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Review

The predictive capacity of *in vitro* preclinical models to evaluate drugs for the treatment of retinoblastoma

Irina L. Sinenko [a,b], Roland C. Turnell-Ritson [a], Francis L. Munier [b,*], Paul J. Dyson [a]

[a] Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015, Lausanne, Switzerland
[b] Jules-Gonin Eye Hospital, Fondation Asile des Aveugles, University of Lausanne, CH-1004, Lausanne, Switzerland

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ABSTRACT

Retinoblastoma is a rare childhood cancer of the eye. Of the small number of drugs are used to treat retinoblastoma, all have been repurposed from drugs developed for other conditions. In order to find drugs or drug combinations better suited to the improved treatment of retinoblastoma, reliable predictive models are required, which facilitate the challenging transition from *in vitro* studies to clinical trials. In this review, the research performed to date on the development of 2D and 3D *in vitro* models for retinoblastoma is presented. Most of this research was undertaken with a view to better biological understanding of retinoblastoma, and we discuss the potential for these models to be applied to drug screening. Future research directions for streamlined drug discovery are considered and evaluated, and many promising avenues identified.

1. Introduction

Retinoblastoma is the most common eye cancer worldwide (Kivelä, 2009), and while today patient survival is greater than 95% in high-income countries, the disease remains lethal in the majority of cases in lower-income countries due to the lack of access to health care (The Global Retinoblastoma Study Group, 2022; Wong et al., 2022). Retinoblastoma is a relatively uncommon disease with around 8000 new cases registered globally every year (Fernandes et al., 2018; Kivelä, 2009; MacCarthy et al., 2006) with an incidence that depends on both the birth rate and population size (Kivelä, 2009).

The first attempt to describe the mechanism of retinoblastoma tumourigenesis was made by Knudson, using the "two-hit" hypothesis (Knudson, 1971). The target of two-step mutational inactivation in retinoblastoma was attributed to the tumour suppressor gene *RB1* (Friend et al., 1986). Our current understanding of retinoblastoma progression is still incomplete, but appears to include a small number of genomic and epigenetic alterations (Kooi et al., 2016; Zhang et al., 2012). The cell-of-origin is considered to be the red/green cone photoreceptor precursor. (Xu et al., 2014). Recently, two retinoblastoma subtypes have been discovered: subtype 1 corresponds to the expression of mature cone markers and has few genetic alterations apart from *RB1* inactivation. In contrast, the more aggressive subtype 2 tumours express less differentiated cone markers, together with neuronal/ganglion cell markers as well as slightly more numerous genetic alterations (Liu et al., 2021).

Historically, the first treatment option that became available for retinoblastoma was enucleation of the eye, which is still used today in cases where diagnosis has been significantly delayed (Appukuttan et al., 2013). The first attempts at conservative management, using radiotherapy, were explored at the turn of the 20th century, and widely used until the 1990s (Stallard, 1952) when such treatment was found to cause secondary non-ocular neoplasms, particularly in young infants (Moll et al., 2001). Radiotherapy was then virtually banned from the armamentarium, and replaced by first line chemotherapy using carboplatin, etoposide and vincristine, followed by focal treatment, such as cryotherapy, hyperthermia, photocoagulation, or brachytherapy (Hamel et al., 2000; Rodriguez-Galindo et al., 2003; Lumbroso-Le Rouic et al., 2008; Shields et al., 2020; Shields and Shields, 2010). In order to mitigate the systemic toxicity caused by chemotherapy, and to overcome treatment resistance of tumour cells disseminated from the retina to adjacent ocular compartments, *i.e.* seeding, due to poor drug concentrations achieved in the seeding compartments, more targeted therapies have been developed, primarily using the drugs melphalan and topotecan *via* new administration routes, such as ophthalmic artery, intra-vitreous or intracameral injections, significantly improving eye survival (Mendoza and Grossniklaus, 2016; Munier et al., 2013).

The limited number of cases, lack of access to disease management in developing countries (Rajeshuni et al., 2019), and the young age of

* Corresponding author. Jules Gonin Eye Hospital, Av. de France 15, CH-1004, Lausanne, Switzerland.
E-mail address: francis.munier@fa2.ch (F.L. Munier).

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Abbreviations

3D	three-dimensional
CAM	chick chorioallantoic membrane
CEV	carboplatin-etoposide-vincristine
CNS	central nervous system
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FDA	food and drug administration
GFAP	glial fibrillary acidic protein
hESCs	human embryonic stem cells
hPSCs	human pluripotent stem cells
MAP2	microtubule associated protein 2
OCT4	octamer-binding transcription factor 4
Rb	retinoblastoma
RB1	retinoblastoma gene
SYP	synaptophysin

patients, are all obstacles for the development and implementation of new treatment modalities and the evaluation of new drugs.

The standard retinoblastoma chemotherapy drugs currently employed in the clinic, *i.e.* carboplatin, etoposide, vincristine, melphalan and topotecan, were not initially developed for the disease, but were repurposed years after their first approval for other types of cancer. These drugs were selected based on their performance in other paediatric malignancies in a series of pilot studies conducted by several groups (Cancela et al., 2020; Chan et al., 2005). Multicentric trials have remained virtually absent from the landscape of retinoblastoma. Complete pharmacological/pharmacokinetic profiles were established only after clinical use of a drug (Schaiquevich et al., 2017). Melphalan, a DNA alkylating agent, was primarily used as a melanoma treatment (Clifford et al., 1963), and carboplatin, another DNA-binding compound, displays a beneficial effect on central nervous system (CNS) tumours, as well as head, neck and ovarian carcinomas (Douek et al., 1991; Ohnuma et al., 1984; Walker et al., 1985). The topoisomerase inhibitors topotecan and etoposide have primarily been used against ovarian cancer (McNeil, 1996) and lymphomas/leukaemia (Issell and Crooke, 1979), respectively. Vincristine was discovered to cause myelosuppression, and therefore found its first application in acute leukaemia treatment (Evans et al., 1963; Karon et al., 1962). Furthermore, combinations of these chemotherapeutic agents (carboplatin-etoposide-vincristine = CEV), have been then used as the current standard-of-care for two decades (Gutierrez and Crooke, 1979; White, 1991).

