Expert Opinion

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Tumour-selective drug delivery via folate receptor-targeted liposomes

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Tumour cell-targeted liposomal delivery has the potential to enhance the therapeutic efficacy and reduce the toxicity of anticancer agents. Folate receptor (FR) expression is frequently amplified among human malignancies. FR is, therefore, potentially useful as a tumour marker for targeted drug delivery. FR-mediated liposomal delivery has been shown to enhance the antitumour efficacy of doxorubicin both *in vitro* and *in vivo,* and to overcome P-glycoprotein-mediated multi-drug resistance. In addition, FR-targeted liposomes have shown utility as effective delivery vehicles of genes and antisense oligodeoxyribonucleotides to FR(+) tumour cells. Both solid tumours and leukaemias can potentially benefit from FR-targeted drug delivery. Multiple mechanisms might contribute to greater therapeutic efficacy for FR-targeted liposomes, such as FR-dependent cytotoxicity and antiangiogenic activity. Further investigation of this promising drug delivery strategy is clearly warranted.

Keywords: angiogenesis, antisense oligodeoxyribonucleotides, chemotherapy, folate receptor, gene therapy, liposome, multi-drug resistance, targeted drug delivery

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1. Introduction

In the late nineteenth century, German bacteriologist P Ehrlich coined the term 'magic bullet' to describe drugs that could selectively kill pathogenic cells without damaging healthy cells [1]. Given the marginal therapeutic index of most anticancer drugs, improving their selectivity for tumour cell via targeted delivery is an attractive approach to enhance their therapeutic effectiveness.

Liposomes were first studied as a model for the lipid bilayer membrane structure [2]. They were, however, quickly recognised as a potential drug carrier due to their biocompatibility and ability to incorporate hydrophilic compounds into its aqueous core and hydrophobic agents into the lipid bilayer. Liposomes exhibit prolonged systemic circulation time and are mostly cleared by the phagocytic cells, which constitute the reticuloendothelial system (RES). Liposomal delivery of chemotherapy agents reduces normal tissue toxicity by lowering free drug concentration in the plasma and enhances antitumour activity by preferential accumulation in solid tumour, due to increased permeability of tumour endothelium and reduced lymphatic drainage; also known as the enhanced permeability and retention (EPR) effect. The composition of liposomes can be adjusted to improve their pharmacokinetic or drug-carrying properties. For example, incorporating polyethyleneglycol (PEG)-derivatised lipid in liposomes reduces RES clearance and increases EPR-mediated delivery [3]; incorporating cationic lipids allows liposomes to form electrostatic complexes with plasmid DNA, thereby providing a means for gene delivery [4,5]; and adopting a pH-sensitive fusogenic lipid composition can facilitate endosomal/lysosomal release of the entrapped agents following cellular

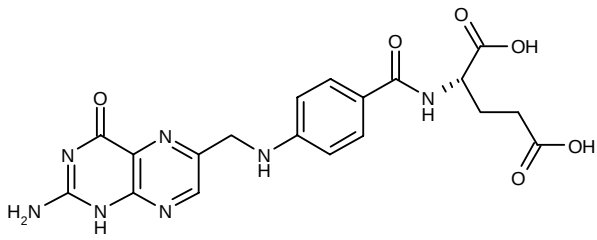

Figure 1. Structure of folic acid.

internalisation of the liposomes [6,7]. Furthermore, a number of targeting ligands, including: antibodies or antibody fragments [8,9]; growth factors [10]; transferrin [11] and vitamin folate [12], have been conjugated to liposomes to achieve selective targeting of tumour cells.

Folic acid is a water-soluble B vitamin (Figure 1), which is essential for *de novo* nucleotide synthesis and one-carbon transfer reactions [13]. Cellular transport of folate is primarily mediated by the reduced folate carrier (RFC), which has only moderate affinity for folate (K_d in the µM range). Meanwhile, a high-affinity folate receptor (FR) for folate is frequently elevated among human tumours and generally absent in most normal tissues. Therefore, FR is potentially useful as a cellular marker for tumour-targeted drug delivery, including targeted liposomal delivery. FR-targeted drug delivery has been the subject of a number of recent reviews [14-18]. This review will focus on the recent progress of FR-targeted liposomes as a tumour-selective drug delivery vehicle.

2. Folate receptor expression and distribution

Human FR, a 38 – 40 kDa *N*-glycosylated protein, has three subtypes: FR-α, -β and -γ/γ´ [19-22]. FR-α and -β are glycosylphospatidylinositol (GPI)-anchored membrane proteins, whereas FR-γ/γ´, lacking the GPI anchor, is a constitutively secretary isoform [23]. The three FR isoforms share ~ 70% primary sequence homology [24,25] and all exhibit high affinity for folic acid (K_d ~ 0.1 nM for FR-α, ~ 1 nM for FR-β and ~ 0.4 nM for FR-γ). However, FR-α and FR-β exhibit differential stereospecificities [26,27]. The FR-α isoform binds physiological disastereomer (6S)-5-methyltetrahydrofolate (5-MeTHF) with higher affinity, whereas the FR-β isoform preferentially binds non-physiological disastereomer (6R)-5-MeTHF [26,27].

