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In vitro and *In vivo* evaluation of folate-mediated tumor cell targeting of Cytrabine

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ABSTRACT

The folate receptor (FR) is a confirmed tumor-associated antigen that binds folate and folate–drug conjugates with very high affinity and shuttles these bound molecules inside cells via an endocytic mechanism. Using folate (or an analog thereof) as the ligand, a wide variety of drug payloads can be delivered to FR-positive cells, ranging from small radioactive imaging agents up
to large DNA-containing formulations. For therapeutic purposes, attachment of small molecular weight, highly potent agents to folate is a novel approach. We exploited this background for our research.

Cytarabine, also known as Cytosine arabinoside is an efficient antineoplastic agent. Its mode of action is due to its rapid conversion into cytosine arabinoside triphosphosphate, which damages DNA when the cell cycle holds in the S phase. Cytosine arabinoside also inhibits both DNA and RNA polymerases and nucleotide reductase enzymes needed for DNA synthesis.

We thereof synthesize the conjugate (Cyt-Fol), by attaching free $-NH_2$ of cytarabine to activated -COOH group of folate in 1:1 equivalents. The novel compound (Cyt-Fol) was characterized by different spectroscopic techniques (UV, NMR, and MS). The compound was labeled with ^{99m}Tc by direct labeling method using stannous chloride as reducing agent at optimized conditions to achieve the maximum labeling efficiency (>95%). It forms stable complex with ^{99m}Tc with high radiochemical purity (98%). K_D was found to be 2.21. ± 0.07 by receptor ligand assay on human tumor cell line KB. Cytotoxicity was determined by MTT assay, and at IC50 flow cytometry and DNA fragmentation was performed on KB cell line. Blood kinetic study showed a quick wash out from the circulation with biphasic pattern showing more than 70% clearance within 10 minutes. The KB cell line tumors in mice were readily identifiable in the γ images and revealed major accumulation of

radiotracer at the tumor site. The ^{99m}Tc-Folate-Cytrabine conjugate have promising utility as a receptor specific radiopharmaceutical for imaging and therapy of neoplastic tissues known to over express folate-binding protein.

Key words: *Folate receptor, Cytarabine, Drug delivery*

I.INTRODUCTION

The days of indiscriminate drug delivery into healthy and pathologic tissues alike are probably near an end. With regulatory approvals of drugs such as Herceptin (trastuzumab) for the treatment of metastatic breast cancer and Gleevec (imatinib mesylate) for the treatment of chronic myelogenous leukemia, a new class of therapeutics, based on selective targeting to pathologic components, has been clinically validated. Within this growing class of targeted pharmaceuticals, folate-drug conjugates constitute a well studied example of a distinct subclass of receptor targeted therapeutics (1).

The exploitation of folate receptor (FR)-mediated drug delivery has been referred to as a molecular Trojan horse approach whereby drugs attached to folate are shuttled inside a targeted FR-positive cell in a stealth-like fashion**.** Tumour-cell-membrane-associated (TCMA) folate receptor is an interesting molecular target for selective targeted delivery of drugs (2, 3). Evidence on the over-expression of folate receptor in a wide variety of tumours, including breast, cervical, colorectal, renal, nasopharyngeal and ovarian cancers than in normal tissues (4), has prompted search for promising folate-conjugated radiometal chelates as potential imaging agents. Conjugates of folic acid are preferred targeting ligands to radiolabel monoclonal antibody against folate receptors mainly due to their small size, lack of immunogenicity, and rapid clearance from the blood stream and well-defined simple conjugation chemistry. Most of the Folic acid conjugates have been shown to bind the folate receptors with high affinity and are taken up by tumour cells via folate receptor mediated endocytosis (5, 6). Folate-conjugation, thus presents a useful method for receptor-mediated drug delivery into receptor-positive tumour cells.

Folate is important for survival of all dividing cells since it is essential for the synthesis of a number of amino acids as well as nucleic acids (7). Folate absorption into cells involves transport of a small ionic molecule across lipid membranes. This is accomplished principally by an anion transporter (8, 9). Folate transporters typically have a low affinity towards folate (Kd in the range of Am– mM). Aprobenecid-sensitive transporter has been shown to mediate folate absorption at the plasma membrane and possibly in endocytic compartments. While folate transport into the cytoplasm is ultimately mediated by a transporter-based mechanism, membrane folate receptors (FRs) that bind folate at nanomolar affinities will certainly increase membrane proximal concentrations of folates in the vicinity of the transporters (10). This is likely to substantially enhance the efficiency of delivery of folates into cells under physiological concentrations (low nanomolar) of this molecule.

The human membrane FR has three isoforms, the α- and β-isoforms are glycosylphosphatidylinositol (GPI)-anchored whereas the γ isoform is a soluble protein (11). FRs are expressed in a limited number of normal tissues but is overexpressed in a large number of epithelial malignancies. FR-a synthesis, at least in part, appears to be under endogenous folate and hormonal control in some cells, but much less is known about the control of function; mice lacking specific FRs show developmental abnormalities (12, 13). FR-α and β are expressed in placental tissue (14). FR isoforms are expressed in normal tissues like lungs, thyroid, kidney, choroids plexus (15) and placenta (11). FR- α is also expressed in the buccal carcinoma cell line, KB cells (14). The FR is often used as a diagnostic marker of various cancerous cell types, being present in predominant cancers like ovarian cancer and brain tumors (16, 17).

Initial folate targeting studies were conducted with radiolabeled and fluorescent proteins covalently attached to folic acid (18). To date, however, folate conjugates of radiopharmaceutical agents (19-21), MRI contrast agents (22), low molecular weight chemotherapeutic agents [13–17], antisense oligonucleotides and ribozymes (23-25), proteins and protein toxins (18, 26, 27), immuno-therapeutic agents (28), liposomes with entrapped drugs (29, 30), drugloaded nanoparticles (31), and plasmids (32) have all been successfully delivered to FR-expressing cancer cells. The expression of FR isoforms as well as folate transporters in carcinomas makes it an attractive anticancer target.

Cytarabine is one of the most effective anticancer agents used for a wide variety of proliferating mammalian cells in culture. Cytarabine is an antimetabolite, which are very similar to normal substances within the cell. When the cells incorporate these substances into the cellular metabolism, they are unable to divide. Anti-metabolites masquerade as purine or pyrimidine - which become the building blocks of DNA. They prevent these substances becoming incorporated in to DNA during the "S" phase (of the cell cycle), stopping normal development and division from the G1 phase to the S-phase. Cytarabine is metabolized intracellularly into its active triphosphate form (cytosine arabinoside triphosphate). This metabolite then damages DNA by multiple mechanisms, including the inhibition of alpha-DNA polymerase, inhibition of DNA repair through an effect on beta-DNA polymerase, and incorporation into DNA. Cytotoxicity is highly specific for the S phase of the cell cycle. The narrow therapeutic index, high volume of distribution and poor tissue specificity requires cytarabine to be delivered as a conjugate with biological vector for the specific action (33, 34).

