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CONSENSUS PROTOCOLS FOR IN VITRO TESTING OF NANOMATERIALS

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1. Models and methods in cancer nanomedicine: general considerations

Nanotechnologies offer high expectations in oncology as they can provide new, safer, and more effective solutions for the delivery of anticancer therapies. Nanoparticle-based delivery can offer a significant reduction of the toxic effects of chemotherapeutic drugs, increasing their tolerability and efficacy. Many different classes of nanoparticles have been developed for drug delivery and are currently on the market. These include polymeric, inorganic, and lipid-based nanoparticles, each class having its own specific properties. The overall advantages of nanomedicines are depicted in Figure 1.

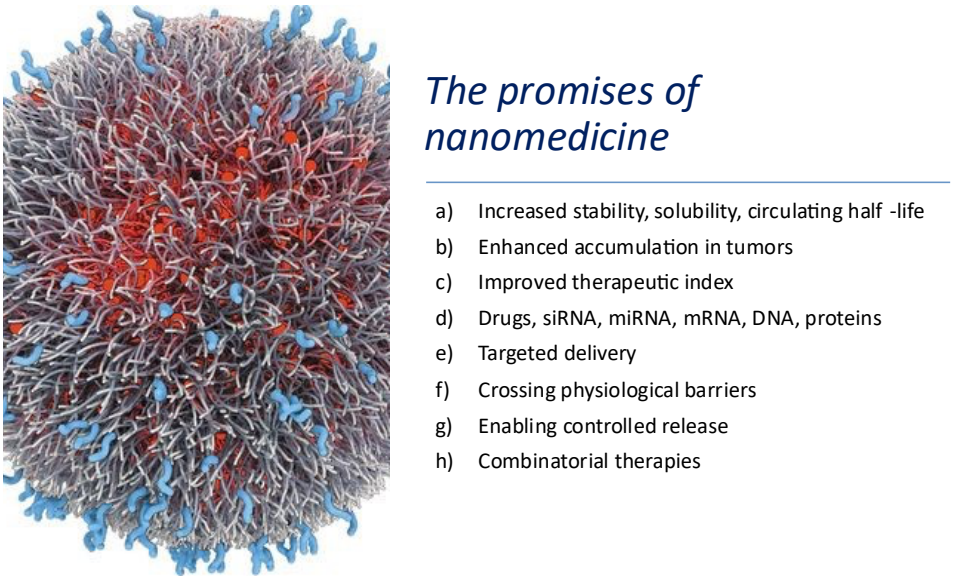

Figure 1. The promises of nanomedicine.

To date, several cancer nanotherapeutics are used in clinics, and many more are under investigation in clinical trials and preclinical studies. However, numerous delivery systems that are effective in preclinical models fail in clinical trials. Thus, the improvement of cancer nanomedicines to increase safety and efficacy remains a major challenge in the field. In addition, the improvement in the chemical composition, design, and biological compatibility of nanoparticles, needs to be matched by the use of new, more stringent, and rigorous approaches for preclinical and clinical investigation.

The objective of this document is to gather and review information on the current approaches for studying nanomedicines for oncological applications in preclinical models and propose ideas and guidelines for future improvement.

Nanomedicines are considered a sort of magic bullet that can be loaded with any drug and can deliver it right to the tumor cells sparing any other cell or tissue in the body. This simplified view has been disappointingly contradicted by the data and the limited number of nanomedicines approved for clinical use. However, research in this field has improved our knowledge of the biological barriers to overcome to reach the target and is providing new solutions and a basis for improvement. Researchers are increasingly aware of the challenges they must face to move nanomedicines to clinical practice in oncology. Indeed, the development of cancer nanomedicines shares many of the same challenges faced in targeted anticancer drug discovery and development in oncology. Overall, the main obstacles to the success of targeted anticancer drugs ad cancer nanomedicines are related to the complexity and diversity of cancer biology and include specific aspects linked to inter- and intra-tumor heterogeneity and the continuous evolution of the disease (Figure 2).

