WG4 - DELIVERABLE D4.1

Formulation of guidelines and documents for translation of nanomedicines from bench to bed/market

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Contacts (Action Chair and Vice Chair)	Prof. Sabrina Pricl sabrina.pricl@dia.units.it Dr Nazende Günday Türeli n.guenday-tuereli@mybiotech.de
EC COST Officer	Dr. Lucia Forzi

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AUTHORS

Lead Authors	Enrico Catalano, Maria Eugenia Riveiro, Nazende Günday-Türeli, Ivana Vinkovic Vrcek
Reviewers	Sabrina Pricl

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4.1.2. SOFT NANOMEDICINES

4.1.1.1. Identification of main common barriers for translation of nanomedicines to cancer patients

Nanomedicines have emerged as a promising strategy for cancer treatment, offering the potential for enhanced efficacy and reduced toxicity compared to traditional therapies. However, the translation of nanomedicines from the laboratory to the clinic remains a challenge, and there are several common barriers that must be overcome to ensure their successful development and delivery to cancer patients. One of the main barriers is the complexity of nanomedicine design and manufacturing, which can make it difficult to produce reproducible and scalable formulations. Another barrier is the lack of standardized preclinical models and assays that accurately reflect the clinical setting, which can hinder the prediction of efficacy and safety in humans. Additionally, regulatory requirements and approval pathways for nanomedicines can be unclear or uncertain, adding to the time and cost involved in bringing them to market. Finally, the high cost of nanomedicine production and limited reimbursement for these therapies can also be significant barriers to their widespread adoption. Addressing these barriers will be critical for advancing the translation of nanomedicines to cancer patients and realizing the full potential of this innovative approach to cancer treatment.

Cancer diseases are highly heterogeneous. Thus, custom solutions to each type must be developed with suitably designed nanomedicines with clinically relevant efficacy and safety. Common barriers for translation of nanomedicines to cancer patients can be listed as the lack of batch-to-batch reproducibility, stability, complex manufacturing methods with special equipment and regulatory guidance on novel starting materials. The pre-clinical studies, prerequisites to clinical trials, can only be relied on, if the nanomedicine is produced by employing control strategies at already early stages of the development – leading to batch-to-batch reproducibility independent of how complex a manufacturing protocol is. This would enable early identification of critical quality attributes (CQAs) which are to be evaluated not only by using physicochemical characterization methods but also by in-vitro and in-vivo methods that are clinically relevant. The methods for identifying and controlling the CQAs must be also easy to translate to regulated environment – standardization, validation and verification.

Nano medicinal products are mostly run under the guidelines governing the conventional formulations in a regulatory context. The existing guidance documents, for conventional formulations are expected to be applied for pharmaceutical nano products, nevertheless this comes with difficulties in translating nano specific features of the formulations not only for manufacturing but also for quality and safety testing. Thus, developers usually lost vast amount of time and resources trying to ensure that their products are characterized to fulfil the expectations of the regulatory authorities. The cost and time needed till market is intimidating for most of the main players in the nanomedicine field.

4.1.1.2. Translational relevance of the unique PK/PD features of nanodrugs carrying anti-cancer agents

Researchers face many difficulties in establishing evidence to extrapolate results from one level of development to another, for example, from an in vitro demonstration phase to an in vivo demonstration phase. Most cytotoxic anticancer drugs are characterized by a narrow therapeutic index, high toxicity profile and large degree of inter-patient PK variability. The narrow therapeutics margins of standard cytotoxics and the issue of low intratumor diffusion have fostered the development of nanoparticles (NPs)[1]. In this sense, developing NPs to improve the specificity of anticancer agents towards tumor tissues and to better control drug delivery is a rising strategy. However, the attrition rate when developing NPs is particularly high and several promising forms showing excellent behavior and efficacy in preclinical studies failed to succeed in subsequent first-in-man studies or later clinical trials. The topic of pharmacokinetic variability is a major and largely underestimated issue with NPs.

Despite increasing efforts and resources in developing nanocarriers, little is known about their actual pharmacokinetics. Paradoxically, the expected higher therapeutic efficacy is mostly based upon improved pharmacokinetics (i.e., reduced clearance, higher specificity towards target organs)[2]. However, the few data made available regarding nanoparticles (for example for liposomes) pharmacokinetics have reported higher interpatient variability as compared with standard drugs[3], as if behavior of nanoparticles in the body could be both more targeted and less predictable.

In oncology, it has been extensively documented that a high inter-patient PK variability is observed with many anticancer agents, which it makes difficult to predict the patient's response to a particular drug[4] . If we consider the multitude of properties that make a carrier or liposomal agent unique from the active small molecule drug that is contained within the nano-carrier, it can be expected that this complexity will lead to a higher significant variability in the PK and PD of the carrier respect to the drug as single agent.

It has been also described that the physical properties of the carrier, the mononuclear phagocytic system (MPS), the presence of tumors in the liver, an enhanced permeability and retention effect, drug-drug interactions, age, and gender contribute in varying degrees to NPs' pharmacokinetic variability and pharmacodynamic endpoints in patients[5]. Future studies must also further evaluate the sources of PK variability and develop additional tools to accurately measure and predict PK and PD variability in patients administered with NPs agents. In the present section, we provide an in-depth analysis of the characteristics of NPs related to their PK and PD output. A comprehensive understanding of the development and regulatory relevance of the PK and PD properties of NPs will contribute to its further clinical progression.

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PHARMACOKINETICS (PK) OF NPS

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME) CONSIDERATIONS. Pharmacokinetic (PK) studies concerning NPs are scarce, and the lack of guidelines for nanomedicines makes it difficult to evaluate this parameter[6]. PK comprises four processes: absorption, distribution, metabolism, and excretion. To understand the pharmacology of NPs, it is essential to analyze and quantify: the area under the curve (AUC), clearance (CL), the volume of distribution (VL), mean elimination time (t1/2), maximum plasma concentration (Cmax), and CL of the kidneys and the mononuclear phagocyte system (MPS).

ROUTES OF ADMINISTRATION. Specific issues of the route of administration should be considered when assessing the safety of a drug product containing nanomaterials and may warrant special assessment in addition to the nonclinical studies normally conducted in support of drug product development. The following are examples of considerations for commonly used administration routes:

1. Topically Applied Products. Increased hair follicle penetration or distribution to local lymph nodes is a possibility for nanomaterials[7]. In addition, nanomaterials can interact with sunlight differently than larger size particles and this can impact the interaction of light with the skin. Penetration of a nanomaterial through the skin in human patients can be impacted by the condition of the skin (e.g., intact, damaged, diseased). The evaluation of effects and exposures achieved in the nonclinical studies should consider this impact.
2. Subcutaneous Administration. Materials introduced below the stratum corneum can possess an increased sensitization potential compared to some other (e.g., dermal) routes. It has been reported that nanomaterials injected subcutaneously can enhance sensitization to other allergens[8]. The biological fate of non-soluble nanomaterials should be considered.
3. Inhalation. Local/respiratory toxicity of nanomaterials can differ from larger particles, as lung deposition, distribution in respiratory tissues, and systemic bioavailability is different. With decreasing particle diameter, a given mass of particles will have a larger specific surface area, and the volume of single particles is lower. Furthermore, agglomerates of particles will have different densities than solid particles. On an equal mass-dose basis, nanosized particles caused greater pulmonary inflammation in rats than their non-nanosized counterparts[9]. In addition, the biological fate (accumulation/translocation, clearance) of non-soluble carrier nanomaterials should be considered.
4. Intravenous Products. Drug products containing nanomaterials can have a different tissue distribution of the therapeutic moiety and a different half-life compared to the same drug products without nanomaterials. In addition, changes in hemocompatability can occur. Hemocompatibility refers to the compatibility of a substance with blood, including its ability to circulate without causing adverse effects on the blood cells or coagulation system. Nanoparticles used in oncology, such as liposomes, polymer nanoparticles, and metallic nanoparticles, can interact with blood components, which can affect their hemocompatibility. For example, nanoparticles can activate blood cells and the coagulation system, leading to thrombosis, inflammation, and other adverse effects. Strategies to improve the hemocompatibility of nanomedicines include surface modifications of nanoparticles

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to reduce their interaction with blood components, using biocompatible materials to construct nanoparticles, and optimizing the size, shape, and charge of nanoparticles to minimize their interactions with blood cells.

5. Oral Products. For orally administered drug products, use of nanomaterial ingredients is often intended to increase bioavailability of the therapeutic moiety. Other than possible local effects and an increased absorbed dose (which should be detected with existing methods), if the oral toxicology studies with a micrometer scale material were adequate, new effects are not expected for soluble drugs. If an insoluble nanomaterial is included in an oral product, toxicology studies should take this into consideration and include assessment of tissues where such materials might accumulate.

ABSORPTION. The rate and extent of absorption depend on the physiological environment and the properties of the NPs. Nanoformulations cross physiological and physical barriers that selectively inhibit the flow of molecules affecting the bioavailability of NPs. Size, surface charge, and shape greatly influence cellular uptake [10]. In the case of the pulmonary route the contact area is greater favoring absorption [11].

After oral administration, the bioavailability of the drug is mainly impacted by the absorption process. Actually, the stability of the drug after contact with biological media, its solubility, and its permeability are key parameters to monitor. The encapsulation of the drug molecules in NP may help to master these parameters and many examples can be found showing oral bioavailability enhancement after encapsulation in nanocarriers [12] but specific issues may also appear. For example, smaller NPs have greater intercellular transport by follicle epithelia and if the surface charge is positive there will be greater transport in mucosal and epithelial cells[13]. In the oral route, the negative surface charge has a higher absorption at the gastrointestinal membrane, and in the small intestine, it is related to size [14]. Gastric or intestinal fluids are well-known stress for free molecules because of the pH of the media and the presence of enzymes such as lipases or peptidases. If encapsulation is proposed to protect active drugs, NPs should remain intact after contact with gastric or intestinal fluid with a minimal encapsulated drug leakage. To evaluate gastrointestinal stability of lipid nanocapsules (LNC), Roger E, et al. [15], studied the size of LNC after 3 hours of contact with a simulated gastric (FaSSIF-V2) and intestinal media (FeSSIF-V2). The LNCs size was not modified after 3h in the gastric medium but quickly destroyed in the intestinal medium because of pancreatic lipase. In addition, Singh A, et al. [16] studied the stability of PEGylated liposomes vs. conventional liposomes after 2 hours of exposure to the harsh conditions of the gastrointestinal environment. They compared the release of the drug from those 2 formulations and observed that if the drug remained in PEGylated liposomes after 2 hours, the drug was totally released with conventional liposomes. For polymeric NP, Tobio M, et al. [17] have also demonstrated that a PEG coating of poly (lactic acid) (PLA) NP increases protection against digestive fluids.

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DISTRIBUTION. Physicochemical properties influence distribution (interaction with biological barriers and proteins), while composition (silica, polymers, proteins, metals, lipids), size, morphology, surface charge, and hydrophobicity impact biodistribution [18]. Their properties at biological barriers such as immune system, epithelium and mucosa, and blood–brain barrier mark the distribution process [19]. For example, silica NPs have a higher affinity for the lungs and distribute better in the liver [20] . The size determines prolonged distribution; the smallest limit in NPs for renal filtration is between 5.5 nm and 10 nm [21].

