Tumor-targeted drug delivery by folate conjugated amphiphiles

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Abstract
A series of amphiphilic conjugates of folic acid and aliphatic fatty acids was designed to serve as targeted drug delivery carriers for anticancer agents. The effectiveness of targeted delivery of amphiphile was evaluated using folate receptor positive HeLa cells and Caco-2 cells that do not express folate receptor. Wild type HeLa cells were found to have 40-fold greater folate receptor - (FRα) expression than Caco-2 cells. Amphiphile uptake was studied by the internalization of 7-amino-4-carboxymethyl coumarin labeled fluorescent amphiphilic conjugate in FRα knockdown HeLa, and Caco-2 cells at 37°C and 4°C, respectively. siRNA specific for FRα was used to knockdown the receptor in HeLa cells by 75% and these modified cells were used as the control in determining the specificity of amphiphiles uptake. Wild type HeLa cells internalized twice as much fluorescent amphiphiles as compared to all other treatment groups at 37°C. Paclitaxel, a lipophilic antitumor agent was used as a model compound to evaluate the efficacy of three homologous series of amphiphile conjugates as targeted carriers in HeLa and Caco-2 cells. The amphiphiles were non-toxic to both cell lines at the concentrations lower than 100µM. Amphiphilic micelle containing paclitaxel exhibited significantly lower IC₅₀ values in HeLa cells when compared to free drug and untargeted amphiphile micelles.

The data from the current studies demonstrated the feasibility of using folic acid conjugated amphiphiles to selectively deliver drugs to FRα positive cancer cells.

Keywords : Folate receptor, targeted delivery, amphiphiles, paclitaxel

Introduction
Treatment using anticancer drugs is limited due to their intolerable side effects. Conventional chemotherapeutic agents kill cells by disrupting the cell division. Their action is non-specific and cause serious damage to even non-tumor cells. An ideal cancer therapy should specifically direct drugs to target cancer cells and kill them preferentially or selectively while avoiding damage to the normal cells (1). One approach to achieving this goal is by targeted drug therapy.

Targeting can be categorized as either passive or active based on the absence or presence of site-directing ligands (2). Passive targeting generally takes advantage of natural anatomical structures or physiological processes to direct drugs to the site of action. Active targeting requires site-directing ligands to bind and interact with target site. Examples of targeted biopharmaceuticals include antibody conjugates and folate-bearing conjugates which utilize ligand-receptor interactions (3).
The folate receptor - (FRα) is a 38.5 kD glycosphatidyl inositol anchored protein with a high affinity \([k_D = 10^{-9} \text{M}]\) for folic acid (4). The folate receptors are diffusely distributed on the cell surface but are functional when concentrated in the caveolae (5). After binding to folic acid, the receptor is internalized by a non-endocytic process and is recycled back to the cell surface after dissociation from the substrate (6-8). The receptor mediated pathway of folate uptake and the expression levels of the receptor itself is regulated by intracellular levels of folic acid (9). The FRα is overexpressed consistently in nonmucinous ovarian carcinomas and tumors of epithelial lineage in endometrium, lung, breast, renal cells and brain metastases (10). Thus the therapeutic advantage of targeting the folate receptors is due to their overexpression, often twenty times more, in these types of malignancies than in epithelial cells or fibroblasts (11). Folate receptor targeting can be achieved with monoclonal antibodies or folate itself (12). Folic acid conjugated radioisotopes have been successfully targeted to ovarian cancer, the second most common gynecologic cancer, for the purposes of prognosis and imaging (13-14). Folate receptor targeting has also been performed by conjugating folic acid to drug delivery systems like liposomes and nanoparticles whereby higher payloads of therapeutic agents could be delivered to the tumor cells (15-18).

Self-assembled amphiphilic molecules in the form of micelles have gained recognition as drug carriers due to their small size, tendency to evade phagocytosis by mononuclear phagocyte system, and prolonged circulation time (19-20). Other advantages of micellar drug carrier include ease of formulation, increased solubility of poorly soluble drugs, enhanced penetration of drug through cell membrane, and passive accumulation in regions of leaky vasculature (19). Drugs encapsulated inside the micelle are protected from inactivation by biological surrounding. Micellar drug carrier can be made to specifically target cancer cells by attaching specific ligands on outer layer of micelles. The shell of the micelle containing folate binding moiety will exhibit enhanced binding, due to multivalent “cluster effect”, to several folate receptors localized in caveolae to help deliver the drug molecule to the site of action (21).

In this study, amphiphiles synthesized using folic acid were used to deliver a model lipophilic anticancer molecule, paclitaxel, to folate receptor expressing HeLa cells. The amphiphile mediated delivery allows a simple formulation approach and may be extended to other lipophilic antitumor molecules intended for folate expressing tumors.

