Revisiting Old Drugs as Novel Agents for Retinoblastoma: In Vitro and In Vivo Antitumor Activity of Cardenolides

Christophe Antczak,1 Carolyn Kloepping,1 Constantin Radu,1 Thorsten Genski,2 Lutz Müller-Kubrt,2 Karsten Siems,2 Elisa de Stanchina,3 David H. Abramson,4 and Hakim Djaballahb

Purpose. Intra-arterial delivery of chemotherapeutic agents offers a new and exciting opportunity for the treatment of advanced intraocular retinoblastoma. It allows local delivery of relatively high doses of chemotherapy agents while bypassing general blood circulation. For this reason, this study was undertaken to revisit some of the FDA-approved drugs for the treatment of retinoblastoma.

Methods. High-throughput screening (HTS) of 2640 approved drugs and bioactive compounds resulted in the identification of cytotoxic agents with potent activity toward both the Y79 and RB355 human retinoblastoma cell lines. Subsequent profiling of the drug candidates was performed in a panel of ocular cancer cell lines. Induction of apoptosis in Y79 cells was assessed by immunofluorescence detection of activated caspase-3. Therapeutic effect was evaluated in a xenograft model of retinoblastoma.

Results. Several FDA-approved drugs were identified that showed potent cytotoxic activity toward retinoblastoma cell lines in vitro. Among them were several cardiac glycosides, a class of cardenolides historically associated with the prevention and treatment of congestive heart failure. Caspase-3 activation studies provided an insight into the mechanism of action of cardenolides in retinoblastoma cells. When tested in a xenograft model of retinoblastoma, the cardenolide ouabain induced complete tumor regression in the treated mice.

Conclusions. Cardenolides were identified as a new class of antitumor agents for the treatment of retinoblastoma. Members of this class of cardiotoxic drugs could be repositioned for retinoblastoma if administered locally via direct intra-arterial infusion. (Invest Ophthalmol Vis Sci. 2009;50:3065–3073) DOI:10.1167/iovs.08-3158

Retinoblastoma constitutes the most common primary ocular tumor of childhood, affecting approximately 5000 to 8000 children worldwide each year. Although the current survival rate associated with retinoblastoma is approximately 90% in developing countries, in some cases successful treatment can be achieved only by enucleation. Furthermore, current treatment modalities are limited by their toxicity. Traditionally, tumor reduction is achieved by external beam radiotherapy or chemotherapy, before local treatment such as thermotherapy, cryotherapy, radioactive plaque, or brachytherapy.1,2 Complications may arise from the use of radiotherapy and systemic chemotherapy. The long-term effects of external beam radiotherapy can include cataracts, radiation retinopathy, impaired vision, and temporal bone suppression.3 Radiation also increases the incidence of second cancers in genetically primed patients, especially those under the age of 1 year.3 Because of various mechanisms of action, chemotherapy is synergistic and is best used in combination with the standard three-drug regimen comprising carboplatin, etoposide, and vincristine.2 Systemic chemotherapy-related side effects include cytopenia, neutropenia, gastrointestinal distress, and neurotoxicity for vincristine.3–5 In addition, an increased risk for the development of second malignant neoplasms has been linked to the use of platinum-based drugs for the treatment of childhood malignancies, and secondary leukemias have been reported in retinoblastoma patients treated with etoposide.8–10 In summary, the limitations of current therapeutic approaches used to treat retinoblastoma, sometimes necessitating enucleation for effective treatment, underline the urgency of developing new and effective therapies.

There has been extensive research aimed at developing alternative agents for retinoblastoma that lack the risks associated with current chemotherapy. A series of studies have investigated the potential of calcitriol (vitamin D) and its derivatives as antiproliferative agents.11–14 However, the mortality of treated animals due to hypercalcemia, remains an issue. Another example is Nutlin-3, a small-molecule inhibitor of Mdm2-p53 interaction.15 Early preclinical studies have shown that Nutlin 3 induces apoptosis in two retinoblastoma cell lines.16,17 Nutlin-3 was also found to synergistically kill retinoblastoma cells in combination with topotecan, but had little effect when used alone.18 Novel effective treatments for retinoblastoma have yet to emerge from those studies.

