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PRECLINICAL MODELS IN ONCOLOGICAL PHARMACOLOGY: LIMITS AND ADVANTAGES

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SUMMARY

A wide range of experimental tumour models, each with distinct advantages and disadvantages, is nowadays available. Due to the inherent differences in their complexity and functionality, the choice of the model is usually dependent on the application. Thus, to advance specific knowledge, one has to choose and use appropriate models, which complexity is largely dependent on the hypotheses to test, that is on the objectives. Whatever the model chosen, the complexity of cancer is such that none of them will be able to fully represent it. *In vitro* tumour models have provided important tools for cancer research and still serve as low-cost screening platforms for drugs. The improved understanding of cancer as "organ system" has pushed for increased accuracy and physiological relevance of *in vitro* tumour models that have in parallel increased in complexity, diversifying their output parameters as they progressed in view to recapitulate the most critical aspects such as the dimensionality of cell cultures (2D *versus* 3D), the mechanical stimuli, the multicellular interactions, the immune interactions and the soluble signalling.
Animal models represent the *in vivo* counterpart to cell lines and are commonly used for studies during the preclinical investigation of cancer therapy to determine the efficacy and safety of novel drugs. They are superior to *in vitro* models in terms of physiological relevance offering imitation of parental tumours and a heterogeneous microenvironment as part of an interacting complex biochemical system. In the present review we describe advantages and limits of major preclinical models used in Oncological Pharmacology.

Key words
Oncology; cell lines; 3D models; engineered mouse models; zebrafish models; immunocompromised mouse models.

Impact statement
Integration of *in vitro*, *in vivo* and *in silico* cancer tumor models is vital to obtain relevant data predictive for efficacy and toxicity in Oncological Pharmacology.

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INTRODUCTION

The past decades witnessed a significant advancement of the knowledge of the molecular bases of carcinogenesis and mechanisms of cancer growth, and unveiled the determining role of the immune system in these processes. However, despite scientific advances in the understanding of cancer biology, novel anti-cancer drugs are struggling to emerge and the success rate of oncology drug development is the lowest among all therapeutic areas (1). Preclinical studies using mice have been extensively used to study the aetiology and pathophysiology of human cancers, and are essential for anticancer drug development. However, due to their poor predictive value, only approximately 5% of anticancer drugs are approved for the market, even if preclinical studies are successful (2-4), major reason behind this low success rate is the poor prediction of clinical efficacy and toxicity profiles from animal and *in vitro* experiments. This is related to the extreme complexity of tumours that are not just a heap of cells that proliferate without control (5). Cancer formation and progression are complex multistep processes involving genetic, epigenetic, and metabolic alterations. In tumour masses heterogeneous cancer cells reside in complex microenvironments in which they are intertwined with other cell types – among which endothelial, hematopoietic, stromal and immune cells – extracellular matrix components (ECM), and subjected to a variety of physical and chemical stimuli that drive cell behaviour towards transformation and malignancy (6-8). Interactions within the tumour microenvironment (TME) also help create metabolic changes, such as hypoxic environment and nutrient gradients, which further contribute to the heterogeneity of cancer cells. This view has increased the ability to identify crucial features of the cancer cells and provided more cues for the identification of relevant drug targets. However, the complexity often hinders the ability to detail relationships, and an in-depth understanding of this intricate interplay is limited by current model systems, which fail to substantiate findings and to elicit sufficient reproducibility.

It is important to understand that each type of cancer model possesses inherent advantages and limitations (9). Different animal and human cancer models should be used at the various stages of the drug discovery process according to the features of the new therapeutic agent under investigation. Using a variety of pre-clinical models can better mirror the human cancer heterogeneity and define the drug doses and schedules to be investigated in clinical trials (10, 11).

In this review, the advantages and limits of the main preclinical oncology models are reported focusing on two-dimensional (2D) and 3D cellular models, immunocompromised and immunocompetent mouse models, zebrafish models and integrated mathematical approaches.

IN VITRO MODELS

The goal of *in vitro* research in cancer pharmacology is to develop a model that can simulate the drug response of a tumor before administering it to animals and patients. There are different *in vitro* models that can be used: computational models, *in silico* models, animal and human cell models and three-dimensional (3D) models. Not necessarily, in testing a drug to verify its efficacy in oncology, it is necessary to use all the mentioned models but it would be advisable to be able to exploit more than one model before moving on to *in vivo* experimentation. In fact, the fostering and extending of the *in vitro* approaches meet the directives of European legislation on the use of animal models in experimentation since well match the replacement, reduction and refinement of the 3R principle.

2D culture models

Two-dimensional (2D) cell cultures represent a simple and widely used experimental model in research. However, they have limitations, such as the inability to reconstruct the correct three-dimensional organization and the com-

plexity of the tumor microenvironment (TME) of human tissue. To date, the *in vitro* model systems are not sufficently predictive, because they do not take into account tumor complexity and heterogeneity and do not reproduce the complex cellular and microenvironmental networks that are crucially involved in the initiation, persistence, invasion and recurrence of the tumor (11, 12).

