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## FOLATE CONJUGATES: A BOON IN THE ANTI-CANCER THERAPEUTICS

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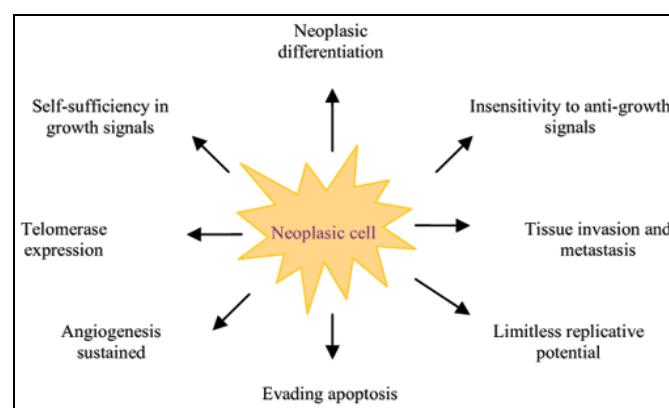
**ABSTRACT:** Targeted delivery system is a promising strategy for improving cancer treatment and diagnostics. Molecular targeted drug delivery system has the potential to maximize the cancer treatment efficacy and minimize the toxicity to normal cells. Folate receptor (FR), an established ovarian cancer marker, is frequently found to be overexpressed in other major epithelial tumors. Overexpression of FR- $\beta$  in myeloid leukemia as & in inflammatory macrophages, including tumor associated macrophages indicates exploitation of FR for targeted drug delivery. Active research taking place in the field of FR based tumor drug targeting has led to development of FR targeted radioimaging agents and chemotherapeutic agents of highly significant value in the field of oncology. FR is targeted by using conjugates of folic acid or anti FR antibodies. Though, both these techniques have their own advantages & disadvantages. To date, promising folate targeted antibodies, Farletuzumab & the low molecular weight folate conjugates like EC20, EC145 etc. have moved from phase II to phase III trials. This review focuses on all the developments made so far from discovery of the FR as a potential drug target as well as radioimaging agents which provide clear insights to this targeted drug delivery system of the future.

**INTRODUCTION:** Cancer is the second most leading cause of death in US and in industrialized countries. Carcinogenesis is a multistep process in which the DNA exposed to mutagens causing mutations in genes which may further cause uncontrolled proliferation of mutated cells and incorporation of the mutation in the body cell leading to tumorigenesis<sup>1</sup>. (**Fig.1** and **Fig.2**) The complex mechanisms involved in carcinogenesis contributes to difficulty in delivering antitumor agents.

The heterogeneous blood supply, interstitial hypertension, relatively long transport distances in the interstitium and cellular heterogeneities are the physiological factors that contribute to poor delivery of therapeutic agents to tumors<sup>2</sup>.

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**FIG.1: MALIGNANT CELL CHARACTERISTICS**

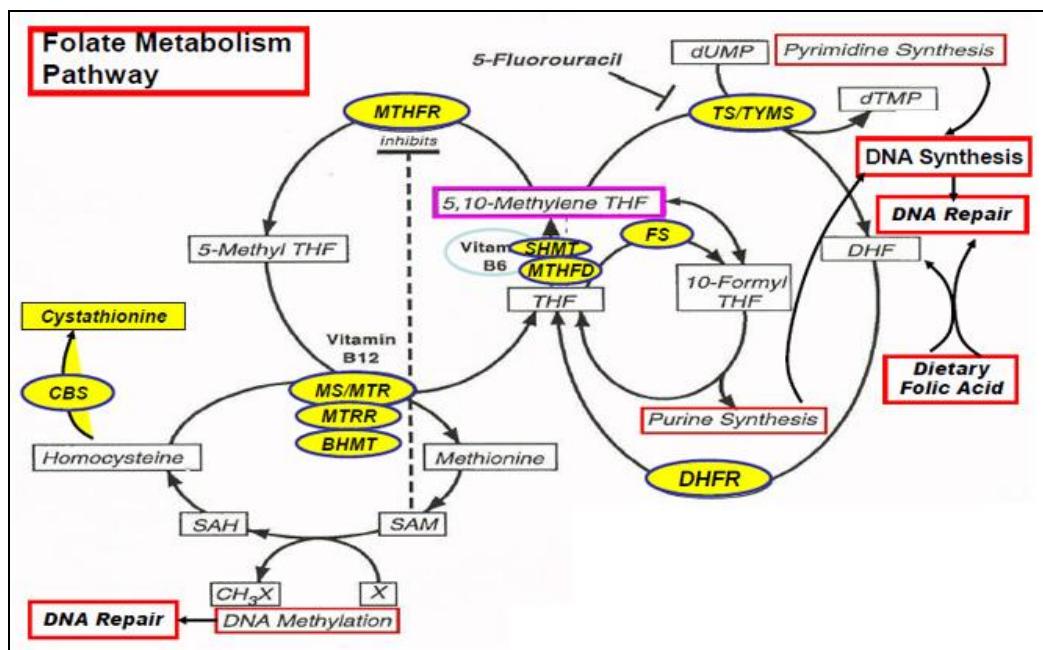


FIG.2: FOLATE METABOLISM PATHWAY

It has been observed that a linear increase in the fraction of cells killed by conventional chemotherapy often requires an exponential increase in drug dose. However, because of toxic side effects of non-targeted chemotherapy to normal cells, only limited quantities of chemotherapeutic agents can generally be administered, frequently allowing a small fraction of the more resistant cancer cells to survive<sup>3</sup>. The above stated reasons led the scientists to explore specific chemotherapeutic delivery systems.

The two distinct classes of such targeted therapeutics are now in clinic i) inhibitors that are specific for enzyme/ processes uniquely important for cancer cell survival and/or proliferation, and ii) nonspecific cytotoxic drugs that achieve tumor specificity by virtue of their attachment to targeting ligands that bind selectively to cancer cells. Imatinib<sup>4, 5</sup>, gefitinib<sup>6</sup>, and erlotinib<sup>7</sup>, and monoclonal antibodies like trastuzumab<sup>8, 9</sup>, rituximab and bevacizumab<sup>10, 11</sup> are the examples of former class of pharmaceuticals. Mylotarg, a monoclonal antibody to CD33 antigen linked to Calicheamicin<sup>12</sup> and a fusion of pseudomonas exotoxin (PE38) and IL-2 that is to IL-2R over expressing malignant cells<sup>13, 14</sup> are the examples of latter class of targeted ligands. Although the protein and monoclonal antibody targeted drugs have improved specificity but compromises in tumor penetration due to their

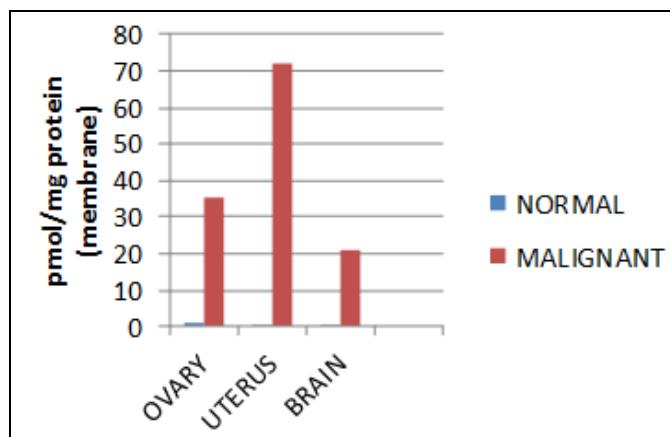
larger sizes<sup>15</sup>. To avoid this limitation, a new class of low molecular weight targeted ligands such as folic acid<sup>16-20</sup>, vitamin B-12<sup>21, 22</sup>, bombesin<sup>23, 24</sup>, LHRH<sup>25, 26</sup>, and GnRH<sup>27, 28</sup> has emerged that penetrate tumors more efficiently. Herein this review, we aim to address the role of folic acid in targeting cancer cells, discovery and development of folic acid as an efficient targeted ligand and to predict the future directions of folate mediated therapeutics.

### Basic Aspects:

#### Folic Acid and Cancer:

Folic acid is water soluble B vitamin that is present in cells as a family of structurally related and metabolically interconvertible enzyme cofactors that are necessary for the synthesis of purine and thymidine nucleotides and for the synthesis of methionine from homocysteine<sup>47, 48</sup>. Folate mediates a metabolic system comprising of several interdependent metabolic pathways that use the cofactor tetrahydrofolate to chemically activate single carbons for cellular biosynthetic reactions. Folate metabolism is compartmentalised in the cytoplasm and mitochondria, with each compartment containing near equal concentrations of folate cofactors. In the cytoplasm, tetrahydrofolate carries one carbon units for the synthesis of three products: i) 10-formyltetrahydrofolate is required for the synthesis of the purine ring (-2 and -8 carbons); ii)

methylene tetrahydrofolate is required for the remethylation of homocysteine to methionine<sup>29</sup> (**Fig. 3**). Methionine can be converted to S-adenosylmethionine (SAM), which serves as a cofactor for numerous methylation reactions including the methylation of DNA, RNA, proteins and neurotransmitters among many other products<sup>30</sup>.



**FIG. 3: COMPARISON OF THE LEVELS OF FOLATE RECEPTOR EXPRESSION BETWEEN NORMAL AND MALIGNANT HUMAN TISSUES. ALL MALIGNANT TISSUES WERE CLASSIFIED AS MEDIUM TO HIGH GRADE TUMORS. FOR EACH PAIR OF NORMAL AND MALIGNANT TISSUES, 100 mg TOTAL PROTEIN FROM CRUDE MEMBRANE PREPARATIONS WAS ISOLATED AND ASSAYED FOR SPECIFIC BINDING OF 3H-FOLIC ACID.**

Impaired folate metabolism is associated with risk for developmental anomalies and disease including neural tube defects<sup>31, 32</sup>, cardiovascular disease<sup>33-35</sup>, and cancer<sup>36-40</sup>. Folate deficiency and polymorphisms in folate dependent enzymes modify risk for colon and other cancers<sup>34, 39, 41</sup>.