Intra-arterial chemotherapy is an entirely new approach for the treatment of advanced intraocular retinoblastoma consisting of the selective ophthalmic artery infusion of chemotherapeutics.18 In a first study of melphalan, a dramatic response to the treatment was observed with a locally administered dose of one tenth of the usual systemic dose of the chemotherapy agent.18 Presumably, local intra-arterial delivery of melphalan, by allowing to bypass the bloodstream, was responsible for the improved efficacy and diminished toxicity observed in this study. Intra-arterial chemotherapy therefore constitutes an ex-
citing new technique that opens the way to the use of previously neglected chemotherapeutic agents due to their high systemic toxicity for the treatment of retinoblastoma. For this reason, we sought to revisit approved drugs and known bioactive compounds to identify potent agents for retinoblastoma to be administered by local intra-arterial infusion.

In this article, we describe the results of the first chemical screen specifically aimed at identifying alternative chemotherapeutic agents for retinoblastoma. We identified potent agents for retinoblastoma cells among a library of 2640 mostly off-patent compounds consisting of marketed drugs, bioactive compounds in various therapeutic areas, toxic substances, and natural products. Of note, we found that the newly identified agents for retinoblastoma belong to well-described pharmacologic classes, some agents currently being used in the clinic. In this study, we characterized the potency of the newly identified drug candidates in different cellular models of retinoblastoma, and we further evaluate cardenolides as a novel potent class of agents for retinoblastoma. We assessed the in vivo efficacy of the cardenolide ouabain in a xenograft model of retinoblastoma, a drug historically used for the treatment of myocardial infarction. Based on our results, we propose that our strategy may lead to alternative potent treatments for retinoblastoma, with globe-conserving strategies in mind.

METHODS

Cell Lines and Tissue Culture

The human retinoblastoma cell lines Y79 and WERI-Rb-1 were purchased from the American Type Culture Collection (Manassas, VA). The human retinoblastoma cell line RB555 originally established by Brenda Gallic (University of Toronto) and the luciferase-expressing Y79Luc cell line were kindly provided by Michael Dyer (Saint Jude Children’s Research Hospital). The human uveal melanoma cell lines C918 and Mum2b originally established by Mary Hendrix (University of Iowa) were generously provided by Daniel Albert (University of Wisconsin). The cell lines Y79, WERI-Rb-1, and RB555 were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 20% (vol/vol) fetal bovine serum (Omega Scientific, Tarzana, CA), 1% (vol/vol) penicillin-streptomycin (Gibco, Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO). The human retinoblastoma cell lines Y79 and WERI-Rb-1 were purchased from the American Type Culture Collection (Manassas, VA). The human retinoblastoma cell line RB355 originally established by Scott Kuch (Mayo Clinic, Rochester, MN) was generously provided by Daniel Albert (University of Wisconsin). The human retinoblastoma cell line RB355 was grown in RPMI 1640 (Invitrogen) with 10% (vol/vol) fetal bovine serum (Omega Scientific, Tarzana, CA), 1% (vol/vol) penicillin-streptomycin (Gibco, Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO). The human uveal melanoma cell lines C918 and Mum2b were grown in RPMI 1640 (Invitrogen) with 10% (vol/vol) fetal bovine serum (Omega Scientific, Tarzana, CA), 1% (vol/vol) penicillin-streptomycin (Gibco, Invitrogen), and 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO).

Figure 1: Schematic of the experimental setup for the pilot screen. For each compound, the dose–response study was performed in triplicate, using a 12-point doubling dilutions with 100 µM compound concentration as the upper limit. The dose–response curve for each set of data was fitted separately, and the two IC50 values obtained were averaged. For compounds having an IC50 below 0.5 M, the dose–response study was repeated using dilutions starting at 0.1 M for more accurate determination of the IC50.

