Published as:
Paszko, E.; Senge, M. O. (2012):
Immunoliposomes.
Current Medicinal Chemistry **19**, 5239–5277.

Immunoliposomes

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Abstract:
Since their discovery by Bangham about 50 years ago, liposomes have become promising tools in drug delivery systems. This has increased the therapeutic index of many drugs, and offers improved drug targeting and controlled release. In order to further improve the specificity of liposomes for malignant tissues, targeted liposomal formulations have been developed which represent the next step of liposomal drug delivery in medical treatment. Antibodies and antibody fragments are the most widely used targeting moieties for liposomes due to the high specificity for their target antigens. This has given rise to a new class of drug delivery vehicles, the so-called immunoliposomes. Immunoliposomes are generated by coupling of antibodies to the liposomal surface and allow for an active tissue targeting through binding to tumor cell-specific receptors. Such antibody modified liposomes are attracting great interest for their potential use in specific drug delivery to cancer cells, gene therapy, drug delivery through blood brain barrier, or molecular imaging. Thus far, immunoliposomes show promising results *in vitro* and *in vivo* and appear to be effective systems for improvements in cancer treatment. This review covers the literature of the past decade with special emphasis on *in vitro* and *in vivo* studies.

Abbreviations: Ab - antibody; AC – analyte capture zone; AML – acute myeloid leukemia; AS – ODNs – antisense oligonucleotides; BBB – blood brain barrier; BF-ELIP – bifunctional echogenic liposomes; BV – bee venom; CFU – colony forming units; CSC – cancer stem cell; DHFR – Dihydrofolate reductase; DOPE – phospholipid dioleoyl phosphatidylethanolamine; DOTAP – *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate; Dox – doxorubicin; ECL – electrochemiluminescence; EGFR – epidermal growth factor receptor; ELIP – echogenic liposomes; EPR - enhanced permeability and retention effect; FUdR – 5-fluoro-2'-deoxyuridine; GD_2 – disialoganglioside; GFP – green fluorescent protein; GTP - γ-glutamyl transpeptidase; GUSB - *β*-glucuronidase; HA – hemagglutinin; HDAC – histone deacetylase; HER2 – human epidermal growth factor receptor 2; HIR – human insulin receptor; HoKc – histidine-lysine peptide; HPR – synthetic retinoid fenretinide; HRP – horseradish peroxidase; ICAM-1 - intercellular adhesion molecule 1; ICT – immunochromatographic test; IHC – immunohistochemistry; IL– immunoliposome; ILSA – immunoliposome sandwich assay; iRNA – interference RNA; IT – immunotoxin; IVUS - intravascular ultrasound; LUV – large unilamellar vesicle; LPD – liposome-polycation-DNA complex; LVT – left ventricular thrombus; mAb – monoclonal antibody; MB – methylene blue; MGSV - mean gray scale value; MT1-MMP - type-1 matrix metalloproteinase; MTD – maximum tolerated dose; MTH – molecular Trojan Horse; 4-NC – 4-chloro-1-naphthol; NHL – Non-Hodgkin's lymphoma; ODN – oligodeoxynucleotides; PBV – peptides in bee venom; pCMV – human cytomegalovirus promoter; PD – Parkinson's Disease; PE – *Pseudomonas* exotoxin A; PEG – polyethylene glycol; pGFAP – glial fibrillary acidic protein promoter; PMMA – poly(methyl methacrylate); PS – polystyrene; RES – reticuloendothelial system; Ru – ruthenium; RTK – receptor tyrosine kinase; scid – severe combined immunodeficient; shRNA – short hairpin RNA; siRNA – small interfering RNA; scFv – single chain antibody fragment; SEB – enterotoxin B; SMIL – stealth monensin immunoliposomes; SUV – small unilamellar vehicles; SRB - sulforhodamine B; Tf – transferrin; TfR – transferrin receptor; TGF – transforming growth factor; TH – tyrosine hydroxylase; THL – Trojan Horse liposome; VCAM-1 - vascular cell adhesion molecule; VCR – vincristine; VSMC - vascular smooth muscle cells;

Key words: liposomes; nanocarriers; drug delivery; targeting; cancer therapy; antibody; immunoliposomes; nano vehicles; pro-drugs

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1. INTRODUCTION

Current efforts in drug development and delivery continue in all areas of clinical research. In many cases, these are driven by individual approaches such as small molecule drug design, natural products, pharmaceutical developments, gene therapy, immune stimulation, and more. As a result of advances made in chemistry, materials science, molecular biology and immunology the borders between these areas have opened leading to new therapeutic approaches based on a merger of individual subject fields. One such example is the concept of immunoliposomes, which combines the use of small molecules encapsulated in liposomal nanocarriers with biological targeting techniques.

Since Bangham *et al.* discovered liposomes more than 50 years ago they have become a promising tool as a delivery system in many areas of medicine, biology, and chemistry [1]. Liposomes are lipid vehicles consisting of one or more concentric phospholipid bilayers, containing an aqueous phase inside and between bilayers. The phospholipids typically used for the preparation of liposomes are comprised of a hydrophilic head group and two hydrophobic chains, which cause the liposomes to encapsulate both hydrophilic and hydrophobic drugs [2,3]. Types and size of liposomes range from vesicles <100 nm (small unilamellar vesicles) to multilamellar and oligolamellar vesicles with diameters >1 μm. Liposomes have several attractive features and can be prepared in various sizes, used to encapsulate almost any material, and are water soluble. Thus, they circumvent many problems associated with small molecule drugs derived from organic chemistry.

The biological fate of liposomes is determined mainly by their size with nano-sized liposomes accumulating in pathological areas as a result of the enhanced permeability and retention effect (EPR) which is based on the fact that the vasculature in pathological areas is 'leaky', unlike normal healthy tissue [4,5]. Conventional liposomes consist of naturally occurring phospholipids and cholesterol, which are essential for lowering the membrane permeability and imparting better stability (cholesterol also modulates membrane-protein interactions). These compounds are rapidly removed from the circulation after administration by macrophages of the reticuloendothelial system (RES) [6]. The circulatory half-life of conventional liposomes can be significantly modulated by using functionalized lipids in their construction and such formulations are named either long-circulation-, sterically stabilized-, or Stealth® liposomes, and demonstrate less reactivity toward serum proteins as well as less susceptability to RES uptake [7]. The most widely used polymeric steric stabilizer is polyethylene glycol (PEG) which is water soluble and exhibits protein resistance, low toxicity, non-immunogenicity and antigenicity [8].

However, despite their promise and wide use in medicine, it is necessary to improve their biodistribution, pharmacological properties, and *in vivo* targeting [9]. A promising strategy to enhance the therapeutic effect of pharmaceutical agents in the target tissue is to deliver specifically liposomes to the target area and so targeted liposomes with ligands attached to their surface, which recognize cellular surface antigens or receptors in target tissues, have been developed [10,11]. In principle, any target binding biological unit can be used, and antibodies or antibody fragments, vitamins, glycoproteins, peptides, oligonucleotides, polysaccharides and others have been attached to liposomes to this effect. Due to their high specificity, liposomes with antibodies attached to their surface as targeting ligands, the so-called immunoliposomes, have emerged as one of the most promising classes for medical applications [12,13,14,15,16]. Historically, the first example of an immunoliposome (IL) was prepared by Torchilin and used to localize in acute canine myocardial infarction [17]. Here, we outline developments in this area during the last decade and focus primarily on cell biological studies; the literature has been covered in depth from 2000 onwards.

2. PREPARATION OF IMMUNOLIPOSOMES

2.1 Liposomes

Liposomes can be prepared *via* different methods the simplest of which is *lipid hydration* where they are made by hydrating a thin lipid film in an organic solvent, which is then removed by film deposition *via* rotary evaporation. After removal of all the residual solvent, the solid lipid mixture is hydrated with an aqueous buffer at a temperature above the gel-liquid crystalline transition temperature of the lipid causing the lipids to swell and hydrate spontaneously and leading to the formation of multilamellar vesicle liposomes.. The *ultrasound method* prepares small unilamellar vesicles using ultrasonication of aqueous dispersions of phospholipids. However, degradation of the phospholipids and encapsulated compounds is a potential drawback. Access to large unilamellar (LUV) and oligolamellar vesicles can be granted by *reverse phase evaporation*, where the lipids are added to a round bottom flask and the solvent is removed using distillation. Subsequently, the lipids are re-dissolved in the organic phase resulting in vesicle formation after which evaporation of the solvent to a semisolid gel, followed by removal of any

non-encapsulated material, completes the process. This method has the ability to encapsulate large macromolecules with high efficiency. Another widely used method for encapsulation of proteins is *freeze thaw extrusion*, where the liposomes are formed in a thin lipid film with the solute to be entrapped present until the film is suspended. The resulting multilamellar vesicles are frozen in water and rotated followed by multiple extrusions, several freeze-thaw-cycles, and further extrusions. This process ruptures and defuses the SUVs resulting in increased sizeafter refusion to form LUVs. The *dehydration-rehydration method* is mainly used for oligolamellar vesicles. More details are given in respective reviews [18,19,20].

2.2 Conjugation Methods

Antibodies or other ligands can be attached to liposomes either before or after their preparation. Binding is achieved either covalently or noncovalently and the various chemical strategies for the attachment of antibodies and antibody fragments have been reviewed recently [21]. A brief account of the possible strategies follows. The ligand is bound to the liposomes surface through hydrophobic anchor groups with functional groups [22]. The lipid can be synthesized prior to its incorporation into the liposome's bilayer, and a reaction is carried out between the ligand and the anchor followed by mixing the resulting ligand with the other constituents of the liposomes [23]. Alternatively, the anchor may be be previously incorporated in the liposomal membrane and then a coupling reaction is carried out on the surface of vehicles [24]. The type and place of attachment can affect the pharmacokinetics and therefore must be taken into account for drug development [25,26].
Coupling of ligands to the surface of liposomes is often performed using thioether bonds, *e.g.*, *via* reaction of thiols and maleimide groups [27]. The sulfhydryl group is present in many proteins, however, often the number of -SH groups is low or zero and they need to be generated from existing disulfide bonds or created by adding heterobifunctional crosslinking agents [21,22]. *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP) and succinimidyl-*S*-acetylthioacetate (SATA) are some of the most popular crosslinking agents. For example, after modification with SPDP, a protein can be treated with dithiothreitol for disulfide to thiol reduction. Then, a terminal -SH group can be conjugated with a functionalized anchor containing sulfhydryl groups, such as *N*-(4-(*p*-maleimidophenyl)butyryl)phosphatidylethanolamine (MPB-PE) [21,22,28,29,30].
Antibodies can be also attached to sterically stabilized long-circulation liposomal membranes in parallel with, or else at the distal end of the polyethylene glycol chain. The latter approach circumvents diminished binding and is more efficient [31,32,33,34]. Many modifications of the PEG chain allow for the coupling of ligands. One example is maleimide-PEG (Mal-PEG) where antibodies are thiolated and linked to liposomes containing MAL-PEG-DSPE (maleimide-polyethyleneglycol-distearoylphosphatidylethanolamine) through thioether bonds [35]. Its main advantage is a high coupling efficiency and formation of a stable covalent bond. However, functionalization and thiolation of whole antibodies may occur randomly and in multiple locations and results in a random orientation of the antibody on the IL surface and causes faster clearance from circulation [35,36]. The hydrazide PEG (Hz-PEG) method is based on oxidation of carbohydrates in the F_c region of the whole antibody to reactive aldehydes and then formation of hydrazone bonds with hydrazide groups on the distal end of PEG chain [22,36,37]. Another method involves crosslinking of carboxylic acid functions on the surface of nanocarriers and primary amines of the ligands. For example, a lipid with a carboxylic acid group at the distal end of the PEG chain, distearoyl-N-(3-carboxypropionoyl poly (ethylene glycol)succinyl)phosphatidylethanolamine DSPE-PEG-COOH, is commonly used [22,38]. Likewise, introduction of an additional cysteine at the Ab C-terminus for linking is possible [39,40]. Antibodies can be also attached to the PEG-terminus of liposomes using an amphiphilic PEG derivative such as *p*-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE). pNP-PEG-PE is incorporated into liposomes *via* its phospholipid residues and binds primary amino group containing ligands through its water exposed pNP groups giving a stable, non-toxic carbamate bond [41].
A recent development is the use of recombinant antibody binding proteins derived from streptococcal protein G via site-directed mutagenesis. This lipoprotein can be incorporated into the liposomes by simple mixing with lipid and can function as a binding site for antibodies to yield functional IL [42].

2.3 Delivery and Basic Structures

Liposomes are lipid vehicles of one or more concentric phospholipid bilayers, containing an aqueous phase inside and between bilayers. The phospholipids typically used for preparing liposomes are composed of a hydrophilic head group and two hydrophobic chains that assure the liposomes are able to encapsulate both hydrophilic and hydrophobic drugs [18]. Conventional liposomes are rapidly removed from the circulation, after systemic administration, by macrophages of the reticuloendothelial system (RES). Their circulatory half-life can be

significantly increased using functionalized lipids and the resulting long circulation liposomes, sterically stabilized liposomes or STEALTH® liposomes demonstrate less reactivity with serum proteins and hence evade opsonization and RES uptake. Improvements in the liposomal targeting of tumor cells can be achieved through conjugation of specific ligands to the liposomal surface [43,44]. Antibodies and antibody fragments are the most widely used targeting moieties for liposomes as they are highly specific for their target antigens and combined antibody and lipid vehicles are known as immunoliposomes [36]. Antibodies can be attached to the liposome surface *via* three different means. Conjugation of an antibody to the surface of conventional (PEG-free) liposomes results in type A immunoliposomes. These show effective specific binding to target cells *in vitro* but the *in vivo* efficiency is often low due to enhanced uptake through RES [45]. Attaching antibodies directly to the membrane of the long circulating liposomes in parallel with PEG yields type B immunoliposomes. Here, the RES uptake is reduced, however, the steric barrier of PEG decreases the efficiency of protein couplings and target recognition [38]. To overcome this problem, antibodies can be attached to the distal end of the PEG chain, forming type C of the immunoliposomes. This leads to liposomal formulations with improved circulation and better antibody accessibility [38,43,46] (Figure 1).

<Figure 1>

Conventional liposomes with encapsulated molecules passively target tissues or organs based on the enhanced permeability and retention effect (EPR), which stems from the fact that the vasculature in pathological areas is ‘‘leaky’’, unlike in normal healthy tissue. The pore size of tumors varies from 100 to 780 nm and allows the spontaneous accumulation of nanomaterials in interstitial tumor tissue [43,47]. Stealth® liposomes and ligand Stealth® liposomes use the same mechanism for tumor targeting. Only ligand targeted liposomes can selectively bind to the target tissue *via* the surface attached specific ligand [36]. The ligand specifity may even be modulated post-identification through molecular evolution [48]. For the delivery of immunoliposomes to the target area, two phases must be considered (Figure 2). The transport phase, where ILs travel from the administration site to the desired tissue and the effector phase, during which immunoliposomes bind specifically to an antigen or receptor that triggers receptor-mediated endocytosis where the whole particle, including the therapeutic molecules encapsulated inside the vehicle, is internalized into the target cell [36]. The latter may be promoted by fusion competent antibodies [49]. The targeted delivery using IL can address two different targeting sites. One is a readily accessible site such as the vascular endothelial surface. More inaccessible target sites such as the blood-brain-barrier may be targeted, too. However, here only a long circulating IL would be expected to reach the required area and this will be discussed later in this review.

<Figure 2>

3. MOLECULAR IMAGING

3.1 Magnetic Resonance Imaging

Molecular imaging is crucial for the *in vivo* characterization and measurement of biological processes at the cellular and subcellular level [2] and can also be used for image guided drug delivery [50]. One of the most fundamental problems with this technique is to acquire target diagnostic signals with sufficient intensity over the background. Thus, a sufficient quantity of the contrast agent needs to accumulate in the target area and not in normal, healthy tissue. Ideally, accumulation of paramagnetic contrast agents should be rapid to minimize the diagnostic exposure time [51]. Typically, most contrast agents consist of chelate complexes due to the toxicity and poor solubility of free paramagnetic heavy metal cations at physiologic pH [52]. Gadolinium complexes are most often employed, and suitable ligands include diethylene triamine pentaacetic acid (DTPA) [52,53] or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA), amongst others [54].

Although many contrast agents are small water soluble compounds that can easily across the endothelial barrier of blood vessels, they have a short lifetime and contrast enhancement is quite low [51,52]. To increase accumulation of contrast agents in the target area, they have been encapsulated into particulate drug delivery vesicles such as liposomes, which are able to entrap multiple moieties in the membrane and the aqueous phase. [2,51,55]. Gd-DTPA was one of the first heavy metal derivatives entrapped inside liposomes [53]. However, low molecular weight paramagnetic compounds can leak from liposomes *in vivo* [56]. In order to circumvent this, carriers with membranotropic chelating agents such as DTPA-stearylamine, DTPA-phosphatidyl ethanolamine or amphiphilic acylated paramagnetic complexes of Mn or Gd were prepared [57]. These agents increase the longitudinal relaxation rates (R1) of surrounding water protons, resulting in increased intensity of the tissue signal [56]. Other procedures to optimize the contrast agents included increasing the quantity of the membrane-bound metal atoms per vehicle using polychelating amphiphilic polymers [52,56].

The next logical step was to attempt a specific delivery of the contrast agents to the desired diagnostic targets such as the cell surface antigens of receptors [2,12,19]. Antibodies such as mAb 2C5 [58,59,60], anti-HER mAb [61] or anti-ICAM-1 mAb [62] attached to the surface of drug-loaded liposomes dramatically increased their tumor accumulation and therapeutic efficacy. These can be used for drug delivery, imaging applications and even the delivery of photodynamic therapy agents [60]. Thus, Gd loaded PEGylated liposomes with membrane incorporated polylysine based polychelating amphiphilic polymers were prepared with the monoclonal antibody 2C5 [52,56]. These systems are able to specifically recognize various cancer cells *via* their surface bound nucleosomes released during apoptosis [63]. A strong, bright fluorescence signal from the tumor treated with 2C5-modified liposomes was observed in *in vivo* MR images and indicated the significantly higher tumor accumulation of IL compared to unmodified nanocarriers. Confocal fluorescence microscopy confirmed the strong binding of antibody modified vehicles to the surface of tumor cells [56].

A similar system used ^{111}In loaded IL as tumor specific contrast agents for the γ-scintigraphic visualization of model tumors in mice [51]. These antibody modified liposomes showed significantly higher accumulation in tumor tissue than the neighboring areas after different times of administration in mice with either murine Lewis lung carcinoma (LLC) or human HT-29 tumors. In addition, the 2C5 coupled liposomes demonstrated better, clearer, and faster imaging of the target area compared to unmodified carriers [51]. A related study investigated the *in vivo* biodistribution and tumor accumulation in LLC, murine breast adenocarcinoma (4T1), human mammary adenocarcinoma (BT-20) and human estrogen receptor sensitive breast carcinoma (MCF-7) [64]. A three to eight fold increase in specific binding and prolonged circulation times was found for the IL. Whole body gamma scintigraphic imaging of mice implanted with different tumors was achieved significantly faster (6 h post injection for 2C5 IL, 24 h for non-modified carriers) with better *in vivo* tumor visualization with the ^{111}In-2C5 IL than the 2C5-free formulations.

PEGylated Gd-DTPA-based paramagnetic ILs, modified with the monoclonal antibody H18/7 against human E-selectin, were design by Mulder *et al.* [65]. They stimulated human umbilical vein endothelial cells (HUVEC) with cytokines such as IL-1β or TNFα to up-regulate the expression of the adhesion molecule E-selectin/CD62E. They then demonstrated *via* MRI and fluorescence spectroscopy that H18/7antibody modified PEGylated liposomes show E-selectin dependent association of the prepared nanocarriers to stimulated HUVEC. Such systems may present useful diagnostic tools for the detection of atherosclerosis, angiogenesis or inflammation. In a similar context, ILs conjugated with the anti-intercellular adhesion molecule 1 (ICAM-1) were evaluated as agents for the early detection of atherosclerotic plaques [62]. Fluorescence microscopy and flow cytometry confirmed the expression of ICAM-1 on the surface of activated human coronary artery endothelial cells. Even though nonactivated cells also expressed a certain level of ICAM-1 protein, binding of the anti-ICAM-1 IL to the activated cells was four times greater. ILs with encapsulated iodinated contrast agent demonstrated specific binding of anti-ICAM-1 to ICAM-1. In practical terms, this indicates the potential to develop a noninvasive computer tomographic angiographic detection technique for atheriosclerosic plaques.

MRI contrast agents delivering ILs were also developed for improved early detection of small lung cancers [66]. Early detection of lung cancer using chest radiography and computed tomography of the chest can be problematic, especially for small lung nodules [67]. Moreover, recent reports have associated an increased cancer risk with higher radiation doses from computer tomography (CT) [68]. Here, Freedman *et al.* developed an IL with anti-transferrin receptor single-chain antibody fragment (TfRscFv) and encapsulated gadopentetate dimeglumine (Magnevist®). This complex was able to increase the resolution and image intensity in a mouse model of primary cancer [69]. The IL efficiently targeted and delivered the agent to lung nodules. The MRI enhancement of nodules was much better with the nano complex than with the free agent that is currently used in the clinic. The authors suggest that this promising approach will allow most benign nodules to be differentiated from potentially malignant nodules at a size five times smaller than with current contrast-enhanced CT [66].

Feng *et al.* [70] developed a different concept using *Gaussia princeps* luciferase for detecting the distribution of ILs. This luciferase has been validated as a reporter gene for *in vivo* imaging applications [71]. ILs were prepared with a fusion protein consisting of protein A (ZZ)-linked to Gaussia luciferase. Bioluminescence and fluorescent analyses indicated that the fused protein conjugated with anti-epidermal growth factor receptor (EGFR) monoclonal antibody (Glase-ZZ-His-mAb) was efficiently delivered into glioma cells expressing EGFR mutant EGFRvIII *in vivo* and *in vitro* [70].

Recent studies with ILs also employ quantum dots and are currently *en vogue* as alternatives to organic dyes as fluorescent markers but still have limitations such as specificity, biocompatibility, or toxicity [72,73]. Conjugation of quantum dots to ILs seems a promising and logical strategy for *in vivo* tumor imaging, including integration of targeting and drug delivery. The cellular binding of quantum dots conjugated to anti-HER IL targeted to human breast carcinoma cell lines with HER2 over expression showed greater specific uptake compared to cells lacking

HER2 [74] as well as greater uptake in comparison to free quantum dots and non-targeted QD-liposomes at matching concentrations. Confocal microscopy demonstrated that unmodified QD-liposomes are only minimally internalized by HER2 over-expressing cells and the free QDs were found to be in close association with the cell surface. However, the conjugated QD accumulated mostly in the perinuclear area, consistent with an endosomal routing [74]. In a similar context, superparamagnetic iron oxide (SPIO) nanoparticles have also been made accessible for use in IL systems [75].

3.2 Electron Paramagnetic Resonance Imaging

Electron paramagnetic resonance (EPR) imaging is a magnetic resonance imaging modality used to visualize magnetic species, such as nitroxides, *in vivo* [81]. The use of nitroxides for cellular imaging offers several advantages such as resistance to bioreduction and a lifetime in cells for sufficient for imaging [61,76].They can be encapsulated in liposomes at high concentrations (>100 mM) and be delivered to cells through endocytosis [61,77]. Liposomes with high concentrations of encapsulated nitroxides are attractive nanocarriers, due to the self-quenching of nitroxides at high concentration. The intact liposomes are spectroscopically dark during circulation and give only a minimal background signal. After endocytosis the liposomes are degraded and release the entrapped nitroxides, which are then diluted in the intracellular space. This restores the nitroxide spectral signal and gives a visible EPR signal [61,77].
A specific targeting of ILs has been used with an Hc7 cell line derived from MCF7 breast tumor cells expressing HER2. Sterically stabilized liposomes with encapsulated nitroxides and coupled to anti-HER2 antibody were able to accumulate nitroxides intracellularly in sufficient concentration for EPR imaging. The circulation time was found to be in the order of days compared to hours for conventional vesicles and the IL carrier systems were more stable with maximal self-quenching of the encapsulated nitroxides [61,78].