Most NPs studied have a diameter in the size range of 30–200 nm, whereas some NPs such as quantum dots and gold NPs can be much smaller with a hydrodynamic diameter of just a few nm [22]. The gaps between endothelial cells are normally less than 2 nm, which restrict the ability of most NPs to exit blood following i.v. injection. The NPs may, however, pass through fenestrae with a diameter up to 200 nm in the endothelium of liver, spleen and bone marrow; kidneys have fenestrae of 20–30 nm, whereas other tissues have very small (diameter less than 6 nm) or no fenestrae [23]. Importantly, there is a leakier endothelium in inflammation areas and in solid tumors where the NPs may exit from blood. The most effective way for NPs to reach tumors following i.v. administration has for many years been regarded to be passive enrichment in tumors due to the so-called Enhanced Permeability and Retention (EPR) effect [24]. Thus, the accumulation is caused by the combination of an increased extravasation (enhanced permeability) and a decreased drainage by the lymphoid system (increased retention). It was recently proposed that NPs reached tumors by an active transport across the endothelial cell layer, but more studies are needed to clarify how most NPs pass the endothelial cell layer and enter tumor tissue [25].

Different studies performed using gold NPs of 15 to 250 nm in mice and rats, showed that the largest NPs were mainly taken up by liver and spleen, whereas the smallest NPs were present in most tissues[26,27]. The smallest NPs, which are not rapidly taken up liver/spleen, will circulate for a longer time and can thus be endocytosed by different cells. The rate of endocytosis differs between cell types and can be very fast for some cells. Thus, macrophages ingest 25% of their own volume every hour, whereas fibroblasts endocytose at approximately one third the rate of macrophages. Based on these considerations and the knowledge about cellular uptake of NPs [28], it is likely that the optimal size of NPs for drug delivery is in the range 20–200 nm.

Upon contact with biological fluids, the surface of the NPs is surrounded by proteins, forming a structure called protein corona that alters the size of the NPs by changing the surface charge and

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making it anionic [29] To predict the NPs behavior and circulation time, the description of the concentration and type of proteins attached to the NPs surface should be performed. To characterize and modelize the distribution, it is important to determine the dynamics of corona formation since it is necessary to conduct protein binding corrections to predict the PK of nanobiopharmaceuticals accurately. Note that modifications of proteins concentration related to physiological changes due to unhealthy tissues may prevent NPs from reaching the target tissue [30]. Accordingly, NP-to-tumor delivery efficiency is likely to be the first part of the problem, as the intratumoural kinetics, interactions and fate of nanomedicines are also important for the use of nanomedicine in cancer applications. The literature lacks sufficient studies to make a full evaluation in this regard.

A very small amount of the injected dose of NPs ends up in the tumors. In a meta-analysis with data from more than hundred preclinical studies, it was calculated that only ~1% of the IV injected dose reached the tumor [31]. As discussed by McNeil S [32], the "NP-tumor accumulation" parameter could not be as a good surrogate for NP efficacy or therapeutic index as it was previously suggested by Wilheim, S et al. [33]. In the case of NPs delivering small molecule drugs, it seems intuitive that the higher NPs loading in the tumour tissue, the more active pharmaceutical ingredient (API) is delivered. Whereas API exists in encapsulated, free and protein-bound forms, free API is the bioactive form that can induce a cancer-killing effect. McNeil S [34], postulates that it is misleading to place a huge importance on this "tumor-accumulation" parameter in lieu of classical pharmacological evaluation of drugs. In contrast to the analysis performed by Wilheim, S et al. [35] , where evaluation of the nanoparticle's API is the most relevant consideration and is required to compare critical factors such as PK, toxicity, and efficacy among formulations.

Liposomal doxorubicin (Doxil) is a historical example to bring further clinical perspective about tumor accumulation. While less than 1% of the injected dose of Doxil is detectable in the tumour, yet this is many times greater than an equivalent dose of traditionally formulated doxorubicin [36]. Clinically, liposomal doxorubicin also dramatically enhances the length of tumour exposure with a half-time life that is nearly fivefold greater and a CL that is three orders of magnitude slower than traditionally formulated doxorubicin. These combined effects confer an improved anticancer treatment for patients with significantly reduced cardiotoxicity [37]. The Doxil example points out how nanoformulations can advantageously alter the PK and toxicity profile of a drug to improve the quality of life for the patient.

For traditional drug delivery systems, the accumulation of drugs in healthy and malignant tissues could be correlated with toxicity and efficacy. Some previous studies have indicated that a higher tumor AUC is correlated with a better therapeutic efficacy. However, Cui J, et al. [38] designed and evaluated two pegylated liposomal doxorubicin formulations (PLD-75 and PLD-100), which had the same lipid/drug ratio and bilayer composition, but different size and internal ammonium sulfate

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concentration. This study revealed that the drug was released at a faster rate from the small size formulation PLD-75, whereas the plasma PK of PLD-75 was similar to that of PLD-100. The tumor AUC after administration of PLD-100 and PLD-75 were 1285.3 µg.h/g and 762.0 µg.h/g, respectively. Maximum drug levels achieved in the tumors were 33.80 µg/g (for PLD-100) and 20.85 µg/g (for PLD-75), and peak tumor concentration was achieved faster in PLD-75 group. Despite of these PK results, enhanced drug accumulation did not result in an increased antineoplastic effect for PLD-75. Furthermore, their biodistribution behavior in tumor-bearing mice was significantly different, the drug deposition was reduced in normal tissues of animals receiving PLD-75 respect to PLD-100. As for toxicity studies, PLD-75 caused more rapid and severe body weight loss even though drug accumulation in healthy tissues was reduced. This study suggests that AUC values of liposomal drugs will not be predictive of their toxicity and efficacy [39].

Accumulation in the tumour is certainly an important factor for nanomedicines that rely on an external stimulus. Nanoparticles that contain an imaging agent and/or have a non-chemical mechanism of action (for example, laser-induced thermal ablation), require a sufficient concentration of particles in the tumour to transduce the stimulus into therapeutic/diagnostic signal.

Another aspect that adds complexity to NPS, is the development of antibody/peptide-conjugated NPS. Antibodies or peptides have been widely used to provide targeting ability and to enhance bioactivity owing to their high specificity, availability, and diversity. Recent advances in biotechnology and nanotechnology permit site-specific engineering of antibodies and their conjugation to the surfaces of NPs in various orientations through chemical conjugations and physical adhesions. It has been described that less than 1% of the injected dose of soluble antibodies are retained in tumors. Thus, by conjugating targeting molecules to NPs should not significantly increase in the accumulation of NPs in tumor tissues. However, such targeting molecules may still be important for uptake into cells and thus improve the therapeutic effect [40]. If conjugated-NPs display differences in the biodistribution respect to their non-conjugated counterparts, it could be due to changes in surface charge or hydrophobicity. Furthermore, based on the low fraction of injected NPs that ends up in tumors, one should not expect to see significant different biodistributions in small animals whether they are tumor-bearing or not.

The main goal of drug nanoencapsulation is to modify the distribution of the drug, for targeting purposes, to enhance its activity and/or to lower its toxicity. Thus, this step of the PK process should be clearly assessed. Once again, regular PK models fail to describe with good accuracy the dynamics of nanoparticle distribution, which depends on the interaction of particles with body fluids and tissues all along the distribution process. Physicochemical parameters of the NP are indeed changing after contact with body fluids and are responsible for the fate of the nanocarrier *in vivo*. Most of the published studies rely on the monitoring of drug blood-concentration along time, which is relevant for non-encapsulated drugs but is not accurate for nanomedicines as stated before. To consider the equilibrium between the free and the encapsulated drug during PK modeling is a requirement to knock down the barriers that currently impair the clinical translatability of nanomedicine treatments.

Other aspect to be analyzed is that regular “classical” PK parameters are not directly valid for NPs PK studies. For example, encapsulated and free drugs are present at the same time from the initial time point (T0), in the blood circulation because in many cases even in the containers there is an equilibrium between free and encapsulated drug. Because of this duality, the regular determination

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of Volume of distribution (Vd) from the blood at T0 is not accurate nor informative. If one calculates the Vd from the total drug (encapsulated plus free drug) the interpretation will be complex as both entities do not display the same diffusion patterns. The impossibility to determine an accurate Vd has some consequences on modeling because, in standard models, Vd is used to calculate other parameters such as drug total clearance. This example shows that regular modeling is not relevant for PK determination of nanomedicines.

Moreover, it has been extensively shown that the circulation time increases with decreasing hydrodynamic diameter of metal-based NPs with polyethylene glycol (PEG) [41]. It should be noted that whereas it is the size of the metal core that determine the MRI signal intensity for iron oxide NPs, it is the hydrodynamic diameter that is important regarding circulation half-life and penetration into tissue. Thus, the length of the PEG chains conjugated to the NPs, will increase the circulation time in blood and also the tissue penetration.

In the recent FDA's guideline "Drug products, including biological products, that contain nanomaterials [42]" it has been establish that to conduct biodistribution studies of nanomaterials, it may be necessary for the material to be labeled in some manner (e.g., radiolabeled, fluorescence) to allow for enhanced detection in vivo. Impact of such labeling of nanomaterials in the study on biodistribution should be considered in the results analysis and interpretation.

During recent years, major improvements have been obtained in the methods used for biodistribution, metabolism and excretion evaluation of NPs, which most often are performed after labelling with radioactive isotopes or fluorescent molecules. Up to date, only ICP-MS measurements of stable inorganic NPs can be used to obtain quantitative measurements of the amounts of NPs in selected organs/tissues. Anyhow, fluorescent labeling is the most common method to study the biodistribution of NPs in preclinical models, but as discussed by Skotland T, et al. [43] there are several pitfalls with this approach. Labelling with radioisotopes and imaging using PET or SPECT have several advantages regarding quantification, but also with such analyses it is not possible to make sure whether the label is still connected to the injected substance at the time of analysis and whether the labelling method has changed the surface properties and thus the biodistribution of the injected particles.

ELIMINATION. This phase comprises the metabolization and the excretion processes. The clearance of NPs is very similar to that of conventional drugs, and they have two main routes of elimination: renal and hepatobiliary filtration [44]. In the case of nanomedicines, several PK parameters associated with the metabolism and drug elimination of the API, are not directly applicable for NPs. For example, NPs are not metabolized using the classical hepatic pathways, there is a specific process for NP degradation mainly linked to liver-NP interactions. The liver acts as a biological filtration system that sequesters 30-99% of administered NPs from the bloodstream, as described by Zhang Y, et al. [45] , causing a reduced delivery to the targeted diseased tissue and potential increased toxicity at the hepatic level. Internalization is largely associated with surface charge, shape, or size

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45 Zhang YN, Poon W, Tavares AJ, McGilvray ID, Chan WCW. Nanoparticle-liver interactions: Cellular uptake and hepatobiliary elimination. J Control Release. 2016 Oct 28; 240:332-348.

and thus also associated with composition of protein corona because it has an impact on those three characteristics.

