Increased ERK activation and cellular drug accumulation in the enhanced cytotoxicity of folate receptor-targeted liposomal carboplatin

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Abstract. Folate receptor-targeted (FRT) liposomes for carboplatin were developed and evaluated in FR-positive and FR-negative cell lines, KB and A549, respectively, for their cytotoxic effects. Significant enhancement in carboplatin potency and intracellular drug accumulation was observed in KB cells when treated with FRT liposomes, compared to free drug and non-targeted liposomes. No enhancement was observed in the FR-negative A549 cells. The increase in carboplatin potency was hypothesized to be associated with an increase in the formation of DNA-platinum adducts resulted from an increase in cellular accumulation of the drug. Surprisingly, FRT carboplatin liposomes showed significantly lower levels of DNA-platinum adducts in comparison to free drug. To elucidate this discrepancy, activation of extracellular signal-regulated protein kinase (ERK) was probed, which has been suggested as an alternative mechanism of carboplatin action. FRT liposomes loaded with carboplatin exhibited the highest level of ERK phosphorylation, and the cytotoxic effect of FRT carboplatin liposomes could be reversed by the MEK/ERK inhibitors, U0126 and PD98059. Importantly, empty FRT liposomes could significantly increase ERK phosphorylation in a concentration-dependent manner without causing toxicity to cells. For the first time, increased potency of carboplatin delivered by FRT liposomes was found to be associated with other molecular targets in addition to DNA-platinum adduct formation. Collectively, the current study suggests a novel mechanism by which FRT liposomes could sensitize cancer cells to drug treatment via modulation of ERK-related cell survival signals.

Introduction

During the past two decades, chemotherapeutic regimens have used platinum (Pt)-based anti-cancer compounds. The cytotoxic effect of Pt-based drugs is thought to be mediated primarily through the formation of DNA-Pt adducts that results in the inhibition of DNA synthesis and subsequent cell death by apoptosis (1,2). Carboplatin is an analogue of cisplatin with improved potency and reduced toxicity (3), and is widely used as a first-line or second-line drug for advanced ovarian cancer of epithelial origin, small cell and non-small cell lung cancer, and less extensively in testicular cancer, head and neck cancer, Ewing's sarcoma, leukemia and brain cancer (4,5). In most cases, carboplatin is the drug of choice over cisplatin due to its markedly reduced toxicity to the kidney and nervous system, yet showing similar cytotoxicity towards cancer cells (3). Despite these advantages, the use of carboplatin remains associated with adverse effects including myelosuppression, anemia, peripheral neuropathy, and stomach pain (6).

Advances in the development of nanoscaled drug delivery systems that are capable of altering the distribution of systemically administered conventional chemotherapeutic agents have resulted in anti-cancer formulations with improved toxicity profiles and/or therapeutic efficacy. Among the various types of drug delivery systems, liposomal delivery is an established platform in the formulation of various anti-cancer chemotherapeutics, giving rise to clinically approved products or those that are in various phases of development (7,8). Given its importance in the delivery of anti-cancer agents, liposomal delivery systems have been developed for the Pt-based compounds (8,9). Among the formulations, SPI-077, which is the PEGylated liposomal cisplatin formulation, was the first to enter early-phase clinical trials; however, this formulation could not succeed due to insufficient release of the drug from the liposome carrier despite high liposomal drug accumulation in the tumour site (10,11). This necessitated the development of liposomal systems which could be specifically internalised by the cancer cells, thereby delivering the drug payload directly to the molecular targets within the cancer cells (12,13).

Intracellular drug delivery was achieved through receptor-mediated endocytosis of liposomes conjugated with targeting ligands which include small chemical moieties, antibodies, antibody fragments and peptides (14,15). Among the targeted liposomal systems, the folate receptor targeted (FRT) liposomes have received much attention owing to their ability to target the FR-overexpressing tumors (16-18). Folate receptors are found to be overexpressed in tumors of epithelial origin but mostly absent in normal tissues (19), and they have emerged as important molecular targets with the recent development of the mono-

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clonal antibody, farletuzumab, which has been demonstrated with clinical efficacy in early phase trials (20,21). Targeting the FR using folate as the targeting ligand on liposomes has several advantages, including small molecular size, chemical and functional stability, and potential lack of immunogenic response (16,22).

In this study, FRT liposomal formulations of carboplatin were developed and evaluated for their cytotoxicity in the established FR-overexpressing KB cancer cells. It is anticipated that an optimised FRT liposomal formulation of carboplatin would exhibit enhanced therapeutic activity in FR overexpressing cancer cells as compared to free carboplatin or PEGylated liposomal carboplatin. This increase in carboplatin potency is hypothesized to be associated with an increase in the formation of DNA-Pt adducts resulted from an increase in cellular accumulation of the drug. Surprisingly, increased potency of carboplatin through FRT liposome delivery was found to be associated with other molecular targets in addition to DNA-Pt adduct formation. For the first time, FRT liposomes have been demonstrated to modulate ERK-related cell survival signalling and sensitized cancer cells to carboplatin.

