CHAPTER V.
Discussion and Perspectives

I. MAIN ACHIEVEMENTS OF THE THESIS

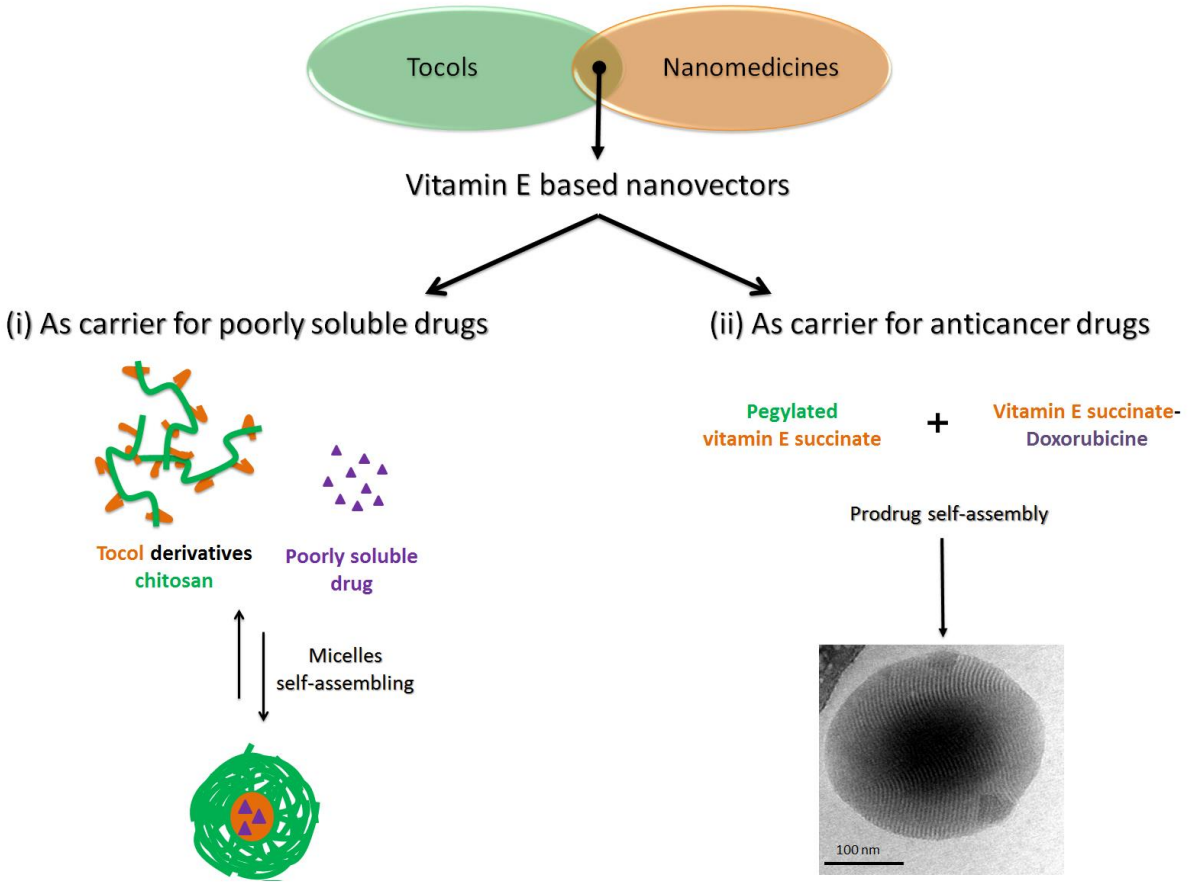

Figure 1. Vitamin E-based nanovectors (i) as carrier for poorly soluble drugs and (ii) as carrier for anticancer drugs.

