

## Supporting Information

### Cationic Folate-Mediated Liposomal Delivery of Bis-Arylidene Oxindole Induces Efficient Melanoma Tumor Regression

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## **Synthesis of FA8:**

**(R)-N-(2-(4-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzamido)-4-carboxybutanamido)ethyl)-N-methyl-N-octyloctan-1-aminium chloride (lipid 1, Scheme I)**

**Synthesis of tert-butyl (2-(dioctylamino) ethyl) carbonate (Compound-2):** Monoboc-protected ethylenediamine (Compound-1, 1.8g) was added to Ethyl Acetate (20ml). To this mixture K<sub>2</sub>CO<sub>3</sub> (6.4 g , 4eq) followed by 1-Bromo octane was added and stirred at 65 °C for 48 h. Reaction mass was filtered and the ethyl acetate was evaporated to get crude. Purification is done by column chromatographic separation using 60-120 mesh silica gel and 1% Methanol-Chloroform (v/v) as eluent. The separation yielded compound-2 as colourless liquid (3g, 70% yield).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ/ppm = 0.9 [m, 6H, CH<sub>3</sub>-(CH<sub>2</sub>)<sub>7</sub>-]; 1.1–1.6 [bs, 20H, -(CH<sub>2</sub>)<sub>10</sub>-; m, 9H, CO-O-C(CH<sub>3</sub>)<sub>3</sub>]; 1.5 [m, 4H, -CH<sub>2</sub>-N-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>]; 2.3–2.5 [m, 4, -CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-]; 2.5 [t, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-]; 3.2 [m, 1H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-]; 3.6 [t, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-];

ESIMS: m/z = 386 [M+1]<sup>+</sup> for C<sub>23</sub>H<sub>48</sub>N<sub>2</sub>O<sub>2</sub>

**Synthesis of N-(2-((tert-butoxycarbonyl)amino)ethyl)-N-methyl-N-octyloctan-1-aminium iodide (3):** Compound-2, 1.8g was added to Methyl Iodide (5ml) and stirred at room temperature for 12h. Methyl Iodide was evaporated to get crude. Purification is done by column chromatographic separation using 60-120 mesh silica gel and 2% Methanol-Chloroform (v/v) as

eluent. The separation yielded compound-3 as white solid (2.5g, 80% yield). .

$^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ ):  $\delta/\text{ppm}$  = 0.9 [m, 6H,  $\text{CH}_3$ -( $\text{CH}_2$ ) $_7$ -]; 1.1-1.6 [bs, 20H, -( $\text{CH}_2$ ) $_{10}$ -; m, 9H, CO-O-C( $\text{CH}_3$ ) $_3$ ]; 1.5[m, 4H, - $\text{CH}_2$ -N- $\text{CH}_2$ -( $\text{CH}_2$ ) $_4$  ]; 3.2-3.3 [s,3, - $\text{CH}_2$ -N( $\text{CH}_3$ )-( $\text{CH}_2$ ) $_2$ -( $\text{CH}_2$ ) $_2$ -]; 3.4-3.6 [m, 4H, - $\text{CH}_2$ -N( $\text{CH}_3$ )-( $\text{CH}_2$ ) $_2$ -( $\text{CH}_2$ ) $_2$ -]; 3.6-3.8[m, 4H, -NH- $\text{CH}_2$ - $\text{CH}_2$ -N-( $\text{CH}_2$ ) $_2$ -];  
ESIMS :  $m/z$ = 400  $[\text{M}+1]^+$  for  $\text{C}_{24}\text{H}_{51}\text{N}_2\text{O}_2$

### Synthesis of N1-methyl-N1,N1-dioctylethane-1,2-diaminium chloride (4)

Compound-3, 2g was added was dissolved in 2 mL dry  $\text{CHCl}_3$ , 1 mL of TFA was added and the mixture at  $0^\circ\text{C}$  was allowed to stir for 3 h. TFA was removed with nitrogen flow and the residue was subjected to chloride ion exchange chromatography over amberlyst A-26 chloride ion exchange resin. The compound-4 obtained after chloride ion exchange was almost pure (1.2 g, 80% yield) directly continued to next step.

ESIMS:  $m/z$ = 300  $[\text{M}+1]^+$  for  $\text{C}_{19}\text{H}_{43}\text{N}_2$ .

### Synthesis of FA8:

Folic Acid, 1g was dissolved in DMSO (10ml), to this mixture HATU (1.03g, 1.2eq) was added. To this solution mixture of compound-4 (0.67g, 1 eq) and Triethylamine (0.459g, 2eq) in DMSO (5ml) was added. Reaction mass was stirred for overnight. 2N HCl 30 ml was added to reaction mass followed by filtration to get crude solid. Crude product was purified by using 60-120 mesh silica gel and 15% Methanol-Chloroform (v/v) as eluent. Yellow solid was then dissolved in acetone and filtered to get pure FA8 ( 0.6g, 36%. Yield).

