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Review

Doxil® — The first FDA-approved nano-drug: Lessons learned

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ABSTRACT

Doxil®, the first FDA-approved nano-drug (1995), is based on three unrelated principles: (i) prolonged drug circulation time and avoidance of the RES due to the use of PEGylated nano-liposomes; (ii) high and stable remote loading of doxorubicin driven by a transmembrane ammonium sulfate gradient, which also allows for drug release at the tumor; and (iii) having the liposome lipid bilayer in a "liquid ordered" phase composed of the high-T_m (53 °C) phosphatidylcholine, and cholesterol. Due to the EPR effect, Doxil is "passively targeted" to tumors and its doxorubicin is released and becomes available to tumor cells by as yet unknown means. This review summarizes historical and scientific perspectives of Doxil development and lessons learned from its development and 20 years of its use. It demonstrates the obligatory need for applying an understanding of the cross talk between physicochemical, nano-technological, and biological principles. However, in spite of the large reward, ~2 years after Doxil-related patents expired, there is still no FDA-approved generic "Doxil" available.

Contents

1. What led to Doxil® development: OLV-DOX

Development of Doxil was initiated in Israel and the USA ~14 years ago when it became evident in a "first in man" (FIM) clinical trial by

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Gabizon and Barenholz that a "first generation" liposomal doxorubicin did not justify further clinical development despite an elevation of drug MTD (rev. in [1]). In this FIM trial we used negatively charged, medium-size oligolamellar liposomes (OLV) composed of two low-T_m (fluid) phospholipids [the zwitterionic egg-derived phosphatidylcholine (EPC), the negatively charged egg-derived phosphatidylglycerol (EPG)], and cholesterol. In these OLV the doxorubicin was membrane associated and passively loaded during the lipid hydration. This liposomal doxorubicin (DOX) is referred to as OLV-DOX (for more information on the OLV-DOX formulation development, characterization, performance, and clinical experience see [2–17], and reviewed in [1]).

In this FIM we also determined the patients' plasma PK of doxorubicin and of phosphatidylglycerol (PG), a phospholipid that is not normally present in human plasma and therefore was used as the liposome marker of the OLV-DOX. From the ratio between the DOX PK and the PG PK we calculated the drug release rate in human plasma in vivo [14,15]. We also determined the OLV biodistribution (BD) by imaging of ^{111}In-remotely-loaded OLV (^{111}In-OLV, [14]). These studies clearly demonstrated that the clearance of DOX when delivered as OLV-DOX is a composite of two processes: (i) clearance of liposomes containing DOX by the RES, predominantly liver and spleen, but not the liver tumor, which is avoided by these OLV; and (ii) clearance of free DOX released fast from liposomes in plasma. The analysis, which includes PK of total drug (DOX), liposome-associated DOX, and liposome markers (PG and ^{111}In-OLV), suggests that both processes operate in human patients and that factors such as the patient's liver function may affect their relative contribution [14,15].

These PK, BD, and imaging data suggest that the reduced clinical toxicity of OLV-DOX results from a somewhat lower peak level of free drug and possibly some changes in the tissue distribution of the liposomes, with a partial shift toward drug accumulation in the RES at the expense of other tissues. The main limitations of the therapeutic strategy based on OLV-DOX, as revealed by this study, are the significant drug leakage and preferential RES uptake.

These shortcomings are probably the result of the basic inferior formulation physicochemical characteristics given below.

(i) Drug location in the liposome bilayer as opposed to encapsulation in the liposome aqueous interior. Bilayer-associated drug may be more accessible to be released to the plasma upon dilution and to associate with plasma proteins [10,11,15] This process is determined by the drug membrane/medium partition coefficient, which in the case of doxorubicin is not high enough to retain the drug during the major dilution the OLV-DOX undergo as a result of intravenous slow infusion to humans [12,15,18,19]. We demonstrated that the discrepancy between the successful therapeutic efficacy in mice and the failure in the human studies is a result of the very large difference in plasma volume (compare, ~1 mL in mice with >3500 mL in humans and in mice and human size). The association of doxorubicin with liposomes is related to the liposome membrane/aqueous medium (plasma) partition coefficient (Kp). Therefore, slow infusion of the liposomes will result in an immediate very large dilution of 3500-fold for each mL that reaches the plasma, compared with only a 5-fold dilution with the i.v. bolus injection of the same liposomes to mice. The fast free drug clearance from plasma keeps this huge dilution effect active throughout all the time of the infusion [12,15,18–20]. The burst of drug leakage shortly after injection into patients (Fig. 4 in [14]) is compatible with the dilution release effect.

(ii) The presence of a high mole fraction of PG in the liposome bilayer may accelerate uptake by the RES [13]; it may also induce complement activation [21–23].

(iii) The liposome size is too large to allow for extravasation in extrahepatic tissues [24] and to take advantage of the enhanced permeability and retention (EPR) effect that was first described by Matsumura and Maeda [25] and reviewed by Maeda et al. [26]. This effect may allow for selective accumulation of nanoparticluates in tumors due to tumor (but not normal healthy tissue) being rich in porous blood capillaries that are permeable to particles of 100 nm and smaller. In addition, the tumor tissue is poor in lymphatic drainage, which enables prolonged retention of the nanoparticles there, followed by local (tumor) drug release and/or for the liposomes to be taken up by the tumor cells. Therefore, the fact that the same dose-limiting bone marrow toxicity was observed with OLV-DOX and with doxorubicin administrated as is (standard care) is not surprising and can be assigned to the large extent of fast drug leakage from circulating liposomes.

In view of the OLV-DOX fast plasma drug release and the changes in tissue distribution and bioavailability, it is uncertain whether the somewhat increased tolerated dosage of OLV-DOX (over free, non-liposomal DOX) will result in an enhanced antitumor activity. The liposomes used in this clinical study are cleared fast by the RES of liver and spleen and to a lesser extent by the bone marrow. These human studies suggest that the mechanism of antitumor activity of OLV-DOX is complex, and presumably results from exposure of tumor cells to drug leaking from circulating liposomes and drug released from the RES. Obviously, drug leakage from circulating liposomes is undesirable since it resulted in unwanted cardiotoxicity. Regarding drug release from the RES, the clinical conditions most likely to benefit from this approach are limited. This approach should not work for treatment of solid tumors, as in most solid tumors drug exposure in relation to dosage may be suboptimal. The OLV-DOX is expected to be highly sensitive to factors such as RES/liver function, site of tumor involvement, and proximity of tumor cells to RES cells.

The failure of this OLV-DOX used in humans had some basic "take home lessons" that led us to the development of a liposomal doxorubicin formulation that should be less toxic and more efficacious than free DOX in humans. The failure of OLV-DOX served as the main driving force and as the basis for Doxil® development.

Our failure with OLV-DOX supported the 1980s' overall low expectation of liposomes as a broad spectrum drug delivery system. This disappointment was summarized in an almost "lethal" paper (to the medical application of liposomes) in *Cancer Research* by Poste et al. [27], which states categorically that: "The inability of liposomes to escape from continuous capillaries and their rapid uptake by circulating and fixed phagocytic cells calls into question the feasibility of using liposomes to 'target' drugs to cells in extravascular tissues".

This and Poste's 1983 publication [28] were "catastrophic" to the medical application of liposomes as it led the scientific community as well as the major grant agencies, the pharmaceutical industry, and the venture capital community to lose interest in this field. It took 10 more years and a few real clinical successes for the field to recover and gain back some trust that enabled the development of more than a dozen FDA-approved liposomal drugs from 1995 to the present.

In planning our advanced liposomal anticancer drug, Liposome Technology Inc. (LTI), Gabizon, and I decided to stay with doxorubicin as the cancer chemotherapeutic agent of choice as most of our considerations (medical, scientific, and practical) for the selection of this drug [1] were still valid. Doxorubicin, like many other anthracyclines, is produced by one of the *Streptomyces* bacteria (*Streptomyces peucetius* var. *caesius*). It was discovered in the 1960s near the Adriatic Sea, which explains the source of the brand name Adriamycin, and showed significant anticancer activity [29–31]. Doxorubicin acts on the nucleic acids of dividing cells by two main mechanisms of action. Firstly, it inhibits DNA and RNA synthesis by intercalating between base pairs of the DNA strands, thus preventing the replication and transcription in rapidly-growing cancer cells. This mechanism is based on the chemistry and physics of the doxorubicin molecule (its positively charged mannose amine that binds efficiently to the negatively charged nucleic acid phosphate diester groups and the excellent fit of the drug anthroquinone planar ring structure for intercalation into the double-stranded DNA). All together, these structural features lead to high affinity of the drug to double stranded nucleic acids in a way that is not dependent on cell metabolism. The high affinity to

DNA in vivo is easily measured physically from the fast quenching of the drug fluorescence upon its binding to the nuclear DNA. The DOX primary amino group, when combined with the drug amphiphacy, makes this drug a good candidate for remote loading into pre-formed liposomes, a property which enabled the successful development of Doxil (see below, and rev. in [32–34]). Secondly, doxorubicin inhibits the enzyme topoisomerase II, preventing the relaxing of super-coiled DNA, which is an additional way for blocking DNA transcription and replication. An additional major biological effect is that doxorubicin forms iron-mediated free radicals that cause oxidative damage to DNA, proteins, and cell membrane lipids. Especially sensitive to this effect are the mitochondrial membranes due to their high level of the negatively charged phospholipid diphosphatidylglycerol (cardiolipin), to which doxorubicin has higher affinity than to other phospholipids. This higher affinity to cardiolipin (which resembles the affinity to DNA) was the basis of Rahman and coworkers' [35–38] selection of cardiolipin as their formulation's negatively charged lipid. This oxidative induced damaging effect is now considered one of the main reasons for doxorubicin's toxicities and side effects. The fact that heart muscle is enriched in mitochondria explains in part the drug's high cardiotoxicity. Standard care treatment by conventional doxorubicin is done by i.v. administration of a relatively high drug dose, in the range of 10 to 50 mg/m^2 (rev. in [39–42]).

The most important clinical consideration for doxorubicin selection as a chemotherapeutic is that this drug is considered one of the most effective anticancer drugs ever developed, and therefore it became one of the main "first line" anticancer drugs almost from its discovery and it remains so till today. It is effective against more types of cancer (including leukemias, lymphomas, and breast, uterine, ovarian, and lung cancers) than any other class of chemotherapy agents [39–41].

However, like most other chemotherapeutic drugs, doxorubicin has toxicities and side effects attached to its use. Its most dangerous toxicity is the cumulative dose-dependent cardiotoxicity (irreversible congestive heart failure), which considerably limits drug usefulness (upper accumulative dose allowed is 550 mg/m^2). Its other side effects include severe myelosuppression, nausea and vomiting, mucocutaneous effects (stomatitis, alopecia, severe local tissue damage, and hyperpigmentation of skin overlying veins used for drug injection) [40,42–44].

It is the combination of doxorubicin's clinical use for such a broad spectrum of tumor types and the very large number of patients treated with it, together with its major deficiencies of dose limiting and accumulating dose limiting toxicities that made it very appealing and attractive to us (and many others) for selecting doxorubicin as the drug of choice for delivery by liposomes.

The scientific supportive reasons were that the drug's chemistry and physicochemical properties were well established, as were drug stability [17,45], and drug ADME (Absorption, Distribution, Metabolism, and Excretion) was common knowledge [46].

Our practical reasons that led us to select doxorubicin as the drug of choice include its distinct spectral (absorbance and fluorescence) properties, which allow easy and accurate quantification of doxorubicin level, its chemical degradation, its state of aggregation, as well as changes in its local environment (pH and level of hydrophobicity). Doxorubicin's reasonably high molar extinction at 486 nm (12,500 OD/M) allows for its quantification spectrophotometrically, and when combined with diode array HPLC enables following certain doxorubicin degradation products. Another major practical advantage of doxorubicin is its long wavelength (>550 nm), and high quantum yield fluorescence emission. The use of fluorescence detection increases the limit of detection by more than 100 fold. The fluorescence excitation and emission spectra are distinguished from each other and both are sensitive to the environment (pH, salt, etc.). This enables following doxorubicin PK and BD for long periods of time [12,15,47–51].