FRs display differential tissue distribution patterns. The distribution of FRs has been measured by various methods including immunohistochemical staining [28], western analysis, reverse transcription–polymerase chain reaction (RT-PCR) analysis [29], and ^{3}H–folic acid binding [30]. FR-α is expressed in certain normal tissues, such as placenta, kidney (proximal tubules), fallopian tube and choroids plexus, among which the expression is restricted to the luminal surface of epithelial cells, where it is inaccessible to blood circulation [31]. In contrast, many malignant tissues, especially the ovary, nasopharyngeal, cervical and chorio carcinomas, but not sarcomas, consistently and uniformly express high levels of FR-α, which is accessible via the bloodstream [32,33].

FR-β expression in normal tissue is found only on placental and haematopoietic cells. Mature neutrophils in peripheral blood express fivefold higher FR-β in its inactive form (non-folate binding) than do myelomonocytic cells in the marrow [29]. Therefore, FR-β can serve as a differentiation maker in the myelomoncytic lineage [29]. In contrast, FR-β expression is amplified in its active form in activated monocytes and macrophages [34]. More interestingly, functional FR-β is expressed in ~ 70% acute myelogenous leukaemia (AML), which makes it a potential marker for targeting drugs to AML [29,35].

The soluble FR-γ/γ´, mainly expressed at low levels in certain haematopoietic cells, has a relatively insignificant role in FR-targeted drug delivery. However, it may serve as potential serum markers for certain haematopoietic malignancies [23,36].

The frequent overexpression among human tumours and highly restricted distribution among normal tissues suggest that both FR-α and -β can potentially be exploited as a tumour-specific cell surface marker that can be used in the targeted delivery of cancer therapeutics.

3. Folate as a tumour-targeting ligand

Mammals are incapable of *de novo* biosynthesis of folate and rely on exogenous folate sources. Folic acid retains high affinity for the FR following derivatisation via its γ-carboxyl, thus making it a potentially useful tumour-targeting ligand. FR-targeting has been evaluated for enhancing tumour cell-selective delivery of a wide variety of therapeutic agents. These include: radiopharmaceuticals [37]; chemotherapeutics [38]; antisense oligodeoxyribonucleotides [10,39]; prodrug-converting enzymes [40]; anti-T-cell antibody [41]; magnetic resonance imaging (MRI) and optical contrast agents [42,43]; boronated neutron capture therapy agents [44], immunogenic hapten [45]; gene transfer vectors [46]; nanoparticles [47]; and liposomal drug carriers [12]. ^{111}In–diethylenetriamine pentaacetic acid (DTPA)–folate has been evaluated clinically as an imaging agent for detecting recurrent ovarian carcinomas. Preliminary results showed a sensitivity of 85% and a specificity of 82% for identifying malignant tumours in the study [48]. These findings demonstrated that FR-specific tumour uptake of a folate conjugate can occur despite the presence of physiological levels of folate and FR in the circulation and suggest that targeting the FR in ovarian cancer is potentially feasible in the clinic.

Using folic acid as a ligand for targeted drug delivery has several advantages compared with polypeptide-based targeting ligands: lack of immunogenicity; unlimited availability; functional stability; high affinity to FR (K_d ~ 10^{-10} M); low molecular weight; and defined conjugation chemistry. Moreover, folate conjugates can be efficiently and non-destructively internalised into cells via FR-mediated endocytosis [12,49-51].

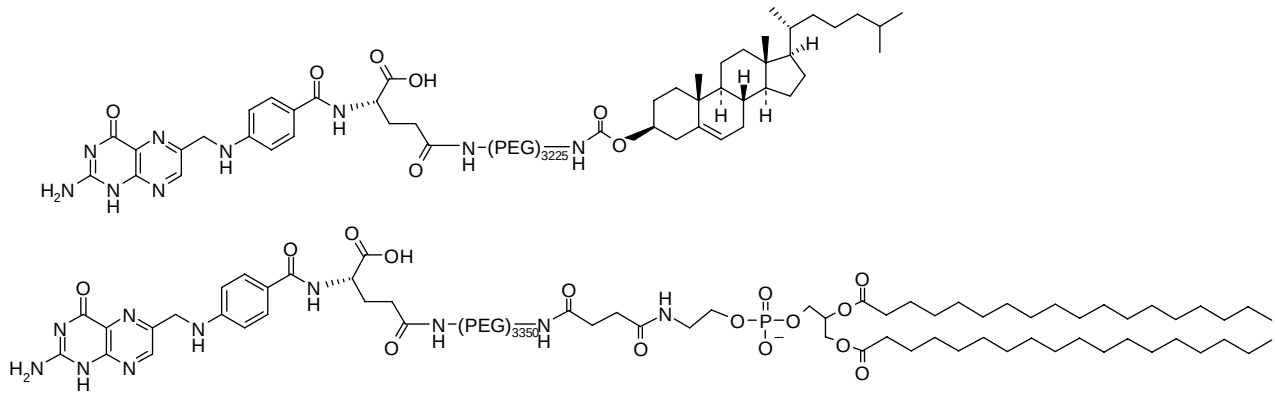

Figure 2. Structures of (A) folate–PEG–cholesterol and (B) folate–PEG–DSPE.
DSPE: Distearoylphosphatidylethanolamine; PEG: Polyethyleneglycol.