**Materials and Methods**

**Materials**

Dialyzed and heat inactivated fetal bovine serum, folate free Dulbecco’s minimum essential medium (DMEM), chemicals for polyacrylamide gel electrophoresis and alkaline phosphatase coupled rabbit anti goat secondary antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Polyvinylidene difluoride (PVDF) membrane for blotting, Immobilon P, was purchased from Millipore Corp. Goat polyclonal anti human FRα antibody and siRNA for human FRα were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was purchased from Ambion. Bicinchoninic acid protein assay kit and stripping buffer were purchased from Pierce. Spectrofluorimetry was performed on RF5301 Shimadzu spectrofluorimeter (Shimadzu Corp. Japan). A Nikon Eclipse TE 200 epifluorescent microscope (Nikon Corp. Japan) was used in visualization of cells treated with aminocoumarin labeled amphiphiles. The folate labeled amphiphiles and the fluorophore labeled amphiphile were

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synthesized based on the synthetic strategy of Luo et al (Fig. 1) (22).

**Cell culture**

HeLa and Caco-2 cells were maintained in folate free Dulbecco’s minimum essential medium (DMEM) (Sigma) supplemented with 10% fetal calf serum which resulted in a final concentration of 5-methyl-tetrahydrofolate of 9nM (23). The cells were incubated in low folate media at 37°C in 5% CO₂.

**Figure 1.** Chemical structures of different series of amphiphilic conjugates of folic acid with aliphatic carboxylic acids.

Drug delivery by folate conjugated amphiphiles
Western Blot analysis

Cells were lysed in a buffer containing 10mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 2mM EDTA, dithiothreitol (DTT), 10% (v/v) glycerol, 1mg/ml leupeptin and 1mM phenylmethanesulphonylfluoride (PMSF) by four freeze thaw cycles followed by three 10sec pulses of sonication. One hundred micrograms of total protein was resolved on 8% SDS PAGE. The protein blotted on to a polyvinylidene fluoride (PVDF) membrane was first incubated with goat polyclonal anti–human FR antibody overnight at 4°C, followed by incubation for 1 hour with alkaline phosphatase coupled rabbit anti-goat antibody. The Lumiphos substrate was then washed off and the membrane was treated with stripping buffer for 15 minutes at room temperature. The membrane was then probed with mouse monoclonal antibodies to glyceraldehyde phosphate dehydrogenase (GAPDH) followed by goat anti-mouse alkaline phosphatase labeled secondary antibody and detected by autoradiography. The receptor protein signal was normalized with the GAPDH signal from the corresponding lanes.

Folic acid receptor (FR-) knockdown by siRNA

HeLa cells (3 x 10^5) were seeded in each well of a 6-well plate and were grown for 24h. Cells were either transfected with FR- siRNA or scrambled siRNA using Lipofectamine Plus reagent, or left untreated. Cells were harvested after 2 days of growth and folate receptor levels were quantified using Western Blot analysis. The transfected cells were passed into fresh 60 mm plates and the folate receptor levels were quantified after 3 days of growth.

Internalization of fluorescent tagged amphiphiles

Internalization of fluorescent tagged amphiphilic molecule FDACC (Fig. 1) was used to study the uptake of these amphiphilic conjugates into folate receptor overexpressing cells. Wild type HeLa cells, FR- specific siRNA transfected HeLa cells (48h after transfection) and Caco-2 cells were plated in six well plates with 3 x 10^5 cells per well. After 24h of growth in folate depleted DMEM, the cells were washed twice in HBSS and treated with 20 µM of FDACC for 30 minutes. A triplicate set of each cell type was maintained at 37°C and 4°C, respectively. The cells were scraped off the plates and lysed in HEDG buffer with three freeze-thaw cycles. The lysates were read in a spectrofluorimeter with exitation/emission 350/450 nm. Protein content of the lysates was measured by BCA assay and the fluorescence from the treated groups was normalized with their respective protein concentrations. For taking fluorescence microscopic images, HeLa and Caco-2 cells were also grown on acid treated (1N HCl for 1h) cover slips and treated with 20µM FDACC in HBSS for 30 minutes. The cells were then washed twice in HBSS and fixed in 4% formalin. They were then observed under an epifluorescent microscope with UV excitation.

