Folic-Acid-Functionalized Doxil with Aqueous Sorbate Core Increases Cellular Uptake and Cytotoxicity

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A. Specific Aims

Doxorubicin is a successful ionizable drug and a key member of the anthracycline member of anticancer drugs. Its mechanism of action is intercalation of DNA, which is known to inherently target rapidly dividing cells with a higher utilization rate of the topoisomerase enzyme. Much effort and attention has been paid to the delivery and targeting of Doxorubicin, as development of improved targeting and delivery methods will allow for comparable local cytotoxic effects with a lower dose of Doxorubicin. A lower required dose has the potential to lower costs to consumers while reducing the systemic toxicity associated with Doxorubicin treatment.

Doxil is the PEGylated formulation of Doxorubicin. The PEGylation prevents uptake by the reticuloendothelial system and allows for a higher residence time at the tumor site. The use of the liposome also provides an opportunity for “decoration” of the surface of the carrier to target upregulated receptors on cancer cells, such as Folic Acid (FA) receptors. The predominant form of Doxorubicin at physiological pH carries a positive charge (Fulop). The bioavailability of ionizable drugs has been shown to be enhanced in some cases where there is a hydrophobic barrier involved. The mechanism behind this effect is thought to be the shielding of ion charge by the other pair member charge. This causes an increase in the lipophilicity of the ion pair relative to the separate ions.

Sorbic acid is a nontoxic acid that is frequently used in the food industry to preserve food. It is known for inhibiting bacterial growth while being nontoxic to humans. It has also been shown to participate in forming ion pairs with positively-charged drugs like Doxorubicin, as it exists as sorbate ion at extracellular pH.

We propose to assess the merit of formulating FA-decorated Doxil with sorbate in the aqueous core (FA-S-Doxil). Specifically, we hypothesize that ion pair formation at the liposome inner boundary will increase the lipophilicity of Doxil and increase the loading capacity of the liposome. Additionally, we expect that ion-pairs will have better cellular uptake characteristics from FA-liposomes than free Doxorubicin or Doxil. The following Specific Aims are designed to test this hypothesis, and to determine the potential of Sorbic acid to function as a drug targeting mechanism.

1. Create and characterize FA-S-Doxil liposomes.
2. To determine the effects of the sorbic acid core concentration on the release profile of doxorubicin from FA-Doxil.
3. Measure the loading efficiency of FA-S-Doxil.
4. To determine the level of cellular uptake of sorbic acid-Doxil ion pairs relative to unaltered Doxil and FA-Doxil.
5. Measure the cytotoxicity of FA-S-Doxil as a function of core sorbic acid concentration.
6. To determine the effect of sorbic acid core concentration on tumor growth rates in a local tumor in vivo.

B. Research Strategy

Doxorubicin is an anthracycline drug that intercalates DNA and causes apoptosis through its blocking of DNA synthesis and replication. The mechanism is naturally targeted at rapidly dividing cells, such as hair follicles and cancer cells. However, Doxorubicin has been shown to have adverse systemic effects including cardiotoxicity and recalcitrant heart failure, especially in high cumulative doses (Zhu, et al.) These adverse effects have prompted an intense search for targeting and delivery methods which will lower the total cumulative dose required for effective treatment of neoplasms. One such breakthrough was the invention of Doxil, a drug widely hailed as one of the first successes of nanomedicine. Doxil is a formulation in which Doxorubicin is encased in a PEGylated liposome.

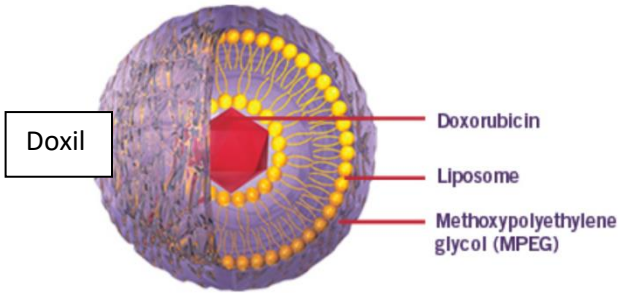
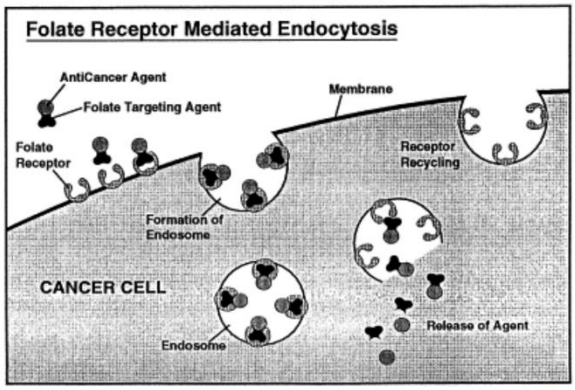


Figure 1: Doxil Structure

Doxil is a PEGylated liposome containing Doxorubicin.