Materials and methods

Materials. 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{1000}$] (ammonium salt) (DSPE-PEG$_{1000}$) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-$_{2000}$] (ammonium salt) (DSPE-PEG$_{2000}$-folate) were obtained from Avanti Polar Lipid (Alabaster, AL, USA). DilC18 (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate) was obtained from Invitrogen (Carlsbad, CA, USA). U0126 was purchased from Cell Signalling Technology (Beverly, MA, USA) and PD98059 was from Calbiochem (San Diego, CA, USA). Anti-human FRα (Mov18) was from Alexis (San Diego), anti-pERK42/44 was from Cell Signalling Techonlogy and anti-β-actin was from Sigma Aldrich (St. Louis, MO, USA). Anti-mouse Alexa-Fluor 594 antibody was from Invitrogen. Goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase were from Pierce Biotechnology (Rockford, IL, USA). Carboplatin (powdered drug), platinum atomic absorption standard solution, Sephadex G-50 size exclusion gel and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Cell lines. KB cells (FR positive human oral carcinoma cell line) and A549 cells (FR negative lung cancer cell line) were obtained from American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured as monolayers in 75-cm^2 tissue culture flasks in folate-free RPMI-1640 media (Gibco Grand Island, NY, USA), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), and were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

Preparation of liposomes. All liposome formulations were prepared by extrusion method as described by Mayer *et al* (23). Lipids were individually dissolved in chloroform and then mixed together in the appropriate proportions. The fluorescent lipid, DiI, was incorporated at 0.5 mol % in the liposome composition. Chloroform was subsequently evaporated under a stream of nitrogen gas, and the sample was then subjected to vacuum for ~3 h to remove the residual solvent. The resultant lipid film was hydrated with gentle stirring for 1 h in 0.9% NaCl (pH 7.5) so that the final lipid concentration was 80 mg/ml. The resultant multilamellar vesicles (MLVs) were extruded ten times through 80 and 100-nm polycarbonate membranes using a heated thermobarrel extruder (Northern Lipids, Vancouver, BC, Canada). Both hydration and extrusion were performed at a temperature of 55±2˚C. The mean diameter and polydispersity of each liposome batch was analyzed by ZetaSizer model 3000 HS (Malvern Instruments, Malvern, UK) at 632 nm.

Drug loading and analysis. For loading carboplatin into the pre-formed liposomes, the passive equilibration method was employed (24). All liposomal samples, drug-loaded or empty, were stored at 2-8˚C subsequently for future studies. Briefly, pre-warmed carboplatin powder was mixed with the liposomes at a drug-to-lipid (D/L) weight ratio of 0.25:1 and was incubated at 55±2˚C with gentle mixing for the required time. At indicated time-points, the liposomal formulations were separated from the un-encapsulated drug using 1-ml Sephadex G-50 spin column pre-equilibrated with 0.9% NaCl (pH 7.5) by centrifugation at 680 x g for 3 min. The liposome eluted in the void was collected and further analysed for final drug and lipid content described as follows. The lipid concentrations of empty and drug-loaded liposomes were determined by the phosphate assay (25,26). Carboplatin content was analysed by atomic absorption spectroscopy (AAS) (27), using a Perkin-Elmer analyst 100 model (Perkin-Elmer, Waltham, MA, USA) attached to a graphite tube atomiser (Perkin-Elmer analyst 100 HGA 800 model). The instrument was operated at a wavelength of 265.9 nm following the sequential temperature of 90˚C for 30 sec, 120˚C for 10 sec, 1100˚C for 15 sec, 2700˚C for 5 sec (reading time) and 30˚C for 40 sec. The standard curve was plotted using a commercially available platinum standard solution. All measurements were made in duplicates.

In vitro cytotoxicity assay. For MTT assay, cells were seeded in 96-well plates at a density of 3,000 cells and 5,000 cells per well for KB cells and A549 cells, respectively, and allowed to attach overnight. On the following day, the culture medium was replaced with 200 μl fresh medium containing the required concentrations of carboplatin in free or liposome-encapsulated form. Free folic acid at 1 mM was included as specified. The plates were further incubated for 72 h at 37˚C. MTT (1 mg/ml) was added to the cells, and after 4 h of incubation, the precipitated purple formazan was solubilized in DMSO. Absorbance at 570 nm was determined using a Tecan Infinite M2000 plate reader (Tecan Group Ltd., Männedorf, Switzerland). Cancer cell viability was calculated from the absorbance values based on the following equation:

$$\% \text{ cell viability} = [(Abs_{test} - Abs_{blank})/(Abs_{untreated\ control} - Abs_{blank})] \times 100\%$$

where Abs_{test}, Abs_{blank}, and $Abs_{untreated\ control}$ represent the absorbance readings from the drug-treated wells, wells with only media and the untreated wells, respectively. The IC_{50} (half maximal inhibitory concentration) values were calculated from

the cell viability data using CalcuSyn 3.0 software (Biosoft, Cambridge, UK).