Since the drugs used in retinoblastoma treatment have not been designed specifically for retinoblastoma, this suggests that better drugs, or drug combinations, are yet to be discovered. Alternative drugs may function better than existing ones when applied together with such approaches.

Drug evaluation for new compounds is a complicated process that can take years (Walters, 1992), though the development of new treatment modalities can be achieved *via* a range of different strategies. (Organizing Committee for the Workshop on Health and Medicine et al., 2004) Molecular approaches, such as proteomics, genomics and transcriptomics, identify new targets through an understanding of the cellular mechanisms underlying the disease phenotypes of interest, and may indicate biomarkers for disease-relevant targets and/or earlier diagnosis (Lindsay, 2003; Sun et al., 2020). These approaches can provide insight into upregulated or downregulated genes, and drugs targeting these biomolecules, already in use to treat other diseases, may be repurposed (March-Vila et al., 2017). Another complementary strategy – high-throughput screening of drug libraries – can facilitate such repurposing, and is advantageous since the drugs in many libraries are already clinically approved for other diseases. Thus, a large body of data concerning their action, toxicity and side effects already exists. Repurposing existing drugs reduces the drug evaluation timeline; however, *in vitro* studies remain a crucial step for identifying drugs to repurpose and for delineating other key features.

While drug repurposing implies meticulous screening of large numbers of compounds from different primary indications, drugs repurposed for paediatric oncology were already used in children as a primary indication (Blatt and Corey, 2013). For example, melphalan was approved by the food and drug administration (FDA) in 1964 for the treatment of multiple myeloma in adults, and later its antitumour activity towards rhabdomyosarcoma and Hodgkin's disease in children was also demonstrated (Belasco et al., 1987; Glimelius and Lahn, 2011). The cytotoxicity of melphalan towards retinoblastoma cells was assessed using a clonogenic assay (Inomata and Kaneko, 1987), and *in vivo* safety studies (Ueda et al., 1995) provided the rational for its use in patients (Schaiquevich et al., 2017). Due to severe toxicity when administrated systemically, melphalan is administered locally, by intraarterial or intravitreous injections, and can also be combined with other chemotherapeutics, such as topotecan, when needed (Al Kofide and Al-Sharif, 2019).

In order to successfully repurpose a drug approved for a different application, its efficacy towards an appropriate preclinical disease model should be shown and validated. The type 2 diabetes drug metformin, despite demonstrating antitumour activity towards retinoblastoma cell lines *in vitro*, failed to suppress tumour growth in a retinoblastoma xenograft model based on the Y79 cell line at pharmacological levels (Brodowska et al., 2014). This failure can be explained by cancer cell specific toxicity, difficulties in drug dose translation from *in vitro* to *in vivo* studies, a nonoptimal treatment regimen and/or the involvement of unexpected pathways when used in combination with adjuvant chemotherapeutics (as is typically the case in the clinic). Attention to biologically and clinically relevant conditions is needed to overcome these challenges. In the case of a repurposed drug demonstrating efficacy in preclinical studies, it can proceed directly to phase II/III clinical trials for evaluation of its efficacy in humans, as the safety profile (typically investigated in phase I clinical trials), is already established. If the new indication is close to the primary one, *e.g.*, repurposing for another cancer type in patients of the same population, the existing dosage and regimen can be applied as a starting point for treatment optimisation. This preliminary knowledge significantly shortens the total drug evaluation timeline.

Overall, for newly developed drugs, about 12% of drug candidates passing preclinical studies enter clinical trials, and about 10% of drugs entering phase I clinical trials are ultimately approved by the FDA (Hay et al., 2014; Van Norman, 2019). Information about the stage-by-stage success/failure rates, especially for the *in vitro* to *in vivo* transition, is very limited. The reasons for such a low preclinical to clinical transition success rate include limitations in relevant response criteria (Johnson et al., 2001), interspecies pharmacology (Peterson and Houghton, 2004), frequent failure of animal models to recapitulate all aspects of a human disease (Harrison, 2013), and ethics-related restrictions on the planning and performing of such experiments (Winston, 2013). In addition, animal experiments take 4–5 years, and the cost of drug development has increased exponentially over the last 50 years (Meigs et al., 2018). All of these limitations combined with the necessity of animal toxicity testing as the basis of approval for further clinical investigation of drug candidates lead to both the need for more careful drug candidate selection in *in vitro* studies in order to decrease the number of animals involved as well as a call for advanced *in vitro* drug screening platforms capable of providing drug responses more relevant for the further translation (Edmondson et al., 2014; Van Norman, 2020).

