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Progress in Small Molecule Therapeutics for the Treatment of Retinoblastoma

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Abstract

While mortality is low for intraocular retinoblastoma patients in the developed world who receive aggressive multimodal therapy, partial or full loss of vision occurs in approximately 50% of patients with advanced bilateral retinoblastoma. Therapies that preserve vision and reduce late effects are needed. Because clinical trials for retinoblastoma are difficult due to the young age of the patient population and relative rarity of the disease, robust preclinical testing of new therapies is critical. The last decade has seen advances towards identifying new therapies including the development of animal models of retinoblastoma for preclinical testing, progress in local drug delivery to reach intraocular targets, and improved understanding of the underlying biological mechanisms that give rise to retinoblastoma. This review discusses advances in these areas, with a focus on discovery and development of small molecules for the treatment of retinoblastoma, including novel targeted therapeutics such as inhibitors of the MDMX-p53 interaction (nutlin-3a), histone deacetylase (HDAC) inhibitors, and spleen tyrosine kinase (SYK) inhibitors.

Keywords

Retinoblastoma; Ocular Drug Delivery; Chemoreduction

1. Introduction

Retinoblastoma is a rare childhood cancer of the retina. Each year, approximately 300 new cases of retinoblastoma are diagnosed in the United States and 5,000 – 8,000 cases are diagnosed worldwide.^[1] Untreated retinoblastoma is always fatal within two years, due to intracranial extension and disease dissemination.^[2] With timely diagnosis and aggressive

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multimodal therapy, cure rates for retinoblastoma in the developed world are high (~95%). Currently available efficacious treatments include photocoagulation,^[3–4] thermotherapy,^[5] cryotherapy,^[6–7] chemotherapy (intravenous and/or local),^[8] plaque radiotherapy,^[9–10] external beam radiotherapy (EBRT),^[11–12] and enucleation (surgical removal of the eye).^[13–14] Reviews of management strategies for retinoblastoma have been published.^[2, 15–17] The choice to utilize chemotherapy depends on tumor laterality and disease stage (Table 1). While enucleation is usually the treatment of choice for late-stage unilateral retinoblastoma, in cases of bilateral retinoblastoma the use of upfront chemotherapy (termed chemoreduction) followed by aggressive focal therapy can increase the rate of eye salvage, particularly when one eye has already been enucleated.^[17–18]

As of the writing of this article, there exists no standard chemotherapy regimen for retinoblastoma, as different experts favor different combinations. Three classes of agents are generally employed in combination therapy for retinoblastoma: DNA crosslinking agents (carboplatin, cisplatin), DNA topoisomerase II inhibitors (etoposide, teniposide), and vinca alkaloids (vincristine). The most commonly used combination in recent studies is vincristine, etoposide, and carboplatin (VCE).^[17–19] To eliminate the potential oncogenic risk of etoposide, the two-drug combination of vincristine and carboplatin has been used at some centers.^[17,20] As with the overall management strategy, the choice of chemotherapy drug combination and schedule are dependent on the laterality and stage of the tumor, as well as the preferences of the clinical team (Table 1). Early stage tumors (Group A) are generally well managed with focal therapies alone. Although chemotherapy with adjuvant local treatments is often adequate for early stage tumors (Groups B, C and D), enucleation (surgical removal of the eye) plays a major role in the treatment of late stage retinoblastoma (Group E). Chemotherapy may also be used in combination with enucleation in the treatment of Group E tumors where there is evidence of metastasis (Table 1).

Current retinoblastoma treatment can produce side and late effects.^[21–23] Side effects of focal therapies may include vitreous seeding, retinal tears and radiation retinopathy.^[24] Partial or full loss of vision occurs in approximately 50% of patients with advanced bilateral retinoblastoma.^[25] Vision loss has a major potential impact on patient quality of life: blindness has been ranked by WHO experts in the penultimate class of increasing disability severity (Class VI, the same class as paraplegia).^[26] There are also other significant late effects, including facial malformations and increased incidence of secondary malignancies due to radiation and etoposide.^[27–30]

For these reasons, therapies are needed that preserve vision and reduce late effects of therapy without compromising the currently high cure rate.^[31] Fortunately, the field has recently seen several critical advances towards achieving this targeted balance between patient survival and improved quality of life. First, there have been significant advances in developing local drug delivery approaches for reaching intraocular targets.^[32] Local delivery has the potential to increase exposure at the intended site of action while reducing overall systemic exposure, improving both efficacy and tolerability and reducing late effects.^[18,33] Local delivery is gaining acceptance in delivery of chemotherapy agents for intraocular tumors like retinoblastoma.^[18,34] Second, several useful transgenic and xenograft animal models of retinoblastoma have been developed in the past decade to prioritize candidates for

clinical testing.^[19,24,35–36] Finally, major advances have recently been made in understanding the biology of retinoblastoma, leading to identification of several promising druggable molecular targets.^[24,31] Leveraging this new understanding to identify and develop novel, targeted therapeutics with superior selectivity could lead to therapies with better efficacy and safety profiles than the currently used broad-spectrum chemotherapy agents. There is tremendous potential to combine spatial targeting (i.e. local delivery to increase intraocular exposure while lowering systemic exposure) with molecular targeting (i.e. use of small molecules that target pathways specific to retinoblastoma), utilizing the robust, clinically relevant preclinical models to prioritize these novel therapies.

For this review, we will briefly discuss advances in local ocular drug delivery and preclinical models of retinoblastoma, focusing predominantly on progress in the identification and development of novel small molecule therapeutics for retinoblastoma. We will highlight compounds which have shown evidence of efficacy in patients (clinical), compounds which have evidence of efficacy in animal models, but not patients (pre-clinical), and compounds which have some evidence of efficacy *in vitro* or in biochemical assays, but have not progressed to *in vivo* testing (pre-vivo).

2. Progress in Delivery of Retinoblastoma Therapeutics

Due to the unique physiology of the eye, one of the primary obstacles in developing drugs for retinoblastoma is the difficulty of achieving sufficient exposure in the tumor.^[37] Anatomical and physiological constraints like the blood retinal barrier (BRB)^[38] limit effective delivery to the eye from systemic routes of administration (i.e. oral and intravenous). Using high doses to overcome this limited BRB penetration can cause systemic toxicity.^[39–40] Treatment of vitreous seeding is particularly challenging due to the lack of vasculature in the vitreous.^[37] Even when compounds do reach the target site, rapid clearance from the eye often results in short intraocular residence times.^[41]

Recently, local delivery approaches have been proposed to overcome these challenges, as they have the potential to increase exposure at the intended site of action while reducing overall systemic exposure, improving both efficacy and tolerability. The eye is particularly well-suited to local delivery because, in contrast to the rest of the central nervous system, it is physically accessible without surgery.^[37,40] Locally delivered broad spectrum chemotherapeutic agents such as melphalan and carboplatin have shown efficacy for retinoblastoma in the clinic.^[18] Local delivery routes for ocular therapeutics generally fall into four categories: topical (transcorneal), periocular (transcleral), intravitreal (direct injection), and intra-arterial infusion (Figure 1). Several review articles on delivery routes for the posterior eye have been published.^[18, 39, 42–44] Local delivery can be further enhanced by combining compounds with novel formulations, biodegradable carriers (such as hydrogels or particulate systems), or sustained release implants.^[32,39,45] Because local routes are gaining acceptance, particular emphasis will be placed on retinoblastoma clinical and preclinical studies that have investigated periocular, intravitreal, or intra-arterial delivery to provide context for how advances in ocular delivery impact retinoblastoma specifically.

2.1 Systemic Route

The primary regimen for treatment of retinoblastoma is currently the intravenous combination of vincristine, carboplatin, etoposide (VCE), which was introduced in the 1990s ([18]). VCE is effective in managing early-stage retinoblastoma, but may not control advanced retinoblastoma, with success generally correlating to tumor stage. Shield et al. report that chemoreduction (6 courses of VCE) successfully treated 100% of group A, 93% of group B, 90% of group C, and 47% of group D eyes.[46] Berry et al. also report a 47% cure rate (26 of 55 eyes) in Group D eyes treated with VCE.[47] Similarly, Rodriguez-Galindo et al. report that chemoreduction (8 courses vincristine and carboplatin) is effective for early stage disease, but that more aggressive treatments are needed for patients with advanced intraocular disease.[20] Factors associated with failure of VCE chemoreduction (defined as unresponsive or recurrent disease) include older patient age, greater tumor thickness' and presence of vitreous seeding.[48] The latter is a characteristic of Group C or D tumors. In a retrospective review, Shields et al. found that Group E retinoblastoma managed with chemoreduction alone showed significantly more need for enucleation or therapeutic radiotherapy than eyes treated with chemoreduction combined with low-dose prophylactic external beam radiotherapy.[49] Regimens for adjuvant chemotherapy (i.e. chemotherapy after enucleation that is administered in the presence of high-risk pathological features and in the presence of overt orbital disease) include VDC (vincristine, cyclophosphamide, and doxorubicin or idarubicin)[50], VCE, or a combination of the two with alternating courses.[16] Like VCE, VDC is administered intravenously.[16,50]

One of the major challenges in treating retinoblastoma via the systemic route is the blood retinal barrier (BRB). Structurally and functionally similar to the blood brain barrier (BBB), the BRB hinders movement of the majority of drugs from the systemic vasculature to the posterior eye segment, including the retina, choroid and vitreous. Penetration into the eye from the vasculature is typically low, and systemic toxicity may limit the use of dose-escalation to overcome limited penetration. Preclinical studies suggest that vincristine, carboplatin, etoposide and topotecan all reach therapeutically effective concentrations via the systemic route,[51–52] but for many agents, innovative drug delivery techniques must be employed to achieve therapeutically relevant intraocular concentrations without systemic toxicity.[51] Because single agent therapies are uncommon for retinoblastoma, this need is particularly critical for combinations with overlapping systemic toxicities.[52]

It should be noted that systemic chemotherapy may be needed not only to control the intraocular tumor, but also to prevent or treat metastatic disease.[53] As such, many local delivery approaches may be worth considering more as adjuvant therapies to combine with intravenous chemotherapy, rather than replacement therapies. It is also worth noting that even if a local delivery route is successful in increasing intraocular concentration compared with systemic delivery, this may not always result in an improvement in efficacy. In some cases the assumption that more drug into the eye results in more tumor cell death may not be valid, as a threshold concentration may exist above which no further efficacy is observed (i.e., a plateau effect). It is also possible to increase intraocular concentration compared with systemic delivery using a local delivery route, but still fall short of the concentration required for efficacy.[43]

