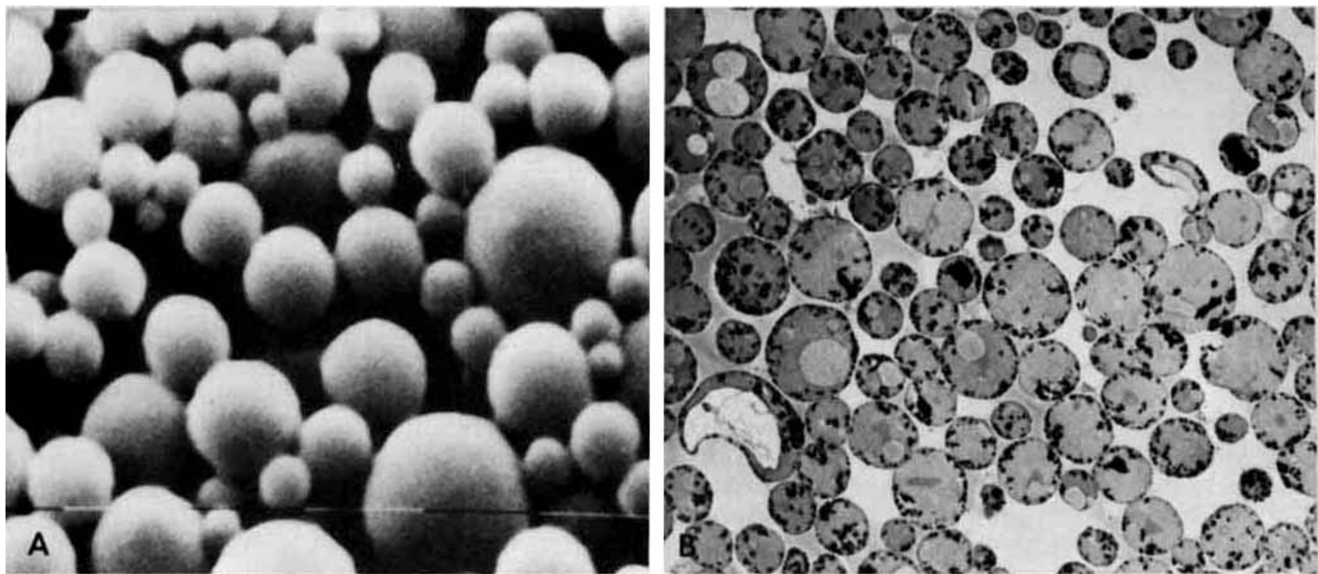

Figure 1—*Scanning electron photomicrograph (A) of microspheres showing an average diameter of ~1 μm. The largest sphere in the photomicrograph is 1.5 μm. Calibration bars represent 1 μm (×15,000). Transmission electron microscopy (B) reveals albumin microspheres containing clusters of Fe_3O_4 in a peripheral orientation (×8500). Drug is entrapped within the albumin matrix.*

subsequent decrease in clearance by mononuclear phagocytes. Restriction of the microspheres within the capillary circulation is essential for two reasons. First, drug diffusion occurs maximally in capillaries and venules; second, the microspheres from the microvasculature must transit into the extravascular space. By doing so, an extra-vascular depot would be created for sustained drug release within the target area. The drug released from the microspheres would saturate the tumor environment with a high local drug concentration. In addition, there is the possibility that the microspheres could be internalized by adjacent tumor cells as a result of increased tumor cell phagocytic activity (52, 53). By this mechanism, cytotoxic drugs released from microspheres would do so intracellularly and in high concentration resulting in cell death. Problems of drug resistance due to the inability of the drug to be transported across the cell membrane may be surmounted.

Preparation—Magnetically responsive albumin microspheres are prepared by a water-in-oil phase separation emulsion polymerization (54). Essentially, this consists of forming, in a small aqueous volume, a solution of albumin and water soluble chemotherapeutic agent (*e.g.*, adriamycin) to which an aqueous suspension of magnetite (Fe_3O_4) is added. This aqueous suspension is then added to a larger volume of oil and the mixture homogenized to generate proteinaceous spheres. These spheres are hardened either by heating the oil above 100° or by adding hydrophobic cross-linking agents to the emulsion. Microspheres are isolated by sequential washings in ether to remove residual oil and are stored as a powder at 4°.