2. Classical cell culture

While mouse models were formerly used as the primary screening method of drug candidates (DeVita and Chu, 2008; Ireson et al., 2019;

Ross and Wilson, 2014), cell culture is now used as the primary step to identify promising compounds (Jedrzejczak-Silicka, 2017; Kitaeva et al., 2020). This approach helps in the implementation of the "3R" strategy – Replace, Reduce, Refine – for using animals in research (Jaroch et al., 2018). The first attempts to culture retinoblastoma cells from a primary source – an enucleated eye – were performed in the 1960s (Huang et al., 1970; Yoneda and Van Herick, 1963). Despite the low success rate of primary tissue culturing (11–20% of tumour specimens were sub-cultured for an extended period of time), important characteristics of retinoblastoma *in vitro* cultures were reported. Correlations between fast cell growth and the loss of morphology and functional characteristics of the original tissue were established (Yoneda and Van Herick, 1963). The chromosome count of retinoblastoma cells was reported to be between 36 and 60. Different cell types in retinoblastoma culture were described, including glial (and microglial), ganglion and fibrocyte cells, which suggests that all of these cells may be aberrant variations of one malignant retinoblastoma cell type (Huang et al., 1970). Subsequently, retinoblastoma cells cultured from primary sources revealed irregular, rounded shapes with significant variability in the sizes of both the cells and nuclei (Fang et al., 2006; Mendoza et al., 2015). In parallel, systematic studies on the culture of mammalian and human healthy retinal tissues were reported (Hansson and Sourander, 1964; Liss and Wolter, 1961). Growth patterns between retinal and retinoblastoma cells were similar – cells grew in rosettes (of which there are two classic types: the Flexner-Wintersteiner rosette, corresponding to early retinal differentiation, and the Homer Wright rosette, which is typical for neuroblastic differentiation, and both are of a neural nature (Fig. 1).

Further investigation and optimisation of cell culture processes resulted in the establishment of the first human retinoblastoma cell line, Y79 (Reid et al., 1974). The cultured tumour cells were reported to possess ultrastructural characteristics identical to those in the original tumours.

Cytogenetically, Y79 cells are a hyper triploid cell line with varying numbers of minute chromosomes. The second established retinoblastoma cell line, WERI-Rb1 (McFall et al., 1977), is morphologically similar to Y79, and also grows as a suspension of loose cell aggregates. In xenograft models, WERI-Rb1 produces non-metastatic ocular tumours, while tumours from Y79 mimic invasive and metastatic disease (Chevez-Barrios et al., 2000).

With the methods of cell line establishment from retinoblastoma tumours in development, other cell lines, derived from unilateral and bilateral retinoblastomas associated with different *RB1* gene mutations and/or additional non-*RB1* mutations, have been shown to express neuronal phenotypes unlike cell lines that have been in culture for extended periods (Griegel et al., 1990a). Among all of the established retinoblastoma cell lines, Y79 remains the most fast-growing, which makes it more suitable for tumour growth-suppression experiments, whereas WERI-Rb1 depicts low endogenous cell death levels (Busch et al., 2014). For these reasons, these two cell lines are the most frequently used to test drug candidates (Brodowska et al., 2014; Lee et al., 2011; Shao et al., 2017; Zhang et al., 2017; Zhou et al., 2020). Additionally, retinoblastoma cell lines have been reported to lose their ability to form rosettes after significant lengths of time in culture. Optimisation of the original human tumour clonogenic assay has allowed successful colony formation from both cell lines. Confirmation of the Y79 cell line as an invasive and metastatic model was obtained using the chick chorioallantoic membrane (CAM) model (Busch et al., 2014). The CAM model was also used to demonstrate the non-metastatic nature of WERI-Rb1 (Busch et al., 2014). More recently, MYCN oncogene status has been associated with tumour formation, and both WERI-Rb1 and Y79 were reported to show MYCN gain (Schwermer et al., 2019).

Etoposide-resistant Y79 and WERI-Rb1 cell lines have been generated by continuous treatment with consecutively increasing concentrations of etoposide or cisplatin until the IC_{50} of the resistant cell line was at least 10-fold higher than that of the parental cell line. These resistant cell lines possess significantly higher growth kinetics and form greater numbers of tumours of larger weight in the CAM assay compared to the original Y79 and WERI-Rb1 cell lines (Busch et al., 2018). Cisplatin-resistant Y79 and WERI-Rb1 cell lines display increased apoptotic rates and reduced proliferation rates, whereas tumour formation capacity in the CAM model does not significantly change relative to non-resistant cell lines. The components of the extracellular matrix have been hypothesized to play a key role in the formation of chemoresistance of Y79 and WERI-Rb1 etoposide-resistant cell lines (Reinhard et al., 2020). Aside from several studies on probable mechanisms for promoting or inhibiting chemoresistance in chemoresistant retinoblastoma cell lines, the use of such cell lines for treatment discovery has been limited (Kong et al., 2020; Yang et al., 2020). One of the few successful examples showed that the etoposide-resistant WERI-Rb1 and the non-resistant WERI-Rb1 cell lines had a comparable sensitivity towards verteporfin when applied with photodynamic therapy, suggesting a possible treatment (Stephan et al., 2008).

The variety of retinoblastoma cell lines currently available (those most commonly used are summarised in Table 1), together with relatively simple handling, provides great opportunities to researchers for drug candidate evaluation – from cytotoxicity and selectivity studies to the elucidation of mechanisms of action, proliferative states, senescence states and cell death types. The ease of performing such studies with cell cultures makes them a convenient tool for the characterisation of drug candidates at a cellular level, as well as for high-throughput techniques.