2.2 Local Delivery Routes

2.2.1 Intravitreal—Because macromolecules have very limited penetration of both the BRB and local ocular tissues, intravitreal injection has emerged as the method of choice for delivering biological therapeutics for the treatment of macular degeneration.^[55] Several studies have been carried out to evaluate the pharmacokinetics of antiviral agents, steroids, and monoclonal antibodies following intravitreal injection.^[39] This approach has also shown some promise for retinoblastoma treatment; chemotherapy agents delivered using this route include thiotepa,^[55] melphalan,^[56–57] and methotrexate.^[58] The invasiveness of intravitreal injection is a drawback to the route: puncture of the globe is ideally to be avoided as it can introduce intraocular infection^[59–60] and provide an opportunity for an otherwise confined tumor to spread beyond the globe.^[57,61] Though improved methodology has reduced concerns regarding tumor spread due to globe puncture,^[57, 61] repeat injections may be required to sustain therapeutic concentrations. Short retention time remains a major limitation for this route, with smaller drugs tending to clear more rapidly from the vitreous.^[39,43] The other limitation of the intravitreal route is the potential for retinal or other local toxicity due to the high initial drug concentration that typically results from a bolus injection.^[18] Combining intravitreal injection with sustained release approaches may reduce injection frequency and improve intraocular exposure profiles (i.e. increase retention time and reduce concentration spikes), which would make this route more attractive for small molecule drugs.^[39]

2.2.2. Intra-arterial—Intra-arterial delivery (selective ophthalmic artery infusion)^[33,62–63] has been gaining popularity as a method for local delivery of retinoblastoma therapeutics, particularly the alkylating agent melphalan.^[62–64] Though melphalan is the most commonly employed agent for intra-arterial infusion, this approach has also been investigated for delivery of methotrexate,^[63] carboplatin,^[63] digoxin,^[65] and topotecan.^[66] One report has been published utilizing three drugs simultaneously via intra-arterial infusion (melphalan, topotecan and carboplatin). Of the 26 eyes treated, 23 of them (88%) avoided both enucleation and external beam radiotherapy.^[67]

Studies generally report high efficacy for intra-arterial chemotherapy (IAC),^[18,24,62, 67–68] but there are also several reports on potential ocular toxicities associated with this route. The primary toxicity is compromise of the local ocular vasculature (ophthalmic artery, retinal artery, or choroidal vessels).^[18,33,69–73] Vascular compromise can cause poor visual outcome, effectively negating many of the benefits of avoiding enucleation. Steinle et al. attribute this local vascular toxicity to effects of melphalan and carboplatin on endothelial cells (described later), and consequences related to the method itself: acid-induced toxicity due to the low pH of the formulation used for infusion (pH 5.0–5.5), mechanical stress on the vasculature resulting from the pulsatile flow of the infusion, inflammatory response triggered by drug precipitation, or some combination of these effects.^[73] Intra-arterial infusion of both carboplatin and melphalan causes acute toxicity in the ocular vasculature of non-human primates^[69–70] and induces significant biochemical changes in primary human retinal endothelial cells,^[73] suggesting that, while IA infusion may be safe for some drugs, the route must utilize the proper chemotherapy agent and dose. For IA to be successful, the

administration protocol, drug, and dosage employed must retain vascular system functionality.

2.2.3. Periocular—Although intravitreal injection and intra-arterial infusion are both effective local delivery routes, less invasive methods that achieve similarly high ratios of local to systemic exposure without disrupting globe integrity are highly desirable. Transscleral delivery has been proposed to avoid the risks of intravitreal injection while retaining the advantages of local delivery.^[74]

Periocular injection is an attractive approach for transscleral delivery, as it takes advantage of the sclera's large surface area and high permeability to small molecules and does not puncture the globe.^[74–76] Injection into the periocular space places the drug in close proximity to the sclera, allowing compound to diffuse across the local ocular tissues into the vitreous. Drug can be injected into the periocular space (the region surrounding the globe). Periocular injection site determines if an injection is classified as retrobulbar (above or below the orbit), peribulbar (in the muscle cone), subtenon (between the Tenon's capsule and the sclera), or subconjunctival injection (between the conjunctiva and sclera) (Figure 1).

Transcleral drug delivery is hindered by three potential types of barriers: static, dynamic and metabolic.^[43–44] The static barriers are the anatomical layers of the globe (conjunctiva and sclera). However, these barriers are not a major factor for diffusion of small molecules as the sclera is well hydrated and relatively permeable. Degradation of drug by endogenous enzymes (metabolic barriers) is also not expected to delay diffusion substantially, as the nearly acellular sclera is relatively metabolically inert.^[43] Melanin binding may play a role in drug distribution, but its impact on intraocular pharmacokinetics (PK) has not been extensively explored for transcleral or any other local ocular delivery route. Removal of drug from the episcleral space by the highly vascularized conjunctiva and lymphatic vessels (dynamic barriers) are believed to be the primary barrier to transcleral delivery.^[43] Ex vivo and *in vitro* studies suggest that physical chemical properties of the compound (including size and solubility), and its formulation, impact transcleral diffusion and intraocular pharmacokinetics following periocular delivery.^[54, 77]

Periocular injection of carboplatin has been extensively investigated, aiming to both increase intraocular exposure and reduce systemic exposure. Clinically, periocular injection of carboplatin has been used as an adjunct to IVC to increase the intraocular concentration of carboplatin.^[18, 78–79] Comparing pharmacokinetics following a single subconjunctival injection of carboplatin (5 mg in 0.4 mL) or a single intravenous injection of carboplatin (18.7 mg/kg of body weight) in rabbits showed significantly higher levels of carboplatin in the intraocular tissues (retina, choroid, vitreous and optic nerve) (vitreous area under the concentration-time curve from 1 to 24h approximately 5.5-fold higher and $C_{\max, \text{vitreous}}$ approximately 4-fold higher) and significantly lower plasma concentrations (plasma area under the concentration-time curve from 1 to 24h approximately 17-fold lower and $C_{\max, \text{plasma}}$ approximately 19-fold lower) from subconjunctival compared to systemic delivery, despite the fact that the total periocular dose was approx. 12-fold lower than the total IV dose.^[79] In the same study there was no evidence of local or systemic toxicity following subconjunctival injections, although it is worth noting that histopathological

analysis of enucleated patient eyes has revealed significant ocular toxicity may result from periocular delivery of carboplatin.^[80] Using a murine model of retinoblastoma, Hayden *et al.* demonstrated dose-dependent reduction of tumor burden after treatment with subconjunctival injection of carboplatin.^[81]

Unlike carboplatin, topotecan exhibits high ocular penetration from the systemic route and achieves roughly comparable intraocular and systemic exposure in rabbits when delivered through either route (periocular or intravenous injection).^[82] Though topotecan can be delivered either via subconjunctival or systemic route, the latter allows for a more-clinically relevant daily administration schedule. The advantage of combining subconjunctival carboplatin (CBP) with systemic topotecan (TPT) is that, despite overlapping systemic toxicities (particularly myelosuppression), these two agents can be co-administered. This approach provides therapeutically relevant intraocular concentrations of both agents within the eye and allows continued chemotherapeutic exposure for several days, as TPT can be administered daily. The combination of subconjunctival carboplatin and systemic topotecan was effective and safe in a murine model of retinoblastoma.^[52] The evidence that local carboplatin delivery reduces systemic exposure and increases intraocular exposure, has led to periocular carboplatin injection combined with systemic VCE emerging as one approach to chemoreduction (see Clinical Trials NCT02319486, NCT02137928 and NCT00186888). In addition to combining local carboplatin with systemic VCE, a small study has been published examining intravitreal injection of carboplatin, preceded by a subconjunctival dose of carboplatin, in patients with bilateral advanced retinoblastoma as an approach to reduce spread beyond the globe.^[83]

2.2.4. Topical—Historically, drugs delivered to the surface of the eye via drops or ointment were believed to reach the interior of the eye primarily through transcorneal diffusion with limited penetration to the posterior eye. Recently it has become apparent that, rather than being restricted to the cornea, drugs applied to the surface of the eye may reach both the retina and vitreous via transcleral diffusion. Transcleral diffusion represents a more promising path to the retina than transcorneal diffusion as the conjunctival surface occupies a greater surface area, the sclera generally has a leakier epithelium than the cornea, and compounds have a shorter diffusion path length to traverse.^[43] Enhancing corneal drug permeability (which can cause toxicological complications)^[39] may not be needed to reach the posterior segment. However, further work is needed to understand how drug properties (such as log P, solubility and molecular weight) and formulation (such as combination with additives and particle size) drive retinal exposure following topical delivery. To our knowledge, there have been no studies on topical delivery of chemotherapy agents for retinoblastoma.

Although there is a widely held belief that the majority of a topically delivered dose is absorbed into systemic circulation rather than into the globe, a systemic review of the literature reveals relatively few PK studies, suggesting that this belief may be over generalized. Unlike intravitreal, intra-arterial or periocular injections, topical formulations can be self-administered. This is less invasive, more convenient and less expensive for patients,^[39] but there are compliance concerns, particularly with very young patient

populations and in potentially life- and/or sight-threatening disorders.^[43] Therefore, the PK consequences of topical delivery are an area needing further study.

2.2.5. Biomaterial Carriers for Local Delivery—Although limited depot effects can be achieved from extraorbital injections for some compounds, sustained drug delivery to the eye is generally not expected from this route,^[44] suggesting that repeated injections would be required to maintain therapeutic drug concentrations. However, children with retinoblastoma can only receive a subconjunctival injection once every 3 weeks – the schedule under which they undergo examination under anesthesia.^[34] Sustained release formulations or implantable systems may increase the duration of local drug release, reducing the frequency of administration. Injectable carriers like in situ forming hydrogels and nano- or micro-particle suspensions are preferable to implants, as they do not require retrieval at the completion of therapy.

Biodegradable carriers have been examined for topotecan and carboplatin due to concerns related to pharmacokinetics and ocular toxicity, respectively. Although locally delivered topotecan does not appear to induce local side effects,^[84] similar ratios of systemic to intraocular exposure are seen for either periocular or IV injection, minimizing the benefit of local delivery.^[52,82] In contrast, periocular administration of carboplatin significantly increases the ratio of intraocular to systemic exposure,^[79] but exposure of local tissues to high peak concentrations appears to cause significant local toxicity, including ischemic necrosis and atrophy of the optic nerve,^[85] fibrosis of the orbital soft tissue and reduced ocular motility,^[86] and transient periorbital edema.^[87] Administering drugs in combination with an injectable biomaterial carrier such as fibrin sealant or micro-particle encapsulation offers a potential solution to both these limitations.