For the above reasons, we were not the only ones to select anthracyclines as the anticancer drugs of choice. All 3 liposome-dedicated US start-up companies were competing in developing 3 different liposomal athracycline formulations. Vestar (later changing its name to Nextar) in Pasadena, CA, developed DaunoXome, which was sold by Gilead Pharmaceutical and recently acquired by Galen. The Liposome Company (TLC) in Princeton, NJ, developed Myocet (now sold by Zenous Pharma Sopherion Therapeutics). Gabizon and I with Liposome Technology Inc. (LTI) in Menlo Park, CA, developed Doxil, which was produced by the Ben Venue CMO plant in Ohio, USA, until the production site was shut down after FDA/EMA testing at the end of 2011 (see more details in Section 7 below). Doxil was sold in the USA by LTI, which in 1996 changed its name to Sequus, which was bought by ALZA. The latter was bought by Johnson & Johnson, which until the recent shortage sold Doxil (=Caelyx) worldwide.

None of the above three liposome companies (Nextar, TLC, and LTI) survived. Currently, Doxil is by far the most successful product of these three.

By 1987, for the reasons described above, it became evident to us that our OLV-DOX formulation would not become a viable product. Based on the lessons learned from this failure in the clinical trial, we came up with guidelines that were expected to overcome at least most of the deficiencies demonstrated by our OLV-DOX (see above). The combination of the 200–500 nm size distribution and the negatively charged and "fluid" liposomes resulted in fast uptake of these liposomes by the RES; (namely, there was no RES avoidance). Not less important is the fact that in the liver the liposomes were not taken up by tumors, but by the RES macrophages [14]. Finally these liposomes reached the liver with a very low level of drug due to the fast drug release upon i.v. injection [14,15]. This fast release is explained by fast dilution induced release of most of the drug load in human plasma as discussed above. In addition such large liposomes could not take advantage of the extravasation typical of nano-particulates (having long circulation time) via the porous blood vessels of the tumor tissue. This unique microanatomy can be used as the Achilles heel of the cancer tissue for selective accumulation of macromolecules and nanoparticles in the tumor tissue (also referred to as passive tumor targeting). The latter unique extravasation was described first by Matsumura and Maeda [25] and referred to as the enhanced permeability and retention (EPR) effect.

The microanatomy of tumor blood vessels and its relevance to tumor therapy were studied extensively also by Jain and coworkers [52] while Bassermann [53] described the changes in vascular pattern of tumors and surrounding tissues during different phases of metastatic growth. Jain and coworkers also point out the high interstitial pressure in tumors (but not in healthy tissues), which reduces the diffusion of low molecular weight drugs from blood vessels into the tumors, thereby reducing the therapeutic efficacy and increasing toxicity of the chemotherapy [54].

The upside of the OLV-DOX development and clinical trial was that we were encouraged and confident that if we overcame the major obstacles discovered during the clinical trial the development of a viable liposomal doxorubicin formulation will be feasible. Actually "with the food came more appetite" as we decided to look for a totally alternative liposomal doxorubicin formulation which will be able to reach most metastatic solid tumors and will not be limited to liver-residing tumors.

2. Development of Doxil

2.1. Liposomal doxorubicin: the desired product profile

We decided that in order for the liposomal doxorubicin product to become an anticancer FDA and EMA approved drug the product should be characterized by the following features:

The liposomes used should be at the nano-scale (nano-liposomes) so they will be able to take advantage of the EPR effect and extravasate from the blood vessels at the tumor into the tumor tissue. Such liposomes can be considered as a "Nano-Drug".

However going nano imposes a major challenge of achieving a sufficient level and stability of drug loading. This issue is related to

the very small (nano) volume of the nano-liposomes. For doxorubicin this may be an especially difficult obstacle to overcome due to the high dose needed to achieve therapeutic efficacy (routine treatment by i.v. doxorubicin is 10 to 50 mg/m^2 [39–42,44]). In addition, the physicochemical properties and especially the low doxorubicin solubility are unfavorable to achieve sufficient passive loading into a 100-nm liposome's intraliposome aqueous phase. Therefore novel drug loading approaches were needed.

- In order to be efficacious the liposomes should reach the tumors loaded with a therapeutically high enough drug level.
- The drug PK and BD should be controlled by the nano-liposomes, namely the liposomal drug should demonstrate a highly prolonged plasma circulation time which is determined by the nano-liposomal carrier's prolonged circulation time in order to enable drug tumor accumulation.
- Drug should be available to tumor cells either by drug release at the tumor site or by the drug loaded nano-liposomes being internalized by the tumor cells.

Table 1 describes the requirements we found necessary to achieve therapeutically efficacious passively targeted drug-loaded liposomes for tumor treatment.

2.2. How and where Doxil was developed

To achieve all the above and a viable liposomal doxorubicin product, LTI used and combined two different novel ideas that matured into two novel technologies that resulted in two very different and independent patent families. The first one deals with the drug loading into nano-liposomes in a way that meets all the above needs, and the second, enables prolonging nano-liposomes' plasma circulation time and RES avoidance. Both technologies were not tried before.

In order to save time, all 4 aspects described in Table 1 were investigated in parallel at 4 different locations: LTI labs at Menlo Park, CA, by LTI scientists; Papahadjopoulos' lab at UCSF by Gabizon (continued later at Gabizon's lab at Hadassah University Hospital in Jerusalem); Terry Allen's lab at the University of Alberta in Canada; and at my lab at the Hebrew University-Hadassah Medical School in Jerusalem.

Table 1
The requirements to achieve therapeutically efficacious passively targeted drug loaded liposomes and means to fulfill them. For relevant references to Table 1 see Barenholz [34].

	Main requirements to achieve therapeutically efficacious passive targeting of liposomes to cancer tissues	Physicochemical and biophysical solutions used to meet the requirements
	Requirements	Solutions
1.	Extended circulation time in intact form in the human plasma	Development of sterically stabilized liposomes (SSL) composed of high T_m phospholipids, cholesterol, and a lipopolymer such as ^{2000}PEG-DSPE
2.	Sufficient levels and stable loading of drug in order for long circulating nano-liposomes to reach disease site with liposomes loaded with drug at a level needed to achieve therapeutic efficacy [$t_{1/2}$ of drug release in blood should be longer than circulation $t_{1/2}$]	Use of pH or ammonium ion gradients for remote (active) loading of amphipathic weak bases or acids into long-circulating nano-liposomes
3.	Extravasation into diseased tissue (tumor)	Using small enough (<120 nm, preferably <100 nm) nano-liposomes in order to efficiently extravasate through the gaps in the tumor vasculature (taking advantage of the EPR effect)
4.	Getting active drug into target cells	Releasing drug from liposomes through selective drug leakage at site due to diseased tissue properties, or using: collapsible ion gradient, or liposomes sensitive to secretory phospholipases, or by applying physical means such as heat [thermosensitive nSSL or use of radiofrequency (RF)] or ultrasound or by internalization due to active targeting.

LTI, Terry Allen, and Alberto Gabizon/Dimitri Papahadjopoulos worked on achieving liposomes having extended circulation and RES avoidance, which due to being at the nano-range size can take advantage of the EPR effect. The EPR effect was expected to result in a selective nano-particulates extravasation from the tumor capillaries to the tumor tissue. The liposomes with prolonged circulation time and RES avoidance were termed by Dr. Frank Martin of LTI "Stealth®" liposomes and this unique property of liposomes was referred to as "Stealthness", which means unseen or unrecognized as particulates by the RES.

At the same time I and my student Gilad Haran (now a Professor at the Weizmann Institute) developed a novel remote and stable loading method of amphipathic weak bases such as doxorubicin into nano-liposomes. This method met all the expectations described in Table 1 above [48,55,56] as it enabled the Doxil nano-liposomes to reach the tumor site loaded with sufficient level of drug and drug release that are needed to achieve therapeutic efficacy in humans [1,50,51]. This loading enabled intratumoral drug release and was not a "dead end" as was the case for "Stealth cisplatin", which did not enable drug bioavailability to the tumor cells, (rev. in [1,19,20,34,50,51]).

2.3. Remote loading of doxorubicin into nSSL to form Doxil

2.3.1. The need for remote loading

For liposome formulations designed for metastatic tumor treatment, intravenous (i.v.) administration is the only option. This requires the use of nano-liposomes for which high and stable (during storage and in circulation) loading are a must. This was not an easy task, due to the combination of very small nano aqueous volume of the nano-liposomes and the high dose of doxorubicin (~50 mg/m^2) needed to achieve therapy. To overcome these obstacles the intra-liposome drug concentration has to reach the range of hundreds of mM. However this is impossible to reach by passive loading due to the poor drug solubility. When the loading is poor, so will be the drug/lipid ratio. This means that either therapeutic levels of drug cannot be reached or therapeutic use of such liposomes will require administering very large amounts of lipids. In addition, when the loading is inefficient there is a great loss of the active agent and a need to remove unloaded drug. Therefore, the use of liposomes as a vehicle becomes inefficient as well as uneconomical.

A careful analysis of the available loading approaches that existed at that time (1986/1987) revealed clearly that the remote (active) loading approach is the only option to achieve a viable formulation, and in many cases the only way to achieve the desired intra-liposome drug concentration, usually defined as drug to lipid mole ratio [48] and rev. in [1,19,20,34].

Deamer and coworkers [57,58] were the first to demonstrate remote loading of amphipathic weak bases (such as catecholamines) by a pH gradient. This approach was extensively used by Cullis and coworkers for many amphipathic weak bases including doxorubicin [59]. Their studies on doxorubicin remote loaded into liposomes by pH gradient led to the development of Myocet by The Liposome Company (TLC) in Princeton.

We in Jerusalem used another remote loading approach, which is based on a transmembrane gradient of ammonium sulfate: $[(NH_4)_2SO_4]_{liposome} \gg [(NH_4)_2SO_4]_{medium}$ that acts as the driving force for the efficient and stable remote loading of amphipathic weak bases into preformed nano-liposomes [20,34,48,55,56]. This drug loading approach is based on the strategy of fabricating nano-liposomes that exhibit a transmembrane intra-liposome high/extra-liposome medium low ion gradient, which acts as the driving force for the remote loading of amphipathic weak base drugs. Amphipathic weak acids can be remote loaded by a similar approach in which the driving force is a transmembrane gradient of calcium acetate (rev. in [32–33]).

Since the application of this approach to Doxil, which was initiated in 1987/1988, [48,55,56,60], our remote loading approaches for loading amphipathic weak bases or acids were successfully employed for other drugs

[20,32–34,48,61,62]. The transmembrane ion gradients can be described as nano-chemical loading engines pre-fabricated into the liposomes, which then exhibit the desired pH and/or ion gradient. These nano-engines are achieved by using salts composed of either weak bases (e.g., ammonium sulfate) or weak acids (e.g., acetic acid). The degree of ionization of these compounds is pH dependent, their ionized species (i.e., ammonium and acetate) have a very low permeability coefficient and octanol-to-buffer partition coefficient and therefore they do not, or only very slowly, transverse the liposome lipid bilayer, while their un-ionized species have high permeability and octanol-to-buffer partition coefficients (exemplified by ammonia gas and acetic acid) and therefore these un-ionized species can diffuse relatively fast across the lipid bilayer and reach the intraliposome aqueous phase (rev. in [20,32–34]). The magnitude of the intraliposome high/external medium low transmembrane gradient of such ions is the driving force for remote loading, as they can be exchanged with amphipathic drugs (weak acids with the liposomal acetate ion and weak bases with the liposomal ammonium ion). The counterion of the gradient-forming ion (e.g., sulfate in the case of ammonium or calcium in the case of acetate gradient) can be selected so that it will control the state of aggregation and precipitation/crystallization of the drug-counterion salt in the intraliposome aqueous phase, thereby contributing to control the efficiency and stability of remote loading, as well as drug release rate at various temperatures [20,63,64].

