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Folate-mediated targeting: from diagnostics to drug and gene delivery

Christopher P. Leamon and Philip S. Low

The covalent attachment of the vitamin folic acid to almost any molecule yields a conjugate that can be endocytosed into folate receptor-bearing cells. Because folate receptors are significantly overexpressed in the majority of human cancers, this methodology is currently being investigated for the selective delivery of imaging and therapeutic agents to tumor tissue. Phase I and II clinical studies for the first folate-containing imaging agent were initiated in 1999, and clinical trials of folate-targeted therapeutic agents should soon follow. This review will summarize folate-mediated drug delivery and highlight those techniques undergoing active preclinical or clinical investigation.

***Christopher P. Leamon**
Endocyte Pharmaceuticals
1205 Kent Avenue
West Lafayette
IN 47906, USA
tel: +1 765 463 7175
fax: +1 765 463 9271
e-mail: cleamon@endocyte.com
Philip S. Low
Department of Chemistry
Purdue University
West Lafayette
IN 47907-1393
USA

▼ Folates are low molecular weight pterin-based vitamins required by eukaryotic cells for one-carbon metabolism and *de novo* nucleotide synthesis. Because animal cells lack key enzymes of the folate biosynthetic pathway, their survival and proliferation are dependent on their ability to acquire and utilize this vitamin. Thus, effective mechanisms for capturing exogenous folates are needed to sustain life.

The structure of folic acid, the form of folate added to fortified foods and vitamin supplements, is shown in Fig. 1. Owing to the two carboxylate moieties positioned at the distal end of the folate molecule, passive membrane permeability at physiological temperature and pH is minimal. To circumvent this obstacle, nature has evolved two mechanisms for the cellular internalization of the vitamin. The first mechanism involves a low-affinity (K_D ~1–5 μM) membrane-spanning protein that transports reduced folates directly into the cell cytosol[1]. The second mechanism uses a high affinity (K_D ~100 pM) glycoprotein receptor, generally referred to as the folate receptor (FR), which preferentially mediates the uptake of oxidized forms of folate (e.g. folic acid) into the cell by endocytosis[2]. Although researchers have previously questioned its role in folate metabolism, the FR is now believed to mediate the cellular uptake of folates under physiological conditions[1,3]. A recent study has shown that FR-knockout mice develop fatal morphogenetic abnormalities, implying that FRs also perform significant functions during embryonic growth[4].

The folate transporter is present on virtually all cells, whereas the high-affinity FR is expressed at high levels mainly on cancer cells. For example, epithelial cancers of the ovary, mammary gland, colon, lung, prostate, nose, throat and brain have all been reported to express elevated levels of the FR[5–13]. However, FR has also been found at significant levels in normal choroid plexus and placenta, and at low levels in normal lung, thyroid and kidney[6]. Overexpression of FR by cancer cells might provide the cells with a growth advantage relative to neighboring normal tissue. Indeed, highly undifferentiated metastatic cancers express considerably more FR than their localized, low-grade counterparts[12].

From a mechanistic perspective, the FR functions to concentrate exogenous folates and various derivatives into the cell cytosol by endocytosis[2]. The term endocytosis refers to the process whereby the plasma membrane invaginates and eventually forms a distinct intracellular compartment. As depicted in Fig. 2, the endocytic vesicles (endosomes) that contain the FR–folate complex rapidly become acidified to ~pH 5 and thereby allow the FR to release the folate molecule[14]. At this point, cytosolic entry of the vitamin can occur by:

(1) direct membrane translocation of the protonated vitamin species;
(2) anion exchange-assisted transport of the vitamin out of the endosome[15]; and
(3) simple leakage of the folate during imperfect membrane fusion events[16].

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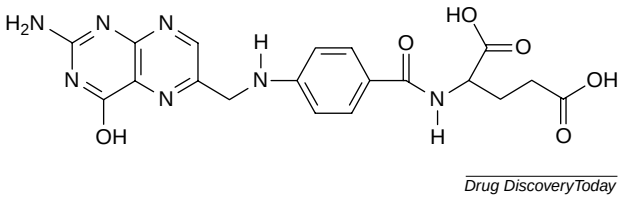

Figure 1. Structure of folic acid.