Notwithstanding these advantages, a potential pitfall for folate ligand-targeting for low-molecular-weight conjugates is high renal uptake due to FR-α expression in the apical membrane of kidney proximal tubules [52]. This site is, however, inaccessible to high-molecular-weight agents or liposomes, which cannot pass through the glomerular membrane due to their size [31].

4. Folate receptor-targeted liposomes

To prepare FR-targeted liposomes, the folate ligand may be incorporated into the liposomal bilayer by three different methods:

- during liposome preparation by mixing a lipophilic folate derivative with other lipid components
- by derivatisation of the distal termini of functionalised polyethylene glycol (PEG)-lipids in intact liposomes
- by post-insertion of the folate ligand into preformed liposomes [12,53].

The lipophilic anchor for the folate ligand can be either a phospholipid or cholesterol. Two lipophilic folate derivatives, folate–PEG–distearoylphosphatidylethanolamine (DSPE) and folate–PEG–cholesterol (Figure 2), have been synthesised as ready-to-use ligands for preparing FR-targeted liposomes [54,55]. To avoid steric hindrance caused by pegylated lipids (e.g., PEG_{2000}–DSPE) in long circulating liposomal formulations, a long spacer (PEG_{3350}) is preferred between the hydrophobic anchor and folic acid [12,54,56].

5. Folate receptor-targeted liposomes for delivery to FR(+) cancer cells *in vitro*

Numerous *in vitro* studies have been reported evaluating receptor-specific uptake of targeted liposomes by FR-expressing cells. For example, when co-cultured FR(+) HeLa and FR- WI38 were treated with FR-targeted liposomes encapsulating calcein, a fluorescence dye, only HeLa cells showed internalisation of FR-targeted liposomes [54]. In an FR-blocking study, uptake of FR-targeted liposomes was reduced by ~ 70% in the presence of 1 mM free folic acid. In contrast, no reduction in cellular uptake of these liposomes was observed in the presence of the physiological concentration (20 nM) of 5-metyltetrahydrofolate [54]. FR-targeted liposomes loaded with doxorubicin have been evaluated in FR(+) KB cells (Figure 3). The uptake of FR-targeted liposomal doxorubicin was 45-fold higher than non-targeted liposomes and 1.6-fold higher than free doxorubicin, and the cytotoxicity was 86- and 2.7-fold greater, respectively [54]. FR-targeted liposomes with a long spacer between the ligand and the lipid anchor (PEG_{3350} for folate ligand) that also incorporated PEG_{2000}–DSPE showed a 37-fold higher internalisation rate than the corresponding FR-targeted liposomes with a short spacer (PEG_{2000}) between the ligand and the lipid anchor [12,56]. Therefore, a longer spacer between the ligand and the lipid anchor appeared to be more effective in overcoming steric hindrance on the surface of the liposomes caused by PEG_{2000}–DSPE [12,56].

Novel formulations of FR-targeted liposomes have also been developed to improve intracellular drug delivery. FR-targeted pH-sensitive liposome, entrapping 200 mM anticancer agent araC, showed ~ 17-fold higher cytotoxicity in FR(+) KB cells compared with araC delivered via FR-targeted non-pH-sensitive liposomes. FR-mediated endocytosis leads to internalisation of FR-targeted liposomes into an acidic compartment, and the pH-sensitive liposomes undergo acid-triggered destabilisation and endosomal drug release [57]. In a separate report, FR-targeted liposomes composed mostly of DDPIsC, an acid labile lipid, were evaluated for the delivery of chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4^{4-}$), a water-soluble photosensitiser. These liposomes showed substantially greater phototoxicity than free $AlPcS_4^{4-}$ and non-targeted liposomal $AlPcS_4^{4-}$ against FR(+) KB cells [58].

