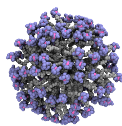

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EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

WG3 - DELIVERABLE 3.2

CONSENSUS PROTOCOLS FOR IN VIVO TESTING OF NANOMATERIALS

Action working group	WG3
Deliverable Nature	Report
Deliverable Identifier	D3.2
Dissemination level	Public
Contractual date of Delivery	31-12-2022
Project website	www.nano2clinic.eu
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1. In vivo preclinical models

The development of nanotechnologies has opened exciting avenues in the area of drug delivery in the field of oncology. The preclinical development of nanomedicines requires their validation using in vivo models. To examine the effects of a novel treatment in *vivo*, an appropriate *animal model* is essential. Murine models are the most eligible animal models in preclinical pharmacologic trials (Figure 1).

Figure 1. Biological endpoints gained from studies with in vivo models.

Genetically engineered mice (GEM) with induced mutations in an immune-competent allow to investigate therapeutic compounds and interactions between cancer cells and the immune system. Nevertheless, the GEM may not perfectly resemble the human diseases' complexity and complementary in vivo models to study human cancer cells are required. This need is fulfilled by implanting human cancer cells in immune-compromised mice. Importantly, engrafts of human and murine cells can be carried subcutaneously and orthotopically. Independently from the site of engraftment, cancer cells may be marked with luciferase or fluorescent tags to facilitate in vivo detection.

In GEM models, such as *PB-Cre; Pten$^{fl/fl}$; R26^{ERG}* and *Alb-Cre; Pten$^{fl/fl}$*, the expression of the Cre-recombinase enzyme leads to the excision of flagged DNA sequences. Importantly, the re-recombinase expression is driven by tissues specific promoters limiting the genetic modification to specific cell types. This approach allows generating specific tumor models that can be employed for in vivo studies as well as sources of murine tumor cells with a defined genetic background. The explanted tumor cells derived from GEMs can be used in 2D or 3D culture systems, or in vivo by implantation in immune-competent host animals (allografts).

Notably, it is important to remember that tumors are often heterogeneous in their cell composition. This heterogeneity is explained by the existence of a hierarchical organization of cancer cells where at the top a subpopulation of cancer with stemness properties is positioned. Cancer stem cells are characterized by the ability to self-renew and progress in cellular subpopulations with different proliferative, tumorigenic and cell phenotypes. Importantly, the differentiation program can be reversible leading to phenotypic plasticity, which can strongly affect treatment response and interaction with the microenvironment. For these reasons, it is important to characterize the different cellular components within tumors to plan an adequate treatment strategy.

Primary tumors often evolve into aggressive and lethal metastases. Models of metastatic disease are of fundamental importance for testing cancer nanomedicines. Liver is one of the most common sites of metastasis for a wide range of solid tumors and is associated with high cancer related lethality. Mouse models of liver metastases are either transplantable or genetically modified. Transplantable models are faster to generate. They involve the injection of cancer cells into the spleen or direct implantation into the liver parenchyma. Intrasplenic injection results in the formation of liver tumors by drainage into the hepatic portal vein via the splenic vein. Intrahepatic injection is a well-established method to generate local lesions after injection. The tumors gradually grow locally and metastasize throughout the liver via intrahepatic vessels. Intrasplenic and intrahepatic injection routes are also valid methods to establish orthotopic models of liver cancer.
In addition, the intravenous and intracardiac injections can reproduce metastatic dissemination to multiple organs, like bone, liver, brain and lung. Together, these methods allow to compare the growth properties of primary and metastatic tumors in the liver and other organs. They also provide valuable tools to study the growth and metastatic dissemination of human tumor cells using immune-compromised mice. On the other hand, implantation of mouse-derived tumor cells (i.e., allografts) in immune-competent mice will be the primary approach to studying the development of primary and metastatic tumors and the impact of the microenvironment and immunological state of the host. Establishing these models in immune-competent mice will also permit addressing the role of the immune microenvironment in therapeutic response and resistance.

Importantly, lesions generated by injected cells can be monitored with ultrasound technology (such as Vevo3100 Fujfilm). Moreover, when possible, cells can be tagged with luciferase and fluorescent protein (e.g. mCherry) before engraftment in order to detect the cells in vivo using optical imaging systems (e.g. IVIS PerkinElemer and NewtonFT500 Witec). With this approach, fluorescent-tagged cells can be isolated ex vivo from the different cellular components constituting the tumor

parenchyma. The ability to follow cancer cells and nanoparticles systemically in vivo provides added value to this research.

In conclusion, Figure 2 summarizes and compare the biological and pharmacological endpoints from in vitro and in vivo studies.

2D *in vitro* models: biological and pharmacological endpoints

- Target expression and essentiality
- Uptake
- Cell toxicity
- Cellular sublocalization
- Biochemical and functional studies
- Biomarker identification
- Tumor heterogeneity
- Treatment resistance

3D *in vitro* models: biological and pharmacological endpoints

- Target maintenance expression
- Uptake
- Cell toxicity
- Biochemical and functional studies
- Biomarker identification
- 3D diffusion
- Structure disruption
- Tumor microenvironment (TME) studies

In vivo models: biological and pharmacological endpoints

- Survival
- Systemic toxicity and side effects
- Immunogenicity
- Antitumor effect
- Antimetastatic effect
- Biochemica studies
- TME assessment
- Systemic biodistribution
- Tumor distribution
- PK and PD
- Biomarker validation

Figure 2. Summary and comparison of biological and pharmacological endpoints from in vitro and in vivo studies.