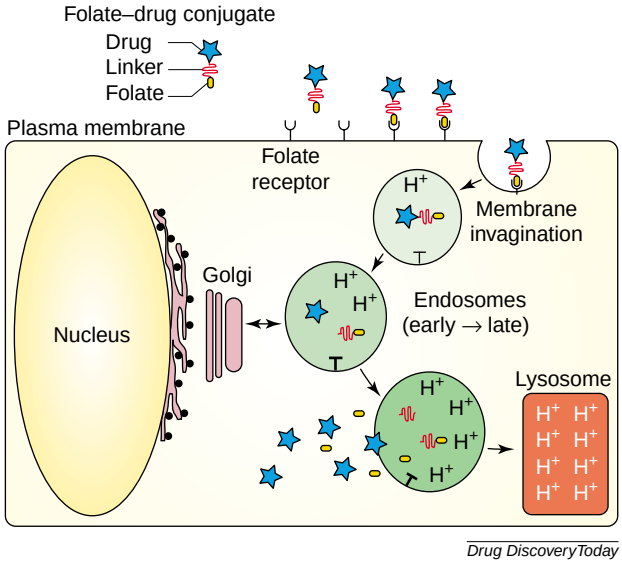

Figure 2. Endocytosis of folate–drug conjugates. Exogenous folate–drug conjugates bind specifically to folate receptors (FRs) presented on the surface of a target cell. The plasma membrane invaginates around the folate–FR complex to form an intracellular vesicle that is commonly referred to as an endosome. As the lumen of the maturing endosome acidifies to ~pH 5, owing to the action of resident proton pumps, the FR releases the folate (or folate conjugate) into the cytosol.

It has been known for nearly a decade that simple covalent attachment of folic acid to virtually any macromolecule produces a conjugate that can be internalized into FR-bearing cells in an identical fashion to that of free folic acid[17]. Numerous publications have emerged to elucidate the mechanism and to further define the advantages as well as the limitations of this technology. To date, folate conjugates of radiopharmaceutical agents[18–23], MRI contrast agents[24], low molecular weight chemotherapeutic agents[25], antisense oligonucleotides and ribozymes[26–30], proteins and protein toxins[17,31–35], immunotherapeutic agents[36–39], liposomes with entrapped drugs[40–44] and plasmids[45–51] have all been successfully delivered to FR-expressing cancer cells. The objective of this review will be to focus on only a few of the aforementioned techniques with regard to their currently known preclinical or clinical investigations, as well as to provide greater insight into the use of folate-mediated macromolecule delivery.

Radiodiagnostic imaging

The field of nuclear medicine has been revitalized with the advent of tissue-specific radiopharmaceutical-targeting technologies. Ligands capable of concentrating at pathological sites have been derivatised with chelator–radionuclide complexes and used as non-invasive probes for diagnostic imaging purposes. For instance, monoclonal antibodies, somatostatin analogs, vasoactive intestinal peptides and folic acid have all been used as ligands to localize radionuclides to tumors[18–23,52–54]. Applications with monoclonal antibodies, and truncated derivatives thereof, initially received the most attention because it was believed that precise, or perhaps superior, tumor-specific targeting might be easily achieved. Unfortunately, this approach was subsequently found to be technically challenging and inferior to other methods, because:

(1) antibodies have prolonged circulation times owing to their large molecular size (an unfavorable trait for imaging purposes);
(2) antibodies can be immunogenic, forcing their laborious humanization whenever multiple doses were anticipated;
(3) antibodies are expensive to produce; and
(4) tumor to non-target tissue ratios (T:NT) of antibody-linked radionuclides were suboptimal[55–57].

Thus, more focus has recently been directed towards the use of smaller tumor-specific ligands that do not suffer from such limitations.