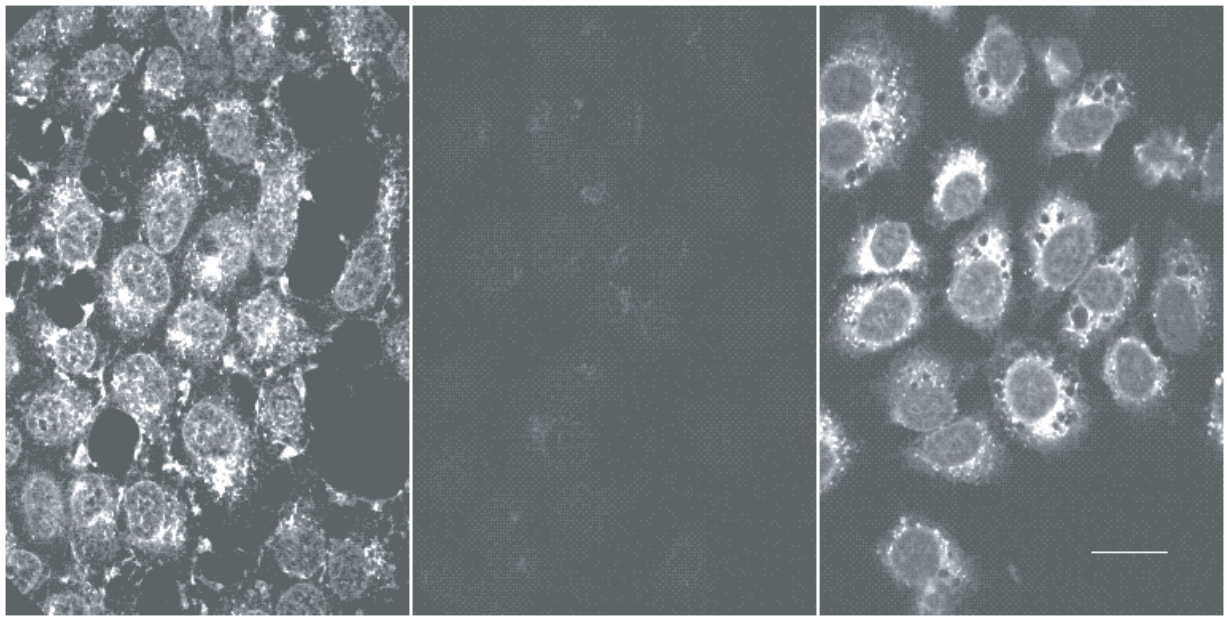

Figure 3. Confocal micrographs of KB carcinoma cells following incubation with various doxorubicin formulations. Adapted from LEE RJ, LOW PS, Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro*. *Biochim. Biophys. Acta* **1233**(2):134-144 Copyright © (1995), with permission from Elsevier [54].

6. Folate receptor-targeted liposomal delivery bypasses multi-drug resistance

Multi-drug resistance (MDR) is a major clinical problem in chemotherapy treatment of cancer. MDR is frequently due to upregulation in tumour cells of plasma membrane pumps, such as P-glycoprotein (Pgp) that actively pumps out cytotoxic agents [59]. Liposomal delivery of drugs has been shown to bypass MDR by possibly reducing exposure to the membrane efflux pump *in vitro* in leukaemia cells [60,61] and some solid tumour cells, including breast, ovarian and small-cell lung carcinoma cells [62]. FR-targeted liposomal delivery also shows the ability to overcome Pgp-mediated efflux in FR(+) cancer cells [63,64]. In a recent study, an MDR FR(+) murine lung cell line (M109R-HiFR) was treated with either free doxorubicin or FR-targeted liposomal doxorubicin. Verapamil, a Pgp inhibitor, greatly enhanced the cellular uptake of free doxorubicin, which otherwise would have been rapidly effluxed, while exhibiting no significant effect on the cellular accumulation of FR-targeted liposomes. Cellular fractionation analysis showed higher doxorubicin concentration in the nuclear fraction of cells treated with FR-targeted liposomal doxorubicin compared with the cells treated with free doxorubicin. Furthermore, FR-targeted doxorubicin showed greater tumour inhibitory activity than non-targeted liposomal doxorubicin and free doxorubicin in an *in vivo* adoptive study [63]. Another study found thermosensitive FR-targeted doxorubicin combined with hyperthermia was 4.8 times more effective than free doxorubicin on the MDR KB85 cells [64]. These results suggest that FR-targeted liposomal delivery are more effective in circumventing the Pgp-mediated MDR than non-targeted liposomal delivery in FR(+) tumours.

7. Folate receptor-targeted liposomal delivery to solid tumour

7.1 EPR effect and intratumoural drug distribution

The ligand-mediated 'active' targeting of drug carriers may also benefit from the unique characteristics of tumour vasculature, which have led to the approach of passive targeting of drugs based on the enhanced permeability and retention (EPR) effect. Pegylated FR-targeted liposomes exhibited extended systemic circulation time similar to pegylated non-targeted liposomes, which is a prerequisite of passive targeting (Figure 4a) [65]. It is, therefore, reasonable to anticipate that FR-targeted liposomes can utilise the same 'openings' (endothelial gaps and fenestrations in tumour microvasculature) to extravasate and reach the tumour cells. In a recent study from the authors' laboratory, FR-targeted liposomes labelled with ^{111}In were administered intravenously to C57BL/6 mice engrafted with FR(+) 24JK-FBP cells. Besides accumulation in the reticuloendothelial system (liver and spleen), relative high levels were found in tumours [55]. Furthermore, FR-targeted liposomal doxorubicin showed greater antitumour efficacy in a FR(+) KB cell BALB/c murine xenograft model. Similar plasma clearance kinetics were observed for the targeted and the non-targeted liposomes (Figure 5). Mice that received FR-targeted liposomal doxorubicin exhibited greater tumour growth inhibition and longer lifespan than those that received non-targeted liposomal