The FR is a known biomarker protein that’s expressed in high quantities by many primary and metastatic cancers, but not on most normal cells. Recognizing this attribute, we have focused our efforts on the development of small molecule targeting systems, based on FA, to deliver covalently-linked therapeutic agents to cells that express the FR.In view of this we carried out the present study by conjugation folic acid to neoplastic agent, Cytarabine, for its targeted delivery for enhancing the therapeutic value for targeted drug delivery system.

II.MATERIALS AND METHOD

Chemicals: Cytarabine was a kind gift from Baroda University, Folic acid, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), dimethylsulfoxide (DMSO), ethanol, acetonitrile, stannous chloride dihydrated ($SnCl_2.2H_2O$), RNase-A, propidium iodide, were purchased from Sigma-Aldrich Co. ^{99m}Tc was procured from Regional Center for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India.

Cell Culture: Monolayer cultures of human oral carcinoma KB cells were maintained at 37^0C in a humidified CO_2 incubator (5% CO_2, 95% air) in DMEM (Sigma, USA) supplemented with 10% foetal calf serum (GIBCO, USA), 50 U/ml penicillin, 50µg/ml streptomycin sulfate and 2µg/ml nystatin. Cells were routinely subcultured twice a week using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA.

Instrumentation: The ^1H NMR spectra were determined by using Bruker Spectro spin. Tetramethylsilane was used as internal standard. Mass spectrum (FAB^+MS) was recorded on a JEOL SX 102/DA-6000 Mass spectrometer using m-nitrobenzyl alcohol (NBA) as the matrix. HPLC analyses were performed on a Waters Chromatograph efficient with 600s coupled to a Waters 2487 photodiode array UV detector. The C-18 RP Beckman column (5µ, 1mm x 1.25cm) and shimadzu ODS-AQ (5µ, 4.6 mm x 250 mm) column were used applying elution system described in the text. The receptor binding data were analysed using iterative curve-fitting program EQUILIBRATE software from graphpad. Radioimaging and biodistribution studies were done using a planner gamma camera fitted with parallel collimator (ECIL, INDIA) and γ-scintillation counter (GRS230, ECIL). Cell cycle progression was studied by FACS Caliber (Becton-Dickinson & Co., USA) flow-cytometer using the Cell Quest (version 3.0.1; Becton-Dickinson & Co., USA) and Mod fit LT (version 2.0; verify software House, Inc., USA) softwares for acquisition and analysis

Animal Models: Animal protocols have been approved by Institutional Animal ethics Committee. New Zealand Rabbits, athymic mice, albino Balb/c mice were used for blood clearance, imaging and biodistribution. Mice and rabbits were housed under conditions of controlled temperature of 22 ± 2^0C and normal diet. Athymic mice were inoculated subcutaneous with 0.1 ml of cell suspension (1×10^6 KB cells) into the right thigh under sterilized conditions.

Synthesis of Folic acid conjugated Cytarabine (Fol-Cyt)

Radiochemical synthesis of [^{99m}Tc]-Cyt-Fol.

Conjugate Cyt-Fol (2mg) was dissolved in a shielded vial and stannous chloride (30µl; 1mg dissolved in N_2 purged 1ml 10 % acetic acid) was added followed by addition of freshly eluted (< 1 h old) ^{99m}Technetium pertechnetate (82 MBq; 200µl). The pH of the reaction mixture was adjusted to 7 with 0.1 M Na_2CO_3 and purged with N_2, shaken to mix. The vial was allowed to stand for 20 minutes at room temperature (25^{o}C).

Radiochemical purity of ^{99m}Tc-Cyt-Fol conjugate

The number of ligand molecules involved in complexation with ^{99m}Tc was determined by ascending thin layer chromatography on ITLC-SG (Paul Gelman, USA) strips using 100% acetone as developing solvent and simultaneously in pyridine: acetic acid: water (PAW) (3 : 5 : 1.5) and saline. Each TLC was cut in 0.5-cm segments and counts of each segment were taken. By using this method percentage of free $Na^{99m}TcO_4$-, reduced ^{99m}Tc and the complex formed between ^{99m}Tc and folate conjugate could be calculated. ^{99m}Tc-Cyt-Fol conjugate remained at the origin and free technetium traveled with the solvent front in acetone.

The radiolabeled drug was injected on HPLC using solvent gradient (eluant A, 10mM ammonium acetate buffer at pH 8; eluant B, acetonitrile gradient, 0 min at 4% B, 10min at 12% B and 15min 15% B at a flow rate of 0.5ml/min) and fractions were collected and radioactivity was counted on automated gamma counter.

Human serum stability evaluation

Human serum was prepared by allowing blood collected from healthy volunteers to clot for 1h at 37°C in a humidified incubator maintained at 5 % carbon dioxide, 95 % air. Then the samples were centrifuged at 400g and the serum was filtered through 0.22-micron syringe filter into sterile plastic culture tubes. The radiolabeled Cyt-folate and folate serum samples were immediately placed in a CO_2 chamber incubated at 37°C and then analyzed to check for any dissociation of the complex. Percentage of free pertechnetate at a particular time point that was estimated using acetone and pyridine, acetic acid and water (PAW) (3: 5: 1.5) as mobile phase, represented percentage dissociation of the complex at that particular time point in serum.

Receptor Binding Studies

The specificity of folate conjugates to bind to cell surface receptors on tumor cells were examined by receptor binding assays on human mouth and nasopharyngeal cell line (KB) grown in normal DMEM (10% serum, 10 µM folic acid). Monolayer culture of the cell line was washed with acidic phosphate buffer saline (pH 3.5) to remove endogenous folates and excess free folate present in the culture medium. The cell line cultures were then incubated for 40 min with ^{99m}Tc-Cyt-folate at 37°C in HBSS containing various concentrations (0.001 µM – 20 µM) in the absence and presence of the 100 folds excess unlabeled folic acid for estimation of total binding and non-specific binding respectively. Specific binding was obtained by subtracting non-specific binding from total

binding. At the end of each experiment, the cells were washed with cold PBS and 0.9% saline 7-8 times. The cell-associated radioactivity was determined by γ- scintillation counting. Scatchard plot analysis was done using EQUILIBRATE software from graph pad.