$^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ +  $\text{DMSO}-d_6$ ):  $\delta/\text{ppm}$  = 0.9 [m, 5.5H,  $\text{CH}_3$ -( $\text{CH}_2$ ) $_7$ -]; 1.1–1.4 [bs, 20H, -( $\text{CH}_2$ ) $_{10}$ -]; 1.5[m, 4H, - $\text{CH}_2$ -N $^+$ ( $\text{CH}_3$ )-( $\text{CH}_2$ ) $_2$ -( $\text{CH}_2$ ) $_2$ - ]; 3–3.2 [s,3, - $\text{CH}_2$ -N $^+$ ( $\text{CH}_3$ )-( $\text{CH}_2$ ) $_2$ -( $\text{CH}_2$ ) $_2$ -];

3.4-3.6 { m, 8.1H, [-CH<sub>2</sub>-N<sup>+</sup>(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-], [ -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-] }, 4.4-4.6 [m, 3.1H, FA- 3C (-CH), FA- 8C(-Ph-NH-(CH<sub>2</sub>)-]: 6.6-6.7 [m, 2.8 H, FA- 5C&7C]: 7.8 [m, 2.5H, FA- 4C & 6C]: 8.8-10 [s, 1.3H, FA- 9C]:

**ESI MS** : m/z= 723[M+1]<sup>+</sup> for C<sub>38</sub>H<sub>60</sub>N<sub>9</sub>O<sub>5</sub>.

## Synthesis of FA12:

**Synthesis of (R)-N-(2-(4-(4-(((2-amino-4-oxo-3,4 dihydropteridin-6-yl)methyl)amino)benzamido)-4-carboxybutanamido)ethyl)-N-dodecyl-N-methyldodecan-1-aminium chloride**

FA12 synthesized following the same procedure followed for lipid **8**. The <sup>1</sup>H NMR and ESIMS spectral data are given below.

### Compound-2 (Scheme I)

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ/ppm = 0.9 [m, 6H, CH<sub>3</sub>-(CH<sub>2</sub>)<sub>7</sub>-]; 1.1-1.6 [bs, 36H, -(CH<sub>2</sub>)<sub>18</sub>-]; m, 9H, CO-O-C(CH<sub>3</sub>)<sub>3</sub>]; 1.5 [m, 4H, -CH<sub>2</sub>-N-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub> ]; 2.3-2.5 [m, 4, -CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-]; 2.5 [t, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>- ]; 3.2 [m, 1H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>- ]; 3.6 [t, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-];

ESI MS : m/z= 498 [M+1]<sup>+</sup> for C<sub>31</sub>H<sub>64</sub>N<sub>2</sub>O<sub>2</sub>

### Compound-3 (Scheme I)

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ/ppm = 0.9 [m, 6H, CH<sub>3</sub>-(CH<sub>2</sub>)<sub>7</sub>-]; 1.1-1.6 [bs, 36H, -(CH<sub>2</sub>)<sub>18</sub>-]; m, 9H, CO-O-C(CH<sub>3</sub>)<sub>3</sub>]; 1.5 [m, 4H, -CH<sub>2</sub>-N-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub> ]; 3.2-3.3 [s, 3, -CH<sub>2</sub>-N(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-]; 3.4-3.6 [m, 4H, -CH<sub>2</sub>-N(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-]; 3.6-3.8 [m, 4H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-];

ESIMS : m/z= 512[M+1]<sup>+</sup> for C<sub>32</sub>H<sub>67</sub>N<sub>2</sub>O<sub>2</sub>

### Compound-4 (Scheme I)

ESIMS : m/z= 412 [M+1]<sup>+</sup> for C<sub>27</sub>H<sub>59</sub>N<sub>2</sub>

**FA12 :**

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> + DMSO-d<sub>6</sub>):** δ/ppm = 0.9 [m, 8.5H, -(CH<sub>2</sub>)<sub>7</sub>-CH<sub>3</sub>]; 1.1-1.4 [bs, 30.8H, -(CH<sub>2</sub>)<sub>18</sub>-(CH<sub>3</sub>)<sub>2</sub>]; 1.5 [m, 4H, -CH<sub>2</sub>-N<sup>+</sup>(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-]; 2-2.4 [m, 4H, (CO)-CH(NH)-(CH<sub>2</sub>)<sub>2</sub>-CO-NH ], 3-3.2 [s, 3.6, -CH<sub>2</sub>-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>-]; 3.4-3.6 { m, 10.2 H, [-CH<sub>2</sub>-N<sup>+</sup>(CH<sub>3</sub>)-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>-], [-NH-CH<sub>2</sub>-CH<sub>2</sub>-N-(CH<sub>2</sub>)<sub>2</sub>-]}, 4.4 [m, 0.7H, FA- 3C(-CH-)]; 4.6 [s, 1.2 H, FA- 8C(-Ph-NH-(CH<sub>2</sub>)-)]; 6.6-6.7 [m, 1.2 H, FA- 5C&7C]; 7.8 [m, 1.2H, FA- 4C & 6C]; 8.8-10 [s, 1H, FA- 9C];

**ESI MS :** m/z = 835 [M+1]<sup>+</sup> for C<sub>46</sub>H<sub>76</sub>N<sub>9</sub>O<sub>5</sub>.

**Synthesis of FA8 NC:****Synthesis of N1,N1-dioctylethane-1,2-diaminium**

Compound-2 (n=5), 2g was added was dissolved in 2 mL dry CHCl<sub>3</sub>, 1 mL of TFA was added and the mixture at 0°C was allowed to stir for 3 h. TFA was removed with nitrogen flow and the residue was subjected to chloride ion exchange chromatography over amberlyst A-26 chloride ion exchange resin. Obtained N1,N1-dioctylethane-1,2-diaminium compound after chloride ion exchange was almost pure (1.2 g, 80% yield) directly continued to next step.