Though *in vitro* 2D models are considered as "simple" relative to 3D and *in vivo* models, all existing drug approved began their journey to the clinic targeting classical 2D adherent cell culture models in the laboratory. Although exceptions exist, drugs that have little effect on the viability of cancer cells grown in these conditions often do not have efficacy in more realistic 3D *in vitro* and *in vivo* models. Therefore, this approach has great utility in initial high-throughput screens to identify potential hits worth additional investigation. Conventional 2D culture allows for a variety of phenotypes and striking features of cancers to be interrogated, including cytotoxicity, proliferation, mobility, invasiveness, adaptability to hypoxic microenvironments, protein expression and pathway plasticity, drug sensitivity studies, drug durability, molecular characterization, and genomic/genetic characterization.

Of additional benefit, monocultures are free from contaminating cells, which allows for a clear understanding of what is occurring specifically within cancers cells relative to other cell types in the TME in response to a given insult, which can be accomplished through co-culture using transwell plates.

Tumor immortalized cell lines

Tumor immortalized cell lines were established over 30 years ago and are still commonly used as *in vitro* model for drug screening (13-15). Since then, a huge variety of animal or human cancer cell lines is available in bioresource centers, such as the ATCC (American Type Culture Collection) (16). Such established cell lines, associated with simple and low-cost maintenance, have been widely useful for elucidating the progressive events leading to malignancy and the mechanisms of tumor cellular physiology (17). Recently, Nusinow et al. (2020) have screened the cell lines in the Cancer Cell Line Encyclopedia (CCLE) through quantitative profiling of thousands of proteins, hence these data represent a new robust resource to explore cellular behavior and facilitate cancer research (18).

Although meaningful insights can be gained from 2D culture, drawbacks to this approach include lack of heterogeneity and the existence of phenotypes that are observed on plastic that do not reflect cancer behavior *in vivo*, and vice versa. For example, melanoma cells proliferate much more rapidly *in vitro* than *in vivo*, likely due to high serum concentrations and stiffness of support (*i.e.*, plastic) (19). Genetic drift also occurs in long-term passaged cells which can lead to the high variability and reduced reproducibility of the results, due to adaptations to the non-physiological conditions (*i.e.*, oxygen and nutrient levels) in 2D cell culture.

Human cancer-derived cell lines are a simple and quick resource and an important model for *in vitro* cancer research. Thanks to these models it has been possible to understand much of the biology of the neoplastic cell and they have been crucial for the screening of antineoplastic drugs and to identify potential new treatments. Although, encouraging results have been obtained, for example from a study using sequencing data and microarray expression profiles from 947 human cancer cell lines, coupled with drug responses for 24 anticancer drugs across 479 cell lines. The study underlined that the cell lines "may provide representative genetic proxies for primary tumors in many cancer types" (17). So, not all cancer cell lines have the same value as tumor models. Another important problem to take into consideration, that could represent a criticality in the use of cell cultures as an experimental model, is the incorrect identification of the cell line. An important step to bypass this problem was to create publicly searchable databases through standardized protocols for the

authentication of human cell lines using short tandem repeat profiling (20). In the first decade of the 2000s, the advent of omic technologies allowed the characterization of tumors at the molecular level which, in addition to revealing the great genetic heterogeneity of tumors, changed the focus from conventional chemotherapeutic agents towards targeted therapy. The specific modification of the gene structure of cells in culture, through the insertion of missing genes or the selective block of overexpressed genes, gives us the possibility to mimic in the laboratory what happens in a specific tumor. Today, different strategies are widely used to study the morphological and functional effects associated with the activity of single genes: it is possible to generate cell lines that can produce substantially any type of gene product through transfection and/or infection techniques with suitable vectors (plasmid or retrovirus). Furthermore, specific methods of gene suppression (antisense RNA, small interfering RNA) allow to easily and selectively inhibit the activity of single genes. To study the complex mechanisms to be put in place to bypass multi-drug resistance, it is possible to create *in vitro* models of resistance to conventional and target antitumor drugs and to use human immortalized cancer cell lines derived from cancer patients showing primary resistance. However, the identification of promising molecules must be subsequently validated in other experimental models, for example, on primary cultures of similar or different derivation compared to the cells initially used in the screening program.

The number of passages a cell line undergoes can lead to such extensive modifications in its characteristics that no longer reflect the tumor from which it was derived, as in the case of aneuploidy. Cell lines no longer retain the tumor heterogeneity present in primary cancer and do not contain the relevant components of the TME. One of the most relevant advantages in pharmacology is the use of cell cultures to study the effects of molecules on human cells, exposing the cells directly to the substances to be tested at different concentrations, having reproducible results in a short time. Furthermore, the loss of components involved in homeostatic regulation *in vivo* also occurs.

Primary cell culture

Primary cultures are made up of cells that arise from a tissue or organ, extracted from both animal or human explant. These cells are able to duplicate only for a limited number of passages, then undergo senescence regardless of the presence of metabolites appropriate for growth. However they represent an excellent study model as they retain most of the *in vivo* cell characteristics. In the oncology field, for this reason, primary cultures are the closest model to *in vivo*, but they are also more delicate for the growing conditions that require (21).