The main objective of this thesis was to develop vitamin-E based nanocarriers to take advantage of the peculiar properties of vitamin E and nanomedicine (i) to enhance oral administration of poorly soluble drugs and (ii) to improve the efficacy of anticancer drugs (Figure 1). We chose to use tocols to overcome drug delivery issues for three reasons: (i) their biocompatibility, (ii) their biological activity and (iii) their tocophily. The main features of the two carriers we developed are summarized in the table below (Table 1).

Firstly, we have synthesized novel vitamin E derivatives of chitosan from which tocopherol succinate glycol chitosan (GC-TOS) conjugates which spontaneously formed micelles in aqueous media. The physico-chemical and morphological analysis of the GC-TOS micelles showed that the GC-TOS micelles have a low CMC, a polymer concentration-dependent size and were positively charged. GC-TOS was non-cytotoxic at concentrations up to 10 mg/mL on a Caco-2 cell model. GC-TOS micelles were able to encapsulate itraconazole and

ketoconazole, two model class II drugs. GC-TOS micelles increased by 3.4 fold the apparent permeation coefficient of ketoconazole across a Caco-2 cell monolayer.

Table 1. Characteristics of the two vitamin-E based nanovectors, polymeric micelles and prodrug nanoparticles.

Vitamin-E based nanocarrier	Polymeric micelles	Prodrug nanoparticles
Carrier composition	GC-TOS	N-DOX-TOS (4 mg/mL) $TPGS_{2000}$ (2 mg/mL)
Administration route	Oral	IV
Drug	Poorly soluble drugs	Chemotherapeutics
Synthesis ease	+	+
Formulation ease	+/-	+
Batch reproductibility	+/-	+
Diameter	202-566 nm	234 nm
PDI	0.4	0.1
Zeta-potential	9 mV	−4 mV
Stability	Low	High (>6 months, 4°C)
Drug loading	Low (2.2% w/w max)	Very high (34 wt %)

Secondly we have synthesized novel vitamin E prodrugs of cytarabine and doxorubicin. The prodrug of DOX and vitamin E, N-tocopherol succinate-doxorubicin (N-DOX-TOS), demonstrated the ability to self-assemble into nanoparticles with high drug payload in presence of D-α-tocopheryl polyethylene glycol 2000 succinate ($TPGS_{2000}$) as stabilizer. *In vitro* studies showed significant cytotoxic activity and cellular uptake of N-DOX-TOS nanoparticles. To our knowledge, no other vitamin E derivatives showing this physico-chemical behavior has been described in the literature highlighting the originality of the study.

II. KEY QUESTIONS

The main query to be addressed is to assess if the new carriers developed take fully advantage of the peculiar properties of vitamin E derivatives. For this purpose I discuss three questions based on our results, the literature and my personal opinion.

II.1. DO OUR VITAMIN E-BASED NANOCARRIERS SHOW POTENTIAL APPLICATION IN DRUG DELIVERY?

Two types of vitamin E carriers were studied for our purpose: (i) polymeric micelles to solubilize poorly soluble drugs and (ii) self-assembled prodrugs to overcome chemotherapy issues. Still, one question needs to be addressed: which of these carriers showed potential application in drug delivery?

First, the ease to synthesize and formulate the nanocarriers needs to be discussed. The synthesis of the conjugates was done by using a classical carbodiimide coupling method to bind the carboxylic acid of TOS to the amino group of the aminosugar of chitosan and doxorubicin. The formulation of GC-TOS loaded micelles requires the use of a GRAS organic solvent, ethanol (*1*). The N-DOX-TOS nanoparticle formulation requires the use of acetone which was found to have “slight health hazards” by a U.S-European study (*2*). Ethanol and acetone are not expected to represent safety issues since they are both evaporated during the formulation process. The preparation of the nanoparticles is much more rapid for N-DOX-TOS nanoparticles and requires only one step in comparison to GC-TOS loaded micelles.