**Synthesis of (R)-2-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzamido)-5-((2-(dioctylamino)ethyl)amino)-5-oxopentanoic acid**

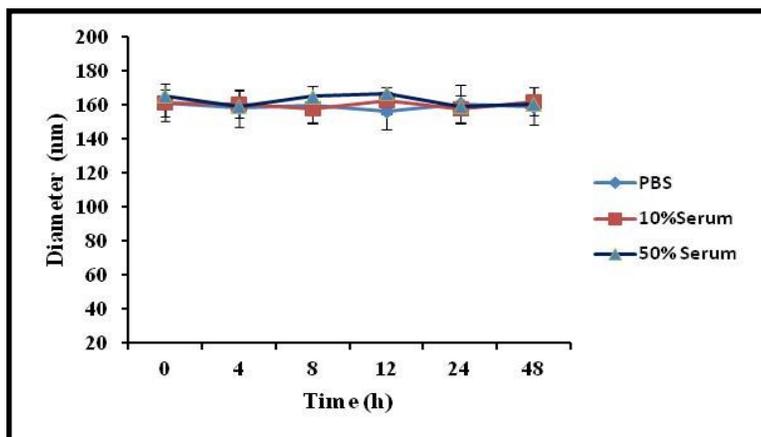
FA8 NC is synthesized by following procedure. Folic Acid, 1g was dissolved in DMSO (10ml), to this mixture HATU (1.03g, 1.2eq) was added. To this solution mixture of above prepared compound N1,N1-dioctylethane-1,2-diaminium (0.75g, 1 eq) and Triethylamine (0.459g, 2eq) in DMSO (5ml) was added. Reaction mass was stirred for overnight. 2N HCl 30 ml was added to reaction mass followed by filtration to get crude solid. Crude product was purified by using 60-120 mesh silica gel and 15% Methanol-Chloroform (v/v) as eluent. Yellow solid was then dissolved in acetone and filtered to get pure FA8NC ( 0.6 g, 36%. Yield).

ESIMS: m/z = 709 [M+1]<sup>+</sup> for C<sub>37</sub>H<sub>57</sub>N<sub>9</sub>O<sub>5</sub>.

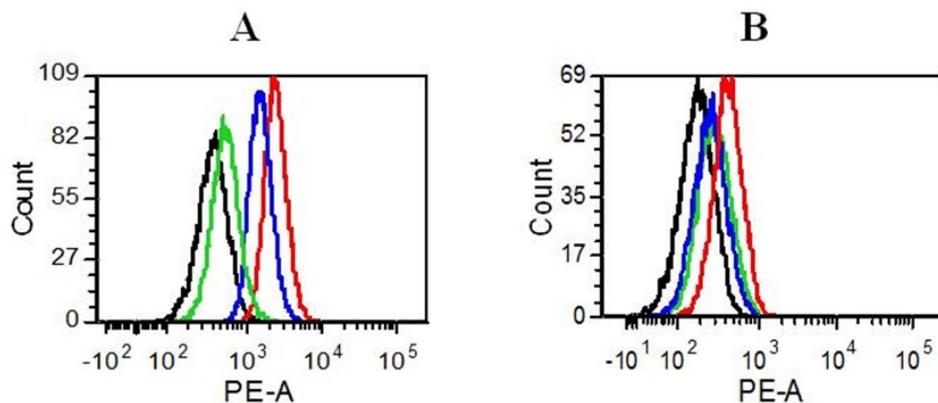
**Sizes, Zeta potentials ( $\xi$ ) of the NME2-associated liposomes carrying FA8, FA12 or FA8 NC.**

Liposomes of	Hydrodynamic diameter (nm)	Zeta Potentials (mV)	Poly Dispersity Index (PDI)
FA8	158±7	16±3.2	0.35
FA12	96 ±8	19.6± 5.1	0.19
FA8NC	167±8	12 ±2.8	0.32
FA8 (PEGYLATED)	170 ± 5	16.5±3.3	0.3

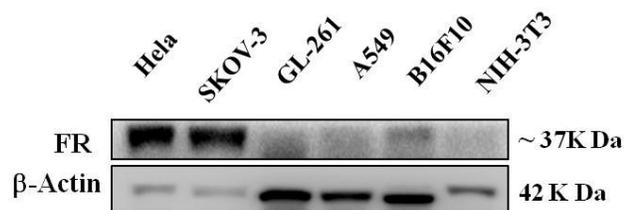
**Table S1.** Hydrodynamic Diameters (nm) and Zeta Potentials (mV) of the liposomes of FA8 and FA12 (containing 1 mM of each lipid) in DI water.



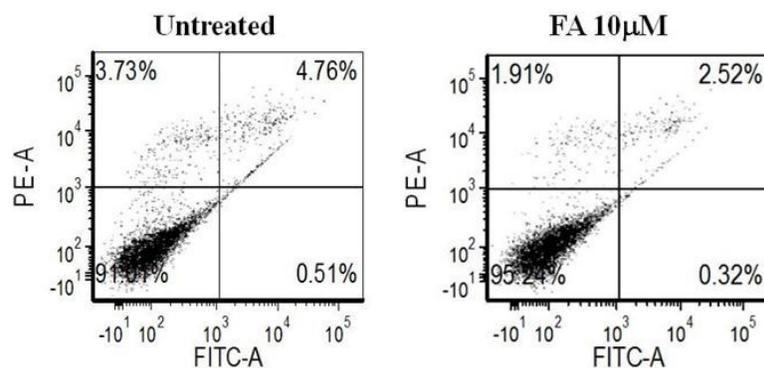
**Fig. S1** Liposome stability studies in PBS and different percentage of serum.



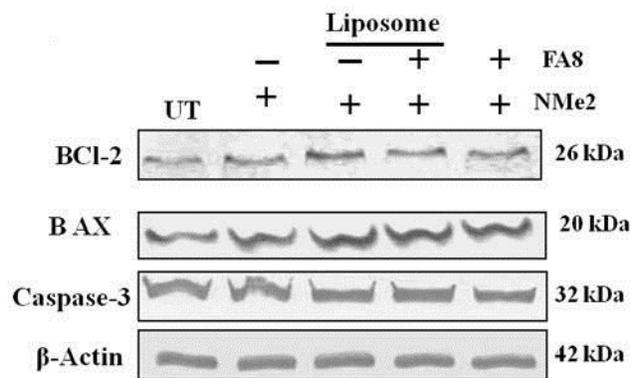
**Fig. S2** Cellular uptake study by Flow cytometry analysis of Rh-PE labelled liposomes of lipids FA8 & FA12 and non-targeting control liposome treated in A) B16F10 & B) A549 cells . Black & green represents Untreated and Non-targeting control liposome treated. Red & Blue represents liposomes of FA8 & FA12 treated cells respectively.