For cell viability assay in the presence of MEK/ERK inhibitors, 5,000 cells per well were plated 24 h prior to the assay and treated as above with or without 10 μM U0126 or 20 μM PD98059. Relative increase in viable cell fraction was calculated by the following equation:

$$\text{Relative increase in viable cell fraction (\%)} = (\text{Viable cell fraction}_{\text{with ERK inhibitor}} - \text{Viable cell fraction}_{\text{without ERK inhibitor}})/\text{Viable cell fraction}_{\text{without ERK inhibitor}} \times 100\%$$

Cellular uptake of fluorescent liposomes. KB cells ($1.5x10^6$) were plated in 35-mm dish and allowed to attach overnight. The following day, 0.005% of FRT-DiI liposomes or NT-DiI liposomes were added to the cells and incubated at 37˚C for 4 and 24 h, respectively. Free folic acid (1 mM) was added to determine the effect of FR blockage. At the end of incubation, cells were washed three times with cold PBS to remove unbound liposomes and using Nikon Eclipse TE2000-U microscope (Nikon Instruments Inc., Melville, NY, USA). Images were captured using Nikon digital camera DXM 1200.

Cellular platinum uptake. KB cells ($1.5x10^6$) and A549 cells were plated in 35-mm dish respectively, and allowed to attach overnight. The following day, 250 μM of the free or liposome-encapsulated carboplatin were added to the cells. After treatment for 24 h, the cells were harvested and pelleted at 1,200 rpm for 3 min, followed by two washes with PBS. Cell count was performed, and $1x10^6$ cells KB cells or $1x10^5$ cells A549 cells were digested in 50 μl concentrated nitric acid, respectively, followed by heating at 85˚C for 1 h. Cell debris was removed by centrifugation at 13,000 rpm for 15 min. Sample was then diluted 8-fold with water before the determination of platinum content by AAS.

DNA adducts formation. KB cells ($1.5x10^6$) were plated in 35-mm dish. The following day, 250 μM of the free or liposome-encapsulated carboplatin was added to the cells and treated for 48 h. The cells were washed with PBS and lysed in 600 μl nuclear lysis buffer (10 mM Tris pH 8.2, 400 mM NaCl, 2 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) for 4 h at 37˚C. Saturated NaCl solution (340 μl) was then added to the cell lysate, followed by vigorous shaking and centrifugation at 13,000 rpm for 30 min. The supernatant containing the DNA was transferred to a fresh tube containing 1.2 ml absolute ethanol and mixed gently. DNA was pelleted by centrifugation at 4,000 rpm for 30 min and washed three times with 70% ethanol. Finally, the DNA pellet was air-dried, dissolved in water and quantified at absorbance 260 nm by UV spectrophotometry. DNA purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm, and a ratio of 1.7-1.9 indicates pure DNA. For DNA-platinum adduct formation, DNA was diluted with 4% nitric acid to a final concentration of 1% nitric acid, and heated at 85˚C for 1 h. Insoluble particles were removed by centrifugation at 13,000 rpm for 15 min. The platinum content of the sample was then determined by AAS.

Western blot analysis. KB cells were plated in 6-well plate at a density of $1.5x10^6$ cells per well and allowed to attach overnight. The following day, cells were treated with 250 μM carboplatin in free or liposome-encapsulated form for 24 h. In another study, the cells were treated with increasing concentrations of carboplatin (62.5, 125 and 250 μM) encapsulated in FRT liposomes in the presence or absence of 50 μM MEK/ERK inhibitors, U0126 or PD98059. Cells were also treated with 1.25-10 mM non-drug loaded FRT liposomes. After treatment, floating cells and attached cells were lysed using ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (v/v) NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% (v/v) glycerol, protease inhibitor cocktail). Cell debris was removed by centrifugation at 20,000 x g for 20 min at 4˚C. Protein was quantified using Bio-Rad protein assay dye reagent, and equal amount of protein (50 μg) was electrophorosed on 10% SDS-polyacrylamide gels, followed by transferring onto nitrocellulose membrane. Membrane was blocked with 5% milk in Tris-buffered saline with Tween-20 (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20) before probing with primary antibody according to the manufacturer's instructions. The membrane was then probed with the corresponding secondary antibody conjugated with horseradish peroxidase for 1 h. Protein bands were detected by enhanced ECL reagent (Pierce) and visualized by autoradiography film (Thermo Scientific, Waltham, MA, USA). For re-probing, blots were stripped with Restore Western Blot stripping buffer (Thermo Scientific). For densitometric analysis, the relative intensity of the bands representing pERK were quantified using Image Guage 4 software and were normalized to the actin bands.