In regular PK analysis, the elimination phase is described by different PK parameters: half-life, mean retention time (MRT) and clearance. For terminal half-life determination, the issue is the same and interpreting a half-life that includes free and encapsulated drug may be very tricky especially if the parameter is compared with a standard injectable or oral formulation without any encapsulation. In this aspect, the mean retention time (MRT) may be much more relevant as it allows a fair comparison between different formulations of the same drug after non-compartmental analysis. MRT is a global value of the meantime a molecule will stay in the studied tissue or media; it is only calculated from the area under the blood concentration curve (AUC) and its shape described by area under the first moment curve (AUMC). Total clearance calculation in the case of nanomedicine should also only be calculated as the ratio of dose and AUC using non-compartmental analysis by keeping in mind that in the case of nanomedicine the result will be different if free drug is considered or if total of free and encapsulated drug is considered. If modeling is involved to determine those elimination parameters, the carrier with its encapsulated drug should be considered as a supplementary compartment. In the model, the stability of the carrier should also be considered as it has an impact on the release of the free drug and thus on its elimination. In fact, the released drug can follow the elimination process, which may not be the same for the encapsulated drug still protected from the major elimination processes (for example, metabolization or kidney filtration).

After NP administration, as it was already discussed above, proteins that are present in different biological media cover NPs to form a protein corona. This step called opsonization can be considered as the first step of elimination during which the NP is tagged by proteins to allow its recognition by specific cells. Indeed, NPs covered with this protein corona generate different cell interactions in the blood such as complement activation or uptake by Kupffer cells in the liver. The second step of NP elimination is the result of these interactions which lead to a degradation of NP by the immune system. It clearly appears that the liver plays an essential role in the elimination of NP especially in the Kupffer cells by micropinocytosis, clathrin-mediated endocytosis or caveolin-mediated endocytosis [46]. In this sense, the NP size can affect endocytosis mechanisms. For example, caveolin-mediated endocytosis occurs in NPS of 20-100 nm, whereas clathrin-mediated endocytosis for 100-350 nm NPs, and micropinocytosis for larger NPs [47]. In this respect it is worth to note that phagocytosis of NPs is more associated with specific recognizable proteins present on their surface rather than the quantity of binding proteins [48].

Interactions with the mononuclear phagocytic system (MPS) lead to NP clearance from blood and sequestration in organs of MPS: liver, spleen, and lymph nodes. It is one of the most important barriers to NP therapeutic efficiency [49]. To limit organ uptake and immune system-related clearance, the most common strategy consists in masking the nanoparticle through surface pegylation [50]. Polyethylene glycol (PEG) added on NP surface reduces uptake by MPS and reduces renal clearance. PEG-coating decreases the formation of protein corona, but this can induce production of anti-PEG antibodies and activation of complement proteins. This mechanism of immunogenicity, which leads

46 Zhang YN, Poon W, Tavares AJ, McGilvray ID, Chan WCW. Nanoparticle-liver interactions: Cellular uptake and hepatobiliary elimination. J Control Release. 2016 Oct 28; 240:332-348.

47 Walkey CD, Olsen JB, Guo H, Emili A, Chan WC. Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. J Am Chem Soc. 2012 Feb 1;134(4):2139-47.

48 Nguyen VH, Lee BJ. Protein corona: a new approach for nanomedicine design. Int J Nanomedicine. 2017 Apr 18;12: 3137-3151.

49 Peng C, Huang Y, Zheng J. Renal clearable nanocarriers: Overcoming the physiological barriers for precise drug delivery and clearance. J Control Release. 2020 Jun 10; 322:64-80.

50 Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev. 2016 Apr 1;99(Pt A):28-51.

to an accelerated blood clearance phenomenon, is well established in preclinical studies but it is unclear on the clinical situation [51].

As mentioned above, a way to improve the PK profile on the elimination phase consists of adding PEG on the NP surface to reduce renal clearance. NP renal elimination is also associated with protein-corona formation as it plays a major role in increasing particle diameter (hydrodynamic diameter) which becomes considerably larger as compared to its in vitro measurement. Consequences on blood clearance are important because NP diameter is associated with cut-off glomerular filtration linked to the elimination of the drug still being encapsulated from blood, so this can affect its blood half-life [52]. Small NPs can be excreted through kidneys if being small enough to pass through the kidney filtration units called glomeruli. Choi H, et al. demonstrated that for NPs with a hydrodynamic diameter of 5 nm, almost 50% of the injected dose in mice follow glomerular filtration and rapid renal excretion, whereas there was less than 20% excretion of 9 nm NPs. While NPs just slightly larger than 9 nm seem to end up in the liver [53]. Moreover, renal elimination is also influenced by NP charge because the glomerular basement membrane is negatively charged, so positively charged NPs are easier filtered, whereas negative NPs have longer circulation times [54].

In a recent review, Peng C et al. discussed about the design of renal clearable nanomedicines, which it turns will reduce MPS uptake and long-term accumulation in vital organs which could induce hepatic or spleen toxicity [55]. Injecting substances that are excreted rapidly in urine may be beneficial for imaging as it is important to reduce the signal from the surrounding tissue to better visualize a diseased area. Thus, most contrast agents used in the clinic are low molecular weight substances that are almost completely excreted in urine within 24 h post dosing. On the other hand, for NPs bearing drugs to have a therapeutic effect, it will be an advantage to have the NPs circulating for a longer time to get as much drug as possible to the diseased area. Also, a prolonged exposure to the drug after injection is likely to increase the therapeutic effect. Certainly, more knowledge about the interaction between NPs and the animal/human body is required to bring more of these products into clinical use.

REGULATION OF PHARMACOKINETIC STUDIES

Even though, FDA has not adopted a regulatory definition of nanotechnology, it has been established that these products will be approved using the same tools as for approval of other products. New drug products that contain nanomaterials should be as thoroughly tested as for any new drug product. For pharmacokinetic regulation, ICH guidelines such as ICH S6 (preclinical safety evaluation of biotechnology-derived pharmaceuticals), ICH S3B (pharmacokinetics: guidance for repeated dose tissue distribution studies), and ICH M3 (R2) are used [56, 57, 58]. In addition, the FDA guidelines for the industry for liposomal products and the EMA and the Japanese Ministry of Health,

51 Hashimoto Y, Shimizu T, Abu Lila AS, Ishida T, Kiwada H. Relationship between the concentration of anti-polyethylene glycol (PEG) immunoglobulin M (IgM) and the intensity of the accelerated blood clearance (ABC) phenomenon against PEGylated liposomes in mice. Biol Pharm Bull. 2015;38(3):417-24.

52 Longmire M, Choyke PL, Kobayashi H. Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. Nanomedicine (Lond). 2008 Oct;3(5):703-17.

53 Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV. Renal clearance of quantum dots. Nat Biotechnol. 2007 Oct;25(10):1165-70.

54 Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm. 2008 Jul-Aug;5(4):505-15.

55 Peng C, Huang Y, Zheng J. Renal clearable nanocarriers: Overcoming the physiological barriers for precise drug delivery and clearance. J Control Release. 2020 Jun 10; 322:64-80.

56 ICH Expert Working Group. S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 1997.

57 ICH Expert Working Group. M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 2010.

58 ICH Expert Working Group. S3B Pharmacokinetics: Repeated Dose Tissue Distribution Studies; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 1995.

Labor and Welfare (MHLW) reflection papers on liposomes and micelles include some recommendations to consider in the PK of nanoformulations [59] . The Nanotechnology Characterization Laboratory (NCL) mentions that PK studies are performed at single and repeated doses, planning tissue distribution studies, and comparing bioequivalence [60]. According to agencies guidelines the parameters to be monitored are the same for conventional drugs. As an additional tool, regulatory agencies have recommended incorporating quantitative and rational approaches such as the pharmacokinetic/pharmacodynamic (PK/PD) modeling. This qualitative tool characterizes the relationship between pharmacokinetics and pharmacodynamics in a time-dependent manner. Well-designed PK/PD modelling provides a better understanding of the exposure–effect relationship and allows them to obtain benchmark PKs to reach the maximum efficacy response with reduced toxicity. This interactive process offers a rational approach in hypothetical modelling that can support the optimization of pharmacokinetic assays in nano-drugs [61].

At the same time, depending on the purpose of the nanoformulation, agencies may require additional data. Regulatory agencies require detailed studies to know the nano-drug's precise disposition, analyzing the encapsulated, non-encapsulated and total drug (encapsulated plus non-encapsulated). In recent years, it has become necessary to present the results of the concentrations of bound drug (non-encapsulated bound to plasma proteins) and unbound drug (non-encapsulated that has not bound to plasma proteins) because non-linear protein binding changes the PK profile of the nanoformulation. In this sense, The NCL has recently developed stable isotope tracer ultrafiltration assay (SITUA) for nanomedicine fractionation suited for distinguishing each fraction and comparing the carrier/drug/protein interactions [62].

PHARMACODYNAMICS: EFFICACY AND SAFETY

Pharmacodynamics is the study of the biochemical and physiological effects of drugs on the body, and it plays a crucial role in understanding its efficacy and safety. Nanomedicines can improve the efficacy of cancer treatments in several ways. First, they can increase the bioavailability and retention of drugs in tumor tissues, allowing for more effective targeting of cancer cells while minimizing toxicity to healthy cells. Second, nanomedicines can enhance the delivery of therapeutic agents to the intracellular compartments of cancer cells, leading to improved drug uptake and efficacy. Third, nanomedicines can enable the combination of different drugs in a single formulation, which can synergistically enhance the anti-cancer effects and reduce the emergence of drug resistance.

For the therapeutic effect, it is not the particles themselves that are important, but the active pharmaceutical ingredient they carry and that the active substances get access to their targets. The drug could have left the particles before the time-point for the biodistribution measurement, or it may still be entrapped within the particles in a form where it is not able to reach its target. Thus, more NPs reaching a target does not mean that more drug is available at the target, as it was discussed by McNeil S [63]. Also, even if only very little of the injected dose is reaching the target, it may still be enough to give a good therapeutic effect. For example, although less than 1% of the

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[63] McNeil, S. Evaluation of nanomedicines: stick to the basics. Nat Rev Mater 1, 2016. 16073.

injected dose of Doxil/Caelyx (the first NP-based product that reached the market; liposomes containing doxorubicin) was detected in tumors, that was still much more than detected in tumors following injection of free doxorubicin [64]. The data published have showed that most NPs end up in liver, spleen and lymph nodes. Moreover, different studies have reported accumulation of NPs in lungs and kidneys, although these signals should be carefully evaluated considering the NPs technology [65].