Cytotoxicity of Fol-Cyt

Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Exponentially growing cells were plated in a 96-well microtitre plate at a uniform cell density of 10,000 cells/well 24 h before treatment. Cells were treated with varying concentrations of the conjugate **(**mM– µM range) for various time intervals viz., 24 h, 48 h, 72 h and MTT assays were performed. At the end of treatment, negative control and treated cells were incubated with MTT at a final concentration of 0.05 mg/mL for 2 h at 37°C and the medium was removed. The cells were lysed and the formazan crystals were dissolved using 150 µL of DMSO. Optical density is measured on 150 µl of extracts at 570 nm (reference filter: 630 nm). Mitochondrial activity was expressed as percentage of viability compared to negative control (mean +/- SD of triplicate cultures). % of viability = [OD (570 nm - 630 nm) test product / OD (570 nm - 630 nm) negative control] x 100.

Macrocolony Assay

Monolayer cultures of KB cell lines were trypsinized and 100 to1000 cells were plated depending upon the concentrations of the drug in 60 mm petridishes and incubated at 37°C in 5% CO_2 humidified atmosphere for 8 days. Colonies were fixed in methanol and stained with 1% crystal violet. Colonies containing more than 50 cells were counted.

DNA Ladder

The qualitative damage to genomic DNA estimated by agarose gel electrophoresis. After drug treatment, cells were washed twice with PBS. The cells were lysed by adding lysis buffer(1% NP40 in 20mM EDTA, 50mM Tris.Hcl pH7.8) for 10 min on ice and centrifuged at 10,000X g for 10 min to obtain supernatants of DNA fragmented fractions. They were then incubated for 1 hr with RNase A (final Conc. 10µg/ml) at 37^{o}C, before being digested for 30 min with proteinase K(final Conc. 100µg/ml) at 56^{o}C to obtain cell lysate. DNA was precipitated with 96% ethanol for 24 hrs at -80^{o}C. DNA samples were electrophoresed on 1.4% agarose gel containing ethydium bromide (final Conc. 0.16µg/ml). Visualized under UV light and photographed.

Flow Cytometry

A quantitative measure of cell cycle distribution was obtained by flow-cytometric analysis of DNA histograms. At 24 h treated cells (at IC50 Conc.) with appropriate controls were detached by trypsinization and fixed in 70% chilled ethanol and stored atleast overnight at -4-8° C. Following fixation cells were washed twice with PBS,

and incubated with 200 µg/ml RNase-A for 30 min at 37° C, and stained using 50 µg/ml propidium iodide in PBS at 4° C for 30 min at a density of 0.5 million cells/ml. Cell cycle distribution was studied with the help of FACS Caliber (Becton-Dickinson & Co., USA) flow-cytometer using the Cell Quest (version 3.0.1; Becton-Dickinson & Co., USA) and Mod fit LT (version 2.0; verify software House, Inc., USA) softwares for acquisition and analysis. A minimum of 10,000 cells per sample was analyzed.

Tumor Transplantation

The inbred Swiss albino strain 'A' female mice (10-12 weeks) used in these studies were obtained from the Institute's central animal facility and weighed 20-25g at the time of tumor implantation. The Ehrlich ascites tumor (EAT) cells obtained from Institute for Biophysics, University of Frankfrut, Germany were maintained by serial passage of tumor cell suspension in the peritoneal cavity of strain 'A' mice (1). Subcutaneous tumors were implanted by intra-muscular injection of $15*10^6$ cells (in 0.1-0.15 ml volume) into right hind leg. Tumor volume was calculated using the formula: $V= \pi/6 (d_1 * d_2 * d_3)$, where d_1, d_2 and d_3 are the three orthogonal diameters measured with the help of a caliper. Experiments were performed when the tumor had attained a volume of 300-400 mm3 (5-6 days after implantation). Animals were sacrificed when the tumor reached a volume of approximately 5000 mm3 to avoid tumor burden related discomfort to the animal as per the UKCCCR guidelines for the welfare of animals in experimental neoplasia (2). All experiments were conducted according to the guidelines established by CPCEA, Indian National Science Academy (INSA) (3) and European Society of animal handling (4) after obtaining the permission from the institute animal ethics committee.

Tumor Regression

Mice bearing subcutaneous tumor were treated with the conjugate intravenously through the tail vein. The injection volume of the the treatment were prepared in normal saline was 0.1-0.15 ml.then animals were kept under observation, tumor volume was measured ar every alternate day using vernier caliper. Tumor volume was calculated using the formula : $V= \pi/6 (d_1 * d_2 * d_3)$, where d_1, d_2 and d_3 are the three orthogonal diameters measured with the help of a calipers.

Blood kinetics

In normal rabbit, 300µl of the complex Cyt-Fol (11.1MBq activity) was injected intravenously through the dorsal ear vein. Blood was with- drawn from the other ear vein at different time intervals starting from 5min to 24 h. Persistence of activity in the circulation was calculated, assuming total blood volume as 7% of the body weight.

Scintigraphy in tumor bearing nude mice

Tumor imaging was performed in KB cell line implanted tumor bearing nude mice administering 100 µL of the labeled conjugate Cyt-Fol((40 µg, 2.96 MBq activity). Images were taken using planar γ-camera equipped with pinhole collimator. Images were obtained at different time intervals starting from 15 min to 24 h after post injection.

Biodistribution in KB cell line implanted tumor bearing nude mice

Human oral carcinoma KB cell line that over expresses the folate receptor was injected subcutaneous in the thigh of the right hind leg of nude mice. When the tumors were easily palpable and approximately of 0.17g, mice were used for biodistribution study. An intravenous injection of ^{99m}Tc-Cyt-Fol conjugate in a volume of 100µL (3.7MBq activity) was injected through the tail vein of each mice. Mice were dissected at 1 h, 4 h and 24 h post injection; different tissues were taken out, weighed and counted in a γ- counter calibrated for ^{99m}Tc energy. Uptake of the radiotracer in each tissue was calculated and expressed as percentage injected dose per gram of the tissue (%ID/g) (Table 1). Institutional Animal Ethics Committee has approved animal protocols.

Result

Synthesis of Fol-Cyt.

The synthesis of the bioligand (Fol-Cyt) was done by performing the reaction between the activated –COOH of Folic acid to the –NH2 of the cytarabine. Fig. represents the chemical scheme of the conjugation between the two. The final product (Fol-Cyt) was obtained in 75% yield, purified using HPLC and Characterized by ESI$^+$MS and NMR.