4. BIOANALYTICAL ASSAYS BASED ON IMMUNOLIPOSOMES

A bioanalytical assay is a procedure for identifying and quantifying the amount of a biological substance based on a specific and functional response to an analytical test [79]. Thus, immunological approaches play an important role in this area. All immunoassay formats rely on the presence of a directly or indirectly detectable label; for example, easily detectable fluorescent or radioactive molecules or enzymes where the latter generates a measurable amount of product inversely proportional to the unknown concentration of antigen [20]. Bioanalytical assays can be applied in many fields, such as for the determination of toxins or allergens in food samples [80].
A typical example for the use of ILs in bioanalytical assays is the detection of food pathogens, *e.g. Escherichia coli* O157:H7 a newly emerging food borne pathogen resulting in severe illness and even death [81]. A major problem associated with *E. coli* O157:H7 contamination of food and water is the problem of quick and precise detection of the contamination. A newly optimized assay for the detection of *E. coli* used immunomagnetic beads specific for *E. coli* O157:H7 in combination with liposomes containing sulforhodamine B (SRB) coated with anti-*E. coli* O157:H7 antibodies [82,83]. The optimized protocol consisted of the filtration or centrifugation of samples, incubation of the filter membrane or pellet with anti-*E. coli* O157:H7 immunomagnetic beads in a special growth medium, and then isolation of the resulting O157:H7-immunomagnetic bead complex by magnetic separation. After washing and incubation with antibody modified liposomes with entrapped SRB, the final immunomagnetic bead-*E. coli* O157:H7-IL complex was again isolated by magnetic separation and then washed and lysed to release SRB. An assay can be completed in less than 8 h and has a detection limit of less than 1 CFU (colony-forming units) of *E. coli* O157:H7 in 1 ml of various aqueous matrices. This was a significant improvement on standard microbiological methods, which are less sensitive and require several days for completion [83].
Another method to detect this bacterium used a colorimetric immunoassay with an IL encapsulating sulforhodamine B. This system was developed by Park and Durst and uses a sandwich immunoassay containing a wicking reagent composed of anti-*E. coli* O157:H7 antibody coupled with liposomes entrapping dye and a plastic backed nitrocellulose strip with a measurement zone and had a detection limit of 10^4 CFU.ml^{-1}. The time required for an assay was only 8 minutes [84]. A similar test developed by Ho and Hsu also gave promising results [85] whilst an analogous system was developed for the detection of *Emilia amylovora*, the causative agent for fire blight an important plant disease [86].
Immunoassays using immunoliposomes were also used to detect *Salmonella*, one of the most important food borne pathogens in humans, causing an increased mortality rate due to Salmonellosis. Among many kinds of *Salmonella* species, *Salmonella typhimurium* and *Salmonella enteritidis* are the most common food borne pathogenic bacteria [87]. Here, Junghee and Kim developed an immunoassay to detect *Salmonella* using immunomagnetic separation

and liposomes coated with anti-*Salmonella* IgG, again encapsulating sulforhodamine B; the same method as described above for *Escherichia coli* O157:H7 detection [88]. The assay was used for the detection of *Salmonella enteritidis* and *Salmonella typhimurium* and measured the fluorescence intensity of SRB produced from an immunomagnetic bead *Salmonella*-LS complex and yielded detection limits of 2.7×10^5 CFU.ml^{-1} for *Salmonella enteritidis* and 5.2×10^3 for *Salmonella typhimurium*. The test required 10 h for completion compared to 3-4 days for conventional plating tests [88].

An improved liposome-based immunoassay for *Salmonella typhimurium* used purified anti-*Salmonella* IgG antibodies obtained from rabbits injected with formalin-killed cells of *S. typhimurium*, conjugated with liposomes encapsulating SRB. Results showed that the assay was highly sensitive and specific for the analyzed substance with a detection limit of 10^3 CFU.ml^{-1}. This is a rather simple and inexpensive technique, which does not require any sophisticated equipment [87]. Next, this newly developed immunochromatographic strip assay was compared with a commercial *Salmonella* kit using gold nanoparticles [89]. The assay consists of an IL solution, sterically diluted cultures of *S. typhimurium* and a nitrocellulose test strip with two coated line zones of antibodies. The lower zone is referred to as the *Salmonella* antigen capture zone (test line) and upper zone functions as a positive control (control line). Upon immersion of the test strip in a reaction mixture of the IL solution and *Salmonella* test cultures the mixture is adsorbed and migrates along the strip. *Salmonella* is bound to the antigen capture zone of the membrane and produces a purple color band, whereas unbound IL continue to migrate to the upper end and are bound in the control zone. The color density of the antigen capture zone was directly proportional to the amount of the pathogen in the test sample. The whole test could be completed in less than 10 min and the detection limit was found to be 100 CFU.ml^{-1} compared to 10^7 for the commercial immunochromatographic test. A related, earlier test by Ho *et al.* used liposomes tagged with anti-*Salmonella* antibody and entrapped methyl blue. No significant cross reactivity to nonspecific pathogenic organisms such as *E. coli* O157:H7 or *Listeria* was observed and the detection limit was 1.680 cells for heat-killed *S. typhimurium* with 99.7 % confidence [90]. Note, that protein G-liposomal nanovesicles in conjunction with the respective antibodies can be used for the simultaneous detection of *E. coli* O157:H7, *Salmonella*, and *Listeria* monocytogenes [91].

ILs with entrapped sulforhodamine B can also be used to detect enterotoxin B (SEB). Enterotoxin B is a toxin produced by *Staphylococcus aureus* that is stable over a wide range of pH (4-10), highly heat-resistant, and is involved in food poisoning, allergies and autoimmune diseases; SEBtherefore has potential use as a biological weapon due to its stability, high toxicity and simple production [92,93]. An improved immunochromatographic test used ILs as labels for the detection of SEB applied an air drying treatment to destroy the structure of the liposomes, resulting in release and local dispersion of the fluorescent dye. The fluorescence detection gave a 15-fold increase in sensitivity compared to visual detection of colored labels. The detection limit was ~20 pg.ml^{-1} and a single test required only 30 minutes [94].

Similarly, a liposome-based immunoaffinity chromatographic assay was developed for the quantification of immunoglobulin E in human serum [95]. IgE is an antibody subclass that triggers the immune reaction and is found only in mammals. Humans typically have a very low serum level, while its concentration is much higher in patients with allergic asthma, atopic dermatitis or other immunno deficiency related diseases. Here, the analytical method was based on a competitive reaction between standards (or serum samples) containing IgE and IgE modified immunoliposomes with encapsulated SRB for a limited number of antigen binding sites on nitrocellulose test strips with the immobilized anti-IgE antibodies in the antigen capture zone. The color intensity was indirectly proportional to the number of IgE units in the sample. The detection limit was 0.37 ng of IgE (equivalent to 20 µl of an 18.5 ng.ml^{-1} solution) with 99.7 % confidence. In comparison to commercially available ELISA kits, the new analysis could be completed within 30 min and did not require any of the washing and incubation steps common for ELISA procedures [95].

Kumada *et al.* developed a new liposome immunoblotting assay using antibody-coupled liposomes encapsulating horseradish peroxidase (HRP) [96]. As substrate, they chose 4–chloro-1-naphthol (4-CN) which can permeate the phospholipid membrane of the liposome. Upon reaction with HRP a colored product is formed which can no longer penetrate through the membrane. The product precipitates and accumulates in the liposomes and is detected *in situ*, providing a clear signal. Using a combination of liposomes and a polyvinylidene difluoride membrane, with an average diameter of 350 nm and pore sizes of 450 nm, respectively, it was possible to detect 0.02 ng of IgM in a sample. In comparison, conventional immunoblotting assays with antibody conjugated HRP had a detection limit of 2 ng of IgM. Moreover, the color of the liposome immunoblotting assay was stable for longer periods than those conventionally used [96].

This kind of immunoblotting assay was also used as a colony lift immunoassay for rapid screening of colonies secreting active scFv [97]. A direct and indirect blotting assay for the detection of active scFv secreted from colonies of *E. coli* on selective agar plates was applied using antibody modified liposomes encapsulating HRP. In direct

blotting, the antigen coated hydrophobic membrane was placed directly on the plate. In the indirect method, the hydrophilic membrane was inserted between the antigen coated hydrophobic membrane and an agar plate in order to prevent direct contact of cells with antigen coated membranes. The blotted membrane was then incubated with anti-IgG IL entrapping HRP (IL assay) or with anti-IgG HRP (conventional immunoblotting assay). The former gave a much stronger signal for scFv secreting colonies compared to the latter [97].

Immunoliposomes encapsulating horse radish peroxidase were also used to improve the signal intensity and detection sensitivity of enzyme immunoassays. Here, non-porous poly(methyl methacrylate) (PMMA) and polystyrene surfaces were used together with HRP conjugated anti-rabbit IgG and anti-rabbit IgG antibody coupled with liposomes. The liposome immunoassay gave a stronger color signal than the enzyme immunoassay on both surfaces, especially at low concentrations [98].

Another approach for the use of ILs in bioanalytical assays utilized antibody modified liposomes which encapsulated a ruthenium complex with two aminobutyl moieties for the detection of hemagglutinin molecules. The latter play an important role in influenza virus infection since the hemagglutinin molecule is situated on the envelope membrane surface of the virus [99]. To improve the detection of these molecules a biosensor combining electrochemiluminescence and an anti-HA IL entrapping the ruthenium complex in the aqueous phase was developed. Highly sensitive detection was observed through electrochemiluminescence of the Ru complex adsorbed onto gold electrodes on which hemagglutinin was immobilized followed by competitive immunoreactions. Optimum measurement conditions allowed detection in a concentration range of 3×10^{-13} to 4×10^{-11} g.ml^{-1} [99].

Other assay systems have been reported for the detection of bovine serum albumin (BSA) based on the electrochemiluminescence of a Ru complex deposited onto a silver surface. One system had a sensitivity comparable to ELISA tests [100]. Likewise, a system for the detection of microcystins, i.e., cyclic heptapeptides produced by cyanobacteria, which cause acute and chronic toxicity, was developed [101]. Even biomarkers can now be quantified using IL approaches. For example, Nucin-15 (MUC-16) an established ovarian cancer marker used to follow the disease during or after treatment for epithelial ovarian cancer can be determined with an electrochemical immunosensor system [102].

5. ECHOGENIC IMMUNOLIPOSOMES

Echogenic liposomes (ELIP) are phospholipid bilayer vehicles that contain gas bubbles inside the liposomes and have potential as ultrasound contrast agents or drug delivering system [103]. Microbubbles of micrometer to nm sizes effectively scatter ultrasound waves due to the large acoustic impedance differences between the gas and the surrounding liquid. Albunex® was the first such agent approved by the FDAand uses sonicated human serum albumin to encapsulate and stabilize air microbubbles and finds use as a transpulmonary ultrasound contrast agent [104,105,106].

Acoustically reflective liposomes can be also prepared without inclusion of gas or other agents relying on an optimal lipid composition and using a dehydration/rehydration method [107]. *E.g.*, high concentrations of phosphatidylethanolamine (PE) (4-12%), low concentrations of phosphatidylglycerol (PG) (1-3%), and constant cholesterol content (30%) gave the highest echogenicity level. Such high echogenic formulations are composed of a multilamellar structure with well separated bilayers, whereas low echogenicity was associated with a thick, unseparated multilayer in a single liposome. The echogenicity of such liposomes is not lost or decreased at body temperature or in the presence of blood components and was found suitable for testing upon conjugation to tissue specific antibodies for local image enhancement, diagnosis, and drug delivery.

By now, there are several reported examples of the use of echogenic liposomes, including immunoliposome formulations. For example, they were used for the ultrasound detection and enhancement of active molecular components of the endothelium and atheroma [108]. In this case, ELIP produced in the absence of air, with small size <1 μm, were coupled with anti-vascular cell adhesion molecule (VCAM-1), anti-intracellular adhesion molecule-1 (ICAM-1), anti-fibrin, anti-fibrinogen or anti-tissue factor (TF) and injected into Yucatan miniswine. The arteries imaged with intravascular ultrasound (IVUS) showed rapid (5 min after administration of antibody modified liposomes) and specific ultrasound signals of the molecular endothelial/atheroma targets. Immunohistochemistry (IHC) confirmed the presence of molecular expression.

Echogenic immunoliposomes conjugated with anti ICAM-1 antibody were also used for the 3D volumetric IVUS visualization and improved detection of early inflammatory arterial atheroma [109]. After injection of antibody modified echogenic immunoliposomes into miniswine the average mean gray scale value (MGSV), a quantitative measurement of the acoustic enhancement obtained from all the arterial segments, increased by appoximately 44% compared with the saline control. Furthermore, anti-ICAM-1 ELIPs were superior in detecting the early-stage

plaque volume where the average increase of the measured plaque/atheroma volume by echogenic IL versus baseline was 24%. Plaque volumes after administration of echogenic liposomes coupled with non-specific IgG control or non-targeted formulations did not differ.
Ultrasound enhanced drug-gene delivery to vascular smooth muscle cells (VSMCs) using ELIP as a vector has also been demonstrated [110]. Liposomes with entrapped calcein as a surrogate for small molecules were conjugated with anti-smooth muscle cell line actin antibody and injected into miniswine. The right-sided arteries were exposed to ultrasound and increased uptake in all layers of the arterial wall, especially in the arterial media which exhibits poor drug and gene delivery, was observed after a brief exposure to 1-MHz continuous wave ultrasound, compared to very little calcein uptake without ultrasound treatment.
Likewise, bifunctional echogenic immunoliposomes (BF-ELIP) were successfully prepared and used for the delivery of stem cells to porcine arterial wall [111]. They were generated by anchoring two antibodies on the liposome surface. Here, monoclonal antibody directed against ICAM-1 antigen presented on the inflammatory endothelium covering atheroma and rabbit polyclonal antibody specific for CD34 antigen on the surface of stem cells were used. The authors noted significantly increased stem cell attachment and penetration, particularly in the aortic segments, in *ex vivo* experiments on $CD34^+$ stem cells from adult bone marrow incubated with ICAM-1 expressing endothelium of the aorta from swine preloaded with BF-ELIP. The presence of BF-ELIP-bound $CD43^+$ cells in the intimal compartments of the arterial walls was confirmed by fluorescence and electron microscopy and clearly validated this approach.
Recently, a new systemic left ventricular thrombus (LVT) model was developed for the determination of ultrasound enhancement of systemic structures after intravenous injection of ELIP coupled with anti-fibrinogen antibody. This is unlike other methods, where ELIP delivery has been intra-arterial [112], in that the echogenic antibody modified liposomes were injected into dogs through the left femoral vein. No hemodynamic effects on the mean arterial blood pressure, heart rate, or the mean pulmonary arterial pressure were observed. Anti-fibrinogen ELIP delivered intravenously proceeded through a single-step process, successfully traversing the lungs and rapidly increasing epicardial and transthoracic ultrasound images (within 5 to 10 min after administration). No hemodynamic effects were observed, which raises the possibility to extend applications of these contrast agents beyond local/arterial injection for broader clinical applications.

6. IMMUNOLIPOSOMES IN GENE THERAPY

6.1 Lipoplexes

The direct targeting of cancer cells with gene therapy is a very promising method of cancer treatment [113,114] and various delivery systems, including liposomes, have been used for gene delivery [115,116]. Recent progress is evidenced by the development of non-viral pharmaceutical formulations of genes for *in vivo* therapy and the most extensively used are the co-called lipoplexes [113,114]. Lipoplexes are complexes comprised of cationic liposomes and DNA that offer many advantages over viral systems for gene delivery. Most significantly, lipoplex complexes lack immunogenicity and due to their noninfectious nature carry no risk of evolving into new classes of infectious pathogens [114,117].
One example for such an approach is the preparation of liposome-DNA complexes coupled with anti-TfR antibody fragment (scFv) by Xu *et al.* [114]. In previous work, cationic immunolipoplexes with a single chain antibody fragment (scFV) against TfR were used and showed promising efficacy for *p53* tumor gene therapy in a human breast cancer metastasis model. However, due to low yields for lipid-tagged scFv progress was limited [118]. The scFv-cys immunolipoplexes significantly enhanced the binding to tumor cells compared to nonmodified complex or ones coupled only with single chain antibody fragment (scFv). They achieved higher transfection efficiencies *in vitro* in various human tumor models including lung (H358), liver (Hep3B), colon (HT29), prostate (DU145), head (JSQ-3) and breast cancer (MDA-MB-435). The ability of the scFv-cys immunolipoplexes to specifically deliver the *p53*gene to tumor tissue was also examined in nude mice bearing DU145 xenograft models which demonstrated high level expression of exogenous wild-type p53. A nude mouse model bearing two human tumor xenografts (JSQ-3 and DU145) treated with scFv-Lip complexed with a plasmid containing another reporter gene coding for GFP (green fluorescent protein) showed a high level of expression of the exogenous GFP gene, whereas only minimal expression was found in animals injected with non-targeted liposomes carrying GFP gene. The anti-TfR scFv-immunolipoplexes also showed better reporter gene expression than liposomes targeted by transferrin itself, which is consistent with *in vitro* studies [114].
Polyethylene glycol stabilized liposome-DNA complexes conjugated with RI7 or 8D3 monoconal antibody against the mouse transferrin receptor on the neuroblastoma Neuro 2A mouse cell lines induced transfection of these cells.

Without the conjugated vector, or in presence of an excess of free free RI7 mAb, no transfection was observed. No signs of toxicity from the antibody modified polyethylene glycol-stabilized liposome-DNA complex or excess RI7 MAbs were noticed in cultured N2A cells [119]. In another study cationic liposome/DNA complexes were prepared, followed by the addition of anti HER2-Fab'PEG3400-DOPE conjugates [120]. Various lipoplex formulations were tested on SK-BR3 human breast cancer cells using a luciferase reporter gene assay to determine enhanced gene delivery of anti-HER2 conjugates. The immunolipoplexes demonstrated a >10-fold increase in luciferase activity compared to the control lipoplexes. Similarly, a 6-fold enhancement of the transfection efficiency in SK-BR3 cells was found. No such increase was observed in HER2 negative MCF-7 cells. Flow cytometry analysis demonstrated significantly higher expression of the green fluorescent protein (GFP) in SK-BR3 cells transfected with immunolipoplexes compared to control lipoplexes or naked DNA alone.
Similarly, liposome/DNA complexes were conjugated with anti-transferrin receptor monoclonal antibody (OKT9) or with anti E-selectin monoclonal antibody (1.2B6) [121]. Flow cytometry analysis showed that the liposomes/DNA-OKT9 complexes, labeled with Cy5 and incubated with E.A.hy926 cells (a human endothelial cell which expresses high levels of human TfR), exhibited a 10-fold higher level of binding than untagged liposomes and faster internalization than the liposome/DNA complex. This study also revealed an increased transfection efficiency of liposomes to cells expressing the appropriate antigens as well as efficient gene delivery (pCMV/*β*-gal) to corneal and vascular tissue *ex vivo* when analyzed by measurements of β-galactosidase activity with the ONPG (O-nitrophenylgalactosidase) colorimetric assay.
In a different approach, pegylated immuno-lipopolyplexes (PILP) employing DNA/polyethylenimine polyplexes, anionic liposomes composed of POPC, (DSPE)-PEG2000 and (DSPE)-PEG2000-biotin, using streptavidin-mAB conjugating through the biotin group located at the distal end of the PEG spacer as targeting antibody, have been studied. The vector was highly effective in protecting the DNA from enzyme digestion and was stable in particle size and zeta potential. Antibody modified pegylated lipoplexes exhibited high efficiency in gene delivery to SMMC-7721 liver cancer cells with no significant cytotoxicity. Intravenous administration of the PILP resulted in tumor and liver targeted gene expression of the reporter genes EGFP and luciferase, as opposed to the lung targeted gene expression obtained with PEI/DNA complexes, causing no cytokine production and liver injury [122].
Low cationic liposome–plasmid DNA complexes modified with cell-penetrating transactivating transcriptional activator peptide (TATp) and/or with monoclonal anti-myosin monoclonal antibody 2G4 (mAb 2G4) specific toward cardiac myosin were developed for targeted gene delivery to ischemic myocardium. Double targeted lipoplexes enhanced transfection *in vitro* in the hypoxic cardiomyocytes but not in normoxic cells where the intracellular myosin is not exposed. It was also noticed that 2G4-modified lipoplexes without TATp provided the same level of gene expression as the double-targeted lipoplexes, suggesting that the 2G4 antibody plays a dominant role in transfecting hypoxic cells. *In vivo* experiments demonstrated an increased accumulation of mAb 2G4-modified TATp lipoplexes in the ischemic rat myocardium and significantly enhanced increased GFP gene expression in the ischemic cardiomyocytes [123].