Though the biochemical mechanisms that account for association between folate deficiency and cancer risk are not yet established but two mechanisms have been proposed: 1) increased rates of uracil misincorporation into DNA resulting from impaired deoxythymidine monophosphate (dTMP) synthesis and, 2) decreased DNA methylation resulting from depressed SAM synthesis<sup>39</sup>. Uracil misincorporation not only increases DNA mutation rates but also increases DNA mutation rates but also increases DNA instability<sup>34, 39, 40, 42</sup>. Folate deficiency in the cells makes them more susceptible to the effects of radiation and alkylating agents<sup>42, 43</sup>. Folate deficiency also influences DNA methylation density. Genome wide DNA hypo

methylation occurs in nearly all cancers, and precedes mutational and chromosomal abnormalities that occur as the cancer progresses<sup>34</sup>. DNA hypo methylation has been proposed to alter chromatin structure and therapy increase mutational rates that contribute to the hyper mutable phenotype that is associated with the initiation and/or progression of cell transformation<sup>44</sup>. Alterations in DNA methylation density also influence gene expression. Microarray studies have indicated that about 10% of murine genes are regulated by DNA methylation<sup>45</sup>. DNA methylation within the 5' promoter region generally correlates inversely with levels of gene expression<sup>46</sup>. Paradoxically, genomic DNA hypo methylation occurs in parallel with allelic specific DA hyper methylation. Tumor suppressor genes seem to be particularly sensitive to methylation silencing tumor progression.

#### Structure and function of Folate receptors:

Three well characterised isoforms of human folate receptors (FR) i.e. FR- $\alpha$ , FR- $\beta$  and FR- $\gamma/\gamma'$ , that are ~70-80% identical in amino acid sequence, but distinct in their expression patterns have been found<sup>49</sup>, FR- $\alpha$  and FR- $\beta$  are found to be attached to glycosylphosphatidylinositol (GPI) membrane anchor, therefore, referred as membrane associated proteins. However, FR- $\alpha$  differs from FR- $\beta$  because of its higher affinity for the circulating folate coenzyme (6S)-5-methyltetrahydrofolate and its opposite stereo specificity for reduced folate coenzymes<sup>50</sup>. FR- $\alpha$  also binds folic acid and physiologic folates with slightly higher affinity ( $k_D \sim 1\text{nM}$ )<sup>52</sup>. FR- $\gamma$  and a truncated form of the protein, FR- $\gamma'$ , lack the GPI anchor and are constitutively secreted in barely detectable amounts as soluble forms of human FR. The binding affinity of the secreted FR- $\gamma$  for folic acid is reportedly to be 0.4nM<sup>53</sup>.

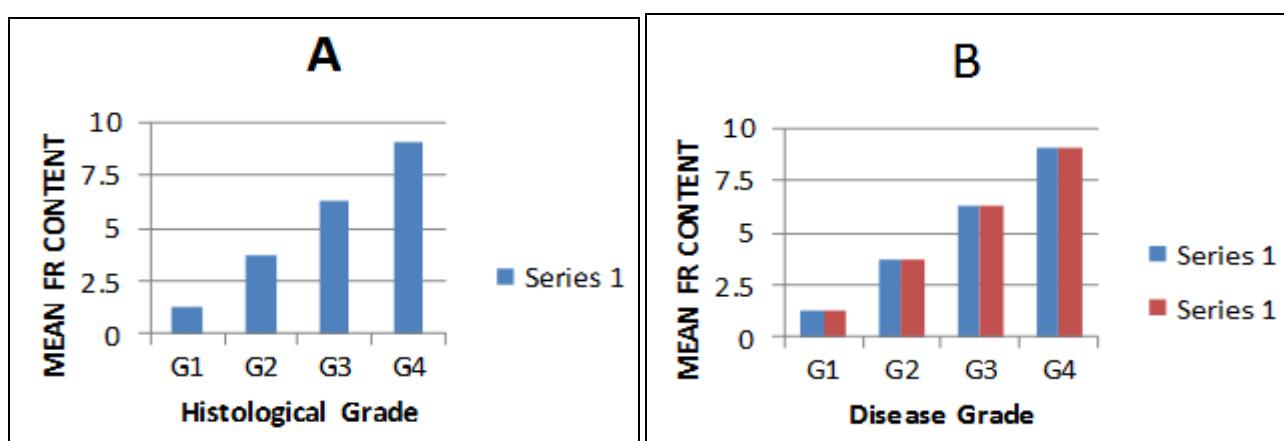
#### Expression of Folate Receptor in Normal and Malignant Cells:

FR isoform expression is tissue specific and differentiation dependent<sup>49, 17</sup>. FR- $\alpha$  is present at only low levels on normal epithelia with an exception of a few normal tissues (kidney, placenta and choroid plexus), but often elevated in malignant tissues of epithelial origin particularly the ovary<sup>18</sup>, uterus<sup>54</sup>, endometrium<sup>56</sup>, brain<sup>57</sup>, kidney<sup>54</sup>, head and neck<sup>54</sup> and mesothelium<sup>58</sup>.

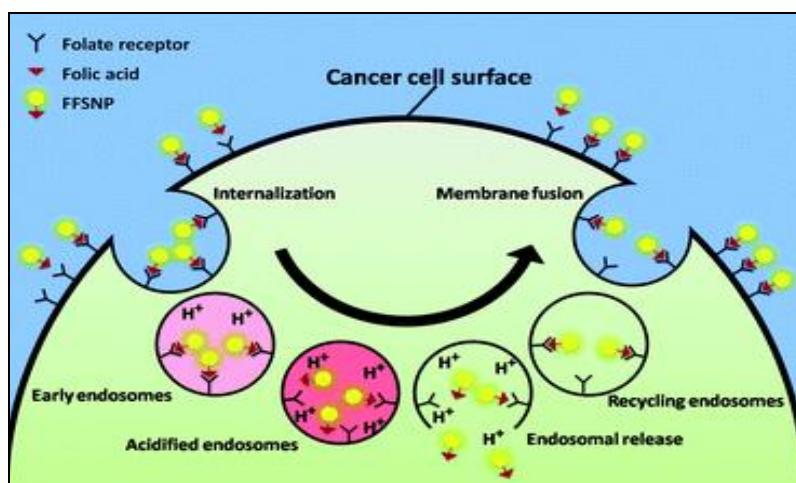
The difference in FR- $\alpha$  expression in normal and malignant tissues of the same origin as measured by  $^{3}\text{H}$ - folic acid binding to crude plasma membrane preparations can often be quite striking, showing levels of upregulation approaching two orders of magnitude<sup>17</sup> (Fig. 4). The degree of FR- $\alpha$  overexpression in patients diagnosed with epithelial ovarian cancer is further correlated with a higher histological grade and more advanced stage of the cancer<sup>18</sup>, suggesting a possible need for elevated folates in more rapidly growing tumors (Fig. 5). A correlation between the degree of FR expression and resistance to standard chemotherapy has also been reported<sup>59</sup>, that is tumors resistant to standard chemotherapy commonly have higher levels of FR. Based on the above stated correlations, it can be conceived that the tumors of advanced stage, higher grade and resistant to standard chemotherapy are most readily targeted by folate linked drugs.

FR- $\beta$ , most commonly expressed in hematopoietic and non-epithelial cells, such as spleen and thymus<sup>17</sup>, is elevated in some malignancies of non-epithelial origin including myelogenous leukemias and sarcomas<sup>49, 60</sup>. The FR- $\beta$  isoforms detected on hematopoietic stem/precursor cells and differentiated cells of myeloid lineages are expressed in an inactive form (exhibits no affinity for folates)<sup>61</sup>. In fact, a functional FR- $\beta$  isoform is found to express on activated (but not resting) macrophages<sup>62, 63</sup>.

FR- $\gamma$  and  $\gamma'$  are also thought to be specific for hematopoietic tissues, particularly lymphoid cells, and are expressed only at very low levels<sup>49</sup>. The secreted forms of the FR may be used as potential serum markers for certain hematopoietic malignancies<sup>49</sup>.



**FIG. 4: OVEREXPRESSION OF THE FOLATE RECEPTOR IN OVARIAN CANCER IS ASSOCIATED WITH A HIGHER HISTOLOGIC GRADE (A) AND MORE ADVANCED STAGE (B) OF THE DISEASE. FROZEN TISSUE SAMPLES WERE MECHANICALLY DISAGGREGATED TO PREPARE SINGLE CELL SUSPENSIONS FOR CYTOFLUORIMETRIC ANALYSIS USING AN ANTI-FR MONOCLONAL ANTIBODY. THE MEAN FR CONTENT REPRESENTS RECEPTOR-ASSOCIATED FLUORESCENCE DIVIDED BY ISOTOPIC CONTROL FLUORESCENCE.**



**FIG.5: FOLATE RECEPTOR MEDIATED ENDOCYTOSIS**

A concern of toxicity to proximal tubules of kidney and choroid plexus of the brain has arisen regarding the use of FR targeted therapeutic agents because of relatively high levels of expression of FR in these tissues<sup>64</sup>. With the help of immune histochemical techniques and <sup>125</sup>I-folate autoradiography, it has been noted that FR is seen only in the apical/luminal or urine facing surface of the tubule cells, where it probably assists in reabsorption of folates from the urine<sup>64-66</sup>.

Thus, folate targeted macromolecules should encounter kidney FR only in individuals suffering from proteinuria and other kidney dysfunctions. Similarly, FR's in the brain appear to be concentrated on the brain side of the blood brain barrier<sup>65</sup>, where they may function to retain the vitamin within cerebrospinal fluid. Based on these and related observations, there is currently no evidence that FR targeted macromolecule therapeutics should damage normal tissues with elevated levels of FR expression.

#### **Accidental Discovery of Folate as a Ligand for Specific Targeting:**

The discovery of vitamin mediated drug targeting was totally fortuitous. A former graduate student, Mark Horn, was assigned the task of demonstrating that receptor mediated endocytosis could occur in plant kingdom, despite current dogma claiming the opposite. Mark linked biotin to his elicitors (molecules known to bind plant cells and elicit multiple disease resistance pathways) and followed their endocytosis with fluorescent streptavidin. The data revealed that the biotinylated elicitors entered plant cells through receptor mediated endocytosis but control studies with unrelated molecules did not behave as anticipated<sup>54, 55</sup>. Thus, biotin when linked to animal proteins such as bovine serum albumin (BSA), insulin, ribonuclease and immunoglobulin G (IgG) was also found to enter the plant cells, even though their non-biotinylated counterparts remained completely extracellular. This observation led to the accidental discovery that biotin could ferry attached proteins into live cells<sup>55</sup>.

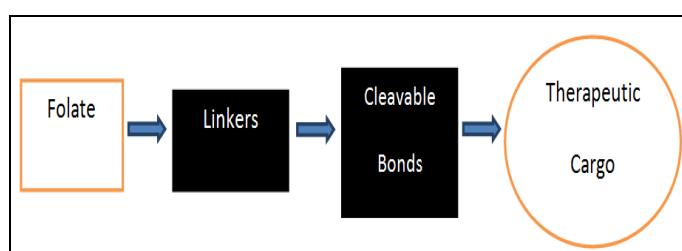
This observation arose the question whether biotin could also ferry attached proteins into animal cells, which are otherwise impermeable into animal cells. Uptake of biotin linked bovine serum albumin was

further evaluated into several available animal cell cultures. It was found that biotin mediated internalisation occurred in some of the tested animal cells (bovine sperm, PC12 cells etc.) only. Further studies were conducted with riboflavin instead of biotin which also revealed limited internalisation of riboflavin mediated proteins in animal cell cultures<sup>67</sup>. An experiment conducted by another graduate, Christopher Leamon demonstrated that folic acid could ferry otherwise impermeable macromolecules in variety of animal cells, which formed the basis for use of folic acid as an important ligand for delivery of macromolecular therapeutics<sup>68</sup>. Although at the time of this discovery, we were unaware of the overexpression of the FR in malignant cells and activated macrophages.