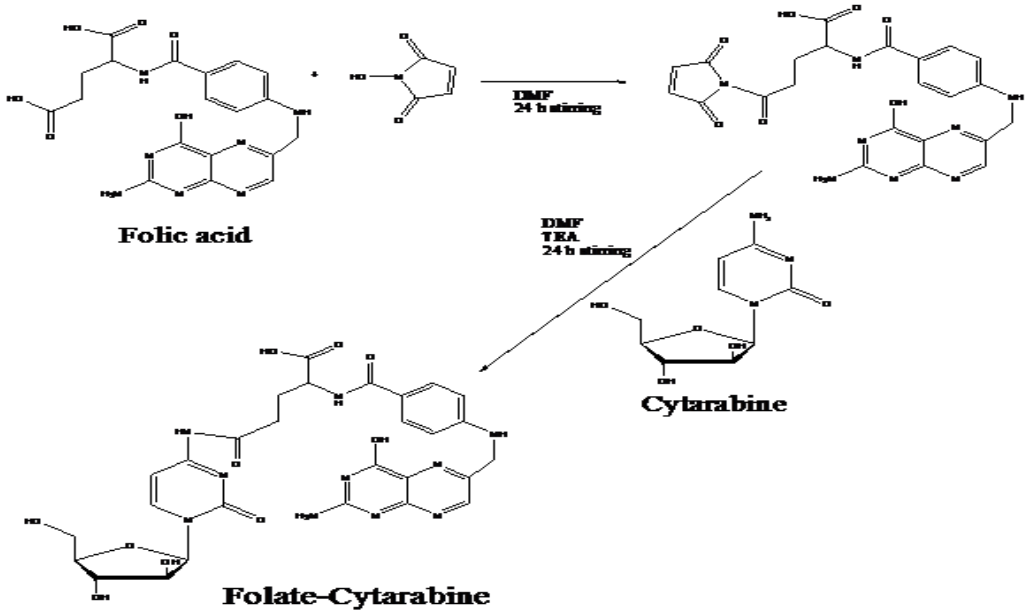

Figure 1: Chemical Scheme for Conjugation of Cytarabine to activated Folic Acid.

Quality control of labeled folate conjugate

The labeled complex remained at the point of spotting and $^{99m}TcO_4^-$ moved towards the solvent front in acetone. The yield of free and complexed ligand, thus, could be estimated. The labeling yield was found to be greater than 95%, as determined by different solvent systems chromatographically. The reaction mixture kept in saline for various time intervals carried out in *in vitro* stability studies. Percentage radiolabeling was calculated for 0, 2, 4, 6 and 24 h. Even up to 24 h labeling efficiency was found to be 95.67% implying that the labeled folate conjugate was relatively more stable as compared to unmodified labeled folic acid.

Human serum stability evaluation

The folate conjugate was successfully labeled with ^{99m}Technetium with more than 97% labeling efficiency as determined by instant thin layer chromatography using different solvent systems. It was sufficiently stable up to 24 h as only 3% of the radiolabeled drug dissociated in serum at 24 h (Fig.2).

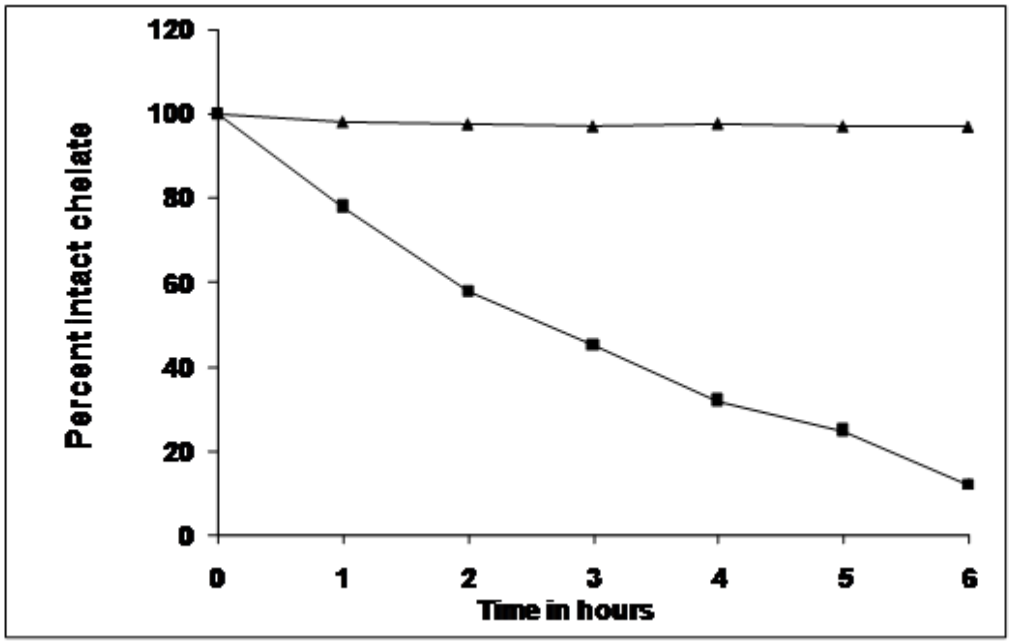

Figure 2: In-vitro human Serum stability study of ^{99m}Tc-Fol-Cyt (▲) and ^{99m}Tc-Fol (■) under physiological conditions.

Receptor Binding

The ability of folate conjugate to bind FRs on the surface of tumor cell line, KB, was examined by saturation binding assay-using ^{99m}Tc-Fol-Cyt as the labeled ligand. Non-specific binding was determined using 100-fold excess of unlabeled folic acid. Examination of binding curves showed storable binding of the labeled folate conjugate. The half maximal binding was found to be 1.5 μM (Fig.3). Fig.4 shows the Scatchard plot analysis,

revealing the affinity of the labeled conjugate on KB tumor cell line. K_D was found to be 3.46 ± 0.02 (mean ± S.E.).