Others

Finally, through immunohistochemical techniques on paraffinized tissues but also molecular characterization on fresh tumor tissues, it is also possible to identify and correlate the histogenetic classification of tumors to determine the site of origin of metastatic tumors and to identify molecules with prognostic and/or therapeutic significance, identify the patients who can benefit from specific treatments (e.g., molecularly targeted "targeted therapy", immunotherapy) and monitor the response to these therapies and the onset of possible resistance.

3D cell culture models

The newly developed 3D *in vitro* culture methods are more tightly to *in vivo* condition. One of the first 3D cultures of human tumor stem cells was carried out in soft agar by Hamburg and Salmon in the 1970s (22). Since then, several different types of 3D models, such as spheroids (23) and organoids (24), have been developed and extensively improved to simulate a plethora of diseases, including cancer.

Firstly, non-scaffold-based 3D cultures were derived from established monolayer cell lines and were named spheroids. Such spherical aggregates of malignant cells, *i.e.* multicellular

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tumor spheroids, were used as *in vitro* models of tumor microregions and avascular stage of tumor growth (25). Spheroids reproduce the main features of solid tumours such as 3D architecture, cellular heterogeneity, cell-cell signalling, extracellular matrix deposition, growth kinetics, gene expression patterns and drug resistance mechanisms (26).

More recently, organoid technology has revealed a great potential in opening up new opportunities for the development of novel therapies in cancer research (27-29). The first organoid model was established by using intestinal stem cells that express single leucine-rich repeat-containing G protein-coupled receptors 5 (LGR5) (30). Sato *et al*. demonstrated that intestinal crypt-villus units are self-organizing structures, which are built from a single stem cell (30). Unlike spheroids, organoids can be expanded long term and exhibit a more advanced and organized architecture that recapitulate the genetic and structural features of the native organ, hence the name "mini-organs" (30, 31). It is worthy to note that organoids can be cryopreserved and remain genetically and phenotypically stable.

Stem cells or multipotent progenitor cells derived from human biopsies were expanded *in vitro* to obtain stem cell-derived organoid models and have allowed to develop highly reproducible and long-lived organoids from single cells (30, 32, 33). As such, organoids can be generated from adult stem cells (ASCs) (34), embryonic stem cells (ESCs) (35), and induced pluripotent stem cells (iPSCs) (36). However, the efficiency of stem cell-based tumor organoids from patients may depend on the cancer type and the presence or absence of specific oncogenic alterations, potentially choosing for the outgrowth of tumour subclones and loss of the genetic heterogeneity of the tumour it is derived from (38). Therefore, it appears more helpful to develop tumour organoids directly from tumors than to use stem cells. Actually, organoids can be directly generated from the patient tumour tissue following surgery or endoscopic biopsy to obtain patient-derived organoids (PDOs) (see for review 39).

PDOs, defined as multicellular extracellular matrix (ECM)-dependent units, recapitulate accurately histopathologic and genomic profiles of the tissue of origin while maintaining genomic stability throughout passaging (27, 40). Huge pools of PDOs are generated and biobanked, offering new possibilities as powerful preclinical models able to account for interpatient variability (29, 39). Indeed, patients' tumors have genetic and epigenetics changes that promote cancer growth and spread, thus their response to the treatment is significantly different.

So far, organoid technology is revolutionizing many areas of science, including cancer research and precision medicine. However, one of the intrinsic limitations of organoid culture is the lack of stroma, blood vessels and immune cells (11). As cancer immunotherapy is holding a pivotal role in the clinic, researchers have recently shown that epithelial organoid cultures can be used in co-culture conditions or may also be integrated into organ-on-a-chip platforms, allowing the build-up of more complex culture systems (41-44).

Table I summarizes the main advantages and disadvantages of all described *in vitro* models.

IN VIVO MODELS

Immunocompetent mouse and rat models: syngeneic, genetically engineered mouse and carcinogen-induced models

Ectopic and orthotopic syngeneic models

Immunocompetent syngeneic models are characterized by the inoculation of cancer-derived cell lines or by the implant of cancer tissues into genetically identical hosts, namely syngeneic mice or rats, at the same (orthotopic) or different (ectopic) anatomic cancer site. Syngeneic ectopic models (SEMs) are useful for the *in vivo* screening of new chemical entities

Table I. Advantages and disadvantages of 2D models used in studies of Oncological Pharmacology.