Doxil reduced cardiac and liver related side effects compared to free Doxorubicin due to the liposome size (~ 100nm). It also took advantage of the Enhance Permeability and Retention effect in which leaky tumor vasculature combined with reduced lymphatic presence in tumors naturally increases the residence time of liposomes and other similarly-sized particles (Green). However, Doxil is not free from its own side effects, which still include inference with the pumping action of the heart as well as irritation and cracking of the hands and feet, known as Hand and Foot Syndrome. It is believed that this new side effects comes from the liposome having a higher preference for residing in the skin compared to free doxorubicin.

In light of the unique side effects and still present risks associated with Doxil, there is a clear incentive to improve targeting and release mechanisms in order to reduce systemic side effects for a given dose. For patients with a history of heart defects, an improved targeting and release breakthrough would mean that they may be able to receive a Doxil regiment that may have been deemed to risky in the past when a higher dose would have been required. New methods of targeting would thus decrease side effects and open the treatment to new individuals. For this reason, it is a very active area of research with hundreds of new ideas being explored. Some themes of the field include prodrugs which exploit the unique oxidative and acidic properties of the tumor environment, and decoration of a nanocarrier (e.g. liposomes) with high tumor cell affinity molecules.



Wang, S. & Low, P.S. Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. J Control Release 53, 39-48 (1998).

Figure 2: Folic Acid Targeting Schematic

One of the most recent developments is the conjugation of Folic Acid to the surface of the a nanocarrier in order to increase the affinity for cancer cells which often upregulate folic acid receptors on their surface (Lu, Zhao, Huang, et al). Folic acid can also be conjugated with PEGylated liposomes as well (Wang, et al). The folic acid PEGylated liposomes have been shown to have high affinity for tumor cells compared to PEGylated liposomes without folic acid.

There have also been extensive efforts to take advantage of the special low-pH environment present in tumors as well as intracellularly in order to target Doxorubicin.

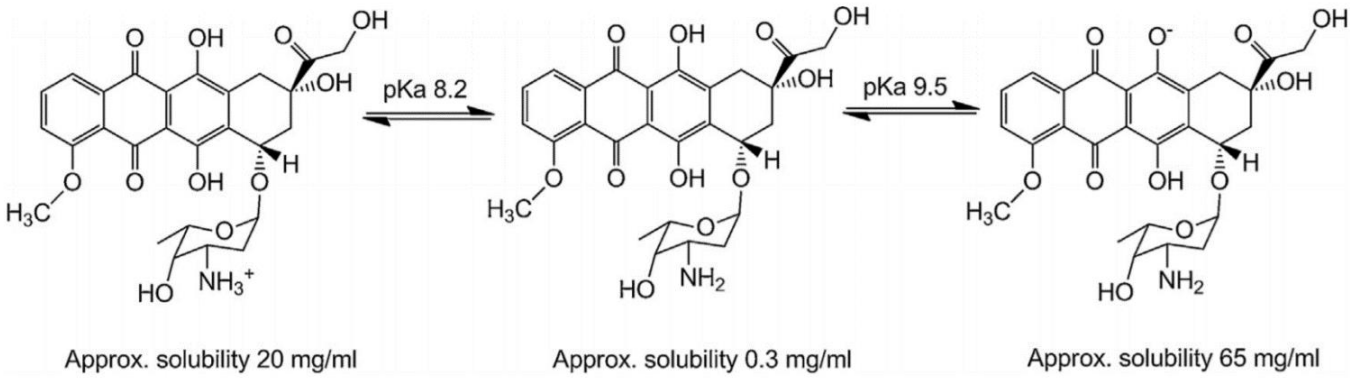


Figure 3: Acid-Base Properties of Doxorubicin.

At physiological pH < 8.2, Doxorubicin carries a positive charge. In its neutral state, it becomes extremely water insoluble and will precipitate out of solution (Fulop, et al).