The development of biomarkers, imaging studies, and companion diagnostics addressing PD and trafficking can identify optimal nanomedicine candidates and potential responders to reduce failure rates in late-stage clinical development. For example, studies that visualized radioisotopes within liposomal drug products by PET or SPECT imaging after administration to patients with solid tumors established particle accumulation and retention at tumor sites [66]. Similarly, MRI imaging of patients following ferumoxytol administration, a 30-nm iron oxide particle with contrast properties, indicated particle accumulation in tumors and substantial variability in tumor uptake [67]. In addition, preliminary data from a small number of patients suggested that the uptake of ferumoxytol or ^{64}Cu-labeled HER2-targeted liposomes in tumors may correlate with intratumoral drug concentration and treatment response measurements. Similarly, it was demonstrated that clinical benefit from BIND-014 treatment (a docetaxel-loaded PSMA targeted PEG-polylactic acid nanoparticle) suggested a correlation between prostate-specific membrane antigen (PSMA) expression and therapeutic response in prostate cancer patients [68]. For example, the use of biomarkers for patient stratification has contributed to the successful clinical development and approval of four antibody–drug conjugates; however, a lack of biomarkers has been noted as the reason behind the failure of cancer nanomedicines based on liposomes, polymeric nanoparticles, and micelles (including CRLX101 [camptothecin loaded PEG-cyclodextrin nanoparticles], or NK105 [paclitaxel-loaded PEG-polyaspartate-based micelles]) [69].

While nanomedicines have the potential to improve the safety of cancer treatments, they also pose unique safety challenges that must be carefully considered. One of the main concerns is the potential for nanomedicines to accumulate in healthy tissues and organs, leading to off-target effects and toxicity. Additionally, the size and surface properties of nanoparticles can influence their interactions with biological systems and potentially trigger immune responses or adverse effects. For example, nanomedicines such as carbon nanotubes or quantum dots entail additional concerns. Potential toxicity and deleterious immunological effects observed in preclinical/clinical studies may compromise their future translation[70].

In the recent release FDA's guideline for NPs [71] it has been stated that components that are non-biodegradable can accumulate and persist longer than biodegradable components and can consequently produce effects related to chronic exposure to these components. A nanomaterial can

[64] Gabizon A, Chemla M, Tzemach D, Horowitz AT, Goren D. Liposome longevity and stability in circulation: effects on the in vivo delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. J Drug Target. 1996;3(5):391-8.

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68 Low S, Hoff D Von, Mita M, Burris H, Eisenberg P, Hart L, et al. Abstract 911: Prostate-specific membrane antigen (PSMA) expression as a potential patient selection marker in patients with refractory solid tumors administered BIND-014, a PSMA-targeted nanoparticle containing docetaxel. 2014. p.911–911.

69 Đorđević S, Gonzalez MM, Conejos-Sánchez I, Carreira B, Pozzi S, Acúrcio RC, Satchi-Fainaro R, Florindo HF, Vicent MJ. Current hurdles to the translation of nanomedicines from bench to the clinic. Drug Deliv Transl Res. 2022 Mar;12(3):500-525.

70 Đorđević S, Gonzalez MM, Conejos-Sánchez I, Carreira B, Pozzi S, Acúrcio RC, Satchi-Fainaro R, Florindo HF, Vicent MJ. Current hurdles to the translation of nanomedicines from bench to the clinic. Drug Deliv Transl Res. 2022 Mar;12(3):500-525.

[71] "Drug products, including biological products, that contain nanomaterials guidance for industry". U.S. Department of Health and Human Services. Food and Drug Administration Center for Drug Evaluation and Research (CDER). Center for Biologics Evaluation and Research (CBER). April 2022. FDA-2017-D-0759. https://www.fda.gov/media/157812/download.

sometimes cross biological barriers in greater amounts than the larger particle size version. This can lead to increased safety concerns in some cases, such as increased penetration of the blood-brain barrier, or the placenta[72]. The biological fate of the nanomaterials, including those that function as drug carriers, and their potential impact on safety should be determined. Since most NPs used for drug delivery are larger than 10 nm, it is very important, both for safety issues and for formal regulatory aspects, that the NPs are degradable and thus hopefully excreted. This is not a problem for NPs made of endogenous substances, like liposomes or albumin-based NPs. Neither a problem with iron oxide-based NPs, as such superparamagnetic particles are biodegradable and have been safely used as contrast agents for magnetic resonance imaging (MRI) for more than 20 years [73]. However, there are in fact very few data demonstrating degradation and excretion of most other NPs under development the last years.

Regulatory agencies consider preclinical toxicity tests for small-molecule drugs useful for nanomedicines when conducted in at least two animal models, over extended treatment periods, and multiple doses. The battery of tests includes acute and repeat-dose studies, safety pharmacology, genotoxicity, developmental toxicity, immunotoxicity, and carcinogenicity, typically employing two animal species (usually rat and dogs) [74]. The most used rodent species for preclinical studies are mice and rats, and there are conflicting results when extrapolating to humans [75]. For example, these species are not good for testing the pyrogenic potential because of their resistance to endotoxins. The most suitable species to evaluate the overall toxicity profile f NPs are non-human primates, which are closer to human physiology and genetics; however, they usually are not as accessible due to their high maintenance cost and ethically related issues. In this context, the use of valid animal species is central for assessing both the positive and the detrimental effects of NPs, thereby predicting their possible risks for human and environmental health. In this sense, they will allow us to understand the safety and to implement safe-by-design nanotechnologies.

Challenges for the evaluations of ADME/toxicity in nanomedicine development include interactions with the immune and/or hematological systems [76]. It is equally important to perform these studies on in vivo models because the assessment of immunological effects in vitro is limited. Endotoxin contamination interferes with the detection of nanomedicine-induced toxicity by inducing a non-specific immune response [77]. Contamination of the systems with endotoxins or lipopolysaccharide (LPS) is a challenge for the immunological characterization of nanoformulations [78]. Another challenge related to endotoxin levels is depyrogenation. Due to the complexity of NPs and their easy tendency to alter their properties, each technique for endotoxin removal has its own merits and drawbacks [79]. There are several depyrogenation methods for nanoformulations: UV irradiation, filtration, ethylene oxide treatment, formaldehyde treatment, and autoclaving. However, these

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methods cause aggregation and changes in particle morphology, stability, and size distribution or clogging of filter pores [80].

The complement activation cascade plays a crucial role in immunological side effects, and nanomedicine-blood cell interactions may contribute. For example, sometimes, the induction of complement activation related pseudo allergy (CARPA) is simply not evaluated or overlooked during the preclinical stage of the nanomedicine development; however, this might result in hypersensitivity reactions in patients during clinical trials [81]. In some assays, the mechanism by which the particles trigger the observed immune response is not yet understood. The complexity of NPs' systems generates the necessity to use many tests to determine their hematological profile [82]. Therefore, understanding how nanomedicines interact with coagulation factors, as complement activation can be dose-limiting, remains an essential task, while evaluations of organ function, phagocyte activation, oxidative burst, cytokine release, hemolysis, thrombogenicity, effects related to protein corona, and antigenicity can inform on nanomedicine toxicity. The NCL has suggested in vivo-in vitro correlation (IVIVC) methods to determine acute toxicities by hemolysis, complement activation, pyrogenicity, cytokine induction, and MPS uptake; however, the determination of thrombogenicity, myelosuppression, immunosuppression, and hypersensitivity is more complex compared with small drugs due to the differential PK/PD and biodistribution of nanomedicines given the absence of reliable models [83]. Therefore, each distinct soft nanomedicine requires specific studies (Tables 1-3).

It can be stated that individual components of nanoparticles and excipients commonly used in formulations are often not immunologically inert and contribute to the overall immune responses to nanotechnology-formulated products. The use of in silico modeling for the prediction of nanomedicine-induced immunogenicity has been proposed as a "personalized safety" approach. With few exceptions, animal cells or models do not entirely predict human immune reactivity, and patient-derived xenografts in immunodeficient mice usually lack relevance regarding immunogenic evaluations. The preferred implementation of immunocompetent models may accelerate clinical translatability given the affinity of nanosized materials for immune cells[84]. Understanding the immunological compatibility of nanoformulations and their effects on hematological parameters is now recognized as an important step in the preclinical development of nanomedicines since the occurrence of immunological adverse events is responsible for 15% of drug failure in early clinical stages.

REGULATION OF PHARMACODYNAMICS STUDIES

The development of nanomedicines for oncology involves a complex regulatory framework that requires rigorous testing to ensure safety and efficacy. The pharmacodynamics (PD) studies of nanomedicines in oncology play a crucial role in assessing the biological effects of the drug and its mechanism of action and are therefore an important component of the regulatory approval process. In summary, the PD studies of nanomedicines in oncology require careful consideration of several regulatory aspects to ensure the safety and efficacy of the drug. These aspects include preclinical

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studies, clinical trial design, biomarkers, regulatory guidelines, and pharmacovigilance. During last years, regulatory agencies such as the FDA and EMA (European Medicines Agency) require evidence of target engagement to approve a new drug. In this sense, identification, and validation of target engagement (biomarkers) became crucial during preclinical PD studies. The combination of robust target engagement and well-qualified disease-related biomarkers enhances understanding of the mechanism of action, ties together preclinical and clinical data, enables the assessment of target engagement, facilitates early proof of concept and dose focusing, and increases the efficiency of early clinical development with improved quality of decision making. Significant progress in biomarker discovery, validation, and qualification has increased drug-development decision making and became almost mandatory for became regulatory applications.

In the United States, the FDA provides guidance for the development of nanotechnology-based products, including nanomedicines. According to the FDA, PD studies for nanomedicines should focus on the mechanism of action, target engagement, and pharmacological effects of the drug. The agency recommends that PD studies be conducted in preclinical models using appropriate biomarkers to assess the drug's activity. In addition to FDA's requirements, the EMA calls researchers for using imaging techniques to evaluate the drug's distribution, accumulation, and retention in tumors and other tissues.

The regulatory guidelines for PD studies of nanomedicines in oncology vary by region, but there are several important guidelines that researchers should consider when designing and conducting these studies. Some of the relevant guidelines are ICH S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; ICH M3(R2) Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, from the FDA's guidance it should be referred to "Drug Products, Including Biological Products, That Contain Nanomaterials", "Drug Development and Approval Process for Manufacturing and Controls Documentation", "Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products" and "Safety Testing of Drug Metabolites". MHLW has issued guidelines related to the safety and toxicity of nanomedicines, such as "Guidelines for the Quality, Safety, and Efficacy Assurance of Nanopharmaceuticals" and "Guidelines for the Nonclinical Safety Evaluation of Nanopharmaceuticals".

The toxicity of nanoformulations is one of the most important challenges limiting the clinical translation of NPs. Regulatory agencies ensure that any nanomedicine must demonstrate a rigorous safety profile based on multiple key factors, such as physicochemical properties and route of administration [85]. Regulatory authorities view nanomedicines on a case-by case basis when gathering safety data. Perspective articles provide guidelines on the development of specific nanoparticle-based drug delivery systems [86]. Only around 5% of initially evaluated entities (including INDs, nanomedicines, and non-nanomedicine-based therapeutics) lead to the submission of a New Drug Application (NDA) and market authorization [87]. The strategies employed by regulatory authorities to evaluate nanomedicine safety/toxicity and compatibility are often adapted from "conventional" medicinal products [88]. From a regulator's perspective, the API of a nanomedicine dictates the specifications analyzed within the regulatory context; however, the multicomponent nature of nanomedicines raises toxicity concerns. Developers should plan what data they need to collect, such as the most relevant parameters influencing nano-drugs' short- and long-term toxicity.