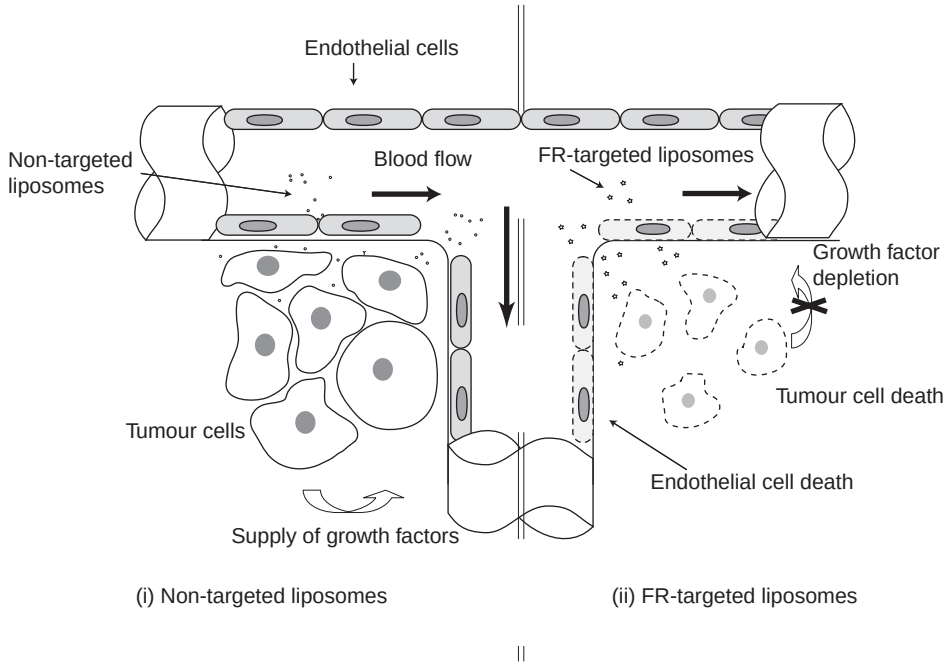

Figure 4a. Mechanisms for direct and indirect inhibition of tumour cells and endothelial cells. Non-targeted liposomes extravasate into tumour through the leaky blood vessels, but most of them remain in the extracellular interstitial space. (i) FR-targeted liposomes exert direct cytotoxicity towards the tumour cells close to the blood vessel and endothelial cells. (ii) Indirectly, distal tumour cells are killed by antiangiogenic effect, whereas the endothelial cells were killed by bystander effect of released free drug and growth factor depletion due to tumour cell death.
FR: Folate receptor.

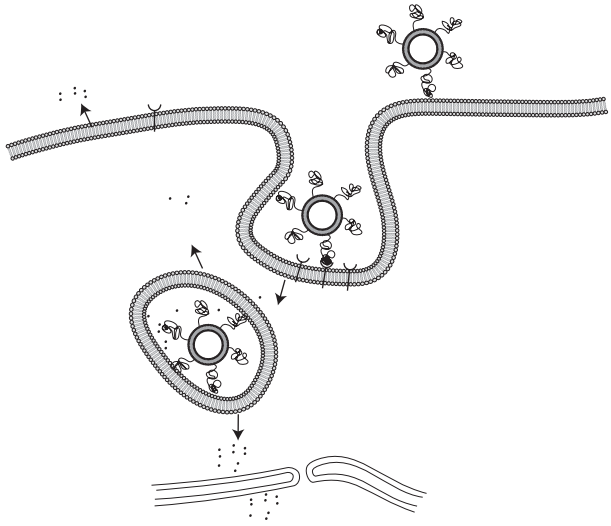

Figure 4b. A schematic diagram of the FR-mediated endocytosis pathway.
FR: Folate receptor.

doxorubicin [65] (Figure 6). The improved efficacy might be due to the recognition and internalisation of FR-targeted liposomes by the FR on the tumour cells following extravasation from the tumour vessels, which led to more efficient killing of the tumour cells [65]. In a recent study using the J6456 ascitic tumour model, overall liposome deposition in tumours was shown to be similar for FR-targeted and non-targeted liposomes [66]. However, FR-targeted liposomes appeared to have higher tumour cell-association than non-targeted liposomes [66]. These studies suggest that even though FR targeting might not significantly alter the overall biodistribution of liposomes in solid tumours, greater therapeutic efficacy might be possible due to increased FR-dependent uptake by the targeted tumour cells.

7.2 Potential role of antiangiogenic effect in targeted liposomal drug delivery

The breakdown of tumour vasculature by perivascular accumulation of liposomes and local release of drug might provide an additional antitumour mechanism. The limitations of rates of diffusion and convection pose a much greater barrier for the intratumoural distribution of liposomes than for conventional drugs. Intratumoural distribution of liposomes is, therefore, likely to be limited to the cell layers that are immediately adjacent to the blood vessel, as indicated by a recent study using animal tumour models [67]. Importantly, the necrosis induced by liposome-entrapped drug might influence the secondary distribution of the liposomes. For example, a recent study appeared to show increased vascular permeability following injection of liposomal doxorubicin in a rat 9L glioma model based on blood oxygenation level-dependent functional MRI [68,69].