Although nanoparticle formulation has been explored for carboplatin,^[88] fibrin glue is by far the most commonly investigated injectable biodegradable carrier for delivery of retinoblastoma chemotherapy agents and candidate therapeutics.^[75–76, 89–92] Mixing carboplatin with fibrin enhances clot formation.^[93] Carboplatin and topotecan released from fibrin depots have both been shown to retain their bioactivity against retinoblastoma cells in culture.^[90,93] Fibrin also provided sustained release of carboplatin both in an *ex vivo* transcleral flux study and in an ocular PK study conducted in rabbits. *In vivo*, fibrin sealant provided sustained delivery of carboplatin to the ocular tissues for up to 2 weeks, while in solution carboplatin clears rapidly.^[75] Using a transgenic mouse model of retinoblastoma, Van Quill *et al* demonstrated that a single injection of low-dose carboplatin in fibrin sealant (0.66 mg total in 30 microliters) was sufficient to induce complete or near-complete intraocular tumor regression in 91% of eyes, with no associated histologic evidence of toxicity.^[89] Similar results have been reported for topotecan combined with fibrin in both preclinical models^[90] and patients.^[91–92] Fibrin-sealant mediated local delivery of topotecan, and general use of fibrin sealant in retinoblastoma have both been reviewed elsewhere.^[76]

3. Clinical Progress in Retinoblastoma Therapeutics

The *de facto* standard-of-care agents for retinoblastoma are carboplatin, vincristine and etoposide (Figure 2 a–c). As these agents have been extensively reviewed elsewhere,^[8,15–16,18, 94] we have elected to focus on alternatives to VCE in this review. Novel approaches to delivery of these standard of care agents (including periocular delivery of carboplatin and combination of chemotherapy agents with fibrin sealant) are discussed in the previous section.

3.1 Topotecan

Topoisomerase inhibition is highly effective in retinoblastoma chemoreduction,^[95] but the most commonly used topoisomerase inhibitor, etoposide, is believed to cause secondary malignancies in retinoblastoma patients.^[96] To address this issue, topotecan was examined in animal models of retinoblastoma.^[51] Topotecan (Figure 2d) is a topoisomerase I inhibitor that has been FDA-approved for use against gynecological cancers and small cell lung carcinoma.^[91] *In vitro*, topotecan induced a dose- and time- dependent reduction of retinoblastoma cell viability, accompanied by an increase in caspase activation,^[90] and was approx. 60-fold more potent than etoposide.^[52] Topotecan achieved therapeutically relevant levels in the vitreous following systemic delivery, and the combination of topotecan and carboplatin was more effective than VCE in the preclinical rodent models of retinoblastoma. Therefore, topotecan represents a promising potential replacement for etoposide in combination therapy for retinoblastoma.^[52]

While topotecan is effective and does not carry the risk of secondary malignancies, it does cause hematotoxicity, especially when combined with platinum derivatives.^[51] Novel formulation and local routes have been extensively evaluated to minimize these systemic side-effects, as have alternative schedules.

As described above, periocular administration of topotecan appears to offer no benefit over systemic delivery in terms of reducing systemic exposure or increasing local exposure and is associated with a more constrained administration schedule.^[52,82] In addition to combining topotecan with fibrin sealant (as described in the section on Biomaterial Carriers for Local Delivery), other novel approaches proposed to reduce topotecan clearance from the subconjunctival space and improve its' PK profile include encapsulation in an episcleral biocompatible polymer implant reservoir and induction of local vasoconstriction via co-administration of agents like adrenaline.^[97]

Other local routes may increase the ratio of intraocular exposure to intraocular exposure for topotecan delivery. For example intravitreal injection of topotecan to rabbits gave a 50-fold increase in vitreal AUC compared to systemic administration.^[98] Likewise, both intra-arterial and periocular administration to pigs gave significantly improved intraocular PK. However, IAC resulted in a higher vitreous concentration and a higher ratio of vitreous AUC to systemic AUC than periocular injection.^[66] Note that these studies assume a higher intraocular exposure will translate to greater tumor-killing efficacy, which, as described previously, may not be the case.

Based on these promising preclinical results, topotecan was examined clinically. A 21-day continuous infusion of 0.3 mg/m²/day was well tolerated in pediatric patients, while at a dose of 0.4 mg/m²/day myelosuppression was reported to be the dose-limiting toxicity.^[99] A 30-min infusion at a dose of 2 mg/m²/d for five consecutive days every third week for a total of 16 cycles to patients with relapsed/refractory metastatic and intraocular retinoblastoma gave significant numbers of partial responses and stable disease.^[100] A Phase I study of periocular topotecan demonstrated that up to 2 mg of periocular topotecan could be administered safely, with no dose limiting toxicity being found.^[84] A retrospective case review concluded that periocular injection of topotecan in fibrin could successfully induce tumor regression sufficient to allow successful focal therapy.^[91] These studies indicate topotecan is an extremely promising agent for retinoblastoma treatment, particularly when combined with novel delivery approaches.

3.2. Anthracyclines

Anthracyclines are a class of drugs that kill cancer cells via intercalation of DNA and topoisomerase inhibition^[101–102] and are active against extraocular/metastatic retinoblastoma. Doxorubicin (Figure 2e) is used to treat several cancers, but causes long term cardiac toxicity and myelosuppression.^[16, 103] Another anthracycline, idarubicin (Figure 2f) exhibits superior BBB penetration and less cardiotoxicity than doxorubicin.^[103–105]

Because their BRB penetration is limited, clinical investigation of doxorubicin and idarubicin has largely focused on cases of extraocular/metastatic retinoblastoma, which is rare in the developed world. An early study with intravenous doxorubicin showed both partial and complete responses.^[106] A larger study demonstrated an overall response rate of 60%, with bone marrow sites (where idarubicin achieves high exposure) being completely cleared of tumor, while all patients with CNS sites (where exposure may be more limited due by the BBB) exhibited progressive disease.^[103] These results suggest that anthracyclines are active in retinoblastoma, but that activity is dependent on achieving sufficient concentrations at the target site.

The combination of doxorubicin's insufficient BRB penetration and dose-limiting myelosuppression and cardiotoxicity have driven efforts to develop novel drug targeting systems to improve intraocular and reduce systemic exposure. Doxorubicin encapsulated in poly- β -hydroxybutyrate microspheres, administered to rabbits via intravitreal injection, provided detectable levels of doxorubicin that were sustained 10 days post injection.^[107] Additionally, encapsulation lowered peak doxorubicin levels in the ocular tissues compared with free doxorubicin. Likewise, *ex vivo* transscleral diffusion studies with doxorubicin encapsulated in poly(lactide-co-glycolide) (PLGA) polymer nanoparticles or liposomes (Doxil®, Tibotec Therapeutics) showed that doxorubicin readily diffused across isolated human sclera and that encapsulation (either in PLGA or liposomes) slowed delivery.^[102] This suggests local transcleral routes such as periocular or topical may be feasible for doxorubicin delivery, but it is worth noting that the dynamic barriers to transcleral delivery (i.e. conjunctival and choroidal blood flow and lymphatic drainage) are not accounted for in the *ex vivo* transcleral flux model.

Taken together these studies show anthracyclines are active against extraocular retinoblastoma and may be similarly effective for intraocular disease if sufficient concentrations can be achieved through novel administration approaches. These results, though limited, support further development of local delivery approaches for doxorubicin, idarubicin or other anthracyclines for treating retinoblastoma.

3.3 Melphalan

Melphalan (Figure 2g) was initially identified as a candidate for retinoblastoma based upon its activity in a clonogenic assay against both fresh primary cells and 2 cultured lines (Y79 and WERI). In this study melphalan reduced colony formation by more than 70% in the largest proportion of cells tested.^[108] To our knowledge, there is no additional published *in vitro* data available concerning the effects of melphalan in retinoblastoma cells. The rationale for the choice of intra-arterial infusion over other local delivery routes is unclear and, due to the logistical difficulty of testing intra-arterial infusion in animal models, there is a relative scarcity of preclinical data on intra-arterial infusion of melphalan. Long-term efficacy or toxicities of intra-arterial melphalan in animal models of RB have not been reported.

Despite evidence of vascular toxicity^[73] and an overall lack of *in vitro* or preclinical data, melphalan is the most commonly used locally delivered chemotherapy agent for retinoblastoma. In Japan and the US, more than 1400 patients have been treated with intra-arterial melphalan.^[24] There is both a high rate of tumor response to IA melphalan (75–90%) and a decrease in enucleation rates (Fig. 3a–b).^[24] For example, in one study 7 of 9 eyes destined for enucleation were salvaged by IA melphalan (Fig. 3a–b).^[62] Other studies have concluded that IA infusion of melphalan is safe and effective and recommended it be combined with other chemotherapy agents.^[68] Some have reported that even one or two cycles of IA melphalan achieved tumor control in advanced retinoblastoma.^[109] However, significant local side effects are also often observed,^[64, 66, 110] including loss of vision.^[64] Toxicology studies in primate models have demonstrated that IA melphalan induces prevalent, acute local vascular toxicities including ophthalmic artery thrombosis, sectoral choroidal nonperfusion, and retinal and vitreous hemorrhages^[69–71, 73, 111] (Figure 3c–d). Steinle et al. attribute this local toxicity to both the mode of administration (as described above, IA infusion may cause vascular toxicity due to decreased pH, pulsatile flow, and drug precipitation in the vasculature) and the choice of chemotherapy agent.^[73] The authors modeled the effects of melphalan *in vitro* with REC, a primary human retinal microvascular endothelial cell line) and found that, at the high concentration encountered during IA infusion, melphalan increased cell death of REC compared to controls, triggered cellular migration, proliferation, and apoptosis in the surviving cells, and increased expression of adhesion proteins (intracellular adhesion molecule-1 [ICAM-1] and soluble chemotactic factors (IL-8)) and monocytic adhesion to human retinal endothelial cells.^[73]

Intravitreal injection has also been evaluated for melphalan delivery. Although IA melphalan caused no systemic toxicity in humans or rabbits, it did cause significant retinal toxicity at high doses,^[112] with subsequent work showing that lower, safer doses only achieved incomplete control (3 of 7 cases at long-term follow-up).^[110] Despite the recent popularity

of intra-arterial melphalan for retinoblastoma treatment, studies now suggest that further characterization of toxicity is needed in order to adequately weigh the risks and benefits.