Model	Advantages	Disadvantages
2D immortalized cell lines	• simple and low-cost maintenance; • long term culture; • amenable to genetic manipulation; • mainly valuable for drug screening and gene expression patterns.	do not reproduce: – the complex cellular; – the microenvironmental networks of tumor.
2D primary cells	• simple and low-cost maintenance; • mainly valuable for drug screening and gene expression patterns; • may be collected from transgenic mice.	• limited culture period, • do not reproduce: – the complex cellular; – the microenvironmental networks of tumor.
Spheroids	• reproduce 3D architecture, • cell-cell signalling; • polarized cell-matrix interactions; • growth kinetic; • gene expression patterns; mainly valuable for drug resistance mechanism.	• limited culture period; • low capability to reproduce the tumor histopathologic and genomic profile; • scarce reproducibility among different techniques.
Stem cell-derived organoids	• well-established culture protocols for many tissues; • long-term culture with progressive differentiation; • can be cryopreserved and remain genetically and phenotypically stable; • amenable to genetic manipulation.	• expensive culture method; • may lose the genetic heterogeneity of the tumour it is derived from; • access to tissue may be difficult or limited.
Patient-derived organoids	• capture interpatient genetic heterogeneity; • recapitulate accurately histopathologic and genomic profiles of the tissue of origin while maintaining genomic stability throughout passaging; • mainly valuable for precision medicine.	• expensive culture method; • access to tissue may be difficult or limited; • do not model tumour immune involvement (amenable to organoid-immune cell co-culture systems).

(NCEs) allowing a reproducible and quantitative evaluation of their overall tolerability, anticancer efficacy and pharmacokinetics (PK)/pharmacodynamics (PD) relationships. However, SEMs own restricted clinical predictability due to the loss of cancer cells and tumor-associated stromal cells heterogeneity and incomplete genotypic and phenotypic similarities to human cancers (45, 46).

On the other hands, syngeneic orthotopic models (SOMs) better mirror the complex cell-cell interactions of the primary TME resulting in more reliable phenotypic and metastatic profiles. Due to their whole immune system, SOMs are crucial for evaluating the impact of new therapeutic agents on cancer local and metastatic invasiveness and occurrence of stromal-related cancer resistance (47). However, as SEMs, SOMs do not reflect the heterogeneity of tumour cells. Therefore, it is crucial to define which genetic profile each model most closely depicts to strengthen the response predictivity to the various therapeutic agents (48). Moreover, SOMs being technically more challenging compared to SEMs with highly variable rates of cancer take and development times are not suitable for the initial evaluation of NCEs in widespread screening. Differently from sc or ip SEMs in which cancer growth can be easily evaluated quantitatively by electronic calipers, in SOMs imaging techniques (*e.g.*, bioluminescent imaging, magnetic resonance imaging, positron emission tomography and ultrasonography) have to be used.

Genetically engineered mouse models

Immunocompetent genetic engineered mouse models (GEMMs) are characterized by the generation of cancer genetic abnormalities that can be modulated in a systemic or in a spatial-temporal (conditional) manner. Allowing the study of the pharmacological modulation of specific mutations, these engineered models are closer both at genotypic and phenotypic levels to human cancers (49). In these non conditional models, autochthonous cancers develop spontaneously and in the appropriate microenvironment in mice. Therefore, the effects of NCEs on immune response, and local and systemic metastatic spread can be efficiently studied with a significant predictive relevance. However, most of GEMMs are characterized by an asynchronous cancer development resulting in variability in cancer occurrence, frequency and growth. Further, GEMMS are obtained in out-bred murine strains whose genetic backgrounds may be nonuniform influencing the pharmacokinetic, the efficacy and safety profiles of anticancer agents. These limitations can be overcome by conditional GEMMs that allow the specific control of gene expression at both time and tissue level (50, 51).

On the other hand, GEMMs are highly time and cost consuming for assessing statistically significant data and generally require imaging techniques for monitoring the anticancer efficacy of NCEs under investigation. Gene editing technologies, such as the CRISPR/Cas9 system, have significantly reduced the time needed to establish a GEM model (52). Generally, GEMMs are suitable for the later phases of the drug discovery process evaluating selected leads that deserve to be investigated in specific clinically appropriate GEMMs for supporting the translational process.

Carcinogen-induced models

The carcinogen-induced models are among the oldest pre-clinical models used (53). These autochthonous models are characterized by the multistage and time-dependent development of solid and hematological cancers in response to carcinogens in immunocompetent mice and rats. These models are produced in out-bred strains of mice and rats characterized by different genetic backgrounds and high reproducible phenotype. Cancer development relies on the protocol used and in particular by the dose and the schedule of carcinogens. A peculiar feature is the similarity of these models to the multistage progression of human cancers (*i.e.*, hyperplasia, dysplasia, pre-malignant lesion, well differentiated cancer and invasive-poorly differentiated cancer). However, the carcinogen exposure can be rather severe compared to carcinogen-related human cancers (48).

These models are of value in understating the stage of cancer development that is more sensitive to the therapeutic agent under investigation. The models need initial low manipulation and costs, but their extended timelines are associated with safety concerns and increasing costs for animal care and maintenance. Therefore, these models have been replaced by xenograft and GEM models in early stage of drug discovery process. To note that these models have been pivotal in pointing out and supporting the cancer immunoediting theory (54).