Second, the batch-to-batch reproducibility is of primary concern for natural polymers like chitosan as it might alter the pharmaceutical properties of the polymer-based carrier and thus the biological response. The chitosan batch-to-batch variability refers to the variability which can affect the degree of deacetylation and the molecular weight of the polymer and the impurity level (*3, 4*). It is mainly due to seasonality of raw materials, freshness and quality of the biomaterials from which it is obtained, difficulties in process control and analytical methods. Efforts have been made but up to now international official standard to determine physico-chemical properties of chitosan have not been determined yet (*4*). On

the other hand, the vitamin-E prodrug batch-to-batch variability is limited due to a closely controlled synthesis, easier analytical characterization and formulation.

Third, differences between the two carriers stand in their physico-chemical parameters. N-DOX-TOS nanoparticles have a size of 234 nm with a narrow polydispersity at high drug concentration unlike GC-TOS micelles which form 566 nm micellar aggregates when concentration reaches 1mg/mL of GC-TOS. The low loading efficiency of GC-TOS micelles would limit the oral administration of drugs requiring high daily doses of GC-TOS. Zeta-potential of N-DOX-TOS nanoparticles is nearly neutral. This is an important feature since nanoparticles should be neutral or negatively charged for successful evasion of the renal elimination as the kidneys are able of filtering positively charged particles (*5*). Zeta-potential of GC-TOS micelles was positive with no sign of cytotoxicity *in vitro* which is essential for orally administered nanoparticles. Moreover, positively charged nanoparticles are able to interact with negative cell membranes of enterocytes favoring mucoadhesion in the gastrointestinal tract.

In my opinion, vitamin E-based prodrug nanoparticles have better features for further development and offered more possibilities of customization as co-delivery of drugs, active targeting strategies or scaling up than GC-TOS.

II.2. IS AMPHIPHILIC CHITOSAN AN EFFECTIVE OPTION FOR POORLY SOLUBLE DRUG DELIVERY?

Polymeric micelles are a promising approach in drug delivery as they have a good stability and they can increase the solubility of poorly soluble drugs by several orders of magnitude. Moreover, polymeric micelles are able to protect the drug from degradation and/or inactivation due to its entrapment of the drug in the inner core (*6*). Amphiphilic chitosan have been widely used as polymeric micelles, mostly for oral delivery of poorly soluble drugs or for IV chemotherapy (*7*). Thus, regarding to our results and the literature, I would like to assess the effectiveness of amphiphilic chitosan for drug delivery.

Amphiphilic chitosan are potentially biologically compatible polymers. Chitosan is a polycationic biodegradable polymer. Chitosan has good mucoadhesive properties due to its positive charge, which increases the adhesion to mucosa and so the time of contact for drug

penetration (*7*). The tunable aspect of chitosan is one of its major assets. Chitosan of different molecular weight can be produced by using an acidic degradation method (*8*). The presence of reactive groups allows chemical modification to develop amphiphilic polymers. Its amino groups are easily functionalizable with various substitution degrees by biocompatible hydrophobic moiety as highlighted in our review. Moreover, the hydroxyl groups can be used to modulate the hydrophilicity of the polymer by grafting glycol or PEG chains (*7*). The type of hydrophilic moeity is another important parameter to consider when synthesizing amphiphilic chitosan. One of the main concerns related to amphiphilic chitosan is aggregation as shown in our study. Glycol chitosan is a good alternative to chitosan as the glycol groups increased the water solubility of chitosan which is only soluble in acidic aqueous solutions. More recently, pegylated amphiphilic chitosan have been developed to enhance the stability of formulation (*7, 9*). The versatility of the chitosan is a major asset to optimize the drug loading and the biological profile of the nanocarrier. Nevertheless, batch-to-batch reproducibility is a major drawback when using chitosan derivatives as drug delivery system as discussed in the previous section.

The preparation of most drug-loaded amphiphilic chitosan nanoparticle requires complex method, organic solvents and long period of time. For example, the preparation of PTX loaded in 5β-cholanic acid modified glycol chitosan nanoparticles required methanol and dialysis method for 2 days followed by centrifugation and filtration (*10*). The preparation of GC-TOS micelles does not required complex methods but still needs more than 24 h. This is a major drawback for scaling up perspectives.