It is important to note that the successful application of this nano-chemical engine benefits from the very small trapped aqueous volume of nano-liposomes (i.e., $2.209E+5\ nm^3$ for a 37.5-nm radius liposome), which supports faster and higher accumulation, as well as intraliposome precipitation of drug-counterion salt in crystalline or non-crystalline (amorphous) forms.

A very important question is how to select amphipathic weak base drugs that can be remote loaded, and especially, remote loaded by transmembrane ammonium sulfate gradient. The answer to these questions requires drug classification.

2.3.2. Drug classification: relevancy to the development of drug delivery systems (DDS)

In 1986 we looked for a simple way to classify drugs by their physicochemical features in a way that will enable the formulators to predict for which DDS they are most suitable and which drug loading approach should be used to load the drug into the DDS [18,19], and in the case of liposomes as the DDS of choice, to predict if the desired drug is suited for a remote loading approach. At that time, with very little available information, we came up with an oversimplified approach and classified all agents into 3 categories based on their oil/buffer and octanol/buffer partition coefficients (Kp). Category I, which fits mainly the oil phase of emulsions and microemulsions, are molecules having high oil/buffer Kp, which are considered highly lipophilic; these molecules do not fit liposomes as their carrier. Category II molecules, having low oil/buffer partition coefficient and medium to high octanol/buffer Kp; are amphipathic in nature. Category III molecules, having very low values in both partition coefficients, are, by definition, water-soluble. For some of the molecules, those which are amphipathic weak acids or bases, the classification between groups II and III is pH dependent, as these molecules can be ionized and charged or non-ionized and not charged. Only at the pHs when ionized, these molecules are at least to some degree water soluble [18–20,32–34]. Although the use of octanol/water partition coefficient to determine suitability of molecules to reside in a lipid bilayer is controversial, it is well established that it is indicative of agent transmembrane diffusion rate and permeability coefficient (as discussed by Stein [65]), and therefore it is relevant to loading efficiency, loading stability, and the drug release profile [65,66].

2.3.3. Remote loading optimization

Liposomes' remote loading by transmembrane gradients is one of the best approaches to achieve the high enough drug level per liposome required for the liposomal drug to be therapeutically efficacious. This "breakthrough" which enabled the approval and clinical use of nano-liposomal drugs such as Doxil, has not been paralleled by an in-depth process understanding that allows predicting loading efficiency of drugs. In our collaboration with Amiram Goldblum and his team at the School of Pharmacy of Hebrew University, we have been applying data-mining algorithms on a databank based on our laboratory's >20 years of liposome research experience on remote loading of 9 different drugs combined with information on basic physical and physicochemical descriptors that include, not only the partition coefficients (logP and/or logD), but also details on apolar and polar surface areas of the desired molecule (and the ratio between the two surface areas), its pKa, and logD at different pHs, as well as characterization of the liposome membrane used. All these data enabled us to build the first model that relates drug physicochemical properties and loading conditions to loading efficiency [32]. This study was a first computation-model-based attempt to enable selection of candidate molecules for remote loading and optimizing loading conditions according to logical considerations. However the small size of our "training set" (9 molecules only) forced us to use a simplified approach to database analysis using the J48 decision tree classification tool in Weka 3.4 software, validated using 10% leave-group-out (LGO) cross validation [32]. In a more recent study [33] we extended the training set to >60 molecules used in 366 loading experiments performed in many laboratories worldwide. This extended information enabled us to develop Quantitative Structure Property Relationship (QSPR) models of liposomes' remotely-loaded drugs. Both experimental conditions and computed chemical descriptors were employed as independent variables to predict the initial drug/lipid ratio (D/L) required to achieve high loading efficiency. Both binary (to distinguish high vs. low initial D/L) and continuous (to predict real D/L values) models were generated using advanced "machine learning" approaches and fivefold external validation. The external prediction accuracy for binary models was as high as 91–96%; for continuous models the mean coefficient R^2 for regression between predicted vs. observed values was 0.76–0.79. We suggest that QSPR models can be used to identify candidate drugs expected to have high remote loading capacity while simultaneously optimizing the design of formulation experiments [33].

In addition such computation-based approaches and modeling should help in designing pro-drugs suitable for remote loading.

2.3.4. Transmembrane ammonium sulfate gradient driven doxorubicin loading into nSSL

For the remote loading of doxorubicin into nSSL we applied the transmembrane ammonium sulfate gradient under conditions that $[(NH_4)_2SO_4]_{lip} \gg [(NH_4)_2SO_4]_{med}$ (lip is the nSSL and med is the extra-liposome medium). Fig. 1 describes the overall mechanism of this loading process. The drug loading is actually a base exchange

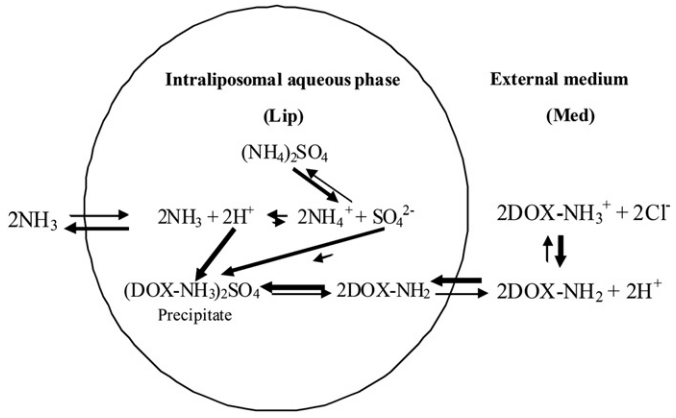

Fig. 1. Doxorubicin remote loading into nSSL exhibiting a transmembrane ammonium ion gradient. ➡ Represents processes occurring during drug loading, → represents processes occurring during drug release.

of the amphipathic weak base drug with the ammonium ions. For doxorubicin >90% drug encapsulation was obtained.

Doxorubicin is accumulated in the intraliposome aqueous phase, where it reaches a concentration >100-fold the drug level in the loading medium (this explains why we refer to it as active loading, as it goes "against" the drug concentration gradient). Based on various spectral analyses including X-ray diffraction [48,56,61,67] almost all the encapsulated doxorubicin is in the intra-liposome aqueous phase and most of it is in the form of aggregated or crystalline $(doxorubicin)_2SO_4$ salt. The loading is actually driven by the transmembrane ammonium ion gradient.

The best way to prove the cardinal and obligatory role of the ammonium ion in the loading of amphipathic weak bases is to use the ionophore nonactine [34], which exchanges ammonium ions with potassium ions ($NH_4^+K^+$). Nonactine does not act as a proton ionophore, has no effect on a proton gradient, which is not related to the ammonium ion gradient and has no effect on remote drug loading driven by a proton gradient. In the presence of nonactine and potassium ions there will be exchange in the intraliposome aqueous phase of NH_4^+ that will be released (effluxed) while being exchanged with K^+ that will be taken up into the liposomes (influxed) so the ammonium ion gradient will collapse and the loading of amphipathic weak bases will be prevented irrespective of the counter-anion that forms the ammonium salt [being either inorganic anions (i.e., chloride, sulfate, phosphate), or organic, low molecular weight (such as citrate or glucoronate) [32,34,64] or polymeric anions (such as dextran sulfate [68], heparin sulfate or sucralfate).

Table 2 [34] demonstrates that the loading of doxorubicin is utilizing 65 to 70% of the ammonium gradient and loading releases 65 to 70% of the encapsulated ammonium. The residual transmembrane ammonium gradient is essential for loading stability. We proved that nonactine induces release of amphipathic weak bases from the nano-liposomes only if the loading was driven by a transmembrane ammonium ion gradient (Fig. 1B). If the remote loading is driven by a pH gradient which is not ammonium ion dependent, nonactine will not cause release of the amphipathic weak base (see Table 2). Nonactine therefore acts differently from the ionophore nigericin, which exchanges between H^+ and K^+. Nigericin prevents amphipathic weak base loading into liposomes for both transmembrane proton and ammonium ion transmembrane gradients (Table 2). Thus, ionophores are important analytical tools to evaluate the role of proton and ion gradients in remote loading [34]. Ionophores are also important tools to prove that the precipitation of the drug-counterion salt is not a dead end and that the drug can be released and be bioavailable and efficacious, as we proved 20 years ago for Doxil [69].

The loading stability in the case of Doxil is a result of the combination of using sulfate as the counter-anion of the ammonium cation and the liposome membranes lipid composition and temperature, which affect the level of $(doxorubicin)_2SO_4$ precipitation.

The transmembrane ammonium-sulfate-gradient-driven drug loading differs from most other remote loading approaches since it neither requires fabrication of liposomes in acidic pH, nor alkalinization of the extra-liposome aqueous phase.

Doxil is a good example of remote loading by an ammonium sulfate gradient under conditions that $[(NH_4)_2SO_4]_{lip} \gg [(NH_4)_2SO_4]_{med}$. Fig. 1 describes the overall mechanism of this loading process. For more details see [20,32,34,48,55,56,60,61,63,67].

The efficiency of loading by this method and its stability are dependent on:

(1) The large (~10^{12}) difference in permeability coefficient of the neutral ammonia (10^{-1} cm/s) and the SO_4^{2-} anion (>10^{-12} cm/s)
(2) The initial pH gradient having the $[H^+]_{lip} \gg [H^+]_{med}$
(3) The low solubility of $(doxorubicin)_2SO_4$ (<2 mM), which also minimizes intraliposomal osmotic pressure and therefore helps to keep liposome integrity
(4) The asymmetry in doxorubicin partition coefficient (K_p) (K_p lip/external med > K_p lip/intra lip med)

Table 2
Characterization of transmembrane ammonium sulfate and pH gradients in ~100 nm nSSL before and after doxorubicin remote loading [1].

Property	Magnitude	Transmembrane proton gradient (ΔpH)
Transmembrane ammonium ion gradient determined by ammonium electrode	$[(NH_4)_2SO_4]_{liposome}/[(NH_4)_2SO_4]_{medium} \geq 1000$	
Intraliposome aqueous pH determined before DOX loading using pyranine preloaded in liposomes	<5.25, being out of the range of the measurement of pH range for pH determination by pyranine (pH 5.3–8.0)	
Determination of transmembrane pH gradient (inner low/outer high) as ΔpH		
± Before DOX loading		
By acridine orange (AO) distribution	96.4% by AO distribution into nano-liposomes	ΔpH ≥ 3.0 pH units
By ^{14}C methylamine (MA) distribution	87.5% by ^{14}C MA distribution into nano-liposomes	ΔpH ≥ 3.0 pH units
+ Nigericine	2.0% by AO distribution into liposomes	ΔpH = ~0.0
	3.0% by ^{M}C ^{14}C MA distribution into liposomes	ΔpH = ~0.0
+ Nonactine	4.0% by AO distribution into liposomes	ΔpH = ~0.0
	3.0% by ^{14}C MA distribution into liposomes	ΔpH = ~0.0
DOX loading		
% DOX loading	≥90.0%	
ΔpH after DOX loading	30–35% by ^{14}C MA distribution into liposomes	~1.0 pH units
ΔpH After DOX loading by ^{14}C MA distribution:		
+ Nonactine	2% by ^{14}C MA distribution	ΔpH = ~0.0
+ Nigericin	2% by ^{14}C MA distribution	ΔpH = ~0.0

Stability, size distribution, level of free drug, and ΔpH remain unaltered for more than 6 months storage at 4 °C. ΔpH for both % ^{14}C MA and % AO are based on calibration curves. For more details see [34].

(K_p oct/external med > K_p oct/intra lip med) [18,20]

K_p is a partition coefficient between the two phases defined in the parentheses; lip = liposome membrane, med = aqueous medium either external or intra-liposomal, oct = bulk octanol phase.