Immunocompromised mouse models: cell-line derived and patient derived xenograft

The simplest tumour models are the xenograft model, in which human cell lines are inoculated into an immunocompromised mouse and tumour growth is monitored with calipers (55). These preclinical models have been widely used, despite their poor predictive validity, as they do not reflect the heterogeneity of tumor cells nor of TME, particularly when heterotopics (tumor cells injected subcutaneously into the flank) rather than orthotopics (tumor cells injected into the corresponding anatomical position). Moreover, these models do not also reflect the multistage process of carcinogenesis, as the inoculation is synchronous. Therefore, it is not expected that pharmacological

data derived from one of these tumour models can predict efficacy in complexity of the human disease. Further, limited is their use for studying the metastatic process and, consequently, the activity of NCEs on the treatment of metastases (56). However, these models can be used to investigate the PK, PD and toxicity of NCEs, their PK-PD relationship (for the optimal dosing regimen selection in clinical trials) and their therapeutic ratio (57).

Patient derived xenograft (PDX) models provide further confidence for decision-making in clinical development and clinical candidate selection (58). In PDX models, resections of human tumours are implanted into immunosuppressed mice (e.g. non-obese diabetic (NOD)/ severe combined immunodeficiency (SCID)). PDX models represent the heterogeneity of tumour cells and TME (59).The tumors are well characterized, both molecularly and histologically, and can be used in validation target and investigation of activity of the new therapeutic agents, as well in selection of patients responsive to a new therapeutic agent (58). Moreover, the PDX models, representing a heterogeneous patient population (60), can be used to perform a preclinical–clinical trial.

Panels of PDX models have been established by academic institutions, research organizations and pharmaceutical companies, and consortia, such as the European EuroPDX Consortium (http://europdx.eu/news-events.html) have been made. Interestingly, in some retrospective studies, the response in PDX models is predictive of clinical response (61). However, there are still several limitations to PDX models, including the high cost (for using and maintaining of the models), the engraftment of human tumors into immunodeficient mouse strains (which cannot be used for testing immunomodulatory agents) and the use of the heterotopic model (subcutaneous tumor injection) rather than the orthotopic equivalent (which is more technically challenging and associated with reduction in engraftment rate) (62).

Recently, in order to use PDX models for the development of immunotherapeutic agents, a number of humanised PDX models (AVATAR) have been generated (63-65). In addition to their use in drug discovery, humanised PDX models can be used to identify patient-specific drug response (66) and novel treatment strategies for patients non responsive to standard-of-care treatment options (67). However, the limitation are still present. In fact, not all tumours are amenable to xenotransplantation, the immune response may not be modelled and the methodology may not allow transplantation of treatment-resistant cells (68). These humanized mouse models also have a limited impact on research into metastasis (69-71).

Zebrafish models

In the last decade, the zebrafish (*Danio rerio*) has become an attractive alternative to mouse for modeling human cancer diseases. The zebrafish possesses unique advantages as a versatile tool in cancer research. Zebrafish generates large numbers of progeny, every week a pair of adult zebrafish produces several hundred fertilized eggs. This aspect offers high confidence in statistical analysis (72, 73). Furthermore, zebrafish have small sizes and require minimal care and husbandry expenses, therefore their maintenance is cheaper compared to mice colony and other mammals (73). Interestingly, a direct comparison of the zebrafish and human protein-coding genes reveals several interesting features: 71.4% of human genes have at least one zebrafish orthologue and, reciprocally, 69% of zebrafish genes have at least one human orthologue. Regarding disease, 82% of human morbid genes can be related to at least one zebrafish orthologue (74). For example, mammals and zebrafish share common molecular pathways of liver tumor progression (75).

The embryos develop externally and are initially transparent, whereas the Casper pigmentation mutant zebrafish (roy$^{-/-}$; nacre$^{-/-}$), developed in 2008, remains transparent throughout adulthood. The transparency makes embryos of zebrafish suitable to direct observe some processes through *in vivo* imaging (76). Be-

sides, zebrafish genetics is easily manipulated allowing us to drive specific homozygosity mutations, producing zebrafish models with genetic defects similar to specific human cancer (74). Cancer progression in these animals recapitulates many aspects of the human disease allowing the identification of genetic and biochemical features of cancer. Regarding drugs, zebrafish efficiently absorbs small molecules directly dissolved in water, allowing easy drug administration (77). Unfortunately, drug delivery using immersion therapy is unable to give the accuracy of dosing and therefore pharmacokinetic studies presents some technical difficulties (78). Nevertheless, a wide range of assays can be carried out in the several tumor models of zebrafish: compound screening and testing, drug reprofiling, target discovery, target validation and toxicological studies (79).

Different approaches assessed by the scientific community, has been developed to induce cancer in zebrafish. Each model presents specific advantages and disadvantages and the best way to select the best approach is to focus on the aim/question to answer in each experimental setting. For instance, cancer in adult zebrafish with a completely developed immune system and organs, represents a more realistic *in vivo* model compared to embryos, which conversely represent a suitable model for live imaging techniques.

There are different methods to induce cancer in zebrafish, a synopsis is reported below.

Chemical carcinogenesis was the first approach used to induce tumor formation in zebrafish. Itemized protocols exist that allow the development of mutant lines without special equipment but simply adding carcinogens to the water. Nevertheless, this approach has a low rate of tumor formation and the tumor is highly heterogeneous for site and type (79).