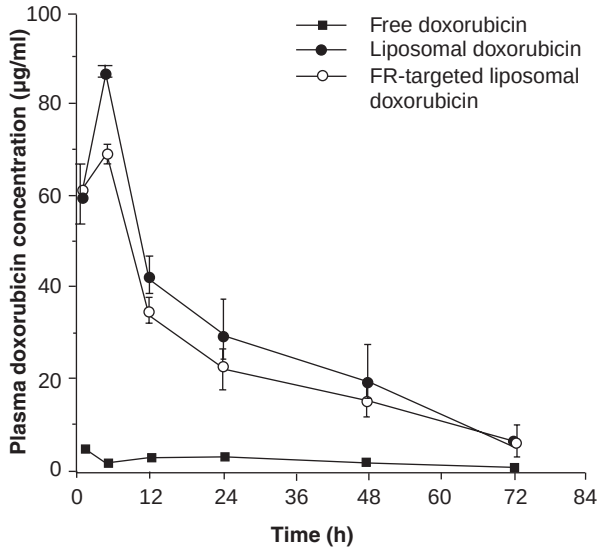

Figure 5. Blood clearance profiles of FR-targeted liposomal doxorubicin, liposomal doxorubicin, and free doxorubicin. Mice were treated with 10 mg/kg of FR-targeted liposomal, liposomal doxorubicin, or free doxorubicin by intraperitoneal injection. Plasma doxorubicin concentrations at various time after injection were determined by a fluorometric assay, as described in [65]. Error bar shown is equal to 1 standard deviation (n = 3). Adapted from PAN XQ, WANG H, LEE RJ: Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model. *Pharm. Res.* **20**(3):417-422, Copyright © 2003, with permission from Springer Science and Business Media [65].

The growth of tumour cells depends on nutrition and oxygen supplied by blood vessels. In turn, initiation and maintenance of blood vessels rely on growth factors provided by tumour or stromal cells [70-73]. Endothelial cells in the tumour are more susceptible to cytotoxic agents and apoptotic effects than quiescent endothelial cells in the normal tissue, due to their increased proliferation rates and lack of p53 mutation [74]. It is possible that FR-targeted liposomes can preferentially accumulate in the proximity of the blood vessels within the tumour and effectively eliminate adjacent tumour cells via active FR-internalisation, as well as destroy the endothelial cells via direct cytotoxicity and bystander effect (Figures 4a and b). In addition, FR-targeted liposomes might exhibit antitumour activity by facilitating tumour cell death via FR-mediated liposomal uptake, which shuts down the supply of vascular growth factors to the endothelial cells. The killing of endothelial cells serves to suffocate the tumour by diminishing oxygen and nutrition supply. The synergistic interplay of cytotoxicity and antiangiogenic activity may be jointly responsible for the observed antitumour efficacy of liposomal chemotherapy *in vivo*. Furthermore, FR-mediated liposomal internalisation may also promote drug release from the liposome, and facilitate secondary intratumoural distribution by diffusion following cell death. Finally, FR-targeted liposomes should also be able to effectively target tumour cells that are in the circulation or micrometastases that are directly accessible from circulation by means of diffusion.

8. Folate receptor liposomal delivery to leukaemia

FR-targeted liposomes can potentially be used as a targeted delivery vehicle to AMLs with amplified FR-β expression. Approximately 70% of AMLs express functional FR-β that is absent in normal haematopoietic cells [29,35]. FR-β can, therefore, serve as a maker for targeted delivery to AML [75]. However, the heterogeneous and variable expression of FR-β poses a potential obstacle to FR-β-targeted therapeutics. The problem can potentially be overcome by selectively inducing FR-β upregulation in the target cells via retinoid receptor ligands [76]. FR-β expression in KG-1 AML cells and primary AML blast cells (FAB-M2 and M4) can be up-regulated by all-trans-retinoic acid (ATRA), and reach steady-state levels that are up to ~ 20-fold higher within a few days [76]. ATRA-induced high FR-β differentiation does not cause terminal differentiation or growth inhibition in these cells. Furthermore, FR-β expression is restricted to the cell lines that are initially FR-β(+). FR-β(-) AML or other tumour cell lines including FR-α(+) cells can not be induced by ATRA to express FR-β [76].