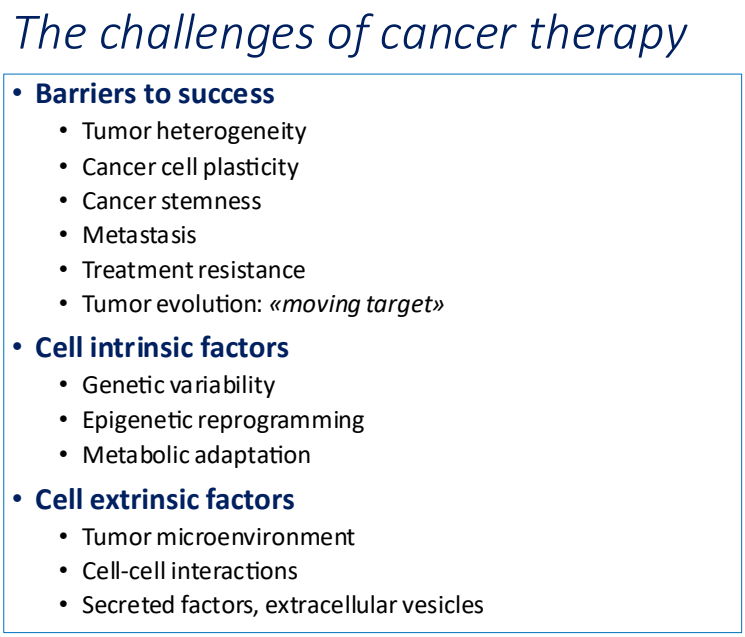

Figure 2. The challenges of cancer therapies.

Importantly, appropriate experimental models and techniques are needed at all steps of the process of preclinical and clinical development to define preclinically the activity, selectivity, and safety of nanomedicines. The choice of the appropriate models to study nanomedicines is a fundamental issue in oncology considering the complexity of the disease. Moreover, cancer nanomedicines should be considered an integral component of personalized medicine strategies (Figure 3). Indeed, nanomaterials can be designed according to the specific needs in terms of therapeutic cargo, tumor target, and the specific objectives in sight. Nanoparticles can be designed to survive in circulation

and release their cargo in a specific environment, tissue, or cell type. In any case, the choice of experimental systems and models is an essential first step.

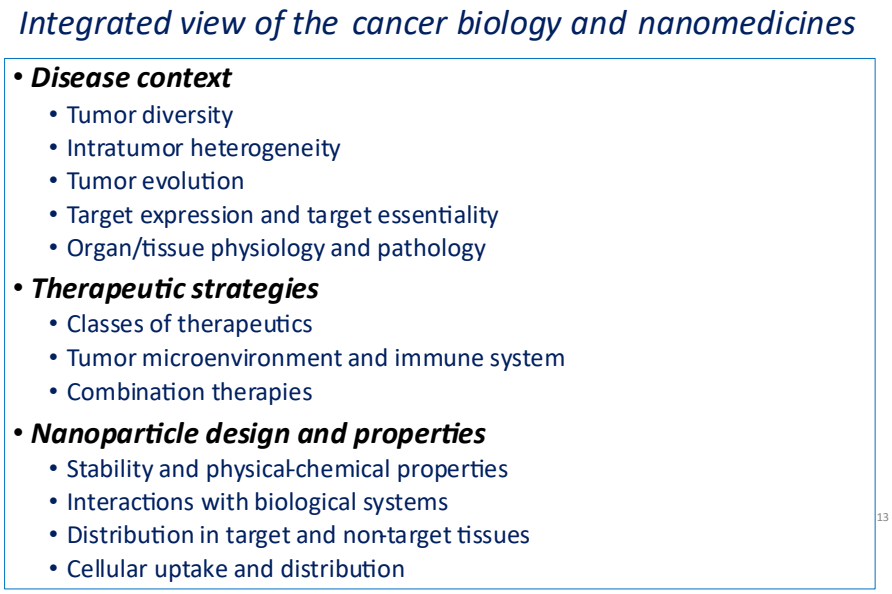

Figure 3. An integrated view of the development of precision cancer nanomedicines.

Typically, cancer nanomedicines delivered intravenously accumulate in the tumor thanks to the enhanced permeability and retention (EPR) effect. The EPR is due to the defective tumor vessels and the impaired lymphatics tissue, which allow the entry and retention of the nanoparticles in the tumor interstitial space. However, the relevance of the EPR is often overestimated. Nanoparticle properties (*e.g.*, size, shape, and surface modifications) influence the EPR effect. Furthermore, heterogeneity in vascular leakiness of tumors and environmental barriers also compromises the EPR and delivery of nanoparticles.

An important aspect in oncology is the need to increase the efficacy, not only prevent toxicity, of the therapy. The development of nanoparticles capable of actively recognizing the target cells and delivering the drug load is expanding. This approach exploits the possibility of decorating the outside of the particles with ligands capable of recognizing specific receptors present on the surface of the target cells. Then, the ligands are expected to guide the delivery of the nanoparticles and favor the release of the drug load in the target cells. In this way, the drug could be released into the tumor cells avoiding normal cells, increasing the specificity and effectiveness of the therapy.