Statistical analyses. All experiments were performed at least three independent times, unless otherwise stated. Results are expressed as mean ± SEM. Statistical analyses were performed with one-way analysis of variance (ANOVA) and the *post hoc* Newman-Keuls test using GraphPad Prism Version 2.00 (San Diego, CA, USA). A $p<0.05$ was considered to be statistically significant.

Results

Characteristics of carboplatin-encapsulated liposome formulations. The lipid compositions used to formulate the liposome systems were as follows: DPPC/DSPE-PEG_{1000} and DPPC/DSPE-PEG_{1000}/DSPE-PEG_{2000}-folate at molar ratios of 95:5 and 95:4.8:0.2, respectively, for NT and FRT liposomes. Carboplatin was loaded into NT and FRT liposomes using an established method based on passive equilibration with an initial drug-to-lipid (D/L) ratio of 0.25:1 (w/w) and incubation time of 60 min (24). The final D/L ratios achieved in the NT liposomes and in the FRT liposomes were 0.062±0.004 (w/w) and 0.057±0.006 (w/w), respectively. All liposomes, before and after drug loading, were within the size range of 100-120 nm with polydispersity indices of <0.25.

Cancer cell viability upon exposure to carboplatin in free form, NT and FRT liposomes. The anti-cancer activity of the various forms of carboplatin was evaluated using two cancer cell lines, the FR-overexpressing KB cells and the FR-negative A549 cells. Expression of FR on cell surface was verified by flow cytometric analysis (data not shown). A concentration-dependent decrease in the viability of KB cells (Fig. 1A) and A549 cells (Fig. 1B)

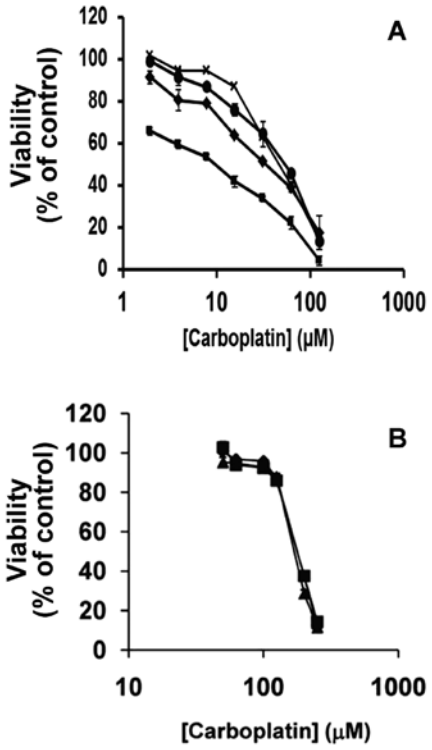

Figure 1. Viability of (A) FR-positive KB cells, and (B) FR-negative A549 cells upon exposure to free carboplatin (●), NT liposome (♦), FRT liposome in the absence (■) and presence (x) of 1 mM free folic acid for 72 h. Free carboplatin was in the form of solution in 0.9% NaCl. At least three independent batches of NT and FRT liposomes were tested on each cell line. Results shown are the mean ± SEM from at least three independent experiments.

was observed when the cells were treated with carboplatin-loaded FRT and NT liposomes as well as with the free drug. Drug-free liposomes did not show significant toxicity towards KB cells or A549 cells, with viability of >85% observed. Based on the cell viability data, the IC_{50} values for FRT carboplatin liposomes, NT carboplatin liposomes and free carboplatin in KB cells were estimated to be 7.2±0.3, 29.4±3.8, and 40.7±4.2 μM, respectively (Table I). This finding demonstrated enhancement in carboplatin potency by 5.7-fold as compared to free drug, and by 4.1-fold as compared to the NT liposomes. Yet, no significant difference was observed in the IC_{50} values among FRT carboplatin liposomes (167.1±2.8 μM), NT carboplatin liposomes (170.9±2.9 μM) and free drug (174.9±2.6 μM) when tested in the FR-negative A549 cells. Importantly, in the presence of 1 mM free folic acid, the IC_{50} values for FRT carboplatin liposomes increased significantly to 48.4±4.6 μM from 7.2±0.3 μM.

