Expert Opinion

Targeted drug delivery via folate receptors

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Targeted delivery via selective cellular markers can potentially increase the efficacy and reduce the toxicity of therapeutic agents. The folate receptor (FR) has two glycosyl phosphatidylinositol (GPI)-anchored isoforms, α and β. FR-α expression is frequently amplified in epithelial cancers, whereas FR-β expression is found in myeloid leukemia and activated macrophages associated with chronic inflammatory diseases. Conjugates of folic acid and anti-FR antibodies can be taken up by cancer cells via receptor-mediated endocytosis, thus providing a mechanism for targeted delivery to FR+ cells. The aim of this article is to provide a brief overview of applications of FR targeting in drug delivery, with an emphasis on the strategy of using folate as a targeting ligand. In order to do this, recent literature is surveyed on targeted delivery via both FR sub-types, as well as new findings on selective receptor upregulation in the targeted cells. A wide variety of molecules and drug carriers, including imaging agents, chemotherapeutic agents, oligonucleotides, proteins, haptens, liposomes, nanoparticles and gene transfer vectors have been conjugated to folate and evaluated for FR-targeted delivery. Substantial targeting efficacy has been found both *in vitro* and *in vivo*. In addition, mechanisms and methods for selective FR upregulation have been uncovered, which might enhance the effectiveness of the FR-targeted delivery strategy. FR-α serves as a useful marker for cancer, whereas FR-β serves as a marker for myeloid leukemia and chronic inflammatory diseases. FR-targeted agents have shown promising efficacy in preclinical models and significant potential for future clinical application in a wide range of diseases.

Keywords: cancer, folate receptor, leukemia, liposomes, targeted drug delivery

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1. Introduction

Targeted drug delivery is a promising strategy to improve the efficacy and safety of therapeutic agents. This is particularly valuable against diseases like cancer, for which dose-limiting toxicity of the drug and the development of drug resistance constitute major barriers to therapeutic success. Among cellular surface targets potentially suitable for use in drug targeting, folate receptor (FR)-α stands out as one of the most promising and one of the most investigated epithelial cancer markers. More recently, the potential importance of FR-β as a cellular target has been increasingly recognized, which extends the range of disease targets to myelogenous leukemia and chronic inflammatory diseases such as rheumatoid arthritis. In this article, a brief overview of published work related to FR-targeting is presented, followed by a discussion of its potential clinical implication.

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2. FRs as selective cellular markers

FRs, also known as folate-binding proteins (FBP), are *N*-glycosylated proteins with high binding affinity to folate. FRs include at least four isoforms, α, β, γ/γ′ and δ. The α, β, and δ isoforms are glycosyl phosphatidylinositol (GPI)-anchored membrane proteins [1-4], whereas FR-γ/γ′ is constitutively secreted by lymphoid cells [5,6]. The affinities of folic acid for the FRs are: FR-α, K_d ~ 0.1 nM [7]; FR- β, K_d ~ 1 nM [8]; and FR-γ, K_d ~ 0.4 nM [4]. FR isoforms display divergent patterns of tissue expression [3]. FR-δ has been found to be expressed on regulatory T cells and has recently been proposed as a potential therapeutic target [9]. However, most of the literature to date has been focused on FR-α and FR-β. These two isoforms share high amino acid sequence identity (~ 70%) and are distinguishable by differential affinities for folic acid and stereoisomers of reduced folates.

It is important to note that unlike the reduced folate carrier (RFC), which mediates transmembrane folate transport, has a K_d in the μM range and is ubiquitously expressed, FR is not normally required for cellular survival and expression of FRs is highly restricted among tissues. The > 10^3-fold higher affinity of FRs for folate enables *in vivo* targeting of the FRs via folate conjugation without concerns over potential interference from the much weaker RFC binding. In other words, distribution of FR-targeted agents is unlikely to be affected by the presence of RFC in non-target tissues.

FR expression, both tissue-specific and differentiation dependent, has been studied by immunohistochemical staining, reverse transcription-polymerase chain reaction (RT-PCR), Western blotting and ^{3}H-folic acid binding, in both normal and malignant tissues [10,11]. Functional FR expression is low or absent in most normal tissues, with the exception of FR-α expression in the luminal surface of certain epithelial cells [11], where it has limited accessibility from the bloodstream. FR-α is expressed on the apical membrane of epithelial cells in the kidney proximal tubules and mediates transport of folate or folate conjugates through transcytosis [12].