Chronologically, the first folic acid conjugate described for *in vivo* tumor imaging was a histamine derivative containing ^{125}-Iodine (^{125}I; Ref. 58). Although impressive tumor images were obtained with this conjugate, it was not considered to be a relevant clinical candidate owing to the long-lived ^{125}I radionuclide component. Subsequent reports described the synthesis and use of folate-deferoxamine for tumor targeting[18,19]. Deferoxamine chelates ^{67}-Gallium (^{67}Ga), a gamma-emitting radionuclide that has a half-life of 78 h. Favorable pharmacokinetic biodistribution profiles and high T:NT ratios were obtained with the folate–deferoxamine conjugate in a tumor-bearing nude mouse model. Unfortunately, partial hepatobiliary clearance was observed, and further preclinical development was stopped because of anticipated problems in accurately imaging regio-abdominal locations in humans. This obstacle was easily overcome, however, by simply replacing the deferoxamine chelator with diethylenetriamine penta-acetic acid (DTPA), an efficient chelator of ^{111}-Indium (^{111}In; 68 h half-life). ^{111}In-DTPA–folate displayed very similar tumor-accumulating

properties to its deferoxamine predecessor, but its primary route of elimination was confirmed to be via the kidneys[20]. Thus, ^{111}In-DTPA–folate was selected for development, and Phase I and II clinical trials were initiated at several locations in the USA in 1999.

As described by Mathias and Green, a clinical kit formulation was developed for convenient, routine compounding of ^{111}In-DTPA–folate[59]. Patients suspected of having ovarian cancer received an approximate 2 mg intravenous dose of the radiopharmaceutical containing 5 mCi of ^{111}In. Following a 4 h time period to allow for renal clearance of non-tissue-bound radioactive material, whole-body single-photon-emission computerized tomographic (SPECT) images were taken to identify the location of the probe. Subsequently, invasive biopsies of the suspicious tissue were performed for unequivocal pathological identification. Representative SPECT images from two enrolled patients are shown in Fig. 3. The images displayed in Fig. 3a demonstrate that the ^{111}In-DTPA–folate probe accumulates in FR-positive kidneys and to a lesser degree in the liver. However, the lower abdominal mass, which was first identified by computerized tomography (CT), also accumulated the probe. A subsequent biopsy confirmed that the mass was malignant ovarian cancer. By contrast, the images obtained from a second patient (Fig. 3b) primarily revealed kidney uptake. No abdominal uptake of the probe was noticed despite the fact that CT confirmed the presence of a very large mass in that region. Interestingly, a subsequent biopsy confirmed that the mass from this patient was benign. Following the treatment of more than 38 enrolled subjects, a pattern of positive uptake of ^{111}In-DTPA–folate in malignant masses (including metastatic abdominal lesions) and negative uptake in benign masses has now been confirmed with a predictive sensitivity of ~94% (Endocyte, West Lafayette, IN, USA, unpublished).

^{111}In-DTPA–folate was never intended for development as a final product, mainly because the field of radiodiagnostic imaging has been transitioning towards using ^{99m}Technetium (^{99m}Tc) -based probes. Nevertheless, clinical evaluation of ^{111}In-DTPA–folate was continued for the purpose of obtaining valuable human biodistribution data while the development of a new ^{99m}Tc-based folate conjugate was concurrently underway. ^{99m}Tc has been adopted as the preferred radionuclide for diagnostic imaging because:

(1) the radionuclide can easily be obtained in a clinical laboratory from commercially available molybdenum generators;
(2) the cost of producing large amounts of ^{99m}Tc is insignificant compared with the cost of ^{111}In; and
(3) ^{99m}Tc has a much shorter half-life (6 h), which allows higher radionuclide doses (~25 mCi) to be administered, yielding high resolution images without the risk of hazardous radiation exposure to vital organs.