4. Pre-clinical Progress in Retinoblastoma Therapeutics

4.1 Preclinical Models of Retinoblastoma

Y79 (the first retinoblastoma cell line established) is by far the most frequently utilized model for *in vitro* testing of drug candidates, although other retinoblastoma cell lines are also employed, including Weri and RB355.^[113–116] Because drug sensitivity can vary amongst the cell lines, testing drugs in multiple, rather than any single cell line, may provide a more complete picture of a compound's potential success as a therapeutic agent. Inclusion of a non-transformed or non-retinoblastoma cell line is also helpful for interpreting *in vitro* results, as it provides an estimate of compound selectivity and can identify potential toxicity concerns. Examples of non-retinoblastoma cell lines that have been used in past studies include BJ (normal human fibroblasts)^[54, 117] and Jurkat.^[31, 117] The human retinal pigment epithelial cell line ARPE-19 is commonly used as an *in vitro* model of ocular toxicity,^[118–119] and may also be helpful in predicting local toxicity.

Though *in vitro* screening is a useful tool to identify candidate drugs and therapeutic targets of interest, further testing is critical before progressing to clinical trials for retinoblastoma. As previously mentioned, retinoblastoma is a relatively rare disease (approx. 300 cases annually in the US). Due to the small number of patients only a very limited number of clinical trials can be run and these may take years to complete.^[19] The unique challenges of reaching the intraocular target also necessitate preclinical testing in animal models prior to progressing to patients as insufficient intraocular exposure may result in lack of *in vivo* efficacy for a compound that shows evidence of efficacy *in vitro*.^[34, 54] Historically, the lack of animal models that faithfully recapitulate the disease has precluded preclinical testing of retinoblastoma therapeutic candidates. However, there have been major advances in this area in the past decade, which have recently been reviewed.^[19, 24, 35–36] There are currently two major types of rodent models of retinoblastoma used for preclinical drug testing: GEMM (genetically engineered mouse models) and xenograft models.

GEMM employed in testing novel retinoblastoma therapeutics have included the LH_{BETA}T_{ag} model^[120–122] and knockout models including the MDMX (*Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};MDMX^{Tg}*) and p53TKO (*Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};p53^{Lox/Lox}*) mouse models.^[31, 34] In the LH_{BETA}T_{ag} model, retinal tumor development is induced by overexpression of the simian virus T antigen (SV40-Tag) driven by the promoter of the luteinizing hormone β -subunit gene (LH β), causing animals to develop bilateral, heritable retinoblastoma.^[122]

While many studies have employed a xenograft model based on subcutaneous injection of cultured retinoblastoma cells to evaluate novel retinoblastoma therapeutics,^[123–126] results from these studies are difficult to interpret as one of the primary challenges of treating retinal disease is reaching the intraocular target site. Thus, while these studies provide some proof-of-concept for the efficacy of a given compound, they do not address whether or not the compound is likely to work in the eye after systemic administration. To address this

need, two rodent orthotopic xenograft models have been developed: a mouse model based on injection of SJ39 (human tumor cells) into the eyes of immunocompromised mice and a rat model based on injection of luciferase labeled Y79 cells (Y79-Luc cells) into the vitreous of newborn rats. These xenograft models offer critical advantages over subcutaneous flank xenografts: they more faithfully recapitulate the human disease because the transplanted cells grow in a developmental environment similar to that of the human retinal tumors^[127] and the challenges of intraocular delivery are accounted for during preclinical testing.^[54] In the Y79-Luc xenograft model, tumor progression or response can be tracked using Xenogen imaging (tumor burden is directly proportional to the detected signal after I.P. injection of luciferin,^[52,128] which allows for relatively facile evaluation of drug efficacy *in vivo*. The SJ39 xenograft model employs tissue taken directly from a patient's eye after enucleation and recapitulates aggressive tumor progression and incomplete response to standard of care chemotherapy regimens.^[34]

4.2 Pharmacokinetics in Preclinical Retinoblastoma Studies

Generally speaking, pharmacokinetics (PK) studies have been rarely included in published retinoblastoma preclinical studies (see Table 2). However, exposure is important to consider when evaluating therapeutic candidates for retinoblastoma due to the challenges associated with reaching the intraocular target site. We have recently shown that the spleen tyrosine kinase (SYK) inhibitor R406, despite killing retinoblastoma cells *in vitro*, fails to produce efficacy *in vivo*, as a result of insufficient intraocular exposure.^[54] Additionally, despite the potential advantages of local delivery routes, not all drugs are amenable to all delivery routes. Topotecan efficiently crosses the BRB, and equivalent intraocular pharmacokinetic (PK) profiles result from either systemic or local topotecan delivery.^[52,82] In contrast, carboplatin^[79,87] and nutlin-3a^[34] cannot effectively be delivered systemically due to insufficient penetration of the BRB. In these cases, subconjunctival injection has been a significantly more effective delivery route. Exposure data from *in vivo* PK studies can be helpful in identifying optimal route for a given compound, and previous studies have demonstrated the importance of using these results to inform drug-specific route selection for intraocular targets. One of the primary challenges in PK data-guided development for ocular diseases is the disconnect in animal models used: rabbits are considered the gold standard for ocular exposure studies, but the majority of efficacy studies for retinoblastoma are conducted using mouse models (see Table 2). There is no data comparing exposure in rabbits with exposure in mice, and no consensus as to which best models drug behavior in humans. The goal of achieving an exposure profile in tumor-bearing mice that matches an exposure profile that was efficacious *in vitro* may be scuttled by the assumption that no species-specific differences exist between mice, rabbits and humans, an assumption which is very likely to be false for intraocular targets. This lack of an evidence-based best practices preclinical model currently represents a major limitation in ocular drug development. Because local delivery routes tend to achieve higher local concentrations, ocular toxicity measures including observation (typically accompanied by scoring using the Draize scoring system), tonometry, electroretinograms (ERG), and histopathology may also be helpful in preclinical evaluation. Approaches to evaluate ocular toxicity in preclinical studies have recently been reviewed.^[32]

Thus, in addition to evaluating efficacy, thorough characterization of pharmacokinetics and toxicity is strongly recommended in order to focus clinical efforts where they will have the most impact. Below, we discuss several potential developmental therapeutics for retinoblastoma, in the light of these issues. The *in vivo* studies on candidate retinoblastoma therapeutics covered in this review are all summarized in Table 2, including animal model used, whether or not intraocular exposure data was included in the study and dosing information (including route of administration, vehicle, dose and schedule).

4.3 Cardenolide Glycosides

A recent high throughput screen in Y79 and RB355 cell lines, using a library of 2640 compounds including marketed drugs and natural products, identified several FDA approved drugs that inhibited growth of retinoblastoma cell lines in culture.^[126] From this study, the cardiac glycosides emerged as a drug class of particular interest, with *in vitro* EC₅₀ values (the concentration at which half the maximum effect is observed) in the range of 0.5–5 μ M in retinoblastoma cells. Further testing of two FDA-approved cardenolide glycosides traditionally used in the treatment of congestive heart failure -- ouabain and digoxin (Figure 4a–b) -- in a Y79 flank xenograft mouse model revealed that continuous subcutaneous infusion of ouabain over 19 days from an implanted osmotic minipump (0.5 μ L per hour of 31.5 mg/mL of ouabain) induced complete tumor regression with no accompanying weight loss.^[65] Based on these preclinical findings, intra-arterial and systemic oral digoxin were evaluated in a patient with unilateral retinoblastoma who had previously failed intra-arterial chemotherapy. In this patient, oral digoxin failed to impact the tumor, but the intra-arterial infusion produced what the authors describe as a “modest but measurable response” that they suggest was limited by their inability to sustain therapeutic exposure in the eye.^[65] If novel local delivery approaches can achieve sufficient intraocular exposure, there may be a role for cardenolide glycosides in the treatment of retinoblastoma. However, it is worth noting that there has been no mechanistic explanation reported for efficacy of cardenolide glycosides in retinoblastoma, and these compounds are common hits in cellular screens, particularly as cancer therapies.^[129–132] Digoxin and ouabain may simply be promiscuous compounds,^[133] and considerably less attractive than therapeutic candidates which kill selectively based on mechanisms specific to retinoblastoma.

4.3 MDMX/MDM2-p53 Interaction Inhibition

The p53 pathway is inactivated in nearly all cancer types. Although 50% of cancers have direct mutations to TP53 (the gene that encodes p53), a subset of cancers retain wild-type p53.^[134–136] In these cases wild-type p53 is inactivated through overexpression of its regulatory proteins or misregulation of downstream genes. MDM2 binds p53 directly, blocking its transcriptional activity, promoting its nuclear export, and targeting it for proteolytic degradation. The role of MDMX is less well understood, but MDMX is amplified in 65–70% of human retinoblastomas, and has been shown to contribute to tumor formation by suppressing p53.^[134] Deregulation of MDM2 and MDMX allows tumors that express wild-type p53 to escape apoptosis. Consequently, specific inhibition of the MDM2/MDMX-p53 interaction represents a promising therapeutic approach for retinoblastoma that may be effective in treating p53 wild-type tumors^[34,134] (Fig. 5a).

A structure-based approach led to identification of nutlin-3a (2-piperazinone, 4-[(4*S*, 5*R*)-4,5-bis(4-chlorophenyl)-4,5-dihydro- 2-[4 methoxy-2-(1-methylethoxy)phenyl]-1*H*-imidazol-1-yl]carbonyl]-) (Fig. 4c), a small molecule that inhibits the MDM2-p53 interaction by binding the p53 binding pocket of MDM2.^[137] Nutlin-3a also binds MDMX and prevents the association of both MDM2 and MDMX with p53^[24,136] (Fig. 5a). Nutlin-3a induces cell death in the Y79 and WERI retinoblastoma cell lines at doses of 5- to 10- μ M.^[135] In vitro, exposure to 5 μ M of nutlin-3a increased cytoplasmic and nuclear p53 in retinoblastoma cell lines.^[135]

PK studies showed that systemic administration of nutlin-3a was not an option due to poor penetration of the BRB^[34] (Fig. 5b). A whole body physiologically based pharmacokinetic (PBPK) model based on nutlin-3a tissue concentrations after intravenous and oral dosing in mice confirmed that nutlin-3a has poor BRB penetration following systemic delivery and that achieving therapeutically relevant intraocular exposure is unlikely.^[136] To overcome this limitation, a subconjunctival formulation was developed for nutlin-3a ultimately allowing a ratio of vitreous area under the curve (AUC) to plasma AUC that was 2000-20,000 fold higher than was achievable via systemic delivery^[34] (Fig. 5b). Subconjunctival nutlin-3a combined with systemic topotecan was significantly more effective than the standard of care VCE combination in an orthotopic xenograft murine model of retinoblastoma^[34] (Fig. 5c–d).