#### **Folate Receptor Mediated Drug Delivery:**

Bart Kamen's group at the University of Texas Southwestern Medical Center reported that folates entered cells via a receptor mediated endocytic process<sup>69</sup>. The physiological process that mediates folate targeted delivery is identical to that for the free-vitamin<sup>68, 70</sup>. Exogenous folate drug conjugates bind to externally oriented FRs located on the plasma cell membrane (**Fig. 6**).

This event is highly specific in which folate acts as key which inserts into FR (acts as a lock). Immediately, after binding the plasma membrane surrounding the folate conjugate/FR complex begins to invaginate until a distinct internal vesicle, called an early endosome, forms within the cell. The pH of the vesicle lumen is then dropped to ~5 through the action of proton pumps that are co-localised in the endosome membrane<sup>71</sup>. This acidification is presumed to protonate numerous carboxyl moieties on FR protein thus promoting a conformational change that will enable the folate molecule to be released.



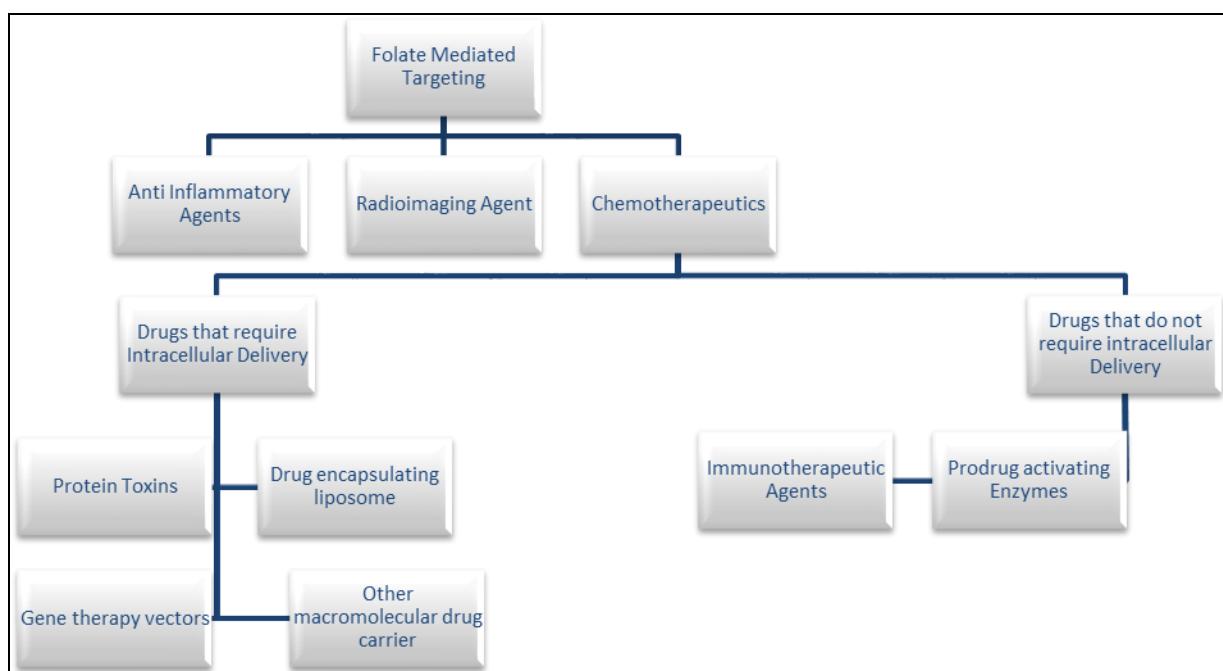
**FIG. 6: FOLATE DRUG CONJUGATES**

**Folate Drug Conjugates:** A typical folate drug conjugate contains 4 modules as depicted in **Fig. 7**. Pteroic acid (Pte) typically functions as Module 1, while the drug moiety is placed in the module 4 position. Very often, a Glu moiety is placed within the linker (module 2) at a position juxtaposed to Pte. Importantly, the combinations of Pte and Glu moieties produce folic acids. Therefore, these molecules are referred as folate conjugates. It has been observed that Glu residue of folic acid is not critical for FR recognition<sup>72, 73</sup>, whereas the Pte core is essential for FR binding. Therefore, this class of targeted molecules can be referred as ‘pteroate conjugates’ the module 3 is reserved for cleavable bonds. The function of the module 2 and module 3 are described below in detail.

**Linkers:** The drug molecule can be linked to the pteroate moiety in many ways. But this linkage

should be done in a manner that assures successful targetability of the drug cargo in the target tissue/cell. For example, if the drug moiety is positioned too close to the Pte core, the overall affinity of the net conjugate for the FR could be reduced or even ablated.

It has been observed that steric interference is more pronounced for small ligands (pteroates) than it is for the larger ligands (proteins, IgG). As such, module 2 functions as a linker to spatially separate the drug from Pte. The composition of the linker can vary widely. It can be derived from peptides, polymers, or even carbohydrates. The linker can be designed to contain functional groups that add to or enhance the physical properties of the overall pteroate– drug conjugate such as water solubility.



**FIG. 7: CLASSIFICATION OF FOLATE DRUG TARGETING ON THE BASIS OF ITS APPLICATION AND ON THE BASIS OF DRUG DELIVERY MECHANISM.**

#### Cleavable Bonds:

It is critical to dissociate the drug cargo from the ligand following endocytosis depending on the nature of the drug’s activity. The drug will show its higher activity only if it is triggered to dissociate from the ligand once it reaches the target tissue/cell. This is accomplished by inserting a cleavable bond at the distal end of the linker and juxtaposed to therapeutic cargo to allow for its

release in original functionality active form. This concept of “programming” molecular separation to occur between the ligand and the “drug” is not new. In fact, this process occurs naturally with many bacterial, plant and fungal protein toxins that bind to receptors on target cells through specialised domains. For many toxins (e.g. Ricin, diphtheria) the toxic subunit is connected to its binding subunit through a single disulphide bond. Although the

toxin is rather stable in the extracellular milieu, a conformational change occurs when it enters the endosome (caused by a drop in luminal pH), which then exposes an inter subunit disulphide bond. Intracellular reduction of this bond follows to efficiently release the toxic subunit inside the cytosol<sup>74</sup>. While the exact mechanism by which the cells reduce such disulphide bonds is not clearly understood, it is known that drug delivery strategies utilizing intra-molecular disulphide bonds for the purpose of the free drug are sometimes successful.

It is well known that endocytic vesicles rapidly acidify to ~pH 5<sup>71</sup>. In this environment, a drug attached to Pte via an acid-sensitive linker (module 3) should be released shortly after entrapment

within the endosome. Studies of the linkage between folic acid and pseudomonas exotoxin (PE 38) demonstrated for the first time the importance of building cleavable linker into the folate targeted cytotoxic agent, an imperative component in future folate conjugate drug designs. This study demonstrated that the potency of folic acid-PE38 conjugate was intricately connected to the type of bond tethering the vitamin to the ribosome inactivating protein. Thus, when the two components were linked via a reducible disulphide bond, full killing potency was observed ( $IC_{50} \sim 10^{-11} M$ ). However, when the components bridged by a thioether bond, its potency decreased by over 4 orders of magnitude<sup>75</sup>.

**TABLE 1: FOLATE RECEPTOR EXPRESSION IN SELECTED SOLID TUMORS DETERMINED PRIMARILY BY IMMUNOHISTOCHEMICAL ANALYSIS OF CANCER TISSUE MICROARRAYS USING A MONOCLONAL IgG TO FR-a.**

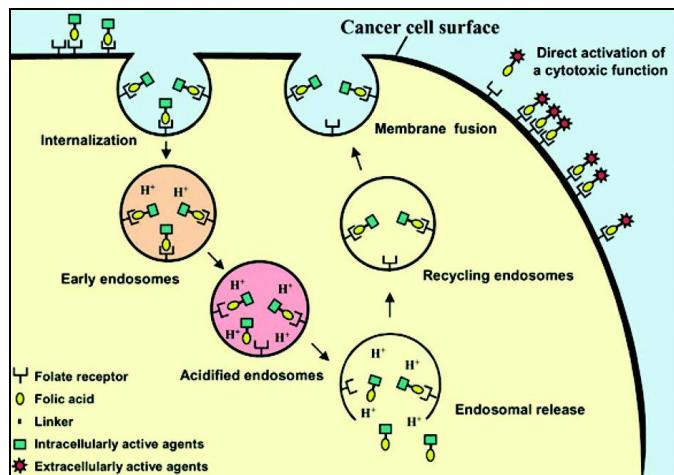
Cancers (solid tumors)	New cases per year in US	%FR	FR-expressing cancers/year
Breast	176 300	48	84 624
Lung	171 600	78	133 848
Uterus	37 400	90	33 660
Colon-rectum	129 400	32	41 408
Ovarian	25 200	90	22 680
Kidney	30 000	75	22 500
Head/neck	39 750	52	20 670
Brain and CNS	16 800	90	15 120
Gastric	26 700	38	10 146
Pancreatic	28 600	13	3718
Endocrine	19 800	14	2772
Testicular	7400	17	1258
Total	708 950		392 404

### Folic Acid Mediated Drugs:

As the FR is overexpressed in various types of tumors, activated macrophages, normal tissues of kidney, choroid plexus of brain, 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.

**Folate Mediated Delivery of Macromolecular Therapeutics:** Applications of the folate targeting for delivery of macromolecular therapeutic agents to cancer cells may be classified into categories as

1) drugs that require intracellular release to exert their cytotoxic/regulatory functions<sup>68</sup>. Examples of macromolecules includes most protein toxins, drug encapsulating liposomes, oligonucleotides, gene therapy vectors, and other colloidal drug carriers; 2) drugs that do not require intracellular loading, but are capable of mediating their functions on the surface of target cell, FR can simply act as a tumor marker that allows concentration of the drug on the tumor cell surface. Examples include prodrug activating enzymes and immunotherapeutic agents that stimulate or redirect the immune system to the cancer cell. (Fig.8). As the folate macromolecular conjugates are not rapidly degraded following internalisation, delivery systems for even the most hydrolytically sensitive macromolecules (e.g. proteins and gene therapy vectors) can potentially be developed.