Following the production of nanoparticles with specific physical and chemical properties with the required surface modifications and therapeutic cargo, the preclinical studies include their testing in biological systems in vitro and confirmation of efficacy and safety using in vivo models. The selection

of the biological systems for in vitro and in vivo validation studies is therefore a central issue in every project aimed at developing new cancer nanomedicines. Studies in vitro are performed with human and murine cell lines and primary cell cultures. There is also increasing interest in 3D cell cultures, like tumor organoids and spheroids, to assess the ability of nanomedicines to target specific cell types and exert functional effects.

Ultimately, to examine the effects of novel treatments in vivo, animal models are essential. Mice are the most common animal model for studying cancer biology and therapy. The need for in vivo models can be fulfilled by implanting human cells in immune-compromised mice. Engrafts can be done subcutaneously facilitating the monitoring of tumor growth. Alternatively, orthotopic and metastatic models can mimic the tissue environment of the primary tumor and metastatic sites. Furthermore, a broad range of genetically modified mice prone to produce tumors orthotopically in the relevant organ is available for testing therapeutics in immune-competent animals. Genetically engineered mice (GEM) are useful for investigating tumor cell responses in the context of an intact immune system. In the case of nanomedicines, GEMs might be particularly apt to investigate the interactions with the local tumor/tissue environment and the impact of the immune system on the efficacy and toxicity of the treatment.

2. 2D cell culture models

A new drug must undergo several steps for the evaluation of safety and activity before approval. Cell-based in vitro assays are essential for drug testing. Cell models are important in nanomedicine for the preliminary assessment of activity before testing in vivo. Furthermore, are useful to understand the mechanisms by which nanoparticles interact with cells. The in vitro assays are generally less expensive than in vivo models and can give more rapidly useful information on the potential of new nanomedicines. Moreover, ethical considerations favor the use of in vitro models to reduce the number of experiments in animals.

The simplest methods to detect a drug's anticancer activity are the cell viability and proliferation assays that measure metabolic changes as a function of the number and activity of viable or nonviable cells. Indeed, cell viability and proliferation are two distinct concepts. Cell viability is a measure of the number of living metabolically active cells while proliferation depends on cell division. Indeed, not all viable cells are in the division stage. Then, a proper detection method can be chosen to evaluate proliferation rate or viability, or both. Colorimetric assays such as SRB or crystal violet are useful to assess the number of viable cells. DNA staining dyes, such as DAPI or Hoechst, or

enzymatic-based probes, such as MTT, XTT, Cell Titer Glo, and JC-1, are used for evaluating cell metabolic activity and cell proliferation.

Other 2D *in vitro* assays are suitable for testing the impact of nanomedicines on tumor cell proliferative capacity and phenotypic features. For example, the colony formation assay probes the effect of the treatment on the ability of single tumor cells to form a colony. Migration and invasion by tumor cells can be measured in Boyden chamber assays.

Much effort has been spent recently to develop new approaches to mimic more closely in vitro some of the in vivo properties of tumors (see below). These 3D models represent intriguing new methods for testing nanomedicines. The 3D tumor-spheroids are enriched in cancer stem cells and can be used to determine the impact of treatments on the tumor-initiating stem-like cell subpopulation. The 3D tumor organoids are useful to recreate the arrangement of tumor cells and better mimic the interactions of tumor cells and components of the microenvironment in a 3D space, mimicking the intact tissue.

All these in vitro systems are suitable for assessing the impact of the treatment on cell proliferation and viability and other biological endpoints and mechanisms. In this context, immunofluorescence (IF) microscopy, based on the use of specific antibodies recognizing the proteins of interest, can provide information on the target expression and localization and the mechanisms of internalization and activity of nanomedicines. Additional information to decipher the drug's molecular activity (e.g., cell death, metabolism, proliferation) can be obtained by flow cytometry.

The in vitro models can allow reproducing some aspects of the tumor microenvironment like extracellular matrix composition and soluble factors to investigate the interactions and impact on the response to nanotherapeutics. In vitro systems are used to to determine the cellular uptake and biodistribution of nanoparticles within cellular compartments. In biological systems, nanoparticles are rapidly covered by the so-called protein corona. The quantity and the type of corona on the nanoparticle surface depends on the intrinsic properties of the nanoparticle (size, shape, surface charges and hydrophobicity) and on the external environment. The presence of the protein corona influences the uptake, internalization, and drug release. Using in vitro models is possible to control the formation of the protein corona and study the influence of variables of the tumor microenvironment. In addition, it is possible to study external factors such as pH, temperature, shear stress and incubation time. Using in vitro models is possible to study the effect of targeting moieties on the uptake of nanomaterials by cancer cells.