However, despite the benefits of classical cell cultures, they have

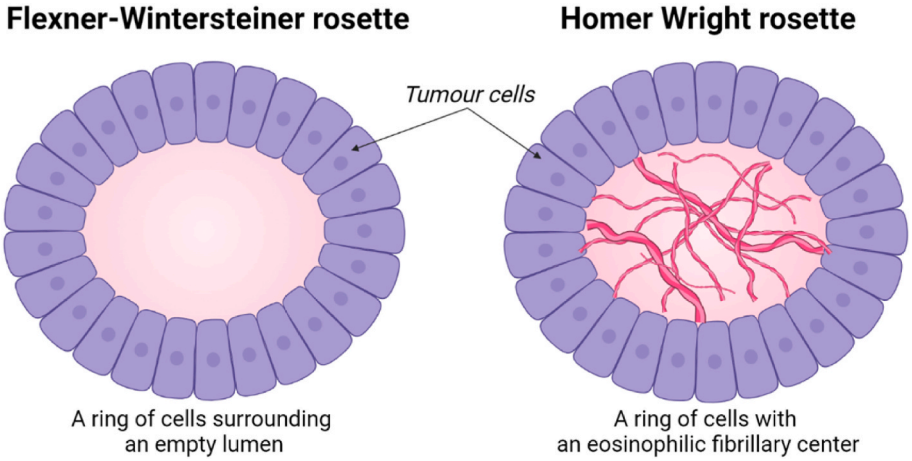

Fig. 1. Commonly found growth patterns of retinoblastoma cells – rosettes. Adapted from reference (Eagle, 2013).

Table 1
The most commonly used retinoblastoma cell lines (both commercial and patient-derived).

Cell line	Commercial	Reference
Y79	Yes	Reid et al. (1974)
WERI-Rb1	Yes	McFall et al. (1977)
RB247C3, RB355, RB383	No	(Squire et al., 1985)
RBL7, RBL13, RBL14, RBL15, RBL18, RBL20, RBL30	No	Griegel et al. (1990b)
HSJD-RBT-1, HSJD-RBVS-1, HSJD-RBT-2, HSJD-RBVS-3, HSJD-RBT-5, HSJD-RBT-7, HSJD-RBT-8, HSJD-RBVS-8	No	Pascual-Pasto et al. (2016)

some major drawbacks. All are typified by a lack of concentration gradients, low levels of organisation, and few cell-cell or cell-matrix interactions, and each of these factors can significantly influence drug penetration and performance, leading to inadequate predictions of drug candidate efficacy and the increased likelihood of failure in *in vivo* studies. Moreover, established cell lines that have been propagated for a long time tend to lose some initial cell features, such as growth patterns and expression of the original phenotype. Furthermore, availability of retinoblastoma cell lines is directly linked to the number of patients that undergo enucleation (as this is the only way to obtain material to establish a cell line) which nowadays is extremely low in developed countries thanks to the high efficacy of treatment strategies and early detection of the disease. To overcome this challenge and potentially increase heterogeneity among available cell cultures, scientists should consider enhanced international collaboration with groups from low-income countries, where retinoblastoma is often diagnosed late, and half of the cases have reached an advanced stage (Fabian et al., 2020). This will facilitate retinoblastoma research around the globe.

3. Three-dimensional (3D) cell culture models

In recent years, 3D cell cultures, referred to variously in the literature as three-dimensional cultures (Winter et al., 2019), spheres (Ma et al., 2011), spheroids (Kuznetsova and Aleksandrova, 2017; Nath and Devi, 2016; Tang et al., 2019), tumourspheres (Bond et al., 2013), tumouroids (Clevers and Tuveson, 2019), and organoids (Clevers, 2016; Hoshino et al., 2017; Mazerik et al., 2018; Saengwimol et al., 2018), have been proposed as better models for mimicking native tissues, reproducing some crucial disease features such as cellular heterogeneity, and the expression of particular genes and proteins that are not expressed in 2D cultures (here, '2D cultures' refers to both adherent and suspension cells). (Corrò et al., 2020).

An ideal 3D culture platform for drug testing and evaluation should present the pathophysiological features of the disease (*e.g.* genomic features, gene/protein expression, therapeutic response), be reliable and provide reproducible results, be adaptive to different treatment modalities (such as the combination of chemotherapy with focal therapy), and be scalable for the high-throughput screening required for testing drug libraries (Decarli et al., 2021). Numerous techniques for the generation of 3D cell cultures for drug evaluation applications are available, including mono- and multicellular cultures, scaffold-free and scaffold-based, low attachment and micropatterned plates, the hanging drop technique, bioprinting, and microfluidic systems (*in vitro* retinoblastoma culture systems are depicted in Figs. 2–4). (Friedrich et al., 2009; Jensen and Teng, 2020; Katt et al., 2016; Langhans, 2018).

Primary cultures best represent the cellular nature of native tumours and are ideal for adapting treatment modalities to specific disease manifestations. It was reported that non-adherent spheres grown in defined medium genotypically match their primary tumour and express neuroendocrine tumour synaptophysin (SYP) and microtubule associated protein 2 (MAP2) markers, whereas adherent cell monolayers are SYP-negative, and express retinal cell markers CD34 and cytokeratin (Fig. 2A). (Bond et al., 2013) These findings emphasise that 3D cultured cells, in contrast to adherent cell cultures, retain more crucial genetic features of the primary tumour. To investigate possible sub-populations of cells that may be relevant to treatment resistance within tumours, a study of the stemness properties in retinoblastoma cells was conducted (Tang et al., 2019). Free-floating spheroids, developed from primary retinoblastoma cells, express the stem-cell markers prominin-1, nestin, and octamer-binding transcription factor 4 (OCT4), while suppressing the mature retinal-cell markers glial fibrillary acidic protein (GFAP), microtubule associated protein 2 (MAP2) and recoverin. These markers demonstrate the immaturity, self-renewal growth in culture and stronger *in vivo* potential of spheroidal retinoblastoma cell cultures compared to established cell lines, which were reported to have only a small number of cancer stem cells (less than 1%) (Seigel et al., 2005). Furthermore, such spheroids with cancer stem-cell-like properties were grown in long-term cultures without the loss of these properties (Ma et al., 2011). These features allow the model to be used for testing and developing drugs aimed at targeting cancer stem-cells.