FR-α is consistently expressed in several carcinomas [11], especially in non-mucinous ovarian carcinomas, uterine carcinomas, testicular choriocarcinomas, ependymomas, and pleural mesotheliomas, and less frequently in breast, colon, and renal cell carcinomas [11]. Correlation of FR-α expression level and histologic grade and response rate to chemotherapy has also been shown in ovarian and breast cancers, suggesting FR-α as a selective marker for cancers and possibly a prognostic marker [13]. Methods for upregulating FR-α expression using anti-estrogens and glucocorticoid agonists have recently been reported [14,15]. The effects of these agents were further enhanced by histone deacetylase (HDAC) inhibitors [15].

FR-β is a differentiation marker in the myelomonocytic lineage during neutrophil maturation [10] and is amplified in activated monocytes and macrophages [16]. However, FR-β in neutrophils is unable to bind folate due to aberrant post-translational modifications [17]. In addition, FR-β is expressed in a functional form in chronic myelogenous leukemia (CML), in 70% of acute myelogenous leukemias (AML) [4,10,18,19] and in activated macrophages associated with rheumatoid arthritis and other chronic inflammatory diseases [20]. FR-β expression is regulated by retinoid receptors and can be upregulated by all-*trans* retinoic acid (ATRA), particularly in combination with HDAC inhibitors [21,22].

Tissue selective expression of FR-α and FR-β and the lack of normal tissue expression of functional and accessible FRs suggest that these can be used as markers for targeted drug delivery to disease cells. On the plasma membrane, FRs enriched in cholesterol-rich lipid rafts, like other GPI-anchored proteins. FR-α endocytosis has been shown to involve caveolae rather than clathrin-coated pits [23]. Folate conjugates have been shown to retain affinity for FRs and interact with the target cells via high affinity binding and are internalized by receptor-mediated endocytosis. The additional possibility of selective FR upregulation in FR expressing cells, but not in tissues where FR expression is undetectable, might further enhance the effectiveness of the targeting strategy.

3. FR targeting for therapeutic delivery – from ovarian cancer to chronic inflammatory diseases

There are two general strategies for targeting FRs: conjugation to anti-FR antibody and conjugation to folate. Early work in FR targeting was based on monoclonal antibodies (mAbs) MOv18 and MOv19, which were murine mAbs of the IgG_1 class raised against a poorly differentiated ovarian carcinoma, recognizing two distinct epitopes [24]. Clinical studies on radioimmunoscintigraphy using ^{131}I-MOv18 were carried out in ovarian cancer patients and showed some efficacy [25]. In addition, α-particle-emitting ^{211}At conjugated MOv18 was found to prolong survival in a murine ascites tumor model developed by i.p.-inoculation of OVCAR-3 ovarian cancer cells [26]. Anti-FR-α/anti-CD3 bispecific antibody has been shown to mediate tumor cell lysis via tumor cell–T-cell cross-linking [27]. MOv19/IL-2 fusion protein was evaluated as an immunotherapy agent against a preclinical model of an FR+ murine tumor and was shown to be effective [28]. MORAb-003, a humanized IgG1 mAb, is currently being developed as a therapeutic antibody for ovarian cancer [29]. It has been shown to efficiently mediate complement-dependant cytotoxicity (CDC), as well as antibody-dependent cellular cytotoxicity (ADCC) and is in Phase II clinical trial. Because mAbs binding to FR is unaffected by the presence of folate or the folate binding capacity of the FR, it has some advantages over folate as a ligand for FR targeting. However, due to the limited availability of mAbs and numerous potential advantages of a low molecular weight

ligand, a vast majority of FR targeting strategies utilize folate as the targeting ligand.