A recent study showed that FR-targeted liposomal doxorubicin was 25-fold more cytotoxic than non-targeted liposomal doxorubicin to FR-β(+) KG-1 cells, and 63-fold more cytotoxic in ATRA-pretreated KG-1 cells [35]. In contrast, FR-β(-) cell lines did not show increased differential cytotoxicity when treated with ATRA [35]. Furthermore, *in vivo* therapeutic activity of FR-targeted liposomal doxorubicin was evaluated in two models, a DBA/2 mouse model containing syngeneic ascites tumour from L1210JF leukaemia cells and a severe combined immunodeficient murine xenograft model with human KG-1 AML cells ascites tumour [35]. In the latter model, FR-targeted liposomal doxorubicin increased the median survival time from 35 to ≥ 80 days. Moreover, mice administered with ATRA and FR-targeted liposomal doxorubicin showed further enhancement in antitumour efficacy, with an increase in cure rate from 12.5 to 60% [35]. As ATRA differentiation therapy is one of the standard treatments for acute promyelocytic leukaemia subtype of AML and liposomal doxorubicin has been approved for solid tumour treatment, the success of combined therapy using FR-targeted liposomal doxorubicin and ATRA in this experiment suggests, therefore, that further clinical studies may be warranted.

9. Folate receptor-targeted liposomes for gene and oligodeoxyribonucleotide delivery

Gene therapy is an emerging therapeutic modality for the treatment of cancers and genetic diseases. Efficient delivery of DNA is the limiting factor in the clinical application of gene

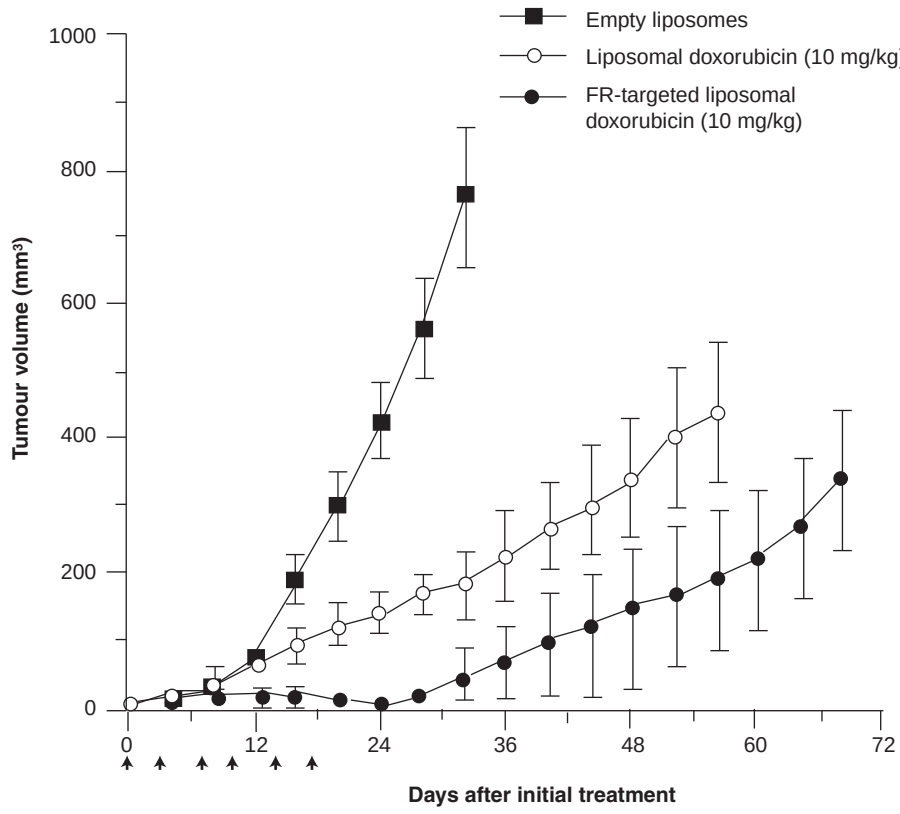

Figure 6. Tumour growth inhibition by FR-targeted liposomal doxorubicin or liposomal doxorubicin. Nude mice with KB tumour xenograft were treated with a series of six intraperitoneal injections (given on every fourth day, as indicated by the arrows) of liposomes containing doxorubicin 10 mg/kg or with unloaded liposomes. Tumour size was measured for each animal on every fourth day starting from the day of the initial treatment. Error bar represents 1 standard deviation (n = 8). Adapted from PAN XQ, WANG H, LEE RJ: Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model. *Pharm. Res.* **20**(3):417-422, Copyright © 2003, with permission from Springer Science and Business Media [65].

therapy. Several FR-targeted vectors have been reported, including folate-modified adenoviruses [46], cationic polymer/DNA complexes (polyplexes) [77], cationic lipid–DNA complexes (lipoplexes) [78,79], and polymer–lipid–DNA ternary complexes (lipopolyplexes) [46,80] that are linked to folate as a targeting ligand [16,48,81-83].

9.1 Folate receptor-targeted liposomes for delivery of plasmid DNA

FR-targeted lipoplexes have shown enhanced *in vitro* and *in vivo* transfection activity in FR(+) tumour cells [84,85]. Cationic liposomes consisting of RPR209120 (a lipopolyamine), dioleoylphosphatidylethanolamine (DOPE) and folate–PEG–cholesterol or folate–PEG–DSPE showed almost 1000-fold higher *in vitro* transfection than non-targeted lipoplexes in FR(+) M109 cell line. *In vivo* study of this formulation also indicated greater gene delivery in the tumour than in normal tissues, even though there was no significantly increased tumour uptake of FR-targeted formulations compared with non-targeted formulations.