Microspheres range in size from 0.2 to 1.35 μm in diameter with an average of 1.0 μm (Fig. 1A). By transmission electron microscopy it was determined that each albumin sphere contains clumps of Fe_3O_4 distributed largely around the periphery of the spheres (Fig. 1B).

Adriamycin was used in early studies of this approach because of its range of efficacy against a variety of tumors but was limited clinically due to its cardiotoxicity. It must be stressed, however, that virtually any water soluble chemotherapeutic agent may be successfully entrapped within the microspheres, giving them a broad potential range of utility.

Since optimal drug delivery *in vivo* necessitates predominantly capillary-level retention of the targeted microspheres, an *in vitro* system capable of simulating linear flow velocities of blood in various sized vessels was used to study the retention of microspheres using various magnetic parameters (55). Results (Fig. 2) demonstrated, at a magnetic field strength of 8000 Oe and using linear flow velocities comparable to those found in the microvasculature (*i.e.*, 0.05 cm/sec), virtually 100% of microspheres were magnetically retained, while microspheres in flow velocities comparable to those in medium sized arteries were not influenced by the magnetic field. Curve B illustrates the shift in the retention curve obtained when more magnetic material was entrapped within the microspheres. Studies on the effect matrix stabilization (chemical cross linking *versus* heating) indicated that the slow release of adriamycin could be manipulated without extensive loss

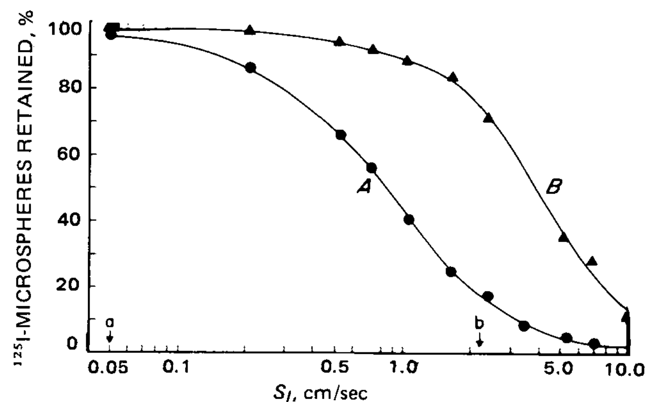

Figure 2—*Magnetic retention of heat-stabilized ^{125}I-microspheres suspended in 0.15* N *NaCl at various rates of laminar flow (S_l = linear flow velocity). Curve A represents microspheres containing 20% Fe_3O_4 (w/w); curve B represents microspheres containing 50% Fe_3O_4. Increased microsphere iron content causes the retention curve to shift to the right. Physiological rates of blood flow are indicated for capillaries (a) and medium-sized arteries (b).*

Table I—*In Vivo* Localization of ^{125}I-Labeled Magnetic Albumin

Magnetic Field Strength, Oersteds	Number of Animals	Distribution Percentages								
		Tail Segment Number				Organ				
		1	2	3	4	Liver	Spleen	Kidney	Lung	Heart
0 (control)	10	0	0	0	0	76–85	3–9	<1	6–19	<1
4000	5	0	0	0–3	0	72–85	3–7	<1	5–18	<1
6000	5	0	0–4	10–25	0	57–70	2–8	<1	6–18	<1
8000	10	0	0–3	37–65	0–2	30–48	2–6	<1	3–17	<1

of drug activity (56).

***In Vivo* Testing**—After determining optimal *in vitro* characteristics, *in vivo* testing was undertaken to assess the validity of magnetic targeting. Initially, attempts were made to target microspheres to normal cutaneous and subcutaneous tissue in the tail of Sprague-Dawley rats (51). For experimental purposes, the tail was demarcated into four approximately equal segments with segment 1 being proximal. A permanent bipolar magnet was placed adjacent to tail segment 3, which was regarded as the target site. The ventral caudal artery was exposed by a cut-down procedure at the base of the tail (segment 1) and a polyethylene catheter was inserted 1.5 cm proximal to the target site. Microspheres labeled with iodine 125 were infused at a rate that corresponded to the rate of blood flow in this artery. After 30 min with the magnet in place, rats were sacrificed and organs counted for iodine 125 gamma radiation. The tail was cut into the designated segments and each was counted individually. In separate experiments animals were sacrificed 24 hr after magnetic localization.