Genome editing approaches represent methods able to inactivate or regulate a specific gene. The most used techniques to edit the genome in zebrafish are TALEs, zinc-finger nucleases, CRISPR/Cas and Morpholino (80-83). Currently, many mutant lines are available and show a good incidence of tumor formation and homogeneity of tumor types. Nevertheless, mutant lines are difficult to maintain and the study of tumors requires histological analysis (79).

Another method is the development of transgenic zebrafish models through microinjecting of exogenous DNA into embryos. This method allows the expression of a gene of interest (for example, oncogene or mutant form of tumor suppressor) in a particular organ or tissue through the use of tissue-specific promoters. The advantages of these approaches consist of a high rate of tumor formation, a temporal and site control of tumor initiation, and, in some cases, the monitoring of tumor progression by fluorescence microscopy. The main disadvantage is the necessity of high experience to overcome the difficulties that arise during the development of the transgenic zebrafish model.

The last developed approach to generate cancer in zebrafish is the transplantation of tumor cells. There are: allogeneic transplantation, *i.e.* the transfer of cells from one individual into another of the same species, xenotransplantation, *i.e.* the implantation of living tumor cells from one species to another and orthotopic transplantation, *i.e.* implanting living tumor cells from one species to another but into xenotransplantation is the transplant into zebrafish embryos of the patient-derived tumor cells (zPDX). This approach has relevant translational potentiality in the precision cancer medicine area because it represents an efficient and fast model to test the patients treatment options in an *in vivo* model having the same molecular, genetic, and clinical characteristics of the patient. Nevertheless, there are also limitations in the transplantation. The main is the necessity to perform an experiment before the development of the adaptive immune system *i.e.* until 21 days of life, to prevent graft rejection. Experiments in adults require immune system ablation through radiation or treatment with dexamethasone, to avoid engraftment rejection. Another limitation is the temperature of

≤ 35 °C, to which larval xenotransplantation experiments are conducted, which is a temperature non-physiological for human cells (78). Finally, orthotopic transplantation is not always possible due to the absence of a concrete organ in zebrafish, such as for breast, lung, or prostate.
Table II summarizes the main advantages and disadvantages of all described *in vivo* models.

Integrated mathematical approaches

The complexity of the system we are considering makes a difficult task to select a unique model that embodies all the desired characteristics. A significant link and enrichment of *in vitro* and *in vivo* systems is provided by *in silico* biological systems, especially integrated mathematical models (84). In fact, mathematical models have the potential to fill in gaps of knowledge left by incomplete experimental models of complex "cancer organs". This has prompted the development of many mathematical models of cancer, which need becomes manifest, as accurate experimental models become more difficult to create. The broad range of mathematical modelling complexity and techniques gives them almost unlimited potential in cancer research (85-89). Models have been created to examine almost any aspect of the complex "cancer system"

Table II. Advantages and disadvantages of *in vivo* tumour models used in studies of Oncological Pharmacology.

Model	Advantages	Disadvantages
Syngeneic models	• Useful for the *in vivo* screening of new compounds (safety, efficacy and PK/PD relationships); • time- and cost-effective models for obtaining drug translational data; • orthotopic models: – useful to investigate the metastatic profiles of tumors – useful to investigate immunotherapeutics.	• Incomplete genotypic and phenotypic similarities to human cancers; • do not reflect the heterogeneity of tumor cells; • orthotopic models require imaging techniques for monitoring anti-cancer drug activity.
Genetic engineered mouse models	• Closer both at genotypic and phenotypic levels to human cancers; • useful to study the pharmacological modulation of specific mutations; • predictive of metastatic potential of tumors; • useful to investigate immune-therapeutics; • suitable for the later phases of the drug discovery process; • useful models for obtaining drug translational data.	• Asynchronous cancer development • difficult to perform powered studies; • non-conditional models suffer of nonuniform genetic backgrounds affecting PK, efficacy, and safety of drugs; • highly time and cost consuming to perform screening experiments; • require imaging techniques for monitoring anticancer drug activity.
Carcinogen-induced models	• Similarity to the multistage progression of human cancers; • useful for tumor-stage sensitivity to anticancer drugs; • low manipulation and costs.	• Severe carcinogen exposure; • safety concerns; • increasing costs for animal care and maintenance.
Cell-derived xenografts	• Useful to investigate the PK, PD and toxicity of anticancer drugs; • useful to investigate the PK-PD relationship and the therapeutic ratio of anticancer drugs.	• Do not reflect the heterogeneity of tumor cells; • do not reflect the tumor cell interactions with microenvironment; • synchronous inoculation.