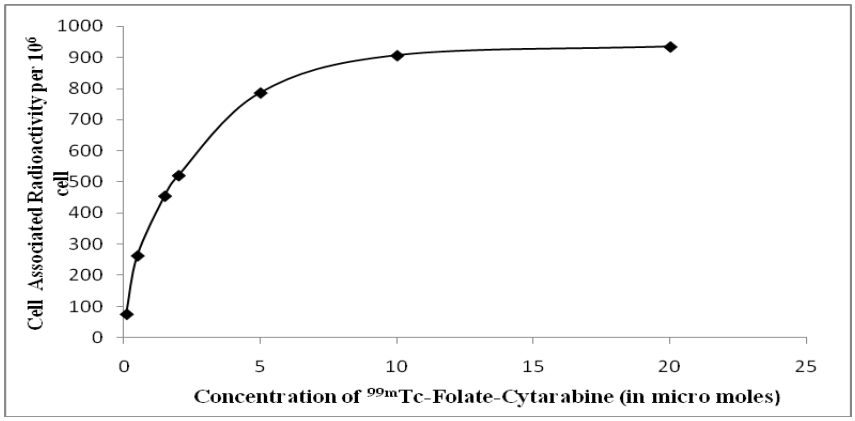

Figure 3: Saturation Binding Assay: Cell Associated $_{99m}$Tc radioactivity per 106 cells following incubation of KB cells with increasing conc. of ^{99m}Tc-Fol-Cyt.Saturation Binding Assay

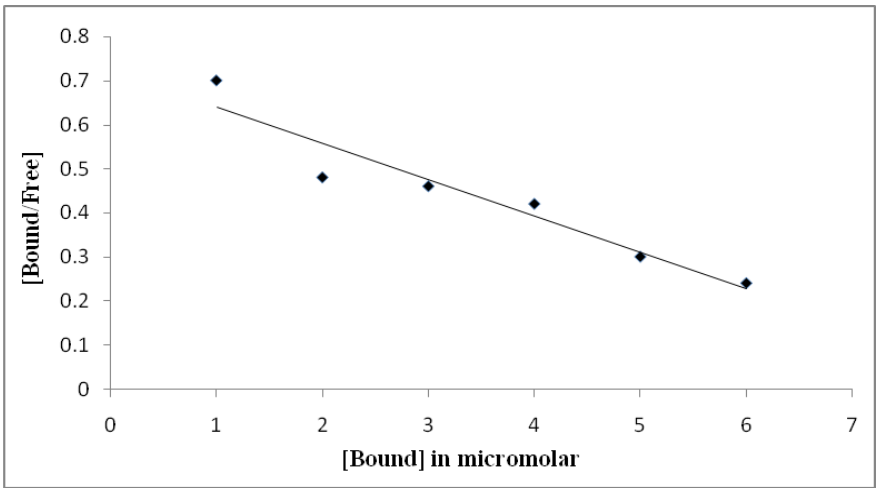

Figure 4: Scatchard plot analysis for Receptor Binding Assay for KB cell line for specific binding to the ratio of bound to free (B/F).

MTT Assay

Metabolic activity was estimated using MTT assay. The surviving fractions of Fol-Cyt were evaluated to determine its dose responsive properties by mitochondrial apoptotic assay. The result determined an enhanced activity which was to be concentration dependent with an IC50 of 10 µM when KB cells were incubated for 24 h for Fol-Cyt. The cytarabine alone could kill 50% of the cells at 100 µM at 24 h (Fig.5). This clearly reveals that the conjugate of cytarabine with folate has a better activity showing effective result at a lower concentration which is 10 folds.

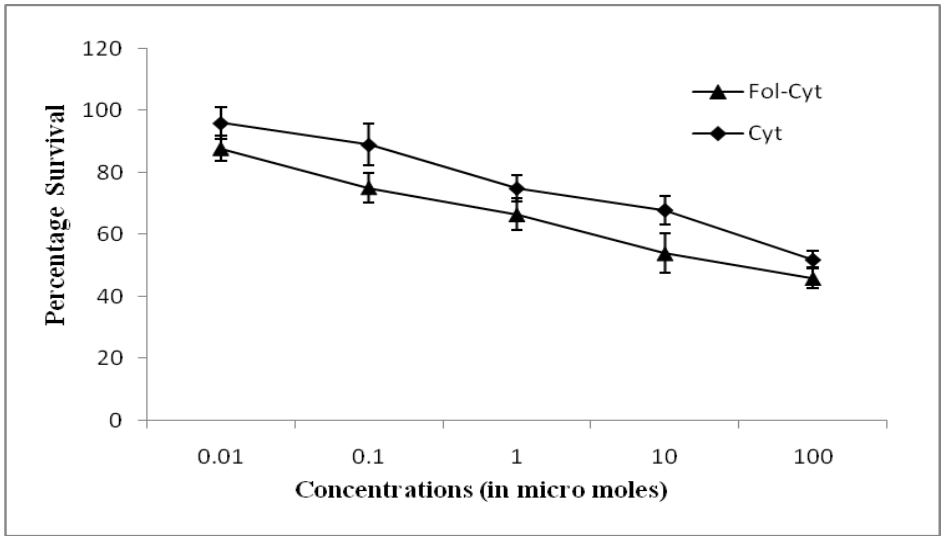

Figure 5: Colorimetric estimation of the mitochondrial activity for cytotoxicity of Fol-Cyt and Fol (MTT assay) at different conc.

Macrocolony

Macrocolony assay carried out in KB cell line, which showed that Fol-Cyt and Cytarabine showed cytotoxicity. Cell survival curves were generated (not shown) and survival fractions were determined shown in Fig.6. The results of macrocolony and MTT shows correlation with each another.

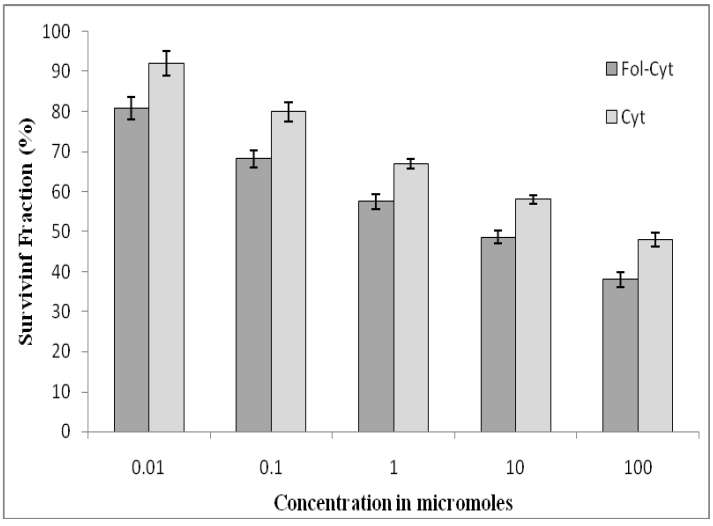

Figure 6: Cytotoxicity estimated by Macrocolony assay for Fol-Cyt and Fol at different conc.