One of the limitations of using micelles is due to their low stability *in vivo*. For example, TPGS displays good solubilization properties but a high CMC (0.2 mg/mL) limiting its potential application as polymeric micelles (*11*). As it was highlighted in chapter III, most of the amphiphilic chitosan derivatives self-assemble in micelles at a low CMC (2-47 µg/mL) meaning they have a sufficient thermodynamic stability to reach the targeted tissue *in vivo*.

Regarding to the biocompatibility, amphiphilic chitosan we synthesized contains vitamin E which is Generally Recognized As Safe (GRAS) and TPGS which is approved as a safe pharmaceutical adjuvant (*11, 12*). Derivatives of TOS have been shown to be biocompatible. For example, polyglutamate-TOS biopolymer (Medusa®) has been proven to be safe and

biodegradable and a drug master file has been submitted to the FDA in the early 2011 (*13*). On the other hand, chitosan/vitamin E succinate copolymer exhibited significant cytotoxicity on cancer cells *in vitro*, with no apparent effect on hemolysis and erythrocyte agglutination (*14*). GC-TOS micelles did not show toxicity *in vitro* up to 10 $mg.mL^{-1}$. LDH measurements confirmed these results while TEER measurements highlighted the ability of GC-TOS to transiently reduce TEER. These data were collected *in vitro* with a monoculture model for a limited period of time and thus present some limitations. However, Caco-2 cells monolayers remains the gold standard as enterocytes model and is a relevant choice to predict the toxicity *in vivo* (*15*). Hence GC-TOS is a potential vehicle for the solubilization of poorly soluble drugs based on the cytotoxicity studies.

The drug loading of our GC-TOS polymeric micelles ranges between 0.14% and 2.4% depending on the BCS class II drug encapsulated. This is a major limitation for pharmaceutical application. We attempted to exploit tocophily to speed up the choice of the best-fitted micellar system for poorly soluble drug. Indeed, vitamin E has the ability to solubilize a variety of water-insoluble compounds depending of their VES parameter. Tocophily has already been used to develop emulsions and other parenteral formulations (*12*). Thus we developed GC-TOS polymer to increase the solubility of poorly soluble drug, particularly the ones with high vitamin E solubility. With 110 µL of vitamin E per gram of GC-TOS, our polymer is not taking effectively advantage of the tocophily property of vitamin E. In comparison, 1 g of $TPGS_{1000}$ contains 300µL of vitamin E. On the other hand, higher substitution degree in GC-TOS led to aggregation of the micellar system. Moreover, the encapsulation of itraconazole, which is tocophilic, was low in comparison with a non-tocophilic drug, ketoconazole. The itraconazole solubility when encapsulated in 1 mg/mL GC-TOS was 4 times lower than its projected solubility suggesting that all the tocopherol is not available for solubilization. In a recent study α-Tocopherol succinate-modified chitosan was synthesized by using a CS of 30 kDa and a substitution degree of 17% corresponding to 285 µL of vitamin E per gram of polymer (*16*). The micellar system was used to carry paclitaxel, another tocophilic drug. The loading efficiency of the micellar system was as high as 7.7%, corresponding to 77µg/mL. In TPGS micellar system, paclitaxel solubility was 38-fold higher than its water solubility at a TPGS concentration of 5 mg/ml, corresponding to 51 µg/mL (*17*). These values are much higher than the projected solubility measured by using the

tocophily parameter as shown in Table 2. These data suggest that the type of carrier might be a critical parameter to take advantage of tocophily.

Table 2. Solubility of itraconazole and pacliitaxel in water and vitamin E and projected solubility of these drugs in GC-TOS micelles, CS-TOS micelles and TPGS1000 (*12, 16, 17*).