**FIG. 8: FOLATE MEDIATED DELIVERY OF MACROMOLECULAR THERAPEUTICS.**

### Folate Mediated Liposomal Carriers for Therapeutic Cargo Delivery:

Liposomes have been exploited as carriers to target larger quantities of drug to cancer cells rapidly. Initial studies with fluorescent liposomes demonstrated that folate directly attached to the liposome lipid head groups did not efficiently bind FR+ cancer cells<sup>76</sup>. Whereas, the introduction of polyethylene glycol as a spacer between folate and liposome led to the entry of this folate PEGylated liposomal conjugate into the cancer cells<sup>76</sup>. With the possibility of loading >30,000 drug molecules into each liposome, the payload potential of these folate targeted liposomes seemed enormous. Unmodified liposomal formulations suffered from short circulation times *in vivo* because of nonspecific uptake by the reticuloendothelial system. This limitation was solved by incorporating ~4% PEGylated lipids into the liposomes, with ~0.1% of the total lipids (folate-PEG-distearoylphosphatidylethanolamine) attached folic acid<sup>77</sup>.

Tumor specific delivery could be enhanced by: i) shortening of the length of the free PEG chains than the folate derivatized chains, ii) restricting the liposomal diameter size to <100nm, iii) using cholesterol and saturated phospholipids as primary building blocks of the liposomes, iv) a pH sensitive or unloading mechanism into the liposomal structure<sup>76-80</sup>. 45fold higher uptake and 85 times greater toxicity was observed when the liposomes were loaded with doxorubicin in folate-PEG-liposomal structures in contrast with their non-targeted conjugates.

Upregulation of FR beta may be selectively induced in AML cells by treatment with *all-trans* retinoic acid (ATRA). In a study, the role of formulation composition in FR-targeted liposomal doxorubicin (DOX) delivery to AML cells was investigated. Formulation containing 0.5 mole % f-PEG-DSPE (folate-polyethylene glycol distearoyl phosphatidylethanolamine) has optimal drug loading properties, efficiency of uptake, and FR-dependent cytotoxicity in FR beta (+) MV4-11 AML cells and also an optimal circulation time relative to the free drug. Drug delivery through the optimal liposomal formulation was enhanced by upregulating FR-beta by treating the AML cells with ATRA<sup>132</sup>.

### Folate Mediated Low Molecular Weight Chemotherapeutic Agents:

Initial studies of folate targeted taxol conjugate, proved the construct to be less potent than anticipated<sup>81</sup>, most likely because of the poor water solubility or slow release of drug from the conjugate. Poor water solubility was known to lead to nonspecific binding to non-targeted cells, and failure to release unmodified drug had been previously shown to lead to drug inactivity<sup>75</sup>. This led to the need to understand thoroughly the physical properties of folate drug conjugates as well as the conditions in FR- mediated endocytic pathways.

The difference in the reducing power between the extracellular and intracellular milieus to induce the selective release of a disulphide linked drug inside its target cell had been exploited. Evidence that this mechanism might be operative was provided by data showing the activity of folate disulphide conjugate of mitomycin C both *in vitro* and *in vivo*<sup>82, 83</sup>. A folate linked fluorescence resonance energy transfer (FRET) construct was prepared to visualise the rate and intracellular location of disulphide reduction, which changed from red to green fluorescence upon reduction of an intramolecular disulphide bond<sup>84</sup>.

Thorough analysis of the behaviour of this conjugate both in cultured cancer cells and live-tumor bearing mice demonstrated that folate-disulphide drug conjugate reduction 1) does not occur in circulation prior to conjugate capture by tumor cells, 2) occurs following endocytosis with a

half time of 6h, 3) begins in endosomes and does not significantly depend upon the redox machinery located on the cell surface, within the lysosome or the Golgi apparatus, 4) occurs independently of endocytic vesicle trafficking along microtubules, and 5) yields products that are subsequently sorted into distinct endosomes and trafficked in different directions<sup>84</sup>.

Another mechanism envisioned to trigger the endosomal release of an active drug from its folate conjugate upon endocytosis involved the decrease in pH commonly observed in late endosomes and lysosomes<sup>71</sup>. A variety of pH sensitive linkers connecting folate to its drug cargo were developed and tested both *in vitro* and *in vivo* to exploit this pH change for drug release<sup>81</sup>. None of the conjugates demonstrated potencies similar to those of the disulphide linked conjugates. A folate FRET conjugate with a pH sensitive linker was constructed to explore the compromised efficacy and found little hydrolysis of pH sensitive linker during endosomal trafficking. Further studies with folate linked pH indicated dyes fortunately revealed that the endosomal compartments visited by monovalent folate conjugates experience pH value only as low as 6.2<sup>85</sup>, whereas, endosomal compartments visited by multivalent folate conjugates experience pH values as low as 5.0 and below which serves as a partial explanation to compromised efficacy.

The rate of FR recycling between the cell surface and its intracellular components serves as an important aspect in delivery of folate mediated drug. Net accumulation of folate conjugates in tumor tissues will depend upon not only the number and accessibility of Fr on the malignant cell surfaces but also the time required for unoccupied receptor to recycle back to the cell surface for additional drug uptake.

It has been found using radioactive conjugates, empty FR+ to unload their cargo and return to the cell surface takes ~8-12h<sup>86</sup>. Given that an average cancer cell will express anywhere from ~ 0 to 10<sup>7</sup> FR/cell, this recycling time constraint suggests that only very potent chemotherapeutic agents could succeed as folate conjugates for the cancer treatment.

### **Folate-Targeted Dendrimers:**

Dendrimers are synthetic, often biocompatible, nonimmunogenic, nanoscaled polymers that can be manufactured to specific sizes and reproducible surface characteristics. Because their surfaces can be densely functionalized, large amounts of diagnostic and/or therapeutic agents can be attached to these dendrimers, rendering them highly compact drug delivery vehicles. The first folate-targeted dendrimers described in the literature consisted of an ammonium-core polyamidoaminecore modified with folate as a targeting molecule and FITC as a fluorescent reporter. This targeted dendrimeric construct was then capped with succinic anhydride to render the remaining surface groups negatively charged to avoid nonspecific cell adsorption<sup>92</sup>. When introduced to FR<sup>+</sup> erythroleukemia cells, the folate-dendrimer particle demonstrated biphasic uptake: rapid uptake within the first minutes (attributed to the dendrimer's initial binding to empty cell surface FR), followed by slower internalization attributed to endocytosis and recycling of FR back to the cell surface.

Redesign of this folate-dendrimer construct to deliver an MRI contrast agent (gadolinium) provided good MRI contrast both *in vitro* and *in vivo* in FR<sup>+</sup> tumor xenografts<sup>92</sup> Quintana et al.<sup>93</sup> then improved the folate-dendrimer design by capping the dendrimer surface amines with hydroxyl and acetamide groups, which enhanced projection of the targeting ligand away from the nanoparticle surface, permitting greater access to cell surface FR. These dendrimers demonstrated increased capture by KB cells and better drug delivery into the same cell type. Importantly, neutralization of the dendrimer's amine surface charge was found to be essential to prevent toxicity and nonspecific uptake of the drug conjugate.

### **Folate Targeted Immunotherapy:**

Folate targeted immunotherapy does not require drug release for therapeutic efficacy. It involves the use of folic acid to deliver a highly immunogenic hapten (low molecular weight immunogen) to the surfaces of the cancer cells to render the malignant cells more "foreign" to the immune system. For this purpose, cancer bearing animals were first vaccinated against fluorescein, after which the immunized animals were treated with folate

fluorescein. Upon injection, the folate conjugate localized to FR expressing cells, decorate their surfaces with  $>10^6$  haptens/cell and lead to their elimination by immune system. As the normal cells lack FR expression, were largely spared from the immune attack. This therapy is found to confer long term immunity against cancer, such that rechallenge with fresh tumor cells invariably led to the rejection of implanted cells without the need for further therapy.

An anti-CD28 folate conjugate has also been tested in a spontaneous mouse brain tumor model where it was also found to prolong animal survival<sup>134</sup>. This treatment has the added benefit of directly activating T-cells without the need for an exogenously added peptide. This method of therapy has been demonstrated to result in complete tumor regression in multiple murine tumor models. It has also led to remission of arthritis in animal models of the disease, where FR+ macrophages are the prominent cause of inflammation.

#### **Folate-Targeted Imaging Agents:**

Due to the over-expression of FR on cancer cells and activated macrophages, folate-targeted imaging agents can serve as noninvasive diagnostic aids to assess the location and severity of FR+ cancers and inflammatory diseases. Consequently, an assortment of folate conjugated imaging agents, including radiopharmaceuticals,<sup>135-143</sup> fluorescent dyes, MRI contrast agents, and PET imaging agents,<sup>134</sup> have been synthesized and tested *in vivo*. In general, both contrast and sensitivity are good, suggesting that many imaging applications of folate targeting may eventually be developed. Although a variety of folate-targeted radionuclides have been used for radio imaging, technetium- 99m (99mTc) has emerged as the preferred diagnostic radionuclide for several reasons. Due to its short half-life (6 h) large radionuclide doses can be administered to yield high resolution images without significant radiation exposure to normal tissues.

Secondly, clinical availability and low cost renders 99mTc attractive compared to other radioisotopes. And thirdly, folate-chelate conjugates that bind 99mTc have been shown to clear rapidly from normal tissues, resulting in increased target tissue to background ratios. Folate targeted 99mTc-

radiopharmaceuticals have been successfully used to image a variety of human cancers as well as to monitor the progression of arthritis in many animal models of the latter disease. Due to development of higher resolution and more sensitive imaging technologies, new opportunities for the design of novel folate-targeted imaging agents have arisen. Unfortunately, the first generation of folate conjugates of MRI and PET imaging agents have met with only limited success. In contrast, folate-targeted near infrared dyes have demonstrated the ability to noninvasively image tumors, atherosclerotic plaque, and arthritic joints through several millimeters of normal tissue. With the aid of such conjugates, it may soon be possible to develop instrumentation for localizing FR+ cancer masses in the course of debulking surgery. As is evident from the diverse nature of folate-linked imaging agents already examined, virtually any new imaging modality could potentially be adapted to folate targeting<sup>143</sup>.