Intracellular accumulation of liposomes and platinum. To elucidate if the enhanced cytotoxicity of the FRT carboplatin liposomes was indeed correlated with an increase in intracellular delivery of carboplatin, intracellular levels of liposomes and Pt were determined and compared among the various formulations of carboplatin. First, cellular uptake of FRT and NT liposomes labelled with the fluorescent lipid DiI was determined in FR-positive KB cells upon 4 h of incubation. As shown in Fig. 2, FRT liposomes showed much higher liposome internalization as compared to NT liposomes, and the internalization of FRT liposomes could be blocked by the presence of 1 mM free folic acid. Next, the intracellular Pt content was determined in cells treated with various forms of carboplatin. Fig. 3A demonstrates significantly higher accumulation of Pt when treating the KB cells with FRT liposomes as compared to the free drug and the NT liposomes ($p<0.05$), with ~2-fold increase over the free drug and 4-fold increase over the NT liposomes. In contrast, cellular Pt accumulation was not statistically different when comparing free drug, NT and FRT formulation in FR-negative A549 cells (Fig. 3B). Taken together, the enhanced cytotoxicity of carboplatin formulated in FRT liposomes was due to FR-mediated endocytosis that resulted in increased cellular Pt content.

DNA-Pt adduct formation. In light of the above finding, it was anticipated that the formation of DNA-Pt adducts would be increased and correlated with the observed enhancement in carboplatin potency. Thus, our next step was to measure DNA-Pt adducts formed in the KB cells. Platination of DNA by carboplatin is comparatively slower than cisplatin (28); therefore, an incubation time of 48 h was chosen for the experiment. Surprisingly, the amount of DNA-Pt adducts formed from FRT carboplatin liposomes was significantly lower than that of the free drug (Fig. 4). These results suggest that the cytotoxicity of FRT carboplatin liposomes might be mediated through some other molecular targets in addition to the formation of DNA-Pt adducts.

Enhanced ERK activation by FRT carboplatin liposomes as an alternative pathway in mediating cytotoxicity. Several studies have reported the role of ERK activation in cisplatin- and carboplatin-induced apoptosis (29-34). It is thus interesting to probe if ERK activation would be observed in KB cells upon exposure

Table I. IC_{50} values of free, NT- and FRT-liposomal carboplatin on FR-positive KB cells and FR-negative A549 cells.

Cell lines	Free carboplatin (μM)	NT liposome (μM)	FRT liposome (μM)	FRT liposome + 1 mM folic acid (μM)
KB	40.7±4.2[a]	29.4±3.8	7.2±0.3[b]	48.4±4.6
A549	174.9±2.6[c]	170.9±2.9	167.1±2.8	-

Cell viability was determined using MTT assay and subsequently analyzed by Calcusyn 3.0 software to estimate IC_{50} values. Results shown are the mean ± SEM from at least three independent experiments. All r-values were >0.9. [a]$p>0.05$ for free form vs NT liposomes; [b]$p<0.05$ for FRT liposomes vs free form and NT liposomes; [c]$p>0.05$ for FRT liposomes vs free form and NT liposomes.

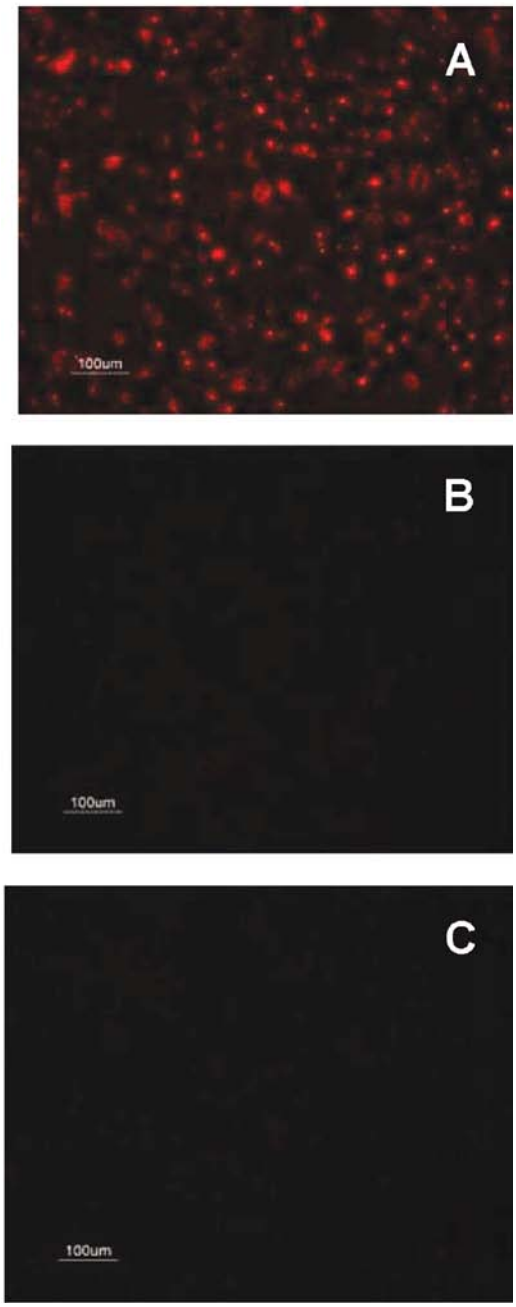

Figure 2. Intracellular accumulation of DiI-labelled FRT liposomes (A), NT liposomes (B), and FRT liposomes in presence of 1 mM free folic acid (C) in KB cells for 4 h. Results shown are representative of at least three independent experiments.