Cytotoxicity Assay for Screening in 384-Well Microtiter Plates

Library compounds were preplated in 1 µL of 1% DMSO (vol/vol) into 1536-well microtiter plates (model 3893; Corning Inc., Corning, NY) using an automated pipetting system (TPS-384 Total Pipetting Solution; Apricot Designs, Monrovia, CA). The cells were added in 8 µL medium to the screening plates with a precision dispenser (Flexdrop; Perkin Elmer). After a 72-hour incubation, 5 µL Alamar Blue was added with the dispenser. The cells were then incubated for another 24 hours, and the fluorescence intensity was read on the imaging system (LEADseeker; Amersham) as previously described. To calculate the IC50 for each compound toward each cell line, the dose–response was assessed in duplicate, using 12-point doubling dilutions with 100 µM compound concentration as the upper limit. The dose–response curve for each set of data was fitted separately, and the two IC50 values obtained were averaged. For compounds having an IC50 below 0.5 M, the dose–response study was repeated using dilutions starting at 0.1 M for more accurate determination of the IC50.

Apoptosis Assay

Y79 cells seeded in culture medium in a 24-well plate were treated with vincristine, etoposide, or ouabain at various concentrations in 1% DMSO (vol/vol) or with 1% DMSO (vol/vol) alone as a carrier control for 48 or 72 hours. After a wash in PBS, the cells were fixed in solution in 4% (vol/vol) cold PBS for 10 minutes. After a wash in PBS, the cells for each condition were dried on a glass slide and washed once with water.

The immunofluorescence detection of cleaved caspase-3 was performed at the Memorial Sloan-Kettering Cancer Center Molecular Cytology Core Facility (Discovery XT processor; Ventana Medical Systems, Tucson, AZ). A rabbit polyclonal Cleaved Caspase 3 (Asp175) antibody (catalog no. 9661L; Cell Signaling, Danvers, MA) was used at a concentration of 0.1 µg/mL. The cells were blocked for 30 minutes in 10% (vol/vol) normal goat serum, 2% (vol/vol) BSA in PBS before incubation with the primary antibody for 3 hours and a subsequent 20-minute incubation with biotinylated goat anti-rabbit IgG (catalog no. PK6101; Vector laboratories, Burlingame, CA) diluted 1:200. The detection was performed with a secondary antibody blocker (Blocker...
In Vivo Studies

Subcutaneous xenograft experiments were performed at the Memorial Sloan-Kettering Cancer Center Antitumor Assessment Core Facility in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Y79LUC cells (10^6) embedded in synthetic matrix (Matrigel; BD Biosciences, San Jose, CA) were injected SC in the right flank of 8-week-old ICR/SCID male mice. Treatment started when the tumors reached approximately 250 mm^3. The mice were randomized into three groups and two mice per group were treated with 10% DMSO (vol/vol) (control group), 1.5 mg/kg ouabain, or 15 mg/kg ouabain. Ouabain was obtained from Sigma-Aldrich (St. Louis, MO) and its chemical identity was confirmed by mass spectrometry. Treatment was performed over a period of 19 days by continuous infusion with osmotic minipumps (model 1007D; Alzet, Cupertino, CA), which provide a delivery rate of 0.5 μL per hour. Ouabain concentration in the minipump was 3.15 mg/mL in the 1.5 mg/kg group and 31.5 mg/mL in the 15 mg/kg group. The minipumps were inserted on the left flank, opposite where the Y79 tumor had been implanted and replaced every week. Tumor size was measured externally two times a week with a caliper. The mouse’s weight was monitored as well as other signs of toxicity throughout the treatment period. Bioluminescent imaging of the tumors was performed once a week and before the mice were killed as follows: The mice were anesthetized with isoflurane inhalation and injected with α-luciferin at 50 mg/kg (Xenogen, Alameda, CA) intraperitoneally; photonic emission was measured with an in vivo imaging system (IVIS 200; Xenogen) with a collection time of 5 seconds.