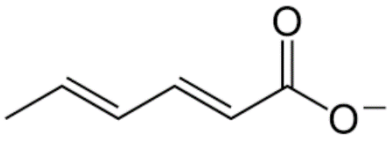


Figure 5: Sorbate Structure

Sorbate (pKa = 4.76) is negative at physiological pH and will form ion-pairs with positively charged drugs like Doxorubicin and Timolol (Higashiyama).

Our idea is that the release mechanism of Doxorubicin from Doxil can be greater facilitated not only by the close contact with tumor cells on account of folate-mediated pinocytosis and by interactions between folic acid receptors and FA on the surface of Doxil, but that it can be furth improved by the introduction of Sorbic Acid into the aqueous core of Doxil. It has bee shown that sorbic acid has been effective in similar studies where a charged drug needs to cross a hydrophobic barrier, such as Timolol crossing the corneal membrane in the eye to relieve glaucoma pressure (Higashiyama). The formation of ion-pairs inside of the aqueous core will zero net charge on the complex as a whole. This in turn will decrease the born or self-energy of the molecule, greatly increasing the favorability of the Doxorubicin-Sorbate complex in the liposomal wall. This should increase the loading efficiency of the drug, as well as facilitate the diffusion of the complex across the liposomal wall and cellular wall when they are drawn into contact by the interaction of the FA on the liposome and the FA receptor on the neoplasm cell. Once inside of the cell, the increased acidity of the environment will protonate the sorbate to a greater degree and encourage the dissolution of complexes into their two component molecules (sorbic acid and Doxorubicin). Doxorubicin will remain predominantly in its protonated form. This approach should decrease systemic side effects due to the increased affinity for residence in the liposomal wall, which should lead to a slower release profile in a strict aqueous environment. Folate mediated endocytosis will be the primary mechanism of uptake.

$$\Delta G = -\frac{N_A z^2 e^2}{8\pi\epsilon_0 r_0}\left(1 - \frac{1}{\epsilon_r}\right)$$

Figure 4: Born Energy of a Charged Species

Our experimental design will revolve around not only testing the hypothesis laid out previously, but on testing the translatability of that hypothesis into marginally more useful and successful therapeutic as defined by metrics which include cellular uptake, size, cytotoxicity, release profile, and tumor growth rates.