	Itraconazole	Paclitaxel
Solubility in water (mg/mL)	10^{-6}	0.00034–0.030
Solubility in 1 mL of vitamin E (mg/mL)	60	11
Projected solubility in 1 mg/mL GC-TOS (µg)	6.5	1.2
Projected solubility in 1 mg/mL CS-TOS (µg)	17	3.1
Projected solubility in 1 mg/mL $TPGS_{1000}$ (µg)	18	3.3

Other amphiphilic chitosan-based drug delivery system have shown promising results with drug loading content as high as 10% wt for 5-β-cholanic acid glycol chitosan (paclitaxel) (*10*), 16% wt for O-octanoyl chitosan-polyethylene glycol (rapamycin) (*9*) and 8% wt for α-Tocopherol succinate-modified chitosan (paclitaxel)(*16*). It was also shown that the degree of deacetylation was a key factor in controlling the drug loading ability of N-phthaloylchitosan-g-mPEG (*18*). Thus, the drug loading potential of amphiphilic chitosan is highly dependent of properties of the chitosan, the hydrophobic moiety and the drug encapsulated.

To my view, amphiphilic chitosan have major limitations to be used as polymeric micelles for poorly soluble drug delivery: (i) chemical modifications remain complex due to the low solubility of chitosan in organic solvents, (ii) the batch-to-batch variability and (iii) the aggregation risk of the micelles. Finally, the specific limitations of the GC-TOS micelles we developed are due to (i) the aggregation of the micelles when increasing polymer concentration and (ii) their poor drug loading ability. These are probably the main reasons to the lack of clinical studies of amphiphilic chitosan in the pharmaceutical field.

II.3. ARE SELF-ASSEMBLED VITAMIN E-BASED PRODRUG AN EFFICIENT OPTION TO OVERCOME CHEMOTHERAPEUTIC LIMITATIONS?

Lipid–based prodrugs have gained attention as they are likely to self-assemble in nanoparticles and have the potential to improve the biopharmaceutical properties of the associated drugs. These compounds have been extensively studied in recent years to overcome chemotherapy drawbacks as nanoparticles have benefits for cancer therapy like the targeting properties and the potential to bypass drug resistance (*19*). Self-assembled

squalene-based prodrugs are one of the most successful examples (*20, 21*). We designed vitamin-E prodrug nanoparticles to take advantage of the biological activities of vitamin E and to develop a nanosystem containing several anticancer agents: (i) chemotherapeutic drug like DOX and (ii) TOS and TPGS which have demonstrated anticancer activity (*22, 23*).

The main limitations of designing self-assembled lipid-based prodrugs are (I) the lack of self-assembly behavior predictability and (ii) the risk of lowering the bioactivity of the drug. We experienced both issues with our vitamin E-based prodrugs. The N-cytarabine-TOS prodrug was not able to self-assemble into nanoparticle even when using TPGS as stabilizer. On the opposite N-DOX-TOS showed the ability to self-assemble into highly structured nanoparticles. These data showed the important role of the drug in the self-assemble property of a prodrug and in the structure of the nanoparticle. N-DOX-TOS nanoparticles showed remarkable physico-chemical properties, i.e. size, charge, stability, drug loading, which could be compatible with clinical use. The N-DOX-TOS nanoparticles were stable up to 4 mg/mL corresponding to a DOX concentration of approximately 2 mg/mL which is in the concentration range of Doxil®, one of the first FDA-approved nanomedicines (*24*).

Our vitamin E-based prodrug nanoparticles and self-assembled squalene-based prodrugs have similar physico-chemical properties. Nonetheless, the presence of PEG chains at the surface of our vitamin E nanomedicine may represent an advantage due to the avoidance of the RES while maintaining a stable supramolecular structure. Couvreur et al. have shown that the incorporation of pegylated cholesterol or pegylated squalene in squalene-based prodrug nanoparticles perturbed the supramolecular organization of the resulting nanoassemblies suggesting they are likely to be quite unstable in the blood stream after administration (*25*).