DNA Ladder Assay

A biochemical hallmark of apoptosis is the characteristic degradation of the genomic DNA by cleavage at the inter nucleosomal sites, generating a 'ladder' of DNA fragments, which can be detected by agarose gel electrophoresis. Apoptosis caused by the conjugated was also tested by performing the ladder assay on KB cells. At IC50 conc. (24 h & 12 h) the KB cells were incubated and the DNA samples were analyzed by 1.5% agarose gel in TBE buffer and visualized by UV-ethidium bromide method. The intensity of apoptotic bands increased with dose dependent-manner. The intensity of apoptotic bands revealed the level of apoptosis induction (Fig.7)

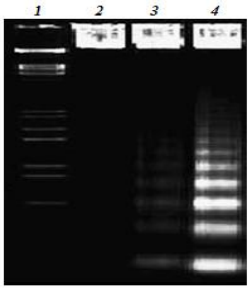

Figure 7: DNA fragmentation induced by Fol-Cyt. Agarose gel electrophoresis showing DNA fragmentation indicative of cell apoptosis induced by Fol-Cyt.
Lane 1: marker; Lane 2: Control; Lane 3: 12 h, Fol-Cyt. (IC50 conc.) Lane 4: 24 h, Fol-Cyt. (IC50 conc.)

Cell Cycle Analysis

Exponentially growing KB cells were treated with different doses of Fol-Cyt and at IC50 and cells were evaluated. Effect of Fol-Cyt induced alterations in the cell cycle progression was studied, thereafter. Fig. 8 depicts the effect of IC50 concentration of Fol-Cyt at 12 h (b) and 24 h (c). At 12 h, a notable percentage of apoptotic cells were observed in comparison to untreated control (a). At the same time point, in comparison to the control group, an increment in the percentage of KB cells in G2 phase cells was also noticed. When cells were allowed to grow in the presence of IC50 concentration of Fol-Cyt upto 24 h, a significant increase in the percentage of apoptotic cells was found in comparison to both untreated control group as well as 12 h treated group. The percentage of G2 phase cells was also found to increase significantly.

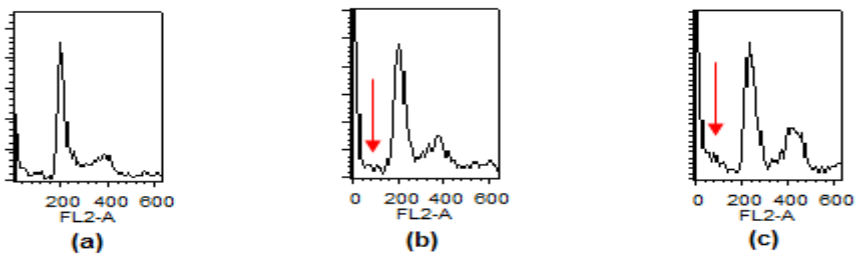

Figure 8: Histograms of Flow Cytometry at IC50 conc. on KB cells at 12 h (b) and 24 h (c) showing apoptosis. (a) is untreated cells (control).

Tumor Regression

In vivo tumor growth inhibition activity of the folate conjugated cytarabine (Fol-Cyt) was evaluated against EAT tumor grafted in Swiss albino strain 'A' mice. The initial growth of tumors in untreated controls was exponential in nature. After the treatment of the drug at successive alternate days, it was observed that the total tumor volume started declining as compared to the control volume. The treated animals survived for six days more. Because only a fraction of Fol-Cyt conjugate can be internalized via the targeted receptor, it is suggested that improved transport or penetration is the reason why conjugate exhibit significantly better cytotoxicity. The conjugate is selective active to the cancer cells expressing the FR. The conjugate showed limited efficacy in reducing tumor growth although it was better than cytrabine alone (Fig.9)

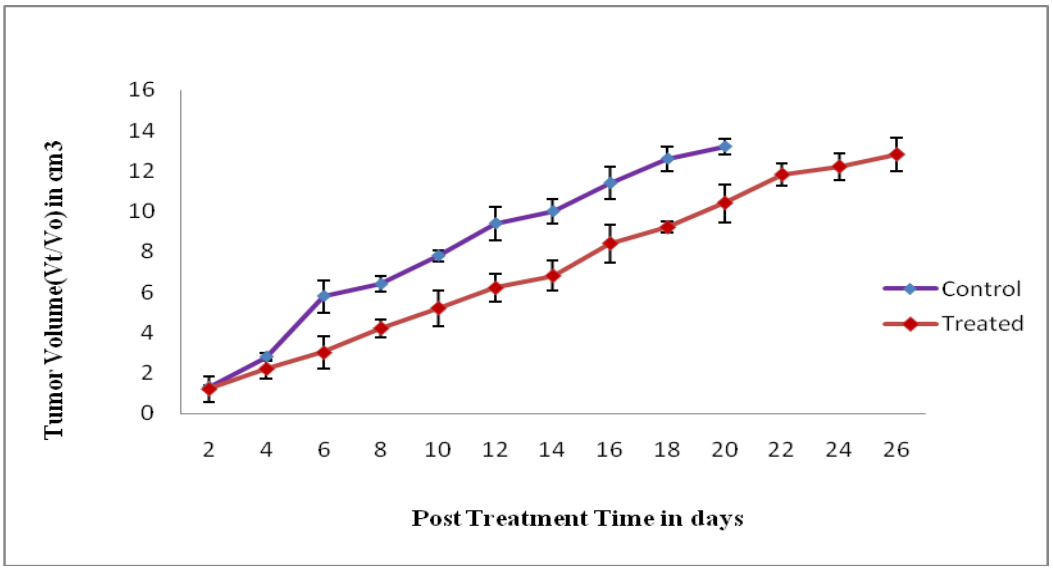

Figure 9: Effect of Fol-Cyt on EAT implanted tumor in Swiss albino strain 'A' mice.

Pharmacokinetics of Fol-Cyt.

In vivo clearance in rabbits revealed that there was a rapid wash out of the conjugate from the circulation as only 15.48% of injected activity remained in the circulation at 30 min. After 30 min the clearance followed a slow pattern and at 24 h approximately 2 % activity persisted in the blood (Fig.10). The biological half-life was found to be $t_{1/2}$ (Fast), 12 min; $t_{1/2}$ (Slow), 2 h and 20 min.