Folate, with a molecular weight of 441, is a vitamin and an oxidized form of folate coenzyme. Compared to [6S]5-methyltetrahydrofolate (5-MTHF), the predominant physiological form of folate, folic acid has a much higher affinity (~ 30x higher) for the FRs. Upon derivatization via one of its carboxyls, folate retains a high affinity for FRs. The K_d of folate conjugates for FRs is usually ~ 10x higher than folic acid, indicating a slight reduction in binding affinity, but still within the nM range. FRs mediate cellular internalization of folate conjugates via receptor-mediated endocytosis. Folate conjugation, therefore, constitutes a method for targeted intracellular delivery of therapeutic agents to FR+ cells. FR-mediated endocytosis appears to follow a non-degradative pathway for folate conjugates and facilitate their intracellular accumulation via receptor recycling. As a low molecular weight physiological ligand, folate is presumably non-immunogenic and is easily derivatized. These desirable properties, combined with its convenient availability, have made folate one of the most studied ligands in targeted drug delivery. A wide range of molecules and drug carriers have been conjugated to folate and evaluated for FR targeting. This has been the subject of numerous recent review articles [30-34].

The following is a brief overview.

3.1 FR-targeted imaging agents

Folate conjugates have been synthesized as FR-targeted contrast agents for radionuclide imaging, magnetic resonance imaging (MRI) and optical imaging [35]. A number of γ-emitting metal chelates have been conjugated to folate, including ^{67}Ga-deferoxamine-folate [36,37], ^{111}In-DTPA-folate [38], ^{99m}Tc-HYNIC-folate [39], ^{99m}Tc-DTPA-folate [40], ^{99m}Tc-EC20-folate [41] and ^{67}Cu-CYCLAM-folate [42]. All of these agents showed FR-dependent cellular uptake *in vitro* and tumor-specific accumulation *in vivo* in murine tumor models. Kidneys also accumulated high levels of the folate conjugate, presumably due to FR expression on the apical membrane of the kidney proximal tubules. ^{111}In-DTPA-folate (FolateScan™) has shown increased tumor localization in FR-α+ tumor mass in a Phase I/II clinical trial in ovarian cancer patients. The results of this study established the feasibility of FR targeting using folate conjugate in a clinical setting and the value of FR-α as an ovarian cancer marker. The tumor cell specificity of FR imaging, however, is limited by the fact that tissues containing activated (FR-β+) macrophages, such as arthritic joints and inflammatory tissues, also accumulated folate conjugates [43]. Besides radionuclides, folate has been conjugated to ^{153}Gd-dendrimers [44] and iron oxide nanoparticles [45] for MRI, and near infrared (NIR) fluorophores and lipid-coated quantum dots for fluorescence optical imaging of FR-α+ tumor cells and/or FR-β+ macrophages associated with tumors and arthritis. Because folate conjugated imaging agents, with the exception of the nanoparticles, are of low molecular weights, they typically distribute rapidly to extravascular target tissues and are subjected to relatively rapid clearance from the blood and non-target tissues. This results in much higher tumor-to-blood and tumor-to-background tissue ratios than macromolecular tumor targeting conjugates such as radiolabeled mAbs, and at a much earlier time-point. This exemplifies the pharmacokinetic advantages of a low molecular weight targeted agent over high molecular weight conjugates. Another important advantage is the presumed lack of immunogenicity of these conjugates, which would enable repeated administration.

3.2 Folate conjugation of chemotherapeutics

Several chemotherapeutic agents have been conjugated to folate for targeted delivery to FR+ cells. Examples are folate conjugates of platinum [46], FdUMP oligomer [47], paclitaxel [48], maytansinoids [49], mitomycin C, vinca alkaloids [50] and a prodrug of thiolate histone deacetylase (HDAC) inhibitor [51]. In addition, a number of folate conjugates of chemotherapeutics have recently been synthesized at Endocyte, Inc., a company specializing in commercialization of folate conjugates for clinical applications. In general, these folate conjugates have shown high affinities for FRs and selective cytotoxicity against FR+ cells. The degree of selectivity depends on the potency of the attached chemotherapeutic agent, linker chemistry and hydrophobicity of the conjugate, which contributes to non-specific cellular uptake. Because the molecular weight of these folate conjugates are within several kDa and frequently ~ 1 kDa, they are able to distribute rapidly to tissues and gain access to FR+ cells and are quickly cleared from the plasma and non-target tissues. As shown in studies with the folate-conjugated radiopharmaceuticals, high level FR-mediated accumulation in the kidneys is expected of these conjugates. Early studies have not shown acute nephrotoxicity, which bodes well for further clinical development of this type of targeted agents. As low molecular weight agents, the folate chemotherapeutics have favorable pharmacokinetic properties that contribute to the rapid achievement of high tumor-to-blood ratio and are presumably non-immunogenic and can be administered repeatedly.