#### **Folate-Targeted Radio Imaging Agents:**

The first report of a folate-targeted radio imaging agent dates back to 1994 when Antich et al. imaged folate receptor positive tumors using an <sup>125</sup>I-labeled folate conjugate<sup>144</sup>. However, because of the long half-life of <sup>125</sup>I, this imaging agent was rapidly replaced by candidates with greater clinical potential. Among these <sup>67</sup>Ga, a gamma-emitting radionuclide with a half-life of 78 h, showed significant promise when targeted in a complex with deferoxamine-folate. The next folate-linked radioimaging agent to be developed, <sup>111</sup>In-DTPA-folate, was found to clear ~97% through the kidneys, displaying little uptake in the peritoneal cavity of healthy individuals.

An <sup>111</sup>In-citrate radiotracer was also shown to have good tumor accumulation (~3% injected dose/g tumor), the tumor to non-malignant tissue ratio was poor (0.3–2.4% injected dose/g tissue for blood, kidney, liver and muscle), confirming the significant benefit associated with ligand targeting. <sup>111</sup>In-DTPA-folate, its use in human imaging was eventually suspended due to the higher cost and longer half-life (~68 h) of <sup>111</sup>In. Based on the considerations above, 99mTc became the radioisotope of choice, not only because of its lower price and shorter half-life (~6 h), but also because of its low energy (140 keV) and facile

chemistry. Not surprisingly, many studies of folate-linked  $^{99m}\text{Tc}$  chelators, including  $^{99m}\text{Tc}$ -6-hydrozinonicotinamide (HYNIC),  $^{99m}\text{Tc}$ -ethylenedicysteine,  $^{99m}\text{Tc}$ -DTPA and  $^{99m}\text{Tc}$ -EC20<sup>144</sup> rapidly appeared.  $^{99m}\text{Tc}$ -EC20, a  $^{99m}\text{Tc}$  complexed to a short folate-linked peptide (Figure 9) was found to both bind FR with high affinity and clear rapidly from the blood ( $t_{1/2} \sim 4$  min). In fact,  $^{99m}\text{Tc}$ -EC20 was also shown to clear primarily through the kidneys, where it was demonstrated to be eliminated in nonmetabolized form.

These qualities, together with its excellent tumor uptake (~17% injected dose/g tissue) and good tumor to blood ratio ( $51 \pm 8.20$ ) rendered EC-20 a good candidate for diagnostic imaging of FR positive tumors. Finally, Müller et al.<sup>145-147</sup> have developed a kit-like formulation for the synthesis of folate conjugated to an organometallic  $^{99m}\text{Tc}$  complex via a picolyamine monoacetic acid (PAMA) bridge. Bio distribution studies in KB tumor-bearing mice have demonstrated that the aforementioned conjugate exhibits good tumor specificity, especially at longer time points.

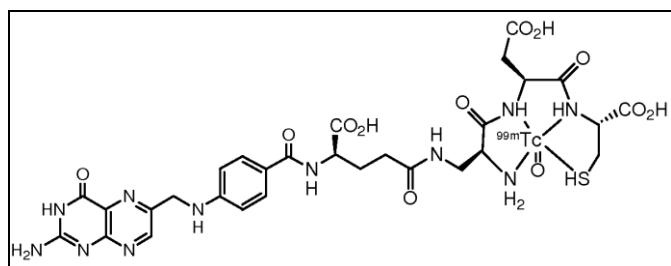


FIG.9:  $^{99m}\text{Tc}$ -EC-20

### Folate-Targeted PET Imaging Agents:

Because of its higher resolution, PET imaging has often become the radio imaging method of choice wherever appropriate instrumentation is available.  $^{18}\text{F}$ -fluoro-2-deoxyglucose ( $^{18}\text{F}$ -FDG), a glucose mimic that is carried into cells by a glucose transporter, shows enhanced tumor uptake because of the higher rate of metabolism of many cancer cells. However, since normal tissues also express glucose transporters and because some normal cells also exhibit high rates of metabolism, false positives can easily occur with  $^{18}\text{F}$ -FDG. To exploit the greater availability and better special resolution of  $^{18}\text{F}$ , Bettio et al.<sup>148</sup> recently conjugated folic acid to  $^{18}\text{F}$ -fluorobenzylamine in a four step reaction (this short reaction sequence is important since the half-life of  $^{18}\text{F}$  is only 110

min).  $^{18}\text{F}$ -FBA-folate was injected into tumor bearing mice, accumulation of the imaging agent was seen to be concentrated at the tumor rim, which was found to contain the majority of viable tumor cells.

### Folate-targeted MRI contrast agents:

A variety of transition metal chelates (e.g. gadolinium) and iron oxide particles are known to enhance MRI contrast by modifying the relaxation of water. In the initial exploration of the use of folate to target an MRI contrast agent, Wiener et al.<sup>149</sup> synthesized a Gd-folate-dendrimer conjugate that was tested in athymic nu/nu tumor-bearing nude mice.

Their results indicated much greater folate conjugate uptake in FR positive than FR negative tumors (3.64 versus 0% injected dose/g tissue), with a tumor to blood ratio of ~13:1. Unfortunately, significant conjugate accumulation was also observed in the liver and spleen (~6% injected dose/g tissue). Choi et al.<sup>150</sup>, for example, synthesized a folate-targeted fluorescent dextran and used this polymer to coat iron oxide particles to permit simultaneous tumor cell visualization by both fluorescence microscopy and MRI. Uptake of the particles by KB cells in culture was found to be FR mediated, since co-incubation with 1 mM free folic acid completely abolished internalization. More recently, Sun et al.<sup>151</sup> developed a folate derivatized iron oxide particle that was coated with PEG (MW=600) to reduce nonspecific scavenging by tissue resident macrophages. In tests with HeLa cells, a significant decrease in signal intensity was observed upon incubation with folate-PEG-particles, decreasing from 231.8 in the absence of conjugate to 16.7 in the presence. Although non targeted PEG-iron particles were also able to reduce MRI signal intensity after 4 h incubation, the magnitude of their effect was significantly reduced (165.8 vs. 231.8).

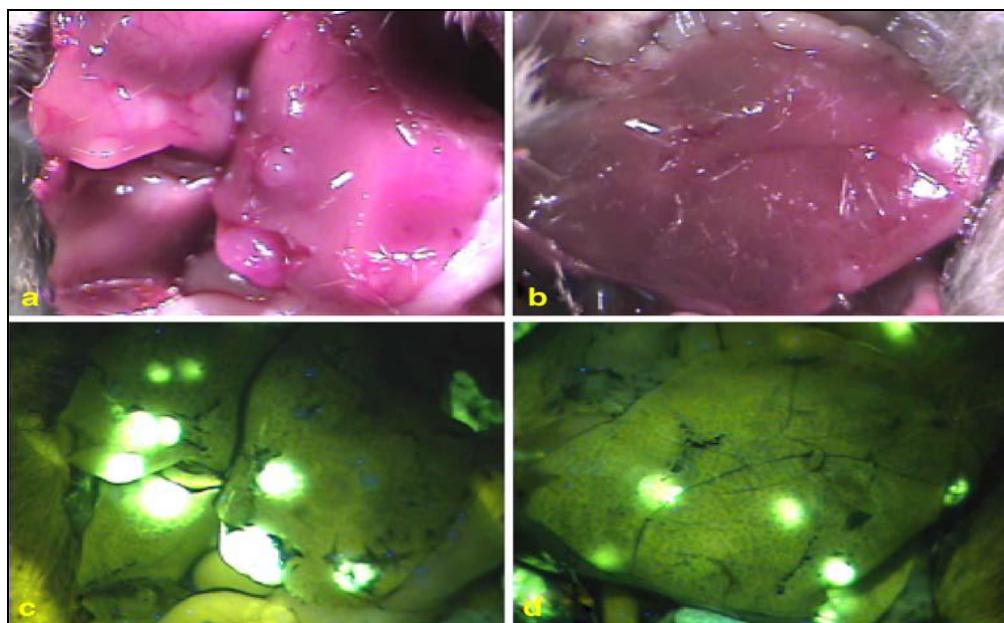
### Folate-Targeted Optical Imaging Agents:

In an effort to contribute to the development of optical imaging modalities, a variety of fluorescent dyes (e.g. fluorescein, Texas Red, the rhodamines, the indocyanines, the Alexa Fluors, the CyX series, etc.) have been conjugated to folic acid and their abilities to detect FR positive tumors have been examined. Kennedy et al.<sup>152</sup> were the first to detect

metastatic disease by fluorescence methods *in vivo* when they observed both single cells and small fluorescent nodules in mouse livers following injection of a folate– fluorescein conjugate into the tail veins of mice with metastatic cancers. (**Fig. 10**) As expected, normal tissues that lacked FR did not bind folate–fluorescein, and thus a good contrast between malignant and healthy tissues was obtained. Although the undesirable optical qualities of fluorescein required surgical opening of the peritoneal cavity in order to obtain the aforementioned images, noninvasive optical images of subcutaneous malignant masses were also collected in a follow-up publication using a folate-linked indocyanine dye.

Tung et al. <sup>153</sup> have linked folate to NIR2 via a hydrophilic spacer and demonstrated that the

conjugate is capable of localizing tumor masses as early as 1 h post i.v. administration. In a follow up study, the same group has shown that tumor: background ratio is not only much greater when folate is used to target the NIR2 dye, but also that the high tumor: background ratio is maintained much longer (up to 48 h) when the dye is folate-targeted <sup>96</sup>. He et al. <sup>154</sup> designed a fluorescent imaging method to noninvasively detect circulating tumor cells as they flowed through the peripheral vasculature. To mimic the clinical situation, FR+ L1210A leukemia cells were first injected i.v. into mice then followed several hours later by injection of folate–rhodamine. Importantly, circulating tumor cells (CTCs) could be easily detected and counted as they flowed through the vasculature of the ear using a multiphoton intravital microscope.



**FIG. 10: FLUORESCENCE IMAGING OF METASTATIC FR POSITIVE TUMOR ON MOUSE LIVER. PANELS (a) AND (b) REPRESENTS THE WHITE LIGHT IMAGES OF TWO MOUSE LIVERS, WHILE PANELS (c) AND (d) REPRESENTS THE SAME TISSUES UNDER FLUORESCENT LIGHT AFTER ADMINISTRATION OF FOLATE–FLUORESCEIN VIA TAIL VEIN INJECTION. IMAGE REPRODUCED FROM REFERENCE <sup>93</sup> WITH PERMISSION FROM THE JOURNAL OF BIOMEDICAL OPTICS**

This suggested that a patient's metastatic disease burden might be accurately quantitated using the patient's blood stream as an *in vivo* flow cytometer. Importantly, necropsy analyses of tissues from the same mice by a trained veterinary pathologist could not detect any metastatic disease until week 5, at which time the first 50 µm nodule was discovered. Subsequently, circulating tumor cells have also been detected using folate–rhodamine in peripheral blood samples from large number of ovarian cancer

patients. Finally, despite their possible toxicities, quantum dots have been explored as possible tools for folate-targeted optical imaging agents due to their high photochemical stability, tunable photoluminescence and good fluorescence quantum yield. For example, Bharali et al. <sup>155</sup> have conjugated InP quantum dots to folic acid and shown the compound to be taken up by KB cells via FR mediated endocytosis.