Model	Advantages	Disadvantages
Patient-derived xenografts	• Reflect heterogeneity of tumour cells and tumor microenvironment; • tumors are well characterized; • useful in validation target; • useful in investigation of activity of anticancer drugs; • useful in selection of patients responsive to new anticancer drugs; • useful to perform a preclinical–clinical trial; • AVATAR useful for testing immunomodulatory agents.	• High cost for maintaining; • generally not useful for testing immune-modulatory agents; • orthotopic models are technically challenging; • many tumors are associated with reduction in engraftment rate; • murine immune response; • treatment-resistant cells may not be transplanted.
Zebrafish	• Small size, easy to maintenance • large progeny/week; • 71.4% of human genes have at least one zebrafish orthologue; • transparent embryos and adults allow *in vivo* visualization of cancer growth and progression (real-time live imaging); • zebrafish genetics is easily manipulated; • easy drug administration.	• Different diet and environment compared to human; • several mammalian organs are not present; • cold-blooded, the physiology is not identic to human; • immersion therapy is unable to give the accuracy of dosing.
Carcinogen-induced tumor in zebrafish	• Simply protocols to add carcinogens to the water; • easy to set up; • no need for special equipment or personal formation.	• Low rate of tumor formation; • high heterogeneity for site and type of tumor.
Mutant lines created by genome editing	• Many mutant lines are available; • good incidence of tumor formation; • homogeneity of tumor types.	• Mutant lines are difficult to maintain; • the study of tumors requires histological analysis.
Transgenic zebrafish	• Allows the expression of a gene of interest in a particular organ or tissue; • a high rate of tumor formation; • temporary and site control of tumor initiation. • If the cells are labeled, the possibility to monitor the tumor progression by fluorescence microscopy.	• The necessity of high experience indeed transgenic lines could be difficult to develop, because of instability.
Transplantation of tumor cells (allogeneic transplantation, orthotopic transplantation, xenotransplantation i.e. the transplanted into zebrafish embryos of the patient-derived tumor cells (zPDX))	• High penetrance; • the number and location of tumor cells are controlled; • rapid tumor progression; • efficient and fast model to test the patient's treatment options in an *in vivo* model having the same molecular, genetic, and clinical characteristics of the patient.	• Require embryos or immune system ablation through radiation or treatment with dexamethasone to avoid engraftment rejection; • cells only survive for a few weeks or days; • experimental temperature is not physiological for human cells; • orthotopic transplantation is not always possible due to the absence of a concrete organ in zebrafish, such as for breast, lung, or prostate cancer.

and used to complement experimental systems with the aim to add additional layers of complexity and to analyse challenging variables not easily studied experimentally. The literature evidences that a rigorous application of mathematical models is extremely valuable in preclinical development. For oncological drugs the key translational objectives concern the safety data and the scientific support for the rationale and biological plausibility of the investigational drug (90). Often the inadequacy and the poor efficacy of the models used to investigate new anti-cancer drugs led to poor understanding of systemic drug toxicity and mechanism of action and then to the failure of the development program. Mathematical models can help the translation of both these key objectives of drug development. Regarding the rationale and biological plausibility, the information on anti-tumour activity associated with an investigational drug, inferred from the xenograft model, can be used in mathematical models to define efficacy and predict clinical anti-tumour response. Regarding the tolerability profile, some of the most common toxicities – myelosuppression, gastrointestinal toxicity and cardiac safety – can be readily described by mathematical models (91-94); in this context the use of proper mathematical models may account for human tolerable exposures, thus rendering preclinical anti-tumour activity highly predictive of the overall response rate in the clinic.

The compelling need to incorporate mathematical models into the drug discovery process at both preclinical and clinical stages is part of the transition in the process of drug discovery and development in oncology we are facing, and is determined by the growing knowledge of basic molecular and cellular mechanisms underlying carcinogenesis and immune-oncology as well as the availability of large amount of data (95, 96). For cytotoxic agents and most of the molecularly targeted antineoplastic agents, mathematical modelling of the preclinical data can now predict the clinical efficacy and toxicity profile with good confidence. However, translation for immune-oncology agents still remains very challenging (90, 97).

The growing use of mathematical modelling in oncology might provide a unifying framework for evaluating the potential of an investigational product. This could help determine the best clinical development strategy and will result in a rationale and more efficient drug development process. The application of mathematical models in oncology drug discovery represents also an example of how efforts between different but potentially complementary disciplines can converge and work in synergy ensuring a more continuing translation of model and analysis techniques (90). Moreover, the integration and increasing application of the *in silico* approach during the drug discovery and development process also comply with ethical requirements and the directives of European legislation on the use of animal models in experimentation since they well match the replacement, reduction and refinement of the 3R principle (98, 99).

In future regards to precision medicine, mathematical models play an increasingly relevant and decisive role aiding the comprehension of complex systems in biomedical research, especially in the oncological field. This evolution towards the introduction of the computational analysis during the development of drugs, by combining *in vitro*, *in vivo* and *in silico* techniques, allows refining experimental programs, providing more accurate and detailed models, ultimately resulting in higher specificity and speed. Overall, this has direct implications for both effective and individualized cancer therapies ultimately achieving better patient survival.