3.3 FR-targeted haptens for immunotherapy

Folate has been conjugated to haptens such as fluorescein isothiocyanate (FITC) [34]. These conjugates are able to selectively target FR+ tumor cells [52] or activated macrophages [43,53], as one would expect. Treatment with FITC–folate treatment combined with pre-immunization with FITC carrier protein, which generates anti-FITC antibodies, can be used as an immunotherapy against cancer and arthritis. This is because FITC–folate can mediate anti-FITC antibody binding to FR+ cells and thus induce antibody-dependent cellular cytotoxicity (ADCC). The therapeutic effects were shown to be further enhanced

with cytokine (IL-2 and INF-α) co-administration [54], which promoted immune effector functions.

3.4 Folate conjugated oligodeoxyribonucleotides (ODNs)

To facilitate targeted delivery to tumor cells, folate has been conjugated to an anti-c-fos antisense ODN [55]. The conjugate exhibited FR-dependent uptake in FR+ cells and inhibited the proliferation of tumor cells [55]. Compared to the alternative strategy of using folate-conjugated drug carriers, which will be described below, these folate conjugates are smaller in size and thus may have pharmacokinetic advantages.

3.5 FR-targeted macromolecular therapeutics

Folate has been conjugated to protein toxins, enzymes and anti-T-cell receptor mAbs [56,57]. Folate is typically conjugated to the protein via N-terminal α-amino and/or lysine ε-amino groups, either directly or via a cleavable linker. Protein toxins, such as momordin, pseudomonas exotoxin and gelonin [58-60] have been conjugated to folate and shown to exhibit selective cytotoxicity toward FR+ cells. Enzymes conjugated to folate include horseradish peroxidase (HRP) as a probe [61], prodrug converting enzyme penicillin-V amidase (PVA) [62] and catalase/superoxide dismutase (SOD) [63]. For T-cell-based immunotherapy, anti-T-cell receptor mAbs and their fragments have been conjugated to folate to facilitate T-cell cross-linking to FR+ tumor cells [26,64-66]. In all cases, the folate conjugates were shown to selectively bind to FR+ tumor cells and/or to mediate selective cytotoxicity. The folate conjugates function similarly to tumor targeting immunoconjugates, but are smaller in size due to the low molecular weight of folate. The potential disadvantage is that the site of folate conjugation typically cannot precisely be controlled, resulting in a heterogeneous population of folate conjugates.

3.6 FR-targeted drug carriers

Various types of drug carriers have been conjugated to folate, including liposomes, lipid nanoparticles, polymeric nanoparticles, polymers and micelles. For targeting of liposomes and nanoparticles, a lengthy PEG-based linker is frequently found to be required, possibly due to the large size of the drug carrier [67]. Multiple folates are typically conjugated to each particle, which enables high affinity multivalent interaction to the FRs. Liposomes and nanoparticles have a long systemic circulation time and are not subjected to renal clearance. However, liposomes and nanoparticles are cleared by the reticuloendothelial system (RES) and have low extravasation rate and limited ability to diffuse within solid tumors. This is likely to limit the achievable tumor-to-blood ratio relative to that of passive targeting of solid tumors based on enhanced permeability and retention (EPR) effect. Moreover, folate-conjugated liposomes and nanoparticles have more rapid clearance via the liver and the spleen. This is most likely due to their greater affinity to RES cells and some level of expression of FR-β on regular macrophages, since clearance of folate liposomes appears to be FR-mediated and was accelerated in mice fed on a folate-free diet [68]. However, for certain disease targets such as AML, where the leukemia cells are FR-β+ and are relatively accessible from the bloodstream, there might be greater opportunity for FR-targeted liposomes and nanoparticles. The advantage of using a drug carrier is the potentially high drug loading capacity of the carrier, the avoidance of direct chemical conjugation with the drug molecule, the avoidance of unintended targeting of FRs in the kidney (for conjugates larger than 50 kDa) and the opportunity for engineering of the drug carrier, for example for long circulation or endosomal drug release. The following are some examples of FR-targeted drug carriers:

FR-targeted liposomes were synthesized by incorporation of lipophilic derivatives of folate, such as folate-polyethyleneglycol (MW 3,350)-distearoyl phosphatidylethanolamine (folate-PEG-DSPE) [69] and folate-PEG-cholesterol (folate-PEG-chol) [70]. The liposomes can further be coated with PEG to promote prolonged systemic circulation. The lipid composition can be adjusted to facilitate stability or drug loading. For example, cationic lipids can be included to enhance loading of ODN drugs. A variety of agents have been loaded into FR-targeted liposomes, including doxorubicin [71-73], daunorubicin [74,75], cytosine arabinoside [76], paclitaxel [77], neutron capturing boronated agents [78,79], photosensitizers [80] and antisense ODNs [81]. Goren *et al.* showed that the delivery of doxorubicin via FR-targeted liposomes bypassed Pgp-dependent drug efflux in drug resistant FR-α+ M109 murine lung carcinoma cells [82]. These data suggested that FR-mediated delivery might overcome drug resistance by FR+ tumors [83]. More recently, FR-targeted liposomal doxorubicin was studied in FR-β+ KG1 and MV4-11 human AML cells and showed enhanced cytotoxicity relative to non-targeted control liposomes [21,73], and the effect was enhanced by selective FR-β upregulation using ATRA [21,73]. Therefore, both FR-α and FR-β are viable targets for folate-conjugated liposomes. FR-targeted liposomal doxorubicin showed increased antitumor activity compared to non-targeted liposomal doxorubicin in an FR+ KB cell nude mouse xenograft model. A possible mechanism for increased therapeutic efficacy for the folate–liposomal doxorubicin in solid tumor is altered intratumoral drug distribution. These liposomes might be more efficiently internalized by FR-α+ tumor cells and/or tumor-infiltrating macrophages that are FR-β+ [84]. This in turn would result in an increase in drug release within the tumor. In contrast, non-targeted liposomal doxorubicin may remain extracellular, be distributed in the interstitial space and release the drug much more slowly. This may result in a bioavailable (free) drug concentration that falls below the minimum effective concentration for inducing tumor cell apoptosis. As shown by very high

IC_{50} values *in vitro*, liposome-entrapped doxorubicin is not very cytotoxic due to drug sequestration. The liposomal drug requires 'activation' by release from the liposomes. Therefore, cellular internalization and the associated drug release may be a critical mechanism of liposomal drug action in solid tumors and a rate-limiting factor in the overall drug delivery pathway. The relative importance of FR expression on tumor cells and tumor infiltrating macrophages and their role in uptake and drug release kinetics of folate-coated liposomes warrant further investigation.

Similar to liposomes, FR-targeted lipid nanoparticles and nanoemulsions, containing an oil phase, have been synthesized using folate-PEG-Chol as FR-targeting ligand and loaded with lipophilic drugs, including paclitaxel and paclitaxel cholesteryl ester [84]. These nanoparticles showed selective cytotoxity against FR+ cells. Folate conjugated pluronic block copolymers were found to be effective in targeted delivery of paclitaxel to multidrug resistant cells [85]. Antisense ODN-PEG-folate was synthesized and formed micelles with cationic lipids to facilitate its delivery [86]. Polymeric micelles were synthesized from cholesterol-grafted poly(*N*-isopropylacrylamide-co-*N,N*-dimethylacrylamide-co-undecenoic acid) and conjugated to folate and paclitaxel [87]. In another study, folate is conjugated to micelles of poly(D,L-lactic-co-glycolic acid) (PLGA) incorporating doxorubicin [88].