Results of these experiments are shown in Table I. Increasing magnetic field strength resulted in increased retention of the drug carrier selectively in tail segment 3, the target site. Using an 8000 Oe magnetic field, ~50% of the infused microspheres were selectively retained in tail segment 3. Animals sacrificed 24 hr after targeting of microspheres demonstrated a similar 50% retention of injected carrier at the target site. No necrosis or evidence of obstructed blood flow was observed in these animals prior to sacrifice. Electron microscopy performed on skin from tail segment 3 demonstrated microspheres within endothelial cells as well as lodged between adjacent endothelial cells (51). Thus, ultrastructural evidence indicated that some targeted microspheres were able to exit from the vascular compartment to set up a potential extravascular drug depot.

The amount of adriamycin at the target site was evaluated in the same rat model system (57). Experimental animals were sacrificed at various time intervals after exposure of the target site to an 8000 Oe magnetic field.

Table II—*In Vivo* Distribution of Carrier-Delivered and Free Adriamycin

Form of Drug	Dose mg/kg	Magnetic Field[a]	Tissue Concentrations, μg/gm[b] Target Tail Skin	Pooled Organs[c]
Carrier-delivered	0.05	0	<1	<1
	0.05	+	3.9	<1
Free	0.05	0	<1	<1
	5.00	0	5.5	15.0

[a] A magnetic field of 8000 Oe was placed over tail segment 3 (target area) for 5 min. [b] Limits of detection, ≤1 μg of drug/g of tissue (wet weight). [c] Organs consisted of liver, spleen, kidneys, lungs, and heart. Adriamycin was found predominantly in the liver.

Adriamycin was solubilized from tail skin and organs and assessed quantitatively by spectrophotofluorometry. In animals sacrificed 5 min after carrier infusion, 3.9 μg of adriamycin/g of tissue was localized in the target tail skin. No drug was detectable in the nontarget tail segments or in the liver. By contrast, a 100-fold higher dose of systemically administered unencapsulated adriamycin was required to obtain comparable local tissue concentrations (Table II). In similar experiments where animals were sacrificed 60 min after carrier infusion, an equivalent adriamycin concentration of 3.7 μg/g was found at the target site with no detectable levels of drug found in adjacent tail segments or in any of the visceral organs examined. In animals receiving 100 times the adriamycin dose (5 mg/kg, iv), the amount of adriamycin at the target site 60 min after administration was only 50% of that delivered by the microspheres. Thus, after 60 min, using 0.1% of the free intravenous dose, adriamycin targeted *via* magnetic microspheres yielded approximately twice the local adriamycin concentration at the preselected site with no detectable systemic distribution as compared to the intravenously administered drug.

The microspheres were next tested for efficacy in the treatment of established rat tumors (58). The ascites form of Yoshida rat sarcoma was chosen due to its known sensitivity to adriamycin and because of its aggressive biological behavior. The solid form of the tumor can be obtained by inoculating tumor cells subcutaneously. Tumor nodules appeared ~3 days after implantation. Average time of death of untreated animals was found to be 16 days post-tumor inoculation.

Prior to microsphere experiments, animals were inoculated with tumor cells in the lateral aspect of the tail and treated with adriamycin intravenously to assess tumor sensitivity to the drug. Animals received either intravenous normal saline, 0.5 mg of adriamycin/kg, or 5.0 mg of adriamycin/kg on days 1, 5, and 9 postinoculation of the tumor. Animals were assessed for tumor size, weight change, and life span.