Another strategy for disease modelling is to focus on the key features that best represent a particular stage or form of the disease. For retinoblastoma, seeding remains a challenging disease manifestation and models mimicking seeding are highly relevant for developing new treatments. Free-floating patient-derived spheroids are able to reproduce the morphology, phenotype and genotype of sphere-class vitreous seeds in patients (Winter et al., 2019) and Y79 cells can mimic the dust class of vitreous seeds. These two models were used to investigate topotecan penetration and activity. The larger the size of the spheres, the longer the time required for the drug to fully penetrate to the core, which correlates with the cytotoxicity, and is consistent with pathological observations of vitreous seeds in patients. Free-floating spheres are therefore a suitable model for assessing the size-dependent drug sensitivity of seeds and for testing new treatment strategies targeting retinoblastoma seeds (Pascual-Pasto et al., 2019; Suresh Babu et al., 2022).

Further development of *in vitro* retinoblastoma models has focused on changing the environment around the cells. Matrigel®, widely used in organoid research, resembles basement membranes (Benton et al., 2014) and promotes the growth of Y79 and WERI-Rb1 cells in suspension, with Y79 cells forming spherical colonies (Albini et al., 1992). During *in vivo* studies, co-injection of Matrigel® with either Y79 or WERI-Rb1 cells into nude mice results in the desired morphology of a native tumour and mRNA expression or the interphotoreceptor retinoid-binding protein, a highly specific retina/retinoblastoma marker (Albini et al., 1992). Retinoblastoma organoids grown from Matrigel®-embedded patient-derived cells retain the histological features and gene/protein expression of the parental seeds, as well as DNA copy-number alterations (Fig. 2, down panel). Furthermore, Matrigel®-embedded organoids exposed to drugs used for the treatment of vitreous seeding revealed that topotecan alone, or the combination of topotecan and melphalan, effectively targeted proliferative tumour cones ($RXR\gamma^{+}$ $Ki67^{+}$) in organoids, blocking mitotic entry. Importantly, the drug responses of organoids were consistent with those of tumour cells at an advanced stage of disease (Saengwimol et al., 2018). Very recently, dissociated organoids were used to identify potential drug candidates by screening 133 FDA-approved drugs (Srimongkol et al., 2023). A multiple tyrosine kinase inhibitor (sunitinib) demonstrated higher suppression of proliferative cones and lowered toxicity compared

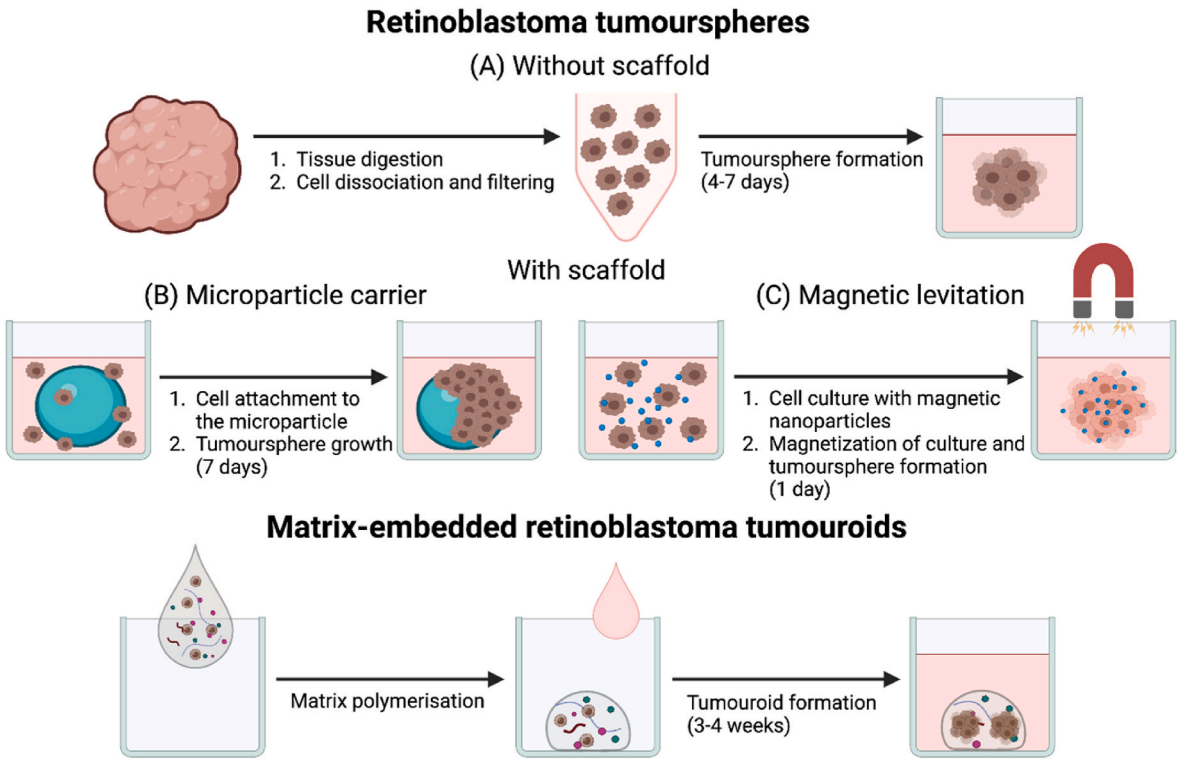

Fig. 2. Three-dimensional retinoblastoma culture systems based on tumour cells, and their methods of preparation (with approximate timescales).