3.7 FR-targeted gene delivery

Numerous studies have been carried out evaluating targeted gene delivery through FRs, using either viral or nonviral vectors. FR targeting has been applied to viral gene transfer vectors. For example, folate-conjugated antibody has been used to alter tropism of adenoviral vectors for targeted gene delivery to FR+ cells [89]. Folate has also been conjugated to baculovirus via a PEG linker for its targeting to FR+ cells [90]. FR-targeted non-viral vectors include cationic polymers, cationic liposomes and pH-sensitive liposomes. These vectors have invariably shown impressive FR-selectivity in cell culture assays and, in addition, shown promising tumor-specific gene transfer activity in several *in vivo* models. In these vectors, folate was either directly linked to a polymer or a lipid component, or indirectly linked via a PEG spacer. The types of formulations included polyplexes, lipoplexes and lipopolyplexes.

For FR-targeted polyplexes, several cationic polymer–folate conjugates have been synthesized for FR-targeted gene delivery. These include poly-L-lysine (PLL)-folate [91,92] PLL-PEG-folate [93], polyethylenimine (PEI)-folate [94], PEI-PEG-folate [94,95], chitosan-folate [96], and pDMAEMA-PEG-folate [97]. Unlike PLL, PEI has inherent endosomal lytic activity and can transfect cells efficiently without the help of another helper component [94]. FR selectivity of the transfection complexes was superseded by non-specific transfection at high positive/negative charge (N/P) ratios, but was shown to be maximized at the neutral charge ratios. It was shown that incorporation of a long PEG spacer between folate and a cationic polymer and a component with endosomal or lysosomal lytic property were both important but might not be essential for efficient FR-selective gene delivery. High FR selective gene transfer can only be obtained at neutral charge ratios, where the *in vitro* transfection activity might not be at a maximum. PEGylation appeared to be a useful method to prolong the systemic circulation time of the FR-targeted polyplexes. Folate conjugation and PEGylation following polyplex formation might be the preferred method since this does not interfere with DNA condensation by the cationic polymer, which is more efficiently accomplished with non-PEGylated cationic polymers.

For FR-targeted lipoplexes, several cationic liposome formulations that incorporate a lipophilic folate derivative as a targeting entity have been studied. Xu *et al.* showed that delivery of p53 gene therapy using cationic liposomes conjugated to folate enhanced the antitumor efficacy of conventional chemo- and radiotherapy [98]. The specific structure of the targeting component used was, however, not described. Folate-PEG-DSPE and folate-PEG-Chol, when combined with a cationic lipid RPR209120 and DOPE, formed lipoplexes with greatly reduced normal tissue gene transfer and efficient *in vivo* tumor gene transfer, although no increase in tumor accumulation was observed compared with non-targeted lipoplexes [99]. In another study, Dauty *et al.* prepared an FR-targeted cationic liposomal vector incorporating 2% folate-PEG-DPPE, and a cationic dithiol detergent as the lipid component and showed efficient FR-dependent cellular uptake and transfection. The disulfide bond in this novel lipid can be reduced in the intracellular environment into surfactant-like molecules, which might facilitate endosomal escape [100].

FR-targeting strategies have also been applied to gene transfer vectors based on lipopolyplexes. LPDI-type lipopolyplexes consist of a ternary complex of cationic liposomes, DNA-condensing polycation and plasmid DNA [101,102]. LPDII-type lipopolyplexes consist of a ternary complex of anionic liposomes, polycation and plasmid DNA. The formulation of LPDII-type vector was reported, in which DNA was first complexed to PLL and then mixed with pH-sensitive anionic liposomes composed of DOPE/CHEMS/folate-PEG-DOPE (6:4:0.01 mole/mole). At low lipid-to-DNA (L/D) ratios, the vectors carried a net positive charge and transfected the cells independent of the FR [101,103]. In contrast, at high L/D ratios, the vectors carried a net negative charge and mediated FR-dependent transfection in FR+ KB cells but not in FR-CHO cells. These first generation LPDII vectors were inactivated by the presence of serum. A variation of this vector formulation was, therefore, developed by Gosselin *et al.* as covalently cross-linked by dithiobis (succinimidylpropionate) (DSP) or dimethyl 3,3′-dithio-*bis*-propionimidate (DTBP), as a DNA condensing agent and a lipid composition of