Only animals receiving the multiple high-dose regimen (5 mg/kg) showed any evidence of tumor sensitivity to the drug as demonstrated by reduction of tumor growth. Furthermore, animals receiving the 5 mg/kg dosage regimen, had an average weight loss of 12 g, demonstrating evidence of systemic toxicity. It should be noted, however, that no difference in life span was noted between any treatment group. In summary, animals receiving multiple doses of adriamycin at 5 mg/kg demonstrated some inhibition of tumor growth; however, these animals also demonstrated evidence of systemic toxicity and no enhancement of life span was noted.

Once tumor sensitivity had been established, experiments were designed to treat the Yoshida sarcoma with adriamycin targeted *via* magnetic microspheres. Again,

Table III—Effect of Magnetic Microspheres Containing Adriamycin on Yoshida Sarcoma-Bearing Rats

	Untreated Control	Adriamycin, 5 mg/kg		Adriamycin 0.5 mg/kg, ia	Placebo Microspheres with Magnet	Microspheres Bearing 0.5 mg/kg Adriamycin	
		iv	ia			No Magnet	With Magnet
Initial tumor size, mm	36.2	19.4	26.8	25.7	29.3	27.8	28.7
Final tumor size, mm	46.4	38.4	41.9	44.5	46.9	48.5	5.0
Deaths, %	90	100	80	100	80	100	0
Regressions, %	11	0	0	0	0	0	92
Total remissions, %	0	0	0	0	0	0	75
Metastases, %	89	100	80	100	80	100	0

animals were inoculated with the tumor cells into the lateral aspect of the tail, but unlike sensitivity experiments, animals were used for experiments 6–8 days *after* tumor implantation. By the time of experimental use, tumors were not only measurable, but occasionally, showed evidence of necrosis. Regardless of the type of therapy given, animals received only single-dose treatment 6–8 days after tumor inoculation.

The experimental group consisted of 12 animals which received drug-bearing microspheres at a dose of 0.5 mg of adriamycin/kg with the tumor exposure to the magnetic field for 30 min. The method of microsphere infusion into the ventral caudal artery was identical to that described previously. The following groups of animals constituted the control groups: 10 animals received placebo microspheres (without drug) infused in the same manner with exposure of the tumor to the magnetic field; another group received drug-bearing microspheres, but no magnetic field was applied. Finally, free adriamycin was administered intra-arterially (*via* the ventral caudal artery) at 0.5 and 5 mg/kg and intravenously at 5 mg/kg. Animals were observed for weight change, tumor size, and death. All animals were sacrificed 29 days after treatment. Complete autopsies were performed and organs were examined both grossly and microscopically for evidence of tumor.

Results of single-dose therapy with magnetically responsive microspheres are presented in Table III. Tumor size increased markedly in all animals treated with free adriamycin, regardless of the administration route or the dose. Control animals receiving either no treatment, placebo microspheres, or drug-bearing microspheres without localization, also demonstrated a significant increase in tumor size. In contrast, there was a significant (92%) decrease in tumor size in animals receiving adriamycin containing microspheres (0.5 mg/kg) with the magnet placed adjacent to the tumor. The weights of all control animals increased during the course of the experiments, indicating no evidence of systemic toxicity. Animals receiving targeted therapy also showed an increase in weight.

In all groups of animals, except those receiving magnetically localized adriamycin microspheres, there was an 80–100% mortality rate and an 80–100% incidence of distant metastases during the 29 days the animals were observed. In contrast, no deaths or metastases occurred in animals treated with the single dose of magnetically targeted microspheres containing adriamycin. Moreover, 75% of the animals in this group had complete tumor remission as confirmed by microscopic examination of the tissues. An additional 17% had significant tumor regression. The remaining animal showed no change in tumor size and was alive at the end of the experimental period. In comparison, there were no remissions in any control group with the exception of a slight decrease in tumor size in one animal from the untreated control group.