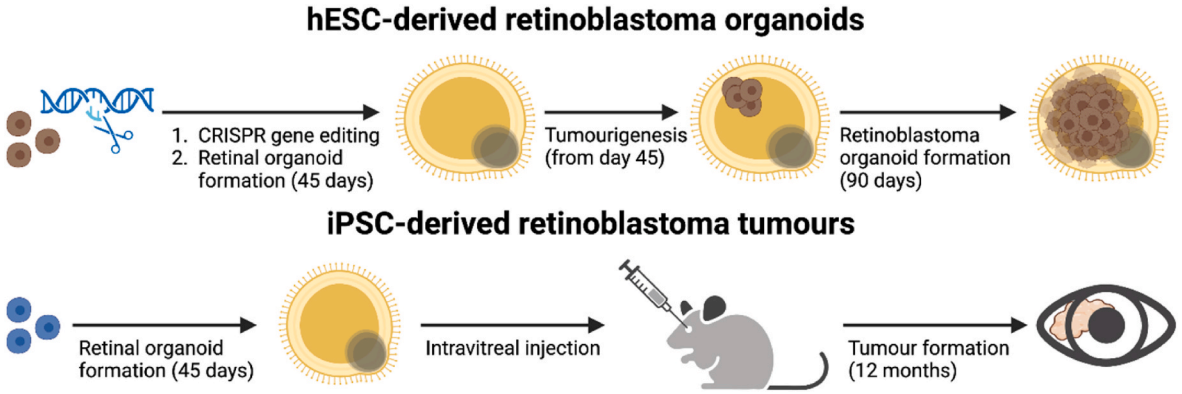

Fig. 3. Human stem cell-based retinoblastoma three-dimensional models, their preparation methods, and approximate timescales. hESC – human embryonic stem cells; iPSC – induced pluripotent stem cells.

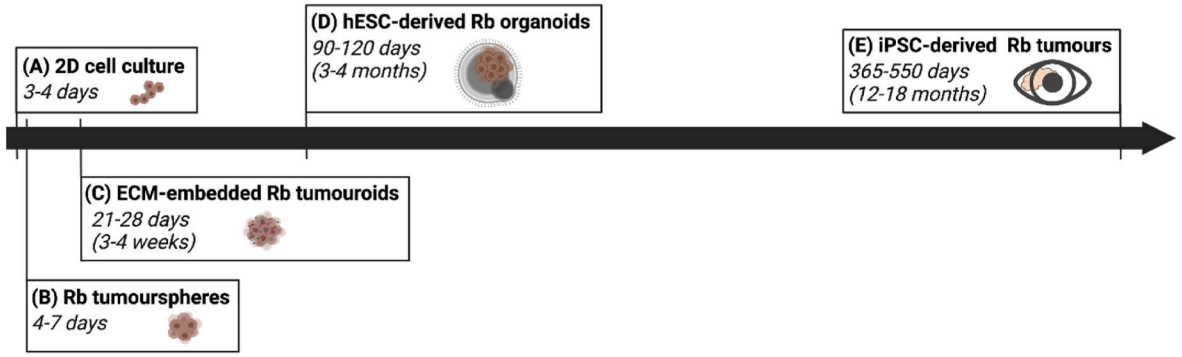

Fig. 4. Various methods for generating advanced retinoblastoma cultures, with representative timelines. iPSCs – induced pluripotent stem cells; hESCs – human pluripotent stem cells; ECM – extracellular matrix; Rb – retinoblastoma.

to melphalan and topotecan, against both *RB1*-deficient and *MYCN*-amplified organoid cultures.

Despite the promising properties of 3D *in vitro* models based on the use of patient-derived cells to recapitulate treatment response in accordance with parental tissue and retain important genomic alterations and gene expression levels, variations between samples, low availability of patient-derived material, and scale-up challenges limit high-throughput drug screening studies. Hence, established cell lines are better suited to high-throughput studies and consequently a scaffold-based model using polymeric microparticles for the growth of Y79 spheres was developed to investigate the efficacy of anticancer drugs (Fig. 2B). (Mitra et al., 2012) This approach supported the growth of established cell lines as tumouroids suitable for high-throughput drug screening. However, whilst the scaffold supported and accelerated the formation of 3D structures, some features, *e.g.*, nutrient gradient and hypoxic regions, were absent. The antiproliferative effect of doxorubicin, etoposide and carboplatin in the 3D model was significantly lower than in the suspension (IC_{50} values were increased by approximately five times). When treating the 3D model with doxorubicin, flow cytometry data demonstrated 4.4-fold lower drug accumulation compared to the suspension cells, accompanied by a 2.3-fold higher collagen content. The latter suggests an increased synthesis of collagen in the 3D model extracellular matrix with the extracellular matrix acting as a barrier to drug diffusion. Microarray and miRNA analysis revealed changes in several genes and miRNA expression in cells grown in the 3D model, which was proposed to influence the environment and drug effects. Such a model emphasises the variation in drug sensitivity due to the more complex cellular/structural organisation in 3D cultures.

Another scaffold-based 3D model based on magnetic levitation (Fig. 2C), (Goldsmith et al., 2018) involves the generation of spheroids by magnetizing a mixture of Y79 cells and nanoshuttles composed of poly-*L*-lysine, iron, and gold. These 3D structures have a similar morphology to retinoblastoma seeds and were used for testing selected treatment conditions. In principle, the model is scalable for higher-throughput screening, and may be interesting for treatment modalities related to the use of magnetic conditions, *e.g.* magnetic hyperthermia.

Retinal organoids, also known as optic vesicles, optic cups or mini-retina, can be generated from stem cells – mouse stem cells, human embryonic stem cells (hESCs) or human pluripotent stem cells (hPSCs) – and differentiated into multiple cell types organised into a micro physiological system (Mazerik et al., 2018). Controlled differentiation, growth, and maturation of organoids allows investigation of a healthy retina, and the development of retinal diseases. However, extensive maintenance, time- and cost-related issues together with genetic variation frequently give inconsistent results with low reproducibility.