The asymmetry in DOX K_p means that the K_p of DOX in the extra-liposomal medium supports influx in a direction opposite to the ammonium sulfate gradient (namely, into the liposomes), while the K_p of DOX in the intraliposomal aqueous phase acts to reduce partition into the membrane, thereby reducing the desorption rate (k_{off}). The reduction in DOX K_p in the intra-liposomal aqueous phase is driven by the ammonium sulfate remaining inside the intraliposomal aqueous phase after DOX remote loading. Therefore, it seems that ammonium sulfate plays a multifactorial role in the remote loading and retention of the loaded drug in the liposomes. For Doxil the interplay between the above four points, when combined with Doxil membrane composition and liposome size, determines liposome performance.

The huge difference in the permeability coefficients (Pd) between the neutral ammonia (Pd = 0.12 cm/s) and the sulfate anion (Pd < 10^{-12} cm/s) combined with the efficient precipitation (gelation) of doxorubicin sulfate in the intraliposome aqueous phase and the low octanol/intraliposome aqueous phase partition coefficient as well as the (above described) asymmetry of DOX K_p all play a major role in the success of Doxil. The type (low molecular weight inorganic or organic, or polymeric) and valency of counter anion that forms the ammonium salt can be used to control the release rate of the liposome remote-loaded amphipathic weak base [20,34,48,64].

The $(DOX)_2SO_4$ long and fiber-like crystals are clearly shown in the Doxil cryo-TEM (Fig. 2A). Doxorubicin intraliposome nanocrystalization does not occur when the bivalent sulfate counterion was

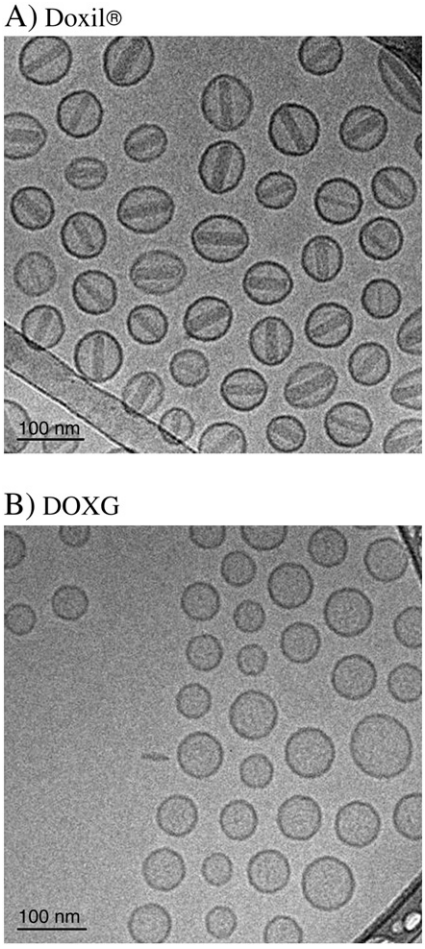

Fig. 2. Comparing cryo-TEMs of (A) commercial Doxil, which is doxorubicin sulfate remote loaded pegylated nano-liposomes and (B) DOXG, which is doxorubicin glucuronate remote loaded pegylated nano-liposomes. Doxorubicin concentration in both cases is 2 mg/mL. For more details on DOXG see [70].

replaced with the monovalent glucuronate counterion (DOXG Fig. 2B). The $(DOX)_2SO_4$ crystal formation is also strongly supported by small X-ray scattering (SAXS) measurements [67]. Accordingly, the Doxil demonstrates a unique reflection at 2.7 nm, which is identical to what was obtained for 30 mg/mL doxorubicin in either ammonium or sodium sulfate (250 mM each). This suggests that this unique reflection is a result of crystallization of $(DOX)_2SO_4$. No such discrete reflections were observed for 30 mg/mL doxorubicin in 250 mM ammonium chloride [67], or for doxorubicin glucuronate ([70] and Fig. 2B). Calculations made suggest that the $(DOX)_2SO_4$ gel is in the form of one-dimensional rods, in agreement with the cryo-TEM. Doxorubicin aggregation is also supported by the increase in 550/470 nm absorbance ratio [48]. Such intraliposome crystallization which results from transmembrane ammonium ion remote loading is not unique to doxorubicin, as the antioxidant nitroxide amphipathic weak base tempamine also shows intraliposome precipitation in the presence of sulfate as a counterion while no precipitation occurs in the presence of glucuronate as a counter-ion. However not all amphipathic weak bases behave like doxorubicn and tempamine; for example, the amphipathic weak base local anesthetic bupivacaine in the form of sulfate salt does not crystallize or precipitate [71,72]. The Doxil cryo-TEM above (Fig. 2A) demonstrates that $(DOX)_2SO_4$ rods touch the vesicle membrane, thereby forcing a vesicle shape change from spherical to non-spherical. Such shape changes do not occur for DOXG nano-liposomes (Fig. 2B), which do not show the presence of intra-liposome drug crystals and remain spherical in shape. However in spite of Doxil's shape change the Doxil liposome's membrane is robust enough to keep the liposome integrity as is indicated from the ability of the Doxil to withstand very high centrifugal forces [67]. This shape change of Doxil may be one of the factors leading to activation of complement by Doxil (see Section 3.3. and [22,23]). For doxorubicin glucuronate remote loaded pegylated nano-liposomes (DOXG) see Section 4 and [70].

Another issue, which may affect therapeutic efficacy and so far was neglected (although it may be highly relevant to drugs such as doxorubicin), is their tendency to self-aggregate at low drug concentrations (reviewed in [17]), forming oligomers of various (mostly low) mer number. Massive precipitation which can be observed by the naked eye occurs only at much higher doxorubicin concentration. The latter is highly dependent on the doxorubicin counter-ion, from >100 mM for glucuronate to 2 mM for sulfate [34,70]. The oligomerization at low doxorubicin concentration results from the stacking of the planar aromatic rings of the anthracycline due to interaction between the π electrons of the planar rings. It occurs for all doxorubicin salts. This self-aggregation is facilitated by increasing ionic strength. Doxorubicin dimers appear already at 1 μM and aggregates' size increases upon increasing doxorubicin concentration [17]. The effect of such oligomerization on therapeutic efficacy is not yet clear. However, based on simple geometric considerations, it is obvious that non-monomeric doxorubicin cannot interact with DNA in the same way as monomeric, and the exact location between the two DNA strands should differ (rev. in [17]). Therefore, the form (monomer versus oligomer) in which the drug is internalized by the tumor cell may be an important factor in drug therapeutic efficacy, with oligomers being less efficacious. The doxorubicin when released from intact Doxil would be released in its uncharged non-protonated form, but in the plasma and the interstitial medium it will be protonated and will form a chloride salt. Due to the large dilution it should be mostly monomeric and/or aggregates of low mer number and therefore the drug should retain close to full biological activity. However the question of what will be the biological activity of doxorubicin taken up by cells as part of intact Doxil in vivo still remain unanswered.

To sum up, while it is clear that the novel development of remote (active) loading driven by the transmembrane ammonium sulfate gradient for the doxorubicin was a breakthrough and one of the main reasons that enabled successful clinical use of Doxil (and its approval by regulatory agencies worldwide) there are still some open questions related to this loading approach which remain unanswered.

2.4. The role of drug release rate (k_{off})

The results of liposome loading when combined with liposome size, structure, lipid composition, and site of injection will determine the liposome bio-fate and rate of drug release in plasma and or tissues reached by the liposomes [18,19]. For example, for i.v.-administrated liposomal drug formulations, only when the drug release (determined by k_{off}) is slower than the liposome clearance (k_c) will the liposome control the drug pharmacokinetics and bio-distribution. When $k_{off} > k_c$, then the ratio k_{off}/k_c is a measure of the rate of drug release in vivo. Controlling this ratio is obligatory to achieve controlled drug release in blood or in the tissues reached by the liposomes. Therefore, this ratio also affects therapeutic efficacy of the liposomal drug. For drugs of fast clearance, when $k_{off} \gg k_c$ the benefits of use of liposomes for drug delivery will be limited for drug solubilization and dispersion but minimal or none for achieving beneficial bio-distribution and controlled drug release. In such cases the performance of the liposomal drug will be similar to that of the free drug. This was exemplified by our first generation "failed" OLV-DOX formulation (see part 1 above). An efficient and functional way to test the release rate is a functional test such as a cytotoxicity test of doxorubicin measuring its IC_{50} in cell culture (in vitro). This was well documented by Horowitz et al. [69], where it was shown that Doxil has about a 2-order-of magnitude higher IC_{50} (lower cytotoxic activity) than free doxorubicin, while as described above and reviewed in Barenholz [1], the IC_{50} value of our failed OLV-DOX was similar to the low IC_{50} value of free doxorubicin. The latter suggests a fast drug release upon the large dilution that occurs in the in vitro

test. This comparison between OLV-DOX and Doxil indicates that Doxil's robust drug retention is the reason for Doxil's high IC_{50} [69].

However, regarding therapeutic efficacy the opposite (a very low k_{off}) is as "bad" or even worse. Namely, when k_{off} is too slow and there is no liposome uptake by the target cells there will be no therapeutic efficacy even if the loaded liposomes will reach and accumulate in the target tissue very efficiently, as the free (bio-available) drug concentration at the target tissue will be too low to have therapeutic efficacy. This case is well exemplified by sterically stabilized cisplatin liposomes (Stealth cisplatin, which do not release the cisplatin [20,73–75]).

Meeting all the requirements of loading of the nano-liposomes, by itself would not be sufficient to achieve the passive targeting of enough of these nano-liposomes to the tumor site. To answer this need LTI developed the PEGylated liposomes (see below). Using doxorubicin remote loaded PEGylated nano-liposomes (Doxil®) enabled achieving a doxorubicin circulation half-life time in humans of ~90 h and doxorubicin presence in the human circulation of >350 h [50,51]. The process leading to the development of PEGylated liposomes is described below.

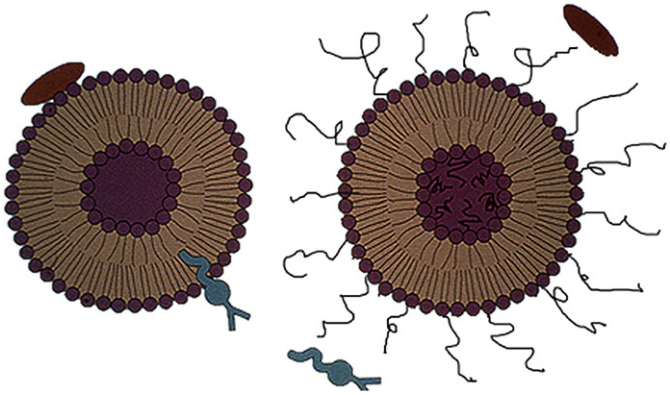

Fig. 3. A cartoon showing a comparison between a conventional liposome (left) and a sterically stabilized (PEGylated) liposome (right).The latter shows lack of insertion of plasma opsonins into its membrane. Courtesy of Dan Lasic.