6.2 Antisense Oligonucleotides

Antisense oligodeoxynucleotides (ODNs) are short pieces of DNA (12–30 nucleotides) that are complementary to a target mRNA and have the ability to block the expression of specific target genes involved in the development of human diseases [124]. However, therapeutic applications of ODNs are limited due to their low physiologic stability, insufficient cellular uptake, lack of tissue specificity and unfavorable pharmacokinetics [125]. Nevertheless, it has been shown that lipid-based systems improve the delivery of ODNs by increasing their stability, cellular uptake and specificity [126]. Moreover, the conjugation of such systems with tumor directed antibodies on their surface resulted in an even more effective delivery of diagnostic molecules to target cells [127].
A typical example is the use of c-myb antisense oligodeoxynucleotides (ODNs) entrapped in cationic liposomes and conjugated with monoclonal antibodies against disialoganglioside (GD_2) [128,129]. The latter is a tumor associated antigen widely expressed by cancer cells of neuroectodermal origin (including melanoma) and with very low expression in normal tissues [130]. Anti-GD_2 ILs demonstrated growth inhibition of neuroblastoma cells *in vitro* by specifically triggering the down-modulation of c-Myb protein expression [128]. The c-Myb protooncogene, a transcription factor gene, is expressed in several solid tumors of different origin, including neuroblastoma, where it is associated with cell proliferation and differentiation [131]. GD_2 targeted liposomes increased ODN uptake by GD_2 positive neuroblastoma cells up to 10-fold, compared to free oligonucleotides. Immunoliposomes carrying c-myb ODNs specifically reduced the expression of c-Myb protein by about 70%. No increased binding or uptake of ODNs was observed in GD_2 negative cells [128]. In subsequent studies, liposomes coated with anti-GD_2 antibodies were loaded with antisense oligonucleotides containing CpG motifs and examined by their ability to stimulate immune

response [132]. Antisense oligonucleotides can also have indirect antitumor effects *via* immune stimulatory mechanisms if they contain nonmethylated cytosine-guanine (CpG) motifs, which are able to induce cytokine secretion and stimulate innate immune responses [133]. Flow cytometric analysis of splenocytes collected from tumor bearing mice and injected with targeted or non-targeted formulations of CpG showed a very rapid increase of CD69 antigen expression on monocytes, B cells, and natural killer (NK) cells induced by the targeted ILs. The non-targeted formulation gave no activation of these cells. Intravenous administration of targeted liposomes encapsulating CpG caused a strong systemic secretion of immunostimulatory cytokines, such as interlekin 12 (IL-12), interferon gamma (IFN-γ), interleukin 1β (IL-1β) or tumor necrosis factor α (TNF-α), in the plasma of neuroblastoma tumor bearing mice in a time-dependent manner [132].
Another strategy used c-myc antisense oligonucleotides encapsulated in anti-GD_2 targeted liposomes for delivering ODNs to melanomas [134]. C-myc is another member of a family of transcription factor genes and functions in cell growth control, differentiation, and apoptosis [134]. GD_2-targeted cationic liposomes with c-myc entrapped inside the vehicles exhibited increased binding and internalization of the ILs in GD_2 expressing melanoma cells *in vitro*, compared to non-targeted formulations. Intriguing results were also observed *in vivo* in mice bearing human melanoma xenografts, where a significant reduction of tumor growth and increased survival was observed. The antitumor effects were associated with down modulation of the expression of the c-myc protein resulting in reduced cell proliferation and increased apoptosis of human melanoma [135]. A related study used a combination therapy with c-myc antisense oligonucleotides and doxorubicin, both encapsulated in GD_2-targeted and non-targeted liposomes [130]. Targeted c-myc formulations sensitized the melanoma (MZ2-MEL) cells to doxorubicin, reducing the IC_{50} by an order of magnitude whereas non-targeted formulations lowered the IC_{50} two-fold, while ODNs alone had no effect. *In vivo* experiments demonstrated a significant inhibition of tumor growth, more pronounced in the case of combination therapy than either treatment alone [130].
Hussain *et al.* reported on liposomes coupled with the humanized single-chain Fv antibody fragment 4D5MOCB targeted to the epithelial cell adhesion molecule (Ep-CAM) and used these to deliver antisense oligonucleotide 4625 specific for bcl-2 and bcl-xL [136]. Ep-CAM is a 40-kDa transmembrane glycoprotein abundantly expressed in many solid tumors, but shows limited expression in normal epithelial cells and is used as a negative prognostic factor in disease progression and survival [136,137,138]. The Ep-CAM targeted IL exhibited specific binding to Ep-CAM overexpressing tumor cells such as the breast adenocarcinoma cell line MCF-7, the ovarian cancer cell line OVCAR-3, and the small cell lung cancer cell line SW2, giving a 10- to 20-fold higher prognosis factor in comparison to non-targeted vehicles. The Ep-CAM negative control gave no specific binding in the non-Hodgkin's lymphoma cell line RL. Anti-EpCAM IL encapsulated antisense oligonucleotide 4625 was efficiently internalized by receptor mediated endocytosis and resulted in down-regulation of bcl-2 and bcl-xL expression at the mRNA and protein level, resulting in increased tumor cell apoptosis. Moreover, in combination therapy, immunoliposomal antisense oligonucleotides increased the sensitivity of Ep-CAM positive tumor cells to doxorubicin [136].

6.3 siRNA Gene Therapy

Small interfering RNA **(**siRNA) are 21-25 nucleotide long, double-stranded RNAs that were discovered in 1998 by Fire *et al.* [139] and have the ability to downregulate genes involved in various human diseases such as cancers, viral infections, and other chronic diseases [140,141]. Gene therapy using siRNA represents a promising and specific method for tumor targeting and thus efficient siRNA delivery to tumor tissue is mandatory for more extensive applications of siRNA *in vivo* [142,143,144].
As in many other cases, ILs can be used as delivery vehicles for siRNA [145]. For example, Gao *et al.* developed a tumor specific, lyophilized, pegylated 3β-[N-(*N*',*N*'-dimethylaminoethane)carbamoyl] cholesterol (DC-CHOL)/dioleoylphosphatidyl ethanolamine (DOPE) immunoliposome (LPIL) conjugated with the Fab' of recombinant humanized anti-HER2 mAb for siRNA delivery [146]. Studies with confocal microscopy confirmed the specific binding to, and internalization of LPIL into, HER2 expressing human breast adenocarcinoma cells (SK-BR3). No significant uptake or internalization was observed in SK-BR cells treated with non-targeting liposomes, nor in breast cancer cells (MCF-7) lacking HER2 expression and treated with both types of vehicles. The experiments revealed that ILs prepared by the lyophilization/rehydration method (LPIL) possess a significantly higher gene silencing ability in HER2 overexpressing SK-BR3 cell lines than antibody modified pegylated liposomes prepared *via* different methods, such as hydration of lipid films or the "post-PEGylation''method, where first the liposomes/siRNA complex is prepared using non-PEGylated liposomes followed by PEGylation [146]. Using RhoA, a member of the Rho family proteins which promote cell invasion [147], as a cancer therapeutic target, it was shown that LPIL encapsulating anti-RhoA siRNA specifically silenced RhoA expression and inhibited cell invasion in SK-BR3 cancer cells whereas non-modified liposomes encapsulating anti-RhoA siRNA exhibited only

very weak RhoA gene silencing and were not able to inhibit cell invasion. No gene silencing and inhibition of cell invasion was observed with MCF-7 cancer cells [146].

In another study, a nano immunoliposome based delivery complex of siRNA encapsulated by a cationic liposome and surface modified with anti-TfR single-chain antibody fragment (TfRscFv) was successfully prepared [148]. The immunoliposome siRNA was able to target specifically and efficiently both primary and metastatic cancers of various types such as prostate, pancreatic, and breast cancer. In further experiments, the efficiency of this complex was enhanced by inclusion of a pH-sensitive histidine-lysine peptide (HoKC) and through encapsulation of modified hybrid (DNA-RNA) anti-HER2 siRNA molecules [149]. *In vitro* studies with Human pancreatic cells (PANC-1) showed that the cell killing ability of the DNA-RNA hybrid form was 50% better than that of the standard RNA-RNA duplex. The difference was even more pronounced when the siRNA hybrid form encapsulated within ILs was compared with modified hybrids (modification of hybrid DNA/antisense siRNA molecule where o-Me substituents have been incorporated in the central region of the sense strand DNA flanks). Moreover, ILs incorporating a pH-sensitive His-Lys peptide carrying modified hybrid siRNA were superior to modified hybrid siRNA entrapped in ILs without HoKC with regard to cell killing *in vitro* in human breast cancer cells (MDA-MB-435). No significant effect of the anti-HER2 modified hybrid siRNA on cell survival was found in the control, a normal human fibroblast cell line (H500). The immunoliposome anti-HER2 siRNA complex was also able to sensitize Human tumor cells to chemotherapeutics such as gemcitabine, silence target genes, and inhibit tumor growth *in vivo* in a PANC-1 xenograft mouse model [149].

Similarly, Zheng *et al.* demonstrated a new method of selectively delivering siRNA to dendritic cells [150]. They used CD40 siRNA containing immunoliposomes coupled with NLDC-145, a monoclonal antibody targeting the endocytic receptor DEC-205 [150,151]. Dendritic cells can act as stimulators of T-cell activation and as regulators of the immune system, which plays a substantial role in preventing autoimmune disease by induction of T regulatory cell generation and other tolerogenic mechanisms [152]. The co-stimulatory molecule CD40, which was used as a target gene, is necessary for bidirectional interaction between T cells and DCs during immune activation [153]. The results demonstrated a specific binding of NLDC-145 modified ILs carrying CD40 siRNA *in vitro* and *in vivo* in contrast to non-modified or coupled with isotype IgG liposomes. Bone marrow derived dendritic cells (BMDCs) transfected with CD40 siRNA ILs exhibited significant gene silencing compared to negative control ILs. A similar effect was found *in vivo* in C57/B6 mice where administration of antibody modified ILs carrying siRNA to mice resulted in selective siRNA uptake by immune organs followed by inhibition of T-cell proliferation in an antigen-specific response was observed [150].

In a different approach, a liposome-polycation-DNA complex conjugated with anti-EGFR Fab' was prepared as an effective nanovector for the delivery of small interfering RNA (siRNA) to MDA-MB-231 breast cancer cells [154]. Here, transfection efficiency was increased in MD-MB-231 and SK-BR3 cell lines, but not in the low EGFR expression cell line MCF-7. The IL also showed enhanced luciferase gene silencing activity in EGFR over expressing MDA-MB-231 cancer cells *in vitro* as well as *in vivo* in MDA-MB-231 tumor xenograft 24 h post intravenous injection. Additionally, the Ab modified liposomes showed significantly higher gene silencing activity *in vivo* than non-modified formulations, which is consistent with the *in vitro* gene silencing assay [154] although it did not yield satisfactory gene silencing activity in hepatocellular carcinoma (HCC). Thus, subsequent studies utilized modifications to develop a superior non-viral vector for siRNA delivery to HCC. These included an increased antibody conjugation *via* post insertion couplings and a reduced pegylation degree [155]. Specific binding affinity tests of these new vehicles were performed *in vitro* with three different HCC cell lines, differing in the level of EGFR-expression (SMMC-7721, LM3, Hep3B). The new antibody modified IL showed significantly increased transfection efficiency in all cell lines compared with non-targeted vehicles. *In vivo* distribution assays demonstrated an enhanced ability of the antibody modified IL to accumulate in EGFR-overexpressing HCC throughout the tumor tissue in a pattern consistent with receptor mediated endocytosis compared to non-modified liposomes. Likewise, anti-EGFR Fab' IL carrying siRNA gave significantly more gene silencing in mice bearing orthotopic HCC than non-targeted formulations [155].

Liposome-polycation-hyaluronic acid nanoparticles modified with tumor-targeting scFv were developed for systemic delivery of siRNA and microRNA into experimental lung metastasis of murine B16F10 melanoma. The scFv targeted nanoparticles carrying siRNA efficiently downregulated target genes such as c-Myc, MDM2 and VEGF in lung metastasis whilst combination delivery of miRNA and siRNA in the liposome-polycation-hyaluronic formulation resulted in an enhanced therapeutic effect [156].

In a different study, liposomes were prepared by combining the dual asymmetric centrifugation method and sterol-based post-insertion technique to couple anti-GD2 antibody for specific delivery of siRNA to neuroblastoma cells [157]. The delivery of siRNA into neuroblastoma cells by anti-GD2-liposomes was shown to occur *via* the

endocytotic pathway and anti-GD2 modified dimethyldioctadecylammonium (bromide salt) liposomes loaded with siRNA specific for VEGF successfully reduced expression of this gene in neuroblastoma cell lines by 30%.
Very recently, a novel method of producing asymmetric liposome particles (ALPs) with highly efficient siRNA encapsulation was developed [158] using two types of lipid inverted micelles. The inner micelle was composed of ionizable cationic 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), which entrapped siRNA, whilst the outer one was composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), DOPE, polyethylene glycol-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (PEG-PE) and cholesterol. After mixing the inverted micelles, ALPs encapsulating >90% siRNA were formed by solvent evaporation and dialysis. These particles, modified with the anti-Human epidermal growth factor receptor antibody, succesfully induced an EGFR-mediated uptake into non-small cell, lung cancer cell lines.

7. BLOOD-BRAIN-BARRIER TARGETING SYSTEMS

7.1 BBB Targeting Immunoliposomes

The blood-brain barrier (BBB) is an active barrier between the circulation and the central nervous system (CNS) which restricts the passage of different substances between the two compartments and plays an important role in the maintenance of the homeostasis of the central nervous system. It has both a barrier and carrier function. The barrier function of the BBB restricts the transport of potentially toxic or harmful substances from the blood to the brain, whereas the carrier function allows the transport of nutrients to the brain and the removal of metabolites [159]. Drug delivery to the brain is hindered by these CNS barriers. Thus, in order to target the brain, drug delivery systems need to pass the BBB or brain capillary endothelial membrane as a first barrier and then cross the tumor cell plasma membrane [160,161, 162]. Liposomes, with their outer lipophilic membrane that increases their permeability across membranes, are promising delivering systems to the brain. Immunoliposomes present an improved liposome technique [163,164] and have been used successfully *in vivo* to achieve targeted delivery to brain tumors [165,166]. Immunoliposomes can deliver the active compounds via receptor-mediated transport. This involves peptide-specific receptors, such as the transferrin (TfR) or insulin receptor (IR), which mediate cerebral uptake. The transport vector across the BBB could be an endogenous peptide, such as insulin or Tf, or else a receptor specific peptidomimetic monoclonal antibody (MAb) presented on the liposome surface. The latter should bind to epitopes on the receptor that are distinct from the binding site of the endogenous ligand resulting in the crossing of the BBB through receptor mediated endocytosis [161,167].
One of the early examples of such studies involved the delivery of daunomycin by targeting the TfR and transcytosis across the BBB using OX-26 IL [165,168]. For rat glioma cells (RG2), which express the transferrin receptor, a specific binding of OX-26 IL was noticed. No cellular uptake was found for ILs conjugated with the unspecific IgG_{2a} control antibody. *In vitro* studies using RBE4 rat brain endothelial cells showed that OX-26 permeated across the RBE4 cell monolayer and that this transport was inhibited by exchanging the OX-26 mAb for an unspecific isotype antibody. Transcytosis OX-26 IL was confirmed *in vivo* by brain perfusion and capillary depletion techniques. The ability to target the TfR with OX-26 coupled ILs was confirmed by *in vitro* studies using the human brain endothelial cell line hCMEC/D3 [169]. Liposomes entrapping fluorescein-isothiocyanate-dextran-4000 (FITC-dextran) or trisodium 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) coupled with OX-26 mAb showed higher uptake by hCMEC/D3 cells compared to the control (non-modified liposomes), as well as the negative control (ILs coupled with IgG_{2a}). Adding a second antibody to the OX-26 IL surface did not yield any significant reduction in cell uptake. The OX-26 immunoliposomes' transport accross the hCMEC/D3 cell monolayer was measured *via* transendothelial electrical resistance and was found to be faster in comparison with two other ILs, one coupled with IgG_{2a}, the other a non-targeted liposome [169].

Likewise, the monoclonal antibody 2C5, specifically recognizing human brain tumor cells, was anchored in liposomes and tested *in vitro* and *in vivo* [58]. These immunoliposomes showed enhanced binding to all tested human brain tumor cell lines such as LN 18, CCF-STTG1, and U-87 MG. The strongest binding was observed in the CCF-STTG1 cell line. Non-targeted formulations and liposomes conjugated with unspecific antibodies did not interact with either cell surface. Experiments *in vivo* with a nude mice subcutaneous brain tumor model using ^{111}In labeled ILs showed a 3-fold higher accumulation of 2C5 IL in the U-87 MG tumor in comparison with the non-specific control formulation [58]. Furthermore, the cytotoxic effect of 2C5 IL loaded with doxorubicin upon U-87 tumor cells *in vitro* was found to be significantly higher than both Doxil® (a commercial doxorubicin-loaded PEGylated liposome) and non-specific IL. Consequently, a significantly improved survival time of mice in an

intracranial brain tumor model established with U-87 MG tumor cells compared to Doxil and IgG formulations, was observed [166].
Another type of IL developed for intracellular delivery to the rat striatum is the Thy 1.1 antibody conjugated with liposomes encapsulating horseradish peroxidase [170]. Thy 1.1 is a membrane protein that is anchored by glycosyl-phosphatidylinositide (GPI) [171]. Antibody modified and non-targeted liposomes were injected into adult Spraque-Dawley rats and their tissues evaluated by light and electron microscopy for intra- and extracellular liposomes. Untargeted liposomes showed less cell specific uptake relative to Thy 1.1 modified vehicles. Light microscopy indicated that the Thy 1.1 IL diffused about 0.5 mm from the injection site into the striatal neurophil within 24 h. Transmission electron microscopy confirmed that immunoliposomes were able to direct substantial *in vivo* delivery of liposomes into neurons and glia within the striatum [170]. A similar system has also been used for kidney targeting [172].
Similarly, Feng *et al.* successfully encapsulated sodium borocaptate (BSH) within nickel lipid liposomes conjugated with anti-EGFR antibody using the antibody affinity motif of protein A (ZZ) as an adaptor [173]. These were used to deliver BSH to glioma cells both *in vitro* and *in vivo*. An *in vitro* immunohistochemical analysis confirmed effective delivery into EGFR expressing glioma cells but not into the EGFR deficient glioma or primary astrocytes. Animal studies established that the IL reached the tumor 4 h after intravenous injection in contrast to non-targeted liposomes.

7.2 Gene Therapy in the Brain

7.1.1 Trojan Horse Liposomes

Trojan horse immunoliposomes (THL) present a promising special concept as a non-invasive, non-viral trans-vascular brain gene delivery system and have been utilized for plasmid DNA encapsulation [174]. Trojan horse immunoliposomes are composed of pegylated immunoliposomes, non-viral gene expression plasmids encapsulated inside vehicles, and endogenous peptide or peptidomimetic monoclonal antibodies that act as a molecular Trojan horse (MTH) [175] and undergo receptor mediated transport through the BBB. Here, a monoclonal antibody binds to a specific receptor located on the BBB and on the brain cellular membrane such as the insulin (IR) or transferrin receptors (TfR), which mediate receptor mediated transcytosis across the BBB followed by endocytosis into brain cells [176].
One example of the application of such systems is the delivery of luciferase and β-galactosidase reporter genes to the murine brain [177]. Exogenous genes encoding for luciferase or β-galactosidase were targeted,using pegylated immunoliposomes conjugated with rat 8D3 mAb, to the mouse transferrin receptor. The Trojan horse ILs were then injected intravenously into adult mice and the tissue-specific expression of different genes in brain and peripheral organs was examined 48 h post administration. A global expression of transgenes in both brain and peripheral tissues was observed. The pattern of expression was parallel to the expression of the neuronal transferrin receptor, which is ubiquitous in brain tissue. Widespread expression of an exogenous gene in brain and peripheral organs was also observed after intravenous administration of Trojan horse immunoliposomes carrying a β-galactosidase gene packaged in an expression plasmid with a short 3'-UTR lacking a heterologous intron (pSV-β-galactosidase). The THLs were conjugated with mAb OX26 targeted to the rat TfR or with IgG_{2a} for isotype control and were then injected into adult male Sprague-Dawley rats where gene expression was measured using β-galactosidase histochemistry. No measurable β-galactosidase gene expression was observed after administration of THL coupled with IgG_{2a}. However, abundant β-galactosidase gene expression in brain and peripheral organs was found after administration of TfR targeted THL and persisted for at least six days after a single dose [178]. In prior work, TfR targeted THLs carrying a luciferase expression vector containing a heterologous intron in the 3'-untranslated region (UTR) were used and demonstrated gene expression in the brain with a peak at 48 hours after a single intravenous administration [179].
Next, the luciferase expression plasmid clone 790 was encapsulated in pegylated immunoliposomes and targeted with three different monoclonal antibodies: the 83-14 murine mAb to the human insulin receptor (HIRmAb-IL), the 528 murine mAb to the human epidermal growth factor receptor (EGFRmAb-IL), and the OX26 murine mAb to the rat transferring receptor (TfRmAb-IL) and additionally, IgG_{2a} – $mIgA_{2a}$-IL was used as isotype control [180]. The level of luciferase gene expression measured in Human U87 glioma cells and rat RG2 glioma cells was much higher after administration of HIRmAb-IL relative to targeting *via* the rat TfR or human EGFR. Human U87 glioma cells treated HIRmAb-IL carrying clone 753 (which lacks the EBNA-1/oriP system) exhibited lower levels of luciferase gene expression compared to that resulting from HIRmAb-IL with 790 clone plasmid DNA. No gene expression was observed after administration of non-specific isotype control $mIgG_{2a}$-IL [180].

Similar results were observed after intravenous administration of plasmid DNA encoding either luciferase or β-galactosidase encapsulated with pegylated ILs conjugated to the mAb for the human insulin receptor (HIR), targeting the Rhesus monkey brain. The HIRmAb-IL carrying the exogenous gene underwent transcytosis through the blood brain barrier followed by endocytosis across the neuronal plasma membrane. The luciferase gene expression was 50-fold higher with the HIRmAb-IL than with the TfRmAb-IL [181]. It was also found that the luciferase gene expression in primate brain decayed with a $t_{1/2}$ of about two days after administration of HIRmAb-IL carrying the luciferase expression plasmid, indicating a parallel degradation of exogenous plasmid DNA [182].
Zhang *et al.* investigated the effect of SV40-*β*-galactosidase encapsulated in pegylated liposomes coupled with mAb targeted to HIR or inside pegylated liposomes without antibody [183]. *In vitro* studies with U87 glioma cells demonstrated a linear increase in the level of *β*-galactosidase histochemical product with time and showed that >90% of the cells were transfected with the exogenous gene when treated with HIR targeted IL compared with non-targeted liposomes. The delivery of the luciferase reporter gene to the U87 glioma cells was tested with HIR targeted IL or with lipofectamine, too. Luciferase gene expression was initially measured for clone 781 driven by the CMV promoter and compared to 790 driven by the SV40 promoter and showed a significant increase in expression of luciferase reporter gene under the influence of the latter. Comparable levels of gene expression were noticed after application of CV40-luciferase plasmid to U87 cells with lipofectamine and immunoliposomes coupled with HIR targeted mAb. A plasmid encoding for EGFR antisense mRNA (clone 882) encapsulated in HIR targeted IL showed a >70% reduction in U87 cell proliferation based on [^{3}H]-thymidine incorporation and gave about an 80% decrease of the immunoreactive EGFR as shown by Western blot [183].
Zhao *et al.* compared the transfection ability and efficiency of liposomes, immunoliposomes, and brain specific immunoliposomes for exogenous gene delivery through the BBB [184]. Conventional liposomes encapsulating pCMV (human cytomegalovirus promoter)-*LacZ* plasmid, immunoliposomes conjugated with OX26 mAb against TfR encapsulating pCMV-*LacZ* plasmid, or brain specific ILs encapsulating pGFAP (glial fibrillary acidic protein promoter)-*LacZ* plasmid coupled with OX26 antibody were injected into rats *via* the venous system. Measurement of *LacZ* mRNA levels and *β*-galactosidase activity in the brain and peripheral organs showed that OX26-pCMV-ILs were able to deliver exogenous genes into the brain at much lower dose (80μg of plasmid) than pCMV-liposomes (300μg of plasmid). In addition, OX26-pCMV-IL demonstrated a high *LacZ* expression in peripheral organs, which suggested that the vector was not organ specific. However, the IL containing brain specific pGFAP-*LacZ* plasmid, which is only activated in glial cells, lead to reduced gene expression in peripheral organs such as heart, liver, and lung. This suggests that expression of an exogenous gene in the target organ is a function of receptor specificity of the ligand and tissue specificity of the promoter [184].
The lysosomal *β*-glucuronidase (GUSB) enzyme was examined for the delivery of non-viral plasmid DNA through the BBB in mice that are null for the *β*-glucuronidase (GUSB) lysosomal enzyme, which is a model for type VII mucopolysaccharidosis [185]. A human GUSB expression plasmid, designated pCMV-GUSB, was genetically engineered and encapsulated in Trojan horse liposomes coupled with 8D3 mAb targeted to the mouse TfR. Upon application to fibroblast cell cultures from GUSB null mice a >50-fold higher enzyme activity compared to untreated fibroblasts was observed. This effect persisted for about two weeks following a single application. The GUSB enzyme activity was also measured *in vivo* after intravenous administration of ILs carrying pCMV-GUSB into adult GUSB null mice. A 10-fold increased GUSB enzyme activity was observed in organs with high microvascular TfR such as brain, liver, spleen, lung and kidney, but not in the heart [185].
Pegylated ILs targeted with anti-mouse transferrin monoclonal antibody 8D3 were also used for the delivery of polyethylenimine(PEI)/ODN polyplexes into the brain [186]. Its *in vitro* cellular uptake was studied in the mouse brain endothelial cell line bEnd5 by measuring the amount of cell associated ^{32} P-ODN after application of the targeted or non-targeted liposomes with the polyplex. Antibody modified ILs resulted in a significant increase of cells with associated and internalized ^{32}P-ODN. The 8D3 modified IL also gave a significant pharmacological effect when a specific transcription factor decoy to NK-κ was delivered. In addition, liposomes conjugated with antibody showed prolonged circulation in the blood after administration and decreased clearance compared to PEI/ODN without specific antibody. Similar results were found *in vivo* in male Balb/c mice [186].