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The determination of the concentration and median lethal dose (LC50 and LD50), the lowest concentration that causes a noticeable effect on the organism (LOEC), and the maximum concentration at which no observable effect is present on an organism (NOEC) allow the safety of nanomaterials to be assessed. Their assessment should also include a consistent set of data at the different organ and toxicological endpoints to evaluate the individual components and the complete formulation [89].

As the biodistribution of nanomedicines possesses a different profile from the parental API, uptake in specific organs may promote local overexposure. Furthermore, beyond the intrinsic toxicity of the bioactive agent and the nanomedicine as a whole drug product, the multiple components may also induce unexpected toxicities, e.g., excipients lacking adequate testing in humans. Therefore, all components, including the drug-free nanocarrier and the whole construct, must be considered in preclinical PK/PD studies at different doses if they have not been previously approved.

Regulatory agencies have pointed out that nanoformulations should not solely be analyzed from a conventional chemical point of view because they exhibit physicochemical properties that make their analysis more complex. There have been several attempts to harmonize toxicological procedures using various initiatives (scientific opinions, guidelines, and regulations) such as ICH and OECD guidelines, ISO, ASTM and FDA, EMA, and MHLW guidelines. The OECD states that each test should comply with Good Laboratory Practice (GLP) under the standard section on safety studies [90]. ICH M3 (R2), ICH S6 (R1), S8, ICH S4, and ICH S9 refer to safety tests that may apply to nanoparticles. It is convenient to use the standardized assays for toxicokinetics of NMs (applicable to NPs) (ISO/TR 22019: 2019), the Toxicity Screening method for NPs in 3D cultures (ISO/AWI TS 22455), genotoxicity (ISO/TR 10993-22: 2017) [91,92,93]. For chronic toxicity testing, it should be referred to the ICH S4, while ICH S9 for anticancer drugs, and the multidisciplinary guideline M3 (R2) for non-clinical safety studies [94, 95, 96].

For biological entities such as proteins, peptides, or antibodies, an innovative product must follow the regulations defined for biological medicinal products and NCEs [97]. Regulatory guidance documents for the non-clinical evaluation of anticancer agents, such as the ICH S9 guideline [98], represent the starting point. These guidelines include toxicological evaluation in rodent and non-rodent species but recommend a limited evaluation of the parent drug and carrier. Concerning non-rodent species, several canine studies have revealed unusual sensitivities to nanoparticles or

[89] Sharma A, Madhunapantula SV, Robertson GP. Toxicological considerations when creating nanoparticle-based drugs and drug delivery systems. Expert Opin Drug Metab Toxicol. 2012 Jan;8(1):47-69.

[90] Organisation for Economic Co-Operation and Development. Guidance Document on Good In Vitro Method Practices (GIVIMP); Organisation for Economic Co-Operation and Development: Paris, France, 2018.

[91] ICH Expert Working Group. S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 1997.

[92] European Committee for Human Medicinal Products. Reflection Paper Providing an Overview of the Current Regulatory Testing Requirements for Medicinal Products for Human Use and Opportunities for Implementation of the 3Rs; European Medicines Agency Pre-Authorisation Evaluation of Medicines for Human Use: Amsterdam, The Netherlands, 2018.

[93] 227. ICH Expert Working Group. S8 Immunotoxicity Studies for Human Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 2006.

[94] ICH Expert Working Group. M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 2010.

[95] 309. ICH Expert Working Group. S9 Nonclinical Evaluation for Anticancer Pharmaceuticals; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 2008.

[96] 310. ICH Expert Working Group. S4 Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing); International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Brussels, Belgium, 1999.

[97] CH guideline Q11 on development and manufacture of drug substances (chemical entities and biotechnological/ biological entities) EMA/CHMP/ICH/425213/2011. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q11-development-manufacture-drug-substances-chemical-entities-biotechnological/biological-entities_en.pdf

[98] CH guideline S9 on nonclinical evaluation for anticancer pharmaceuticals EMA/CHMP/ICH/646107/2008. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-s9-non-clinical-evaluation-anticancer-pharmaceuticals-step-5_en.pdf

components such as polysorbate surfactants [99]. Due to these findings, phase I studies of Abraxane and BIND-014 employed toxicology studies in non-human primates, while initial studies of the parent compounds (paclitaxel and docetaxel, respectively) employed canine models.

Box 1. Summary of the major PK and PD challenges hampering nanomedicine regulation
○ High PK Variability in patients
○ Challenges for assessing the ratio toxicity/efficacy
○ Lack of a unified definition or classification of nanomedicines/nanomaterials
○ Lack of agreed regulations
○ Analytical methods differ for each nanomaterial → needs to define specific validation protocols and standards
○ PK profiles diverge from "single-agent" constituent materials
○ Stability, Drug Loading, Drug Releases issues
○ Current in vitro and pre-clinical toxicological studies fail to mimic in vivo complexity
○ Complex systemic biodistribution and fate
○ Non-Integrated immunotoxicity testing approaches early in the program

The three important requirements for the agencies are endotoxin levels, sterility, and depyrogenation. An emerging method for assessment of endotoxin content in drugs is the recombinant Factor C (rFC) activation method (highly sensitive and quantitative), recently included in July 2020 in the tenth edition of the European Pharmacopoeia [100]. The most widely accepted assay by agencies for detecting endotoxin in nanoparticles is the Limulus Amebocyte Lysate (LAL) assay. However, this assay has several limitations and a second assay such as the endotoxin detection assay based on ELISA technology (EndoLISA), might be necessary to confirm the results [101] .

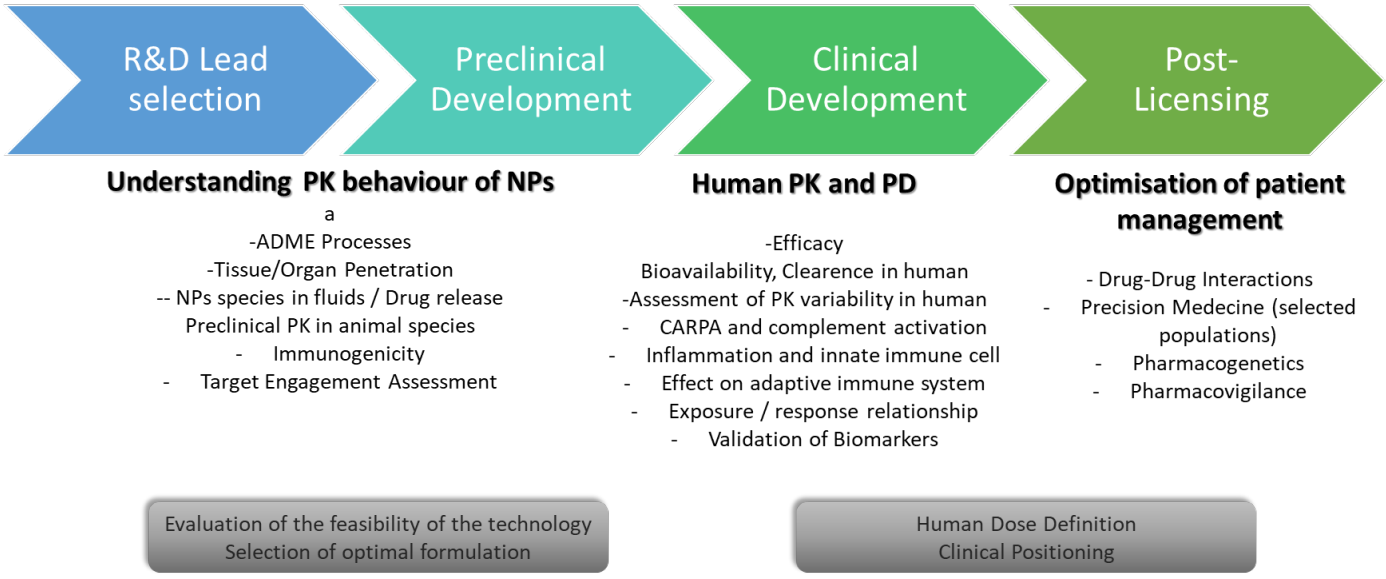
The immunotoxicity assessment of NPs has no specific regulatory framework or regulatory guidance. The good practices rely on the ICH S6 and S8 guidelines [102]. If the nanoformulation contains a low molecular weight drug, the ICH Section 8 should be used as reference, whereas the formulation contains a biotechnology-derived the ICH S6 should be followed. However, there is a question of whether ICH S8 immunotoxicity assays are a reliable assessment tool for NPs. For example, ICH S8 lacks guidelines for testing CARPA induction, hypersensitivity, inflammasome activation, and myelosuppression. As discussed by Halamoda-Kenzaoui, B et al. [103], no standards are available so far for the evaluation of the interaction of nanomedicines with the immune system. In this sense, agencies have recommended using ISO and ASTM standards. Whereas, ASTM includes analysis of hemolytic properties, the standard test for colony formation of mouse granulocytes and macrophages, and a quantitative test method for the chemoattractant capacity of a nanoparticulate material in vitro (ASTM E56-2525-08-2013, ASTM WK60373). the ISO standards contain a standardized framework for the detection of immunotoxicity for NPs (ISO/TS: 10993-20). In vitro

[99] Masini E, Planchenault J, Pezziardi F, Gautier P, Gagnol JP. Histamine-releasing properties of Polysorbate 80 in vitro and in vivo: correlation with its hypotensive action in the dog. Agents Actions. 1985 Sep;16(6):470-7.
[100] Council of Europe. European Pharmacopoeia, 10th ed.; Council of Europe: Strasbourg, France, 2021.
[101] Steinová, J.; Bob˘cíková, K.; Hristov, D.R.; Ševc ° u, A. Evaluation of two different methods for endotoxin detection in nanoparticle suspensions. In Proceedings of the Nanocon 2016, Brno, Czech Republic, 19–21 October 2016.
[102] Giannakou C, Park MV, de Jong WH, van Loveren H, Vandebriel RJ, Geertsma RE. A comparison of immunotoxic effects of nanomedicinal products with regulatory immunotoxicity testing requirements. Int J Nanomedicine. 2016 Jun 22;11:2935-52.
[103] Halamoda-Kenzaoui B, Holzwarth U, Roebben G, Bogni A, Bremer-Hoffmann S. Mapping of the available standards against the regulatory needs for nanomedicines. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2019 Jan;11(1):e1531.

immunotoxicity can be analyzed by detecting cytokine release, interferon levels, phagocytosis or leukocyte proliferation. Moreover, the immunology team at the NCL has published seven protocols for the evaluation of immunotoxicology aspects of nanomedicines, including complement activation and oxidative stress in T lymphocytes, antigen presentation and stimulation, and the detection of naturally occurring antibodies to PEG. For example, variations in shape, size, and composition of nucleic acid nanoparticles induce distinct immunostimulatory profiles [104]. Recent studies have described how the nanosized carrier employed for delivery provides an additional means to tailor nanomedicine immunorecognition, for example, lipid-based platform versus dendrimers display differences in cytokine induction [105].