4.4 Epigenetic Mechanisms: Spleen Tyrosine Kinase (SYK) and Histone Deacetylase (HDAC) Inhibition

4.4.1 SYK Inhibition—Recent characterization of the genetic and epigenetic landscape of retinoblastoma revealed that, while the retinoblastoma genome is more stable than previously believed, RB1 loss leads to epigenetic deregulation of key cancer pathways. Retinoblastoma may therefore arise as a result of non-genetic mechanisms of cancer pathway deregulation.^[31]

One promising target arising from this study was induction of expression of the proto-oncogene spleen tyrosine kinase (SYK). Though not expressed in normal human retinas, SYK was found to be upregulated in 100% (82/82) of the retinoblastoma samples evaluated (Fig. 6a) and was shown to be required for retinoblastoma cell survival.^[31] Inhibition of SYK with the small-molecule inhibitors BAY-61–3606 (Fig. 4d) and R406 (Fig. 4e) caused caspase-mediated cell death in the retinoblastoma cell lines Weri and Y79 but not the control cell line Jurkat). *In vivo*, BAY-61–3606 delivered via subconjunctival injection in combination with systemic topotecan was efficacious in blocking human orthotopic retinoblastoma xenograft proliferation^[31] (Fig. 6b).

Because BAY-61–3606 is not in clinical development, R406, and its orally available prodrug fostamatinib (R788) (Fig. 4f), which have advanced into late-phase clinical trials for oral therapy of autoimmune disorders, were evaluated.^[54] Though R406 effectively induced caspase-mediated apoptosis in retinoblastoma cells in culture, intraocular exposure was insufficient to reduce tumor burden in a human orthotopic xenograft mouse model of retinoblastoma (Fig. 6c–d). Maximal vitreal concentration was 10-fold lower than the minimal concentration required to kill retinoblastoma cells *in vitro*. Pharmacokinetic

characterization of various alternate routes (including subconjunctival, topical and intravitreal administration of various formulations) revealed that none reached the PK performance required for retinoblastoma cell death in culture. It was concluded that R406 was not a viable clinical candidate for the treatment of retinoblastoma.^[54]

Although both SYK inhibitors tested so far have limitations that make them unattractive clinical candidates, these studies have provided evidence that targeting this pathway can be efficacious in retinoblastoma. SYK remains a promising target, as there are many other small molecule SYK inhibitors with diverse physiochemical properties being developed, particularly for autoimmune disorders and blood cancers.^[138–141] In addition, previous studies of SYK inhibition have implicated a number of downstream signaling molecules as mediators of the SYK survival signal,^[141–142] including the Bcl-2 family of proteins (Fig. 6e). Consistent with previous findings in B-CLL cells,^[142–143] inhibition of SYK in retinoblastoma reduced expression of MCL-1, the only member of the Bcl-2 family of pro-survival proteins that is upregulated in retinoblastoma.^[31] This suggests that the small molecule Bcl-2 inhibitors (particularly MCL-1 inhibitors) currently in development for other cancers^[144–145] may provide another option for targeting this pathway in retinoblastoma.

4.4.2 HDAC Inhibition—HDAC inhibitors (HDACi) are a promising new class of anticancer therapies with three being FDA approved: vorinostat (Zolinza; Merck), for the treatment of cutaneous T cell lymphoma (CTCL); romidepsin (Istodax; Celgene), for the treatment of CTCL and peripheral T cell lymphoma (PTCL); and belinostat (Beleodaq; Spectrum Pharmaceuticals), for the treatment of PTCL. Several other HDAC inhibitors are in development for various diseases, including cancer, neurological diseases, and immune disorders.^[146]

Several characteristics of HDAC inhibitors make them promising candidates for retinoblastoma. First, although the retinoblastoma genome is relatively stable, the epigenetic profile exhibits profound deregulation relative to that of normal retinoblasts.^[31] This suggests that HDAC inhibitors, which modify gene expression via epigenetic regulation, may be efficacious as targeted retinoblastoma therapeutics. Second, HDAC inhibitors exhibit tumor cell-selective cytotoxic effects and tumor cells with deregulated E2F1 activity are particularly sensitive to HDAC inhibition.^[147] Loss of the RB protein leads to increased E2F activity, suggesting HDAC inhibition is rational for retinoblastoma treatment. Finally, several studies have reported that HDACi potentiate the cancer cell-killing effects of other therapeutics, including agents currently used to treat retinoblastoma.^[148]

In vitro, there is dose dependent reduction of retinoblastoma cell survival following treatment with trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), or MS-275.^[148] In follow up studies, MS-275 (entinostat) (Fig. 4g) was efficacious in two preclinical animal models of retinoblastoma (transgenic mouse and rat ocular xenografts) when administered every other day via IP injection.^[148] Intraocular PK data was not reported in this study, but the authors did show a pharmacodynamic response with increased acetyl-histone levels in the retina 4 hours post-administration. Direct intraocular administration of 1 μ L of 10 μ M MS-275 did not alter ocular tissue morphology in mice.^[148] Although more

preclinical evaluation is needed before HDAC inhibitors can advance to the clinic, inhibition of HDAC represents a promising new target in retinoblastoma.

4.5 Tubulin Altering Molecules

As previously described, vincristine is a frontline chemoreduction agent for retinoblastoma. The mechanism of action of vincristine is inhibition of microtubule assembly,^[149] which suggests retinoblastoma tumor cells might exhibit similar sensitivity to other tubulin-altering compounds. Paclitaxel (Fig. 4h) is a taxane that induces marked apoptosis in tumor cells through effects on the tubulin dynamics.^[150–151] Paclitaxel has been approved for the treatment of ovarian cancer, breast cancer, and non-small cell lung cancer. There is some limited *in vitro* and *in vivo* data to suggest paclitaxel's potential as a retinoblastoma therapeutic. Several studies have reported that Y79 cells are sensitive to low doses of paclitaxel.^[153–156]

When examined in the LH beta-Tag transgenic mouse model of retinoblastoma (Fig. 7a–c), subconjunctival paclitaxel (two injections given 72h apart of doses ranging from 15.2 µg to 0.5 mg) significantly reduced tumor burden at all doses tested compared with control eyes (Fig. 7a–c) and produced mild, transient ocular toxicity. A subconjunctival dose of 0.25 mg paclitaxel completely cleared tumor burden (Fig. 7c) but induced ocular toxicities that ameliorated with time. At higher concentrations, more severe ocular toxicities were observed.^[157]

The primary impediments to pursuing paclitaxel as a retinoblastoma therapeutic appear to be toxicity^[157–158] and formulation (to overcome its poor water solubility, paclitaxel is commonly formulated in Cremaphor which can induce side effects).^[159] However, the formulation concerns can potentially be overcome. Toxicity concerns may likewise be addressed through careful therapeutic index determination: given the high potency of relatively low doses of paclitaxel in retinoblastoma cell lines (IC₅₀ in Y79 for 48 hrs exposure = 3.5 nM),^[156] the high concentrations of paclitaxel shown to induce toxicity *in vivo* (0.25 mg per eye) may be considerably larger than the dose needed to reduce tumor burden. Combining paclitaxel with other agents might enable further dose-reduction, as it has in other cancers. Combining paclitaxel with Beta-Lapachone (a new investigational anticancer agent)^[155] (D'Anneo *et al.*, 2010) and RNAi-mediated silencing of stathmin^[156] both reduced Y79 sensitivity to sub-nanomolar levels of paclitaxel. Additional data (including intraocular exposure of paclitaxel and related compounds for local delivery routes) is needed to evaluate the suitability of taxels for retinoblastoma.

4.6 Vitamin D analogs

Observations that calcium deposits were often associated with regions of dying cells in retinoblastoma led to the proposal of using vitamin D as a therapeutic. The role of vitamin D in cancer therapy has been extensively reviewed.^[160] This theory lay quiescent due to the potential toxicity of vitamin D and the lack of a preclinical model of retinoblastoma.^[161] However in the past decade, several vitamin D analogs, including calcitriol (Fig. 4i) have been investigated using both *in vitro* (Fig. 8a–b) and *in vivo* models of retinoblastoma (Fig. 8d). Vitamin D receptor mRNAs were detectable in Y-79 cells, LH beta-Tag tumors, and

human retinoblastoma specimens using RT-PCR.^[162] Though the mechanism of action of vitamin D analogs in retinoblastoma is unknown, death of Y79 cells following treatment with vitamin D analogs is associated with the upregulation of both p53 and p21,^[161] and accompanied by a concentration-dependent increase in Bax protein and a reduction in Bcl-2 content (Fig. 8a).^[163] Vitamin D analogs might also be exerting anti-retinoblastoma tumor effects via targeting of the hedgehog signaling pathway, as has been described for other cancers.^[164–165]

The FDA approved vitamin D analog calcitriol (1,25-dihydroxyvitamin D₃) (Fig. 4i) has been extensively studied for retinoblastoma. Calcitriol inhibited Y79 growth *in vitro*^[162] and reduced tumor burden in both xenografts (prepared by subcutaneous injection of Y79 cells) (Fig. 8c) and transgenic mouse models of retinoblastoma.^[122–125, 161–162] Despite its efficacy in these models, the use of calcitriol as a retinoblastoma therapeutic is strongly limited by its systemic toxicity, including elevated calcium levels in the plasma (hypercalcemia) and nephrotoxicity.

To address mortality of treated animals due to hypercalcemia, other vitamin D analogs have been examined, including 1,25-dihydroxy-16-ene-23-yne vitamin D₃ (16,23-D₃),^[124–124,161] 1 α -hydroxyvitamin D₂ (doxercalciferol),^[124, 162] and 2-methylene-19-nor-(20S)-1 α -hydroxybismopregnacalciferol (becocalcidiol).^[166] Although these compounds all exhibited less toxicity than calcitriol, they all either still induced hypercalcemia or failed to reduce tumor burden in transgenic mice. Most published *in vivo* studies have examined systemic delivery (typically IP injection in a mineral oil injection). Local delivery approaches might provide a solution to toxicity concerns.