7.1.2 Delivery of Tyrosine Hydroxylase

The brain delivery of tyrosine hydroxylase (TH) therapeutic genes with Trojan horse immunoliposomes for a transvascular gene therapy of Parkinson's disease presents another interesting application [187,188]. The TH expression plasmid was encapsulated in pegylated immunoliposomes coupled with murine OX26 mAb targeted to the rat TfR or with the mouse IgG_{2a} isotype control. In parallel, rats were lesioned with the neurotoxin 6-hydroxydopamine injected into the medial forebrain bundle, which results in an experimental Parkinson's disease

model. Only the TfRmAb targeted-IL showed specificity and was able to undergo receptor-mediated transcytosis across the BBB and receptor mediated endocytosis into neurons behind the BBB by accessing the TfR. The efficacy of the tyrosine hydroxylase gene therapy was verified by measuring the stratial enzyme activity and by the rotational behavior of rats in response to intraperitoneal apomorphine. This gene therapy resulted in a complete normalization of stratial tyrosine hydroxylase enzyme activity ipsilateral to the lesion and gave an 82% reduction of abnormal rotational behavior [187,188]. The long term, weekly administration of TH expression plasmids encapsulated in TfR mAb targeting Trojan horse ILs did not show any toxic side effects in rats, nor were changes in serum chemistry, organ histology, body weight or inflammatory reactions observed in the brain [189].

7.1.3 RNAi Delivery to the Brain

Another promising strategy is the development of antisense therapeutics which knock down gene expression post-transcriptionally is the use of RNA interference (RNAi) [176]. The delivery of short hairpin RNA (shRNA) was demonstrated in a brain tumor model using an anti-luciferase shRNA expression plasmid encapsulated in pegylated immunoliposomes conjugated with mouse OX26 mAb targeted to the rat TfR [190]. Short hairpin RNA is an artificial form of RNA interference which recognizes target mRNA via base-pairing interactions and has the ability to induce gene silencing in mammalian cells [191]. C6 rat glioma cells were transfected with the luciferase gene and implanted in the caudate-putamen nucleus of adult male Fisher CD344 rats, which resulted in the generation of luciferase-expressing brain cancer. Next, Trojan horse ILs carrying clone 952 plasmid DNA were injected intravenously into the tumor bearing rats and the luciferase activity was measured in the tumor and the contralateral brain. The TfRmAb targeted-ILs were able to inhibit 90% of the luciferase gene expression in the brain tumor and this effect lasted for at least five days after a single injection. No luciferase enzyme activity was observed in the contralateral brain [190].
RNAi based gene therapy was also used to target the human epidermal growth factor receptor in experimental brain cancer in severe combined immunodeficient (scid) mice [192]. The epidermal growth factor is expressed in many tumors including the brain and plays a pro-angiogenic role in cancer growth. Thus, knock down of the tumor EGFR is one of the goals of brain gene therapy [193]. An expression plasmid encoding a short hairpin RNA targeting a specific sequence (nucleotides 2529-2557) within EGFR mRNA was encapsulated in pegylated immunoliposomes coupled with two receptor-specific mAb: the murine 83-14 mAb targeting the human insulin receptor (HIR) and rat 8D3 mAb targeting the mouse TfR. Delivery of the RNAi expression plasmid gave 95% suppression of EGFR function. An intravenous administration of the Trojan horse IL resulted in knock-down of immunoreactive EGFR in the tumor *in vivo* and resulted in an 88% increase in survival time of scid adult mice implanted with human U87 glioma cells [192]. Similar results were obtained with identical Trojan horse liposomes using the luciferase expression plasmid DNA encapsulated in pegylated immunoliposomes [194].

8. CANCER THERAPY

8.1 Breast Cancer

8.1.1 anti-HER2

Most IL studies on breast cancer target the human epidermal growth factor receptor 2 encoded by gene ERBB2. This is commonly referred to as HER2 and is amplified in approximately 18-20% of breast cancers [195]. Some level of HER2 overexpression is documented in many other types of cancer, including those of the prostate, brain, bladder, lungs, and gastric system [196,197,198]. The HER2 protooncogene encodes a 1255 amino acid, 185 kDa receptor tyrosine kinase (RTK). It is a member of the class I RTK family, along with the epidermal growth factor receptor (EGFR) HER3 (ErbB3), and HER4 (ErbB4) [199]. Women with breast cancers that overexpress HER2 exhibit an aggressive form of the disease with significantly shortened overall survival. Studies indicate that the amplification of HER2 plays a direct role in the pathogenesis of this cancer and thus it is an attractive target for drug delivery [200]. The receptor is located in the cell membrane and can be targeted easily with commercially available antibodies [197,201,202]. After binding to HER2 the receptor-antibody complex is internalized into the cell endosome providing selective drug delivery into the cell cytoplasm [196,197,199].
Park *et al.* generated anti-HER2 immunoliposomes using long circulating liposomes coupled with monoclonal antibody anti-HER2 to provide targeted drug delivery to HER2 overexpressing cells [29,199,203]. They showed that anti-HER2 ILs bound efficiently to and were internalized into HER2 overexpressing cells, resulting in an intracellular drug delivery which was 700-fold greater than with non-targeted sterically stabilized lipsosomes. Using

HER2-overexpressing xenograft models (BT-474/MSKCC, BT-474/SF, MDA-MB-453 and MCF/HER2 implanted subcutaneously in the back or flank of nude mice) and doxorubicin (Dox) encapsulated in liposomes linked with anti-HER2 they demonstrated anticancer activity including growth inhibition, regression, and cure. The IL with Dox gave better results than other treatment conditions such as: free Dox, liposomal Dox, free anti-HER2 mAb trastuzumab as well as combination therapies of free Dox with free mAb or liposomal Dox together with free mAb [199,204].

A similar approach was used with breast cancer cells overexpressing HER2 (BT-474 and SK-BR-3) and MDA-MB-231, which expresses low levels of HER2, using an IL conjugated with anti HER2 antibody trastuzumab (Herceptin®) and encapsulated paclitaxel [205,206]. The latter is a taxane widely used in chemotherapy and highly effective against breast cancer, ovarian carcinoma, head & neck cancers, and non-small-cell lung cancer. The antibody modified liposomes showed significantly higher cellular uptake of paclitaxel compared to nonmodified liposomes in the HER2 overexpressing cancer cells. In MDA-MB-231 cells, the difference between targeted and non-targeted vehicles was not noticeable. Furthermore, immunoliposomes demonstrated significantly higher tissue distribution of paclitaxel in *in vivo* studies and better antitumor efficacy than non-targeted liposomes. Both carrier systems showed similar tumor tissue distribution and antitumor activity in cells with low levels of HER2 [205].

Likewise, melittin, which has recently shown anticancer properties [207], could be transported into human breast cancer cells using pegylated ILs linked with trastuzumab [208]. These ILs were able to decrease cancer cell viability in a dose-response dependent manner and in correlation to the level of HER2 expression in various Human breast cancer cells. Maximum efficacy was achieved against MCF7/HER2 and SK-BR3 cells which exhibit a high level of HER2 expression on their surface. Moreover, the incorporation of melittin into the immunoliposome increased its cytotoxic efficacy by about four times and provided better selectivity [208].

Other examples are immunoliposomal formulations of vincristine and vinblastine linked with anti-HER2 scFv F5 antibody, which were tested with the human mammary carcinoma cell lines SK-BR3 and BT474-M2. *In vitro*, anti-HER2 ILs with vincristine exhibited a dramatically improved cytotoxicity. The IC_{50} for the immunoliposomal formulation of vincristine was 253-fold lower in SK-BR3 cells and 63-fold lower in BT474-M2 cells compared to non-targeted formulations. *In vivo* anti-tumor efficacy of various vincristine formulations was studied in mice with HER2-overexpressing BT474-M2 xenografts. The ILs were the most active of all formulations tested, with five of nine mice showing complete tumor regression, and significant improvements in efficacy [209]. A facile ELISA test for the quantification of F5 in plasma has been developed [210].

Anti-HER2 antibody modified liposomes can be also used to deliver immunotoxins to cancer cells. Here, PE38KDEL-loaded anti-HER2 PEGylated liposomes were constructed with Fab' of recombinant humanized anti-HER2 monoclonal antibody (anti-HER2 Fab') [211]. PE38KDEL is a 38 kDa mutant form of *Pseudomonas* exotoxin A (PE) and exhibits superior anti-tumor activity and less non-specific toxicity than free PE [212]. *In vitro* studies were carried out on Human breast cancer cell lines and SK-BR3 cells which were most sensitive to antibody modified vehicles (IC_{50} = 36.63 pM) in comparison to MDA-MB-231 cells (IC_{50} = 291.92 pM), and MCF-7 (IC_{50} > 2000 pM). Incubation of all three cell lines with control immunotoxins PE-HER (PE38KDEL : rhuMAbHER2= 1:1 molar ratio) gave similar results, which suggests that the binding ability and specificity of the anti-HER IL and control immunotoxins to HER2 antigen is critical for cytotoxicity induction. However, targeted liposomes demonstrated better cytotoxicity against SK-BR3 cells (IC_{50} = 36.63±11.67 pM) than PE-HER (IC_{50} = 83.98±18.68 pM). Similar results were obtained with MDA-MB-231 cells [211].

Antisense oligonucleotides, which inhibit gene expression, are also promising therapeutic tools for cancer therapy [213]. In one study, Rodriguez *et al.* tried to target antisense oligonucleotides against dihydrofolate reductase RNA in Human breast cancer cells that overexpress HER2. They used ILs that contained a specific p185/HER2 Ab to improve the sensitivity of the oligonucleotide treatment [214]. Dihydrofolate reductase is involved in the *de novo* synthesis of thymidine, purines, and glycine, is required for cell proliferation, and is inhibited by methotrexate. ILs were prepared based on the high affinity between streptavidin and biotin and the use of biotinylated antibodies. First, streptavidin was covalently attached to DOPE and mixed with the cationic liposome *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) complexed with the antisense oligonucleotide. A second approach was based on the binding of streptavidin to Ab and oligonucleotide, both biotinylated, and the latter complexed with DOTAP. The full ILs were more toxic than the antisense oligonucleotide in the absence of the antibody, thus indicating the increased sensitivity of the treatment [214].

HER2 overexpressing cancers are also potentially good candidates for treatment with histone deacetylase (HDAC) inhibitors. These down-regulate the receptor, decrease synthesis of new HER2 transcripts, increase proteosomal degradation of the overexpressed receptor protein, increase the decay of mature ErbB2 mRNA, and sensitize HER2-overexpressing cells to a variety of antitumor agents, including trastuzumab [215,216]. ILs encapsulating a potent hydroxamate-based HDAC inhibitor (LAQ824) were successfully prepared and were specifically taken up into

HER2 receptor-overexpressing human breast cancer BT474 xenografts [216]. Antibody modified LAQ824 showed greater *in vitro* cytotoxicity against SKBR3 breast carcinoma cells than non-conjugated liposomes and the free drug. Enhanced cytotoxic activity was also observed *in vivo* against BT474 xenografts. Both targeted and non-targeted IL formulations were effective in retarding tumor growth, the former being more effective. Treatment effects on fully grown BT474 xenografts were tested by measuring histone acetylation and HER2 mRNA levels. Only a small increase in acetylation was observed for the tumors treated with free LAQ824. Substantially increased levels of histone 4 (H4) acetylation were observed after treatment with liposomal and immunoliposomal LAQ824. RNA purified from the same treated samples and analyzed by Northern blotting showed significantly reduced amounts of HER2 mRNA 24 h after treatment with liposomal and immunoliposomal formulations and was more pronounced in IL-LAQ824. No significant difference was noticed after treatment with free LAQ824 [216].
Recently, a new immunoliposome targeting the heparin-binding epidermal growth factor-like growth factor (HB-EGF) was developed [217]. HB-EGF stimulates the growth of various cells in an autocrine or paracrine manner and is highly expressed in many cancer cells, including breast cancer [217,218]. Doxorubicin loaded liposomes coupled with the Fab' fragment of anti-human HB-EGF mAb injected intravenously into mice bearing MDA-MB-231 cancer cells not only resulted in a strong suppression of the tumor growth but regression, too [217].

8.1.2 anti-HER2 and Hyperthermia

Hyperthermia is an alternative treatment modality and is based on the direct cell killing effect of temperatures above 42.5 °C, an effect that can be enhanced by repeated heating. A modern approach was described by Kikumori *et al.* who constructed trastuzumab linked ILs, which contained magnetite nanoparticles [219]. Such agents generate heat in an alternating magnetic field and have the advantage that they can be activated several times over as tumor cells are heated without damaging the surrounding healthy tissues [219,220]. In contrast to conventional hyperthermic techniques, these agents allow the use of temperatures of up to 45 °C without substantial collateral damage. In conventional hyperthermia ($T \approx 42$ °C) the treated tumor cells undergo apoptosis, whilst here cancer cells undergo necrosis [219,221]. Using longitudinal insertion into the cancer nodules, almost all injected magnetite was delivered to the cells overexpressing HER2 after 48 h, while HER2 negative tumors were virtually cleared in the same period. This indicates that retention of magnetite is dependent on conjugation to the anti HER2 antibody. Tumor growth was almost entirely suppressed by just two hyperthermic treatments and this demonstrates the promise of the approach as an alternative therapeutic modality for refractory breast cancer [219]. Similar positive results were observed during *in vitro* tests with magnetic cationic liposomes linked with Herceptin® or rituxan. SK-Br3 cells showed uptake of 60% of the magnetite nanoparticles and subsequently stimulated hyperthermia resulted in strong cytotoxic effects [220].
Kullberg *et al.* enhanced the specificity of drug delivery to tumor tissue by using a two-component delivery system that required the interaction of two different liposomes within the same endosome for cytoplasmic delivery. As base material they used a thermosensitive IL conjugated with the anti HER2 mAb trastuzumab. These ILs release their content upon heating to above body temperature. This intriguing two-component approach is based on separate delivery vehicles, which differ only in the substance that they encapsulate, which interact within endosomes of the target cell before actual delivery of the drug. Thus, after the receptor-antibody complex is internalized into the cell endosome, two endosomes may fuse together to form larger vehicles. Localized hyperthermia may then cause the thermosensitive liposomes to release the two substances into the one fused endosome, where they can interact to deliver the drug into the cell cytoplasm. An experiment using the internalization of two sets of thermosensitive ILs with either a fluorescent rhodamine or calcein probe revealed a significantly increased specificity for cells overexpressing the HER2 receptor. The data indicated that a two-component therapy was seven- to nine fold more effective in targeting HER2 overexpressing cells, compared to one-component delivery. Note, for the calcein system that the tumor:tissue ratio was 35:1 [197].

8.1.3 anti-EGFR

A second target used in cancer studies is the epidermal growth factor receptor (EGFR, ErbB). It is involved in the pathogenesis of many tumors and is a proven target for cancer therapy [222]. Anti-EGFR ILs were designed using Fab' fragments of mAb C225 (cetuximab), covalently linked to liposomes containing an anticancer drug [223]. Similarly, a folate-folate binding protein has been used to construct C225 IL [224]. Anti-EGFR immunoliposomes showed long circulation and stable drug retention, irrespective of the mAb fragment present, as well as efficient accumulation in tumors. The ILs internalized extensively within tumor cells (92% of cells compared to <5% for non-targeted liposomes) indicating different mechanisms of delivery at the cellular level. Anti-EGFR ILs bind and

internalize in EGFR-overexpressing tumor cells *in vitro* [225] and *in vivo* [223]. The antitumor effects of anti-EGFR IL were significant and superior to all other treatments, including the corresponding free or non-targeted drugs such as doxorubicin, epirubicin, and vinorelbine [223].

8.1.4 anti-RON Receptor Tyrosine Kinase

The RON receptor tyrosine kinase is a member of the Met proto-oncogene family and is mainly expressed at relatively low levels in various epithelial cells. However, overexpression is often observed in colon and breast cancer cells and thus it presents a suitable target [226,227]. Immunoliposomes loaded with doxorubicin were formulated followed by post insertion of mAb Zt/g4, Zt/c1, or their Fab fragments, which are specific for the RON receptor tyrosine kinase [228]. Flow cytometry analysis showed that both Zt/g4 or Zt/c1-IL bind to cancer cells and cause RON internalization resulting in significant uptake of the targeted liposomes. This uptake was confirmed by confocal fluorescence spectroscopy. Both types of IL demonstrated increased cytotoxicity *in vitro* in T-47D breast cancer cells with significant reduction of the IC_{50} compared to liposomes conjugated with IgG as control. Interestingly, Zt/g4 IL encapsulated doxorubicin also showed effectiveness in killing HCC1937 cancer cells, a cell line which is insensitive to pegylated liposomal doxorubicin. The efficiency of Fab fragments for targeted delivery was relatively low compared to whole antibodies linked to doxorubicin containing liposomes. Thus, anti-RON antibody-directed drug delivery is an effective means to deliver cytotoxic drugs [228].

8.2 Colon Cancer

Colorectal cancer is the second most common cause of cancer related deaths after lung and breast cancer [229]. Surgery is the only treatment option that can efficiently remove primary tumors. However, the prognosis of many patients following surgery is poor and 50-60% of all patients develop metastases within the next 5 years. Chemotherapy using fluoropyrimidines such as 5-fluorouracil (5-FU) or 5-fluoro-2'-deoxyuridine (FUdR) is often applied in combination with modulators such as leucovorin, methotrexate or interferon, but with only a minimal increase in survival rates. These therapies are limited by short circulatory half-life and severe toxicity towards rapidly proliferating tissues such as the bone marrow or GI tract. The application of targeted drug delivery may offer improvements through the limitation of side effects, increased tumor specificity and prolonged circulatory half-life [229].

One example of an IL in colon cancer therapy was the conjugation of Fab' fragments of recombinant humanized anti-TAG-72 monoclonal antibody (HuCC49) to sterically stabilized liposomes encapsulating plasmid DNA (pDNA) [230]. This IL was able to adhere to the surface of TAG-72 overexpressing LS174T human colon cancer cells more efficiently than conventional liposomes. Intravenously administered antibody modified liposomes were superior in delivering the transgenes pCMVLuc or pEGFP-N1 to xenografted tumor tissues LS174 T overexpressing TAG-72 than non-targeted vehicles. Conventional cationic liposomes became deposited in pulmonary capillary beds due to aggregation with serum proteins, whereas ILs with electrically neutral surfaces effectively passed through the lungs and also avoided RES in the liver. This novel immunoliposome anti-TAG-72 was also able to inhibit LS174T tumor growth *in vivo* better than the corresponding free pDNA or empty anti-TAG-72 IL [230].

Another type of tumor-specific IL was prepared by coupling anti-BCG (anti-*Mycobacterium bovis*) mAb to pH-sensitive fusogenic liposomes modified with succinylated polyglycidol [231]. These liposomes showed higher selectivity for colon carcinoma 26 cells, which share a common antigen with BCG, in comparison to conventional liposomes. The presence of 50 µg of free antibody decreased cell binding of BCG-sucPG liposomes, which suggest that the binding of antibody modified vehicles to colon cancer 26 cells is receptor specific. Uptake into colon 26 cells after binding appears to be endocytotic [231].

On the other hand, Koning *et al.* suggested that the enhanced uptake by cancer cells is due to tumor associated macrophages [229,232,233,234]. They conjugated mAb CC52, which is specific for CC531 rat colon carcinoma, to PEG liposomes, either in a random orientation *via* a lipid anchor (MPB-PEG-liposomes) or uniformly oriented at the distal end of the PEG chain (Hz-PEG-liposomes) and containing a dipalmitoylated derivative of the anti-cancer drug FUdR (FUdR-dP) as a prodrug in the bilayers. Type B immunoliposomes decreased the amount of antibody that could be coupled compared to type A ILs. The lower level of Ab with the presence of PEG molecules on type B vehicles resulted in a lower level of association with the tumor cells than type A ILs. CC52 Ab attached to the distal end of polyethylene glycol chain (type C IL) significantly enhanced the association with CC531 cells, even with a relatively low antibody density [229]. They also compared liposomes with varying amounts of Ab CC52 with regard to their target-binding capacity and uptake by Kupffer cells. Together with the splenic macrophages the latter are largely responsible for competitive removal of liposomes from the circulation [233]. The balance between the

association with target cells and association with Kupffer cells appears to shift toward the latter when the amount of Ab was increased. Sterically stabilized liposomes with a relatively low number of Ab attached to the distal end of the PEG chain demonstrated a significantly higher ratio of uptake by cancer cells relative to Kupffer cells and gave better targeting to cancer tissue than other ILs [233].

It was suggested that tumor growth in the body influences the pharmacokinetics of ILs. High Ab density on the liposomal surface increased elimination from the circulation and concomitantly increased uptake by, most of all, the liver. It was shown that ILs retain the anticancer prodrug FUdR-dP during circulation. The uptake of vehicles by metastatic colon tumor nodules in the liver was enhanced *via* Ab attachment to levels comparable to uptake in liver tissue. Thus, long-circulating ILs can localize to a considerable extent in liver metastatic nodules. Moreover, increased uptake of antibody-modified liposomes in the tumor can be achieved with tumor specific and tumor irrelevant antibodies alike. This may indicate that uptake of immunoliposomes by metastatic tumor nodules is mostly mediated with tumor macrophages rather than through specific interaction with tumor cells.

This was further supported by experiments with a ^{14}C-cholesteryloleate labeled bilayer. Upon IL internalization, intralysosomal degradation and release of the labeled ^{14}C-cholesteryloleate from the cells would occur [235]. Nevertheless, only a very low level of the label was detected in the medium after 24 h incubation, indicating that cell-bound targeted liposomes were hardly internalized by CC531 cells. Similar results were obtained using inhibitors of endocytosis and lysosomal degradation, which had no effect on the association of CC52 modified vehicles with CC531 cells [234]. In contrast, incorporated FUdR-dP was readily and almost completely hydrolyzed by tumor cells. This suggests that the uptake of the prodrug proceeds independently of the IL internalization. FUdR or its metabolites appeared predominantly in the medium whilst only 10-20% remained intracellularly, which indicates that hydrolysis occurs intracellularly [229]. It was also demonstrated that after transfer of the FUdR-dP prodrug to the cell membrane it is internalized *via* endocytosis or pinocytosis and then hydrolyzed in the lysosomes to active FUdR [229].

A different approach was taken by Wang *et al.* in order to enhance the radiosensitivity of Human colon cancer cells by docetaxel [236]. Docetaxel ILs were prepared by coupling antibodies against carcinoembryonic antigen (CEA) to cyanuric chloride at the distal end of PEG chain. Cytotoxicity studies of the targeted liposomes were carried out with a LoVo adenocarcinoma cell line expressing CEA. Anti-CEA Ab modified liposomes showed efficient and specific cytotoxicity for LoVo cells and enhanced the effects of docetaxel and led to radiosensitization; apoptosis, monitored by flow cytometry, was significantly higher in LoVo cells due to the combined effects of IL, docetaxel, and radiation. Immunochemical tests demonstrated that survivin was localized mainly in the cytoplasm and a semiquantitive analysis indicated that low survivin expression was associated with higher radiosensitivity after treatment with anti-CEA IL combined with irradiation. Thus, CEA expressing cells are radiosensitized through decreased expression of survivin [236].