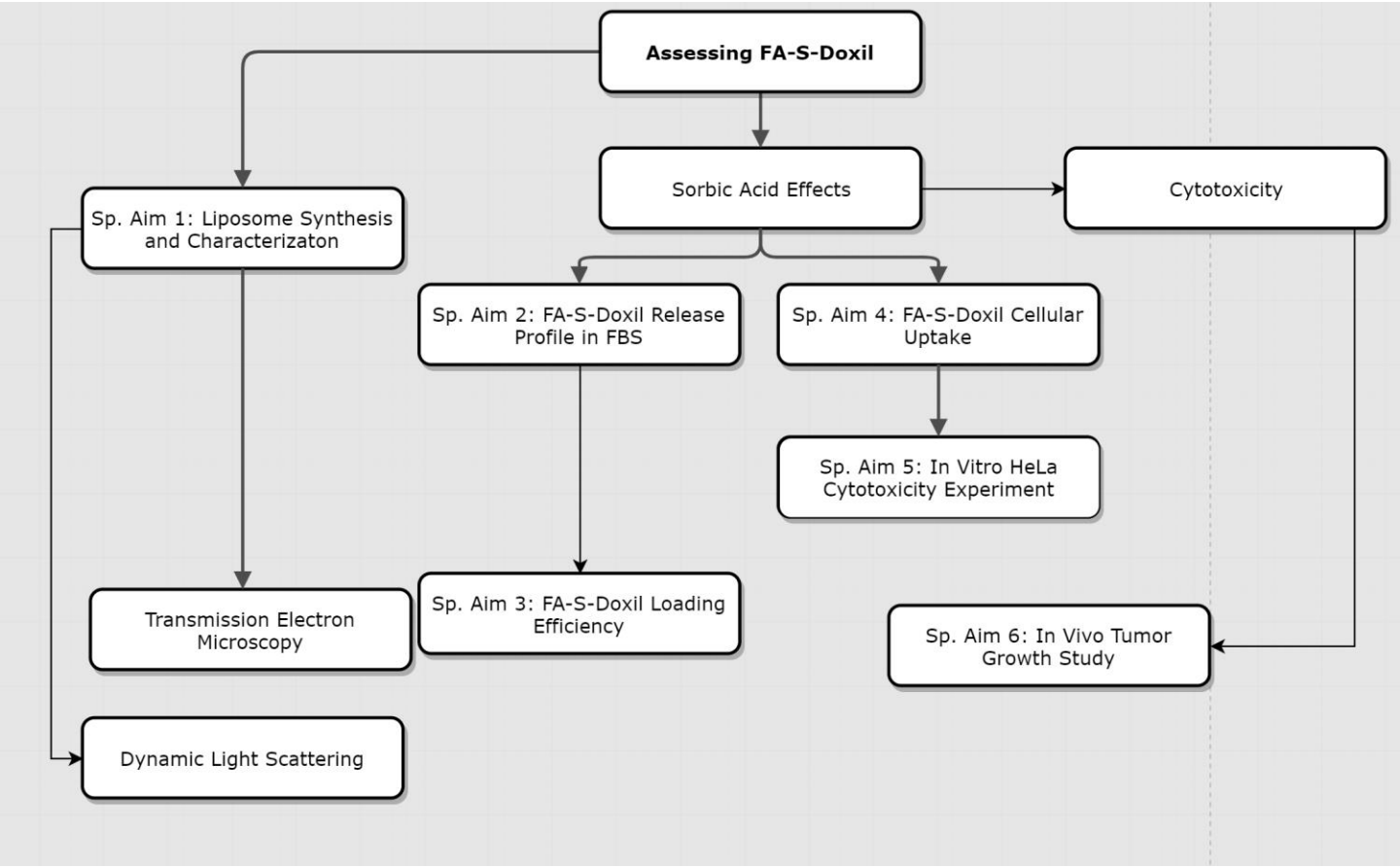


Figure 6: Experimental Overview:

A series of experiments designed to assess the therapeutic value of FA-S-Doxil as compared to Doxil. Doxil is still a competitive drug in the market today and a reasonable benchmark, so it will be used frequently as a control.

FA-S-Dox will be synthesized by thin-film hydration and extrusion of PEGylated FA - distearoylphosphatidylethanolamine and be subsequently rehydrated with an aqueous sorbic acid and Doxorubicin solution. The resulting solution will be extruded through a series of polycarbonate membranes with pore size 50-200 nm. This will be analogous to the method used by Wang et al.

After formulation of the FA-S-Dox, the size will be determined through standard Dynamic Light Scattering. This will allow us to determine the size distribution. It has been shown by Wang et al that the functionalization of the liposome with FA does not have a significant effect on the hydrodynamic radius of Doxil. We would expect an analogous result here. In ideal conditions, the FA-S-Dox would be monodisperse and roughly the same size as Doxil, allowing FA-S-Dox to take full advantage of the EPR effect to achieve passive targeting. The results of the DLS will be confirmed visually by the implementation of a Transmission Electron Microscopy (TEM) image. This will allow us to build a visual representation of the size dispersion.