4.7 Angiogenesis Inhibition

Angiogenesis (the formation and proliferation of new blood vessels from preexisting capillaries) is a tightly regulated process that normally only occurs during developmental and repair processes. During tumor growth and metastasis, the balance of pro- and anti-angiogenic factors becomes perturbed, often through overexpression of proangiogenic factors like vascular endothelial growth factor (VEGF). The discovery that this process (termed the “angiogenic switch”) is critical to tumor growth has led to the development of many anti-angiogenic agents to prevent solid tumor progression.^[167] Retinoblastoma is a well vascularized tumor that is dependent on its vascular supply^[168–169] and VEGF is highly expressed in retinoblastoma cells (Fig. 9a) and retinoblastoma patients,^[168] suggesting a potential role for anti-angiogenic compounds in the treatment of retinoblastoma.

In addition to development of anti-angiogenic drugs for cancer, there is growing interest in their use for the numerous ophthalmic diseases that feature pathological neovascularization, including retinopathy of prematurity (ROP), diabetic retinopathy (DR) and age-related macular degeneration (AMD).^[43] Given their shared intraocular target site, some of these drug candidates might ultimately also prove useful in treating intraocular tumors.

Anti-VEGF protein macromolecules are currently under investigation in various solid tumors and ophthalmologic diseases.^[43,168] Y79 xenografted mice responded well to anti-VEGF monoclonal antibody bevacizumab (Avastin; Genentech/Roche, Inc.) when given

twice weekly for 4 weeks. Bevacizumab caused a 2-fold reduction in tumor microvessel density (determined by immunohistochemistry) which caused a 75% reduction in the growth of the retinoblastomas (Fig. 8c) without producing significant systemic toxicity.^[168] Though systemically applied bevacizumab is unlikely to reach the intraocular target, this study provides an important proof of concept for the use of angiogenesis inhibitors in retinoblastoma.

Although intravitreal injection of VEGF antibodies has proven successful for AMD,^[43] the invasiveness of the procedure has led to interest in developing small molecule angiogenesis inhibitors. Subconjunctival combretastatin A-4 phosphate (CA-4P) (a water-soluble vasculature-targeting pro-drug) (Fig. 8a) induced an extensive, dose-dependent reduction of vessel density and significant tumor reduction in treated eyes compared with control eyes in a transgenic mouse model of retinoblastoma (simian virus-40 T-antigen– positive mice) (Fig. 8d–f).^[170] Furthermore, there was no evidence of corneal, lenticular, choroidal, or retinal toxicity.^[170] Similarly, subconjunctival anecortave acetate significantly reduced tumor burden in the LH_{BETA}T_{AG} mouse model of retinoblastoma, particularly when combined with carboplatin.^[171] Combining both agents enabled use of a sub-therapeutic carboplatin dose without loss of therapeutic efficacy. A recent screen for compounds that impacted vascular development in the zebrafish retina identified albendazole, a tubulin targeted drug, as slowing the progression of ocular tumors.^[172] Calcitriol (discussed in greater detail in the section on vitamin D analogs) has also been shown to inhibit retinal neovascularization.^[173]

Though angiogenesis-inhibitors appear to be safe for adult retinae,^[174–175] concerns have been raised regarding their use in pediatric patients due to the potential impact of angiogenesis-inhibition in developing eyes.^[176] There is some limited data to suggest that anti-VEGF agents may cautiously be used in developing retinae: bevacizumab did not induce any changes in cell death or proliferation in early developing rat retinal explants maintained in organotypic culture,^[176] and no electroretinographic or morphological abnormalities were detected in 11- or 35- day old rabbits who received intravitreal injections of bevacizumab.^[177] Given the importance of vasculature in retinal function, more thorough investigation of the developmental toxicology of angiogenesis inhibitors is certainly warranted before they are pursued as retinoblastoma therapeutics.

4.8. Neurotransmitter Pathway Disruption

Neurotransmitter receptors, transporters, and biosynthetic enzymes are expressed in human retinoblastoma, and targeted disruption of these pathways reduces retinoblastoma growth *in vivo* and *in vitro*.^[178] Based on their observation that retinoblastomas express genes characteristic of monoaminergic amacrine cells, 13 well characterized pharmacological agents that target the major neurotransmitter pathways were tested *in vitro* revealing that broadly-acting monoamine transporter inhibitors blocked proliferation of retinoblastoma cell lines (Weri, Y79 and RB355), with fluphenazine (Fig. 4k) and chlorpromazine (Fig. 4l) being the most potent. Though there is a rationale for their activity in retinoblastoma (a cancer that arises in a neurological tissue), these agents are, like the cardenolide glycosides, frequent hits in cancer repurposing and may prove to be efficacious due to their promiscuity rather than a retinoblastoma-specific mechanism. Follow up studies of these two agents in a

murine orthotopic xenograft model demonstrated that weekly subconjunctival injection of either fluphenazine or chropromazine for 3 consecutive weeks significantly reduced tumor volume, as measured by MRI and histological analysis.^[178] Though there is no PK data for subconjunctival delivery of these agents, local delivery likely reduces systemic exposure to levels below clinically administered systemic doses, suggesting these agents achieved efficacy at clinically relevant doses over a relatively clinically feasible administration schedule (weekly injections for 3 weeks).

4.9. Arsenic Trioxide

Arsenicals have a long history of use in the treatment of leukemia. Arsenic trioxide (As₂O₃; ATO) (Fig. 4m) has received FDA approval for relapsed/refractory acute promyelocytic leukemia (APL).^[179] ATO may be attractive for retinoblastoma as it is believed to act via mechanisms distinct from traditional chemotherapy agents (including inducing generation of reactive oxygen species (ROS) leading to oxidative damage-induced apoptosis) and does not appear to be subject to development of drug resistance.^[180] At high concentrations (5 μM), ATO caused significant growth inhibition of both Y79 and SNUOT-Rb1 retinoblastoma cell lines.^[181] Weekly intravitreal injections of either 0.1 μM or 5 μM ATO in orthotopic xenografts of SNUOT-Rb1 cells in mice reduced tumorigenesis, with the higher dose showing a more pronounced reduction. Non-tumor bearing eyes exhibited no change in retinal thickness and no inflammatory cells in the vitreous, retina, or choroid in response to ATO treatment.^[181]

4.10. EDL-155

An isoquinoline derivative, EDL-155 (Fig. 4n), was unexpectedly found to have high intraocular concentrations after systemic administration.^[182] Subsequently, it was found to be weakly active against Y79 cells in killing retinoblastoma cells *in vitro* and *in vivo*. Despite EDL-155's relatively weak potency in Y79 cells in culture (EC₅₀ 9.1 μM), it was effective *in vivo*. Daily periocular injections of EDL-155 (20 mg/kg/day in 0.1% DMSO in saline) for 4 consecutive days significantly reduced tumor burden (measured by xenogen imaging and confirmed by histopathological analysis) in a Y79-Luc xenograft mouse model of retinoblastoma without inducing any observable toxic side effects.^[182] The authors found a lack of viable mitochondria and evidence of incorporation into autophagosome vesicles in retinoblastoma cells treated with EDL-155, suggesting the compound kills retinoblastoma cells by disrupting mitochondria and inducing autophagy.^[182]

5. In vitro testing

Though the majority of publications that report sensitivity of retinoblastoma cells to novel small molecule therapeutics also include at least some *in vivo* efficacy results, there are a small number of studies on compounds which have, to date, only been evaluated *in vitro*, primarily natural products and hits from high throughput screening efforts.

The antioxidant resveratrol has been shown to induce time and dose-dependent apoptosis in Y79 via the mitochondrial/intrinsic apoptotic pathway.^[183] Another HTS study attempting to identify compounds that potentiate the cytotoxicity of resveratrol in retinoblastoma cell

lines, highlighted additional compounds with *in vitro* activity towards Y79 and RB355, including some that exhibited additive effects when combined with resveratrol.^[184] Another study has suggested that curcumin blocks proteins involved with multidrug resistance in the Y79 cell line.^[185] Co-administration of curcumin with other chemotherapy agents may, like cyclosporine, act as a chemosensitizer to improve retinoblastoma cell sensitivity to chemotherapy.^[185] However, the use of antioxidants must be very cautiously considered in combination therapies for retinoblastoma, as generation of reactive oxygen species (ROS) to increase cellular oxidative-stress has been implicated as an important mechanism of action for many of the novel agents recently investigated.

The same screen that identified the cardiac glycosides described above also identified other reasonably potent compounds in retinoblastoma cell lines, including propachlor, phenylmercuric acetate, pyrithione zinc, primaquine and dihydrogambogic acid.^[126] Okadaic acid and parthenolide induced apoptosis via targeting of the PTEN/Akt/MDM2/p53 pathway in human retinoblastoma Y79 cells. The combination of sub-therapeutic doses of these two compounds was cytotoxic, suggesting some degree of potentiation occurs when they are co-administered.^[153]

In addition to the fact that mechanistic selectivity for retinoblastoma hasn't been established in these cases, it is also worth noting that *in vitro* screening can easily yield false positives, and the compounds described here (high throughput screening hits and antioxidants) may fail to translate to clinically efficacious therapies for that reason.

Though no candidate compounds have been tested, Qu et al. demonstrated that NF- κ B is related to retinoblastoma tumorigenesis and progression through a non-conventional pathway, suggesting NF- κ B may represent another potential therapeutic target for retinoblastoma therapy.^[186]

6. Conclusions

Despite the high cure rate associated with multimodal therapies for retinoblastoma, there is still a pressing need to develop new therapies that preserve vision and avoid the late effects of currently available interventions. We have reviewed the major progress made in animal models for preclinical testing, advances in ocular local drug delivery, and recent progress in the identification of novel therapeutic targets for retinoblastoma. Recent success with local delivery of standard of care broad spectrum chemotherapy agents (including carboplatin, topotecan, melphalan) suggests combining novel targeted therapeutics with local delivery (particularly sustained release carriers to enhance control of local PK) will play an increasingly significant role in development of safe, effective therapies that treat retinoblastoma while preserving vision and minimizing side-effects. However, there are still critical limitations to overcome in translating novel targets and delivery routes to clinically relevant retinoblastoma therapies.