diolein/CHEMS/folate-PEG-Chol (6:4:0.05 m/m) [104]. The disulfide cross-linked PEI can theoretically be reduced in the intracellular environment, which facilitates the cytosolic release of the associated plasmid DNA following cellular entry. Diolein was selected as a helper lipid due to its ability to promote H_{II} phase formation. The resulting FR-targeted vectors exhibited improved gene transfer activity in the presence of serum compared to the DOPE-based FR-targeted LPDII vectors. Furthermore, Shi *et al.* [105] reported efficient gene delivery using an LPDII vector that incorporated PEI as a DNA condensing agent and a cationic/anionic lipid pair, composed of DDAB/CHEMS/Tween-80/folate-PEG-DSPE, as a novel pH-sensitive endosome disruptive component and achieved excellent *in vitro* transfection results in serum containing media. Another FR-targeted LPD II-type formulation was reported by Reddy *et al.* [106,107] In their formulation, PLL/DNA polyplexes with a net positive charge were combined with pH-sensitive liposomes composed of DOPE/cholesterol/*N*-citraconyl-DOPE/folate-PEG-DOPE. The component *N*-citraconyl-DOPE was hydrolyzed at endosomal pH, thereby unmasking the fusogenic properties of DOPE. The resulting vector mediated highly efficient FR-dependent gene transfer. Cationic polymers and cationic liposomes have the ability to form electrostatic complexes with plasmid DNA and facilitate its cellular uptake via charge-mediated interactions. Such a non-specific mechanism of delivery is unlikely to be effective in gene transfer to tumor cells via systemic administration, given the high concentration of plasma proteins and blood cells in the circulation. In contrast, FR-based targeting is a promising mechanism to facilitate tumor-selective gene delivery *in vivo*.

3.8 FR-targeted liposomes for antisense ODN delivery

FR-targeted liposomes were evaluated as a potential vehicle for ODN delivery. In a study by Wang *et al.*, a 15-mer antisense to the EGF receptor gene (AEGFR2) was entrapped in FR-targeted liposomes and evaluated in cultured KB cells [108]. The FR-targeted liposomal ODNs exhibited greater efficacy in cellular growth and EGFR expression inhibition compared to free ODNs and non-targeted liposomal ODNs. A similar system based on liposomal entrapment was evaluated by Leamon *et al.* for the FR-targeted delivery of ^{35}S or FITC-labeled ODNs [109]. The study found that a PEG linker length of 1 kDa between folate and the lipid anchor was sufficient for full FR targeting efficiency. Another study by Rait *et al.* showed that FR-targeted cationic liposomes were more efficient than the non-selective Lipofectin™ transfection agent in delivering anti-HER-2 ODNs into breast cancer cells both *in vitro* and *in vivo* [110,111]. The optimal cationic liposome composition was found to be DDAB/DOPE (1:1). Compared to free ODNs, folate-coated cationic liposomal ODNs exhibited prolonged systemic circulation time and increased tumor localization. Interestingly, treatment with these ODNs resulted in the chemosensitization of the tumor cells, both in culture and in a murine xenograft, to docetaxel, regardless of HER-2 expression status of these cells. A similar study by Zhou *et al.* [112], showed that FR-targeted near-neutral liposomes carrying antisense ODNs to be superior to non-targeted liposomes in ODN delivery in FR+ cells and was unaffected by the presence of serum in the culture media. Recently, a folate-PEG-PEI conjugate has been used for the delivery of siRNA and shown to be highly effective [113].

4. Conclusion

FRs are emerging as valuable cellular markers for prognosis and targeted therapeutic delivery based on receptor upregulation in disease tissues. Initial interest in FR targeting has been concentrated on FR-α, which is frequently elevated in carcinoma cells. The potential importance of FR-β, a myeloid lineage differentiation marker found in AML and activated macrophages is becoming realized as an important target for FR-targeted therapeutics. A large volume of work has been accumulated in the literature on application of FR-targeting on a variety of therapeutic modalities. *In vitro* data uniformly demonstrated selective targeting of FR+ cells. Some promising imaging and therapeutic data have been obtained in preclinical models, although FR-dependent tumor localization is difficult to demonstrate for liposomes and nanoparticles due to their size. Results from clinical trials involving targeted imaging agents and anti-FR therapeutic antibodies in ovarian cancer were also promising. Methods for selective FR upregulation using common clinical agents (dexamethasone, ATRA) have been reported. Properties including small size, low cost, high affinity of folate for FRs and high selectivity of FR tissue distribution, are likely to make folate a versatile targeting ligand for cancer, leukemia and chronic inflammatory diseases.