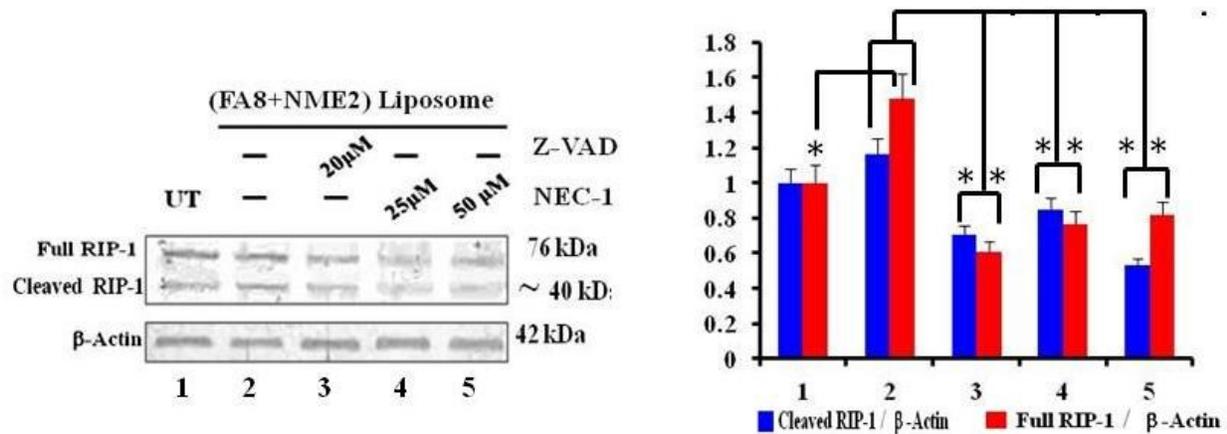
**Fig. S3** Folate receptor expression in different tumor cells.



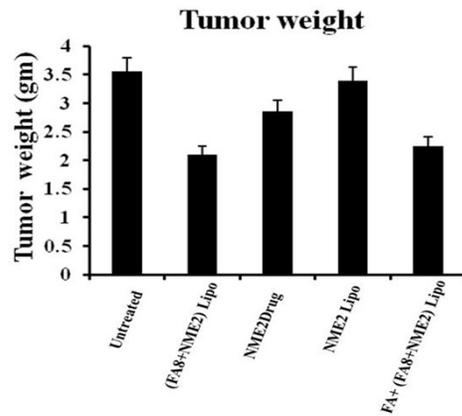
**Fig. S4** Flow cytometry apoptosis analysis in B16F10 cells treated with FA 10 $\mu$ M after 12h of treatment in folate free RPMI media.



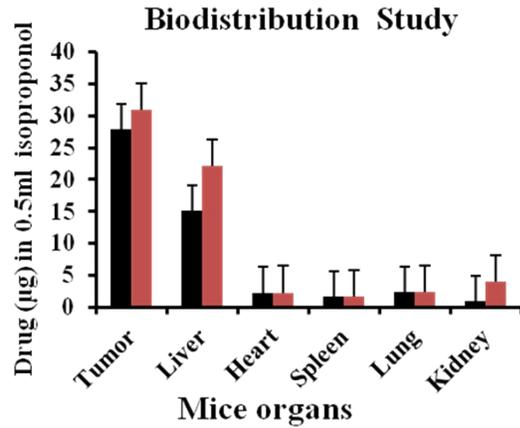
**Fig. S5** Differential expressions of proteins BAX, BCI-2, Caspase 3 in (FA8+NME2 ) liposome treated B16F10.



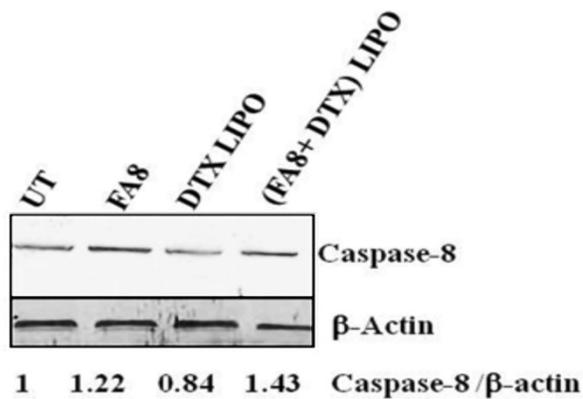
**Fig. S6** RIP is cleaved by a caspase-8. B16F10 cells were pre-treated with 20  $\mu$ M of Z-VAD-FMK (caspase 8 inhibitor) and or RIP-1 protein inhibitor NEC-1 (25  $\mu$ M & 50  $\mu$ M) after 2 h followed by treatment with targeted (FA8 + NME2) liposome formulation for 24 h. Calculated ratio of cleaved RIP-1 with respect to Full RIP-1. In all experiments untreated (UT) cells extract was loaded as a control. \* denotes,  $p < 0.01$ .