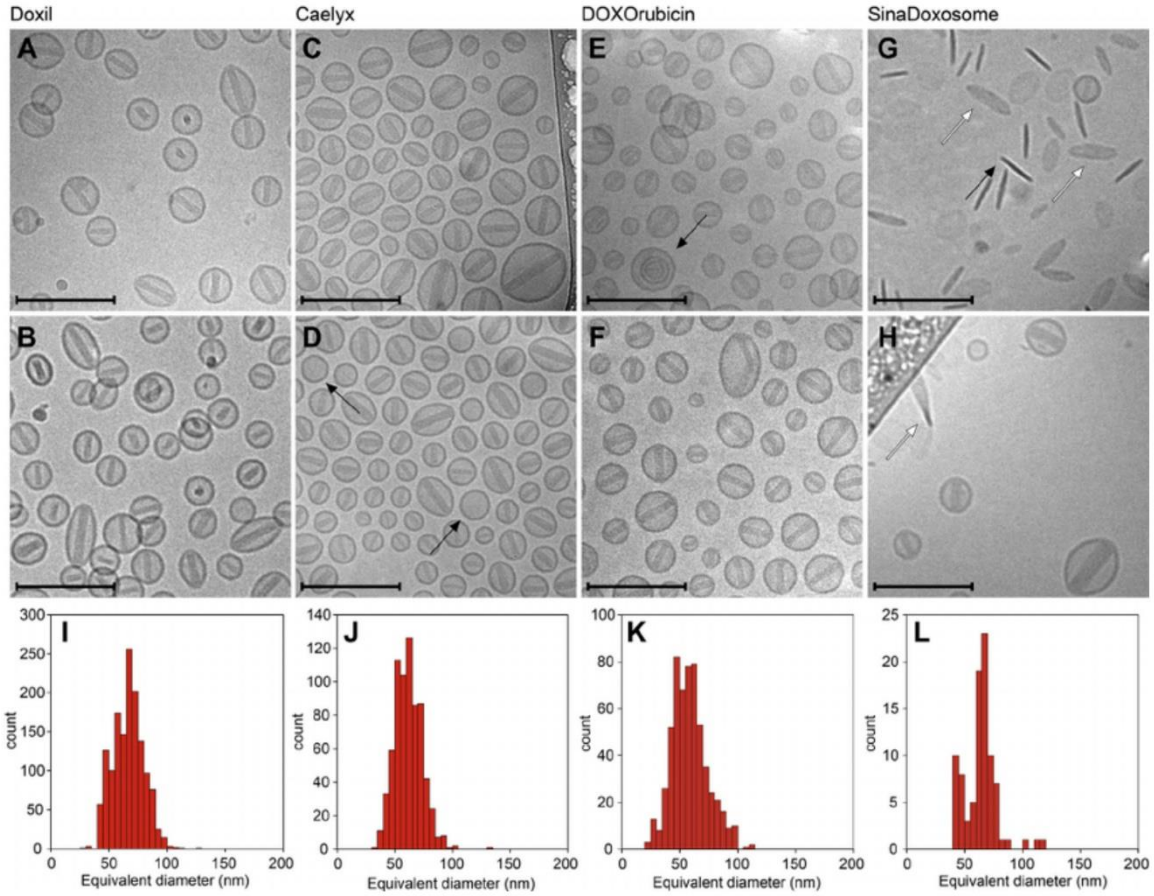


Figure 7: Representative TEM Data

Data that represents the desired output from TEM analysis specified by Specific Aim 1. Photo attributed to Wibroe.

The release profile of FA-S-Doxil will be essential to targeting to ensure that premature release does not occur before EPR targeting and FA-mediated endocytosis can occur. In order to test this, there will be an experiment in which our FA-S-Doxil will be placed in a 10% FBS solution. In parallel, traditional Doxil, free dox, and a 10% FBS solution will act as positive and negative controls. The solution will be sampled each hour and the amount of free Dox will be measured through fluorescent microscopy. Doxorubicin in a fluorophore that is easily quenched. As such, measurements will be taken at sparse time points (each hour) over a 24-hour period.

In order to determine the loading efficiency of the drug as a function of the sorbic acid concentration, de-emulsification will be performed on a series of 10 sample partitioned on the continuum from 0 mg/mL sorbic acid core up to and including the solubility limit for sorbic acid in water. After shaking and treatment with acid, DL% = (weight of dox extracted)/(weight of feeding material). The feeding material includes the liposomal walls as well as the doxorubicin.

A cellular uptake study will be performed in-vitro, again using the 10 samples of different sorbic acid concentrations. The concentration of Doxorubicin in the cells will be quantified by fluorescence and will be tracked over a 24 hours period. Regular Doxil will be included as a control. The total amount of fluorescence will be normalized to with the total (known) amount of doxorubicin.

A cytotoxicity study will be conducted in which an alive-dead stain will be introduced into the culture. This will allow a tracking of the cell survival rate over a series of times for each of 10 samples of varying concentrations of sorbic acid in the core. The zero-concentration case will be FA-Doxil, and regular Doxil will be included as a benchmark. It is hoped that the transmission of free Doxorubicin into

the cells in increased and this translated into an increase in the end-behavior cytotoxicity chart. See figure 8.