In prior experiments performed to assess tumor susceptibility to adriamycin, it was found that the tumor would respond only when the therapeutic regimen consisted of multiple dose intravenous adriamycin (5 mg/kg) given on days 1, 5, and 9 after tumor inoculation. Animals thus treated showed some evidence of tumor regression. However, these animals also experienced weight loss suggestive of toxicity due to the drug. More importantly, survival was not enhanced in animals treated in this fashion compared with untreated controls. In contrast, a single dose of adriamycin, when administered in magnetically responsive microspheres and targeted to the tumor site, achieved 75% total remissions with no deaths or histologic evidence of metastases in experimental animals. These animals were treated after tumor load was evident (6–8 days after tumor inoculation) and with only a single dose of 0.5 mg of adriamycin/kg as compared to multiple-dose therapy. Evidence of tumor necrosis was histologically evident as early as 3 days after treatment.

It is important to note that none of the rats treated with targeted therapy had any histological evidence of metastatic disease. This was perhaps related to the timing of the therapy, though tumor nodules were large and rapidly growing at the time of experimental use. The metastatic potential of the tumor at the time of treatment, however, may well have been limited. It is believed that the spread of metastases begins ~7–8 days after tumor inoculation, with tumor neovascularization beginning at day 2–3[1]. Alternatively, destruction of the tumor cells by adriamycin, along with subsequent decrease in tumor load and inhibited secretion of soluble low molecular weight tumor product inhibitors (59) may have tilted the immunological balance toward an active host response.

Thus, the potential of magnetic microspheres to enhance the therapeutic index of an antineoplastic agent has been demonstrated. The increase in the therapeutic index appears to come from both decreased toxicity and increased efficacy. Nonetheless, several questions remain to be resolved before the advent of clinical use. The first and foremost involves the external magnetic field used in targeting. The magnetic microspheres are held in place by the magnetic field gradient and not just the strength of the magnetic field. Magnetic field gradients drop off dramatically from the magnet pole face. The localization of microspheres to a tumor present subcutaneously allows maximum magnetic field gradients to be imposed on the

[1] Dr. Judah Folkman, personal communication.

target site. Other target sites may be too distant to allow the retention of the magnetic microspheres to take place. The theoretical problem of focusing magnetic field gradients at sites external to the magnet face is formidable. This problem must be solved if widespread clinical use is to be expected.

The second problem is that the magnetic microsphere must be administered intra-arterially as opposed to intravenously. An intravenous administration would result in the clearance of the microspheres by mononuclear phagocytes before reaching the target site. Intra-arterial administration involves a necessary cut-down, and hence some morbidity. This problem may possibly be overcome by altering the surface characteristics of the microspheres to allow them to bypass the phagocytic cells upon intravenous administration. Results in this area would be applicable to liposomes. Finally, this carrier may be applicable only for water soluble antitumor drugs. This problem is relatively minor, since currently, most antitumor drugs are water soluble.

Nonetheless, given the above limitations, the magnetic microspheres have given the most dramatic demonstration to date of the ability to direct antitumor drugs to a target site resulting in increased efficacy of the incorporated drug.

CONCLUSIONS

Drug carriers offer significant promise for the selective delivery of toxic chemotherapeutic agents in the treatment of cancer. Liposomes have been explored to a considerable degree for use as drug carriers in the treatment of neoplasms. Though some interesting results have been obtained in this area, liposomes generally have failed to perform as a practical method for drug delivery *in vivo*. Their usefulness is restricted, mainly due to a lack of target site specificity and their rapid clearance by mononuclear phagocytes in the liver and spleen. Approaches that take advantage of specific cell receptors *via* antibodies or glycolipids show some potential for achieving specificity; however, major obstacles remain in the application of these approaches. In contrast, a magnetically responsive drug delivery system is capable of a significant degree of *in vivo* targeting as well as a controllable release of drug at the microvascular level.

It is the authors' view that magnetically responsive drug carriers, either as a lipid or protein base, may have significant clinical relevance in the treatment of neoplastic disease. Each type of magnetically responsive carrier would have certain advantages. Protein microspheres would be useful for water-soluble drugs and can serve as long-lived localized drug depots. Magnetically responsive lipid base systems would be most applicable to water insoluble antitumor drugs. They may also have a smaller size than the protein microspheres, and hence a longer circulatory lifetime. Whichever type of magnetically responsive carrier is developed, the potential for targeting *via* external magnetic fields offers a new approach to a longstanding challenge in cancer chemotherapy. Although problems remain to be solved, the experimental results to date justify optimism.