2.5. Prolongation of nano-liposome plasma circulation time

Terry Allen at the University of Alberta was the first one to describe long circulating liposomal formulations. She included GM_1 ganglioside in the liposomes, which in mice is acting act as a "steric stabilizer". This means that in mice the inclusion of GM_1 reduces dramatically liposome uptake by the RES, leading to RES avoidance. This led to a prolonged plasma circulation time for the liposomes [76,77]. In 1986 LTI started to support Terry Allen's research on this topic. One year later Alberto Gabizon (in Dmitri Papahadjopoulos' Lab at UCSF) used hydrogenated phosphatidylinositol (abbreviated as HPI) as a steric stabilizer lipid [13]. LTI scientists tried another approach to achieve long-circulating liposomes of various lipid compositions that differed from those studied by Allen and Gabizon. They synthesized and studied novel lipids that were not similar to the well established natural lipid species, and instead they used pegylated phospholipids which are actually lipopolymers [78–81]. All these three groups (that were related to LTI) worked in parallel on nano-liposomes of different lipid compositions focusing on small unilamellar liposomes with narrow unimodal size distribution having a mean size of ~100 nm. These nano-liposomes were prepared by medium pressure extrusion using polycarbnonate filters with defined pore size [82] licensed by UCSF to LTI. These 3 labs used different lipid compositions in order to achieve the same goal of extended circulation time, RES avoidance, and intra-tumor accumulation. Terry Allen achieved it by inclusion of sphingomyelin to rigidify the liposome membrane and GM_1 ganglioside as a steric stabilizer. Alberto Gabizon was using hydrogenated phosphatidylinositol (HPI) as a steric stabilizer, which was included in nano-liposomes composed of "solid" high-T_m lipids mixed with cholesterol [13], while LTI scientists started working with DSPE-PEG; this was Annie Yau-Yang's idea combined with Carl Redmann's chemical synthesis [78,79], rev. in [80,81]. LTI was not the only group to work with PEG-DSPE; at the same time, Vladimir Torchilin and Leaf Huang and their teams joined forces and worked on it too [83], as well as Gregor Cevc's lab in Munich [84]. A comprehensive review on PEGylated liposomes is given in many of the papers in *Stealth Liposomes* [85], a book edited by Dan Lasic and Frank Martin.

The inspiration and motivation to start working with PEGylated lipids like PEG-DSPE came probably from pioneering research in the 1970s by Frank Davis, Abraham Abuchowski, and colleagues who foresaw the potential of the conjugation of polyethylene glycol (PEG) to proteins [86]. Abuchowski founded Enzon Inc., which brought three PEGylated drugs to the market. Various length (350–50,000 Da) chains of PEG polymer are available. Low molecular weight drugs were also PEGylated. However, the main PEGylated products so far are a few proteins and one liposomal formulation, Doxil® (the topic of this review article). For peptides and proteins (including antibody fragments), relatively large PEG polymers of >5000 Da were mainly used. It was found that pegylation helps to improve safety and efficacy as well as to reduce the immunogenicity of many therapeutics [87,88]. The suggested mechanism by which PEGylation "works" is that it is a result of the alterations it produces in the physicochemical properties of the molecule to which the PEG residues are covalently attached. These may include changes in level of hydration, conformation, electrostatic binding, and hydrophobicity/hydrophilicity balance. Increasing the level of hydration of the covalently attached PEG (3 to 4 molecules of water per 1 ethylene oxide oxygen [89,90]) induces changes in structure and leads to increase in the PEG moiety's volume and bulkiness. Altogether, this results in "steric stabilization" which reduces nonspecific protein–protein interaction and nonspecific protein–cell interaction (Fig. 3). These physical and chemical changes increase systemic retention of the therapeutic agent. Also, they can influence the binding affinity of the therapeutic moiety to the cell receptors and can alter the absorption and distribution patterns. PEG polymer has only 2 reactive OH groups (one at each end of the PEG molecule) and, in order to prevent the PEG from inducing intra- and inter-cross linkages, one of these hydroxyl groups is methylated so that the proteins and lipids are PEGylated by methoxy-PEG (mPEG).

The success of PEGylated proteins was the driving force for the successful development of Doxil® as the first FDA-approved liposomal drug and nano-drug (November 17, 1995). In lipids, PEG chains of 350 to 15,000 Da were tried (equivalent to 8 to 334 ethylene oxide units), and various considerations such as the metabolism of the PEGylated lipids and the rate of secretion via the kidneys were used in the decision which PEG length to select for lipid PEGylation and to the preferred choice of a 2000 Da PEG residue.

2.6. Selection of PEGylated nano-liposomes as the basis of Doxil

Each of the labs described above that worked on long circulating liposomes has its publications and patents on its unique liposome formulations. In the early 1990s it became evident that for various scientific and practical reasons (availability, cost, species specificity, etc.), the GM_1-ganglioside-based formulation was excluded from the race [81,91]. At LTI, HPI and PEG-DSPE remained in the race. In order for LTI to decide which of the two lipids will be the one to use in humans, we performed in 1991 a critical comparative PK study in Beagle dogs [92]. Dog was selected due to its much larger plasma volume (~500 mL), which resembles humans much better in evaluating "dilution induced drug release" [14,15] than small rodents with their very small plasma volume (and therefore no major dilution).

The Beagle dog doxorubicin PK study clearly demonstrated the superiority of Doxil, which was based on a 2000 Da PEG-DSPE as a steric stabilizer, to similar nano-liposomes based on HPI as a steric stabilizer, although both liposomal formulations were much superior to free doxorubicin [92].

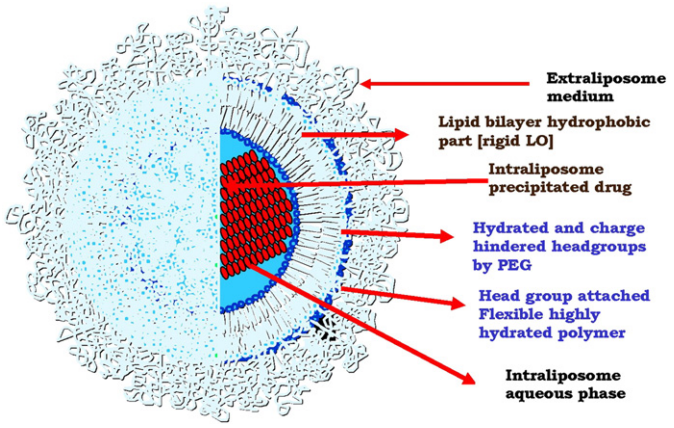

Fig. 4. A cartoon of Doxil® = PEGylated nano (<100 nm) unilamellar liposome. It is based on cryo-TEM, SAXS, WAXS, DLS, compressibility, and doxorubicin absorbance and fluorescence [48,61,67,90].

Using mice peritoneal macrophages (obtained from the ascitic fluid of mice treated with thioglycolate) in vitro, Doxil liposomes show only 40% uptake of liposomes of identical size distribution and lipid compositions but lacking the 5 mol% PEG-DSPE [93]. This reduction in liposome uptake is in direct correlation with the increase in plasma circulation time. The increase in mole% of PEG-DSPE can further reduce macrophage uptake and probably the nSSL circulation time [73,93,94]. For more details on the physicochemical effect of PEG-DSPE on nano-liposomes see [47,95].

2.7. Doxil — each component matters

In Doxil each component matters (Fig. 4) and contributes to the optimized performance!!! Doxil is an excellent example to demonstrate the essential and obligatory role of lipid physical chemistry, lipid biophysics, and nano-technology in the success of liposome-based drugs.

A calculation based on the concentration of Doxil components and on liposome size reveals that 1 mL of the commercial Doxil dispersion contains 2.3×10^{14} liposomes and each liposome contains ~10,000 molecules of doxorubicin, above 95% of which is in the crystalline phase.

3. Doxil performance in humans

3.1. Pharmacokinetics and passive targeting to tumors

In our Jerusalem 1991–1994 "first in man Doxil clinical trial" Doxil demonstrated high and selective tumor localization, published in *Cancer Research* [50]. The data (presented in Fig. 5) are the first proof for the EPR effect induced in tumors by passive targeting in humans [50]. The accumulation of Doxil in humans' tumors was further supported by direct fluorescence microscopy of patient biopsies by Gabizon and coworkers [96].

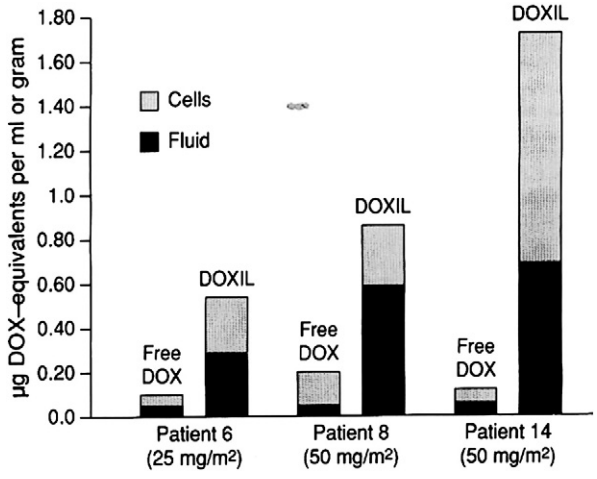

Fig. 5. Doxorubicin levels in patients' tumor biopsies, comparing free DOX and Doxil [100].

The above-cited Jerusalem pilot study includes 53 courses of Doxil (average of 3 per patient, spaced 3 to 4 weeks apart). It was aimed to determine in cancer patients the plasma pharmacokinetics and accumulation of doxorubicin in malignant effusions when given intravenously as Doxil, compared to free (non-liposomal) doxorubicin administered in what was considered then as "standard care". This study clearly showed much higher levels of doxorubicin both in tumor cells and tumor interstitial fluids after Doxil administration than after free doxorubicin administration. Using the cationic ion exchanger Dowex-50 [97,98], we found that more than 98% of the plasma doxorubicin after Doxil i.v. administration is liposome associated. Pharmacokinetics was determined for 25 and 50 mg/m^2 doxorubicin. The plasma elimination time of Doxil followed a bi-exponential curve, with half-lives of 2 and 45 h (median values), most of the dose being cleared from plasma under the longer half-life. A large difference in volume of distribution was also found (4 L for Doxil versus 254 L for free doxorubicin). Similarly, doxorubicin derived from Doxil showed a much slower rate of clearance (0.1 L/h for Doxil vs. 45 L/h for free doxorubicin).

The species of doxorubicin metabolites derived from Doxil doxorubicin in patients' urine were identical to those in patients injected with free doxorubicin; however the overall daily urinary excretion in the Doxil group was significantly reduced. Most encouraging are the results on the levels of drug at the malignant effusions, which were 4 to 16 times higher than after free doxorubicin administration. In addition, after doxorubicin administration, drug levels in the tumors peaked between 3 and 7 days post administration, which means the exposure of the tumor cell to the drug is much longer and at much higher levels than after free doxorubicin administration [50,51,99]. These data are in excellent agreement with our preclinical studies and indicate that stable remote loading of doxorubicin into long-circulating nano-liposomes serves well the objective of passive targeting of doxorubicin to tumors (rev. in [99]).

For further information on the superiority of the pharmacokinetic (PK) performance of Doxil, see [51]. That review summarizes the PK profile in humans at doses between 10 and 80 mg/m^2. The PK has one or two distribution phases: an initial phase, with a half-life of 1–3 h, and a second phase, responsible for most of the clearance, with a half-life of 30–90 h. The AUC after a dose of 50 mg/m^2 is approximately 300-fold greater than that with free drug. Clearance and volume of distribution are drastically reduced (at least 250-fold and 60-fold, respectively). These studies indicate the importance of utilizing the distinct pharmacokinetic parameters of pegylated nano-liposomal doxorubicin in dose scheduling.

3.2. Doxil bio-fate and mechanism of action

Animal data that come from many labs suggest that Doxil extravasates and accumulates as intact liposomes in tumors having "leaky" vasculature. For nano-long-circulating particulates the extravasation is probably the rate-limiting step of accumulation at the tumor tissue. Inside the tumor tissue Doxil liposomes "move" by convection and distribute through the tumor. This is the EPR effect [25,26,54,101]. Free doxorubicin, on the other hand, distributes into all tissues of the body, with tumor interstitial hypertension slowing down diffusion of the free drug from the vasculature to the tumor tissue [101,102]. The overall effect for doxorubicin administered as Doxil is that drug levels at the tumor tissue are higher than for doxorubicin administered as a free drug. In the case of Doxil almost all the plasma circulating drug is measured as liposome associated drug, in which form it also reaches the tumor. However, if there is insufficient drug release at the tumor site there is no efficacy in spite of the superior tumor localization and reduced toxicity. This was well documented in animals [49,75,103,104] and in humans [105,106] for Stealth

cisplatin, which is identical to the Doxil liposome in size distribution and lipid composition but is passively loaded with cisplatin [73,74].