RESULTS

Identification of Alternative Cytotoxic Agents for Retinoblastoma among Known Drugs

We wanted to identify alternative cytotoxic agents for retinoblastoma among known drugs and bioactive agents. To meet this goal, we screened a combined library of 2640 commercially obtained chemicals representing biologically active and structurally diverse compounds from known drugs, experimental bioactives, and natural products, mostly off-patent. We relied for the screen on the use of the well-described cytotoxicity assay based on the reduction of the dye resazurin and commercially sold as Alamar Blue.23 Due to its compatibility with the requirements of high-throughput screening,24 in this assay, the fluorescence emitted by the living cells on metabolism of Alamar Blue is proportional to the number of metabolically active cells. Hence, the cytotoxicity or the cytostaticity of a compound can be assessed relative to that of a control agent. Because we wanted to identify chemical scaffolds with broad activity for retinoblastoma as opposed to compounds cytotoxic toward only one specific retinoblastoma cell line, we adopted a strategy where we screened our combined drug library in parallel against two retinoblastoma cell lines. We chose to use the Y7925 and the RB35526 human cell lines as models of retinoblastoma, because they are among the few well-established human retinoblastoma cell lines available and because we managed to optimize their growth in high-density format (data not shown). Duplicate sets of the combined library of 2640 compounds were tested at 10 μM consecutively the same day for each cell line. After statistical analysis of the duplicate sets of data to assess the reproducibility of the screen and to ensure the absence of systematic error, we calculated the average percentage inhibition for each compound based on high and low controls present on each plate as previously described.27 When we compared the newly generated Y79 and RB355 data sets we found that a large population of the tested compounds was active only toward one of the two cell lines (Fig. 1). This result validates our approach consisting of screening our combined drug library against two cell lines in parallel to select broad-acting compounds. We then compared in a scatterplot the percentage inhibition for each compound in both the Y79 and the RB355 data sets (Fig. 2).

Although most tested compounds had no significant activity in either screen or were active in only one screen, we focused on the population of compounds demonstrating greater than 95% inhibition in both screens to select as positives only those compounds that were likely to have broad activity for retinoblastoma. The chemical structures of the selected 11 positives at 95% inhibition threshold are depicted in Figure 3. We performed cytotoxicity profiling for these 11 positives against the human retinoblastoma cell lines Y79, RB355, and WERI-Rb-1, as well as against the uveal melanoma cell lines C918 and Mum2b. We found that all 11 selected positives had broad and potent cytotoxic activity against these five ocular cancer cell lines with calculated IC_{50} ranging from 40 nM to 27 μM (Table 1). All selected positives were cytotoxic toward at least three of five cell lines, whereas most of them (9/11) were potent against all tested cell lines (Table 1). Of interest, most of the selected positives could be grouped into two well-known pharmacological classes: ion pump effectors (five) and antimicrobial agents (four). The four most potent compounds identified belonged to the pharmacological class of ion pump effectors. Among them was digoxin, which is currently approved by the FDA for the treatment of cardiac arrhythmia and for the prevention of heart failure.