Cytotoxicity studies

In vitro cytotoxicity of the amphiphilic carriers in HeLa and Caco-2 cells was assayed by sulforhodamine B (SRB) assay. In a typical assay procedure 5000 cells are plated in each well of a 96-well plate and incubated for 24h. The cells were then treated with various concentrations of amphiphilic conjugates and further incubated for 48h. The cells were then fixed in 10% trichloroacetic acid for 45 minutes at 4 °C, washed, stained with 0.4% SRB in 1% acetic acid for 30 minutes then rinsed with 1% acetic acid until the washings are colorless. The stained cells are then treated with 200 iL of 10 mM Tris base and the release of cell bound dye was measured at 540 nm in a microplate reader. To evaluate the efficacy of the amphiphilic conjugates in targeting tumor cells, paclitaxel was chosen as a model anticancer agent. In a similar setup as described earlier, the cells were treated
with the drug in a micellar solution of targeted amphiphilic conjugates or treated with the drug in water or treated with the drug in a micellar solution of untargeted amphiphiles. A control group of cells were also grown without any treatment. Percent viability of different treatments relative to control at the end of 48h was calculated using following equation:

\[
\text{Percent Viability} = \frac{\text{Abs. Test} - \text{blank}}{\text{Abs. Control} - \text{blank}} \times 100
\]

where Abs. Test = mean of absorbance from triplicate wells at a single dose
Abs. Control = mean of absorbance from untreated cells

The %viability was plotted with respect to drug concentration and it was fitted to four parameter logistic equation using Sigmaplot® to calculate the drug concentration required for 50% viability in test samples (24).

\[
y = a + \frac{b - a}{1 + 10^{(\log x - \log IC_{50})/d}}
\]

where
a = maximal value of %viability that is attained
b = minimal value of viability% that is attained
d = Hillslope

\[\log IC_{50} = \log \text{of the dose at which 50% of the cells are viable}\]

This concentration, IC_{50}, was taken as a measure of efficacy of the micellar drug delivery system. The IC_{50} parameter calculated from each treatment was then compared to that obtained from free drug treatment and statistical differences between the test and control groups were evaluated by Students t test at an a level of 0.05.

Results and Discussion

Folate receptor - (FRα) expression and knockdown in HeLa cells

FRα expression is known to be dependant on the intracellular levels of folic acid (9). Cells grown in DMEM containing 4 mg/L folic acid often have undetectable level of FRα expression (25). Thus HeLa cells were cultured in folate-free DMEM supplemented with 10% fetal calf serum for three months and the expression level of FRα was compared to Caco-2 cells grown under the same conditions. Level of FRα in HeLa cells was found to be forty times more than the levels in Caco-2 cells (Fig. 2). Therefore, HeLa cells cultured in folate-free DMEM were used for further experiments. HeLa cells were transiently transfected with siRNA specific for FRα with Lipofectamine Plus in OptiMEM for 5h. The transfection medium was replaced with folate-free DMEM and the cells were allowed to grow for 48h. Cells were then passed and assayed for FRα expression by Western Blot analysis after 1 day, 2 days and 3 days of growth. A 75% receptor knockdown was noted following 24h of growth after a single passage of the transfected cells (Fig. 3). In the consecutive time points the receptor knockdown levels decreased to 62% at the end of 3 days of growth after a single passage of the transfected cells (Fig. 3). Lack of significant reduction in GAPDH in the transfected cells suggested that the FRα knockdown in HeLa cells was specific for the receptor.

Cellular uptake of fluorescent labeled amphiphiles

7-Aminocoumarin-4-acetic acid labeled fluorescent amphiphiles FDACC were prepared to study the internalization of the delivery system into target cells. Uptake of the fluorescent labeled amphiphiles was measured at 37 °C and at 4 °C in wild type HeLa cells, FRα knockdown HeLa cells and Caco-2 cells. Perinuclear distribution of the fluorescent probe was observed in wild type HeLa cells (Fig. 4). This observation was
in agreement with the intracellular distribution of FRα as previously shown by Doucette et al (26). The fluorescent tagged amphiphile accumulates more in the HeLa cells than in the Caco-2 cells indicating that the folate receptor targeted drug delivery system can preferentially internalize into folate receptor expressing tumor cells. HeLa cells at 37 °C showed significantly higher amount of fluorescent amphiphile internalization when compared to other groups (Fig. 5). The transport is thus ATP dependant and is expected to slowdown at lower temperatures. In the FRα knockdown cells and Caco-2, small yet significant differences existed between uptake at 37 °C with uptake at 4 °C (Fig. 5). This may be due to a small amount of receptor protein that was present in these cell lines or due to the presence of reduced folate carrier, a trans membrane spanning protein of the solute family class 1 which is often expressed in Caco-2 (27).