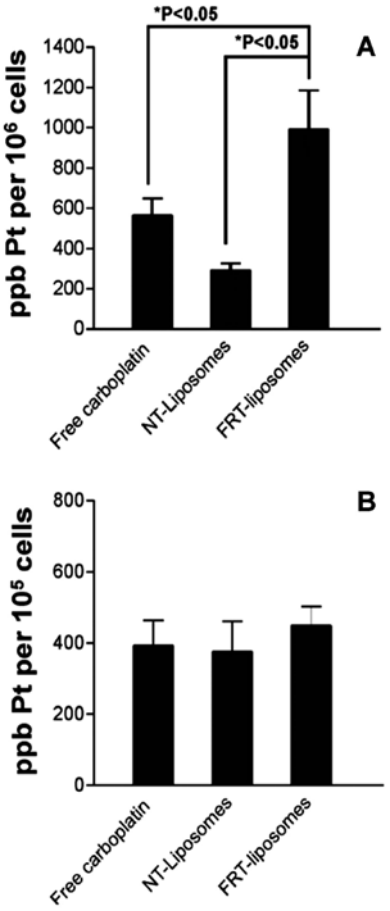

Figure 3. Cellular Pt accumulation in (A) FR-positive KB cells and (B) FR-negative A549 cells for free carboplatin, NT or FRT carboplatin liposomes. Data represent mean ± SEM of four independent experiments.

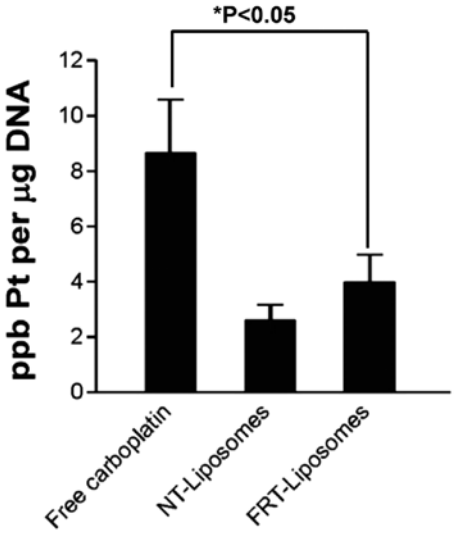

Figure 4. DNA-Pt adduct formation in KB cells for free carboplatin, NT or FRT carboplatin liposomes. Data represent mean ± SEM of five independent experiments.

to FRT carboplatin liposomes, which in turn, mediated the cytotoxic effect of these liposomes. In general, treatment with carboplatin in the three different forms resulted in significantly higher levels of phosphorylated ERK (p-ERK) as compared to control ($p<0.05$, Fig. 5A). Among the three formulations of carboplatin, FRT carboplatin liposomes displayed the highest level of p-ERK, which was significantly higher than free carboplatin and NT carboplatin liposomes ($p<0.05$). Of importance, ERK activation was noticed in drug-free FRT liposomes; therefore, ERK activation was probed in KB cells treated with increasing concentrations of non-drug loaded FRT liposomes. As demonstrated in Fig. 5B, concentration-dependent ERK activation could be observed when KB cells were treated with lipid concentrations of 1.25, 2.5, 5 and 10 mM of FRT liposomes. Furthermore, Fig. 5C showed the activation of ERK was depen-