Li and colleagues demonstrated direct evidence of the "two-hit" hypothesis by generating retinal organoids from hPSCs with monoallelic ($RB1^{m1/wt}$) and biallelic mutation of *RB1* ($RB1^{mt1/mt2}$). Only organoids derived from hPSCs-$RB1^{mt1/mt2}$ showed retinoblastoma tumourigenesis, supporting the notion that inactivation of both copies of the *RB1* gene is essential and sufficient to develop retinoblastoma (Li et al., 2022). By inducing *RB1* mutations into hESCs with CRISPR-Cas9 technology, retinal organoids were shown to reproduce cell state transitions during retinoblastoma tumourigenesis (Fig. 3, top panel). (Kanber et al., 2022; Liu et al., 2020; Rozanska et al., 2022) This model helped to elucidate disease development, and showed that the retinoblastoma cell of origin in these organoids is the maturing cone precursor. Moreover, among the multiple cell types present in the organoids, retinoma-like cells were purported to be intermediate between premalignant cone precursors and tumour cells. As a proof of feasibility of the model, commonly used chemotherapeutics – vincristine, carboplatin, etoposide, melphalan and topotecan – were tested, and led (with the exception of etoposide) to a reduction of the $Ki67^{+}$ proliferative marker and caused cell apoptosis in the organoids (Liu et al., 2020; Rozanska et al., 2022). Unfortunately, due to the long organoid maturation time (120–150 days) and their complex maintenance requirements, further optimisation is needed for them to be considered suitable for routine drug screening and evaluation. However, such a model is suitable for the testing of small numbers of lead drug candidates. In contrast, CRISPR/Cas9-induced $RB1^{-/-}$ hESCs were used for medium-throughput screening of a library comprising 119 FDA-approved chemotherapeutics and compared to control hESCs (Avior et al., 2017). Such a model is as convenient for screening drugs as classical cell culture, but should not be considered a true 3D culture. Studies revealed that from the 119 drugs tested on $RB1^{-/-}$ hESCs, most of the drugs used to treat retinoblastoma in the clinic, such as etoposide, topotecan and vincristine, performed similarly in the $RB1^{-/-}$ and control cells, while mutant cells were more susceptible to carboplatin, potentially due to mitochondrial dysfunction (Avior et al., 2017).

To conclude, 3D retinoblastoma cultures more closely mimic native tumours than traditional cell cultures, and in some cases patient-derived cell culture is challenging or even impossible to obtain. 3D retinoblastoma cultures also display features of parental tissue when grown with patient-derived cells and/or disease manifestation. Several methods are available to generate retinoblastoma tumouroids, giving an opportunity to adapt them to specific research needs. However, studies on the discovery and evaluation of new therapeutics are rare, and to the best of our knowledge there is no case of implementation of such models into the drug discovery pipeline for retinoblastoma. Instead, known drugs already used for retinoblastoma treatment have been tested on 3D models as a proof-of-concept, and the drug response is similar to those of particular disease stages. For the further development of such models, there is therefore a need to scale up and test a larger number of treatment conditions in order to identify new, promising treatment candidates.

4. Hybrid *in vitro-in vivo* models

Another direction for the further evaluation of the usefulness of 3D models is the possibility of transferring them to *in vivo* models and compare points of interest, and although such studies require the use of animals, they could potentially reduce the number of animals needed. However, to the best of our knowledge, limited studies on engrafting whole-mount retinoblastoma tumouroids generated *in vitro* are available. The benefits of such protocols for drug discovery purposes are yet to be reported.

The earliest experiments on retinoblastoma tumour cells or aggregates of cells were transplantations into athymic nude mice (Gallie et al., 1977) and albino CDF rats (Kobayashi et al., 1982) performed in late 1970s. Retinoblastoma specimens taken after enucleation or Y79 cells were injected into the anterior chamber. Fresh tumour cells showed little growth after injection, whereas Y79 cells spread from the intraocular injection site to the orbit, optic nerve and brain (Gallie et al., 1977). The aggressive behaviour of Y79 cells is echoed by the previously mentioned study where their invasiveness was associated with a metastatic phenotype, whereas WERI-Rb1-derived tumours in transgenic Rag-2 knockout immunodeficient mice remain localized in the eye with anterior choroidal invasion occurring only at the late stages (Chevez-Barrios et al., 2000). Both subcutaneous and orthotopic xenograft models (summarised in Table 2) are available for retinoblastoma (del Cerro et al., 1992; Li et al., 2012) with limitations to recapitulate the developmental environment of human retinoblastoma (Laurie et al., 2005). Orthotopic injections of tumour cells into the vitreous or subretinal space result in fast-growing tumours (del Cerro et al., 1992; Kobayashi et al., 1982). Retinoblastoma xenograft models have been extensively used to investigate intraocular pharmacokinetics and toxicity (Bogan et al., 2021a, 2021b; Kaczmarek et al., 2021) – information that is highly valuable for intravitreal drug administration insights – as well as to evaluate some potential treatment candidates with antitumour activity (Burr et al., 2011; Dalgard et al., 2008; Delrish et al., 2021; Laurie et al., 2005; Wu et al., 2018; Xia et al., 2019).

Table 2
Xenograft models of retinoblastoma.