However, this is not the case for Doxil, for which substantial therapeutic efficacy was shown in many tumor animal models and in humans (rev. in [1,50,99,133], and references listed therein). In the case of Doxil, doxorubicin release can be assessed by determining the presence and level of doxorubicin metabolites using HPLC [50]. The reason is that doxorubicin is metabolized only intracellularly [107], therefore the presence of doxorubicin metabolites in the cancer tissue indicates that drug was released from the liposomes and taken up by the cells where it is processed. Already in our Jerusalem 1991 Doxil FIM study [50], we demonstrated by HPLC analysis of human plasma, urine, and tumor effusions, that while in plasma the level of metabolites is very low and almost all the drug there is liposome associated, this is not the case for the urine and tumor effusions, where a relatively large fraction of the drug was transformed into doxorubicin's normal metabolites [50]. However, the mechanism of drug release and its internalization by the tumor cells is not yet known. Two different mechanisms can be suggested to explain Doxil's doxorubicin internalization into tumor cells in vivo: (i) intact Doxil liposomes uptake by cells, followed by intracellular drug release, or (ii), the doxorubicin is released in the tumor interstitial fluid from where it is taken up by the cells as a free drug. The contribution of the intact Doxil uptake by tumor cells must be minimal, as intact cisplatin Stealth nano-liposomes, which have similar lipid composition and size distribution as Doxil, does not show uptake of cisplatin by tumor cells and therefore they lack therapeutic efficacy (see references above). Therefore we are left with the second option of tumor cells' uptake of drug which was released in the tumor interstitium. Factors leading to doxorubicin release from Doxil may include collapse or partial collapse of the ammonium sulfate gradient and/or the destabilization of Doxil liposomes by phospholipases that hydrolyze the liposome phospholipids (see review by Mouritsen and Jørgenson [108]), thereby enabling faster doxorubicin release.

However, there are two major objections to the latter phospholipase-related drug release explanation. The first one is the fact there is no drug release in vivo from Stealth cisplatin which is identical in size and lipid composition to Doxil; the second is that the presence of cholesterol in the liposome membrane inhibits drastically phospholipase activity [108]. Therefore, we are left with the default, which suggests that the collapse of the ammonium sulfate gradient plays a more major role in doxorubicin release of Doxil in vivo. However the latter assumption is as yet unproven and its proof requires further in-depth investigation.

3.3. Doxil tolerability

A detailed report on Doxil (Caelyx) preclinical toxicology was summarized by Working and Dayan [133]. In our 1994 *Cancer Research* publication [50] we demonstrated that in humans overall Doxil is well tolerated and shows a distinct superiority over "standard of care" doxorubicin in most evaluated side effects. This was recently updated by Solomon and Gabizon's [99] review. In general, Doxil improves to a large extent patient daily compliance, and of special importance is the dramatic reduction of cardiotoxicity (when compared to standard care) which allows increasing the accumulated dose and thereby extending treatment duration. Recently a novel major immune modulatory effect of Doxil was discovered in patients. Treatment of ovarian cancer with the standard care first-line platinum-based chemotherapy has a very high record of efficacy at the beginning of the treatment. However, the majority of patients at advanced stages eventually have evidence of recurrent disease and are then treated with carboplatin doublets. Upon re-treatment, > 15% of patients show severe HSRs to the carboplatin treatment. These reactions were fatal in a number of instances, and are mediated by IgG to platinum, explaining some cross-reactivity with cisplatin, and rarely with oxaliplatin. However, when carboplatin is given together with Doxil these reactions were not observed [109]. Moreover, of great interest is the finding in a recently published randomized trial that the doublet carboplatin/Taxol combination is significantly more often associated with carboplatin reactions than the doublet carboplatin/Doxil (Caelyx) combination [110]. Namely, Doxil seems to have an immunosuppressive effect that prevents/reduces the secondary (IgG-mediated) hypersensitivity response to carboplatin.

However, in spite of overall tolerability superiority of Doxil over doxorubicin, two side effects not typical of what is observed for the free drug standard of care treatment were observed for Doxil. The first and more dominant one results in grade 2 or 3 of desquamating dermatitis and is referred to as Palmar Plantar Erythrodysthesia (PPE) or "foot and hand syndrome". The PPE, which was already demonstrated in our first FIM study [50] and recently reviewed by Solomon and Gabizon [99] shows up as redness, tenderness, and peeling of the skin. The prevalence of this side effect limits the Doxil dose that can be given as compared with doxorubicin in the same treatment regimen. One of the bad aspects of the PPE is that its severity increases with dose and was more pronounced for 3-week intervals than 4-week intervals between treatments. So far there is no complete solution to this effect, except the above increase in interval between treatments [99]. The second effect is an infusion-related reaction that shows up as flushing and shortness of breath; it is a unique adverse immune phenomenon that Doxil, like many other nano-systems, can provoke. It is actually a complement activation-related pseudo-allergy (CARPA). CARPA is an acute hypersensitivity, or infusion reaction and called thus because of the causal role in its patho-mechanism of complement activation instead of IgE binding [111], rev. in [22,23]. CARPA can be reduced by slowing the infusion rate and by premedication. More details on Doxil tolerability can be found in [50,99], Doxil homepage (www.doxil.com), and drugs online (www.drugs.com/pro/doxil.html). There is a large probability that reducing these two side effects and especially reducing or overcoming the adverse PPE effect may improve the overall Doxil performance and extend its application.

3.4. Doxil therapeutic indications

This review will not discuss in detail Doxil clinical performance, a topic covered extensively in many publications and reports. Good starting points are the review by Solomon and Gabizon [99] and the two updated highly relevant websites, Doxil homepage (www.doxil.com) and drugs online (www.drugs.com/pro/doxil.html). The Doxil item in Wikipedia entitled "Clinical pharmacology of liposomal anthracyclines: focus on PEGylated liposomal doxorubicin" is also helpful. As of today (March 6, 2012), Doxil showed 501,000 results on Google search, 7590 on Google Scholar and 46,166 on PubMed search.

A summary of indications for Doxil approved by the U.S. FDA and/or European Medicines Evaluation Agency (EMA), with approval year is given below.

- AIDS-related Kaposi's sarcoma: superior efficacy over former conventional therapy (1995).

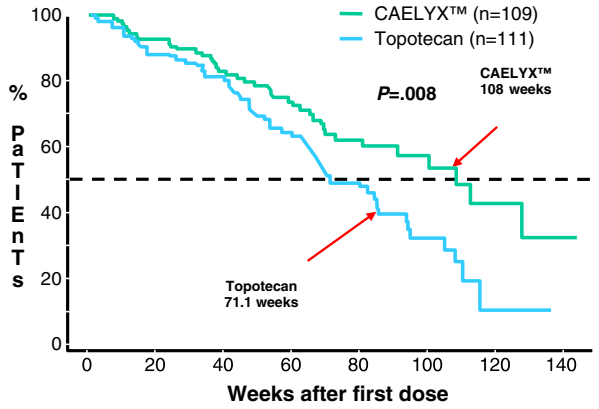

Fig. 6. Caelyx™ (= Doxil) is superior to topotecan in ovarian cancer [1].

- Recurrent ovarian cancer: superior efficacy and improved safety profile over comparator drug (topotecan) (1998), as demonstrated first by Gordon et al. [112] (Fig. 6).
- Metastatic breast cancer: equivalent efficacy and reduced cardiotoxicity compared to free doxorubicin (2003).
- Multiple myeloma: equivalent efficacy and improved safety profile compared to free doxorubicin combo. Superior efficacy in combination with bortezomib over single agent bortezomib (2007).

In addition, regarding cardiac function, Doxil demonstrates major reduction of cardiotoxicity as compared to free doxorubicin in all settings tested (2000).

4. Doxil take home lessons and what will next-generation Doxil-like liposomes look like?

In summary, the anticancer nano-drug Doxil shows superiority to free doxorubicin (standard of care) clinical performance in a variety of neoplastic conditions due to its unique EPR-related pharmacokinetics and bio-distribution, which reduce side effects (especially important is the large reduction in cardiac toxicity) and improve overall patient compliance and quality of life. These, when combined with the way the doxorubicin is remote loaded into the long-circulating nano-liposomes, improve (in certain tumors such as ovarian cancer) the antitumor therapeutic efficacy when compared with conventional doxorubicin. This explains why, of the >12 liposomal drugs approved for clinical use [113], Doxil has the most extensive clinical use.

Based on Doxil success, various novel drug formulations including modified Doxil, or other nano-drugs based on similar Stealth liposomes loaded with other drugs or with drug combinations are now at different stages of development. These novel nano-drug formulations should have reduced (or lack) the side effects of Doxil, PPE and acute infusion reactions. One approach to reduce these is to slightly reduce the half-life of the liposomal doxorubicin by replacing the sulfate counterion of the ammonium used for the remote loading with glucuronate. The use of glucuronate which has a permeability coefficient similar to sulfate but does not induce intra-liposome drug precipitation, results in somewhat shorter circulation time of the doxorubicin, but without loss of therapeutic efficacy in tumor-bearing mice, [34,70]. This relatively small but distinct effect on the PK is expected to lower accumulation of doxorubicin in the skin, thereby reducing severity of PPE. Other ways to extend and improve nano-liposome-based anticancer therapy and to have a better control of drug release (rev. in [34]) can be achieved by (a) the use of external means such as hyperthermia [114,115] or focused ultrasound [75,103,116]; (b) the use of a drug combination by remote loading of two drugs that act synergistically in one liposome [117,118] and (c) the use of a combination of two different treatment modalities, such as Doxil and interleukin-2 (IL-2), in liposome-based immunotherapy [119]. The concept of activating the host immune mechanisms to destroy residual tumor cells after chemotherapy has long been proposed. The use of the DOX-IL-2 combination stems from the fact that doxorubicin, when administered as Doxil, is much less toxic to the immune system than free drug, and therefore IL-2 delivered in liposomes following Doxil is highly efficacious. The idea behind this chemo-immuno treatment combination is that the Doxil will take care of most of the tumor burden while the immunotherapy elicited by the IL-2 will activate the intact immune system enabling it to kill the residual tumor cells [119]. The use of liposomal IL-2 results in lower toxicity of the IL-2 and prolongation of IL-2 circulation time without loss of its potency [120,121].

A very promising approach is the one used recently by Jain and coworkers [122]. Accordingly, losartan, which inhibits collagen I synthesis was used to modify the interstitial tumor environment, leading to increase in Doxil (and other nano-particulates) accumulation in tumors, thereby increasing Doxil therapeutic efficacy. For more options that were used or proposed to improve Doxil performance see Solomon and Gabizon [99].

The story of Doxil development carries two important messages. The first one is that Doxil's successful development opens the way to major improvement in tumor therapy and it served as a gold standard in the new field referred to as Nano-Medicine. The second one is that development of such a complex drug system requires having a highly multidisciplinary team that can deal in an integrative way with the expertise needed [1,19,34,100,123]. Not less important is the recognition that the understanding and optimal utilization of physicochemical principles are crucial to the successful development of such a complex drug product.

5. Doxil historical perspectives

Pre-Doxil era (liver passively targeted by liposomal doxorubicin referred to as OLV-DOX)

1979	Gabizon and Barenholz started their basic research on liposomal doxorubicin
1984	First clinical trials with liposomal doxorubicin (OLV-DOX which differs to a large extent from Doxil)
1985	LTI licensed the OLV-DOX technology and Barenholz and Gabizon 1990, 1991 I.P. on OLV-DOX
1987	Clinical trial of OLV-DOX failed
1988	Barenholz developed and Yissum, R&D Company of the Hebrew University of Jerusalem, Israel patented new concept of doxorubicin remote loading, the basis of Doxil (Barenholz and Haran 1993, 1994); patents were licensed to LTI
1989	LTI patented the Stealth concept and registered Stealth®
1989	Gabizon and LTI start to develop sterically stabilized (Stealth) liposomes
1989	LTI, Gabizon, and Barenholz start Doxil® development
1991–1992	Doxil "First in man" (FIM) clinical trial in Jerusalem
1994	Gabizon and Barenholz first major publication on Doxil clinical trials (*Cancer Research 1994*)
1995	(November 17) Oncologic Drugs Advisory Committee (ODAC) recommended FDA approval of Doxil
1996	First Doxil sales in USA and Europe
2010	(March) US patent expired

6. Doxil I.P. aspects

It is important to note that Doxil® was based on two families of patents. However, there is no direct patent on Doxil. One family covers the transmembrane-driven remote loading of amphipathic weak bases such as doxorubicin [55,56], while the second deals with contribution of the lipopolymer PEG-DSPE as a lipid component of liposome membrane for prolongation liposome circulation time and RES avoidance [78].