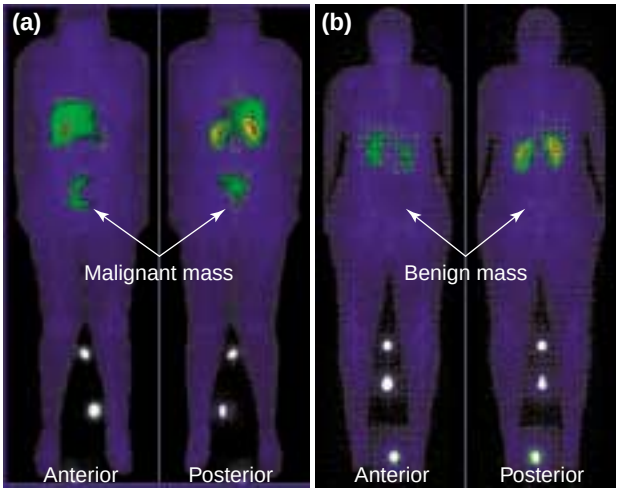

Figure 3. SPECT images of two patients receiving ^{111}In-DTPA–folate. **(a)** Anterior and posterior images of a female patient subsequently diagnosed with malignant ovarian cancer. **(b)** Similar images from a second female patient subsequently diagnosed with a large benign ovarian cyst.
Abbreviations: SPECT, single-photon-emission computerized tomography; DTPA, diethylenetriamine penta-acetic acid.

Several folate-based ^{99m}Tc conjugates have already been described in the literature. For example, folate conjugates of ^{99m}Tc-6-hydrazinonicotinamido-hydrazido[22] (HYNIC), ^{99m}Tc-12-amino-3,3,9,9-tetramethyl-5-oxa-4,8 diaza-2,10-dodecanedinoe dioxime[23] (OXA) and ^{99m}Tc-ethylenedicysteine[21] have all shown promising *in vivo* tumor uptake qualities. Further, a fourth proprietary ^{99m}Tc-folate conjugate, EC20 (Endocyte), has recently entered Phase I clinical trials for the diagnostic imaging of pelvic and abdominal masses in women. Assuming that EC20 clinically performs equally well or better than ^{111}In-DTPA–folate, a valuable non-invasive diagnostic imaging agent for ovarian and possibly other cancers might soon be available.

Cytotoxin targeting

There is clearly tremendous value in a technique that can image a tumor non-invasively in a cancer patient. However, such a technique might also logically be used to deliver toxic agents to those same tumors that concentrated the imaging agent. Consequently, there has been an ongoing effort to identify potent antiproliferative folate–drug conjugates.

The fundamentals of folate–cytotoxin therapy were first appreciated from studies involving the targeted killing of

FR-positive cells using folate conjugates of protein synthesis-inhibiting enzymes[33]. Thus, folate conjugates of various cell-impermeant ribosome-inactivating proteins (e.g. momordin, saporin, ricin A) were found to selectively kill cultured malignant cells *in vitro*. Selectivity was confirmed by several important controls, which demonstrated that:

- the unmodified protein was completely benign to the target cells;
- an excess of free folic acid quantitatively blocked folate–cytotoxin-mediated cell killing; and
- pretreatment of cells with phosphatidylinositol-specific phospholipase C, an enzyme that removes glycosylphosphatidylinositol-linked proteins (similar to the FR) from a cell's surface, effectively blocked folate–cytotoxin-mediated cell killing.

Furthermore, the same folate–cytotoxins could be demonstrated to selectively kill malignant cells when co-cultured in the same dish with non-transformed cells[35]. Typical IC_{50} values of ~1 nM were observed using several FR-expressing cell lines. However, more potent conjugates could be prepared ($IC_{50} \leq 10^{-11}$ M) if protein toxins with inherent endosome escape mechanisms [e.g. *Pseudomonas* exotoxin; (PE)] were used[34]. In fact, even low FR-expressing malignant cells that were unresponsive to folate–momordin were very sensitive to folate–PE. This significant enhancement of the cytotoxic activity of folate–PE was attributed to PE's inherent ability to translocate from intracellular vesicles to the cytosol, a function assigned to its second structural domain[60].

Although it is tempting to speculate on the anti-tumor activity that might result from testing folate–cytotoxin conjugates *in vivo*, practical pharmaceutical considerations have decreased the priority for their development. Instead, folate conjugates of conventional drug molecules are currently being screened for anti-tumor activity. To date, only one report has emerged describing the evaluation of a folate–drug conjugate. Thus, a small molecular weight microtubule-disrupting agent, a maytansinoid, was conjugated via a disulfide-containing linker to folate, and growth inhibition of FR-expressing cells only was observed *in vitro*[25].