3. 3D in vitro models

Organoids are defined as self-organizing three-dimensional cell cultures that faithfully represent the organ from which they are established. Organoid cultures can be initiated from different sources, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (ASCs). A tissue biopsy sample is generally dissociated into single cells or small tissue fragments and plated in organoid-forming conditions. Different methodologies can be employed to induce organoid morphogenesis, however, the most used one is to plate cells embedded in a semisolid extracellular matrix (ECM) and culture them with tissue-specific media, supplemented with a mixture of suitable growth factors. Under these conditions, cells self-assemble and generate small, highly organized 3D structures that recapitulate the arrangement of the tissue of origin. Organoids established from human samples are defined as patient-derived organoids (PDOs), and they reflect the characteristics of the patient tissue from which cells are derived. To increase the stability of the organoids cultures and to have the possibility to establish them without the need for an immediately available patient sample, cells from a patient biopsy can be injected subcutaneously in immunocompromised mice where they grow to form a xenograft, defined as patient-derived xenograft (PDX). This tissue can be in turn digested and plated in organoid-forming conditions to generate PDOs. Organoids can also be established starting from murine tissues, e.g. from mice with a specific genotype, with disease-associated genetic mutations, mice subjected to a treatment, or healthy mice as controls. These 3D structures are defined as mouse-derived organoids (MDOs).

In the last decade, organoids have been extensively studied and are emerging as promising models to overcome the limitations of in vitro studies and bridge the translational gap to in vivo applications. The 2D cell cultures are cheap, easy to maintain, and very suitable for high throughput screening making them ideal for the first phases of experimentation. The in vivo models are very complex and allow an accurate representation of human physiology and pathophysiology. They are expensive, time and resource-consuming and raise ethical concerns. Organoid cultures provide many advantages: first, they comprise a heterogeneous cell population assembled in a well-organized 3D structure and are therefore more representative models than 2D cell lines. They are suitable for high throughput screening and maintain the characteristics of the organ from which they are derived (e.g., PDOs represent patient characteristics and response to treatment). Organoid cultures are relatively cheap and are suitable models for preclinical applications with translational relevance. Organoids are currently employed for a wide variety of applications, from basic research to developmental biology. They can be genetically modified to study disease-associated mutations. They are extensively used for drug development and testing. In this field, they have proven to be suitable models for the preclinical testing of nanomedicines. Different strategies can be used for testing nano-

drugs. However, if our goal is to test a nano-compound on organoids we need to consider the ability of the nanoparticles to penetrate the 3D structure. This feature is influenced by the different physiochemical characteristics of the nanoparticle, including size, shape, stiffness, crosslinking and surface charge.

To verify efficient nanoparticles penetration into the organoids nanoparticles can be loaded with a fluorescent dye and internalization can be verified by confocal microscopy. If we then want to assess the effects on the cells within the organoids, we can choose between a variety of functional assays. A readout of treatment efficacy is loss of cell viability. Assays like the CellTiter-Glow 3D are designed to determine cell viability in 3D organoids and spheroids by measuring the level of ATP in a luminescence-based assay.

Another aspect that can be evaluated when studying a drug's efficacy on organoid cultures is the ability of cells to self-assemble and generate 3D structures. This can be measured by quantifying organoids' numbers and diameters as a readout of cellular proliferation and phenotypic reprogramming. Furthermore, since organoids are complex structures that recapitulate the organization of the tissue of origin, another aspect that can be evaluated when assessing a drug's efficacy is organoid morphology. For instance, different morphologies have been described for prostate cancer organoids that reflect the characteristics of the tissue of origin. Cystic organoids, with a well-conserved lumen, reflect the morphology of a healthy prostate; full, hyperplastic organoids are histologically like prostate adenocarcinoma and organoids with more irregular, bulging structures are described to be models of more advanced stages of the disease.

By observing organoids morphology, we could also assess the invasive potential of tumor cells within the 3D structure. Indeed, those structures with cells sprouting from the organoid surface outwards, defined as slithering, reflect invasive properties and metastatic capabilities. There are different methods to quantify this phenomenon such as counting the number of cells that escape the organoid, measuring the distance reached by invasive cells from the organoids, or measuring the total area covered in time. These 3D structures can also be characterized and observed by confocal microscopy to appreciate their morphology and look for specific characteristics using various cell trackers or antibodies. Besides functional assays, one can dig deeper into the pharmacodynamics of a compound using various molecular assays. For example, one can sort cells and perform single-cell RNAseq to evaluate different cell populations in the organoids culture or extract total RNA and perform qRT-PCR or bulk RNAseq to evaluate changes at the transcriptional level. To look at molecular targets at the protein levels one can extract proteins and perform immunoblotting analysis.