In vitro experiments demonstrated the cytotoxicity of vitamin E derivatives on MCF-7 breast cancer cell lines. The biological activity of the N-DOX-TOS nanoparticles was not statistically lower than doxorubicin *in vitro* after 72h on a MCF-7 breast cancer model. Nevertheless data showed N-DOX-TOS IC50 (1.1µM) was higher than that of DOX (0.08 µM) and the physical mixture of DOX, TPGS and TOS (0.14 µM) after 72h. The higher N-DOX-TOS IC50 was attributed to an incomplete release of DOX from the nanoparticles. This led our team to synthesize an ester derivative of DOX, 14-D-α-tocopherol succinate doxorubicin ester (O-

DOX-TOS), since ester derivatives are known to be less resistant then amido derivatives. This compound is still under investigation.

In summary, the ease to prepare the nanoparticles and the physico-chemical properties of N-DOX-TOS nanoparticles speak up to conduct further studies to assess the *in vivo activity* of our vitamin E-based nanoparticles. Even if there is no advantage in terms of biological activity in comparison to DOX, N-DOX-TOS nanoparticles may have less side effects by taking advantage of the EPR effect. Still, effort needs to be done to optimize this new nanomedicine and to reach our goal, namely to develop a more efficient and less toxic anticancer agent.

III. PERSPECTIVES

Our perspectives focus on Vitamin E based self-assembled doxorubicin prodrug which is the most promising nanosystem we developed. They start with the choice of the optimal prodrug and nanoparticle optimization, following by the *in vitro* assessment and finally *in vivo* evaluations of the anticancer delivery system.

III.1. NANOPARTICLE DESIGN

The synthesis of the DOX-TOS prodrug could be optimized by designing a targeted prodrug with a cleavable link for drug release at the tumor site (*26-31*). Prodrug targeting strategies have endeavored to take advantage of low extracellular pH, elevated enzymes in tumor tissues, hypoxic environment inside the tumor core, and tumor-specific antigens expressed on cell surface (*32*). DOX should be conjugated to TOS through a spacer that ensures the extracellular stability of the prodrug and incorporates a pre-determined breaking point leading to a release of the active drug at the cellular target site (*29, 32*). Acid sensitive linkers might be a good option as they can be incorporated into the prodrug to cleave it under the acidic conditions. Prodrug uptake through endocytitic process is characterized by highly acidic intracellular compartments such as endosomes (pH = 4.0–6.5) (*31*). Examples of acid sensitive linkage used in prodrug design are hydrazine (*32*) (Figure 2). Taking advantage of the pH decrease through pH-sensitive hydrazine link is a common strategy in DOX prodrug design (*33-39*). The hydrazine linkers undergo spontaneous hydrolysis in acidic environment while remaining stable at pH >7 in the general circulation (*29*). Binding TOS to DOX through a hydrazine bond to the doxorubicin C13 carbonyl might be a more effective approach to release both TOS and DOX inside cancer cells.

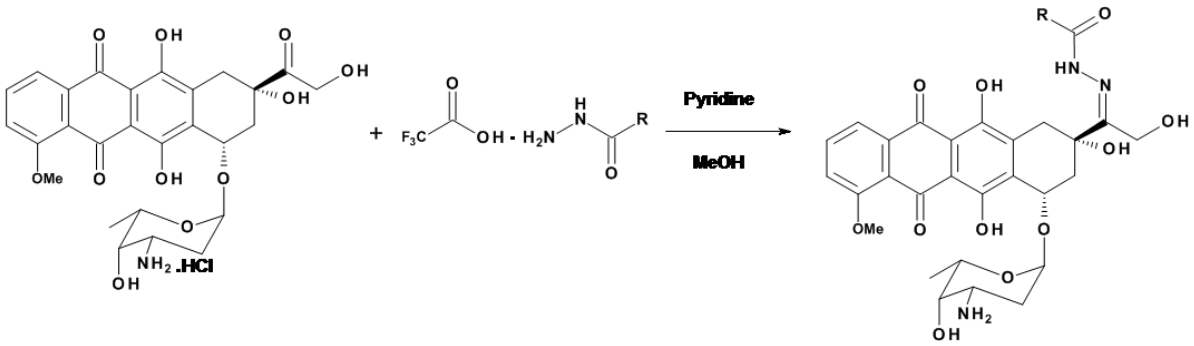

Figure 2. General synthesis scheme of hydrazine derivatives of doxorubicin (32).