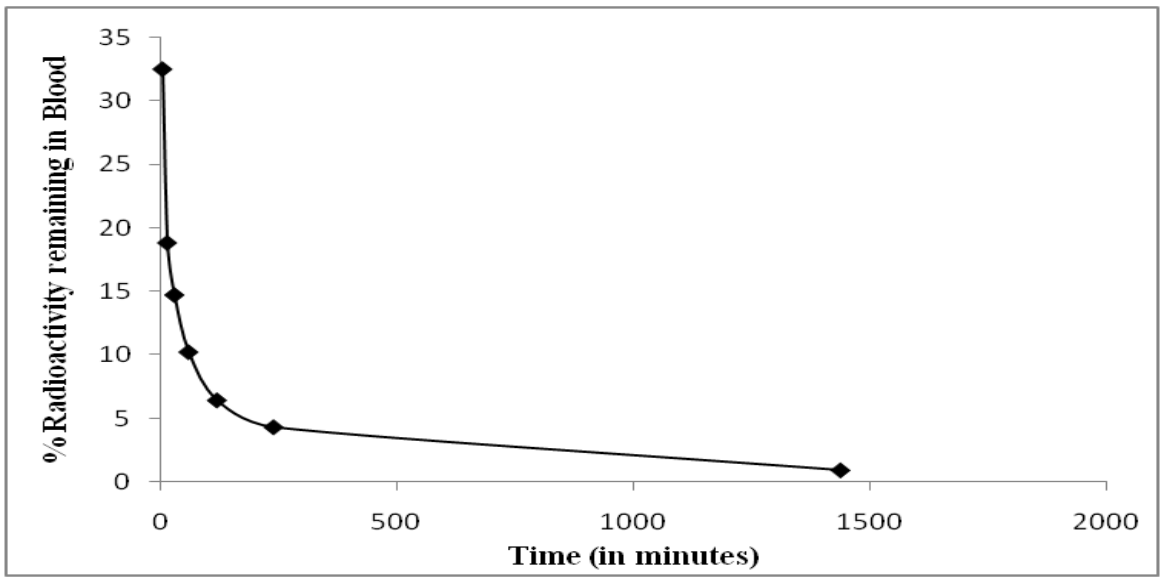

Figure 10: Blood Clearance of ^{99m}Tc-Fol-Cyt (11.1 MBq activity) administered through ear vein in normal rabbit.

Scintigraphy

The ability of the labeled conjugate to target receptors in vivo appears to be demonstrated in a preliminary imaging study. Localization of ^{99m}Tc labelled Fol-Cyt in a single BALB/c mouse bearing an EAT tumor over time, as determined by gamma camera imaging, is presented in Fig.11 at 2 h. Imaging was carried out at different time intervals after administering labeled conjugate intravenously. The mice depicted the beginning of accumulation of activity in tumor at 30 min, which reached to maximum at 2 hour accumulating in tumor. While radiotracer uptake is also apparent in the kidneys, the tumor/background tissue contrast is otherwise quite good.

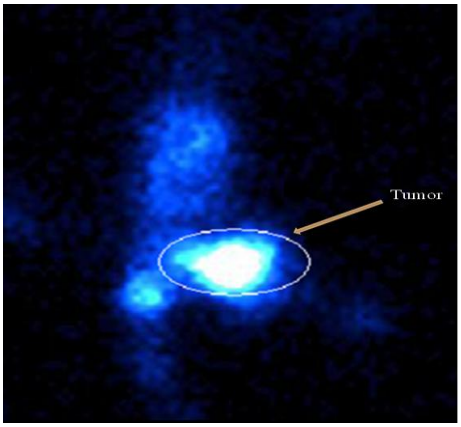

Figure11: Whole body γ-image of BALB/c mice with subcutaneous folate receptor positive tumor in right thigh after 2 h following intravenous administration of ^{99m}Tc-Fol-Cyt.

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Biodistribution

Following intravenous administration to mice, the conjugate ^{99m}Tc-Fol-Cyt, was found to be efficiently cleared from the blood and primarily excreted into the urine. Only 4.42 ± 0.02 % of the injected dose was in the intestines at 4 h post-injection. Athymic nude mice implanted with human KB cell line exhibited major accumulation in liver (18.5%/g) followed by kidneys (15.0%/g) at 2 h showing that the complex is excreted both by hepato-biliary as well as renal routes. Fig.12 shows high tumor accumulation of 4.52 ± 0.12 %ID/g at 2 h that remains high 5.8 ± 1.02 up to 4 h post injection was observed. This uptake was significantly blocked by (>90%) in the animals receiving a high dose of cold folic acid together with the radiolabeled drug (Fig.13). As the background activity gradually cleared from the body, there was increase in target-to-non target ratios.

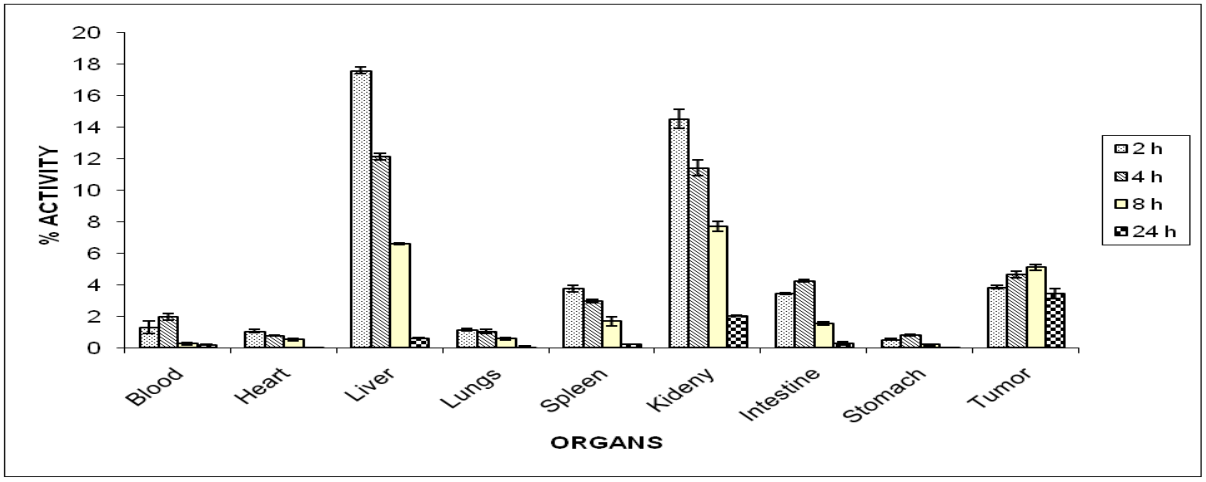

Figure 12: Biodistribution of 99mTc-Fol-Cyt in Balb/c mice with EAT cell tumor implant.