As mentioned before, the RON receptor tyrosine kinase can be used as a cancer target, too. It is also of interest in the context of hypoxia, *i.e.* the reduction of tissue oxygen tension in certain regions of a tumor mass, which plays a key regulatory role in tumor growth and malignant progression [237,238]. Various studies showed that hypoxic cancer cells are highly resistant to apoptotic death and are malignant with aggressive behavior [238]. Antibody-directed RON targeting seems a promising approach for delivery of agents such as doxorubicin to enhance cytotoxicity against hypoxic cancer cells [239]. For example, the Zt/g4 antibody against the RON receptor is highly specific and causes RON dimerization, followed by internalization into the cytoplasm [228]. *In vitro* studies showed that Zt/g4 antibody modified liposomes can partially overcome resistance and are effective cytotoxic agents against hypoxic cancer cells [239]. High level of RON are observed in colon cancer cells under acute hypoxia and this expression is functional in terms of ligand binding, receptor internalization, and uptake of antibody directed ILs. It was shown that RON expression mediates high levels of binding and cytoplasmic internalization of Zt/g4-Dox-IL, which effectively killed hypoxic HCT116 and SW620 cells with reduced IC_{50} values compared to free Dox and pegylated-liposomal Dox. No cytotoxic effect was observed in HCC1937 cells with diminished RON expression under hypoxia. Even though Zt/g4 modified Dox liposomes do not completely overcome the resistance acquired by hypoxic cells, their increased cytotoxicity against hypoxic cancer cells resistant to Dox make them a promising basis for further developments [239].

8.3 Leukemia

Immunoliposomes have been also found useful in the treatment of leukemia, with chemotherapy being the principal treatment modality for this disease. However, recently considerable progress has been achieved in the management of leukemia. Clinically approved antibodies such as alemtuzumab or ritiximab show promising results [240,241]. To

improve targeting and effectiveness of these therapies, liposomes modified with such antibodies have been developed as well.

8.3.1 Standard Immunoliposomes

In order to have a suitable test system Lapalonbella *et al.* developed clones that stably expressed high or low levels of surface CD52 and comparable high levels of human leukocyte antigens HLA-DR, CD19, CD20, CD37, CD40 and CD32 by limiting dilution of the Raji Burkitt's lymphoma cell line [242]. They demonstrated that alemtuzumab is able to induce *in vitro* cell lysis in CD52 high clone Raji cells but not in the CD52 low clone. These cells were also sensitive to antibody mediated cytotoxicity. *In vivo* results indicated that the median survival of mice inoculated with the high CD52 clone and treated with alemtuzumab was significantly increased. For the low CD52 clone no difference was found between trastuzumab and alemtuzumab treated groups. Human CD19+/CD52+ cells were found after 24 d in the bone marrow in high CD52 clone inoculated mice treated with trastuzumab but not with alemtuzumab. No evidence of Human CD19+/CD52+ cells was found in the bone marrow of similarly treated low CD52 clone inoculated mice. Thus, these lymphoma cell clones are very useful models for therapeutic studies and the evaluation of existing and novel antibodies directed against CD52. To determine the possible use of this model for CD52 targeted delivery, the delivery of FAM-labeled oligodeoxyribo nucleotide (FAM-ODN) containing liposomes (alemtuzumab- or trastuzumab-coated IL) was carried out and compared in the high CD52 clone and primary B-CLL cells *in vitro*. Flow cytometric and fluorescence microscopy demonstrated a higher uptake of the alemtuzumab-coated ILs encapsulated FAM-ODN than trastuzumab coated control liposomes [242].
Novel liposomes containing imatinib and coupled with anti-CD19 were developed to efficiently kill positive acute lymphoblastic leukemia cells [243]. Patients with Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL) have poor prognosis despite intensive therapeutic intervention [244]. Targeted molecular therapy has been introduced, and a BCR-ABL tyrosine kinase inhibitor, imatinib, has been proven to be an effective treatment for Ph+ ALL. However, most patients rapidly become resistant to imatinib [245]. Thus, a new delivery system using antibody modified liposomes allows the specific targeting of imatinib, as CD19 is highly expressed only in cells of the B-cell lineage including Ph+ ALL, and is quickly internalized after antibody binding [246]. Therefore, sterically stabilized liposomes were conjugated with antibody anti-CD19 mAb CLB-CD19 to encapsulate imatinib [243]. The study demonstrated that antibody modified liposomes were almost completely internalized into all CD19+ tested cell lines compared to empty liposomes and PEGylated liposomes without antibody. The internalization of ILs was inhibited by the CLB-CD19 antibody in a dose-dependent manner, which strongly indicates that internalization of IL was specifically mediated by the cell surface CD19 molecule. The IC_{50} values of CD19 antibody modified liposomes for five different types of Ph+ ALL cells ranged from 0.02 to 0.9 μM; this was about 11times lower than those of free imatinib and non-modified PEG liposomes. Moreover, they were able to inhibit cobblestone area formation by primary Ph+ ALL cells more significantly than free imatinib or imatinib in non-targeted liposomes. This suggests that these vehicles can used for the treatment of clonogenic leukemic cells, namely leukemic stem cells. The utility of this treatment system for patients with Ph+ ALL acquired resistance was also shown [243].
Another prospect was the use of liposomes coupled with antagonistic humanized monoclonal antibody, milatuzumab, against CD74 [247], a type II transmembrane protein expressed on B cells [248]. Results showed that milatuzumab mediates direct cytotoxicity in CLL cells by a mechanism involving aggregation of the protein on the cell surface. Combining milatuzumab with liposomes improved the cytotoxic response in CLL. Liposomal formulations of milatuzumab induced more cell death in CLL in comparison to liposomes conjugated with non-specific antibody IgG and significantly more than milatuzumab with anti-F_c crosslinker. This cytotoxicity was observed even without packaging the immunoliposome with a chemotherapeutic agent, such as doxorubicin [247].
Similar to other areas ILs with different ligands and encapsulated doxorubicin have been used in leukemia treatment. One of these examples is CD22 targeted PEGylated liposomal doxorubicin for the treatment of non-Hodgkin's lymphoma (NHL). CD22 is a B-lymphocyte-specific glycoprotein expressed by mature B-lymphocytes and anti-CD22 mAb HB22.7 has been effectively coupled to liposomes containing Dox and was found to bind only to CD22 expressing NHL cell lines. The HB22.7 component was internalized upon binding. For CD22+ cells the IC_{50} of immunoliposomes was lower than for pegylated liposomes. No difference was observed between targeted and non-targeted vehicles in the CD22- cell line and both were less toxic than nonliposomal doxorubicin in CD22+ and CD22- cell lines. In addition, the HB22.7 modified liposomes remained bound to the cells and continued drug release and cytotoxicity to the required area, while non-targeted and free Dox were easily separated from the cells [249]. Similarly, a liposomal vehicle coupled with monoclonal antibody HD37-CCH against B-cell CD19 antigen was used and it exhibited an 11-fold higher cytotoxicity to human Burkitt's lymphoma cell lines than non-targeted vehicles [250,251].

Long-circulating liposomes loaded with Dox targeted to the CD34 antigen present on Human myelogenous leukemia KG-1a cells were more problematic. While the ILs showed higher cytotoxicity against KG-1a cancer cells than unmodified liposomes they had only the same effect as the free drug. Endocytosis studies showed that the anti-CD34 My10 mAb modified liposomes were not internalized but remained in the cell membrane and caused a cytotoxic effect through the release of Dox into the space near the membrane [252].
Norcantharidin encapsulated liposomes modified with a novel murine antihuman CD19 mAb 2E8 (2E8-NCTD-liposomes), which specifically recognize and bind CD19+ leukemia cells, were another approach taken by Zhang's group [253]. Norcantharidin is an anti-cancer drug derived from cantharidin, a cancer killing component from Chinese traditional medicine which kills cancer cells by inhibiting phosphatase A2, blocking the cell cycle and inducing apoptosis [254]. The targeting efficiency of the antibody modified liposomes on CD19+ (Nalm-6) cells was significantly higher than in CD19- cells and that of non-targeted formulations. The survival percentage of Nalm-6 cells was two times lower than that of Molt-3 cells treated with 2E8 antibody modified liposomes at the same concentration of norcantharidin, which indicates that novel formulations can decrease non-specific cytotoxicity to CD19- cells [253].
A different strategy used pH-sensitive polymer-based ILs targeting the CD33 antigen for the delivery of 1-β-arabinofuranosylcytosine (ara-C) to Human myeloid leukemia cells [255,256]. 1-β-Arabinofuranosylcytosine is a pyrimidine analog pro-drug which penetrates cells through carrier mediated transporters used by other nucleosides where after internalization it is metabolized to the active triphosphate form, which is cytotoxic [257]. Ara-C is the gold standard for first line treatment of acute myeloid leukemia and is part of induction therapy, often associated with an anthracycline to improve antitumor response [258]. PEGylated liposomes were coupled with the whole anti-CD33 mAb or its Fab' fragments. *In vivo* studies in leukemic mice showed that targeting of pH-sensitive liposomes with the anti-CD33 Fab' fragment was as effective as the whole mAb in recognizing cells expressing CD33. However, the IL with the Fab' fragment exhibited a longer circulation lifetime in normal mice and provided a higher level of ara-C in blood. They were also largely preserved in immunodeficient mice inoculated with leukemic cells [255].
Studies with lenalidomide, an immunomodulatory agent, for the treatment chronic lymphocytic leukemia (CLL) showed that it down regulates the CD20 antigen on the cell surface *via* enhanced internalization without influencing transcription [259]. This agent, which also has anti-proliferative and anti-angiogenic properties, can alter the T- and NK-cell response, downregulates cytokine expression, and has an effect on vascular proliferation through modulation of VEGF [260]. CD20 antigen internalization can also enhance the delivery of an oligonucleotide incorporated into anti-CD20 (rituximab) ILs. Rituximab represent a major therapeutic advance for B-cell malignancies, including CLL and possesses several potential mechanisms of actions such as antibody dependent cellular cytotoxicity (ADCC) [261,262]. Moreover, downregulation of CD20 antigen by lenalidomide in chronic lymphocytic leukemia was accompanied by a reduction of rituximab mediated apoptosis and antibody dependent cellular cytotoxicity. An alternative strategy, which should avoid the antagonism between lenalidomide and rituximab, is to first administer rituximab and then treat with lenalidomide [259].
Immunoliposomes also gave promising results for the delivery of siRNA to CD33 positive acute myeloid leukemia cells [263] or in transfecting plasmid DNA to CD3+ T lymphocytes (Jurkat cells) [264]. The siRNA loaded immunoliposomes or immunolipoplexes showed specific binding to and internalization by CD33 expressing cells and sequence specific silencing of the leukemic AML/MTG8 fusion gene by interference RNA [263]. Similarly, plasmid (pEGlacZ) DNA was condensed with poly-L-lysine (PLK_{99}) to form a polyplex (a cationic polymer - nucleic acid complex) and later mixed with several anionic liposome formulations to form lipopolyplexes (a liposome – cationic polymer – nucleic acid complex). To target the Jurkat cells, lipopolyplexes were coupled with anti-CD3 antibody. Results demonstrated that lipopolyplexes containing PLK_{99}-plasmid DNA showed significant gene transfer activity as monitored by β-galactosidase expression. No transfections occurred when the Jurkat cells were treated with just polyplex. This suggested that the lipid component was essential for gene expression. Likewise, polyplexes and lipopolyplexes with non-CD3 specific antibody had no transfection activity [264]. However, further studies for improvement of the carrier systems are necessary.

8.3.2 Immunotoxins

Immunotoxins (IT) are molecules containing a protein toxin and a covalently coupled ligand which is either an antibody or a growth factor. ITs have several advantages over conventional therapeutic agents such as selectivity for tumor tissue or potential delivery of very strong toxins. However, it is difficult to find a potent toxin without significant nonspecific toxicity as well as an antibody specifically directed to antigens expressed on leukemic cells [265].

To potentiate the activity of ricin based anti-MY9 immunotoxin (anti-MY9-IT) *in vitro*, stealth monensin liposomes conjugated with anti-MY9 antibody were prepared [266]. Monensin is a carboxylic ionophore which can improve the activity of ricin based immunotoxins and it has been reported previously that it is possible to prepare long circulating liposomes conjugated with monensin [267]. The anti-MY9 IT is a blocked ricin which has its nonspecific binding eliminated through chemical blocking of the galactose binding domains of the B-chain. It was developed to selectively kill acute myeloid leukemia (AML) cells [265]. The anti-MY9 (anti-CD33) antibody appears to be a good candidate for targeting of acute myeloid leukemia cells, as previous results showed that anti-CD33 reacts with clonogenic AML cells in more than 80% of all cases but did not react with normal pluripotent cells [265,268]. Singh *et al.* demonstrated that stealth monensin liposomes conjugated with anti-MY9 mAb (SMIL) had a high degree of tumor specificity to CD33 positive cells (HL-60, a Human promyelocytic leukemia cell line), whereas the antigen negative Human lymphoid cell line (Namalwa) did not show any response to the potentiation of anti-MY9-IT with SMIL. Stealth monensin liposomes coupled with nonspecific IgG did not demonstrate any difference in potentiation compared to unconjugated monensin liposomes for anti-MY9-IT, which strongly indicates a high specificity of SMIL to tumor cells [266].
Similar results were obtained in a related study where an anti-MY9 immunotoxin with antibody modified monensin liposomes showed 2070-fold higher cytotoxicity against HL-60 cells compared to a 360-fold potentiation with unconjugated monensin liposomes. No significant cytotoxicity was observed against an unrelated SW 620 Human colon cancer cell line. Neither IT alone nor antibody modified monensin liposomes were able to kill the CD33 antigen negative cells. This shows the high specificity of targeting stealth monensin liposomes coupled with anti-MY9 monoclonal antibody [269].

8.4 Other Cancers

Immunoliposomes have been applied to many other cancer types such as gastric, neuroblastoma [270], colorectal cancer, lung cancer, prostate cancer, and hepatic cancer [271]. The first clinical application of antibody-conjugated liposomes in oncology was the use of MCC-465, an immunoliposome encapsulating doxorubicin coupled with the Fab' fragment of a cancer reactive human mAb (GAH). It presented high binding to cancerous stomach tissues with a positive ratio of >90% [272]. In preclinical studies, MCC-465 demonstrated superior cytotoxic activity against Human stomach cancer cells such as B37 compared to doxorubicin alone or entrapped into non-targeted liposomes [273]. The clinical data demonstrated the stability of the MCC-465 formulation in blood and indicated that conjugation with mAb GAH did not interfere with the stealth effect of the PEG liposomes. Drug tolerance was good and only mild side effects were noticed. No severe skin toxicity was observed, unlike in clinical trials with a similar drug, doxorubicin encapsulated within PEGylated liposomes (doxil) but with no antibody modification [274]. However, the pharmacokinetic parameters were similar to those of doxil but differed from those of free doxorubicin. The maximum tolerated dose of DCC-465 was slightly lower than that of doxil [272].
Recently, ILs encapsulating docetaxel and conjugated with trastuzumab were analyzed in a NCI-N87 xenograft mouse model to determine a potential clinical application for HER2/neu overexpressing gastric cancer [275]. The antibody modified liposomes resulted in a higher inhibition of tumor growth in the xenograft model than either docetaxel alone or non-modified liposomes; no adverse local effect were observed.
Superior results of antibody modified liposomes over non targeted vehicles were also obtained with the GD_2-positive Human neuroblastoma cell line HTLA-230 both *in vitro* and *in vivo* in nude mice. Here, PEGylated liposomes tagged with anti-disialoganglioside (GD_2) mAb and encapsulated doxorubicin or synthetic retinoid fenretinide (HPR) were used [276,277,278]. Antibody modified liposomes demonstrated specific binding, uptake and higher cytotoxic activity than non-targeted vehicles; they also resulted in a significant reduction of tumor growth. Long-term survival was observed in mice treated with immunoliposomes but not in animals injected with the drug alone or encapsulated in vehicles without antibody [276,277]. Similarly, Pastorino *et al.* demonstrated in studies with doxorubicin that liposomal drugs targeted with the Fab' fragment of GD_2 were more effective than whole mAb of GD_2 in all experiments and remained longer in the blood [276]. Doxorubicin loaded liposomes were also conjugated with NGR peptides targeting the angiogenic endothelial cell marker aminopeptidase N (CD13) isoform [279]. This peptide, when coupled to anticancer compounds, is capable of delivering the therapeutic molecule specifically to tumor vessels [280,281]. Tumor regression, destruction of tumor vasculature and prolonged survival of orthotopic neuroblastoma xenografts using NGR targeted ILs were observed. Moreover, the combined use of tumor and vascular targeted liposomal therapies enhanced the cytotoxic effects against aggressive Human neuroblastoma in an animal model and resulted in the dramatic inhibition of tumor endothelial cell density. A combined therapy, where half doses of each single liposomal formulation, but equal total doses of DXR, were used, clearly showed additive activity compared to tumor and vascular targeted formulations administered alone [279].

Immunoliposomes targeting proliferating endothelial cells were developed by adding a single chain F_v fragment (scFv A5) directed against human endoglin (IL5A) [282,283]. Endoglin is a type I membrane glycoprotein located on cell surfaces and is part of the transforming growth factor beta (TGF β) receptor complex. It is an attractive molecule for the targeting of the tumor vasculature and can lead to a long lasting anti-tumor response [284]. ILA5 showed rapid and strong binding to Human endoglin expressing endothelial cells (Human microvascular endothelial cells - HDMEC and Human umbilical vein endothelial cells - HUVEC). No binding was observed with various endoglin negative cell lines and blood lymphocytes. Furthermore, increased *in vitro* cytotoxicity towards endothelial cells was observed with antibody modified liposomes encapsulating doxorubicin compared to non-targeted vehicles or the free drug [283].
In different studies immunoliposomes directed against the vascular cell adhesion molecule 1 (VCAM-1) [285], a transmembrane glycoprotein expressed on activated endothelial cells during inflammation and cancer [286], were examined *in vitro* and *in vivo*. They demonstrated specific binding to activated endothelial cells [287] under static and simulated blood flow conditions.
Promising results were also presented by Hatakeyama *et al.* with doxorubicin loaded immunoliposomes tagged with antibody against the membrane type-1 matrix metalloproteinase (MT1-MMP) [288]. MT1-MMP plays an important role in angiogenesis and is expressed on angiogenic endothelium cells as well as on tumor cells [289,290]. The targeted ILs showed superior cellular uptake *in vitro* into fibrosarcoma cells (HT1080) highly expressing MT1-MMP and resulted in significant suppression of tumor growth in tumor bearing mice [288]. Immunoliposomes conjugated with the Fab' fragment from humanized anti-EGFR mAb EMD72000 (matuzumab) or C225 (cetuximab) likewise exhibited promising results in delivering doxorubicin to colorectal cancer [291]. Various EGFR-overexpressing colorectal cancer cells (DLD1, HTC116, LS174T, HT29, SW480, LS180, and CaCo2) showed specific binding of anti-EGFR ILs, irrespectively whether Fab' of C225 or EMD72000 were presented on the liposomal surface. The immunoliposomal formulation of dox was also significantly more cytotoxic than the corresponding non-targeted liposomal drug [291].
Systemic administration of gemcitabine encapsulating immunoliposomes with anti-EGFR antibodies attached at the distal end of the PEG chain inhibited the growth of non-small cell lung cancer (NSCLC) established *in vivo* [292]. Human non-small cell lung carcinoma RPCI-2E9 expressed β_1 integrins were targeted with doxorubicin loaded liposomes conjugated with Fab' fragment of mAb specific for human β_1 integrins. Results showed a tumor specific binding and efficient internalization of the IL into the lung tumor cells *in vitro* and gave significantly higher cytotoxicity compared with doxorubicin alone, no antibody modified liposomal doxorubicin or control immunoliposomes conjugated with nonspecific Fab'. Studies of a metastatic Human lung tumor xenograft severe combined immunodeficient (SCID) mouse model showed a significant suppression of tumor growth, prevention of the metastatic spread of the tumor, and longer survival times in tumor bearing mice injected with 1F11 modified liposomes [293]. Targeting liposomes with anti-c-Met human single chain antibody fragment (scFv), against c-Met receptor [294], which belongs to a subfamily of receptor tyrosine kinases (RTKs) [295], are a promising approach to deliver doxorubicin to lung cancer *in vitro* and *in vivo*. Moreover, *in vivo* studies with scFv-conjugated quantum dots demonstrated a potential application of this scFv for targeted imaging and early diagnosis of lung cancer [294].
Prostate cancer appears also to be amenable to IL treatment. Here, immunoliposomes tagged with anti-CD166 scFv (H3) monoclonal antibody targeting the antigen CD166 were used successfully [296]. Immunoliposomal formulations of three different drugs (topotecan, vinorelbine, doxorubicin) were effectively taken up by various prostate cancer cell lines, such as Du-145, PC3 or LNCaP, and H3. Immunoliposomal topotecan seems the most cytotoxic in all types of cells and showed a significantly improved cytotoxic activity in comparison with the drug encapsulated in non-targeted liposomes [296]. In related studies, liposomes loaded with doxorubicin and modified with the prostate cell-specific monoclonal antibody 5D4 (mAb 5D4) specifically recognized several different types of prostate cancer cell lines expressing the prostate membrane specific antigen (PSMA) and gave enhanced cytotoxicity compared to the non-targeted dox liposomes *in vitro* [297].
Another intriguing application utilized bee venom. Bee venom plays a prime role in the defense of bee colonies. Its components such as melittin possess anti-proliferative and pro-apoptotic effects [298,299,300]. Peptides in bee venom (PBV) containing mellitin have shown potential use in cancer therapy. For example, a study by Hu *et al.* utilized a sterically stabilized liposomal PBV formulation coupled with the humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25), which effectively bound to Human hepatoma SMMC-7721 tumor cells *in vitro* and *in vivo* [301]. ELISA assays indicated that the hdsFv25 IL had a high degree of tumor specificity and this was confirmed by cytotoxicity studies carried out with SMMC-7721cells and Hela cells *in vitro*. HdsFv25 ILs also gave good results in BALB/c nude mice models, exhibiting a significantly increased antitumor activity resulting in a reduction in tumor size compared to unmodified liposomes [301].

Expression of the RON receptor tyrosine kinase by pancreatic cancer stem cells (CSC) was also exploited as a potential target for immunoliposomes modified with anti-RON mAb Zt/c9 antibody to deliver chemotherapeutics such as doxorubicin [302]. Highly RON expressing pancreatic cancer stem cells expressing CD44, CD24 and ESA markers ($CSC^{+24/44/ESA}$) derived from L3.6pl cells were treated with Zt/c9 IL and showed specific interaction and rapid RON internalization. This resulted in effective uptake of the immunoliposomal formulation of dox and gave lower IC_{50} compared to the nonmodified formulation or liposomes coupled with nonspecific antibody IgG. This effect was more pronounced in combination with small molecule inhibitors such as lapatinib, sunitinib or dasatinib [302].
Antibody modified liposomes also gave positive results in epithelioid (M28) and sarcomatoid (VAMT-1) subtypes of Human mesothelioma when liposomes modified with Human single chain antibody (scFsv) M1 were used [303]. The ILs demonstrated their potential as a promising vector for effective drug delivery into mesothelioma cancer cells and showed highly efficient and rapid internalization into both subtypes of tumor cells *in vitro* and selective tumor targeting *in vivo* compared to non-targeted formulations [303].