We recommend addressing the crucial regulatory information needed for PK/PD assessments of NPs from the early phases of the program (summarized in Tables 1-3). For example, check if accurate, robust and validated methods for the assessment are available for each parameters/characteristic (cost, resources and time can be reduced if standardized and regulatory accepted methods are available, reducing the risk and uncertainties of regulatory approval process). Check if the available guidelines for these parameters are applicable for the developed nanotechnology-based product. As it was extensively reviewed by Halamoda-Kenzaoui B, et al.[106] most of standardized methods that could be relevant for health products are related to particle size, morphology, and surface charge, while no standardized methods are available to assess other characteristics relevant for medical applications, for example drug loading and release kinetics.

Figure. Role of PK/PD evaluation during the development of NPs in oncology



[104] ICH Topic S 8 Immunotoxicity studies for human pharmaceuticals. 2006. https://www.ema.europa.eu/en/ich-s8-immunotoxicity-studies-human-pharmaceuticals-scientific-guideline

[105] Avila YI, Chandler M, Cedrone E, Newton HS, Richardson M, Xu J, Clogston JD, Liptrott NJ, Afonin KA, Dobrovolskaia MA. Induction of Cytokines by Nucleic Acid Nanoparticles (NANPs) Depends on the Type of Delivery Carrier. Molecules. 2021 Jan 27;26(3):652.

[106] Halamoda-Kenzaoui B, Vandebriel RJ, Howarth A, Siccardi M, David CAW, Liptrott NJ, Santin M, Borgos SE, Bremer-Hoffmann S, Caputo F. Methodological needs in the quality and safety characterisation of nanotechnology-based health products: Priorities for method development and standardisation. J Control Release. 2021 Aug 10;336:192-206.

4.1.1.3. Crucial role of understanding the GMP/GLP requirements during early development stages to reduce the attrition in later manufacturing scale-up, IND-enabling studies or clinical studies. How to ensure that the final product fulfills the regulatory agencies specifications

cGMP aims reducing the risks that might be encountered during pharmaceutical production and might have a direct effect on the CQA of final product by identifying critical material attributes (CMA) and critical process parameters (CPP) through testing the final product. Time and resource efficient scale-up and GMP compliant manufacturing would rely on careful selection and screening of starting materials and their suppliers as an integrated part of Quality by Design (QbD).

Considering that the quality of starting materials, active pharmaceutical ingredients or excipients, of a nanomedicine might be critical for efficacy and safety, it is very important to work with these components from suppliers with pharma quality already during the early development stages. Otherwise, one might be forced to completely adapt the nanomedicine formulation and even the manufacturing protocol, which would directly cause delay for entering clinics, since adequate quality, efficacy and safety data (pre-clinical studies) by using representative batches are prerequisites of clearance for clinical trials obtained.

Any material used in the pharmaceutical (nano)drug product should to be manufactured under appropriate manufacturing practices and supplied under good distribution practices. The exact definition of “appropriate” manufacturing and distribution depends on the starting material by its role in the formulation as active or inactive ingredients.

Although excipients are not to be produced under GMP, such substances must ensure quality and safety in a (nano)formulation. Pharmacopoeia describe the quality requirements of formulation components, their analysis methods and the limit of critical properties in a dedicated monograph to assure the safety and quality of the excipient. General monographs also provide the details for equipment minimum requirements, analytical methods and specifications.

CQAs have relevance for clinical success and industrial sustainability of a developed product, as they are direct measures to safety and efficacy. At the end of the scale-up process, the CQAs of nanoformulations must be maintained. Thus, the relationship between manufacturing processes and CQAs should be investigated at early stages and understood. This can be achieved by employing industrially applicable manufacturing steps already during the development phase, given that this doesn’t cause additional costs. For example, repeated centrifugation for purification should already be avoided since at larger scale such manufacturing step is associated with high costs equipment. At clinical and industrial scales such methods are to be replaced by cost-friendly and GMP compliant methods such as Tangential flow filtration (TFF). An early integration of those aspects in the development is a key factor for reducing the time to market.

And overall of those, a control strategy which deals collectively and individually with quality, efficacy and safety is required. ICH Q8 defines Control Strategy as a planned set of controls, derived from current product and process understanding that ensures process performance and product quality. The controls should govern drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control. (ICH Q10).

4.1.1.4. How to extrapolate data obtained with nanomedicines in animals to humans?

- **New statistical-based approaches for translation from preclinical in vitro results of nanopharmaceuticals and predictions' effects on clinical trials**
- **Extrapolation of data obtained in animal models to humans has always presented a challenge for translation to clinical trials and is even more challenging for biopharmaceuticals with nanoparticles**

New statistical-based approaches for translation from preclinical in vitro results of nanopharmaceuticals and predictions' effects on clinical trials

Nanotechnology has conquered a considerable amount of attention from various fields and has become a multidisciplinary subject, where several research ventures have taken place in recent years. This field is expected to affect every sector of our economy and daily life in the near future. The use of statistical methods has also helped the rapid development and validation of nanotechnology in terms of data collection, treatment-effect estimation, hypothesis testing, and quality control [107]. Topics of nanomedicine that can be analyzed through statistics include experimental design, uncertainty modeling, process optimization and monitoring, and areas for future research efforts.

The statistical validation and compliance of data derived from nanomedicine-based solutions is important to allow systematic validation for finding common guidelines [108].

The main methods for performing statistical analysis are enlisted as it follows: mean, standard deviation, regression, hypothesis testing, sample size determination, Bayesian method and statistical inference. The statistical procedures are useful to learn more about the formation processes of nanocomposites. Researchers have reported new progress in developing nanocomposites. Forming processes dictate properties of nanocomposites based on complex chemical and mechanical reactions, where even minor changes of environmental factors or process settings may result in unexpected outcomes [109]. Statistical methods are necessary to improve the performance of experimental runs and sample measurements and the complex relationship between process outcomes and controllable/noise factors.

Statistics can be fundamental to improve low-quality and high-defect processes of nanosyntheses and nanofabrications due to defect rates of nanocomposites that remain rather high. Mass and high-yield production is the key step confronting nanomanufacturing research. Statistical quality control and productivity improvement techniques, which proved to be tremendously helpful in traditional manufacturing, are needed in nanomanufacturing to enhance product quality and improve production efficiency. Statistical modelling helps deal with special data types and processes for developing nanoscale materials that exhibit forms that are different from the usual patterns seen in conventional manufacturing situations [110]. Statistical process control and automatic process control techniques can be used to monitor the process and manufacturing advancements in nanoapplications. Additionally, the possibility of using stochastic partial differential equations plays a role in describing thin-film deposition processes. Extensions of commonly used statistical techniques are required to draw experiments of nanotechnology with good reproducibility and reliability [111]. Beyond these extensions, stochastic modeling of processes of nanopharmaceuticals production and analysis can increase characterization of multilevel or multiscale uncertainties with

[107] Jeng, S.-L.; Lu, J.-C.; and Wang, K. "A Review of Reliability Research on Nanotechnology". IEEE Transactions on Reliability. 2007, 56(3), 401-410.
[108] Jeng, S.-L.; Lu, J.-C.; and Wang, K. "A Review of Reliability Research on Nanotechnology". IEEE Transactions on Reliability. 2007, 56(3), 401-410.
[109] Roco, M. C. (2004). "Nanoscale Science and Engineering: Unifying and Transforming Tools". AIChE Journal. 2007, 50(5), 890-897.
[110] Roco, M. C. (2004). "Nanoscale Science and Engineering: Unifying and Transforming Tools". AIChE Journal. 2007, 50(5), 890-897.
[111] National Nanotechnology Initiative. (2015). "Nanotech Facts". Available at http://www.nano.gov/html/facts/homefacts.html

another valuable topic [112] .To improve the reproducibility of nanoscience-based publications intended for use in biomedical applications, the use of a scientific and statistical-based approach can be related to statistical validation. This includes, details on material characterization (e.g., synthesis, composition, size, shape, dimensions, size dispersity, and aggregation), biological characterization (e.g., cell seeding details, cell characterization, and passage), and experimental protocol details (e.g., culture dimensions, administered dose, method of administration, and delivered dose). In addition to the collection of accurate and valid information on the characterization of nanomedicines using standardized methodologies is a key step toward a more precise understanding and/or prediction of the safety and therapeutic/diagnostic efficacies of nanomedicine products. In other words, although reporting minimum information related to type and results of characterization is essential that is insufficient to guarantee the repeatability and reliability of nanomedicine data. For instance, many characterization techniques such as DLS or zeta potential measurements are highly dependent on experimental conditions and sample preparation details.

Extrapolation of data obtained in animal models to humans has always presented a challenge for translation to clinical trials and is even more challenging for biopharmaceuticals with nanoparticles

Extrapolating data obtained in animal models to humans is a common practice in drug development, including in nanomedicines. Animal models are used to test the safety and efficacy of drugs and to understand the underlying mechanisms of diseases. However, it is important to note that animal models are not perfect representations of human biology and therefore, the extrapolation of data obtained in animal models to humans requires careful consideration.

There are no standardized protocols for adapting physicochemical characterization, manufacturing process, biological activity design, or preclinical and clinical trials, making it difficult to establish the risk–benefit ratio required for any therapeutic product. When extrapolating data obtained in animal models to humans, several factors must be considered, such as physiological differences between animals and humans, differences in metabolism, immune system, and organ structure may affect the safety and efficacy of a drug in humans. Therefore, it is important to carefully evaluate the similarities and differences between animal models and humans before extrapolating data. Another important consideration is the dose and exposure levels used in animal models. physiological, biochemical, and proteomic differences, disease heterogeneity, PK, and bioavailability are different in humans and animals, which makes estimating nanoformulation data more complex [113].

The doses and exposure levels used in animal studies may not be directly applicable to humans, as humans may have different sensitivities or may be exposed to the drug in different ways. Therefore, it is important to carefully evaluate the pharmacokinetics and pharmacodynamics of the drug in humans to determine the appropriate dose and exposure levels for clinical trials.

Almost all research is completed using animal models at some point in the development of a new therapy with the understanding that preclinical results can be successfully translated to humans. Allometric scaling is a method used to estimate the appropriate dose of a drug for a patient based on their body size or weight, which involves using preclinical animal data to predict human PK parameters to determine a clinically relevant starting dose to use in drug development. When it comes to nanomedicines, it is important to consider the unique properties of these particles, such

[112] National Nanotechnology Initiative. (2015). “Nanotech Facts”. Available at http://www.nano.gov/html/facts/homefacts.html

[113] Barré-Sinoussi F, Montagutelli X. Animal models are essential to biological research: issues and perspectives. Future Sci OA. 2015 Nov 1;1(4):FSO63.

as their size and surface charge, which can affect their pharmacokinetics and biodistribution in the body.