It took ~7 $^{1}/_{2}$ years from the submission of these two families of patent applications in 1988/1989 until Doxil's approval in November 1995. Remote loading patents were extended in the USA till March 9, 2010, and therefore Doxil enjoyed 14 years of patent protection in the USA. Currently in most countries there is no I.P. to protect Doxil®.

7. Generic doxorubicin in liposomes (Doxil-like)

The patent protection of Doxil® in the USA has been over since March 2010, and Doxil/Caelyx is selling well (over $600 million annually), so how come there is still no generic PEGylated liposomal doxorubicin (PLD)-like product approved by the FDA or EMA?

In addition to the complexity of FDA approval of generic Doxil, the current situation is even more complex. Ben Venue Laboratories (the sole supplier) has stopped Doxil production because of FDA-cited GMP deficiencies at their facilities. A shortage of Doxil has persisted since summer of 2011; in November there were reportedly ~2700 people in the US alone on a waiting list for Doxil treatment. Ben Venue spokesman Jason Kurtz said that the company does not have a time frame for when manufacturing will resume (for more information on Doxil shortage, see http://www.outsourcing-pharma.com/Contract-Manufacturing/Doxil-supplies-going-further-but-J-J-CMO-uncertainty-remains and many other websites). This shortage makes the "reward" for generic Doxil even more appealing.

The explanation for the lack of generic Doxil is that such a generic product is much more difficult to develop than a simple drug, or even than biologicals such as antibodies, because in addition to what is needed for the approval of generic low molecular weight drugs and biologicals, for approval of generic liposomal drugs, there are additional physical and physicochemical requirements needed.

The complexity of generic Doxil approval is discussed in a recent review by Jiang et al. [124] from the Office of Generic Drugs of the FDA. It is best to cite verbatim the abstract of this paper (with which I fully agree).

> *"One challenge in developing a nanoparticle drug-delivery system is understanding the critical physicochemical properties that may impact its in vivo performance and establishing analytical techniques that can adequately characterize in vitro and in vivo properties. Doxil®/Caelyx®, a PEGylated liposomal doxorubincin (PLD), is one of the leading approved nanoparticle product used in cancer therapy. In this review, we use PLD as an example to illustrate identification of key in vitro and in vivo characteristics. The following characteristics, including liposome composition, state of encapsulated drug, internal environment of liposome, liposome size distribution, lamellarity, grafted polyethylene glycol at the liposome surface, electrical surface potential or charge, and in vitro leakage, are considered critical to demonstrate the supramolecular structure of PLD and ensure consistent drug delivery to cancer tissues. Corresponding analytical techniques are discussed to determine these liposome characteristics. Furthermore, in vivo stability of the PLD can be determined by plasma pharmacokinetics of both free and liposome-encapsulated drug. A better understanding of the critical in vitro and in vivo liposome characteristics together with improvements in analytical technology will enable generic liposome product."*

In February 2010 the FDA issued a non-binding recommendation which relates to Generic Doxorubicin in Liposomes (http://www.fda.gov).

In the present review I will focus only on few special aspects raised which are related to Doxil physico-chemical properties and their relevancy to Doxil performance. For the detailed FDA Generic Doxil guidelines draft see the FDA website (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf). Citations from the FDA document are in italic type while my comments are in regular (roman) type.

> *"The surface-bound methoxypolyethylene glycol (MPEG) polymer coating protects liposomes from clearance by the mononuclear phagocyte system (MPS) and increases blood circulation time. The PEG layer thickness is known to be thermodynamically limited and estimated to be in the order of several nanometers. The PEG layer thickness should be determined."*

The methoxy PEG (2000 Da) residues of the PEG-DSPE can assume either a mushroom (at low PEG-DSPE mole fraction) or at higher mole fraction, transformation into brush conformation, which involves PEG–PEG interaction [47]. The actual measurement of the PEG layer thickness is not easy to perform as the PEG moiety does not show up in cryo-TEM, and its contrast in SAXS measurements is also poor and requires sophisticated methodology and software (for more details see [118], this issue).

- ***Electrical surface potential or charge***

Surface charge on liposomes can affect the clearance, tissue distribution, and cellular uptake. Liposome surface charge should be measured.

Liposome surface potential and zeta potential are not identical as they are measured at different distances from the lipid/water interface (phospholipid headgroup). Both are measured by different methods which are based on two different approaches. The location of the electrical surface potential is fixed at the phospholipid headgroup, while the zeta potential can be at different locations with respect to the phospholipid headgroup. For example, the complete coating of liposomes by PEGylated lipids moves the location of measurement further away from the phospholipid headgroup, thereby reducing the magnitude of the measured liposome zeta potential (a charge-hiding effect). However, the PEG moiety does not affect the electrical surface potential. PEG-DSPE actually introduces a negative surface potential due to its phosphate diester moiety (for more details see [90,95,125]).

- ***In vitro leakage under multiple conditions***

In vitro drug leakage testing to characterize the physical state of the lipid bilayer and encapsulated doxorubicin should be investigated to support a lack of uncontrolled leakage under a range of physiological conditions and equivalent drug delivery to the tumor cells. Below are some examples of proposed conditions.

Assaying doxorubicin in vitro release from Doxil and generic Doxil is essential for determining their chemical and physical stability upon storage and during the prolonged plasma circulation time.

In vitro and in vivo drug release profiles are both dependent on the membrane lipid composition and physical state and the integrity of the membrane lipids as well as the stability of the residual transmembrane ammonium sulfate gradient. Reduction in each of the two can be destructive, as it may reduce the therapeutic efficacy and can even be dangerous since it may result in massive drug release to the plasma. While measuring of lipid integrity is simple and requires simple analytical follow-up of lipid stability, mainly using HPLC with suitable ELSD or Corona detectors or LCMS, however, measuring the magnitude and the stability of residual ammonium gradient is less straightforward. Ammonium ions can be determined either by HPLC or specific electrodes (either as ammonium or as ammonia) [48]. Sulfate ions should be determined by HPLC. Determining the change in sulfate to phospholipid ratio is a good measure of integrity of the Doxil liposome membrane, as sulfate permeability coefficient is very low and much lower than that of ammonium ions (10^5-fold lower) or ammonia gas (10^{11}-fold lower) [20]. It is worth noting that ammonia may be released from the Doxil during long-term storage or during its blood circulation at body temperature (37 °C), resulting in reducing the magnitude of ammonium gradient.

- ***Active liposome loading process with an ammonium sulfate gradient***

In order to meet the compositional equivalence and other equivalence tests, an ANDA sponsor would be expected to use an active loading process with an ammonium sulfate gradient. The major steps include 1) formation of liposomes containing ammonium sulfate, 2) liposome size reduction, 3) creation of ammonium sulfate gradient, and 4) active drug loading. An active loading process uses an ammonium gradient.

FDA is requested to deal with all 4 steps of Doxil fabrication. If all these steps go well, it is expected that more than 95% of the doxorubicin will be liposome encapsulated. The active (remote) loading by transmembrane gradient of ammonium sulfate is the heart of the loading matter. It should enable achieving almost complete and stable drug loading, concomitantly having drug release at the tumor. The ammonium gradient is the driving force for the remote loading [19,20,32–34,48,64]. The transmembrane ammonium ion gradient can be tested specifically by ammonium and/or ammonia electrodes [48], and ammonium ion can also be determined by HPLC. The ammonium ion measurements require special precautions. For determination of intraliposome ammonium concentration, there is a need to determine the trapped aqueous volume. To account for losses during preparation, the ammonium amount has to be quantified per amount of total lipid HSPC (measured by HPLC), or total phospholipids (measured by phosphorus determination). For more details on these QC assay see [16,134].

The doxorubicin in DOXIL is largely in the form of a doxorubicin sulfate precipitate inside the liposome. The generic doxorubicin HCl liposome must contain an equivalent doxorubicin precipitate inside the liposome.

The demand for showing equivalent amount and shape of intraliposome doxorubicin-sulfate precipitate/gel is important as this precipitate/gel is highly relevant for maintaining loading stability during storage and during circulation in blood. It can be determined semi-quantitatively by cryo-TEM and SAXS measurements [61,67], and Fig. 2 above.

The relevance of sulfate as the ammonium counterion is well documented in a recent publication by Mamida et al. [126], who compared Doxil with 6 different PEGylated liposomal doxorubicin formulations based on dextran sulfate (DSAS) as ammonium counterion for their performance in a murine breast cancer model, and in tumor-free monkeys. The change in counterion resulted in a very similar doxorubicin plasma PK to that of Doxil. The therapeutic efficacy expressed as decreases in tumor volume was somewhat better for the DSAS-based formulation, but at the cost of a 3.2-fold increase in aspartate transaminase levels (a marker of hepato-toxicity). Also, a 5.0-fold increase in cardiac troponin I levels (a marker of cardiac toxicity), as well as increase in bone marrow hypocellularity (a marker of bone marrow toxicity), and increase in kidney toxicity [126]. This study demonstrates well what we claim above that for Doxil every detail matters!!!

- ***Internal environment* (*volume*, *pH*, *sulfate and ammonium ion concentration*)**

This can be measured using acridine orange remote loading [48,63].

The internal environment of the liposome, including its volume, pH, sulfate and ammonium concentration, maintains the precipitated doxorubicin. The measurements of total and free concentrations of components (including sulfate ions) described in liposome composition section allow the inference of the internal concentration inside the liposome.

Total and Doxil-encapsulated doxorubicin should be determined after separation between un-encapsulated and encapsulated drug. Free un-encapsulated doxorubicin can be separated and removed from the liposomes by gel permeation chromatography or by Dowex cation exchanger [48,97,98]. This should be followed by doxorubicin determination using the right HPLC procedure.

Sulfate ion is the counterion which is important to fine tuning of loading stability (see [32,48,64]).

Sulfate ion concentration can be determined by HPLC with an appropriate detector (see above).

Satisfying these requirements requires the following: determination of liposome trapped volume from measurement of sulfate ion by HPLC, or less preferably, ammonium ion by ammonium electrode [48] or HPLC. Level of encapsulated ammonium sulfate should be determined after removal of non-encapsulated ammonium sulfate by exhaustive dialysis, and/or gel permeation chromatography, followed by determination of intraliposome sulfate concentration by HPLC.

Doxil intraliposome pH should be determined by the intra-to-extra-liposome partitioning of radioactive methylamine (Table 2 above and [34]). Similar approaches can also be applied. However in the case of Doxil, the use of chromophores or fluorophores for this determination is not straightforward due to the high background absorbance and fluorescence of doxorubicin [34].

- ***Liposome morphology and number of lamellae***

Liposome morphology and lamellarity should be determined, as drug loading, drug retention, and the rate of drug release from the liposomes are influenced by the degree of lamellarity.

Doxil morphology may be relevant to the level of complement activation [22,23,111], while number of liposome lamellae may be relevant to drug release profile. The determination of Doxil shape, lamellarity, and physical state of the intraliposome drug requires the use of cryo-TEM [67] and Fig. 2 above.

- ***Lipid bilayer phase transitions***

Equivalence in lipid bilayer phase transitions will contribute to demonstrating equivalence in bilayer fluidity and uniformity. The phase transition profiles of the lipid ingredients and liposomes should be comparable to those of Doxil.