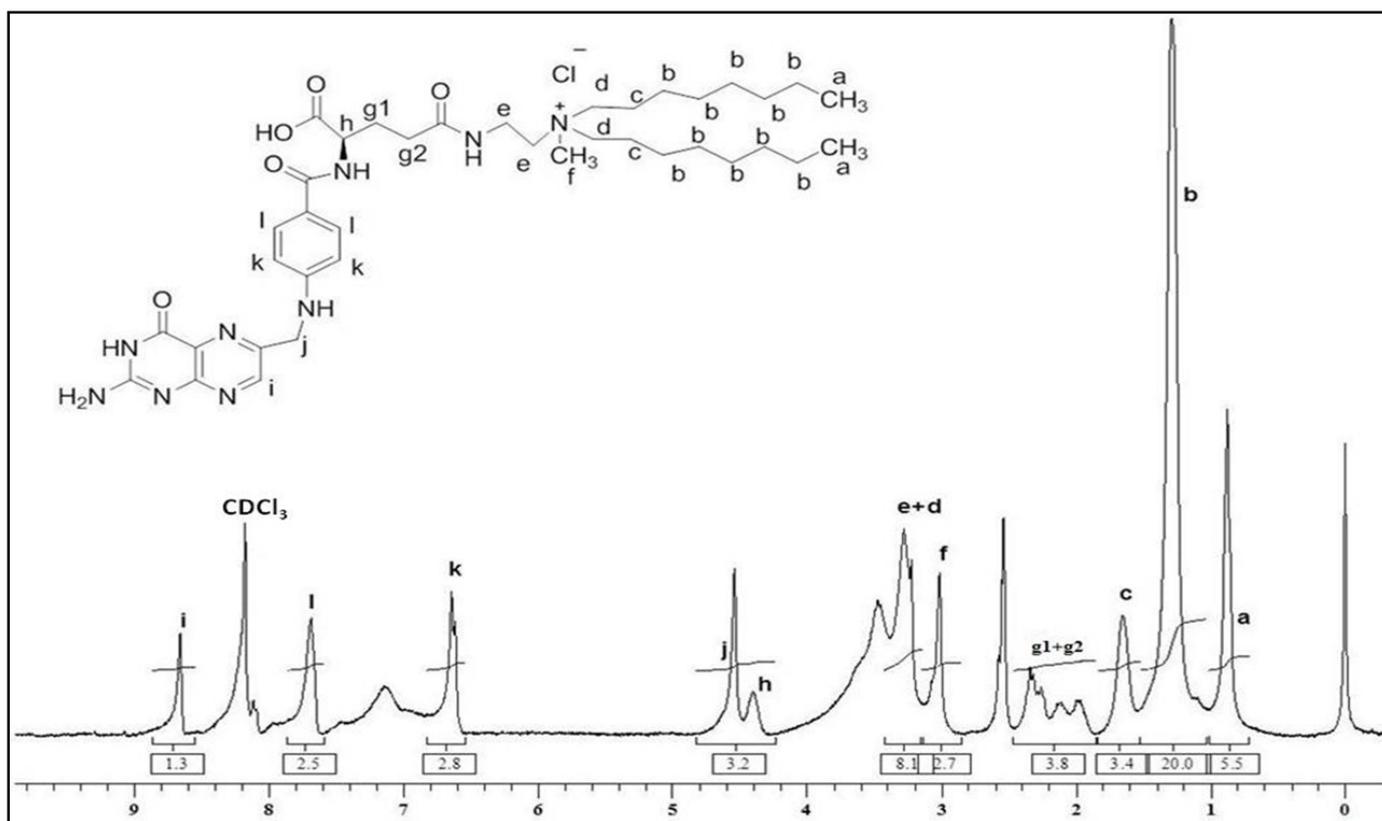
**Fig. S7.** Average tumour weight graph of all mice groups



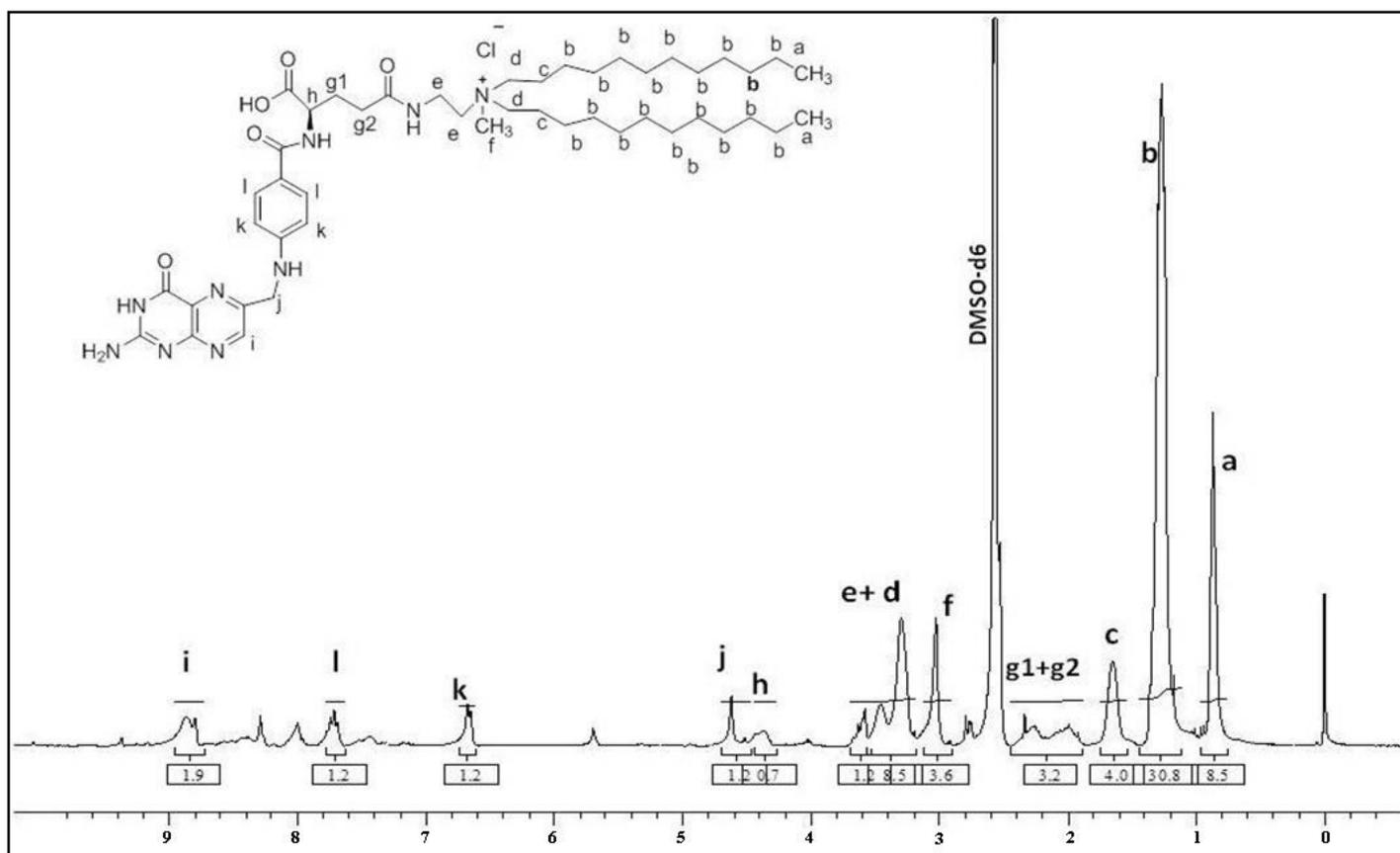
**Fig. S8** Comparison of biodistribution profile of Pegylated (FA8 +NME2) liposome (red) with non-pegylated (FA8+NME2) liposome (black) in melanoma tumor bearing mice after 24h of treatment.