4. Tissues-on-a-chip models

Tissue engineering (TE) is an evolving interdisciplinary field that integrates biology, engineering and medicine. It focuses on the development of biological substitutes to restore, replace, maintain, or enhance tissue and organ function. Cells, growth factors and biomaterial scaffolds are the basic elements of TE, which can also exploit technologies such as biofabrication and bioreactors to generate a tissue construct replicating its native counterpart. The cells could be either progenitor cells that can be differentiated into the specific cells of interest or mature primary cells. Growth factors are essential both to foster cell growth and to induce the differentiation of progenitor cells into their mature phenotype. The engineering scaffold provides the 3D architecture to the developing tissue and can be used to deliver growth factors for new tissue formation. Finally, different technologies can be used to generate a TE constructs, ranging from 3D printing and biofabrication, which can predefine tissue architecture, to microfluidics and bioreactors, useful to provide adequate physicochemical stimuli for construct maturation up to computational simulations, to rationally design the tissue constructs. The typical workflow for producing a TE construct starts from the isolation of cells from a patient biopsy, which then are expanded and seeded on a biomaterial scaffold. The cellularized construct is maintained in controlled conditions until maturation. Once the tissue is ready, it is possible to use it as a model of the real tissue, for investigating pathophysiological mechanisms or testing drugs in a relevant environment.
In this context, the pipeline for discovering a new drug suffers from low efficiency, with many promising compounds that fail in clinical trials after successful preclinical phases, due to unexpected toxicity or low effectiveness. A major cause resides in the inadequacy of available pre-clinical models, consisting in 2D in vitro models and animals. Indeed, 2D in vitro systems are too simplified as compared to complex conditions in vivo and animal physiological and pathological mechanisms do not always match those in humans, resulting in poor predictivity of patient outcomes. To overcome this limitation, TE constructs used as models, are starting to emerge as a promising strategy. They are generally based on microfluidic devices, with the advantage of high control on environmental conditions and small sample volumes, hosting 3D matrices embedding tissue-specific cells. They are produced through soft litography techniques, consisting in the production of a silicon or resin mold, which is then transferred to a PDMS slab that is coupled to a cover glass slide to generate closed channels inside. Cellularized gels and culture medium can then be inserted in the channels, possibly also applying fluid flow with a pump.

Models of bone and muscle tissues with vascularization have been generated, allowing to follow the flow, arrest and transendothelial migration of cancer cells in the tissues. The model showed how

cancer cells migrated more into a bone-like tissue as compared to muscle and that this bone migration was hindered by the addition of adenosine in the environment. With a similar model, it was also demonstrated that the addition of platelets and neutrophils to cancer cells flowing into the vessels increased cancer cell extravasation and that the supplementation of an anti-platelet drug reversed this effect. A further model used in the lab recapitulates the structure of an established bone metastasis, with a perfusable vascular network in which it is possible to flow immune cells such as neutrophils. The vascular network in the model was responsive to the presence of both tumor cells (which decreased its development) and neutrophils (which increased its permeability).

Microfluidics is a powerful technology, however, it has also some drawbacks, such as the limited thickness of the models, thus not really representing the complex architecture of tissues in vivo, and the difficult application of standard analytical techniques due to the scarce biological material involved. Thicker 3D tissue models can be generated, for example mimicking the bone and the muscle tissue. The bone model was based on a fibrin gel containing calcium nanoparticles and embedding vascular cells, osteoblasts, osteoclasts and macrophages. The addition of breast cancer cells led to cancer cell growth and localization in the perivascular niche. Furthermore, the model reproduced the response to anticancer drugs better than a simpler cancer 3D model. Finally, the skeletal muscle model that was developed allowed to achieve the reconstruction of non-planar muscle fibers, intertwined with a vascular network and surrounded by an endomysium layer. With this model, it was possible to recapitulate the main hallmarks of a fibrotic skeletal muscle such as the overproduction of collagen and fibronectin. In conclusion, TE 3D in vitro models can recapitulate pathological mechanisms and drug effects better than standard 2D models, paving the way to new methods for preclinical screening potentially improving the drug discovery process.