Finally, there will be in in-vivo study using the same 10 samples of FA-S-Doxil. Each FA-S-Doxil

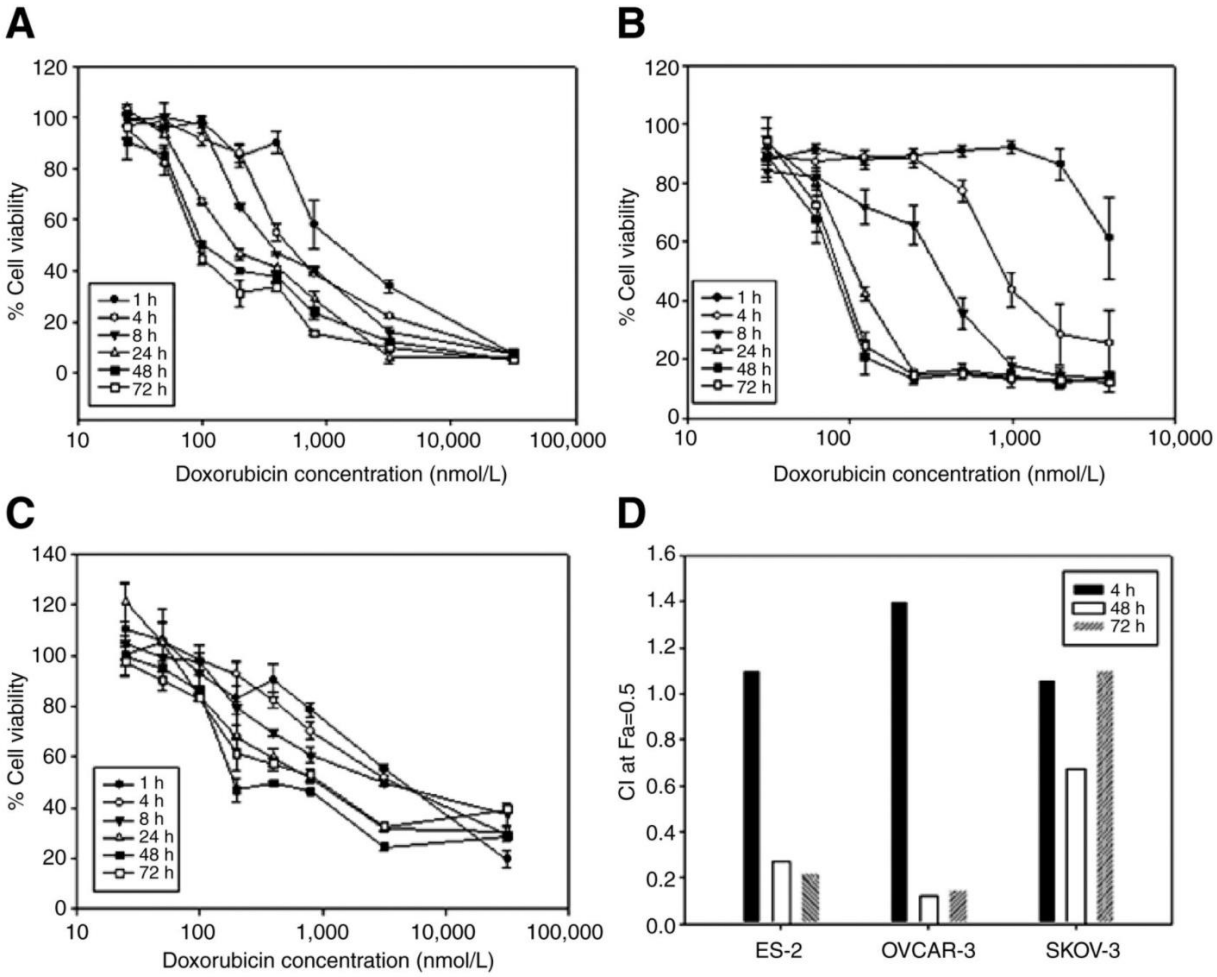


Figure 8: Representative Data for Doxil

A demonstration for the type of cytotoxicity study to be conducted in the case of FA-S-Doxil. Shown for feasibility purposes. Special emphasis to part (A). Attributed to Patankar et al.

core concentration will be tested in triplicate in a Chinese mouse model. A small tumor will be introduced into the leg of the animal. The controls will be no treatment and Doxil. The results will be normalized to the growth rate of the no-treatment case. This will be a crucial step in determining the viability of this modification. Injections will be given in the tail vein every day for 30 days at the same time. In this step, any side effects may manifest themselves. The FA-S-Doxil samples will be subject to a complex pharmacokinetic system in which it will need to prove itself efficacious. Success will be defined as a growth rate that is significantly lower than that shown in the Doxil group. That is the standard that will be required for FDA approval. In addition, mouse weight and death events will be recorded. See figure 9.