**Fig. S9** Caspase-8 protein expression in (FA8+DTX ) liposome treated ( 100nM with respect to DTX) B16F10



**Fig. S10** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> +DMSO-d<sub>6</sub>) spectrum for FA8



**Fig. S11** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> +DMSO-d<sub>6</sub>) spectrum for FA12

ACC-ECK-722 #3-36 RT: 0.03-0.15 AV: 34 NL: 2.17E7  
T: FTMS (1,1) + p ESI Full ms [100.00-2000.00]

722.47040  
C<sub>38</sub> H<sub>60</sub> O<sub>5</sub> N<sub>9</sub> = 722.47119  
13.5 RDBE  
-1.09194 ppm

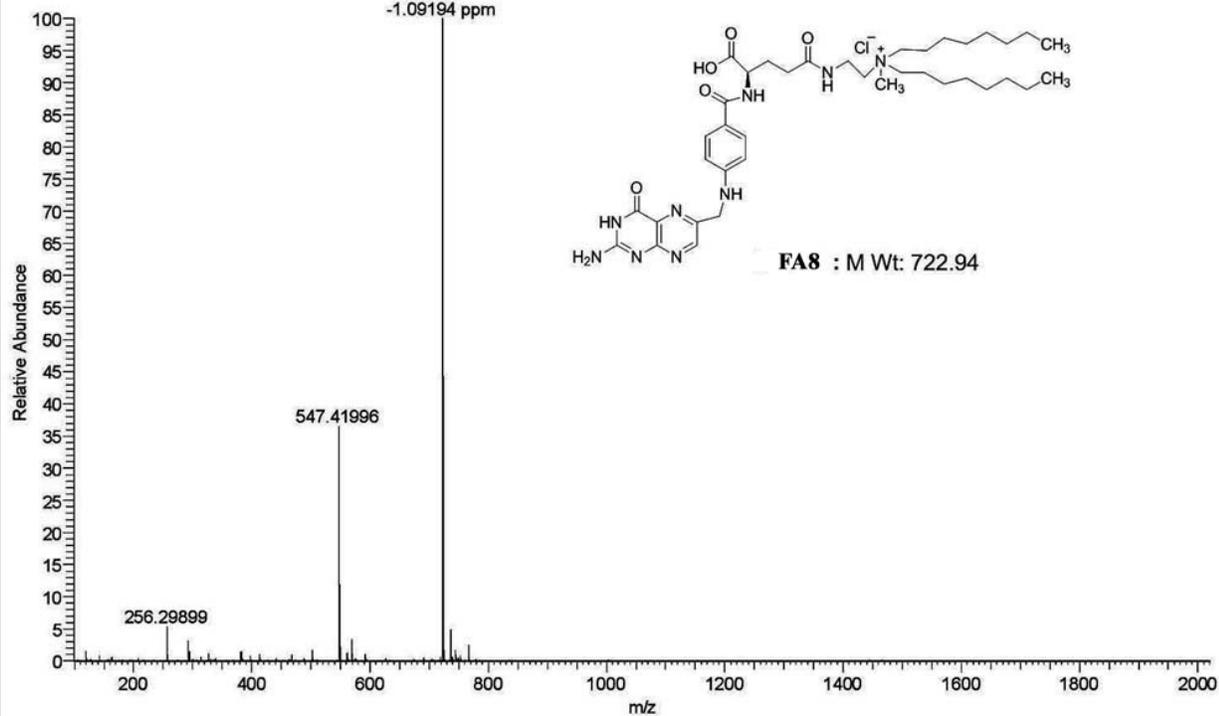


Fig. S12 HRMS for FA8

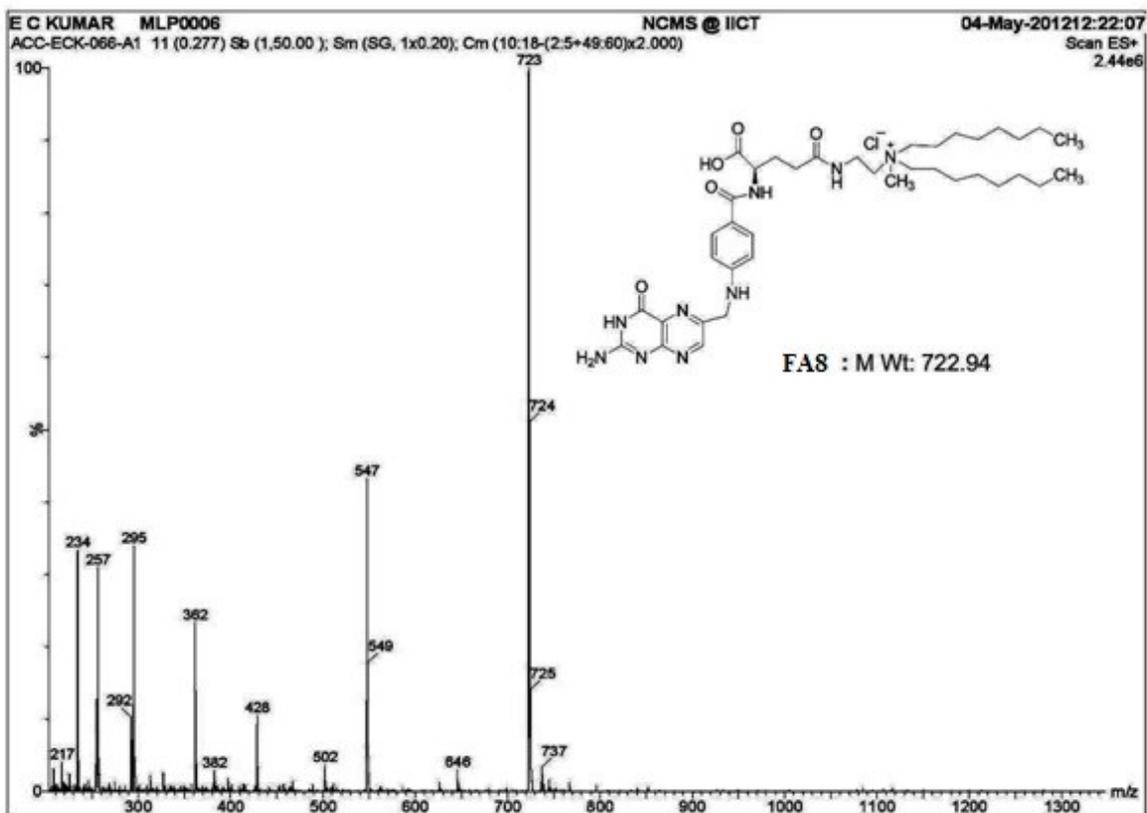


Fig. S13 ESI-MS of FA8

ACC-ECK #3-34 RT: 0.02-0.13 AV: 32 NL: 5.79E6  
T: FTMS (1,1) + p ESI Full ms [100.00-2000.00]