There are several mathematical models that can be used to predict the pharmacokinetics and biodistribution of nanomedicines, including allometric scaling models. Some commonly used models for allometric scaling in nanomedicines include:

1. Surface area-based scaling: This model assumes that the dose of a drug should be scaled based on the surface area of the patient's body. This model has been used for several nanomedicines, including liposomal formulations and nanoparticles.
2. Mass-based scaling: This model assumes that the dose of a drug should be scaled based on the patient's body weight. This model has been used for some nanomedicines, but it may not be appropriate for all nanoparticles due to differences in their pharmacokinetics.
3. Volume-based scaling: This model assumes that the dose of a drug should be scaled based on the patient's body volume. This model has been used for some nanomedicines, but it may not be appropriate for nanoparticles with surface properties that affect their biodistribution.

Ultimately, the most appropriate model for allometric scaling in nanomedicines will depend on the specific properties of the nanoparticle and the intended application. It is important to carefully consider the pharmacokinetics and biodistribution of the nanoparticle in preclinical studies and in human trials to determine the most appropriate dosing strategy. Specific guidance for dosing in nanomedicines is based on large particle drug designs, introducing modifications based on the properties, agglomeration states of NPs, and biodistribution data. The drug delivery dose is the patient's administered amount (mg/kg body weight or surface area). For NPs, it can be stated as the number of particles delivered; however, it is always necessary to consider the amount of drug encapsulated for proper comparisons in animals testing [114].

Allometric scaling is based on a power-log relationship between body weight (W) and drug clearance (CL) among mammals and has been used to compare the disposition of nonliposomal drugs across species. However, allometric scaling techniques have not led to many benefits in translating the distribution of NPs from animal models to humans and there is a lack of literature in this area.

Carol W, et al., conducted a study to use allometric scaling to: (1) compare the disposition of pegylated liposomal drugs across species (in male and female mice, rats, dogs, and patients with refractory solid tumors) and determine the best scaling model and (2) predict PK parameters of pegylated liposomal drugs in humans. Standard allometry demonstrated a relationship between clearance of small molecules and NPs with body, spleen, liver, and kidney weights, total monocyte count, and spleen and liver blood flow. However, using scaling to predict CL of these agents in humans often resulted in differences >30%. Despite a strong correlation between body weight and mononuclear phagocyte system (MPS)-associated variables with CL among preclinical species, the use of the equations did not predict CL [115]. These results show the difficulty of using allometric scaling to determine dosing for humans and how a combination of physiologic factors (as compared to a single variable) may be necessary to improve the predictive quality of this technique. Thus, new methods of allometric scaling and measures of MPS function need to be developed.

In this regard, differences in tumor microenvironment between humans and animal models is due to full or partial presence of the immune system. Lucas A, et al., determined the function of the MPS cells (via phagocytosis assessment) against multiple NPs formulations of anthracyclines, including: PEGylated liposomes, non-PEGylated liposomes, micellar doxorubicin and traditional small molecule doxorubicin in common preclinical models, such as SCID mice, Sprague-Dawley rats, and beagle

[114] Ramos TI, Villacis-Aguirre CA, López-Aguilar KV, Santiago Padilla L, Altamirano C, Toledo JR, Santiago Vispo N. The Hitchhiker's Guide to Human Therapeutic Nanoparticle Development. Pharmaceutics. 2022 Jan 21;14(2):247.

[115] Caron WP, Clewell H, Dedrick R, Ramanathan RK, Davis WL, Yu N, Tonda M, Schellens JH, Beijnen JH, Zamboni WC. Allometric scaling of pegylated liposomal anticancer drugs. J Pharmacokinet Pharmacodyn. 2011 Oct;38(5):653-69.

dogs. MPS screening for mouse and rat blood emulated human MPS behaviors, showing that the greatest reduction in phagocytosis occurred after the ex-vivo addition of small molecule doxorubicin > micellar doxorubicin > non-PEGylated liposomes > PEGylated liposomes (Doxil). This trend was most likely due to cytotoxic effects on monocytes and dendritic cells from the small molecule formulations and increasing protective properties of the various formulations from traditional systemic clearance mechanisms and recognition by the MPS [116]. If we could understand how much NP formulations are cleared by MPS cells, we can better predict how this will translate to humans. In this sense, since MPS is an important factor in the clearance of the NPs, any decrease in its function could lead to safety concerns. One of the most recognized consequences of aging is a decline in immune function. In adults that are over the age of 80, there can be a loss of MPS function which would put them at risk for NP related toxicities [117]. This effect was examined in a Phase II trial with 60 elderly subjects who were treated with the pegylated liposomal doxorubicin, Doxil, for metastatic breast cancer. The results of this study showed that in older patient, Doxil plasma half-life was extended (due to reduced clearance of liposomes by the MPS), which led to a higher incidence of Doxil's most common side effect: palmar-plantar erythrodysesthesia [118].

Having a good model is important to translational work because accurately predicting which components have the greatest effect on PK parameters can make scaling up to humans more of a realistic feat. PK variability and lack of predictive value of allometric models have drastic effects when determining the starting dose of a First-in-human Phase I clinical trials. Much of this difficulty comes from trying to explain the inter-patient PK variability between NP agents. In a meta-analysis by Schell R et al.[119] demonstrated that liposomal agents with a lower clearance have a greater degree of PK variability, suggesting a slower and more variable recognition and uptake of the liposomal agent by the MPS which leads to a higher PK variability. Thus, it should be considered that while engineering liposomal or NPs to attain a lower CL and prolonged circulation time in plasma to achieve higher exposures of drug delivered, it could introduce more PK and PD-toxicity unpredictability. A comprehensive meta-analysis of 9 liposomal and non-liposomal anticancer agents was conducted to determine how the experimental study design and sampling schema might affect the PK variability of liposomal NPs in humans. The results of this analysis demonstrate a significantly higher interpatient PK variability of liposomal agents compared with non-liposomal agents when samples were obtained up to 14 days compared with 24 h. In this study, it was highlighted that due to prolonged systemic exposure of encapsulated drug, PK studies of liposomes must extend sample collection to include later time points (to 7-14 days) than those required with conventional formulations with shorter half-lives to accurately calculate PK parameters and fully characterize PK variability in patients. While this is not a concern for conventional anticancer drugs, many of which are mostly cleared from the body after 24 h, liposomal agents, such as the pegylated liposomal formulation of CKD-602 (a semi-synthetic camptothecin analogue) and the PEGylated liposomal doxorubicin (Doxil), are retained in circulation for much longer time periods (from 7 to 28 days). Consequently, sample collection must be extended to later time points when studying liposomes to ensure accurate characterization of PK parameters of these agents. This study highlights the need for the development of a standardized sampling strategy to all NPs and

[116] Lucas AT, Herity LB, Kornblum ZA, Madden AJ, Gabizon A, Kabanov AV, Ajamie RT, Bender DM, Kulanthaivel P, Sanchez-Felix MV, Havel HA, Zamboni WC. Pharmacokinetic and screening studies of the interaction between mononuclear phagocyte system and nanoparticle formulations and colloid forming drugs. Int J Pharm. 2017 Jun 30;526(1-2):443-454.

[117] Lucas AT, Madden AJ, Zamboni WC. Formulation and physiologic factors affecting the pharmacology of carrier-mediated anticancer agents. Expert Opin Drug Metab Toxicol. 2015;11(9):1419-33.

[118] Sostelly A, Henin E, Chauvenet L, Hardy-Bessard AC, Jestin-Le Tallec V, Kirsher S, Leyronnas C, Ligeza-Poisson C, Ramdane S, Salavt J, Van-Hult S, Vannetzel JM, Freyer G, Tod M, Falandry C. Can we predict chemo-induced hematotoxicity in elderly patients treated with pegylated liposomal doxorubicin? Results of a population-based model derived from the DOGMES phase II trial of the GINECO. J Geriatr Oncol. 2013 Jan;4(1):48-57.

[119] Schell RF, Sidone BJ, Caron WP, Walsh MD, White TF, Zamboni BA, Ramanathan RK, Zamboni WC. Meta-analysis of inter-patient pharmacokinetic variability of liposomal and non-liposomal anticancer agents. Nanomedicine. 2014 Jan;10(1):109-17.

conjugated agents, as a means of reducing inaccurate documentation of PK variability that arises from suboptimal study designs. This variability adds a significant cost to the development of NP agents as the PK variability results will lead to more time and resources needed in an individual study. For example, many more dose escalations were needed for NPs compared to small molecules drugs.

Normally, PK parameters such as clearance and volume of distribution were used to describe the disposition of drugs, regardless of being a small molecule drug or a complex NP. These standard PK parameters work well for characterizing small molecule drugs, but there are concerns that traditional mathematical analyses may not provide enough information about tumor delivery due to the prolonged circulation affecting PK parameters, thus affecting the accuracy to describe NPs [120]. To address this problem, Madden A, at el., evaluated a novel metric utilizing the PK properties of both NPs and matching small molecule payloads in tumor-bearing mice: the relative distribution over time (RDI-OT). RDI-OT is defined as the ratio of tissue drug concentration to plasma drug concentration at each time point. The standard concentration versus time area under the curve values (AUC) of NPs were higher in all tissues and plasma compared with small molecules. However, 8 of 17 small molecules had greater tumor RDI-OT $AUC_{0\text{-last}}$ values than their NPs comparators and all small molecules had greater tumor RDI-OT $AUC_{0\text{-}6\ h}$ values than their NPs comparators. Our results indicate that in mice bearing flank tumor xenografts, small molecules distribute into tumor more efficiently than NPs. While these results seem contradictory, it demonstrates that NPs may not accumulate within tumors, but rather provide a slow release of their small-molecule payload in circulation which can readily distribute into tumor [121]. While this mechanism is novel, it also highlights the need to improve on current analytical techniques to separate individual NP states (such as encapsulated/conjugated, released) within blood, tumor, and tissues to accurately define NP disposition.

Developing NPs to improve the specificity of anticancer agents towards tumor tissues and to better control drug delivery is a rising strategy. However, the attrition rate when developing NPs is particularly high and several promising forms showing excellent behavior and efficacy in preclinical studies failed to succeed in subsequent first-in-man studies or later clinical trials. The topic of pharmacokinetic variability is a major and largely underestimated issue with NPs.

Regulation of NPs is under the control of each country's regulatory authority. The regulations for nanoparticles in clinical trials are not specific for this type of drug and must follow the same rules as conventional drugs, and there are only certain specifications given that come from previous experience from the researchers. Once NPs have reached regulatory agencies evaluation, questions regarding the extrapolation of data obtained in preclinical to clinical trials arise due to the lack of guidelines for assay development and the absence of specific regulatory aspects. There is a lack of controls, comparators, problems with stability, dose calculation, bioequivalence assessment, and biological toxicity demonstration. The failure of several formulations at the clinical stage is due to the lack of specific protocols for physicochemical, biological, and physiological characterization [122]. There is a need to reconsider obtaining a broader data set to address the specificities of the pharmacokinetic and pharmacodynamic profiles of these novel formulations.

Common causes for early clinical failure of nanoformulated drugs include endotoxin contamination, the induction of cytokine storm, hypersensitivity reactions, complement activation,

[120] Piscatelli JA, Ban J, Lucas AT, Zamboni WC. Complex Factors and Challenges that Affect the Pharmacology, Safety and Efficacy of Nanocarrier Drug Delivery Systems. Pharmaceutics. 2021 Jan 17;13(1):114.