HSPC, the main membrane component of Doxil liposomes, has a gel [solid-ordered (SO)] to fluid [liquid-disordered (LD)] phase transition with a Tm ~53 °C [47], and this Tm is not affected much by the ~5 mol% of PEG-DSPE. However, in the presence of the Doxil

high mole% of cholesterol, as expected, there is no SO to LD phase transition [95]. The thermotropic behavior of Doxil can be determined by various methods, the most straightforward and preferred method being differential scanning calorimetry (DSC) [127].

- ***Liposome size distribution***

Liposome size distribution is critical to ensuring equivalent passive targeting. The ANDA sponsor should select the most appropriate particle size analysis method to determine the particle size distributions of both test and reference product. The number of liposome product vials to be studied should not be fewer than 30 for each of the test and reference products (i.e., no fewer than 10 from each of three batches).

Doxil size distribution is a very critical issue, as size may have a large impact on liposome PK and BD, and therefore on therapeutic efficacy and toxicity (see below). Also relevant is the observation that the presence of free drug may induce liposome aggregation and/or formation of drug aggregates. Both types of aggregates may induce complement activation [23,111].

However, size distribution determination is not an easy task and none of the currently available methods gives complete and absolute values. Each of the currently used methods has some pitfalls and/or does not see the full scale [16,128], and references listed therein. The two most commonly used size determination methods for liposomes in the expected size range of Doxil are dynamic light scattering (DLS), and cryo-TEM (the latter being mainly a supportive confirmatory method). Size determination by DLS is based on the determination of diffusion coefficient of nano-particulates, calculated from the exponential decay of the autocorrelation curve of which the radius of the particles are determined. In order to get the best results the instrument that performs the DLS measurement has to get the information about the temperature, refractive index, and viscosity of the solution in which the measurement is performed. Size determination could be a perfect method if the liposome population is very homogeneous in size. But in most situations this is not the case, and the liposome population has a size distribution and therefore there are many autocorrelation curves which have to be deconvoluted to their separate components. This procedure of deconvolution is not simple and requires applying a mathematical model. However there is more than one model available and each of the models may use different mathematics. Even for the same model there is no unique solution. Usually size determination readouts come in 3 different ways: (1) a mean and distribution according to analysis by light intensity (which is much more affected by the larger particles, as intensity is increased by a power of 6 of their size); (2) a mean and distribution of particles by their volume (which again gives more weight to the larger particles); or (3) a mean and distribution by particle number. Only if the three types of readout are very similar can the population be considered homogeneous with respect to size. The higher is the discrepancy between the 3 readouts the more heterogeneous is the liposome population. So when data on size determination are given in order to evaluate them, the evaluator has to know if analysis was corrected for temperature, refractive index, and viscosity and not less important, which of the 3 readouts was used (by intensity, volume, or number). It is preferable to include these 3 readouts in the product description.

Cui et al. evaluated the effect of size distribution on Doxil-like formulations [129]. They prepared smaller than Doxil liposomes (75 nm, 300 mM ammonium sulfate) of lipid composition identical to Doxil. These liposomes showed identical PK parameters in normal mice, however faster drug release for the 75 nm liposomes. Doxil liposomes had higher AUC and Cmax in S-180 sarcoma-bearing mice than the 75 nm Doxil-like liposomes. The 75 nm liposomes were more efficacious therapeutically, but also had a greater toxicity (based on decreases in body weight). However, in this specific publication [129] there is not enough information to evaluate the quality and precision of the size distribution determination. Therefore, it is not recommended to use this information as a go/no go variable.

8. Personal touch

I cannot end this Doxil review without a personal touch. The road to the development of Doxil® covers a major part of my professional career. I have been working on the development of liposomal drugs since 1979. However, I could not perform all my applied multidisciplinary work without the many research years and major efforts I dedicated to basic research in the fields of lipid biochemistry and biophysics. I started to study phospholipid and sphingolipid enzymology when I was a second-year Biology student working, to support my family, in the laboratory of Shimon Gatt, with whom I later performed my graduate studies (M.Sc. and Ph.D. theses). My involvement in lipid biophysics started due to a need to better understand lipid enzymology. Most lipids are not water-soluble and in order to serve as a substrate there is a need to disperse them in an aqueous phase with the aid of detergents (mixed micelles [130], rev. in [131]) or in the form of liposomes [132]. At the time I worked in Gatt's lab, liposomes, which were introduced by Alec Bangham in the mid-1960s, were still in their infancy. Lipid enzymology introduced me to lipid biophysics and therefore I dedicated a large part of my Ph.D. thesis to different aspects of lipid biophysics, under the supervision of Rex Dawson and Peter Quinn of Dawson's lab (lipid monolayers) and in Alec Bangham's lab (liposomes). Both groups were part of the Institute of the Animal Research Council at Babraham, Cambridge, UK. Indeed, my studies at Babraham had an important impact on my Ph.D. thesis and the rest of my scientific career. My Ph.D. thesis was submitted and approved by the Senate of the Hebrew University in 1971. Since then, my independent research was focused on various aspects of lipid biophysics and "liposomology". I was lucky to perform my first and prolonged sabbatical with Tom Thompson at the Department of Biochemistry of the University of Virginia (UVA) Medical School in Charlottesville, VA, (1973–1975) and continued to interact for many years with the UVA team, which included (in addition to Tom Thompson), Chien Huang, Burt Litman, Dov Lichtenberg, Rodney Biltonen, and others. In the 1970s/1980s UVA was one of the world's dominant labs in membrane biophysics. At UVA, liposomes were characterized (like never before) by many physical methods, almost to the level of macromolecules.

In 1979, Alberto Gabizon, (then a young M.D./Ph.D. who arrived to the Oncology Department of Hadassah University Hospital) and I started to work on the development of liposomal doxorubicin formulation for human use (rev. in [1,7]). This direction of my research was intensified in 1984, after a meeting I had with Dimitri Papahadjopoulos from the University of California at San Francisco (UCSF), a long-time friend and colleague "liposomologist". We had met at various scientific conferences since 1973 when I was at UVA. The UVA membrane research group was in tight competition with the UCSF group, although the interests of the two groups were only partially overlapping. The UVA group focused mainly on lipid biophysics and physical chemistry, and the UCSF (Dimitri Papahadjopoulos) group focused more on biologically relevant topics such as fusion, interaction of liposomes with cells, etc. Whenever Dimitri and I met, we talked extensively about science (mainly membrane and liposome research), as well as on culture, art, history, food, and wine. Dimitri kept telling me about Liposome Technology Inc. (LTI), a start-up located at Menlo Park, CA, which focused its R&D in the field of liposome-based diagnostic and medical applications. Dimitri and his previous student, Frank Szoka, were the scientific founders and mentors of LTI. Nick Arvanitidis was convinced by Dimitri

to become LTI CEO, and Frank Martin, another student of Dimitri's, was the first LTI employee. Nick brought with him Sally Davenport, Carl Grove, and Kathy, who had worked in Nick's previous R&D company, to deal with LTI administration. Dimitri asked me if I would be interested to spend a sabbatical at LTI. He told me that it was a great challenge and intellectually very rewarding. He already knew about our efforts in the field of drug delivery, and that we were close to the "first in man" experiment with liposomal doxorubicin, but he was more interested in my knowledge and experience in lipid and liposome biophysics and physical chemistry, as he well understood that this was the heart of the matter of developing liposomal products. I hesitated as, so far, most of my research was academic in nature. Dimitri proposed that I come to his lab at UCSF, give a seminar there, and he would organize my visit at LTI so I would be able to judge for myself whether spending a sabbatical at LTI was of any interest to me. I also got a formal invitation from Nick, LTI CEO, to visit LTI and spend a day there. As things looked serious, I consulted with Hanna, my wife, who supported me and encouraged me to seriously evaluate this interesting proposition. I knew this was not easy for her, as it meant that I would be away from home (in California) for long periods of time, and she would have to take care, alone, of our 4 daughters, the dog, and our home. With her encouragement, I accepted Dimitri's and Nick's offers to visit. My visit at Dimitri's and at LTI was organized for December 1984. My seminar at Dimitri's lab was on glycosphingolipid biophysics, which was followed by discussion with Dimitri's lab people. The next day Dimitri drove me to LTI in Menlo Park, which at that time was a start-up company of ~40 people, where I spent the whole day talking with many company employees. After dinner with Nick and some good wine, Nick and I had a long conversion in which Nick was trying to convince me to spend my sabbatical at LTI. Nick is Greek, and as such he understood well the Mediterranean mentality and way of thinking, so we understood each other very well. Without going into detail and possibly with the aid of the good wine served continuously by Nick, I agreed to seriously consider his proposal. Nick drove me to my San Francisco hotel very late that night. The excitement, together with the 10-h jet lag, made it very difficult for me to sleep.

Returning to Israel, I discussed Nick's proposal with Hanna, and with her support and encouragement, I accepted it. I told Dimitri and Nick that I would not be able to come unless LTI would support our OLV-DOX program in Jerusalem. It took a short time until the LTI board decided to accept my request. Frank Szoka, Dimitri, and Nick called me from the board meeting at 02.00 AM Israel time and woke me up to tell me that LTI had accepted my request. But their condition was that I assured them of my continuous involvement in their relevant research and R&D programs. LTI support meant what was considered a large grant at that time, which would allow us to continue our OLV-DOX research, and especially the "first in man" clinical trial. So, it seemed we had a deal, the small details of which still needed to be finalized. At about that time I was approached by another US company that proposed to license from us the OLV-DOX technology and product. Their proposal was tempting, as it involved what I considered then a large sum of money up front and reasonable royalties. However, this company requested that we would be used only as consultants and not be involved in the research and R&D of the product. I did not like this idea, as we looked upon the product as a "baby" we had to nurture to maturity. I preferred LTI to the other company, as I believed that our day-to-day involvement in the product development was crucial to the program's success. The future would show that I was right. So I convinced Moshe Vigdor, the CEO of Yissum (the Research and Development Company of The Hebrew University of Jerusalem) to accept LTI's proposal. It did not take Nick long to come to Israel and finish the LTI–Yissum first license agreement, which was the basis for a master agreement that continued for 21 years. It started with LTI, with Nick as CEO, and went all the way to Johnson & Johnson. After the approval of Doxil by the FDA there was a change of management at LTI, and Craig Henderson, a top-level oncologist from UCSF (who had been involved in Doxil's clinical development) became LTI CEO. Then the company name was changed to Sequus. Craig and others sold Sequus to ALZA, Mountain View, CA (a major drug delivery company), with Doxil (due to its increasing sales) being one of the main reasons for the deal. It did not take a long time for ALZA to be bought by Johnson & Johnson, which until then was hardly involved in drug delivery systems. Again, Doxil was one of the main reasons for the deal. All the rest is history!!!

During this fantastic very long voyage of 48 wonderful years of active research I met many fascinating people, with whom I interacted and/or collaborated, and many of them remain lifelong friends. The 15 years I worked on liposomal doxorubicin, of which 50% was dedicated to Doxil development, was a unique experience I will never forget. It enabled me to be involved in a very complicated and complex process of drug development and to see its approval worldwide. The reward in terms of satisfaction is unmatched by any of my other achievements. I am trying hard to transfer my experience, part of which is summarized in this review, to many students and others worldwide.

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Most importantly, I want to dedicate this review to my wife, Hanna. Without her encouragement, advice, patience, dedication, and support throughout our 52 years of life together I would not be able to accomplish my part of Doxil development and possibly Doxil would not be developed!

I also want to thank my four daughters, Chagit, Ayelet, Tamar, and Avigail (Abigail), who grew up during the years described in this review; their husbands, Uri, Perri, Ron, and Assaf; and last but not least, our twelve grandchildren, Yael, Yuval, Amit, Omri, Inbar, Mika, Rotem, Guy, Eyal, Gal, Dror, and Kfir, who give us so much joy. Our daughters and grandchildren were my escape during periods of despair.

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