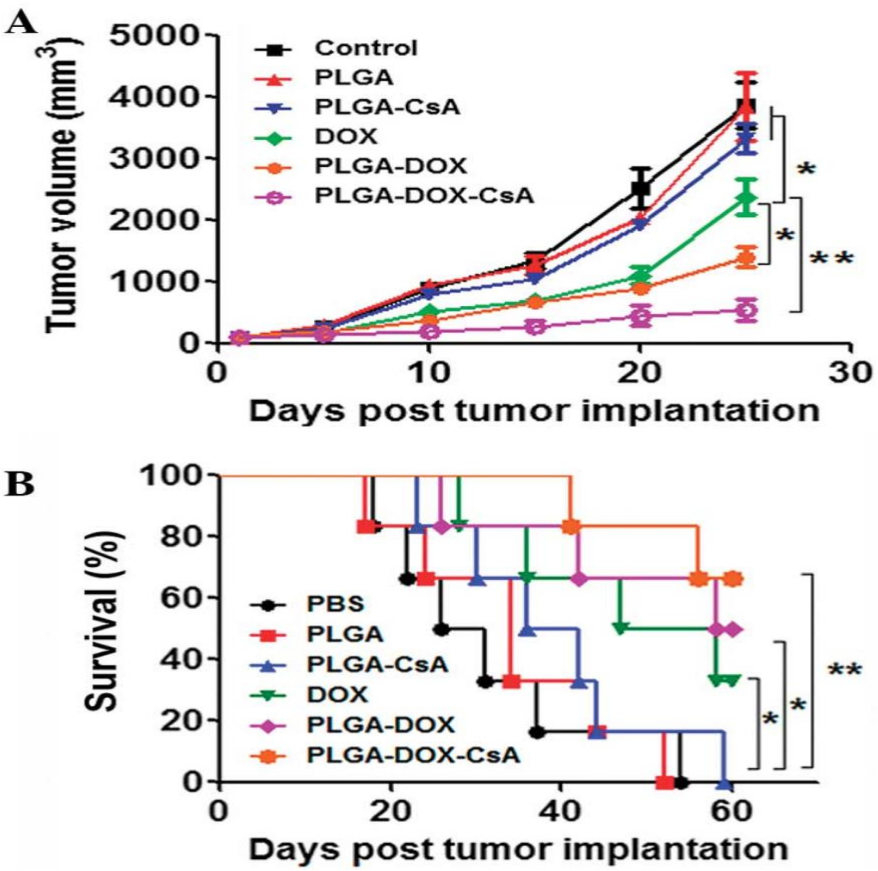


Figure 9: Representative Data for Doxil

A demonstration for the type of in-vivo tumor growth study to be conducted in the case of FA-S-Doxil. Shown for feasibility purposes. Attributed to Xu et al.

In each study, we are hopeful that FA-S-Doxil will have greater uptake, greater targeted cytotoxicity, and greater efficacy. However, there are some potential shortfalls of FA-S-Doxil that may arise, especially in the in-vivo study. It is hypothesized that the reason Doxil patients often have the Hands and Feet Syndrome while injections of free Doxorubicin do not induce it is because of the liposomal delivery being conductive to high residence times in the layers of the skin. If follows from the ion-pair formation that the affinity for the drug to leave the liposome when in contact with the skin membranes will only increase. In this case, it may be desirable to lower the concentration of sorbic acid to a point where there is a balance between the ion-pair and normal Doxorubicin forms of the drug in the core, in order to balance the side effects of Hands and Feet and congestive heart problems associated with free Doxorubicin.

Experiment	Task	Sp. Aim	Year 1	Year 2	Year 3	Year 4
1	FA-S-Doxil Synthesis	1	XXXX	XXXX		
2	FA-S-Doxil TEM/DLS	1	XXXX	XXXX		
3	Release Profile in FBS	2	XXXX			
4	FA-S-Doxil Loading Efficiency	3	XXXX	XXXX		
5	Cellular Uptake Study	4		XXXX	XXXX	
6	Cytotoxicity Experiment	5		XXXX	XXXX	XXXX
7	In Vivo Tumor Growth Study	6		XXXX	XXXX	XXXX

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