Compared with the folate–protein conjugates that destroy target cells with enzymatic activity at only a few molecules per cell[61], the killing of cancer cells with folate–drug conjugates might present a greater challenge, particularly if the inherent cytotoxicity of the attached drug is moderate. As shown in Fig. 4, a possible solution to this problem would be to prepare multi-drug–folate conjugates where one folate molecule would bind to one FR but deliver many copies of therapeutic agent. It is also tempting to speculate on the cytotoxic activity that might result if two or more different drugs with different mechanisms of action were to be incorporated into this design. Although resources have recently been devoted to exploring these alternative approaches, no biological data are available as yet.

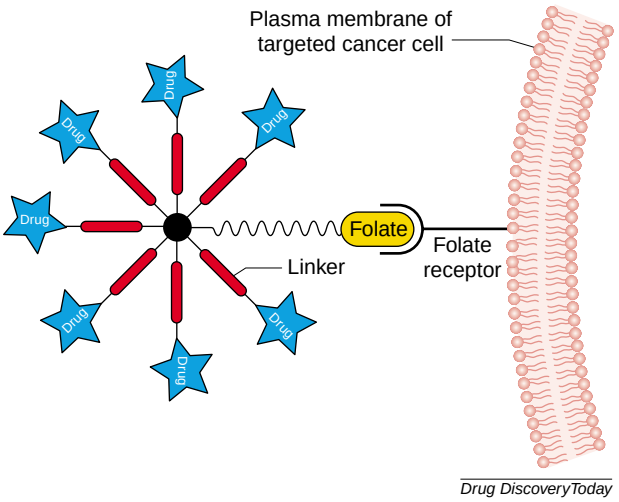

Figure 4. Cell binding of a second-generation multi-drug folate conjugate. Exogenous multi-drug folate conjugate binds specifically to a folate receptor (FR) presented on the surface of a targeted cancer cell.

Gene and oligonucleotide therapy

The use of folate-targeted non-viral polynucleotide vectors for cancer therapy has recently been reviewed[62,63]. Therefore, we shall limit this discussion to highlight general experimental principles, followed by a few recent examples.

Most non-viral gene therapy techniques involve electrostatic complexation of the polyanionic nucleic acid with a cationic component such as monocationic or polycationic lipids, polylysine, polyethylenimine, cationic dendrimers or protamine[64–69]. The purpose of this complexation is to condense the polynucleotide into a small package in order to facilitate cellular entry via endocytosis. However, this electrostatic complexation of the polynucleotide with the synthetic polycation is in itself not sufficient to guarantee successful cell uptake. Thus, the polynucleotide–polycation complex is typically formed under conditions that allow for a residual net positive charge to remain. This positive charge then enables the formulation to electrostatically interact with cell membranes, a process known to promote endocytic clearance of the adherent material[70]. This non-specific approach to intracellular delivery is acceptable when applied under carefully controlled conditions *in vitro*; however, positively charged vectors generally aggregate in serum into supra-micron-sized particles that primarily get trapped within an animal's lungs and to a lesser extent in the spleen and liver[71]. Aggregation can be

decreased if the net charge of the condensed material is neutralized, but the resulting transfection efficiency of the formulations is substantially weakened *in vivo*.

The use of folate to target condensed polynucleotide therapeutics in the presence of serum would theoretically solve both problems. Polynucleotides could be compacted under charge-neutral conditions to reduce the aforementioned serum interference, and folate present within the formulation would efficiently mediate its tumor cell-specific uptake. The following examples are descriptions of two apparently serum-tolerant methods that utilize folate-based formulations for the functional delivery and expression of transgenes in receptor-bearing cancer cells.