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Figure 3. Western blot analysis of siRNA mediated knockdown of FRα in HeLa cells. Each data point represents the mean ± SD of three independent evaluations. The Western blot data shown represents one of the three studies. Lanes 1 and 2 refer to cell lysates from wild type HeLa (HeLa(wt)) and nonsense siRNA transfected HeLa cells (HeLa (NS siRNA)). Lanes 3, 4 and 5 contain cell lysates from FRα specific siRNA transfected HeLa cells at 1 day (HeLa KD 1day), 2 days (HeLa KD 2day), 3 days (HeLa KD 3day) of growth after a single passage following 48h incubation after siRNA transfection. p>0.05 between lanes 1 and 2. p<0.05 between lanes 3,4,5 and 1
Figure 4. Fluorescent micrographs of cells treated with 20µM FDACC showing internalization of the fluorescent labeled amphiphile at the end of 30 mins. incubation at 37°C. - A) wild type HeLa and B) Caco-2

Figure 5. Spectrofluorimetric assay for the amount of FDACC internalized in wild type HeLa, FRα knockdown HeLa and Caco-2 cells at 37°C and at 4°C respectively. For all the groups n=4, p<0.001 between HeLa (37) and all other groups, p<0.05 between treatments at two different temperatures for each cell line.

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Figure 6. Cytotoxicity profiles of drug treatments in wild type HeLa cells with amphiphilic conjugates, with free drug and with untargeted amphiphile (UA). A) FC(n) series B) FDC(n) series C) FPC(n) series D) IC₅₀ values in nM estimated from curve fits of the respective cytotoxicity profiles from each treatment. Each data point in A, B and C is represented as mean±CV% with n=3 and r² > 0.98 for all the fitted curves. In D, p<0.05 between IC₅₀ of free drug treatment or IC₅₀ of drug treatment in presence of UA and IC₅₀ of drug treatment in presence of amphiphilic conjugates. The error bars signify standard error of estimate of IC₅₀ from the curve fit.

Internalization of folate tagged delivery systems is known to follow a caveolar pathway (28). The GPI anchored FR- receptors occur diffusely on the cell membrane but are functional only when associated with caveolae in cholesterol rich domains often referred to as lipid rafts (28). The caveolae form flask shaped invaginations on the cell surface and transform into vesicles when transporting folic acid into the cells (28).

Effect of the targeted delivery system on median lethal dose

The major goal of any tumor targeted delivery system is to achieve cell kill at a lower dose than required for a nontargeted system. It is also important that the delivery system be safe and should not be toxic at the range of concentration that will be used in a delivery system. HeLa cells were used as an in vitro test...
model for tumors expressing folate receptors, whereas, Caco-2 was used as a control. The folate labeled amphiphilic conjugates and the untargeted amphiphiles were tested for their toxicity in HeLa and Caco-2 cells. An average of 20-30% mortality in HeLa and Caco-2 cells were noted after 48h of exposure to 100 μM of any of the folate labeled amphiphilic solutions. Visual observation of the treated cells did not reveal any immediate lysis of the cells within 2-3h of treatment as observed for strong surfactants and detergents (29). HeLa cells, when treated with paclitaxel in a micellar solution of the targeted amphiphiles, exhibited significantly lower IC\textsubscript{50} values than when treated with free drug or with an untargeted amphiphile (Fig. 6 a-d). Similar observations with targeted delivery of anticancer agents have also been demonstrated (30-33). FC(n) series of amphiphilic conjugates resulted in a sixteen fold decrease in the amount of drug required to elicit the IC\textsubscript{50} response as compared with the free drug. Higher homologues of this series of amphiphiles viz FC16 and FC18 could not be used due to their low solubility. Use of FDC(n), FPC(n) and FPLB classes of amphiphiles resulted in an average reduction of IC\textsubscript{50} dose by sixteen, eight and six folds, respectively. Within a homologous series of amphiphiles, an increase in the chain length of the aliphatic acid resulted in a decrease in the IC\textsubscript{50} indicating their involvement in drug permeation into the HeLa cells (Fig. 7). Amphiphilic conjugates with larger aliphatic chain length have lower critical micellation concentration resulting in more number of micelles for the same concentration when compared to one with a smaller aliphatic chain length. For Caco-2 cells the IC\textsubscript{50} of the targeted delivery had no significant difference with the untargeted delivery signifying no contribution of the amphiphiles in intracellular delivery of the drug (Fig. 8 a-d).

![Figure 7](image.png)

**Figure 7.** IC\textsubscript{50} values plotted as a function of size of aliphatic chain length of the amphiphilic conjugates.

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Conclusion

The feasibility of amphiphilic conjugates of folic acid with aliphatic carboxylic acid as micellar carriers for targeted delivery of drugs to tumor cells is demonstrated. The amphiphilic conjugates were actively internalized by a receptor mediated process proving the specificity of this folate tagged delivery system towards the folate overexpressed cells. The amphiphilic conjugates were shown to internalize into the cells and elicited cytotoxic response at a lower concentration and did not show any toxicity within the tested concentration range.

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