## Other Folate-Nanoparticle Drug Delivery Systems:

Folate has also been conjugated to a host of other nanoparticle platforms, including super paramagnetic nanoparticles,<sup>95, 96</sup> gold nanoparticles,<sup>97, 98</sup> magnetite ( $\text{Fe}_3\text{O}_4$ ) nanoparticles,<sup>99</sup> carbon nanotubes,<sup>100, 101</sup> lipoprotein-based nanoplatforms,<sup>102, 103</sup> thermo responsive microgels,<sup>104</sup> bovine serum albumin nanoparticles,<sup>105</sup> and virus capsid proteins.<sup>106, 107</sup>

Because little data are available on the *in vivo* properties of these nanomedicines,<sup>101, 103, 108</sup> their potentials as novel drug carriers cannot be assessed at this juncture.

However, in general, the design and application of such folate-targeted nanoparticles are similar to the folate-targeted nanomedicines described above in that the primary objective focuses on improved delivery of larger payloads and more hydrophobic drugs. Unfortunately, much like their better characterized counterparts, these particles may similarly suffer from compromised penetration into solid tumors and nonspecific uptake by macrophages in the liver, spleen, and other organs of the reticuloendothelial system. Use of PEGylation (or a related coating) to suppress macrophage recognition could conceivably reduce nonspecific uptake, but improvement of penetration into solid tumors will require creative strategies to digest or weaken the dense extracellular matrix that cements solid tumors together.<sup>109</sup>

Clearly, while tumor targeted nanoparticles offer attractive solutions to many problems plaguing small molecule delivery, they simultaneously introduce new obstacles of their own that will require solutions before their full potentials can be realized.

### Folate-targeted drugs in human clinical trials:

The initial folate-targeted drug to be tested in humans was  $^{111}\text{In}$ -DTPA-folate (Fig. 12a), a folate conjugated chelator of  $^{111}\text{In}$  that was demonstrated to bind FR with ~1 nM affinity<sup>123, 124, 125</sup>. As anticipated from the known bio distribution of FR in animals and humans,  $^{111}\text{In}$ -DTPA-folate was found to localize specifically to cancer cells and the kidneys in non-inflamed cancer patients. Because the folate conjugate was observed to clear rapidly from FR-negative tissues with a

t1/2 of <10 min, it became obvious that folate conjugates not captured by FR would be rapidly excreted from the body (97% via the kidneys in the case of  $^{111}\text{In}$ -DTPA-folate). This rapid clearance from FR-negative tissues was considered desirable, since imaging agents that remain in receptor negative tissues contribute only to nonspecific background, and therapeutic agents that are retained in nontargeted tissues simply add to off-target toxicity.

EC20, a folate conjugate of  $^{99}\text{mTc}$  (Fig. 12b), was synthesized, and introduced into the clinic for imaging of cancer patients. EC20 demonstrated similar tumor selectivity and rapid clearance from FR-negative tissues to that seen with  $^{111}\text{In}$ -DTPA-folate.

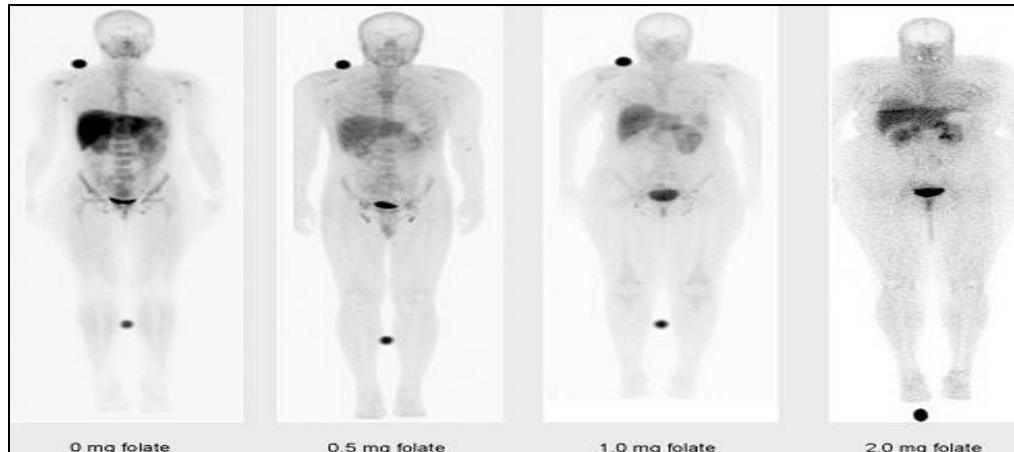
The first folate-targeted therapeutic agent to enter the clinic was EC17, a folate-linked fluorescent hapten that was designed to enhance the immunogenicity of FR-positive tumors (Figure 12c). In animal tumor models, the targeted hapten strategy was found to promote the rapid influx of antibodies and immune cells into the cancer mass, leading to complete elimination of advanced tumors with little or no toxicity to healthy cells.

The fourth folate-targeted drug to enter clinical trials was EC145 (Fig. 12d), a folate conjugate of desacetylvinblastine hydrazide. In contrast to the aforementioned imaging agents and EC17, where release of the folate-conjugated drug was considered undesirable, release of desacetylvinblastine hydrazide was essential for its therapeutic activity. Therefore, a reversible linker was required that would discharge the therapeutic warhead in unmodified form following uptake of the conjugate by the cancer cell.<sup>126-129</sup>

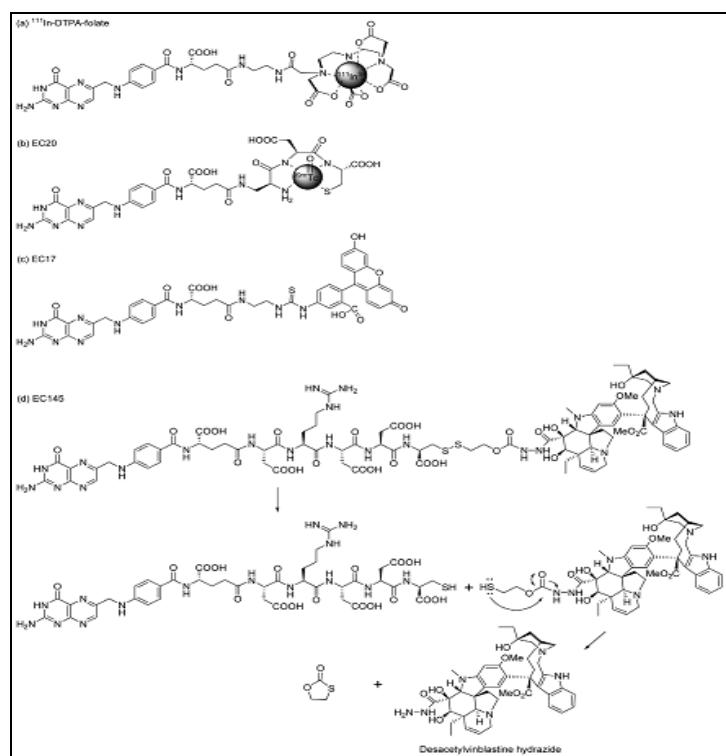
Farletuzumab and vintafolide have shown clinical promise in phase 2 and 3 trials<sup>157, 158</sup>. Farletuzumab is a FRa-specific monoclonal antibody that can induce cell death through complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity<sup>159</sup>. Vintafolide can deliver chemotherapy to FR-expressing cells, as it is a conjugate of folate and the chemotherapeutic agent desacetylvinblastine monohydrate<sup>157</sup>.

99mTc-etalfolatide, a 99mTc-labeled folate conjugate, is in late phase trials in Europe and the United States. It allows noninvasive, whole-body imaging of the FR. 99mTc-etalfolatide takes advantage of the more optimal SPECT imaging characteristics of technetium (half-life of 6 h and 140-keV photon) and has been evaluated in several clinical Trials. No safety concerns have been identified and the only 99mTcetalfolatide-related adverse events were lower abdominal pain, nausea, and vomiting (occurring in, 1% of patients)<sup>159</sup>.

Several phase 2 trials showed that 99mTc-etalfolatide imaging may be able to identify patients who are most likely to benefit from vintafolide. FR expression and 99mTc-etalfolatide physiologic uptake, which occurs in liver, kidneys, spleen, bladder, and, to a lesser extent, bone marrow, may complicate interpretation of FR expression in lesions close to these organs<sup>159</sup> (**Fig.11**). To partially saturate FRs, and to reduce 99mTc-etalfolatide uptake in these organs, a small amount of folic acid is injected before 99mTc etalfolatide administration.



**FIG. 11:** 99mTc-ETARFOLATIDE BIO DISTRIBUTION PATTERN (1 h AFTER INJECTION) AFTER FOLATE PREINJECTION IN HEALTHY VOLUNTEERS. IN ABSENCE OF FOLATE PREINJECTION, 99mTc-ETARFOLATIDE UPTAKE IS SEEN IN LIVER, KIDNEY, SPLEEN, BLADDER, AND BONE MARROW. THIS BACKGROUND ORGAN ACTIVITY IS DECREASED WITH PREINJECTED FOLATE.



**FIG.12: STRUCTURE OF FEW FOLATE CONJUGATES**

An FR $\alpha$  targeted antibody-drug conjugate, IMGN853, composed of three components: 1) an anti FR  $\alpha$  monoclonal antibody that targets FR positive cancer cells, 2) a disulphide based linker, and 3) DM4, a cytotoxic maytansinoid which acts as a microtubule polymerization tubulin inhibitor is being investigated in an ongoing Phase I study in patients with endometrial cancer & epithelial ovarian cancer. Preliminary results show it to be well tolerated<sup>171</sup>.

### **Various Ongoing Applications of Folic Acid in Delivery of Cytotoxic Drugs and as Imaging Agents:**

In the case of cancer, the well-characterized up-regulation of folate receptors on malignant cells to target folate linked pharmaceuticals to cancer tissues *in vivo*. Drugs that have been linked to folic acid for tumor-selective drug delivery to date include (i) protein toxins, (ii) chemotherapeutic agents, (iii) gene therapy vectors, (iv) oligonucleotides (including small interfering RNA (siRNA)), (v) radioimaging agents, (vi) magnetic resonance imaging (MRI) contrast agents, (vii) liposomes with entrapped drugs, (viii) radiotherapeutics agents, (ix) immunotherapeutic agents, and (x) enzyme constructs for prodrug therapy. The folate receptors are also overexpressed on activated (but not resting or quiescent) macrophages. Recognizing that activated macrophages either cause or contribute to such diseases as rheumatoid arthritis, Crohn's disease, atherosclerosis, lupus, inflammatory osteoarthritis, diabetes, ischemia reperfusion injury, glomerulonephritis, sarcoidosis, psoriasis, Sjogren's disease, and vasculitis<sup>87</sup>.