9. HIV TARGETING

Highly active antiretroviral therapy, which combines several antiviral agents has had a dramatic impact upon long term survival rates. However, significant toxicity is still associated with the treatment and serious adverse effects including central nervous system toxicity, hypertriglyceridemia or lipodystrophia [304]. AIDS has become one of the most important infectious diseases. Thus, the selective delivering of antiretroviral drugs to HIV infected cells, resulting in reduced side effects, decreased nonspecific toxicity and an overall improved therapy, is required. HIV type 1 (HIV-1) is an enveloped retrovirus and proteins embedded in the viral envelope enable the virus to attach to and fuse with target cells and play an essential role in the virus replication cycle. These envelope glycoproteins consist of a complex of gp120 and gp41 generated by precursor polypeptide (gp160) and both, especially gp120, are major targets for the anti-viral immune response [104,305].
Several of these proteins have been used as targets for ILs in attempts to find alternative drug treatments. Recently, Clayton *et al.* developed sterically stabilized PEGylated ILs coupled with the Fab' fragment of HIV-gp120 directed monoclonal antibody F105 to deliver the novel HIV-1 protease inhibitor PI1 [306]. FACS and confocal images studies showed effective binding to HIV infected cells but not to uninfected cells. The ILs were readily taken up by HIV-1-infected cells, internalized and localized in the Golgi apparatus over time. This is in agreement with previous studies on the uptake and internalization of the gp-120-specific F105 IgG as a targeting moiety [307]. Accumulation in the Golgi apparatus may suggest a reservoir of antiviral drugs in the cytoplasm of HIV infected cells. Still, due to destabilization of lipid vehicles and drug release, the PI1 should diffuse from the Golgi and distribute throughout the cytoplasm over time. The sustained and strong antiviral activity indicates release and activity inside the cells. The IL delivered greater amounts of the drug to the HIV infected cells and demonstrated longer activity than comparable concentrations of free drug or drug encapsulated in non-modified liposomes. At a concentration of 1 μM of PI1, the targeted liposomes provided sustained 100% inhibition of viral replication over 4 days, whereas free drug and non-targeted liposomes showed approximately 60% and 80% inhibition, respectively. With a concentration of 0.1 μM the effect was even more pronounced (80 %, 10% and 20 % inhibition, respectively) [306].
A different and earlier example of ILs used indinavir encapsulated in sterically stabilized anti-HLA-DR liposomes [308]. $CD4^+$ T lymphocytes, which are the major target for HIV-1 infection, express substantial levels of the HLA-DR determinant of the major histocompatibility complex class II molecules [309]. Anti-HLA-DR IL showed efficient delivery of high concentrations of indinavir to lymphoid tissues with an increase by a factor of 126 compared to the free drug. This confirmed the results of previous mice studies [310]. Moreover, the sterically stabilized ILs accumulated better than conventional ILs, suggesting the importance of PEG for uptake by the lymphatic system [311]. Toxicity studies of repeated administration showed no significant differences in the levels of hepatic enzymes of mice treated with free indinavir or liposomal formulation of the drug compared to baseline and control untreated mice. Histopathological analyses also demonstrated no significant damage to liver and spleen in comparison to the untreated control group. In addition, immunogenicity studies showed, that Fab fragment bearing ILs were less immunogenic than liposomes bearing the entire IgG [308]. Similarly, promising results were obtained in experiments with the same delivery system encapsulating amphotericin B [312]. Amphotericin is effective in inhibiting HIV-1 replication at concentrations of 5-10 $\mu g.ml^{-1}$ [313] and has also been used for antimicrobial ILs [314]. Conventional liposomes containing this drug had no effect on viral infection, although they inhibit the replication of HIV-1 in H9 cells at similar concentrations. The packed anti-HLA-DR IL exhibited high specificity for HLA-DR proteins and inhibited 77% of HIV-1 replication at a concentration 0.5 $\mu g.ml^{-1}$, while the free drug did not show any antiviral activity. Complete inhibition of viral replication was observed following

incubation with the IL formulation at a concentration of 2.5 $\mu g.ml^{-1}$. The same formulation also inhibited the replication of macrophage tropic HLA-DR/NEG HIV-1_{ADA}. Gieseler *et al.* established an IL highly selective for Human myeloid dendritic cells by targeting the DC-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) (CD209) [315]. Flow cytometry showed superior targeting efficacy for DC-SIGN in comparison to other markers such as: CD1a, CD4, CD45R0, and CD38. The authors suggested that the IL reach the same intracellular compartments as HIV-1, where the latter is normally protected from a systemic attack.
A new and promising approach in this area is the immunoliposomal targeting of the lymphocyte function-associated antigen-1 (LFA-1) integrin expressed on all leukocytes combined with the delivery of small interfering RNA [316]. Sterically stabilized LFA-1 integrin-targeting liposomes showed selective *in vivo* uptake of siRNA by T cells and macrophages, which play crucial roles in viral infection and pathogenesis. The siRNA are well protected within the liposomes and can be delivered at high concentration to achieve good therapeutic efficacy without unintended off-target effects in nonleukocyte populations that do not express LFA-1. The *in vivo* administration of anti-CCR5 IL containing siRNA resulted in carrier-specific gene silencing in $CD14^+$ monocytes for up to 10 days. Humanized mice infected with HIV and treated with anti-CCR5 IL demonstrated enhanced resistance to infection as assessed by the reduction in plasma viral load and disease-associated CD4 T-cell loss.

10. CONCLUSIONS

Since their invention half a century ago liposomes have become promising tools for drug delivery [317]. They increased the therapeutic index of many drugs, improved drug targeting and allowed controlled release. Still, a significant problem associated with these first generation systems was their rapid removal from circulation by macrophages of the reticuloendothelial system. This led to the development of a second generation of liposomes, the long circulation liposomes or stealth liposomes. Use of polymeric steric stabilizers allowed the preparation of clinically acceptable systems in high purity, large scale which had low cytoxicity, were non immunogenic and antigenic [2,43,47]. Still, even liposomal systems with prolonged circulation time did not actively target the desired tissue. This gave rise to the third generation of targeted liposomal formulations which represent the current state of the art of liposomal drug delivery in medical treatment [17]. As outlined here the most widely used targeting approach is the use of antibodies and antibody fragments due to the high specificity for their target antigens. As a result an expanding body of information is now available on these so-called immunoliposomes [31,43].
The development of immunoliposomes has resulted in diagnostic and treatment applications in many areas of medicine. For example, they show promising results *in vitro* and *in vivo* and appear to be effective systems for improvements in cancer therapy. The targeted liposomes offer various advantages over classic drugs and one of the most compelling of these is that larger drug amounts can be delivered to the target tissue. Likewise, immunoenzymosomes may result in increased prodrug-drug conversion rates [318]. Liposomes can encapsulate drugs, DNA, siRNA, virions (immunovirosomes) [319] or contrast agents with high load capacity and only a few ligand (targeting) molecules per liposome are required to selectively deliver high payloads of drugs *via* receptor mediated endocytosis [320]. Further improvements of the binding activity are possible through presentation of multiple targeting molecules on the surface of individual liposomes [169]. A promising approach for further developments resides in the potential for additivity and synergy between the signaling antibodies on the liposome surface and the entrapped drug [320]. This can further increase the therapeutic index of the chemoactive drugs [200]. Several examples for this are now available which clearly prove the clinical benefit of using this concept in conjunction with well established small molecule drugs. The reader is referred to the many studies using doxorubicin described above [199,200]. Additionally, antibody modified vehicles can increase the sensitivity of cancer cells to other chemotherapeutics, *e.g.*, through the use of siRNAs [149]. Thus far, many of these studies relied on *in vitro* and *in vivo* models, yet the translational potential is evident.
More and more potential clinical applications than those discussed in some detail above are emerging. A survey of the relevant *in vitro* and *in vivo* studies is given in the Tables. To name only a few, these include myocardial ischemia [321] and other cardiovasculatory diseases [322] and the treatment of Lupus [323]. Even testis targeting studies have been reported and indicate the possibility to target spermatozoa [324]. Another example is the preliminary studies on antimalarial drug delivery [325].
The utility of immunoliposomes is not restricted to "classic" clinical treatment options. Significant potential exists for using them to cross the blood brain barrier [159,160,161] and *in vivo* studies have successfully achieved a targeted delivery to brain tumors *via* receptor-mediated transport [165,166]. Similarly, they can be used for the delivery of contrast agents [65,66], as ultrasound detection devices in the form of echogenic immunoliposomes [109,110], and as reactive components in bioanalytical assays for identifying and quantifying the amount of biological substances [81,83]. In practical terms, these analytical uses will most likely find rapid use in medicine,

while a full fledged immunoliposomal treatment of a disease will require more time for development and approval. However, large scale preparation of the targeting moieties is at hand and continuous advances are made in GMP-compliant processes [326]. Still, the potential of this concept for various applications has clearly been proven and will result in significant advances in treatment and diagnostics in the years to come.

Acknowledgments:
This work was supported by grants from Science Foundation Ireland (SFI P.I. 09/IN.1/B2650) and the Health Research Board (HRB Translational Research Award 2007 TRA/2007/11). We thank Stuart A. MacGowan for help with the manuscript.

Conflict of interest:
None.

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Figures

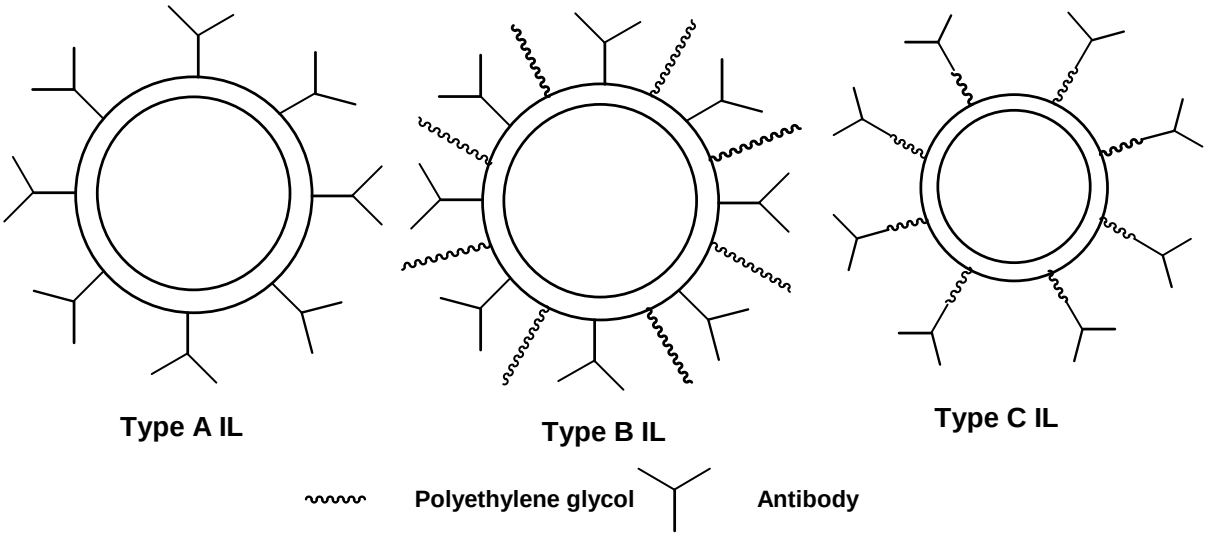

Figure 1. Types of immunoliposomes: A – antibodies attached directly to the PEG free liposome membrane; B − antibodies attached to the liposome membrane in parallel with PEG; C − antibodies are attached at the distal end of PEG chains.

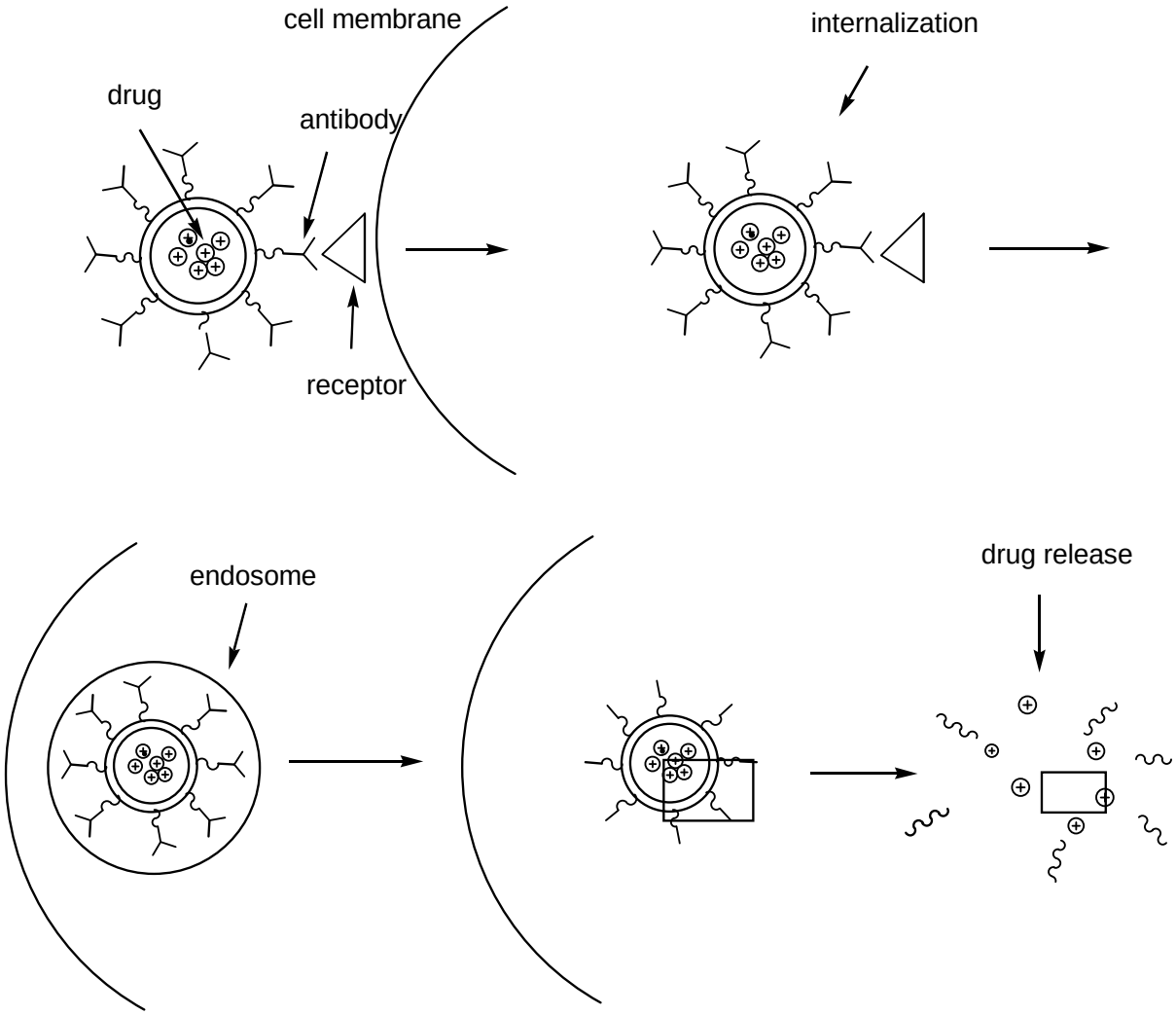

Figure 2. Receptor mediated endocytosis.

Table 1. Immunoliposomes and cell lines used for *in vitro* experiments.

Antibody IL type	Antibody target	Cell type	Cell line	Encapsulated drug / Comments	Year	Ref.
1F11 (Fab')	Human integrin β1 subunit	Human lung cancer	RPCI-2E9/IV4/PSA	Doxorubicin / IL 30times more effective	2000	293
2C5 mAb	Cancer cell specific nucleosomes	Human brain tumor	CCF-STTG1, U-87 MG, LN-18	/ binding study	2005	58
2C5 mAb	Cancer cell specific nucleosomes		B16-F10, HeLa and MCF-7	Doxorubicin / study on IL with pH sensitive bonds and a cell penetrating peptide (TAT) shows increased drug release at low pH	2012	327
2C5	nuclear auto-antibodies (ANAs) with nucleosome (NS)-restricted specificity	Murine Lewis lung carcinoma Murine breast adenocarcinoma Human mammary adenocarcinoma Human oestrogen receptor-sensitive breast carcinoma	LLC 4T1 BT-20 MCF-7	/ ^{111}In-labelled uptake and binding study	2006	64
2C5 mAb	Cancer cell specific nucleosomes	Murine Lewis lung carcinoma Human mammary adenocarcinoma	LLC BT20	Doxorubicin /	2004	59
2C5 mAb	Cancer cell specific nucleosomes	Murine Lewis lung carcinoma Human mammary adenocarcinoma	LLC MCF-7, BT20	$GdCl_3$•$6H_2O$ / MRI contrast agent study	2006	52
2C5 mAb	Cancer cell specific nucleosomes	Human brain tumor, human astrocytoma cells	U-87 MG	Doxorubicin / Doxil modified with antibodies	2007	166
2G4 anti-myosin mAb	intracellular cardiac myosin	rat cardiomyocytes	H9C2	pDNA encoding for GFP/double targeting with cell-penetrating transactivating transcriptional activator (TAT) peptide (TATp) and/or with mAb 2G4	2009	123
4D5MOCB scFv	epithelial cell adhesion molecule (EpCAM)	Human breast carcinoma Ovarian cancer non–Hodgkin's lymphoma Small cell lung cancer	MCF-7 OVCAR-3 RL SW2	Antisense oligonucleotide 4625 specific for both bcl-2 and bcl-xL / IL sensitizes EpCAM positive cells 2- – 5-fold for doxorubicin	2006	136
4D5MOCB scFv	epithelial cell adhesion molecule (EpCAM)	Human breast carcinoma non–Hodgkin's lymphoma Small cell lung cancer	MCF-7 RL SW2	Doxorubicin /	2007	328
5D4 mAb	Prostate cell specific	Human prostate cancer	LNCaP, PC3, DU145	Doxorubicin /	2008	297
anti-3G2	Endothelial KDR receptor	Human endothelial cells	HUVAV	/ good binding to human umbilical vein and microvascular endothelial cells; low binding to NIH-3T3 mouse fibroblast cells	2000	329
anti-BCG mouse	BCG	Mouse colon carcinoma 26 cells	Colon-26	/ pH-sensitive fusogenic liposomes	2002	231
anti-carcinoembryonic antigen	carcinoembryonic antigen	Adenocarcionoma	LoVo	Docetaxel / targeted radiosensitization	2005	236
anti-CC531	Rat colon carcinoma		Isolated rat liver macrophages	/ comparison of different liposomes and IL types	2002	330
anti-CD3	CD3	T lymphocytes	Jurkat	Plasmid pEGlacZ (*Escherichia coli* β-galactosidase reporter gene) / pH-sensitive IL	2002	264
anti-CD19, murine mAb	CD19	Multiple myeloma	ARH77	Doxorubicin / higher binding and cytotoxicity compared to without dox; selective receptor binding in a mixture of	2000	331

				peripheral blood mononuclear cells from multiple myeloma patients		
anti-CD19	CD19	Philadelphia chromosome–positive acute lymphoblastic leukemia	Various lymphoma and leukemia cell lines (OM9;22, KOPN-30, KOPN-57, KOPN-72, SUP-B15, K562, Daudi Burkitt lymphoma, BV173 CML-BC, NB4)	Imanitib (BCR-ABL tyrosine kinase inhibitor) /	2004	243
anti-CD19	CD19	Human Burkitt's lymphoma	Namalwa	Doxorubicin / internalized IL (anti-CD19) show better effect than IL against non internalized epitopes (CD20)	2001	332
anti-CD19	CD19	Human Burkitt's lymphoma	Namalwa	Doxorubicin / similar efficacy of conventional type coupled IL and post-insertion IL	2002	333
anti-CD19 (Fab')	CD19	Human Burkitt's lymphoma	Namalwa	Doxorubicin or vincristine /	2004	334
anti-CD19	CD19	Human Burkitt's lymphoma	Namalwa	Doxorubicin or vincristine / beneficial effect of combination therapy (anti-CD19 + CD20 IL) depends on cell type and drug	2004 2005	335 336
anti-CD19 scFv constructs, scFv HD38-C / scFv HD37-CCCH	CD19	Human Burkitt's lymphoma	RAJI	Doxorubicin /	2007	250
anti-CD19 (HD 37 mAb, HD37 Fab', scFv HD37-CCCH)	CD19	Human Burkitt's lymphoma	RAJI	Doxorubicin / comparison of Ab units, Fab' best	2008	251
anti-CD19 (2E8 mAb)	CD19	Human B-line leukemia stem cells	Nalm-6, Raji, Molt-3, K562	Norcantharidin / test of leukemia stem cell killing drug	2010	253
anti-CD20 (rituximab)	CD20	Chronic lymphocytic leukemia	CLL B-cells isolated from patients with CLL	/ coadministration of lenalidomide downregulates CD20 and results in diminished rituximab-mediated apoptosis and ADCC	2008	259
anti-CD22 mAb (HB22.7)	CD22	Non-Hodgkin's lymphoma (NHL)	Human Burkitt's B-cell lymphoma CRL-1596, T-cell leukemia Jurkat	Doxorubicin /	2010	249
anti-CD22 scFv (Mut HA22)	CD22	Non-Hodgkin's lymphoma (NHL)	Human B-cell lymphoma BJAB, Raji	Doxorubicin / mutant form more active	2010	337
anti-CD33 (anti-MY9 antibody)	CD33		HL60	Monensin /	2001 2001	266 269
anti-CD33 (clone p67.6)	CD33	Leukemia	HL60, KG-1, THP-1	/ ph sensitive IL	2009	256
anti-CD33 (clone p67.6)	CD33	Human myeloid leukemia	HL60	1-β-D-arabinofuranosylcytosine (ara-C) / ph sensitive IL	2010	255
anti-CD33 scFv	CD33	Human acute myeloid leukemia	SKNO-1, Kasumi-1	AML1/MTG8 siRNA /	2010	263
anti-CD38(murine IB4)	CD38				2008	338
anti-CD74 (milatuzumab)	CD74	Chronic lymphocytic leukemia	CLL B-cells isolated from patients with	/ use of milatuzumab in IL	2010	247