The nanoparticle could be improved by using an active targeting strategy. DOX conjugates for selective delivery to tumors through receptor-mediated targeted delivery have been widely studied(*31*) (Figure 3). Folic acid (FOL) conjugated to TPGS could be used for targeted doxorubicin delivery to the cancer cells of folate receptor (FR) overexpression (*40*). FOL has already been successfully grafted to TPGS for the delivery of DOC and DOX through active targeting (*41, 42*). The FR can actively internalize bound-folates through endocytosis and is expressed at different levels in cancers (*43*) (Figure 3). The effectiveness of FA targeted drug delivery systems has previously been shown and some of them have already entered clinical trial process, e.g. FA conjugate of desacetylvinblastine monohydrazide (*41, 42, 44-48*). Incorporating FOL-TPGS into DOX-TOS nanoparticles would allow assessing the complementarity of passive and active targeting strategies. Fol-TPGS/N-DOX-TOS nanoparticles should have at least two advantages in comparison to the FOL-TPGS-DOX prodrug described in the introduction (II.5.2.3): (i) better pharmacokinetic characteristics as the FOL-TPGS-DOX prodrug is not able to self-assemble into nanoparticles; (ii) higher drug payload. *In vitro* experiments would be conducted to assess the effectiveness of active targeting. KB cells, a human nasopharyngeal epidermal carcinoma cell line which stably express FR, would be used as positive target and HT-1080 cell, a human fibrosarcoma cell line which lack FR, would be used as negative control (*49*). The usefulness of active targeting could also be demonstrated by pretreatment of cancer cells with FA before incubation with the FOL nanoparticles (*50*).

The efficacy of chemotherapeutic agents is reduced in cells which have developed multiple drug resistance (MDR). Since DOX-TOS nanoparticles contain a P-gp inhibitor, $TPGS_{2000}$, the cytotoxicity of on DOX-resistant cell line should be studied. P-gp-overexpressing, resistant

MCF-7/adriamycin (MCF-7/ADR) cells have been extensively studied for this purpose (*51-53*). Cytotoxicity would be assessed using tetrazolium salt MTT assay. Verapamil, a powerful P-gp inhibitor, would be used as positive control. This will help to figure out if vitamin E derivatives are attractive MDR sentisizer.