Polylysine and polyethylenimine compaction methods

Folate conjugates of polylysine (pLys) and polyethylenimine (PEI) have been tested *in vitro* for their abilities to deliver both transgenes and oligonucleotides into cells. In general, the folate moiety is observed to enhance cell binding and entry in addition to transgene expression and antisense effects. Because the extent of transgene expression can be enhanced when lysosomotropic reagents are included in the incubation medium, at least some of the endocytosed material probably remains within an organellar compartment[46]. Thus, a built-in method to promote endosome escape could significantly enhance the potency of these particles. Recently, another technical improvement for folate–pLys- and folate–PEI-mediated transfection has been described whereby a polyethyleneglycol (PEG) spacer placed in between the folate moiety and the polycationic backbone was found to dramatically enhance transgene expression relative to the non-PEG-containing polycationic counterparts[48,49] (Ref. 49 available online at www.pharmsci.org). This enhancement was attributed to the ability of the PEG spacer to more effectively present and mediate binding of the folate ligand to FR on the surface of cancer cells, and the technique was found to be dependent on the charge ratio of the complex, PEG size and extent of polycationic derivation with PEG–folate. Furthermore, as shown in Table 1, many human cancer cell lines from a variety of origins were successfully transfected with the pLys–PEG–folate conjugates. Interestingly, we have noted that the transfection efficiency and enhancement of folate-specific transfection varies among the many cell lines tested to date, probably because the expression of FR, degree of intracellular sequestration and levels of metabolic activity will differ between cells.

Cationic lipid formulations

Recently, a folate-containing cationic liposome formulation was optimized for the systemic delivery of plasmid DNA to squamous cell carcinomas of the head and neck as well as breast cancer xenografts[72]. The inclusion of the folate-targeting ligand was found to remarkably enhance the transient *in vivo* transfection of the tumors as demonstrated with both β-galactosidase and wild-type p53-containing plasmids. Furthermore, tumor-bearing test animals treated with the folate-based p53 gene therapeutics were found to be sensitized to subsequent radiotherapy, such that the combination of the systemic targeted gene- and radio-therapies resulted in complete tumor regression[72]. Hopefully, the remarkable anti-tumor effects observed with these formulations in animal models will also be observed clinically.

Table 1. Folate-copolymer mediated transfection of various human cancers *in vitro*[a]

Cell line	Species	Origin	Folate-enhanced transfection (fold increase)
KB	Human	Nasopharyngeal carcinoma	33
HeLa	Human	Cervical carcinoma	30
C33A	Human	Cervical carcinoma	17
H1299	Human	Non-small cell lung carcinoma	150
NCI-H522	Human	Non-small cell lung carcinoma	8
MDA-MB-231	Human	Lung carcinoma	226
A549	Human	Lung carcinoma	1400
HS578t	Human	Mammary carcinoma	221
IGROV	Human	Ovarian carcinoma	46
OVCAR-3	Human	Ovarian carcinoma	26
AN3CA	Human	Endometrial carcinoma	1400

[a]Cells were treated with 20 μg ml^{-1} of polylysine-PEG-folate:luciferase plasmid DNA or polylysine:luciferase plasmid DNA complexes for 48 h and then harvested for luciferase quantitation. The values shown represent the fold increase in transfection mediated by the folate copolymer compared with polylysine alone.

Future directions and concluding remarks

Understandably, most of what is known regarding the mechanistic utility of folate-mediated macromolecule delivery has come from experiments performed *in vitro*. Molecules ranging in size from amino acids to liposome–DNA complexes (<200 nm diameter) have successfully

been targeted to and endocytosed within FR-expressing cells. By contrast, knowledge regarding *in vivo* folate-conjugate tumor targeting is much more limited.

Folate appears to target small molecular weight imaging agents to tumors, as confirmed in both laboratory animal models and in humans[18–20,58] (Fig. 3). However, larger payloads might present greater targeting challenges. For example, the uptake of a bovine serum albumin–folate conjugate into xenographic tumors was found to only be increased 1.4-fold relative to the unmodified protein[73]. This might be accounted for by either low vascular permeability within that tumor model or by serum instability of the conjugate. However, the fact that monoclonal antibodies[74] or liposomes[75] effectively target tumors *in vivo* suggests that tumor penetration is not the limiting factor. Therefore, folate should theoretically be capable of targeting larger cargos to tumors, assuming that the folate moiety is positioned on the macromolecule such that effective binding to the FR is sterically permitted.