			CLL			
anti-CD74 Fab'	CD74	Human B lymphoma	Rajii	Doxorubicin / test of Fab' instead of whole Ab for IL	2007	339
anti-CD105	Human mesenchymal stem cells		MSC	Magnetite nanoparticles / method for magnetic force-based mesenchymal stem cell expansion	2005	340
anti-CD138	CD138, surface hybrid proteoglycan Syndecan-1	Primary effusion lymphoma	BCBL-1	29-mer phosphorothioate oligonucleotide (FITC-ODN) / test transfection study	2010	341
anti-CD166 scFv (H3)	CD166 (MEMD), activated leukocyte cell adhesion molecule	Human prostate cancer	PC3, Du-145, LNCaP	Topotecan /	2007	296
anti-CD209 mAb [derived from clones 120507 (IgG2b)]	CD209, i.e. DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN),	Myeloid dendritic cell	Myeloid dendritic cell monocytes	Calcein / proof of concept for delivery, HIV treatment	2004	315
anti-c-Met scFvs (MS20)	c-Met, hepatocyte growth factor receptor	Human lung cancer	H1993, H520, H460, H441, A549, 293T	Doxorubicin or scFv-conjugated quantum dots /	2011	294
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7, strain 43895	Sulforhodamine B / *E. coli* bioassay	2000	84
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7	Sulforhodamine B / *E. coli* bioassay	2003	82
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7	Sulforhodamine B / *E. coli* bioassay, no interference from *Salmonella* or *Listeria*	2003	85
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7	Sulforhodamine B / *E. coli* microcapillary bioassay	2004	342
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7, strain 43889	Carboxyfluorescein / *E. coli* bioassay	2004	343
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7, strain 43895	Sulforhodamine B / *E. coli* bioassay, determination in apple cider	2004	344
anti-*E. coli* O157:H7	*E. coli*	*Escherichia coli*	O157:H7, strain 43895	Sulforhodamine B / *E. coli* bioassay, immunomaganetic bead-IL-fluorescence assay	2005	83
anti-ED-B (scFV)	ED-B fibronectin	Human colorectal cancer	Caco2	/ binding study	2002	345
anti-EGFR+anti-EGFRvIII (C225 Fab') anti-EGFR (scFv C10)	Epidermal growth factor receptor (EGFR)	glioma cells carcinoma cells EGFRvIII stable transfectants	U87 A-431, MDA-MB-468 NR-6M	Doxorubicin or vinorelbine or methotrexate /	2003	225
anti-EGFR (various Ab types)	Epidermal growth factor receptor (EGFR)	Human colorectal cancer Hman breast cancer	DLD1, HCT116, LS174T, HT29,SW480, LS180, Caco2, Colo205 MDA-MB-468	/ various mAb fragments EMD72000 (matuzumab) or C225 (cetuximab)	2006	291
anti-EGFR (C225)	Epidermal growth factor receptor (EGFR)	Human glioma	U87	Doxorubicin / test of folate-folate binding protein linking procedure	2007	224
anti-EGFR	Epidermal growth factor receptor (EGFR)	Human glioma	U87 ΔEGFR expr. EGFRvIII U87 WT expr. wt EGFR PA U87 (parental) expr. no EGFR	Sodium borocaptate / no binding in PA U87 cells and primary astrocytes	2009	173
anti-EGFR	Epidermal growth factor receptor (EGFR)	Human lung cancer	A549	Gemcitabine / development of pH sensitive IL	2008	346

anti-EGFR with *Gaussia* luciferase	Epidermal growth factor receptor (EGFR)	Human glioma	U87 ΔEGFR expr. EGFRvIII PA U87 (parental) expr. no EGFR.	8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt / only weak fluorescence in PA U87 cells, theranostics approach	2010	70
anti-EGFR (Fab' fragments of the anti-EGFR IgG C225	EGFR	Human breast cancer	BT474	Topotecan / test of various cell lines (MDA-MB231, MDA-MB468)	2010	347
anti-EGFR mAb (Fab')	Epidermal growth factor receptor (EGFR)	Human breast cancer	MDA-MB-231	anti-Luciferase siRNA / enhanced binding affinity and luciferase gene silencing activity	2011	154
anti-EGFR (TLPD-FP75)	Epidermal growth factor receptor (EGFR)	Hepatocellular carcinoma	SMMC-7721	anti-Luciferase siRNA or anti-RhoA siRNA/ silencing of transfected luciferase	2012	155
anti-EGFR	Epidermal growth factor receptor (EGFR)	H&N SCC Murine fibroblasts	UM-SCC-14C NIH 3T3 clone 2.2	insulin-like growth factor 1 receptor (IGF-1R) kinase inhibitor A538 / dual targeting study of EGFR and IGF-1R	2012	348
anti-EGFR mAb	Epidermal growth factor receptor (EGFR)	Non small cell lung carcinoma	A549 NCI-H322 NCI-H460 NIH-3T3	FITC-siRNA-uptake study / siGL3 (siRNA for GL3-luciferase), siTMPR4 (siRNA for TMPRSS4) gene silencing efficiency study	2012	158
anti-GD_2	disialoganglioside GD_2	Neuroblastoma	GI-LI-N, HTLA-230, CAN, LAN-5	c-myb antisense oligonucleodides /	2000 2004 2005	128 349 127
anti-GD_2	disialoganglioside GD_2	Neuroblastoma	GI-LI-N, CAN, IMR-32, HTLA-230	Fenretinide /	2003 2003	277 278
anti-GD_2 (whole) anti-GD_2 (Fab')	disialoganglioside GD_2	Neuroblastoma	CAN, GI-LI-N, HTLA-230, IMR-32	Doxorubicin /	2003	276
anti-GD_2 (Fab')	disialoganglioside GD_2	Neuroblastoma	HTLA-230	Doxorubicin /	2003	350
anti-GD_2	disialoganglioside GD_2	Neuroblastoma	HTLA-230, SH-SY5Y, NXS2	Doxorubicin / combined administration of IL and doxorubicin loaded liposomes targeted against the tumor vasculature via the NGR peptide (angiogenic endothelial cell marker aminopeptidase N)	2006	279
anti-GD_2	disialoganglioside GD_2	Neuroblasoma	Kelly IMR-32 SK-N-AS	siRNA / down regulation of vascular endothelial growth factor A (VEGF-A)	2011	157
anti-GD_2	disialoganglioside GD_2	Melanoma	GI-LI-N, HTLA-230	c-myc antisense oligonucleodides /	2004	349
anti-GFAP	glial fibrillary acidic protein	Olfactory ensheathing glial cells	rats	Dil dye / binding and uptake study	2008	351
anti-HA peptide mAb	Hemagglutinin peptide (TGLRNGITNKVNSVIEKAA)	*Influenca* virus		Bis(2,2′-bipyridine)[4,4′-bis(4-aminobutyl)-2,2′-bipyridine] ruthenium perchlorate / bioassay for influenca virus hemagglutinin	2008	99
anti-hDEC-205	Human DEC205	Dendritic cells	BDC	/ uptake and binding study	2007	151
anti-HER2 polyclonal Ab	HER2	Human breast cancer	SK-BR3	antisense oligonucleotide directed toward the translational start site of dihydrofalate reductase RNA	2002	214
anti-HER2 (rhuMAb HER2-Fab′, scFv C6.5, scFv F5)	HER2	Human breast cancer	SK-BR3		2001	199
anti-HER2, F5 anti-ErbB2 (F5-scFv)	ErbB2 (HER2/*neu*)	Human breast cancer	SK-BR-3	Doxorubicin /	2002	352
anti-HER2 (Fab')	HER2	Human breast cancer	SK-BR3, MCF-	luciferase gene / gene delivery study	2003	120

			7, HeLa			
anti-HER2 F5 scFv	HER2	Human breast cancer	SKBR3	LAQ824 (candidate histone deacetylase (HDAC) inhibitor) /	2005	216
anti-HER2 scFv	HER2	Human breast cancer	SK-BR-3, MCF-7/HER2	Semiconductor quantum dots (+doxorubicin) / uptake study	2008	74
anti-HER2 Fab′	HER2	Human breast cancer	SK-BR3, MDA-MB-231, MCF-7	PE38KDEL (38 kDa mutant form of *Pseudomonas* exotoxin A) / SK-BR3 (IC_{50} = 36.63pM); MDA-MB-231 (IC_{50} = 291.92 pM); MCF-7 (IC_{50} > 2000 pM)	2009	211
anti-HER2 scFv antibody fragment	HER2	Human breast cancer	SK-BR-3, BT474-M2	Vincristine or vinblastine /	2009	209
anti-HER2, recombinant humanized mAb Fab'	HER2	Human breast cancer	SK-BR3 MCF-7	anti-HER1 siRNA or anti-RhoA siRNA/ uptake in SK-BR3 only; good silencing of HER1 gene or RhoA expression and inhibition of cell invasion in SK-BR3	2010	146
anti-HER2 mAb (Trastuzumab)	HER2	Human breast cancer	SK-BR3	Magnetite nanoparticles (Fe_3O_4) / hypothermia therapy	2004	220
anti-HER2 mAb (Trastuzumab)	HER2	Human breast cancer	SK-BR3 BT-474 MDA-MB-231	Paclicatel / significantly higher uptake compared to low HER2 expressing cells (MDA-MB-231)	2007	206
anti-HER2 (Trastuzumab)	HER2	Human breast cancer	SKBr3	Melittin / action of IL appears to be cytolytic; trastuzumab resistant breast cancer cells (JIMT-1) could also be targeted	2010	208
anti-HER2 (Trastuzumab)	HER2	Human breast cancer	Hc7	Dipotassium (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetate or 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl / EPR study of NO spin probes	2010	78
anti-HER2 (Trastuzumab)	HER2	Metastatic ovarian carcinoma	SKOV3-NMP2	^{225}Ac / test of new IL preparation method with rigid liposomes	2008	353
anti-HER2 (Trastuzumab)	HER2	Metastatic ovarian carcinoma	SKOV3-NMP2	Doxorubicin / pH-sensitive IL for better drug release	2009	354
anti-HER2 (Trastuzumab)	HER2	Mammary epithelial cellsw	MTSV1-7, Ce2	Calcein / hyperthermia induced release from thermosensitive IL with listeriolysin O	2010	355
anti-HER2 scFv F5	HER2	Human breast cancer	BT474	Topotecan / test of various cell lines (SKBR3, MDA-MB453, Calu-3, HCC1569, MCF-7)	2010	347
anti-HIV-gp120-directed monoclonal antibody F105, Fab' fragment	HIV-gp120	HIV-1, Human T-cells	HUT78 PM1	HIV-1 protease inhibitor PI1 (substituted benzimidazole sulfonamide) / IL uptake by HIV-1 infected cells	2009	306
anti-HLA-DR Fab′	HLA-DR, HIV surface glycoprotein	Human CD4 T lymphocytes	SUP-T1 HUT-78 RAJI	None / no binding None / high level of binding None / high level of binding	1999	310
anti-HLA-DR Fab′ / anti-HLA-DR IgG	HLA-DR, HIV surface glycoprotein	Human CD4 T lymphocytes	SUP-T1 PM1 (clonal der. of HUT-78	Indinavir / Fab' fragment less active than entire IgG in sterically stabilized IL	2002	308
anti-HLA-DR Fab′	HLA-DR, HIV surface glycoprotein	Human T-lymphocytes	Jurkat E6.1	Amphotericin B / test for HIV application	2000	312
anti-HLA-DR Fab′	HLA-DR, HIV surface glycoprotein	Mouse spleen cells	C3H	None / test for HIV application	2000	311
anti-Human endoglin scFv A5	Human endoglin (CD105)	Human endoglin expressing endothelial cells	HUVEC HDMEC	Doxorubicin	2004	283
anti-Human E-selectin mAb 1.2B6	Human E-selectin	Human endothelial hybridoma	EA.hy 926	Various marker genes /	2003	121
anti-Human E-selectin	Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	/ uptake study; about 25 % of IL were taken up	2001	356
anti-Human E-selectin	Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	Dexamethasone / Dex-IL internalized less by activated HUVAC cells than a dexamethasone anti-E-selectin immunoconjugate	2003	357
anti-Human E-selectin mAb H18/7	Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	Gd-DTPA-bis(stearylamide) as MRI agent, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B	2004	65

				sulfonyl) (rhodamine-PE) for fluorescence indication / use as MRI agent		
anti-Human E-selectin mAb H18/7	Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	Calcein or doxorubicin or VE-cadherin siRNA (Mm_Cdh5_2_HP siRNA) / 10fold better release of calcein with SAINT-C18 cationic amphiphile compared to standard IL	2010	358
anti-Human insulin receptor mAb 8314/stretavidin (8314/SA)	Human insulin receptor (HIR).	Human hepatoblastoma, mouse hepatocellular carcinoma	SMMC-7721 H22	Polyplexes prepared with plasmid DNA and branched polyethylenimine, EGFP plasmid/ transfection study	2010	122
anti-ICAM-1 (CD54)	Intercellular adhesion molecule 1 (ICAM-1)	Human coronary artery endothelial cells	HCAEC, CC-2585	5-[*N*-acetyl-(2,3-dihydroxypropyl)-amino)-*N,N'*-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-benzene-1,3-dicarboxamide (iohexol) / cell uptake study for CT imaging	2009	62
anti-ICAM-1 (CD54)	Intercellular adhesion molecule 1 (ICAM-1)	Primary high endothelial venule cells	HEV	/ thest of new IL for binding to activated endothelial cells with low cellular uptake	2011	359
anti-ICAM-1/anti-E-selectin	Intercellular adhesion molecule 1 (ICAM-1); Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	/ test of effect of lipid raft formation on binding	2010	360
anti-ICAM-1/anti-ELAM	Intercellular adhesion molecule 1; endothelial-leukocyte adhesion molecule-1	Human umbilical vein-derived endothelial cells	HUVEC	/ test of effect of ratio of two targeting Ab, maximal binding at 1:1 ratio	2010	361
anti-IGFI-R Ab 1H7	Insulin-like growth factor	Prostate carcinoma Neuroblastoma	DU145 Kelly	Doxorubicin /	2010	362
anti-Mouse lactate dehydrogenase, LDH-C_4	LDH-C_4	Mouse spermatozoa		/ targeting study	2008	324
anti-Mouse transferrin receptor mAb 8D3	Mouse transferrin	mouse brain endothelial cells	bEnd5	Oligodeoxynucleotides / brain targeting	2009	186
anti-Mouse transferrin receptor mAb 8D3 or RI7	Mouse transferrin	mouse neuroblastoma	Neuro2A	Oligodeoxynucleotides / transfection test	2007	119
anti-Mouse transferrin receptor mAb 8D3	Mouse anti-TfRMAb	Brain tumor	U87	short hairpin RNA directed at nucleotides 2529–2557 within the human EGFR mRNA / RNA interference	2004	192
anti-Mouse transferrin receptor mAb 8D/streptavidin (8D3/SA)	Mouse transferrin receptor	Human hepatoblastoma, mouse hepatocellular carcinoma	SMMC-7721 H22	Polyplexes prepared with plasmid DNA and branched polyethylenimine, EGFP plasmid/ transfection study	2010	122
anti-MPB	MPB	Rat Schwann cells	Schwann	/ binding and uptake study	2007	364
anti-MPB	MPB	Rat Schwann cells	Schwann	miRNA (21 b. p.) blocking MBP synthesis/ specific suppression of target protein	2008	365
anti-MT1-MMP (Fab')	Membrane type-1 matrix metalloproteinase		HT1080	Doxorubicin / MT1-MMP as a target molecule for tumor and neovascularity	2007 2007	288 366
anti-MUC 1	MUC 1 antigen	murine breast adenocarcinoma cell line 410.4 transfected with full-sized construct of the human MUC-1 gene	GZHI	Doxorubicin /	2001	367
anti-OVCAR-3 (Fabβ 323/A3)	Ovcar cells	Human ovarian carcinoma	OVCAR-3	Dox-GA3/ IL with exposed β-glucoronidase = immunoenzymosome; increased prodrug conversion	2003	318
anti-*Salmonella* IgG	*Salmonella*	*Salmonella typhimurium*		/ development of immunoassay for *Salmonella* detection	2012	87
BM1234 mAb	*Plasmodium falciparum*-infected red blood cells [pRBCs]	*Plasmodium falciparum*-infected red blood cells	*Plasmodium falciparum*	Chloroquine / test for antimalerial action	2011	325
BM1232 mAb or BM1234	*Plasmodium falciparum*-infected red blood cells [pRBCs]	*Plasmodium falciparum*-infected red blood cells	*Plasmodium falciparum* 3D7 D10	Chloroquine or quantum dots / test for antimalerial action	2011	368

anti-*Salmonella* common structural antigens	Salmonella common structural antigens	*Salmonella typhimurium*		Methyl blue / *Salmonella* bioassay	2008	90
anti-*Salmonella* IgG, polyclonal goat	Salmonella IgG	*Salmonella typhimurium*		Sulforhodamine B / *Salmonella* bioassay	2008 2011	88 89
anti-SEB mAb mouse	*Staphylococcus* enterotoxin	*Staphylococcus aureus*		Sulforhodamine B / *S. aureus* bioassay	2008	94
anti-VCAM-1	Vascular cell adhesion molecule 1	Human endothelial cells	EA.hy926	/ uptake and binding study	2005	287
anti-VCAM-1	Vascular cell adhesion molecule 1	murine endothelial cells	bEnd.3	/ tumor vessel model	2008	285
anti-VCAM-1/anti-E-selectin	Vascular cell adhesion molecule 1; Human E-selectin	Human umbilical vein-derived endothelial cells	HUVEC	/ test of different rations of Ab on IL	2011	369
anti-VGEFR-2, rat	VGEFR-2	Endothelial cells	293T	/ 7-fold increased binding to cells	2007	370
BLCA-38	Human CaP cells	Human prostate cancer	LNCaP-LN3, DU-145	/ Ab linked to peptide 101, designed around the first 22 amino acids of bee venom, melittin	2004	299
CC52	Rat colon carcinoma	Rat colon adenocarcinoma	CC531		2000	234
CC52	Rat colon carcinoma	Rat colon adenocarcinoma	CC531	5-Fluoro-2′-deoxyuridine dipalmitoyl derivative (FUdR-dP) / uptake model for lipophilic drugs	2002	229
CC52	Rat colon carcinoma	Rat colon adenocarcinoma	CC531 Kupffer cells	/ comparison of different liposome types	2003	233
GAH (cancer-reactive human monoclonal antibody)		Human GI cancer: colon cancer esophageal cancer stomach cancer rectum cancer	 Caco-2, DLD-1, SW620 TE-8 HSC-3, MKN-1, MKN-45, B37 SW837	Doxorubicin / dose-dependent cytotoxicity for GAH-reactive B37 cancer cells	2003	273
GAHr (recombinant cancer-reactive human monoclonal antibody)		Various Human cancer tissue cells	various	Doxorubicin /	2004	371
GC4 scFv (tumor-targeting human monoclonal antibody)	cell-surface antigen associated with the B16F10 tumor	Murine melanoma cells	B16F10	siRNA and miRNA /	2010	156
hdsFv25 (humanized antihepatoma disulfide-stabilized Fv) (PE38)	Hepatoma	Human hepatoma	SMMC 7721	Peptides of bee venom (90% mellittin) /	2006 2006	301 372
hdsFv25 (humanized antihepatoma disulfide-stabilized Fv)	Hepatoma	Human hepatoma	SMMC 7721	Immunotoxin PE38 /	2010	373
HuCC49 (Fab')	humanized antitumor-associated glycoprotein-72 (TAG-72)	Human colon cancer	LS174 T	Plasmids encoding antiangiogenic proteins, such as angiostatin, K1/3, endostatin, saxatilin / *in vitro* transfection study	2008 2012	230 374
J591	FOLH1-expressing cells	Human prostate cancer	LNCaP-LN3, DU-145	/ Ab linked to peptide 101, designed around the first 22 amino acids of bee venom, melittin	2004	299
MES-1	monoclonal rat anti-mouse E-selectin antibody	Endothelial cells	H5V	Dexamethasone /	2008	375
Mesothelioma-targeting human scFv (M1)	Human mesothelioma cell surface antigens	Human sarcomatoid mesothelioma Human epithelioid mesothelioma	VAMT-1 M28	/ identification and testing of internalizing antibodies that target mesothelioma-associated cell surface antigens	2008	376
Mesothelioma-targeting human	Human mesothelioma cell	Human sarcomatoid	VAMT-1	^{111}In-labelled IL / binding and internalization in both subtypes	2011	303

scFv (M1)	surface antigens	mesothelioma Human epithelioid mesothelioma	M28			
MRK-16	P-glycoprotein	Human myelogenous leukemia cells	K-562	Vincristine / increased uptake of drug by adriamycin resistant cells via IL delivery	2001	377
Murine 83–14 mAb	Human insulin receptor	Brain tumor	U87	Human EGFR antisense gene / 70-80 % inhibition	2002	183
Murine 83–14 mAb	Human insulin receptor	Brain tumor	U87	Luciferase gene / comparison of targeting strategies, 100–200-fold higher gene expression compared to targeting to EGFR (528 mAb) transferring receptor (OX26 mAb)	2003	180
Murine 83–14 mAb	Human insulin receptor	Brain tumor	U87	Short hairpin RNA directed at nucleotides 2529–2557 within the human EGFR mRNA / RNA interference	2004	192
Murine 83–14 mAb	Human insulin receptor	Human glioblastoma Murine brain endothelial cells	HTB14 bEnd3	pGL4-13 plasmid DNA / test of DNA encapsulation protocol	2009	378
My10 mAb	CD34	Human myelogenous leukemia	KG-1a	Doxorubicin / IL were not internalized by the KG-1a	2004	252
NLDC-145 mAb	Dendritic cell marker DEC-205	Delivery to dendritic cells	DC cells from bone marrow progenitors (C57BL/6 mice)	CD40 siRNA	2009	150
Ox7	Thy 1.1 antigen	Rat mesangial cells	RMC	/ uptake study	2005	172
Ox26 mAb	Rat transferrin receptor	Rat gliomas cells Rat brain capillary endothelial	RG2 RBE4	/ BBB targeting	2000	165
Ox26 mAb	Rat transferrin receptor	Rat brain capillary endothelial	RBE4	Radiolabelled digoxin / suppression of MDR1 P-glycoprotein	2002	168
Ox26 mAb	Rat transferrin receptor	Rat brain capillary endothelial	RBE4	Daunomycin / 2-3fold higher intracellular concentration	2005	379
Ox26 mAb	Rat transferrin receptor	Rat skeletal muscle cells	L6	/ ILwith bound streptavidin	2004	380
Ox26 mAb (Fab')	Rat transferrin receptor	Hybridoma	Y3.AG.1.2.3.	None	2007	27
Ox26 mAb (Fab')	Rat transferrin receptor	Hybridoma Rat brain capillary endothelial cells	Y3.AG.1.2.3. BCEC	^{188}Re $(S_3CPh)_2(S_2CPh)$ for labeling / less binding to BCEC	2008	381
Ox26 mAb	Transferrin receptor	Immortalized human brain capillary endothelial cells	hCMEC/D3	/ BBB targeting	2011	169
Ox26 mAb + anti-Aβ-MAb	Transferrin receptor + Aβ1-42 peptides	Immortalized human brain capillary endothelial cells	hCMEC/D3	/ BBB targeting, effect of preptreatment with Aβ1-42 peptides	2012	382
Rat IgG or Rabbit IgG			Rat Kupffer cells	/ 3-4 better uptake of rat IgG-IL	2001	383
TfRscFv, anti-transferrin receptor	transferrin receptor	Human bladder cancer	HTB-9	RB94 plasmid, specific for tumors / gene therapy	2008	384
TfRscFv, anti-transferrin receptor	transferrin receptor	Human breast cancer	MDA-MB-435	p53 expression plasmid pCMVp53 /	2001	118
TfRscFv, anti-transferrin receptor	transferrin receptor	Human breast cancer	MDA-MB-435	p53 expression plasmid pCMVp53 /	2002	114
TfRscFv, anti-transferrin receptor	transferrin receptor	Human breast cancer	MDA-MB-435	modified hybrid (DNA-RNA) anti-HER-2 siRNA / IL complex improvement through inclusion of a pH-sensitive histidine-lysine peptide in the complex (scL-HoKC)	2007	149
TfRscFv, anti-transferrin receptor	transferrin receptor	Human breast cancer	MDA-MB-435	GMC-5-193, quinazolinone analogue /	2008	385
TfRscFv, anti-transferrin receptor	transferrin receptor	Human H&N cancer	JSQ-3	p53 expression plasmid pCMVp53 /	2002	114
TfRscFv, anti-transferrin receptor	transferrin receptor	Human lymphoblastic leukemia	K562	Magnevist®, MRI contrast agent /	2006	69
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	DU145	p53 expression plasmid pCMVp53 /	2002	114
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	DU145	Various plasmids /	2004	386
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	DU145	Magnevist®, MRI contrast agent /	2006	69
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	DU145	GMC-5-193, quinazolinone analogue /	2008	385
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	PANC-1	blunt-ended 19-mer short interfering hybrid (siHybrid) (H) comprised of sense-DNA/antisense-RNA targeting HER-2 mRNA + modifications / IC_{50} = 7.8–37nm	2006	387

TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	PANC-1	modified hybrid (DNA-RNA) anti-HER-2 siRNA / IL complex improvement through inclusion of a pH-sensitive histidine-lysine peptide in the complex (scL-HoKC)	2007	149
TfRscFv, anti-transferrin receptor	transferrin receptor	Human prostate cancer	PANC-1	Superparamagnetic iron nanoparticles (SPIO) / uptake study	2008	75
TfRscFv, anti-transferrin receptor	transferrin receptor	Mouse renal cell carcinoma	RenCa	Gadopentetate dimeglumine (gad-d), MRI contrast agent /	2009	66
Zt/c9	RON receptor tyrosine kinase	Pancreatic cancer stem cells	L3.6pl	Doxorubicin / targeting study	2011	302
anti-HER2 (Herzeptin)	HER2	Human breast cancer	BT-474, SKBR-3	Paclitaxel / cytotoxicity better than taxol	2007	205
Zt/g4, Zt/c1 (Fab fragments)	RON receptor tyrosine kinase	Colon and breast cancer	T-47D, HCC1937, MCF-7, SW620, SW837	Doxorubicin / targeting study	2009	228
Zt/g4, mAb	RON receptor tyrosine kinase	Hypoxic colon cancer	HCT116, SW620	Doxorubicin / anti tumor effect dependent on hypoxic RON expression; HCC1937 cells with diminished RON expression under hypoxia were insensitive	2011	239

Table 2. Target tissue and cell types used for *in vivo* experiments with immunoliposomes.