Cardenolide Cytotoxicity in Ocular Cancer Cells

A structural analysis of the positives identified during the screen revealed that the five ion pump effectors that we previously characterized (Table 1: peruvoside, ouabain, neriifolin, digoxin, and digoxigenin all share a common chemical scaffold (Fig. 3A) that corresponds to the core structure of cardenolides. When we performed a structural search for compounds present in our combined library sharing the same scaffold, we identified 19 cardenolides. To our surprise, we found that all of them had induced greater than 75% inhibition toward at least one cell line during the screening and that they constituted 10 of the 29 positives at a threshold of 90% inhibition in both screens (Fig. 2). In addition, 13 (68%) of 19 cardenolides present in our combined library induced greater than 50% inhibition in both screens. This striking observation led us to focus on cardenolides as a new class of antiproliferative agents for retinoblastoma. To explore the structure–activity relationship (SAR) within this chemical class, we constituted a collection of 35 cardenolides and derivatives. We then assessed the dose-response for each compound toward the ocular cancer cell lines Y79, RB355, WERI-Rb-1, and C918. The results of this SAR study are summarized in Figure 4. With 23 of the 35 tested cardenolides (64%) having potent antiproliferative properties toward at least of the two ocular cancer cell lines tested (IC_{50} < 10 μM; Fig. 4A), we confirmed that cardenolides constitute a class of potent and broad-acting agents for retinoblastoma. The most potent compound among the 35 tested cardenolides (derivative 1) had a calculated IC_{50} of 35 and 90 nM toward the cell lines C918 and RB355, respectively (Figs. 4A, 4B). As we investigated the structure activity relationships underlying the potency of cardenolides in our panel of ocular cancer cells, we...
identified a clear trend among the 35 derivatives that we tested: The presence of a glycoside substitutant on the 3-hydroxy group seemed to influence the potency. Indeed, 21 compounds among the 23 most potent derivatives tested (91%) had a glycoside moiety grafted to their 3-hydroxy group (Fig. 4A). On the other hand, a significant proportion of the 12 less potent compounds (42%) did not have any glycoside moiety at this position (Fig. 4A). This observation seems to indicate that the presence of such a glycoside substitutant is beneficial to the broad and potent activity of cardenolides toward ocular cancer cells. Several cardenolides had potent activity across the entire panel of ocular cell lines tested, such as the drug ouabain, which has a long history in the treatment of heart failure28–30 (Fig. 4C).

**Compared Potency of the Cardenolide Ouabain with Known Agents in Cell Models of Retinoblastoma**

We compared the potency of a representative of the cardenolide scaffold to that of known effective agents against retinoblastoma. Namely, we tested the dose–response of the drug ouabain with the human retinoblastoma cell lines Y79 and RB355, and compared its potency to that of vincristine, etoposide, carboplatin, cisplatin, nutlin-3, and calcitriol. We chose ouabain as a representative of cardenolides because it had demonstrated broad and potent activity toward all the tested cell lines (Table 1; Figs. 4A, 4C), and because of its long history as a cardiotonic drug. In our assay, ouabain was the most potent compound toward Y79 cells, with an IC50 of 0.65 μM compared with 11 μM for etoposide and 78 μM for nutlin-3 (Fig. 5A). The activity of vincristine toward Y79 cells reached a plateau at 50% inhibition, which prevented us from calculating an IC50 for this compound. Carboplatin, cisplatin, and calcitriol did not demonstrate any significant activity toward Y79 cells below 100 μM in our assay. Oubain had a similar potency toward RB355 cells with an IC50 of 0.40 μM compared to 1.6 nM for vincristine, 0.97 μM for etoposide and 11 μM for nutlin-3 (Fig. 5B). Cisplatin reached a maximum of 65% inhibition at 100 μM, and neither carboplatin nor calcitriol had any significant activity below 100 μM. These results demonstrate that the in vitro potency of the cardenolide ouabain is comparable to or even greater than the most potent agents for retinoblastoma currently known. That ouabain was equally potent toward these two cell lines suggests that its mechanism of action may be independent of DNA replication or cell division.

**Compared Effect of Ouabain and Clinical Agents on Apoptosis of Y79 Cells**

To determine whether the antiproliferative effect of the drug ouabain is mediated by induction of apoptosis, we performed immunostaining of activated caspase-3 in Y79 cells treated with cardenolides or known agents for retinoblastoma for 72 hours (green channel); treated cells were also stained with Hoechst to image the nuclei (blue channel; Fig. 6). The drug concentrations used in this experiment were previously determined according to a pilot study where treated Y79 cells were live stained with a green fluorescent dye (Yo-Pro; Invitrogen, Carlsbad, CA), which stains apoptotic cells.31 Based on this study, we identified 72 hours as the optimum incubation time

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**Figure 1.** Overall comparative analysis of the Y79 and RB355 screens. The percentage loss of cell viability induced by each tested compound in both screens is represented as a heat map, to identify those compounds potent toward both cell lines.