Table 2 summarizes published preclinical efficacy studies for retinoblastoma candidate therapeutics, and highlights one of the major limitations of currently available preclinical data. As Table 2 shows, the majority of efficacy studies are conducted in mouse models of

retinoblastoma (either transgenic or xenograft) and, despite the unique challenges of reaching the retina and vitreous, these studies generally fail to include exposure data. For some of the compounds evaluated, separate PK studies have been conducted,^[75, 79, 97–98, 107, 187–188] but most of the published PK results have employed rabbits, as they are considered the “gold-standard” species for ocular PK testing. Though species-specific differences in exposure may exist, there has been no attempt to conduct comparison studies or correlate exposure in mice with exposure in rabbits. Many studies also employ dosing schedules that are not clinically relevant, as the frequency of intravitreal injection and subconjunctival injection may be more limited in patients (approx. once every 3 weeks). Much more rigorous PK testing utilizing clinically relevant dosing and schedules is critical in preclinical testing to best inform progression of therapeutic candidates.

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List of Abbreviations

HDAC	histone deacetylase
SYK	spleen tyrosine kinase
ROP	retinopathy of prematurity
DR	diabetic retinopathy
AMD	age-related macular degeneration
VEGF	vascular endothelial growth factor
HTS	high throughput screening
IVC	intravenous chemotherapy
VCE	vincristine, carboplatin, etoposide
IAC	intra-arterial chemotherapy
ERG	electroretinogram
RPE	retinal pigment epithelium
CBP	carboplatin
EBRT	external beam radiation therapy
ETO	etoposide

G-CSF	granulocyte colony-stimulating factor
PD	progressive disease
VCR	vincristine

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Figure 1. Ocular Drug Delivery Routes

Systemic delivery relies on penetration of the blood retinal barrier (BRB) to bring drug to the posterior segment of the eye. Local delivery routes include intra-arterial (perfusion of the ophthalmic artery), intravitreal (direct bolus injection into the vitreous), topical (application of drug to the exterior of the eye) and periocular (injection into the periocular space just outside the globe), including subconjunctival (injection below the conjunctiva).

Figure 2. Chemical structures of standard of clinical retinoblastoma therapeutics

(a) carboplatin, (b) vincristine, (c) etoposide, (d) topotecan, (e) doxorubicin, (f) idarubicin and (g) melphalan

Figure 3. Use of intra-arterial infusion of melphalan to treat retinoblastoma is associated with both high rate of tumor response and prevalent, acute ocular toxicities

(a) Retinoblastoma tumor before and (b) 3 weeks after one 3 mg dose of intra-arterial melphalan (reproduced with permission from Abramson *et al.*, 2008).^[62] (c–d) Real-time retinal observations during intra-arterial melphalan infusion in a nonhuman primate model reveals retinal artery precipitates.^[69]

Figure 4. Chemical structures of candidate retinoblastoma therapeutics investigated in preclinical studies

(a) ouabain (b) digoxin (c) nutlin-3a (d) BAY-61-3606 (e) R406 (f) R788 (g) entinostat (h) paclitaxel (i) calcitriol (j) combretastatin A4 (CA-4P) (k) fluphenazine (l) chlorpromazine (m) arsenic trioxide

Figure 5. Inhibition of the MDMX/MDM2-p53 interaction by nutlin-3a kills retinoblastoma cells in vitro and in vivo

(a) p53-MDMX/MDM2 pathway as a target in retinoblastoma (modified from Brennan et al.^[24] and Nalepa *et al*^[189]) (b) Pharmacokinetics of nutlin-3a following oral administration (filled circles, solid lines) or subconjunctival administration (empty squares, dotted lines). Concentration of nutlin-3a measured at 0.5, 1, 2, 4 and 8 hours in plasma (red lines) and vitreous (black lines). Concentration vs. time plot was used to fit a 2-compartment model to determine the area under the curve (AUC) and calculate the ratio of the AUC in vitreous/plasma for each route of delivery.^[24] (c) Kaplan Meier survival curves for orthotopic xenograft mice receiving no treatment (black line) compared to mice receiving standard of care vincristine/etoposide/carboplatin (VCR/ETO/CBP) (blue line) and compared to those receiving subconjunctival nutlin-3a/systemic topotecan (nutlin-3a/TPT) (red line).^[24] (d) Representative xenogen images for orthotopic xenografts rats treated with subconjunctival nutlin-3 and topotecan for 5 days (reproduced with permission from Laurie *et al*^[134])

Figure 6. SYK inhibition as a therapeutic target for retinoblastoma

(a) Immunohistochemistry of retinoblastoma tissue and normal retina tissue H&E (left, purple) and anti-SYK (right, brown) showing increased expression of SYK in retinoblastoma tissue but not normal retina (reproduced with permission from Zhang *et al*^[31]) (b) Survival curves of orthotopic xenografts (SJRB001X) mice receiving subconjunctival BAY-61-3606 in combination with systemic topotecan (red line, n=20) or untreated (black line). Bay-61-3606 in combination with topotecan improved treatment outcome (reproduced with permission from Zhang *et al*^[31]). (c) Pharmacokinetic behavior of R406 administered following subconjunctival delivery of R406. Concentration of R406 measured at 0.5, 1.0, 2.0, and 4.0 hours in the plasma (dashed line, empty circles) and vitreous (solid line, filled circles) (reproduced with permission from Pritchard *et al*^[54]). (d) Kaplan Meier plot of event free survival (EFS) of orthotopic xenografts (SJ-39) mice receiving subconjunctival R406 in combination with systemic topotecan (dashed black line,

n=20) or untreated (solid gray line, n=10). Due to insufficient intraocular exposure, subconjunctival R406 does not improve provide efficacy in this model (reproduced with permission from Pritchard *et al*^[54]). (e) Downstream targets in the SYK pathway.

Figure 7. Paclitaxel kills Y79 retinoblastoma cells in culture and reduces tumor burden in a transgenic mouse model of retinoblastoma

(a) Dose dependent tumor burden reduction in transgenic mice treated with paclitaxel (doses of paclitaxel represent milligrams in 20 μ L of 100% DMSO administered via subconjunctival injection). Tumor burden determined by taking the ratio of tumor area to total globe area of the histology cross-section with the largest tumor focus. Reductions in tumor burden for saline- and DMSO-treated control eyes were not statistically significant (reproduced with permission from Suárez *et al*^[157]) (b-c) representative histopathologic examination of enucleated globes of transgenic retinoblastoma mice (b) left untreated or (c) treated with 0.25 mg paclitaxel administered via subconjunctival injection (reproduced with permission from Suárez *et al*^[157])

Figure 8. Vitamin D analogs inhibit tumor cell growth *in vitro* and in preclinical models of retinoblastoma

(a) Representative immunoblot analysis of apoptosis-related proteins in Y79 cells incubated for 72 hr with vitamin D analog 1,25-(OH)₂D₃ shows dose-dependent increase of Bax and dose-dependent decrease of Bcl-2 (reproduced with permission from Wagner *et al.*^[163]). (b) Dose response curves of Y79 cells treated with vitamin D analogs (1,25-(OH)₂D₃ (empty diamonds) and the synthetic analogue KH1060 (filled diamonds) (reproduced with permission from Wagner *et al.*^[163]). (c) In vivo tumor volumes in a subcutaneous Y79 xenograft mouse model of retinoblastoma during 5 weeks of treatment with cisplatin,

calcitriol, combination therapy and control. Combination therapy (calcitriol + cisplatin) significantly inhibited tumor growth compared to controls (reproduced with permission from Kulkarni *et al*^[125]).

Figure 9. Inhibition of angiogenesis by anti-VEGF antibody (Bevacizumab) or small molecule angiogenesis inhibitor (CA-4P) inhibits tumor growth in murine models of retinoblastoma (a) Expression of vascular endothelial growth factor (VEGF) in culture medium of Y-79 cells incubated at 37°C in a hypoxic chamber and in a normoxic chamber (reproduced with permission from Lee *et al*^[168]) (b) Inhibition of angiogenesis by treatment with an anti-VEGF antibody (Bevacizumab) produces dose-dependent inhibition of tumor growth in a xenograft model of retinoblastoma in a xenograft model (asterisks and dagger denote a statistically significant difference from the control group) (reproduced with permission from Lee *et al*^[168]). (c) Dose dependent tumor reduction in eyes treated with CA-4P normalized to untreated control eyes reduction (reproduced with permission from Escalona-Benz *et al*^[170]). (d) Representative H&E histopathology sections of an eye treated with 15 mg CA-4P and (e) an untreated control eye (reproduced with permission from Escalona-Benz *et al*^[170]).

Table 1International Classification System and Treatment for Retinoblastoma^[8,16,24]

Group	Globe Salvage Likelihood	Quick Reference	Specific Clinical Characteristics	Treatment
A	Very Favorable (good visual and overall prognosis)	Small tumors away from fovea and disc	<ul style="list-style-type: none"> Tumors 3 mm in greatest dimension confined to the retina, and Located at least 3 mm from the foveola and 1.5 mm from the optic disc 	<ul style="list-style-type: none"> Argon-YAG laser Diode laser-induced hyperthermia Cryotherapy Brachytherapy
B	Favorable (good visual prognosis)	Larger tumor	<ul style="list-style-type: none"> All remaining tumors confined to the retina not in Group A Subretinal fluid (without subretinal seeding) 3 mm from the base of the tumor 	<ul style="list-style-type: none"> VCR + low dose CBP Focal therapy for 2–5 cycles
C	Doubtful (visual prognosis variable)	Localized seeding	<ul style="list-style-type: none"> Local subretinal fluid alone > 3 to 6 mm from the tumor Vitreous seeding or subretinal seeding 3 mm from the tumor 	<ul style="list-style-type: none"> VCR + high dose CBP + ETO + G-CSF, up to 6 cycles Focal therapy Possible local carboplatin
D	Unfavorable (high morbidity from treatment, visual prognosis variable)	Diffuse seeding	<ul style="list-style-type: none"> Subretinal fluid alone > 6 mm from the tumor Vitreous seeding or subretinal seeding > 3 mm from the tumor 	<ul style="list-style-type: none"> VCR + high dose CBP + ETO + G-CSF, up to 6 cycles EBRT Possible local carboplatin
E	Very unfavorable (high morbidity from treatment, no visual potential)	Extensive	<ul style="list-style-type: none"> Presence of one or more of the following poor prognosis features: <ul style="list-style-type: none"> More than 2/3 globe filled with tumor Tumor in anterior segment Tumor in or on the ciliary body Iris neovascularization Neovascular glaucoma Opaque media from hemorrhage Tumor necrosis with aseptic orbital cellulitis Phthisis bulbi 	<ul style="list-style-type: none"> Enucleation Prophylactic 3-agent chemotherapy if high-risk features for disease dissemination observed on consensus pathologic evaluation