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RESEARCH ARTICLES

Properties, Stability, Assay, and Preliminary Pharmacokinetics of the Immunomodulatory 1,2-*O*-Isopropylidene-3-*O*-3′(*N*′,*N*′-dimethylamino-*n*-propyl)-D-glucofuranose Hydrochloride

EDWARD R. GARRETT ˣ, ACHIEL VAN PEER, HELMY MAHROUS, and WALDTRAUT SCHUERMANN

Received May 14, 1981, from *The Beehive, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.* Accepted for publication August 14, 1981.

Abstract □ 1,2-*O*-Isopropylidene-3-*O*-3′(*N*′,*N*′-dimethylamino-*n*-propyl)-D-glucofuranose hydrochloride (I) is a new agent with claimed immunomodulatory action and antiviral activity. Thin-layer chromatographic procedures and identifying tests were developed to separate the drug, its synthetic precursors, and solvolytic products, and were applied to stability studies. It is stable in 0.1 *N* NaOH at 60° where its acid solvolysis product, 3-*O*-3′-(*N*′,*N*′-dimethylamino-*n*-propyl)-D-glucose is readily degraded. The partition coefficient of I ($pK_a' = 9.28$) between chloroform and plasma was 6.4 ± 0.2 *SEM* between pH 10.5 and 11.0. Plasma and urine (0.5 ml) adjusted to pH 11.0 were extracted with 10 ml of chloroform and the extract evaporated. The reconstituted residue in 50 μl of benzene, with the diisopropylaminoethyl analog of I as an internal standard, was derivatized with 50 μl of heptafluorobutyric anhydride at 60° for 45 min and was evaporated and reconstituted in 100 μl of benzene to be assayed for I by GLC with electron capture detection with a sensitivity of 5 ng/0.5 ml of biological fluid. The procedure was applied to pharmacokinetics in the dog and a two-compartment body model was observed with a terminal half-life of 103–130 min. At the 40-mg dose, 60–64% was excreted renally unchanged and 20–34% as unidentified metabolities. At the 200-mg dose 82–85% was excreted renally unchanged and 15–17% as unidentified metabolites. The respective renal clearances of I were 135 and 163 ml/min. The respective total clearances of I were 204 and 191 ml/min. These metabolites were apparently unextracted with chloroform from biological fluids at pH 11 and the liquid scintillation counting (LSC) assay of extracted radiolabeled I appeared synonomous with the GLC assay of I in such fluids.

Keyphrases □ Pharmacokinetics—new immunomodulatory and antiviral agent, dogs □ GLC, electron capture—pharmacokinetics of a new immunomodulatory and antiviral agent, dogs □ Immunomodulatory agent—pharmacokinetics of a new immunomodulatory and antiviral agent, dogs

It has been shown (1–7) that 1,2-*O*-isopropylidene-3-*O*-3′-(*N*′,*N*′-dimethylamino - *n* - propyl)-D-glucofuranose hydrochloride, I, exhibits immunomodulatory action and antiviral activity and that it possesses pro host action in which cellular immune response is augmented and macrophages are activated (8, 9). The advantageous therapeutic action is to potentiate protective responses of the immune system(6) without the inhibition of vital cell actions with their concomitant toxicities (4).

Compound I is a substituted monosaccharide of low toxicity, widely different in structure than the clinically used nonsteroidal anti-inflammatory agents with high incidences of toxicity. An interesting argument for this possible activity of the 3-substituted monosaccharide (1–8) is that it mimics the immunological activity of the cell walls of mycobacteria with its positive cyclic guanosine monophosphate action and without its negative cyclic adenosine monophosphate effect on proliferation.

The procedure for the synthesis of I, is outlined in Scheme I. The diisopropylidene derivative of D-glucose, IV, is prepared by the addition of acetone. It is subsequently conjugated with *N*′,*N*′-dimethylamino propanol, II, in the 3 position. Compound I is then prepared by selective acid hydrolysis of the 5,6-*O*-isopropylidene group.

0022-3549/82/0400-0387$01.00/0
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