Description	Cell source	Animal	Implantation site	Reference
Y79	Y79	Nu/nu mouse	Anterior chamber	Gallie et al. (1977)
Y79	Y79	Nu/nu mouse	Subcutaneous	
Y79	Y79	Nu/+ mouse	Anterior chamber	
Fresh retinoblastoma	Primary culture	Nu/nu mouse	Anterior chamber	
Fresh retinoblastoma	Primary culture	Nu/+ mouse	Anterior chamber	
EXP-5	Retinoblastoma-like explant	CDF albino rat	Intraocular	Kobayashi et al. (1982)
Y79	Y79	Fischer 344 albino rat	Subretinal	del Cerro et al. (1992)
Y79	Y79	Nu/nu mouse	Subcutaneous	Albini et al. (1992)
WERI-Rb1	WERI-Rb1	Nu/nu mouse	Subcutaneous	
WERI-Rb1-Matrigel	WERI-Rb1	SCID mouse	Subcutaneous	Cowell et al. (1997)
Y79 tumour	Y79	transgenic Rag-2 knockout immunodeficient mouse	Intravitreal	Chevez-Barrios et al. (2000)
WERI-Rb1 tumour	WERI-Rb1	transgenic Rag-2 knockout immunodeficient mouse	Intravitreal	
Y79-GFP	GFP-labelled Y79	Newborn Sprague-Dawley rat	Intravitreal	Laurie et al. (2005)
SO–Rb 50	SO–Rb 50	NOD-SCID mouse	Subcutaneous	Li et al. (2012)
HSJD-RBT-2, HSJD-RBT-5, HSJD-RBT-7 HSJD-RBT-8	HSJD-RBT-2, HSJD-RBT-5, HSJD-RBT-7 HSJD-RBT-8	Nu/nu mouse	Intraocular	Pascual-Pasto et al. (2016)
WERI-Rb1	WERI-Rb1	New Zealand white rabbit	Intraocular	Daniels et al. (2018)
SJRB-iPSC-4, SJRB-iPSC-4CR, SJRB-iPSC-8, SJRB-iPSC-8CR	Retinal organoid	C57BL/6 SCID mouse	Intravitreal	Norrie et al. (2021)

Matrigel®, which supports Y79 and WERI-Rb1 cells growth *in vitro*, also enhances the growth of human retinoblastoma subcutaneous transplants *in vivo* when injecting a tumour-cell suspension mixed with Matrigel® into nude mice (Albini et al., 1992). It was shown that WERI-Rb1 cells mixed with Matrigel® resulted in 75% more tumours in SCID mice than without Matrigel® (Cowell et al., 1997).

To assess retinoblastoma formation, organoids were grown from patient-derived *RB1*$^{-/-}$ induced pluripotent stem cells (iPSCs), and after retinal differentiation for 45 days, were engrafted onto immunocompromised mice (Fig. 3, down panel). (Norrie et al., 2021) The tumours thus formed in the mice displayed the same cellular and genomic features as in parental tissues. The overall process took 12–18 months to produce retinoblastoma in this system, making it unsuitably slow for drug evaluation, although the system is highly biologically relevant and can reveal the *RB1*-associated mechanism of retinoblastoma tumourigenesis and its cellular origins (Fig. 4).

5. Conclusions and outlook

3D structures grown from patient-derived specimens or from established cell lines mimic the natural tumour to a greater extent than classical cultures. Their characteristics are closer to the original tumour tissue, and stem-cell-like properties result in self-organisation and growth. Such models can be considered better options for drug screening, due to their improved simulation of drug penetration in a real tumour, avoiding the expenditure of time and resources on further evaluation of non-optimized drugs in *in vivo* studies. In several studies, 3D models were tested with the drugs used for the treatment of retinoblastoma in the clinic and demonstrated biologically relevant responses. Although 3D models seem to be more predictive for drug response than classical cell culture, they are not yet sufficiently widely used for the discovery of new drugs or drug repurposing, but instead have been used for selective screening of treatment conditions and, more often, to answer biological questions. Moreover, when engrafted to animals, 3D structures enhance tumour growth *in vivo*. The more challenging protocols required for the growth of 3D structures need further optimisation/automation to facilitate their implementation in the discovery of new treatments for retinoblastoma. Furthermore, primary tissues, used in the majority of studies which generate 3D models, require access to the source, and can be difficult to obtain. Using primary tissues allows the detailed study of retinoblastoma and its underlying variable genetic make-up, which, on the one hand, may lead to different drug responses, but, on the other, provides the possibility of adapting treatments to different stages of the disease (Aasen and Vergara, 2019), and could ultimately lead to personalized medicines for retinoblastoma.

3D *in vitro* models created using established cell lines, despite losing some of the features of primary cells, have shown their potential for more accurate drug sensitivity prediction. Moreover, as these models can be scaled-up and used for extensive drug screenings, they can serve as an intermediate step between classical culture and selective testing on the primary source-derived or *in vivo* models, thus reducing the number of animals used. At present, 3D models, positioned between *in vitro* and *in vivo* models, are typically only compared with classical *in vitro* models. Limited data is available on the direct comparison of drug responses between 3D and *in vivo* models in drug sensitivity studies. As a 3D model is ultimately an approach to better simulate *in vivo* studies and facilitate the drug development process, a direct comparison of these two approaches is essential to better assess the benefits, applicability and limitations of 3D models. Since retinoblastoma is a rare cancer, the major pharmaceutical companies show little interest, if any, in developing new drugs for the disease. Hence, drug repurposing studies (Cancela et al., 2020), or the synthetic modification of drugs already used to treat retinoblastoma to improve their selectivity (Kerr et al., 1998; Singh et al., 2018), are the mostly likely approaches to further improve treatment protocols. Consequently, the application and further development of the 3D models described herein will play a crucial role in future therapies.

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Declaration of interest

None.

Data availability

No data was used for the research described in the article.

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