**Figure 2.** Comparative analysis of the Y79 and RB355 screens. The percentage loss of cell viability induced by each tested compound in both screens is represented. Red: the 29 positives at a threshold of 90% inhibition in both screens; green: the 19 cardenolides present in the library. The color scheme shows the proportion of cardenolides among all positives as well as their potency.
and selected drug concentrations that maximized the number of apoptotic cells (data not shown). Baseline caspase-3 activation was evaluated with control Y79 cells treated with 1% DMSO (vol/vol; Fig. 6A). We found that vincristine (Fig. 6B) and etoposide (Fig. 6C) induced significant apoptosis in Y79 cells compared with baseline levels, as previously de-

![Chemical structures of selected compounds](https://example.com/structures.png)

**Figure 3.** Summary of the structures for the 11 positives selected at a threshold of 95% inhibition in both screens. (A) Cardenolides; (B) noncardenolides.

**Table 1.** Summary of the 11 Positives Identified in the RB355/Y79 Screening Campaign

<table>
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<tr>
<th>Compound Name</th>
<th>Chemical Class</th>
<th>Pharmacological Class</th>
<th>Therapeutic Use</th>
<th>IC₅₀ (μM)</th>
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<td>Ion pump effector</td>
<td>Prevention of congestive heart failure</td>
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<td>Ion pump effector</td>
<td>Cardiotonic</td>
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<tr>
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<td>Cardenolide</td>
<td>Ion pump effector</td>
<td>Prevention of congestive heart failure, FDA approved</td>
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<td>Antimicrobial</td>
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Positives belonging to the class of cardenolides are shaded. The calculated IC₅₀S for each positive in the ocular cancer cell line cytotoxicity panel are detailed.
scribed. Ouabain in this experiment was used at a concentration of 0.5 μM compared to 100 μM for vincristine and 10 μM for etoposide, because higher concentrations of ouabain eradicated Y79 cells in our pilot study with the nucleic acid dye. At this lower concentration, ouabain still induced significant apoptosis in our assay (Fig. 6D).

**Figure 4.** Structure–activity relationship study for a collection of 35 cardenolides in a panel of four ocular cancer cell lines. (A) Heat map and numerical summary of calculated IC₅₀ values for the 35 cardenolides in the ocular cancer cell line panel. The structure of identified chemical scaffolds is highlighted. (B) Representative dose–response curves generated for derivative 1 in the panel of ocular cancer cell lines. (C) Representative dose–response curves generated for the drug ouabain in the panel of ocular cancer cell lines.
Assessment of the In Vivo Efficacy of Ouabain in a Xenograft Model of Retinoblastoma

We investigated the therapeutic effect of the drug ouabain in a mouse xenograft model of retinoblastoma. Three groups of two 8-week-old ICR/SCID male mice bearing Y79 tumors implanted in the flank were treated with vehicle only or with 1.5 or 15 mg/kg ouabain. The mice were continuously infused subcutaneously with an osmotic minipump delivery system, to mimic the local delivery that intra-arterial chemotherapy allows. Evaluation of the tumor burden by bioluminescent imaging shows that ouabain at 15 mg/kg rapidly induced a dramatic decrease in tumor size leading to complete tumor regression (as assessed by bioluminescence imaging) after 14 days of treatment (Fig. 7). In comparison, tumors in the vehicle-treated control group continuously grew, necessitating euthanizing the animals at day 19. Quantification of tumor size confirmed this result: the average tumor size for the control group reached 1000 mm³ at day 14 and the tumors kept growing, whereas in both animals treated with 15 mg/kg ouabain, the tumors were nearly eradicated by day 14 (18 mm³ average size Fig. 8A). At a lower dose of 1.5 mg/kg, ouabain seemed to reduce the tumor burden compared with that in the control group (Fig. 8A). Throughout the treatment period, the average body weight of treated and control animals did not differ significantly, indicating that even at the high dose of 15 mg/kg, ouabain did not induce any significant toxicity (Fig. 8B).