A potential limitation for using folate to deliver highly cytotoxic drugs to cancer cells is that FR-positive kidneys might undesirably accumulate the same drug. Folates accumulate in the proximal tubules for the probable purpose of kidney-assisted transcellular reabsorption[76,77], and the extent to which the proximal tubule cells directly utilize these scavenged folates is not certain. Thus, it is not yet known whether folate–drug conjugates captured by the proximal tubules will damage renal function. Furthermore, although folate conjugates of small molecular weight imaging agents accumulate within the proximal tubules[19,20], kidney retention of larger folate conjugates might not be significant, because macromolecules >40 Å in radius are not effectively filtered from the blood during kidney transit[78]. Regardless, suitable methods for reducing the kidney uptake of smaller folate conjugates are still needed. Several kidney-blocking approaches have, in fact, already been proposed[62]. For example, the use of kidney brush-border-specific hydrolysable linkers, and a subsequent chase dose of free folic acid, are worthy of further investigation.

Various methods are available for identifying FRs in fresh or frozen tissues. For instance, an *in vitro* ^{125}I-folic acid binding assay has been used to quantify the relative levels (fmol mg^{-1} total protein) of FR in tissues[79], whereas immunohistochemical or radioimmunoassay techniques using anti-FR monoclonal antibodies have been used to qualitatively assess the uniformity of FR expression[11,80]. Both techniques are currently in practice for assessing the extent of FR overexpression in human cancers. It is hoped that the cumulative data obtained from these analyses will lead to a better understanding of the types of human diseases, including cancer, which might favorably respond to folate-conjugate therapies.

After ten years of exploratory research and development, folate-conjugate technology is still considered to have significant therapeutic value. Success from ongoing clinical investigations with folate-radiodiagnostic imaging agents, in addition to encouraging preclinical therapeutic studies (P.S. Low *et al.*, unpublished), have sustained the interest of both academic and pharmaceutical scientists. As depicted in Fig. 5, one possible strategy for clinically applying this technology begins after the confirmation of an undefinable mass by CT. A folate-linked radiodiagnostic agent (FolateScan; Endocyte) would then be used to determine whether or not the mass expressed functional FR. If the scan were negative, the patient would probably undergo exploratory surgery and/or conventional chemotherapy. However, if the scan were positive, the patient would be eligible for folate-targeted chemotherapy. Because many FR-positive cancers are associated with poor clinical outcomes, this alternative therapeutic approach may bring about a radical improvement in some patients' prognoses.

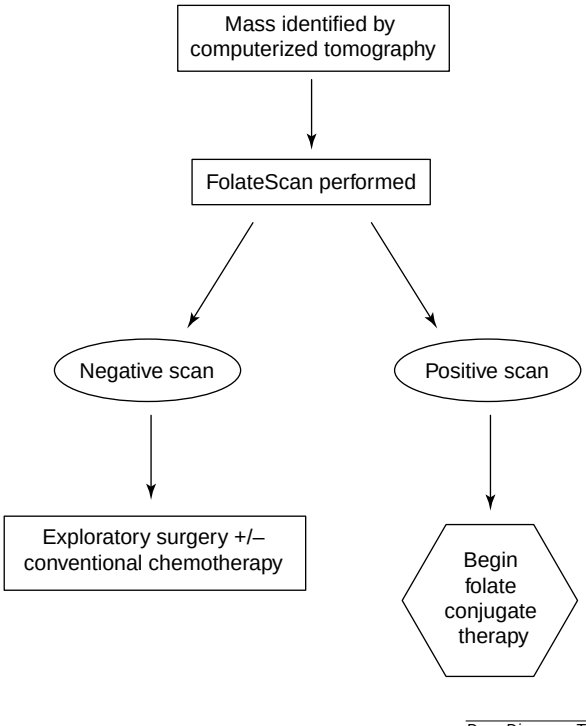

Figure 5. Possible clinical strategy for applying folate technology. Patients that present with an abdominal mass, as confirmed by computerized tomography (CT), could be administered with a folate-radiopharmaceutical imaging agent (FolateScan) to determine whether the tissue mass expresses the folate receptor. If the FolateScan were negative, the patient would probably undergo exploratory surgery and/or chemotherapy. If the FolateScan were positive, however, the patient might then be eligible to receive folate–drug conjugate therapy.

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