Folate receptors (FR) may be of use for targeted delivery of cytotoxic drugs in invasive urothelial carcinoma (iUC), for which improved therapy is needed. FR expression and function in iUC were explored and the antitumor activity and toxicity of a folate-targeted vinblastine conjugate were evaluated in dogs with naturally occurring iUC, an excellent model for human iUC. FR immunohistochemistry was carried out on iUC and normal human and dog bladder tissues together with nuclear scintigraphy in dogs to monitor iUC folate uptake. It was found that folate-targeted therapy holds considerable promise for treating

iUC, where FR-b may be important in addition to FR-a<sup>88</sup>.

Tumor specific intraoperative fluorescence imaging may improve staging and debulking efforts in cytoreductive surgery and thereby improve prognosis. Patients with ovarian cancer, intraoperative tumor specific fluorescence imaging with a FR $\alpha$ -targeted fluorescent agent showcased the potential applications in patients with ovarian cancer for improved intraoperative staging and more radical cytoreductive surgery<sup>89</sup>. Also in an experiment conducted to show whether chemotherapy has any effect on the expression of folate receptor-alpha in ovarian cancer showed that there is no alteration in the expression rates and folate-targeted agents may have a place in the treatment for ovarian cancer, before as well as after chemotherapy<sup>90</sup>.

Folate receptor beta (FR $\beta$ ) is only detectable in placenta and limited to some hematopoietic cells of myeloid lineage in healthy people. FR $\beta$  is over-expressed in activated macrophages in autoimmune diseases and some cancer cells. In a study aimed to develop an FR $\beta$ -specific human monoclonal antibody (mAb) that could be used as a therapeutic agent to treat rheumatoid arthritis and other autoimmune diseases, as well as FR $\beta$  positive cancers it was concluded that unlike folate-drug conjugates, m909 (a functional recombinant FR $\beta$  protein produced in insect cells and used as antigen to isolate a mAb) selectively binds to FR $\beta$ , does not recognize FR $\alpha$ , and has at least one effector function. m09 alone has potential to eliminate FR $\beta$  positive cells. Because m909 does not compete with folate for receptor binding, it can be used with folate-drug conjugates in a combination therapy. m909 can also be a valuable research reagent<sup>113</sup>.

The advent of targeted therapies has offered a new treatment paradigm for lung cancer, but currently validated and emerging drugs are effective in only a small minority of lung cancers, predominantly adenocarcinomas. Folate receptors can serve as targets for drugs attached to folate and are overexpressed in many cancers. It has been shown that large percentage of lung cancers; including squamous cell carcinomas in addition to adenocarcinomas, strongly express folate receptor. This suggests that folate-linked targeted therapy

can potentially be used to treat the majority of lung cancers, both adenocarcinomas and, particularly, squamous cell carcinomas, that do not respond to current targeted therapies<sup>117</sup>.

Folate and folate-linked drugs bind to FRs with nanomolar to subnanomolar affinities. Because cells lacking FR exhibit no affinity for folate-linked drugs, only cells that express accessible FR are observed to take up appreciable levels of folate-targeted drugs. Not surprisingly, lungs of healthy individuals display no uptake of folate-targeted radioimaging agents, whereas lungs of many cancer patients display localized uptake at sites of malignant disease. Based on the high affinity of folate conjugates for FR, 4 FR-targeted therapeutic agents and 1 FR-targeted imaging agent are currently undergoing human clinical trials. The folate-targeted therapeutic agents include a highly immunogenic hapten, a modified vinblastine<sup>114</sup>, an epothilone, and a dual warhead folate conjugate containing both desacetylvinblastine and mitomycin C in the same molecule<sup>115</sup>. The imaging agent is comprised of folate linked to a 99mTc chelating agent<sup>116</sup>.

Folate receptor alpha (FR $\alpha$ ), encoded by folate receptor 1 (adult) gene, has emerged as a cancer biomarker and potential therapeutic target. In addition, its expression in tumors may offer prognostic information. In a study conducted to analyze the expression pattern of FR $\alpha$  in resected colorectal cancer liver metastases utilizing immunohistochemistry as the detection method it was found that there is overexpression of FR $\alpha$  in a subset of hepatic metastases<sup>118</sup>. Both the primary and metastatic tumors have a similar pattern of FR $\alpha$  expression. FR $\alpha$  expression is present in a subset of resected colorectal cancer liver metastases, and is independently associated with early death after hepatic resection. Studies on the use of FR $\alpha$ -targeted strategies in resectable stage-IV colorectal cancer patients and on evaluation of FR $\alpha$  as a cancer prognostic marker are justified<sup>119</sup>.

Pro-inflammatory macrophages play a prominent role in such autoimmune diseases as rheumatoid arthritis, Crohn's disease, psoriasis, sarcoidosis, and atherosclerosis. As pro-inflammatory macrophages have also been shown to overexpress a receptor for the vitamin folic acid (i.e., folate

receptor beta; FR- $\beta$ ), folate-linked drugs have been explored for use in imaging and treatment of these same diseases. These FR- $\beta$ + macrophages coexpress markers of alternatively activated (M2-type) macrophages, including the mannose receptor and arginase-1. Folate-conjugated fluorescent dyes and radioimaging agents can be specifically targeted to these asthmatic lung macrophages, with little uptake by macrophages present in healthy lung tissue. FR- $\beta$  is shown to be substantially upregulated on AAMs, which are thought to be intrinsically involved in the development of asthma, idiopathic pulmonary fibrosis, cirrhosis of the liver, and other fibrotic diseases. Because these folate receptors can be selectively targeted with folate-linked imaging agents, such folate conjugates could prove useful in assessing disease severity and response to therapy in affected patients<sup>120</sup>.

Early detection of heart disease is essential for the implementation of intervention strategies that reduce the risk of cardiovascular events. Radio imaging methods that have been explored for this purpose include 18F-FDG, which measures sites of elevated metabolic activity; 99mTc-annexin A5, which reveals regions of enhanced apoptosis and thrombosis; and 99mTc-labeled anti-lectin like oxidized low-density lipoprotein receptor 1 antibody, which detects the lectin like oxidized low-density lipoprotein receptor 1 that is overexpressed on a variety of vasculature associated cells. It was shown that folate targeted chelate of 99mTc, termed 99mTc-EC20 can be used for imaging of folate receptor (FR)-expressing macrophages that accumulate in atherosclerotic plaques, internalize cholesterol-rich lipoprotein particles, and evolve into foam cells that form components of vulnerable atherosclerotic lesions<sup>121</sup>.

99mTc-etafolatide imaging can be used as a prognostic tool because FR expression is prognostic for ovarian and lung cancer<sup>160, 161</sup>. Intraoperative FR imaging with optical probes can improve staging and facilitates cytoreductive surgery<sup>162</sup>. 99mTc-etafolatide imaging may aid in selecting patients for intraoperative fluorescence FR imaging and help to identify deep seated lesions that could be missed by intraoperative optical imaging because of limited signal penetration.

Preclinical studies have demonstrated that <sup>99m</sup>Tc-*tarafolatide* imaging can be used to visualize macrophage activation in atherosclerotic plaques and arthritis and to assess disease activity <sup>163-165</sup>. One clinical study showed that <sup>99m</sup>Tc-*tarafolatide* imaging may be more sensitive than physical examination for assessing disease activity in rheumatoid arthritis patients <sup>166</sup>.

EC131 is a novel conjugate of FA with the potent maytansinoid DM1 drug linked together with a disulfide bond. It has been recently shown that linkers of this type are stable in the circulation for a prolonged period of time, but are cleaved inside the cell, releasing a free cytotoxic derivative of maytansinoid. Notably, intracellular release of disulfide-linked drugs from a FA conjugate was recently shown by real-time imaging using a fluorescence resonance energy transfer technique. FA-drug conjugates constructed with comparable hydrophilic spacers were found to be active against FR-expressing tumors. Similarly, EC131 was found to produce a marked antitumor effect, selectively eradicating FR-expressing tumors in multiple mouse models <sup>130</sup>.

A folate receptor targeted camptothecin prodrug was synthesized using a hydrophilic peptide spacer linked to folate via a releasable disulfide carbonate linker. The disulfide carbonate-peptide spacer confers water solubility to the prodrug without compromising FR binding affinity and provides a release mechanism for rapid unloading of camptothecin within FR + cancer cells. Moreover, this synthetic strategy could prove useful for construction of additional camptothecin prodrugs linked to other targeting ligands <sup>131</sup>.

Glioblastomas are the most malignant forms of brain cancer cells as they are resistant to the chemotherapeutic agents & radioimaging agents caused by enhanced resistance to apoptosis. Many forms of Glioblastomas are folate receptor positive. Davila et al. developed folate targeted apoptosis inducing drugs for glioblastoma treatment using nano-sized protein particles containing cytochrome C (NPs). Protein drug stability problems were countered by covalently decorating the constructs with glycans. Confocal imaging revealed the specific up-take of FITC tagged FA-NPs by GL261 cells, but not by primary cultured astrocytes.

Examination of live brain slices encompassing glioblastoma tumor showed some non-specific accumulation of NPs in healthy tissue in two hours after application of FA-NPs, but 20-times less compared to the tumor area. NPs, used in vitro in concentration of 100 mg/ml, have no cytotoxic effect in astrocytes but cause 40% death in tumor cells. The use of NPs in combination with LY294002 (PI3K/AKT blocker), 50 mM, caused 90% death in tumor cells. In vivo TUNEL investigation of tumors generated in C57Bl/6 mice by implantation of GL261 cells revealed the strong signs of apoptosis after three days of in-site administration of NPs through the micro osmotic pumps without the evident signs of apoptosis in healthy tissue. It can be stated that the designed NPs demonstrate good specificity for GL261 cells, effectively cause the apoptosis in these cells when used in combination with LY294002, and give a good baseline for the development of efficient methods for treating glioblastomas <sup>167</sup>.

Wenchen Li et al developed a multifunctional zwitterionic nanogels using copolymers of ornithine methacrylamide using folic acid for specific drug targeting with fluorescent crosslinking carbon dots (CCDs) <sup>168</sup>. In this construct, the zwitterionic nanogels network served as a functionalizable non-fouling matrix for drug loading, while the introduction of CCDs as cross linkers enabled the real-time tracking and locating of the nanogel. The nanogels showed exceptional stability when incubated in protein solutions and stable fluorescence similar to that of CCDs. Labeled dextran was encapsulated in nanogels as a model drug, and was released in a controlled manner. Importantly, cellular uptake experiments showed that the folic acid – conjugated nanogels can be specifically internalized by the folate receptor – overexpressed cancer cells, but not in normal tissue cells. This type of multifunctional nanogels holds great potential for targeted delivery and simultaneous imaging in cancer therapy due to their great stability, bio imaging capability, excellent biocompatibility, controlled drug release, and selective cell targeting.