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thrombogenicity, and API immunotoxicity. Another difficulty at this stage is the clinical study design where the choice of the appropriate study size, the number of controls, and the timing of therapy administration are challenges whose correct choice determines whether the trial is relevant to test the study hypothesis. Patient selection criteria, dosing regimen, the timing of therapy administration, stages of the condition, and duration time add to the challenges likely to contribute to failures in the translation of NPs therapies [123]. While small molecule phase I dose-escalation studies in human patients evaluate three to five doses, this can rise to as high as fourteen for nanomedicines, which may derive from the initial analysis of starting doses in canine models that can present hypersensitivity to nanomedicines [124]. This trend was highlighted in a meta-analysis by Caron W, et al., in which the group gathered information about the number of dose escalations and patients enrolled in Phase I NPs and small molecules trials. The studies involving NP agents had a significantly greater number of dose levels than studies involving SM agents (7.3 vs 4.1, respectively) [125]. With more dose levels, more resources are needed to account for an increased need for patients. At an average cost of ~$100–150,000 per patient in a phase I trial, the increased number of patients means a significantly higher cost to run these trials.

At later clinical stages, during Phase 2 and Phase 3 clinical trials of novel nanoformulations the main causes of the failure are related to lack of efficacy rather than toxicity. Consequently, there are few trials in clinical research facing numerous regulatory challenges [126]. The question arises as to why nanoparticle-based biopharmaceutical systems fail to achieve marketing approval despite their significant advantages. The challenge lies in the fact that the very properties that make NPs promising have become a challenge for the researchers doing the design and the evaluators at regulatory agencies. Key issues related to clinical development include biological challenges, large-scale manufacturing, biocompatibility and safety, intellectual property, government regulations, and overall cost-effectiveness compared to current therapies [127]. Overall, researchers should interact with regulatory bodies at early stages to compile and organize relevant information for submission to the regulatory reviewers while filing an investigational new drug (IND) application for nanomedicines requesting authorization for clinical trials.

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4.2.1. HARD NANOMEDICINES AND ORNAMENTATION OF NANOPARTICLES

4.1.2.1. Underscore the regulatory challenges of the future wave of cancer nanomedicines

Cancer nanomedicine is considered to be one of the most promising fields of research, with the potential to revolutionize cancer treatment. Nanomedicine involves the application of nanotechnology in the creation of targeted drug delivery systems for cancer treatment. Although the field has shown significant progress in recent years, several regulatory challenges require considerable attention. These challenges range from safety and efficacy to production and manufacturing.

One of the most important challenges in the regulation of cancer nanomedicine is safety. The utilization of nanotechnology in drug delivery systems raises concerns about the potential toxicity of these systems. It has been observed that certain nanoparticles could cause organ-specific toxicity, such as liver and kidney damage, when administered to animals, which highlights the importance of safety evaluation. Toxicity is a major challenge in the manufacturing and commercialization of nanomedicines, and regulatory authorities must thoroughly evaluate the safety data before granting approval.

Another significant regulatory challenge is the efficacy of cancer nanomedicine. While many studies have shown that nanomedicines exhibit promising results in vitro and animal models, the translation of these findings to patients has been limited. Efficacy is a critical component of drug approval, and investigating the effectiveness of nanomedicines in clinical trials is necessary for regulatory approval.

A further challenge in the regulation of cancer nanomedicine is the standardization of manufacturing practices. Manufacturing nanomedicines requires strict quality control and standardization, as several variables can significantly affect the final product's efficacy and safety. Additionally, production scale-up can also be challenging, and the cost of manufacturing must be kept within acceptable limits.

Finally, regulatory bodies must ensure that the regulatory framework is flexible and adapts to advancing science and technological advancements. The rapid development of the field of cancer nanomedicine requires regulatory authorities to stay up to date with new innovations, products, and processes, and to reform the regulatory framework accordingly.

While cancer nanomedicine shows promise, regulatory challenges surrounding safety, efficacy, manufacturing, and regulation persist. Regulatory authorities must overcome these challenges to ensure that nanomedicines are thoroughly evaluated for safety and efficacy, and patients can benefit from these new technologies for targeting therapeutics.

The translation of nanomedicines from the lab level into marketed product faces several challenges, including characterization of physicochemical properties, pharmacodynamics, pharmacokinetics, process control, biocompatibility, and nanotoxicity, scaling-up as well as reproducibility. The challenges of nanomedicine development are in connection with the different requirements from the patient (clinical and therapeutic use), industry (production), and regulatory bodies (authorization process). This paper aims at reviewing the status and regulatory aspects of nano-based drug delivery systems with a focus on the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) regulations. In addition to discussing the risks accompanied by the development of nanomedicine, the potential of following a risk-based methodology from the early stage of the R&D phase is emphasized here to ensure safety and efficacy when developing novel nano-based dosage forms. The R&D of nanomedicines is a complex and multidisciplinary approach, and there are still many challenges in their regulation and legislation. In general, the most

critical considerations for nanomedicines are the product quality assessment (physicochemical characteristics, quality control, manufacturing process) and product safety assessment (pharmacokinetics, biodegradation, accumulation, and nanotoxicity). The paper presents a promising paradigm in the development and marketing authorization of nanomedicines, namely the Quality by Design (QbD) approach. Sufficient knowledge on the quality, safety, and efficacy of nanomedicines is necessary to obtain a significant focus on establishing robust, standardized methods for evaluating the critical quality attributes of nanomedicines. The QbD-based submission is highly recommended and required by the regulatory authorities, enabling a smooth clinical translation of the novel nanomedicines.

4.1.2.2. Integration of cancer nanomedicine with tissue engineering and nanobiotechnology for multidisciplinary approaches for cancer treatment (nanorobots)

Nanomedicine can be considered the step forward to manage therapeutics delivery by nanorobots. Multifunctional nanomedicines have revolutionized the field by providing new nanosystems that tackle multiple disease fronts, mainly via co-delivery of cargos with therapeutic, diagnostic, or theranostic properties, and active targeting of the cargo to the targeted site that can be integrated with tissue engineering and bioengineering and site of lesion. Nanomedicines may play a crucial role with a significant development boom in recent years, but unsatisfactory rates of clinical translation. Thus, this is in line with the urgent need to develop new tools the that exploits the interdisciplinary intertwining of engineering, material sciences and nanotechnologies to create new futuristic solutions for cancer nanomedicine [128].

The physicochemical properties of nanotherapeutics and nanorobots (for example, size, geometry, surface features, elasticity, stiffness, porosity, composition, targeting ligand and drug release kinetics) affect systemic delivery to tumors, thus determining and exploiting the EPR effect and therapeutic outcomes [129]. The "engineered" surface of a nanomedicine product and developed nanorobots interfaces with the biological environment, the precise nature of which will depend on the proposed clinical application, and the route of administration e.g. blood plasma in intravenous delivery. Many nanomedicines approved as products and/or undergoing development include as an integral component of their design either a non-covalent or covalently bound coating. The physico-chemical nature of the nanocarriers, the uniformity of surface coverage, and the coating stability (both in terms of attachment and susceptibility to degradation) will govern the pharmacokinetics, the bio-distribution of the product and its intracellular fate. In addition, the infusion-related reactions observed clinically for certain coated nanomedicines (e.g. iron solutions and PEGylated liposomes) may be due to the physico-chemical properties of the coating material, specific bio-molecular interaction with the coated nanomedicines (e.g. complement activation) and/or cell interaction.

The intrinsic limits of conventional cancer therapies prompted the development and application of various nanotechnologies for more effective and safer cancer treatment, herein referred to as cancer nanomedicine.

Effective systemic delivery of nanotherapeutics to solid tumours requires a deeper understanding of the biological factors involved, such as nanoparticle–protein interaction, blood circulation, extravasation to and interaction with the perivascular tumour microenvironment, tumour tissue

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penetration, tumor cell internalization and intracellular trafficking [130]. Considerable technological success has been achieved in this field, but the main obstacles to nanomedicine becoming a new paradigm in cancer therapy stem from the complexities and heterogeneity of tumour biology, an incomplete understanding of nano–bio interactions and the challenges regarding chemistry, manufacturing and controls required for clinical translation and commercialization. The supramolecular properties of these nanosystems can be precisely and rationally programmed for the design of smart depot vehicles, enabling an efficient in vivo transport and combinatorial therapy, within a single nanomedicine structure. Targeting the tumor microenvironment with nanorobots and the premetastatic niche with nanotechnologies offers another promising strategy for cancer therapy.

From this point of view nanorobotics can help with a new novel engineering approaches to capitalize on our growing understanding of tumor biology and nano-bio interactions to develop more effective nanotherapeutics for cancer patients [131].

Nanorobots are classified such as nanoelectromechanical systems (NEMS) derived from the miniaturization of microelectromechanical systems (MEMS). To create functional nanorobots are essential the stages of designing, architecting, producing, programming, and implementing such biomedical nanotechnology that are all necessary steps of nanorobotics [132].

A large amount of effort is currently being invested in developing precision medicine-based strategies for improving the efficiency of cancer theranostics and modelling, which are envisioned to be more accurate, standardized, localized, and less expensive. To this end, interdisciplinary research fields, such as biomedicine, material sciences, pharmacology, chemistry, tissue engineering, and nanotechnology, must converge for boosting the precision cancer ecosystem. In this regard, precision biomaterials have emerged as a promising strategy to detect, model, and treat cancer more efficiently. These are defined as those biomaterials precisely engineered with specific theranostic functions and bioactive components, with the possibility to be tailored to the cancer patient needs, thus having a vast potential in the increasing demand for more efficient treatments. It appears that nanodrug delivery systems hold great potential to overcome some of the barriers to efficient targeting of cells and molecules in cancer. Nanorobots can have similar size to that of organic human cells and organelles that provides a huge variety of its possible uses in the field of health care and environmental monitoring of microorganisms. Multifunctional applications can be cell healing that is possible with nanorobots that are tiny enough to reach the cells. The envision and creation of artificial cells (nanorobots) that patrol the cardiovascular system, thus, detecting and destroying infections in minute quantities, distinguish cancer cells from healthy ones and kill them in a site-specific way [133]. This nanorobotics platform could be a programmable system with approachable ramifications in medicine, creating a revolutionary replacement from therapy to bar. Chemotherapeutic substances employed in cancer treatment measure disseminates non-specifically throughout the body, where they exert an influence on both malignant and normal cells, nanorobots may be able to deliver these drugs only to the specific target.

A nanorobot can provide with smart chemotherapy solutions for medication administration and give an efficient early dissolution of cancer by targeting only the neoplastic-specific cells and tissues and preventing the surrounding healthy cells from the toxicity of the chemotherapy drugs so being used.

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Nanorobots have the advantage to be engineered as drug transporter for timely dose administration that allow chemical compounds to be kept in the bloodstream for as long as essential, providing expected and controlled pharmacokinetic characteristics for chemotherapy in the therapies for anti-cancer [134]. The clinical use of nanorobots for diagnostic, therapy, and surgery can be achieved by injecting them via an intravenous route. The nanorobots may be getting intravenously injected into the body of the recipient [135].

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