Tissue type	Animal model	Cell line	Antibody IL type	Encapsulated drug / Comments	Year	Ref.
Acute myocardial infarction	Langendorff isolated perfused heart model, CD-1 male rats	–	Myosin-specific monoclonal 2G42D7	/ cardiac cell membrane lesion sealing with cytoskeleton targeted IL resulted in preservation of myocardial viability	2004	388
				/ recovery to normal heart function in Langendorff instrumented adult rat hearts undergoing global ischemia	2007	363
Acute myocardial infarction	Langendorff isolated perfused heart model, CD-1 male rats	–	Myosin-specific monoclonal 2G42D7	ATP / ATP loaded IL increase cardio protection	2006	389
Acute myocardial infarction	Mycordial infarction rat model, male Sprague-Dawley	–	anti-P-selectin	/ targeting study	2007	390
Acute myocardial infarction	Mycordial infarction rat model, male Sprague-Dawley	–	anti-P-selectin	human $VEGF_{165A}$ / significant increase in fractional shortening and improved systolic function	2007	390
Acute myocardial infarction	Rabbits, rabbit hearts with experimental left ventricular myocardial infarction	–	anti-myosin cytoskeletal-antigen specific IL	/ reduction in myocardial injury after treatment	2007	391
Arterial artheroma	Human umbilical arterial endothelial cells infected with *Chlamydia pneumoniae*	*Chlamydia pneumoniae*	Echogenic IL	Azithromycin / effective inhibition of microbial growth in endothelial cells under retention of acoustinc properties	2004	392
Arterial artheroma , left ventricular thrombus model	mongrel dogs	–	rabbit anti-human fibrinogen	echogenic immunoliposomes for ultrasound enhancement of atheroma components	2002	112
Arterial artheroma	Yucatan minipigs with induced artheroma	–	anti-ICMA-1, anti-vascular cell adhesion molecule-1 (VCAM-1), anti-fibrin, anti-fibrinogen, anti-tissue factor (TF) conjugated ELIPs	echogenic immunoliposomes for ultrasound enhancement of atheroma components; comparison of different ELIPs	2004	108
Arterial artheroma	Yucatan minipigs with induced artheroma	–	anti-intercellular adhesion molecule-1 (ICAM-1)	echogenic immunoliposomes for ultrasound enhancement of atheroma components	2010	109
Artheriosclerosis	adult male Sinclair cross miniswine	–	anti-CD34 + anti-ICAM-1 combined	/ echogenic IL delivery to porcine arterial wall, application study	2010	111
Brain, mice	Adult male BALB/c mice	–	8D3, 83-14 (anti-TfRMAb, mouse)	6- to 7-kb expression plasmid encoding either / luciferase or β-galactosidase / gene transfer study	2001	177
Brain, mice	Male MPS type VII GUSB null mice	–	8D3, 83-14 (anti-TfRMAb, mouse)	Human GUSB expression plasmid (pCMV-GUSB) / b-glucuronidase (GUSB) transfection as a model of type VII mucopolysaccharidosis	2008	185
Brain, mice	Male BALB/c mice	–	anti mouse transferrin receptor mAb 8D3	Oligodeoxynucleotides / brain targeting	2009	186
Brain, monkey	Rhesus monkey	–	anti-human insulin receptor	Luciferase gene / gene transfer proof of concept	2003	181
					2006	182
Brain, rats	Rats	–	OX26	/ *in vivo* transcytosis test	2000	165
Brain, rats	male Sprague–Dawley rats	–	OX26 mAb (anti rat-Tf)	6- to 7-kb expression plasmid encoding either / luciferase or β-galactosidase / gene transfer study	2000	179
Brain, rats	adult male Sprague Dawley rats	–	OX26 mAb	6.8 kb pSV-b-galactosidase plasmid / gene transfer study, expression of the exogenous gene in brain, liver, and spleen	2001	178
Brain, rats	Sprague–Dawley rats	–	anti-Thy 1.1	Horseradish peroxidase gene / light and electron microscopic study of IL for intracellular delivery to the rat striatum	2002	170

Brain, rats	adult male Sprague Dawley rats	–	OX26 mAb	7-kb expression plasmid encoding for rat tyrosine hydroxylase / no toxic side effects upon weekly administration	2003	189
Brain, rats	Rats	–	OX26	Various plasmids / transfection study	2010	184
Brain tumor model	Intracranial brain tumor, male Fischer CD344 rats	C6-790	Mouse 83-14 mAb to the human insulin receptor	Oligodeoxynucleotides / brain targeting	2003	190
Brain tumor model	Intracracinal model of brain tumor, female scid mice	U-97	8D3, 83-14 (anti-TfRMAb, mouse)	Nonviral expression plasmid encoding antisense mRNA against the human epidermal growth factor receptor gene (*EGFR*) / 100 % increase in lifespan	2002	194
Brain tumor model	Intracracinal model of brain tumor, female scid mice	U-87	8D3 rat mAb (anti-TfRMAb, mouse) murine 83–14 mAb (anti-human insulin receptor)	Short hairpin RNA directed at nucleotides 2529–2557 within the human EGFR mRNA / RNAi gene therapy	2004	192
Brain tumor model	Subcutaneous Human brain tumor model, female *nu/nu* mice	U-87 MG	mAb 2C5	/ binding study with ^{111}In labeled IL	2005	58
Brain tumor model	Intracracinal model of brain tumor, female nude mice	U-87 MG	mAb 2C5	Doxorubicin / Doxil modified with antibodies	2007	166
Brain tumor model	Brain tumor model, female nude mice (BALB/c Slc-nu/nu)	U87 ΔEGFR	anti-EGFR	Sodium borocaptate (^{10}B labeled)/ test for application for boron neutron capture therapy (BNCT)	2009	173
Brain tumor model	Brain tumor model, female nude mice (BALB/c Slc-nu/nu)	U87 ΔEGFR	anti-EGFR with *Gaussia* luciferase	8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt / theranostics approach, tumor visualization	2010	70
Embolic focal stroke, rats	Rat model		anti-Actin	/ coadministration of IL with tissue plasminogen activator (tPA) significantly reduced tPA-induced hemorrhage	2003	393
Gastric cancer	Gastric cancer xenograft mouse model	NCI-N87	Trastuzumab	Docetaxel /	2011	275
Glioblastoma	Glioblastoma xenograft model; NCR *nu/nu* mice	MDA-MB-468, U87, U87vIII	anti-EGFR (Fab' fragments of cetuximab (IMC-C225)	Doxorubicin or epirubicin or vinorelbine / uptake study	2005	223
Glioblastoma	Female Wistar rats, intracerebral stereotactic implantation glioma cells	C6	anti-GFAP and against the E2 extracellular loop of connexin 43 (MAbE2Cx43)	Dil C18 or Gd–DTPA / uptake study	2012	394
Glomerulonephritis	C57bl/6 female mice, glomerulonephritis was induced by injection of sheep anti-mouse GBM Ab and TNFα		monoclonal rat anti-mouse E-selectin antibody (MES-1)	Dexamethasone / strong reduction in glomerular proinflammatory gene expression, without side effects as with free dexamethasone	2007 2008	395 375
Glomerulonephritis, IgA nephropathy	Male Wistar rats, anti-Thy1.1 nephritis was induced at day 0 by injection of anti-Thy1.1 IgG		OX-7 Fab'	mycophenolate mofetil / single dose results in amelioration of mesangial proliferative glomerulonephritis	2011	396
Hepatocellular carcinoma	Female BALB/c mice	H22	8D3 rat mAb (anti-TfRMAb, mouse)	EGFP or luciferase plasmid / transfection study / inflammatory cytokines	2010	122
Hepatocellular carcinoma	Orthotopic HCC model, BALB/c nude mice	SMMC-7721	anti-EGFR (TLPD-FP75)	anti-luciferase siRNA / silencing of transfected luciferase	2012	155
Hepatocellular carcinoma	Hepatoma model, BALB/c nude mice		anti-VGEFR-2	Doxorubicin / significant delay in tumor growth	2007	370
Hepatocellular carcinoma	Hepatoma model, BALB/c nude mice	SMMC 7721	hdsFv25 (humanized antihepatoma disulfide-stabilized Fv)	Peptides of bee venom (90% mellittin) /	2006	301
Human B lymphoma	SCID xenografts model	Namalwa	anti-CD19 (Fab')	Doxorubicin or vincristine / better therapeutic effect with vincristine	2004	334
Human bladder cancer	xenograft model, female athymic nude mice	HTB-9	TfRscFv, anti-transferrin receptor single-chain antibody fragment	RB94 plasmid, tumor specific / combination with gemcitabine resulted in significant tumor growth inhibition/regression and induction of apoptosis	2008	384

Human breast cancer	female immune competent *Balb/c* mice model	4T1-MUC1	anti-MUC-1	Doxorubicin /	2001	367
Human breast cancer	Breast cancer xenograft model,	BT474	anti-HER2 (various)	Various application examples	2001	199
Human breast cancer	Nude mouse breast cancer metastasis model	MDA-MB-435	TfRscFv, anti-transferrin receptor single-chain antibody fragment	p53 expression plasmid, pCMVp53 /	2001	118
Human breast cancer	Xenograft model, nude mice	BT-474, MDA-MB-453, MCF-7, MCF-7/HER2	anti-HER2 (recombinant human MAb HER2-Fab' or scFv C6.5)	Doxorubicin / reduced regrowth of tumor with dox-IL	2002	204
Human breast cancer	Breast cancer xenograft model, homozygous nude mice (NCR *nu/nu*)	subline of BT474 human breast adenocarcinoma cells (ATCC HTB-20)	F5 anti-ErbB2 (F5-scFv)	Doxorubicin / significant reduction in tumor size	2002	352
Human breast cancer	Breast cancer xenograft model, homozygous nude mice (NCR *nu/nu*)	BT474	F5 anti-ErbB2 (F5-scFv)	/ therapeutic effect is the result of increased intracelluarl delivery rather than tissue accumulation	2006	397
Human breast cancer	Breast cancer xenograft model,	BT474	anti-HER2 F5 scFv	LAQ824 (candidate histone deacetylase (HDAC) inhibitor) /	2005	216
Human breast cancer	Xenograft model, nude mice	MDA-MB-435	TfRscFv, anti-transferrin receptor single-chain antibody fragment	blunt-ended 19-mer short interfering hybrid (siHybrid) and modifications /	2006	387
Human breast cancer	Xenograft model, nude mice	MDA-MB-435	heparin-binding epidermal growth factor-like growth factor (HB-EGF)	Doxorubicin / uptake study	2012	217
Human breast cancer	Xenograft model, female BALB/C *nu/nu* athymic (nude) mice	BT-474 MDAMB-231	anti-HER2 (Herzeptin)	Paclitaxel / significantly higher tumor tissue distribution of paclitaxel in the BT-474 (HER2 overexpressing)	2007	205
Human breast cancer	HER2-overexpressing MCF-7/HER2 xenograft model, mouse	MCF-7/HER2	anti-HER2 scFv	Semiconductor quantum dots (+doxorubicin) / uptake and imaging study	2008	74
Human breast cancer	Xenograft model, female *nu/nu* mice	BT474	Trastuzumab (anti-HER2)	Magnetite nanoparticles (HML) / use in hyperthermia treatment	2009	219
Human breast cancer	Breast cancer xenograft model, NCR *nu/nu* female mice	BT474-M2	anti-HER2 scFv antibody fragment	Vincristine or vinblastine /	2009	209
Human breast cancer	Xenograft model, NCR *nu/nu* female mice	BT474	anti-HER2 scFv F5	Topotecan / improved antitumor activity compared to nontargeted formulations	2010	347
Human breast cancer	Breast cancer, female nude mice (BALB/c)	MDA-MB-231	anti-EGFR mAb (Fab')	anti-Luciferase siRNA / ~20% luciferase gene silencing	2011	154
Human neuroblastoma	murine model of human neuroblastoma	HTLA-230	anti-GD_2 (Fab')	Doxorubicin / long term survivors only with dox+IL combination	2003	350
Human neuroblastoma	murine model of human neuroblastoma	HTLA-230	anti-GD_2	Fenrentenide / completely inhibition of macroscopic and microscopic metastases	2003	278
Human neuroblastoma	murine model of human neuroblastoma	HTLA-230	anti-GD_2	antisense CpG-containing oligonucleotides (TL-asCpG) / combined therapy with antibodies against IL-10 receptor (aIL-10R) results increased percentage of long term survivors, surviving mice were completely cured	2009	398
Human neuroendocrine tumor	Xenografts model, female athymic NMRI v/v mice	BON	anti-IGFI-R	Doxorubicin / test of anti-IGFI-R IL	2010	362
Human non-small cell lung cancer	Xenograft model, female BALB/c *nu/nu* mice	A549	anti-EGFR mAB'	Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; dFdC) /	2009	292
Human pancreatic cancer	orthotopic metastasis xenograft model, female athymic nude mice	PANC-1	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Various plasmids / uptake study	2004	386
Human pancreatic cancer	female athymic nude mice	PANC-1	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Superparamagnetic iron nanoparticles / uptake study 1 mouse	2008	75

Human pancreatic cancer	orthotopic metastasis xenograft model, female athymic nude mice	Capan-1	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Magnevist®, MRI contrast agent	2006	69
Human pancreatic cancer	orthotopic metastasis xenograft model, female athymic nude mice	Capan-1	TfRscFv, anti-transferrin receptor single-chain antibody fragment	modified hybrid siRNA / uptake and localization study	2006	148
Human prostate cancer	orthotopic metastasis xenograft model, female athymic nude mice	DU-145	TfRscFv, anti-transferrin receptor single-chain antibody fragment	p53 expression plasmid, pCMVp53 /	2002	114
Human prostate cancer	orthotopic metastasis xenograft model, female athymic nude mice	DU-145	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Various plasmids / uptake study	2004	386
Human prostate cancer	Athymic BALB/c *nu/nu* male mice xenograft model	LNCaP-LN3, DU-145	J591 or BLCA-38	/ Ab linked to peptide 101, designed around the first 22 amino acids of bee venom, melittin	2004	299
Human prostate cancer	orthotopic metastasis xenograft model, female athymic nude mice	PC-3	TfRscFv, anti-transferrin receptor single-chain antibody fragment	modified hybrid siRNA / uptake and localization study	2006	148
Human stomach cancer	Human stomach cancer xenograft model, male Balb/cAJcl-nu mice	Subcutaneous: MKN-45 Subrenal capsule: HSC-3, B37, WiDr-Tc,SW837, TE-8, Caco-2, DLD-1, SW620, MKN-1, MKN-45	GAH (cancer-reactive human monoclonal antibody)	Doxorubicin / comparison of 10 xenograft models bearing cancer cells with varying GAH reactivity	2003	273
Human stomach cancer	Human stomach cancer xenograft model, male Balb/c nu-nu mice	Subcutaneous: MKN-45	GAH (cancer-reactive human monoclonal antibody)	Tc-99m / biodistribution and uptake study	2009	399
Human stomach cancer		B37	rGAH	Doxorubicin / IL inhibited growth of dox-insensitive stomach cancer cells (B37)	2004	371
Immune system, delivery to dendritic cells	C57/B6 mice	DCs	mAb NLDC-145 (anti-DEC-205)	CD40 siRNA / selective uptake in immune organs and functional immune modulation	2009	150
Immune system	peripheral blood mononuclear cell grafted, Immunodeficient NOD.cg-*Prkdc*^{scid}IL2rg$^{tm/Wjl}$/Sz (NOD/SCID/IL2rγ^{null}) mice	peripheral blood mononuclear cells	TS1/22, anti-human lymphocyte function–associated antigen-1 (LFA-1)	anti-CCR5 siRNA / gene silencing study, humanized mice challenged with HIV after anti-CCR5 siRNA treatment showed enhanced resistance to infection	2010	316
Kidney, rats	Rats, male Wistar		OX7 (anti Thy 1.1)	Doxorubicin / specific targeting to mesangial cells	2005	172
Kidney, rat	Rats, male Wistar		OX7 (anti Thy 1.1)	Doxorubicin / tissue distribution study, highest in spleen, then lung, liver and kidney, heart and brain negative; toxic effects only in renal glomeruli	2008	400
Leishmaniasis	BALB/c mice infected with *Leishmania donovani* AG83 strain	*Leishmania donovani*	anti-51 kDa protein	Doxorubicin / complete elimination of splee parasite	2004	401
Lung cancer	orthotopic metastasis xenograft model, female athymic nude mice	MDA435/LCC6	TfRscFv, anti-transferrin receptor single-chain antibody fragment	modified hybrid siRNA / uptake and localization study	2006	148
Lung cancer	orthotopic metastasis xenograft model, female athymic nude mice	MDA435/LCC6	TfRscFv, anti-transferrin receptor single-chain antibody fragment	GMC-5-193, quinazolinone analogue / IL lowers IC_{50} 3-4fold compared to nontargeted, IL increased the sensitivity of cancer cells to doxorubicin, docetaxel, or mitoxantrone 3- to 30-fold	2008	385
Lung metastasis	Female C57BL/6 mice	B16F10	GC4 scFv	siRNAs against c-Myc, MDM2, and VEGF- / FITC-labeled siRNA-uptake study	2010	156
Lupus erythematosus	Female NZM2328 and (NZM2328 × NOD)F_1 ([NZM/NOD]F_1) mice	lupus glomerulonephritis	anti–α8 integrin	Dil / targeting to glomerular mesangial cells	2008	323
Mouse mammary carcinoma	C3H female mice	MCa-4	anti-E-selectin	Combretastatin / targeting of tumor vasculature in conjunction with fractionated irradiation dosing	2009	402

Mouse mammary carcinoma	*neu*-N transgenic female mice	NT2.5	7.16.4, a mouse anti-Her2/*neu* mAb	^{213}Bi / α-emitter targeting study	2010	403
Melanoma	Transplanted tumor model, C57BL male mice	B16-F10 melanoma cells	cyclo(Arg-Gly-Asp-D-Phe-Cys) (RGD), anti- $\alpha_v\beta_3$, integrin	Combretastatin / targeting of tumor vasculature	2005	404
Murine breast carcinoma	BALB/c mice	4T1	2C5 (anti-ANA with nucleosome restricted specifity)	/ tumor gamma scintigraphy	2006	64
Murine Lewis lung carcinoma	BALB/c miceC57/BL	LLC	2C5 (anti-ANA with nucleosome restricted specifity)	/ tumor gamma scintigraphy	2006	64
Murine lung carcinoma	Lewis lung carcinoma, female C57BL/6J mice	LLC	2C5 (anti-ANA with nucleosome restricted specifity)	$GdCl_3$•$6H_2O$ / MRI application	2008	56
Murine teratocarcinoma	CD-1<R>-nu/nu mice	F9	anti-ED-B (scFV)	2'-deoxy-5-fluorouridylyl-N^4-octadecyl-1-β-*D*-arabinofuranosylcytosine / 62-90 reduction in tumor growth	2002	345
Myocardial ischemia, rats	EMI (experimental myocardial infarction) model in rats		2G4	2G4-modified TATp-lipoplexes fluorescence labeled with Rh-PE /increased accumulation in ischemic rat myocardium/enhanced transfection cardiomiocytes in ischemic zone	2009	123
Neovasculature + tumor	Male BALB/c nude mice	HT1080	anti-MT1-MMP (Fab')	Doxorubicin /	2007	288
Parkison, experimental model	6-hydroxydopamine model of PD in adult male Sprague-Dawley rats	–	Ox26 mAb (Fab')	Tyrosine hydroxylase expression plasmids / brain gene targeting; normalizes striatal tyrosine hydroxylase and reverses motor impairment	2003 2004	187 188
Rat carotoid plaque lesions	Rats, carotid intimal hypertrophy was induced by balloon injury	anti-LOX1	lectin-like oxidized low-density lipoprotein receptor-1	rho-kinase inhibitor fasudil /	2011	405
Rat colon cancer	Rat colon adenocarcinoma, male WAG/Rij rats	CC531 colon adenocarcinoma	CC52	/ uptake study	2000	234
Rat colon cancer	Rat colon adenocarcinoma, male WAG/Rij rats	CC531 colon adenocarcinoma	CC52	5-Fluoro-2′-deoxyuridine dipalmitoyl derivative (FUdR-dP) / uptake study, macrophages involvement	2001	232
Rat inflammatory bowel disease	Rats, dinitrobenzensulfonic acid induced colitis		mouse anti human TfR	/ test study for drug delivery to inflamed gut mucosa	2011	406
Rat model of crescentic glomerulonephritis	Male Wistar rats		anti-Thy 1	NF-κB or scrambled decoy ODN / hemagglutinating virus of Japan (HVJ) liposomes with OX7 gives efficient ODN transfer in rat glomeruli	2002	407
Retina, monkey	Rhesus monkey		83-14 murine mAb human insulin receptor (HIR)	β-galactosidase or luciferase plasmids / specific transport across the blood retina barrier	2003	408
Small cell lung cancer	Tumor xenograft, female CD-1 (ICR *nu/nu*) mice	SW2	4D5MOCB scFv (anti-EpCAM)	Doxorubicin / pharmacokinetics	2007	328
Small lung cancer	metastatic RenCa mouse model, female BALB/c mice	RenCa	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Gadopentetate dimeglumine (gad-d), MRI contrast agent	2009	66
Small lung cancer	B16/F10 mouse model, female C57Bl/6 mice	RenCa	TfRscFv, anti-transferrin receptor single-chain antibody fragment	Gadopentetate dimeglumine (gad-d), MRI contrast agent	2009	66
Testis	male mice [strain CD-1 (ICR)]	–	anti-mouse lactate dehydrogenase (LDH-C_4)	/ targeting study	2008	324
Tissue distribution analysis	female C3H mouse	–	anti-HLA-DR Fab′	None / Tissue distribution analysis; highest accumulation in cervical and brachial lymph nodes	1999	310
Tissue distribution analysis	female C3H mouse	–	anti-HLA-DR Fab′	None / Tissue distribution analysis for HIV treatment	2000	311
Tissue distribution analysis	female C3H mouse	–	anti-HLA-DR Fab′ / IgG	Indinavir/ good delivery to lymphoid tissues	2002	308
Tissue distribution analysis	Male Wistar rats	–	Ox26 mAb (Fab')	^{188}Re $(S_3CPh)_2(S_2CPh)$ for labeling / roughly doubled brain concentration 24 h post-injection compared to non-targeted systems	2008	381
Tumor visualization	Xenograft model, female	murine Lewis lung	mAb 2C5	$^{111}InCl_3$ / γ-scintigraphy	2006	51

	C57BL/6J and nude mice	carcinoma (LLC) Human colon adenocarcinoma (HT-29)				
Vascular smooth muscle cells	Yucatan minipigs	–	anti-SMC actin Ab	/ test of improved uptake of echogenic IL through ultrasound-mediated delivery	2010	110
Vasculature, anti-VCAM-1	Human Colo 677 xenograft, female CD1 nude mice	Colo 677	anti-VCAM-1 (vascular cell adhesion molecule 1)	None / selective targeting of tumor vessels	2008	285