Lipopolyplexes (LPD) are ternary complexes consisting of liposomes complexed with polycations (such as polylysine [PLL], polyethylenimine, protamine and polyamidoamine dendrimers) and condensed plasmid DNA. Condensed DNA complexed with cationic liposomes or anionic liposomes were coined by authors as LPDI or LPDII, respectively [46,86]. FR-targeted LPDI composed of cationic liposomes/protamine/DNA and folate–Cys–PEG–PE(phosphatidylethanolamine) showed greater transfection activity in FR(+) M109 cell line. This formulation also showed 8- to 10-fold higher gene transfer activity *in vivo* compared to the non-targeted control. However, increasing the folate ligand density on liposome surface resulted in decreased gene transfer activity, presumably due to increased steric hindrance of PEG that prevented efficient endosomal release of the vector. The overall positively-charged LPDI might also nonspecifically interact with negatively cell membranes, and result in increased nonspecific gene transfer.

FR-targeted LPDII, first developed by Lee and Huang [46], has a net anionic character and show reduced nonspecific binding to cell membranes and, therefore, increased receptor dependent gene transfer. Preparation of FR-targeted LPDII can be carried out by first condensing DNA with PLL at a ratio of 1:0.75 (w/w) to get an overall slightly positive-charged

DNA/PLL complex, and then mixing with anionic pH-sensitive liposomes composed of DOPE(dioleoyl phosphatidylethanolamine)/CHMES(cholesteryl hemisuccinate)/folate–PEG–DOPE (6:4:0.01 mole/mole). The resulting FR-targeted LPDII showed spherical particles with a mean diameter of ~ 74 ± 14 nm under an electron microscope, and exhibited superior transfection efficiency compared with non-targeted LPDII. Another FR-targeted LPDII developed by Reddy *et al.* [87] comprised DNA–PLL complex and DOPE–cholesterol–C–DOPE (an acid-labile lipid for pH triggered endosomal release)/folate–PEG–DOPE similarly exhibited efficient FR-mediated gene transfer [87].

9.2 Folate receptor-targeted liposomes for delivery of antisense oligodeoxyribonucleotides

Based on electrostatic interaction, negatively-charged antisense oligodeoxyribonucleotides (ODNs) can also be encapsulated into FR-targeted liposomes for selective delivery to FR(+) tumour cells both *in vitro* and *in vivo* [39]. FR-targeted cationic liposomes mediated the delivery of anti-HER-2 antisense oligonucleotide (AS HER-2 ODN), and inhibited cell growth and HER-2 expression [88]. *In vivo* study also showed prolonged stability in blood and increased uptake in tumours [89]. In a separate report, FR-targeted liposomes have also been evaluated as a carrier of antisense ODNs against the epidermal growth factor receptor and shown improved efficacy relative to free- or non-targeted liposomal ODNs [10].

10. Expert opinion and conclusions

Many FR-targeted therapies have been evaluated both *in vitro* and *in vivo* and have consistently shown excellent tumour cell-targeting properties. Radionuclide conjugates of folic acid for imaging of ovarian and endometrial cancer have been evaluated clinically with promising initial results [48]. However, there have not yet been clinical studies evaluating therapeutic potential of FR-targeted liposomes. Recent promising data on FR-targeted liposomes in animal tumour models and similar findings in studies on anti-HER2-immunoliposomes suggests that targeted liposomes are potentially therapeutically superior to non-targeted liposomes, even though the effect of targeting on overall tumour accumulation is usually moderate at best due to the over-riding EPR effect [90]. These data, combined with the many theoretical advantages of FR-targeting, such as non-immunogenicity, Pgp avoidance, and available methods of *in vivo* upregulation, suggest FR-targeted liposomes have great potential for future clinical applications.

Although FR-targeted liposomal delivery has consistently produced satisfying results *in vitro*, fewer *in vivo* studies have been reported so far. For solid tumours, studies seem to suggest that, rather than increasing overall tumour localisation, elevated cellular internalisation, bypassing of Pgp, and antiangiogenic effects might play critical roles for the superior efficacy of targeted liposomes. In addition, there is great rationale for exploring targeted liposomal delivery to leukaemias, due to the relative accessibility of the target cells and the long circulating properties of liposomal drug carriers. The clinical potential of this delivery strategy has yet to be explored. As FR expression in cancer and leukaemia patients is likely to be variable, effective targeting might benefit from co-administration of ATRA (for FR-β upregulation in AMLs) and tamoxifen (for FR-α upregulation in solid tumours). In addition, prescreening of patients using serum FR assay and/or imaging using FR-targeted radiopharmaceuticals might be necessary to determine the potential benefit for targeted therapy. In addition to cancer and leukaemia, FR is also overexpressed among activated macrophages in rheumatoid arthritis, which constitutes another potential disease target for FR-targeted liposomes. Further preclinical and clinical studies are clearly warranted in order to assess the potential role of FR-targeted liposomes in the management of cancer, leukaemia and rheumatoid arthritis.

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