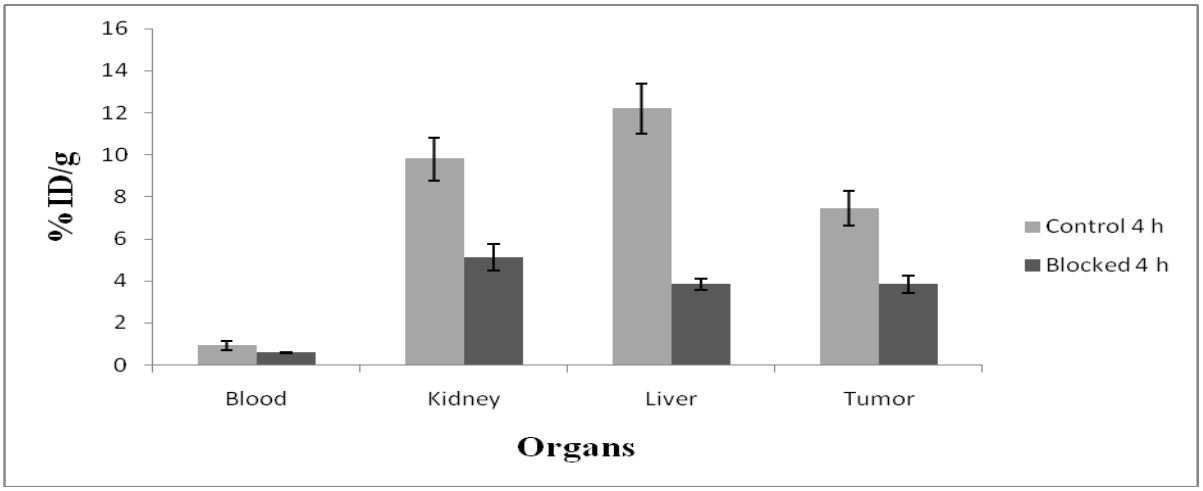

Figure 13: ^{99m}Tc-Fol-Cyt administered through ear vein in EAT grafted Balb/c mice with and without a co-administration of blocking dose at Blood, Kidney, Liver and Tumor site.

III.DISCUSSION

Targeted drug delivery systems promise to expand the therapeutic windows of drugs by increasing delivery to the target tissue as well as the target–non -target tissue ratio. This in turn lead to a reduction in the minimum effective dose of the drug and the accompanying drug toxicity, and an improvement in therapeutic efficacy at equivalent plasma concentrations. Given the often limited number of targeted receptor sites on any given target tissue, targeted delivery is a particularly attractive approach for agents with narrow therapeutic windows and/or are active at very low concentrations. The receptor for vitamin folate has been identified as a marker for ovarian carcinomas and has also been found to be frequently overexpressed in a wide range of other types of tumors, therefore, presents an attractive target for tumor- selective drug delivery.

The toxic side effects associated with the administration of anticancer drugs makes them ideal candidates for site specific delivery. Most small molecule chemotherapeutic agents have a large volume of distribution on intra venous administration which often leads to a narrow therapeutic index due to a high level of toxicity in healthy tissues. Through conjugation of drugs with biological vectors, such as a folic acid, monoclonal antibody, biotin, amino acids etc., the volume of distribution is significantly reduced which results in decreased nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor site. Knowing that folate conjugation enables a drug molecule to target and become endocytosed into FR expressing tumor cells, we initiated a project to construct and evaluate the activities of folate conjugated cytarabine.

The synthesized bioligand yield was 75% which was purified using HPLC which is used for targeted therapeutic applications to folate positive tumors. In vitro human serum stability under physiological conditions suggested that there was least transcomplexation of the labeled bioconjugates. In drug delivery, the balance between the hydrophilic and lipophilic nature has shown to be a major factor in determinig the rate extent of drug absorption. As the drug on labeling is highly lipophilic in nature so the rate of transfer of the drug molecule across biological membranes and to the target site is very rapid.

The fact that labeled folate conjugate is in direct competition with the folate present in the medium, though the endogenous folate was removed by acidic wash, may bring about intriguing possibility of modulating the effectiveness of folate conjugate binding to folate receptor. The amount of receptor activity increases markedly when cells are depleted of folate through growth in folate-depleted medium (35). The alpha carboxylic acid is necessary for receptor binding and the γ-isomer of folate conjugate facilitates the uptake of the modified folic acid into folate receptor positive cells (36). The folate–cytarabine conjugate had shown to retain high specific affinity to the FR in a competitive binding assay on KB cell line. The specific binding of the conjugate was revealed by the Scatchard plot analysis with Kd 2.21. ± 0.07.

Using an MTT cytotoxicity assay, we have also observed that folate–Cyt is able to specifically deliver Cytarabine into KB cells without harming co-cultured normal cells. This targeted agent Fol-Cyt as tested for the ability to induce cytotoxicity in the cancer cell lines using both an assay of mitochondrial activity (in a MTT assay) and a clonogenic assay. IC50 for Fol-Cyt was 10 µM as compared to 100 µM for fol. A direct correlation was found between MTT assay and clonogenic assay. These results were correlated with the DNA ladder and Flow cytometry analysis, which revealed the same pattern of apoptosis at IC50 for 12 h and 24 h treatment. Tumor regression was performed in EAT cell tumor implanted in Balb/c mice. It revealed that there was a delay in the growth of the tumor which received the treatment of 10µM every second day and the survival was increased by 6 days as compared to the control group. Because only a fraction of Fol-Cyt conjugate can be internalized via the targeted receptor, it is suggested that improved transport or penetration is the reason why conjugate exhibit significantly better cytotoxicity.

The blood kinetic profile radiolabeled compound showed its high target uptake with the diagnostically useful target-to-non target ratio in a short period of time. The labeled drug showed fast clearance that resulted in the decrease in the background activity. The high specificity of Fol-Cyt results in selective uptake and distribution of the radiolabeled receptor ligand at the tissues, which are known to contain a substantial concentration of the target receptor that can be visualized in the high quality images, obtained 2 h after administration of Fol-Cyt in animal models. The biodistribution studies showed radioactive drug persisted in stomach and intestines up to 4 h as the radiolabeled folate-conjugate cleared through hepatobiliary and renal routes.

Collectively, these results imply that the use of folate-drug conjugates may be an effective form of chemotherapy that does not cause unwanted injury to normal tissues, including the FR-positive kidneys. These data also support the hypothesis that the FR-positive kidney proximal tubules function primarily to shuttle scavenged folates or folate conjugates back into systemic circulation (via kidney assisted transcellular reabsorption) rather than use them for localized biological consumption

In conclusion, tumor-selective cytarabine bioconjugates were designed, synthesized, and tested in vitro and in vivo. The folate targeting moiety led to in vivo tumor volume reduction and appears to be important in attaining an overall level of cancer control.

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