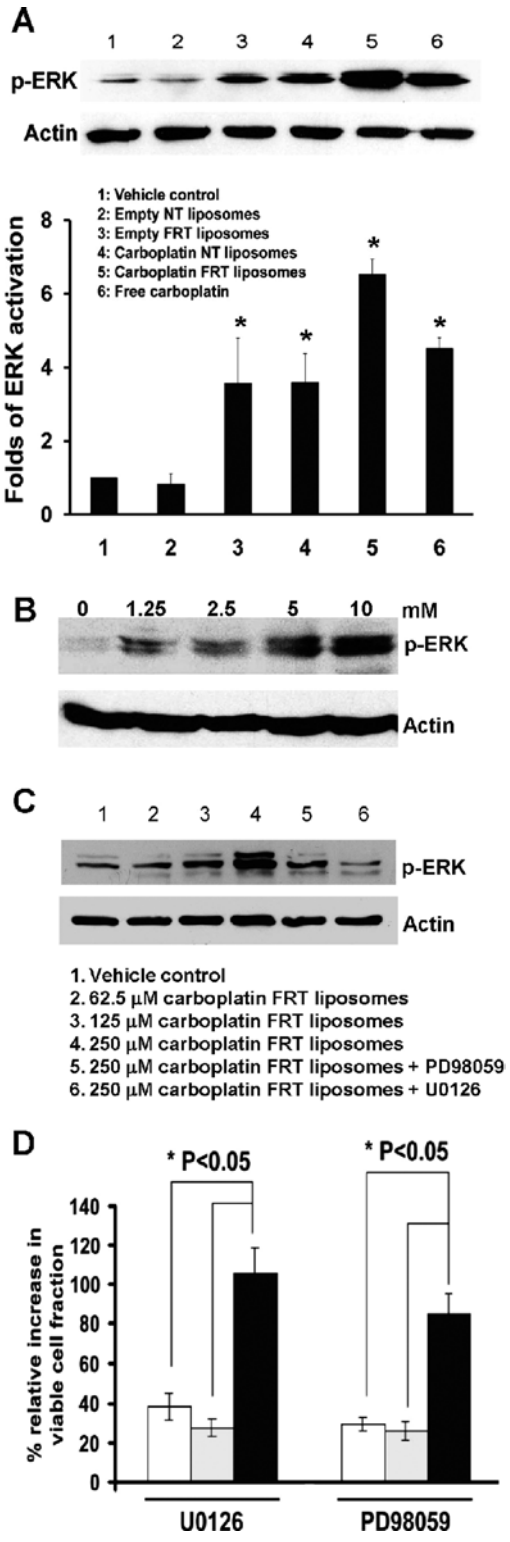

Figure 5. Modulation of ERK activation upon exposure to FRT liposomes in KB cells. (A) ERK activation when KB cells were treated with free carboplatin, NT and FRT liposomes with or without drug encapsulation. (B) Concentration-dependent ERK activation by empty FRT liposomes. (C) ERK activation in KB cells treated with increasing concentrations of FRT carboplatin liposomes which could be blocked by MEK/ERK inhibitors U0126 (10 μM) and PD98095 (20 μM). (D) Reversal of KB cell killing in the presence of the MEK/ERK inhibitors as reflected by the % relative increase in viable cell fraction observed in the three treatment groups: 1) free carboplatin (white), 2) NT carboplatin liposomes (grey), 3) FRT carboplatin liposomes (black). Results shown are the mean ± SEM from at least three independent experiments. For Western blots, β-actin was used as the loading control, and representative blots from three independent experiments are shown. The folds of ERK activation were calculated from the relative intensity of the bands representing p-ERK and normalized to the actin bands.

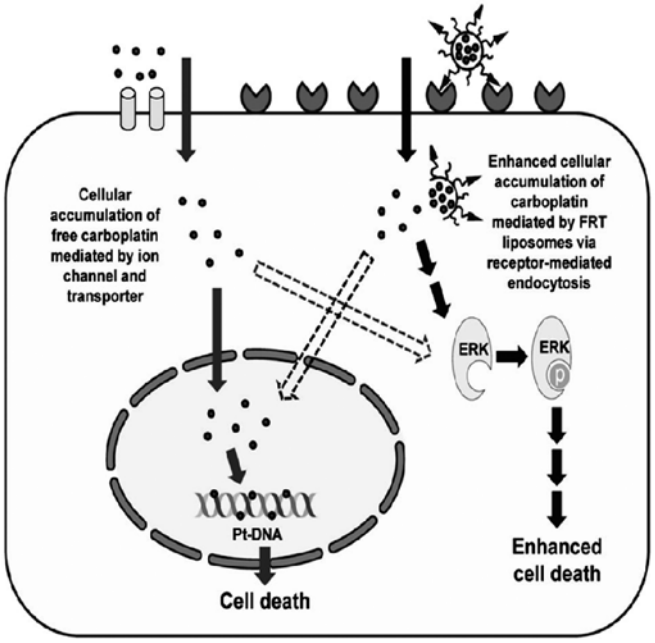

Figure 6. Comparison of the mechanism of action of carboplatin delivered in free form and in FRT liposomes. Solid arrows, major pathway; dashed arrows, minor pathway.

dent on the concentration of carboplatin FRT liposomes, which could be inhibited by concurrent treatment with the MEK/ERK inhibitors, U0126 and PD98059.

To corroborate the finding on ERK activation with cell viability, MTT assay was performed on KB cells treated with carboplatin FRT liposomes, NT liposomes and free drug in presence or absence of the MEK/ERK inhibitors. In general, significant increase in cell viability was observed when the drug-treated cells were incubated with the MEK/ERK inhibitors for the three forms of carboplatin (data not shown). The % relative increase in the viable cell fraction upon ERK inhibition, as calculated by the equation in method section, was determined and compared among the three formulations of carboplatin to delineate the contribution of ERK activation in mediating the cytotoxic effect. As shown in Fig. 5D, FRT carboplatin liposomes displayed the highest increase in viable cell fraction upon ERK inhibition by U0126 and PD98059, which was significantly different from the free drug and NT carboplatin liposomes ($p<0.05$). Taken together, our results suggest that ERK activation plays a major role in mediating the cytotoxicity of FRT carboplatin liposomes rather than DNA-Pt adduct formation. This observation could shed new light on the utility of FRT liposomes in modulating ERK-related cell survival signalling pathways and in enhancing the cytotoxicity of chemotherapeutics.