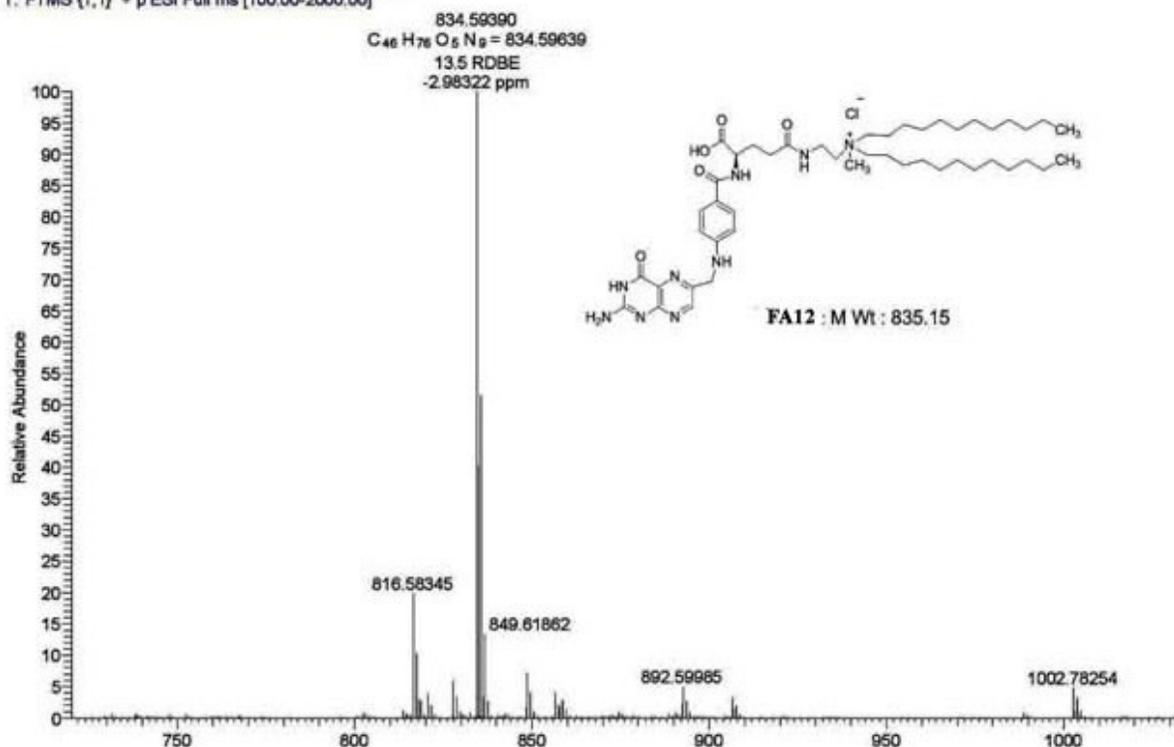


Fig. S14 HRMS for FA12

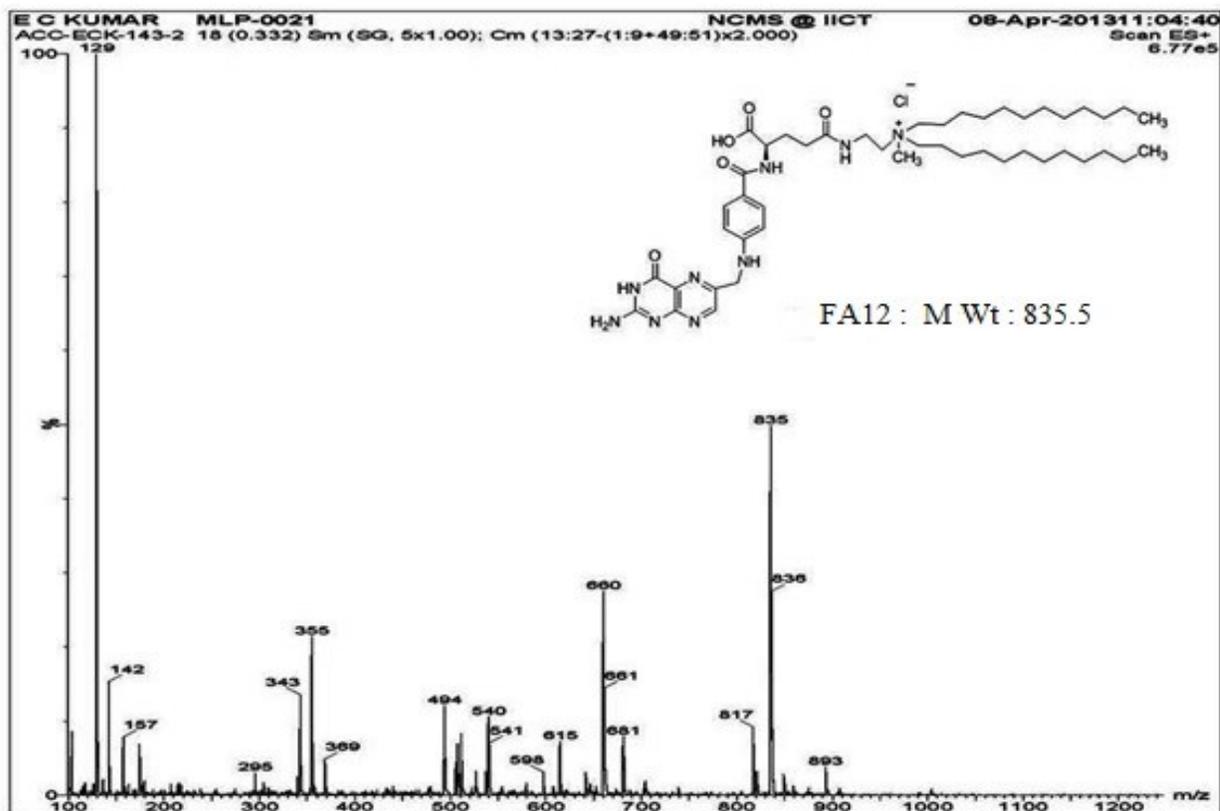