DISCUSSION

Although effective treatments for retinoblastoma exist, they have important limitations. External beam radiation was once
the standard therapy for retinoblastoma, but it has largely been abandoned because of the risk of secondary malignancies. Now that radiation therapy has been replaced by the standard three-drug regimen of carboplatin, vincristine, and etoposide, concerns are emerging that the currently used chemotherapeutic agents for retinoblastoma may play a significant role in the occurrence of secondary acute myelogenous leukemia. Because of the limitations of currently available treatments, extensive research has been aimed at discovering new agents for retinoblastoma. Preclinical studies have been conducted on vitamin D analogues and nutlins, but the safety and efficacy of retinoblastoma. Preclinical studies have been conducted on naturally occurring derivatives of cardenolides with broad and potent activity in our panel of ocular cancer cell lines, which are currently being further investigated. When comparing the in vitro antiproliferative properties of the drug ouabain to known or experimental agents for retinoblastoma, we reported that the potency of ouabain is comparable to agents currently used in clinics. The apparent discrepancy between the potency of vincristine and etoposide toward Y79 and RB355 cells could be explained by the difference in the doubling time of these cells: 45 hours for Y79 cells versus 24 hours for RB355 cells. Because the mechanism of action of etoposide and vincristine relies on DNA replication and cell division, respectively, these drugs most likely demonstrated a greater activity in our assay with RB355 cells because they divide faster than Y79 cells. Furthermore, we demonstrated that ouabain induces apoptosis in Y79 human retinoblastoma cells at a dose of 0.5 μM. This observation is in agreement with previous studies showing that cardenolides induce apoptosis in various cell types. Finally, when we used a local delivery system to assess the therapeutic effect of ouabain in a xenograft model of retinoblastoma, we observed a drastic response leading to complete tumor regression after 14 days of treatment. Even at the high dose of 15 mg/kg ouabain used in this study, no signs of toxicity were observed. Altogether, our results clearly validate our strategy, in that we have identified among other drugs the well-known class of cardenolides as novel agents for retinoblastoma.

Plant extracts from the genus Digitalis containing a mixture of cardenolides have been used to treat congestive heart failure for centuries. Cardenolides such as the drug ouabain have been extensively used in clinics for 200 years as cardiotonics. Cardenolides are still used in clinics today, including the FDA-approved drug digoxin for the treatment of cardiac arrhythmia and for the prevention of congestive heart failure. The antiproliferative properties of cardenolides were initially investigated 40 years ago, but the idea of using them to treat cancer was abandoned because of the narrow therapeutic index of this class of compounds. It was later suggested that the concentration at which cardenolides induce apoptosis in cancer cells may be compatible with their therapeutic use. Clinical evidence seems to confirm this hypothesis, since patients with breast cancer who were treated with digitalis were protected from aggressive disease and benefited from a lower cancer recurrence rate. Nevertheless, there is the potential for cardiovascular toxicity when using this class of drugs systematically. For our purpose, however, cardenolides constitute an exciting new class of agents for retinoblastoma, because we have the opportunity to deliver them locally by selective ophthalmic artery infusion, as recently described in a phase I/II study. In that study, direct infusion into the ophthalmic artery allowed the delivery of high concentrations of melphatlan to the eye with markedly decreased toxicity compared with systemic administration. These promising results indicate that we could deliver high concentrations of cardenolides to the eye by intra-arterial infusion without exposing the patient to potential cardiovascular toxicity. Altogether, our results demonstrate that cardenolides constitute an exciting new class of drug candidates for retinoblastoma. Among this class of drugs, digoxin which is approved by
the FDA for the treatment of cardiac arrhythmia and to prevent congestive heart failure was broadly potent across a panel of human retinoblastoma cell lines (Table 1). In light of the results of our study, we propose that the well-known cardiotonic drug digoxin could be repositioned for the treatment of retinoblastoma if administered locally via direct intra-arterial infusion.

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