Discussion

In this study, an FRT liposomal formulation of carboplatin was developed and evaluated for its cytotoxicity in FR-overexpressing KB cancer cells, with the anticipation that FR-mediated endocytosis of FRT liposomes would increase intracellular accumulation of carboplatin, which in turn, would enhance the formation of DNA-Pt adducts and subsequent cytotoxicity. Several important observations

could be made. First, from the cell viability data (Fig. 1), it is clear that FRT liposomes would not confer enhancement in carboplatin potency when the cancer cells have negligible FR expression. Thus, the improved potency could be attributed to effective internalization of carboplatin into the FR over-expressing KB cells through FRT liposomal delivery, which is supported by data presented in Figs. 2 and 3. Indeed, our data on FRT carboplatin liposomes are in agreement with previous studies involving FRT liposomal delivery of doxorubicin, paclitaxel, docetaxel and 5-FU that conferred 2-3-fold improvement in IC_{50} values when compared to the free drug (35-38).

Surprisingly, the increased cellular accumulation of carboplatin by FRT liposomal delivery did not correlate with increased formation of DNA-Pt adducts. In fact, from our data, carboplatin in free form had higher levels of DNA-Pt adduct formation as compared to FRT liposomes. Pt-based compounds in their free form are reported to act primarily through DNA-Pt adduct formation; thus, it is intriguing to observe a discrepancy between carboplatin potency and DNA-Pt adduct formation in our present study. Aronov *et al* have described similar findings with folate-PEG conjugated carboplatin analogues, supported by data from cellular accumulation of Pt and confocal microscopy (39). In their study, the IC_{50} values of the analogues did not correlate with cellular Pt accumulation or DNA-Pt adduct formation. Thus far, no further studies have been performed to address this discrepancy. Thus, for the first time, we have demonstrated that increased potency of carboplatin through FRT liposomal delivery could be associated with other molecular targets in addition to DNA-Pt adduct formation.

The anti-cancer mechanisms of Pt-based compounds have been described to involve more complex cellular and molecular events in addition to the formation of DNA-Pt adducts. Several research groups have reported the active role of ERK in mediating the cytotoxicity of cisplatin and carboplatin (29-34), and these studies have provided the basis to probe ERK activation as a molecular event in mediating the cytotoxic effect of carboplatin delivered via FRT liposomes. Our results demonstrated significantly higher ERK activation by FRT carboplatin liposomes as compared to free form and NT liposomes. ERK activation upon treatment with carboplatin FRT liposomes was concentration-dependent, and could be reversed in the presence of MEK/ERK inhibitors.

Most importantly, ERK activation could be induced by increasing concentration of empty FRT liposomes (Fig. 5B), suggesting the ability of these liposomes to modulate the ERK-related cell survival signals and sensitize the cancer cells to carboplatin treatment. This is further supported by the results from the concurrent use of MEK/ERK inhibitors with FRT carboplatin liposome treatment which produced the greatest reversal in KB cell killing (Fig. 5D), as reflected by the highest % relative increase in viable cell fraction in the FRT carboplatin liposome group. Of note, other liposomal systems which have been conjugated with monoclonal antibodies such as rituximab or trastuzumab modulated important signalling pathways such as Akt and NF-κB (40). Collectively, these findings suggest that different routes of drug entry could lead to potential shift in the intracellular distribution of carboplatin and its mechanism of action, whereby DNA-Pt adduct formation may not remain as the primary molecular target of carboplatin when delivered in the FRT liposome. Fig. 6 presents the comparison of carboplatin delivered as free form or in FRT liposomes, with ERK activation proposed to be the major molecular event mediating the cytotoxic effect of carboplatin delivered by FRT liposomes. What remains to be elucidated is whether ERK activation would be observed as a molecular event in modulating drug cytotoxicity in other FR-targeted drug formulations.

In conclusion, our studies have shown that the potency of carboplatin delivered by FRT liposomes was significantly enhanced in FR-overexpressing KB cells when the drug was loaded into an FRT liposomal delivery system. For the first time, enhanced cytotoxicity of FRT carboplatin liposomes could be related to increased cellular uptake and ERK activation rather than enhanced DNA-Pt adduct formation. Future studies involving the evaluation of the FRT carboplatin liposomes in animal models are warranted.

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