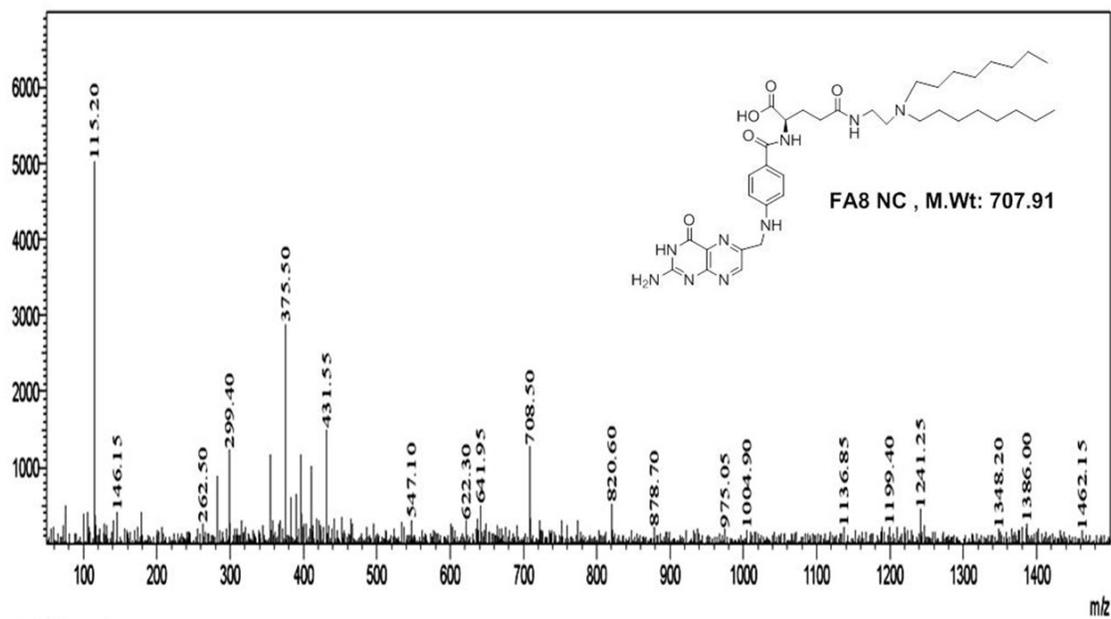


Fig. S15 ESI MS for FA12

Mode: ESI Positive



**Fig. S16** ESI MS for FA8 NC