CONCLUSIONS

There is a wide range of tumour models, each with distinct advantages and disadvantages. It is clear that any model is lacking some aspects of reality. While *in vivo* models can capture the complexity of the metastatic process in a living system, visualization of the individual steps is

challenging and extracting quantitative mechanistic data is usually very difficult. In contrast, *in vitro* models have reduced physiological relevance, capturing only limited aspects of the TME, but allow control of most experimental variables and permit quantitative analysis (100). A further advancement has been introduced with ex-vivo systems developed to improve basic *in vitro* cell cultures while overcoming shortcomings of preclinical animal models and serve as more clinically relevant models to predict drug response in cancer patients.
Looking at the appropriateness of the model and the translatability of the results, relevant data for efficacy and toxicity can be obtained if the preclinical model system integrates *in vitro*, *in vivo* and *in silico* experiments. In fact, the complexity and heterogeneity of cancer, as well as the corresponding vast amount of available data, ask for a systemic approach such as computational modelling (95, 96) . Overall, *in silico* biological systems, especially integrated mathematical models, provide significant link and enrichment of *in vitro* and *in vivo* systems (**figure 1**). In addition, efforts to expand integrative approaches to combine information on multiple levels – molecular, cellular, microenvironmental etc… - will further refine the experimental programs (101-103). Cancer and biomedical science in general will benefit from the combination of *in silico* with *in vitro* and *in vivo* methods resulting in higher specificity and speed, providing more accurate, detailed and refined models that ultimately support prediction and decision-making.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

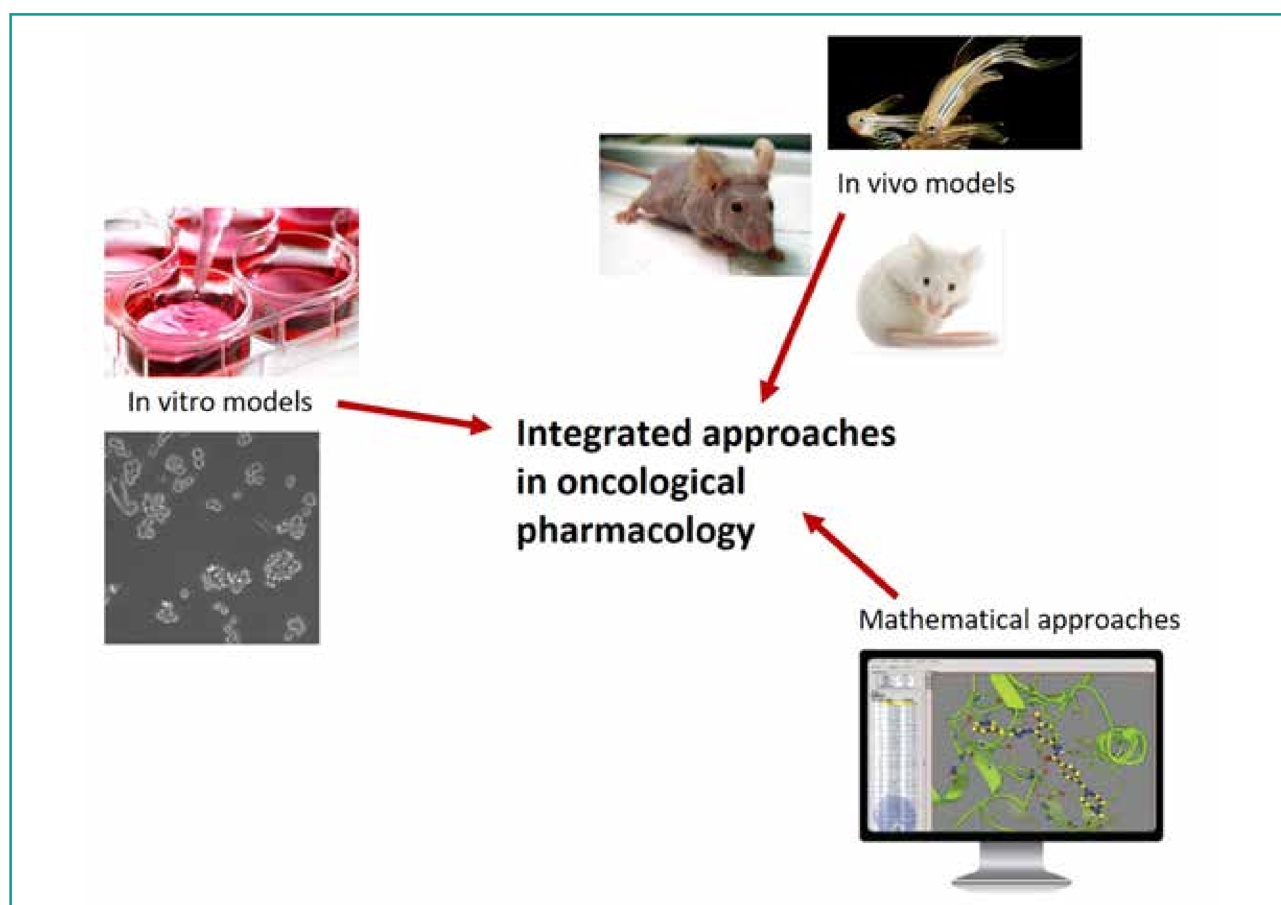

Figure 1. The integrated approaches in Oncological Pharmacology.

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