A folate-receptor-targeted poly (lactide-co-Glycolide) (PLGA)-Polyethylene glycol (PEG) nanoparticle is developed for encapsulation and delivery of disulfiram, an oral aldehyde

dehydrogenase inhibitor with anticancer properties, into breast cancer cells. After a comprehensive characterization of nanoparticles, cell cytotoxicity, apoptosis induction, cellular uptake and intracellular level of reactive oxygen species are analyzed. In vivo acute and chronic toxicity of nanoparticles and their efficacy on inhibition of breast cancer tumor growth is studied. The folate-receptor-targeted nanoparticles are internalized into the cells, induce reactive oxygen species formation, induce apoptosis and inhibit cell proliferation more efficiently compared to the untargeted nanoparticles.

The acute and toxicity test show the maximum dose of disulfiram equivalent of nanoparticles for intravenous injection is 6 mg/kg while show significant decrease in the breast cancer tumor growth rate. It is believed that the developed formulation could be used as a potential vehicle for successful delivery of disulfiram, an old and inexpensive drug, into breast cancer cells and other solid tumors<sup>169</sup>.

Ru(II) polypyridyl complexes have been expected as promising therapeutic agents against cancer owing to its DNA photo cleavage activity. However, the lack of cell selectivity poses a significant obstacle to their practical application. Herein, the strategy combining cell-specific imaging with photo induced cell death based on [Ru(phen)2(dppz)]2+ has been developed by incorporating [Ru(phen)2(dppz)]2+ into folate conjugated liposomes. The cells overexpressing folate receptors could specifically recognize this vehicle and be imaged through the luminescence of [Ru(phen)2(dppz)]2+. Thereafter, the delivered [Ru(phen)2(dppz)]2+ interacted with DNA in cells and led to photo induced cell death. This work provided a possible alternative for cancer diagnosis and therapy<sup>170</sup>.

Folate-NOTA-A1<sup>18</sup>F, a PET imaging agent, a viable alternative to EC20, has been synthesized and underwent pre-clinical evaluation and has been found to have improved radiopharmaceutical properties such as higher resolution, shorter image acquisition time etc. This folate targeted improved imaging agent may proof to be useful in identification, diagnosis and staging of patients with FR expressing tumors<sup>172</sup>.

## Future Directions and Concluding Remarks:

Receptor targeted drug delivery can claim two advantages over non targeted therapies depending on the nature of targeting ligand and properties of therapeutic cargo: 1) receptor targeted delivery can enhance the net drug uptake by pathologic cells, 2) reduce the drug deposition into non pathologic cells, thereby diminishing collateral toxicity to normal tissues. Folate receptor drug targeting is able to achieve both aforementioned advantages. FR mediated endocytosis does not expose the folic acid targeted drug to destruction by lysosomes on internalisation unlike antibody and other ligand targeted technologies. Therefore, macromolecular or hydrolytically sensitive drugs could be delivered by folic acid targeting, since most are inactivated by the lytic enzymes concentrated in lysosomes.

Low level of toxicity to normal tissues is the most significant advantage of folate receptor mediated delivery. Low toxicity profiles could possibly be explained as – i) FR is significantly upregulated on many cancer cells as compared to their non-transformed counterparts. FR expression may even further increase in late stage and high grade neoplasms ii) folate conjugates can be constructed to promote their rapid excretion by integrating with highly hydrophilic linkers if they are not quickly captured by a cell surface FR., iii) very high affinity of folic acid and folic acid conjugates for its receptors ( $k_D \sim 10^{-10}$ M), folate conjugates can be administered at very low concentrations and still saturate all accessible FR. And because of doses of folate conjugates that exceed receptor saturation do not contribute to therapy, folate conjugates need not be administered at toxic concentrations to achieve a clinical response, iv) functional FR that are present on normal tissues are localised primarily to apical surfaces of polarised epithelia, where they are inaccessible to parenterally administered folate conjugates.

Thus, FR in the lungs is present on the membrane surface facing the airways, and in the gut and elsewhere, the receptor faces the lumen of the epithelium. In those locations, access to circulating folate conjugates is blocked, unless the injury to epithelium allows leakage of the conjugate into the luminal compartment. FR expressed on the apical surface of the proximal tubules of kidney appears to serve as a salvage receptor that captures folate in

the urinary filtrate and transcytoses the vitamin back to blood.

In a recent study it has been found that the size of the folate mediated nanoparticles has a significant effect on their rates of penetration into solid tumors. By using folate-PEG rhodamine Conjugates of different sizes it was seen that large sized particles accumulate more slowly and less efficiently in tumor masses than the much smaller ones. The binding affinity of the nanoparticle for tumor FR can be enhanced by increasing the number of targeting ligands attached to each nanocarrier. Thus, multivalent attachments can augment binding avidity when high affinity monovalent interactions cannot be achieved. The folate-targeted nanoparticles can be loaded with large drug payloads to mitigate their reduced accumulation in tumor sites. Higher concentrations of the nanomedicines can be administered to drive greater tumor accumulation<sup>112</sup>.

Folate targeting has some additional advantages like folic acid is inexpensive, nonimmunogenic, could be easily coupled with variety of therapeutic drugs and radioimaging agents as it is found to be stable against organic solvents, acids and bases. The population of FR that does not internalise but rather remains on cell surface may be exploited to retarget the immune system to the pathologic cells.

Folic acid has been frequently exploited to target attached drugs to cells that overexpress a folate receptor (FR). Unfortunately, folic acid and folate-linked drugs bind equally well to both major isoforms of the FR—that is, FR $\alpha$ , which is primarily expressed on malignant cells, and FR- $\beta$ , which is upregulated on activated monocytes and macrophages. Because both major isoforms of FR can be expressed simultaneously in the same organism, folic acid cannot enable selective targeting of therapeutic and imaging agents to either tumor masses or sites of inflammation. In an experiment a reduced and alkylated form of folic acid, N5, N10-dimethyl tetrahydrofolate (DMTHF) that exhibits selectivity for FR- $\alpha$  was injected into mouse bearing FR- $\alpha$ -expressing tumor xenografts and imaged by g-scintigraphy. It was seen that the new targeting ligand was found to bind malignant cells in mice with solid tumor xenografts but not peripheral blood monocytes or inflammatory

macrophages in animal models of atherosclerosis, rheumatoid arthritis, muscle injury, or ulcerative colitis<sup>91</sup>.

The folate receptor (FR) is a GPI anchored cell surface glycoprotein that functions to facilitate folic acid uptake and mediate signal transduction. With the introduction of multiple folate-targeted drugs into the clinic, the question has arisen regarding how frequently a patient can be dosed with a FR-targeted drug or antibody and whether dosing frequency exerts any impact on the availability of FR for subsequent rounds of FR-mediated drug uptake. The rate of internalization and trafficking of most cell surface receptors may depend on multiple parameters, including: (i) the nature of the ligand,<sup>110</sup> (ii) the level of receptor occupancy and (iii) the degree of receptor clustering induced by its binding ligand. Thus, natural agonists commonly promote rapid receptor endocytosis, while antagonists generally do not.

High levels of receptor occupancy may also induce internalization and destruction of some occupied receptors, while low levels of receptor saturation may stimulate greater receptor trafficking to recycling endosomes and subsequent return of the receptor to the cell surface. So in 2012 it was proved that FR occupancy nor does folate conjugate valency have any significant effect on FR levels at the cell surface. FR endocytosis occurs at a constitutive rate, regardless of FR occupancy or cross-linking due to multivalent ligand binding<sup>111</sup>.

The folate mediated targeting has limitations too. Firstly, not all cancers over express a FR. Thus, alternative targeting ligands have to be explored for cancers that lack over expression of FR like prostate, for specific targeting. Second, because of the high affinity of folate for its receptor, some conjugates may release too slowly from the receptor to permit accumulation of a therapeutic dose. This limitation could be overcome by exploiting 1) the difference of reducing power between intracellular and extracellular milieus to develop the easily hydrolysable linkers, 2) the difference in pH between late endosomes and lysosomes to develop the pH sensitive linkers which could be easily hydrolysed. Thirdly, the high affinity of folate for FR could also lead to “binding

site barrier" problems if the size of the therapeutic cargo is too large. This limitation was overcome by either designing therapeutic cargos that do not obstruct the interstitial spaces between cells or by modifying the targeting ligand so that it binds with lower affinity. However, since significant toxicities have not been observed, and since other limitations of folate targeting can generally be avoided with some skill and forethought, the future of successful development of folate linked drugs seem bright.

Recently scientists have experimented for the first time on oligomeric/inorganic hybrid nanoparticles to provide with new type of biomaterials for tumor-targeted imaging with high selectivity. Using one AB2-type small molecule 1, they have prepared oligomeric nanoparticles 1-NPs with a condensation reaction and self-assembly. With functional -NH<sub>2</sub> and -SH groups on their surfaces, 1-NPs could be facilely conjugated with NIR-emitting CdTe/CdS QDs and Mal-FA to synthesize oligomeric/inorganic hybrid nanoparticles 1-NPs-QDs-FA. These nanoparticles have shown excellent targeting property to FR-expressing tumor cells *in vitro* and were successfully applied to selectively imaging FR overexpressing tumors *in vivo*<sup>133</sup>.

Folate receptor-targeted imaging holds great promise for the diagnosis and staging of malignant masses. The fact that FR are expressed on a wide variety of cancers, combined with folate's high affinity for FR, rapid uptake by FR+ tumor tissues, rapid clearance from FR healthy tissues, ease of conjugation to imaging agents, lack of immunogenicity, and low cost renders the vitamin an attractive candidate for use in receptor-targeted cancer imaging. FR-targeted imaging agents should not only find clinical applications in the diagnosis, staging and monitoring of FR+ cancers, but may also aid in selection of patients for FR-targeted therapies.

Finally, since FR has also been recently identified on activated macrophages, the same imaging modalities should readily be applied to the diagnosis and staging of macrophage-mediated inflammatory and autoimmune diseases, including rheumatoid arthritis, Crohn's disease, atherosclerosis, lupus, inflammatory osteoarthritis, ischemia/reperfusion injury, glomerulonephritis, and psoriasis<sup>156</sup>.

Indeed, the time has come to test the concept in clinical trials and let the strategy either thrive or die on its own merits.

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