5. Expert opinion

FR-targeted delivery is a promising strategy for treatment of FR+ tumors, AML and chronic inflammatory diseases involving activated macrophages, such as rheumatoid arthritis. The effectiveness of targeted drug delivery is limited by the abundance of FR on the target cell surface, which is a common limitation in receptor-based targeted delivery strategies. Only high potency agents with nM range or lower effective concentration are potentially suitable for receptor-based targeted delivery. The obstacle of limited FR expression can be partially overcome by receptor modulation, for example, using dexamethasone for FR-α upregulation and ATRA for FR-β upregulation. Plasma folate may compete with folate conjugates for FR binding. However, it appears that FR binding competition from the endogenous form of folate, i.e., [6S]5-MTHF (at 1 – 50 nM in human plasma), which has ~ 30x low affinity for the FR compared to folic acid, should not significantly impede FR

binding of folate conjugates, especially multivalent liposomes and nanoparticles, which typically exhibit a much higher apparent affinity. This is evidenced by tumor localization of ^{111}In-DTPA-folate in ovarian cancer patients. Nevertheless, high dose folate or multivitamin supplementation during therapy may result in supra-physiological plasma folate concentrations and interfere with FR targeting and thus should be avoided.

The molecular weight of a folate conjugate has a profound impact on its pharmacokinetic properties. Low molecular weight folate conjugates, such as folate-radiopharmaceuticals, folate-chemotherapeutics, and folate-hapten, can rapidly access FR+ cells and are rapidly cleared from systemic circulation, resulting in ultra-high target-to-non-target tissue ratios. However, these will also exhibit high kidney accumulation, which may lead to elevated nephrotoxicity. The diagnostic value of folate-imaging agents may be limited by the confounding factor of FR-β expression in activated macrophages associated with inflammatory tissues, which renders the agents less specific for FR-α+ tumor cells. However, since FR expression has been identified as a prognostic factor, *in vivo* detection of FR expression, even qualitatively, could have prognostic value and be used to select patient candidates for FR-targeted therapy to be developed in the future. Folate-liposomes and folate-nanoparticles are much larger in size and have limited accessibility to tumor cells in solid tumors. However, they may still be more efficiently internalized by tumor cells or tumor-infiltrating macrophages, which may be therapeutically beneficial. Accessibility may be a lesser factor for targeting activated macrophages associated with inflammatory diseases and for targeting AML cells.

Clinical success in targeted drug delivery area has so far been limited by the lack of a suitable cellular target and/or a high degree of difficulty in the production of clinical quantities of targeted drug carriers. FR-targeted agents represent excellent candidates for clinical development. Interestingly, although FR-targeting initially was considered as a strategy for tumor-targeted delivery, the disease target has expanded to include AML and chronic inflammatory diseases. Questions to be addressed in future studies include the possible role of receptor upregulation in promoting targeted therapy, both for FR-α and FR-β positive tumors, and the cellular population responsible for enhancement in therapeutic response in solid tumors to FR-targeted drugs, that is the relative roles of tumor infiltrating macrophages and the tumor cells themselves. Since activated macrophages have increased FR-β expression, it is possible that folate-conjugates can effectively target tumor infiltrating macrophages in non-FR expressing tumors, thus extending the FR-targeting strategy to FR-α negative tumors.

The area of folate receptor targeting remains an area of great excitement, suggested by the large number of recent publications and a number of planned clinical trials on FR-targeted mAbs and folate conjugates. One reason for this is the ready availability of folate and the relative ease with which folate can be conjugated to drugs and drug carriers. In fact, folate has routinely been used as a convenient model for studying targeted drug delivery. Despite the high volume of publications on preclinical studies, no clinical study targeting FR for therapeutic purpose has been reported thus far. Given the advantages of the FR targeting strategy, more clinical trials are urgently needed to define the therapeutic role of anti-FR mAbs and folate conjugated drugs and drug carriers. In addition, further preclinical studies are needed to define mechanisms of *in vivo* targeting and the potential of FR-targeted agents for clinical application.

Declaration of interest

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