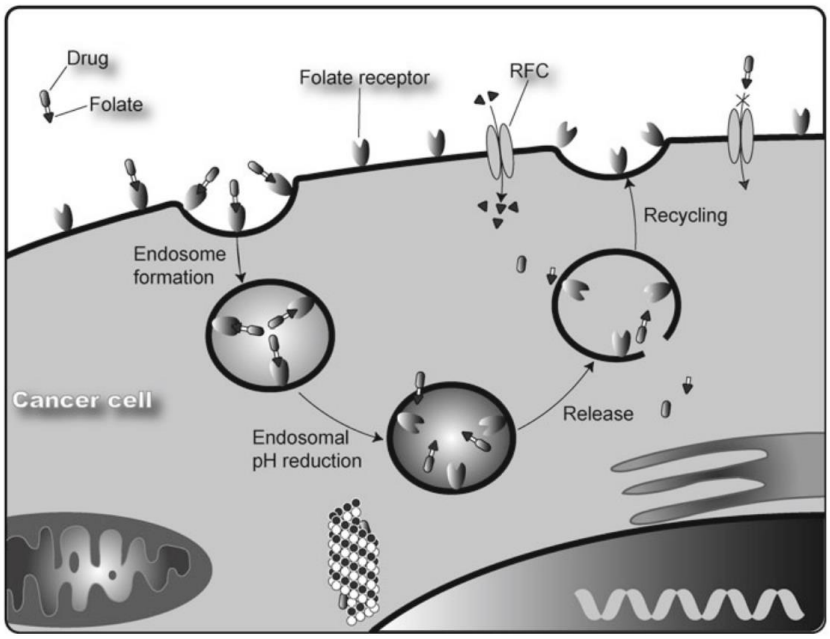

Figure 3. Scheme of FOL-drug conjugate internalization by the FR-mediated endocytosis pathway: (i) FA-drug conjugates bind specifically to the FR protein, (ii)he plasma membrane invaginates around the conjugate:FR complex to form an intracellular vesicle (early endosome); (iv) as the lumen of the maturing endosome acidifies to pH 6, the receptor changes conformation and releases the conjugate (54).

Finally, hydrophobic therapeutics could be encapsulated in the hydrophobic domains of nanocarriers. This could enable multiple drugs to be delivered simultaneously as new combination therapies (*55, 56*). Paclitaxel would be an ideal candidate since it has shown to be highly soluble in vitamin E (11 mg/ml) and exerts its anticancer activities through a different pathway then DOX (*12*). Synergistic effect of co-administration of PTX and DOX has already shown promising results (*57-60*).

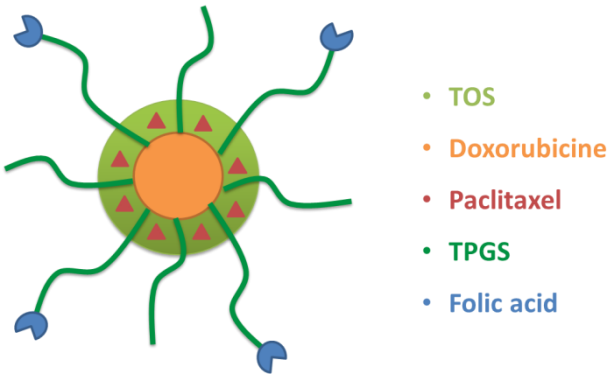

Figure 4. Schematic representation of the fully optimized DOX-TOS nanomedicine.

A fully optimized DOX-TOS nanomedicine would be able to fight tumor trough six ways: (i) passive targeting due to EPR effect; (ii) active targeting mediated by folic-acid TPGS; (iii) P-gp inhibition thanks to TPGS; (iv) anticancer properties of TOS; and chemotherapeutic activities of (v) paclitaxel and (vi) released doxorubicin (Figure 4).

III.2. IN VIVO ASSESSMENT OF VITAMIN E-BASED PRODRUG NANOPARTICLES

In vivo studies would be conducted by using KB tumor-bearing mice which is obtained by subcutaneous inoculation of KB cells into nude mice. This model is relevant because (i) the tumor has reproducible characteristics and over expresses the FR (ii) it is a solid tumor model thus EPR effect can be effective and (iii) folate-targeted liposomal DOX and non-targeted pegylated liposomal DOX have already been studied with this model (*61, 62*).

To assess *in vivo* potential, four formulations should be studied: (i) free DOX, (ii) DOX-TOS nanoparticles, (iii) FA-DOX-TOS nanoparticles, (iv) Doxil®. First maximal tolerated dose should be tested on healthy mice (5, 10, 15, 25, 40 mg/kg) (*63*). Survival and weight variations would be studied 15 days after IV administrationin. Secondly we would study the anticancer activity of the formulations after IV administration in KB tumor-bearing mice. Tumor growth, weight variation, tumor mass and survival rates would be studied (*61, 64*). Thirdly, biodistribution studies of the nanoparticles would be conducted. After 48 h, mice would be sacrificed and DOX concentration in liver, rate, kidneys and heart analyzed (*64*).

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