Abbreviations: CBP = carboplatin, EBRT = external beam radiation therapy, ETO = etoposide, G-CSF = granulocyte colony-stimulating factor, PD = progressive disease, VCR = vincristine

Table 2

Summary of Preclinical Testing of Retinoblastoma Candidate Therapeutics

Animal Model of Retinoblastoma	Therapeutic Target	Therapeutic Candidate	Dosing Information (Route, Formulation, Schedule)	PK Data Included?	Ref.
Xenograft mouse (subcutaneous injection of Y79 cells); fed a vitamin D and calcium restricted diet	Vitamin D analog	1,25-dihydroxy-cholecalciferol (D ₃ , calcitriol)	IP injection, 0.05 µg calcitriol in mineral oil vehicle, daily 5 times a week for 5 weeks	No	[123]
	Vitamin D analog	1,25-dihydroxy-16-ene-23-yne-vitamin D ₃ (12,23-D ₃)	IP injection, 0.5 µg 16,23-D ₃ in mineral oil vehicle, daily 5 times a week for 5 weeks	No	
Xenograft mouse (subcutaneous injection of Y79 cells); fed a vitamin D and calcium restricted diet	Vitamin D analogs	Calcitriol	IP, 0.5 µg in 250 µL ethanol stock diluted in mineral oil, 5 times a week for 5 weeks	No	[161]
		16, 23-D ₃			
Xenograft mouse (subcutaneous injection of Y79 cells); fed a vitamin D and calcium restricted diet	Vitamin D analog	1α-OH-D ₂	Oral gavage, 0.2 µg per mouse in coconut oil, treatment 5 times per week for 5 weeks	No	[124]
		16,23-D ₃	IP, 0.5 µg per mouse in mineral oil, treatment 5 times per week for 5 weeks.	No	
Transgenic mouse (LH-β-Tag)	Vitamin D analog	1α-OH-D ₂	Oral gavage, 0.2 µg per mouse per day in coconut oil, treatment 5 times per week, either 5 weeks, 10 weeks or 15 weeks total	No	
		16,23-D ₃	IP, 0.5 µg per mouse in mineral oil, treatment 5 times per week, either 5 weeks, 10 weeks or 15 weeks total	No	
Xenograft mouse (subcutaneous injection of Y79 cells)	Vitamin D analog	2-methylene-19-nor-(20S)-19-hydroxybishomo pregnacalciferol (2Mbisp)	Oral gavage of either 20 µg/kg, 60 µg/kg, or 180 µg/kg in 0.1 mL of Neobee M-5 oil, daily administration (7	No	[166]

Animal Model of Retinoblastoma	Therapeutic Target	Therapeutic Candidate	Dosing Information (Route, Formulation, Schedule)	PK Data Included?	Ref.
Orthotopic xenograft, mouse (human tumor cells)	Spleen Tyrosine Kinase (SYK) Inhibition	BAY 61-3606	days/week for 5 weeks Single subconjunctival dose of BAY61-3606 (10 μ L of 2 mM in DMSO) on day 1 and daily doses of topotecan (TPT) on days 1–5 for up to six courses (21 days per course)	No	[31]
Orthotopic xenograft, mouse (SJ39 human tumor cells)	SYK Inhibition	R406 (in combination with topotecan)	10 μ L of 8 – 9 nanomoles R406 per eye on day 1, and TPT ^{sys} (0.7 mg/kg per dose, i.p.) on days 1 to 5 very third week	Yes	[54]
Complementary knockout mice MDMX knockout: (<i>Chx10-Cre;Rb^{lox/Lox};p107^{-/-};MDMX^{Tg}</i>) and p53 knockout (<i>Chx10-(6)Cre;Rb^{lox/Lox};p107^{-/-};p53^{lox/Lox}</i>) Orthotopic xenograft, mouse (SJ39 human tumor cells)	MDM2/ MDMX-p53 inhibition	Nutlin-3a (in combination with topotecan)	Combination of local Nutlin-3a and systemic topotecan administered on a 5-day schedule: subconjunctival injection of 25 mM nutlin-3a (10 μ L/eye) on Day 1, and daily IP injections 0.7 mg/kg topotecan on Days 1–5	Yes	[24]
Xenograft mouse (subcutaneous injection of Y79 cells)	Unknown	Ouabain	1.5 or 15 mg/kg in 10% DMSO delivered via continuous subcutaneous infusion from an osmotic minipump (delivery rate = 0.5 μ L per hour) implanted on the opposite flank as the tumor and replaced weekly over a treatment period of 19 days	No	[126]
Orthotopic xenografts rats (luciferase-labeled Y79 cells injected in neonatal rat pup eyes)	Unknown	EDL-155	Periocular injection, 20 mg/kg in 0.01% DMSO in normal saline; daily injections for 4 days	No	[182]

Animal Model of Retinoblastoma	Therapeutic Target	Therapeutic Candidate	Dosing Information (Route, Formulation, Schedule)	PK Data Included?	Ref.
Transgenic mouse (LH- β -Tag)	Histone deacetylase (HDAC) inhibition	MS-275 (Elatinostat)	IP injection, 20 mg/kg MS-275, administered every other day for 21 days	No	[148]
Orthotopic xenografts rats (luciferase-labeled Y79 cells injected in neonatal rat pup eyes)			IP injection, 20 mg/kg MS-275, administered every other day for 13 days	No	
Xenograft mouse (subcutaneous injection of Y79 cells)	Alkylating agent	Cisplatin	IP injection, 50 μ g of cisplatin in 0.1 ml of mineral oil and 0.25 ml saline, administered daily 5 days/week for 5 weeks	No	[125]
	Vitamin D analog	Calcitriol	IP injection, 0.05 μ g of calcitriol in 0.1 ml of mineral oil and 0.25 ml saline, administered daily 5 days/week for 5 weeks	No	
	Not applicable (combination)	Cisplatin + Calcitriol	IP injection, 50 μ g of cisplatin in 0.1 ml of mineral oil and 0.25 ml saline and 0.05 μ g of calcitriol in 0.1 ml of mineral oil and 0.25 ml saline, administered daily 5 days/week for 5 weeks	No	
Transgenic mouse (SV40 Tag)	Angiogenesis inhibition	Combretastatin A-4 phosphate (CA-4P) prodrug	Subconjunctival injection, 0.5, 1.0, 1.5, and 2.0 mg in 20 μ L dissolved in saline, administered every 72 hours for a total of six injections	No	[170]
Transgenic mouse (LH- β -Tag)	Angiogenesis inhibition	Anecortave acetate	Subconjunctival injection, 600, 300, or 150 μ g in 20 μ L manufacturer-provided vehicle, single injection	No	[172]
Transgenic mouse (LH- β -Tag)	Alkylating agent	Carboplatin	Subconjunctival injection, 37.5 or 10 mg/mL of carboplatin loaded into dendritic	No	[88]

Animal Model of Retinoblastoma	Therapeutic Target	Therapeutic Candidate	Dosing Information (Route, Formulation, Schedule)	PK Data Included?	Ref.
			nanoparticles or 10 mg/mL of carboplatin in aqueous solution, single injection		
Transgenic mouse (LH- β -Tag)	Alkylating agent	Carboplatin	Suconjunctival injection, 37.5 or 75 mg/mL carboplatin in 30 μ L fibrin sealant, single injection	No	[89]
Transgenic mouse (SV40 Tag)	Alkylating agent	Carboplatin	Subconjunctival injection, doses of 30, 50, 62.5, 85, 125, 200 or 300 μ g in 25 μ L balanced salt solution, administered twice per week for a total of six injections	No	[81]
Transgenic mouse (LH- β -Tag)	Antimitotic	Paclitaxel	Subconjunctival injection, 0.5, 0.25, 0.125, 0.0526, 0.0313 or 0.0152 mg in 20 μ L of 100% DMSO, two injections delivered 72-hr apart	No	[157]
Transgenic mouse (LH- β -Tag)	Topo-isomerase inhibition	Topotecan	Subconjunctival injection, 3.2 mg/mL in 30 μ L of fibrin sealant (0.1-mg total dose), single injection	No	[90]
Orthotopic xenografts rats (luciferase-labeled Y79 cells injected in neonatal rat pup eyes)	Not applicable (combination)	Topotecan + Carboplatin	Subconjunctival injection of topotecan (10 μ g/eye) on Day 1 and IP injection of carboplatin (10 mg/kg)	Yes	[52]
	Not applicable (combination)	Topotecan + Carboplatin	Subconjunctival carboplatin (100 μ g/eye) on Day 1 and IP injection of topotecan (0.2 mg/kg daily $\times$ 5 days)	Yes	
Transgenic mouse (<i>Chx10-Cre; Rb^{lox/Lox}, p107^{-/-}; p53^{lox/Lox}</i>)	Not applicable (combination)	Topotecan + Carboplatin	Subconjunctival carboplatin (100 μ g/eye) on Day 1 and IP injection of topotecan (0.1 mg/kg or 0.7 mg/kg administered	Yes	

Animal Model of Retinoblastoma	Therapeutic Target	Therapeutic Candidate	Dosing Information (Route, Formulation, Schedule)	PK Data Included?	Ref.
			daily $\times$ 5 days every third week		
Orthotopic xenografts rats (luciferase-labeled Y79 cells injected in neonatal rat pup eyes)	Not applicable (combination)	Topotecan, Carboplatin, Vincristine or Etoposide as single agents; Topotecan + Vincristine; Etoposide + Carboplatin; Topotecan + Carboplatin	All agents delivered via tail vein injection; Topotecan = 2 mg/kg Carboplatin = 70 mg/kg Vincristine = 0.5 mg/kg Etoposide = 10 mg/kg	Yes	[134]
Genetic mouse model (see paper for details)	Not applicable (combination)	Topotecan + Carboplatin; Topotecan + Vincristine	Tail vein injection, 0.1 mg/kg topotecan daily $\times$ 5 days, then once weekly carboplatin (18 mg/kg) or vincristine (0.01 mg/kg); one cycle = 2 weeks of topotecan followed by a week off topotecan; 3-week cycle repeated three times (9 weeks)		
Orthotopic xenograft mouse (SNUOT-Rb1 cells)	Not applicable	Arsenic trioxide (ATO)	Intravitreal injection, 0.1 or 5 μ M, administered weekly	No	[181]
Orthotopic xenograft, mouse (SJ39 human tumor cells)	Monoamine transporter inhibition	Fluphenazine	Subconjunctival injection, dose and vehicle not included, administered weekly for 3 consecutive weeks	No	[178]
		Chpromazine			