3. MATERIALS AND METHODS

3.1. Coulomb-Controlled Iontophoresis device

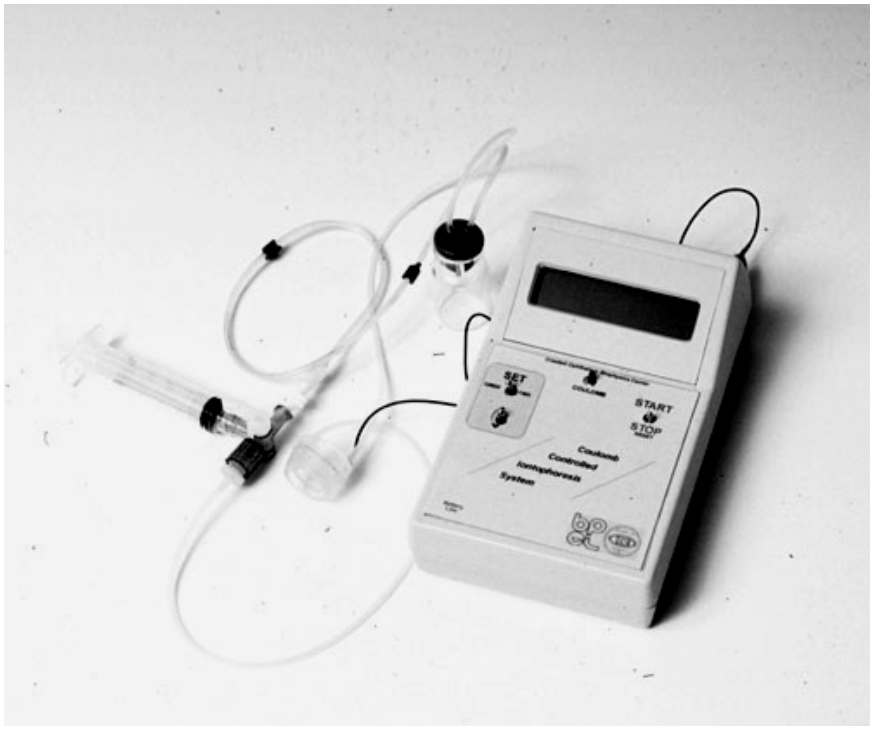
The iontophoresis device used in this research project for ophthalmology was designed and built at the Biophysics Center Bascom Palmer Eye Institute Miami and in Cooperation with OPTIS, France. **(Fig. 5)**

It consists of a 9V battery-operated microprocessor-controlled power unit that delivers a constant direct current for a pre-selected time period to a set of custom-made transcorneal or transscleral electrodes and the return electrode.

To avoid direct metal-tissue contact, the ocular applicator contains a platinum wire recessed in a well and is fully immersed in the drug solution. The wire is shaped to generate a uniform electrical field within the drug solution. The fluidic surface of the well is thus the true and only electrode in contact with the eye tissues. Platinum was selected to avoid oxidation of by-products that could penetrate the tissues and produce potential changes in the electrical impedance. Furthermore a peristaltic pump was used to induce a circulation at a maximum suction pressure of 25 mmHg to ensure a constant drug flow from the drug solution container to the relatively large ocular electrode reservoir [0.5 cc] and thereby maintaining of a constant drug concentration and pH. As the surface area of the applied field is kept constant and as the iontophoretic current is precisely regulated, the current density at the ocular surface remains uniform and constant, and hence a constant flow of charge and drug transfer rate through tissues is achieved.

In contrast to other ocular iontophoresis electrodes a lower current density [$\leq 5mA/cm^2$] can be used to achieve a similar drug transfer as the used CCI ocular probes consist of a relatively large contact surface area [annular surface of the transscleral electrode: $0.5cm^2$]. As a result of the low current density tissue changes can be avoided and thereby its impedance instabilities. In conclusion the electrical field across the ocular epithelium remains constant during the treatment. The applied voltage is continuously adapting to compensate potential variation and intraoperative changes in the tissue impedance that may occur at the return electrode. Poor contact or accidental disruption of the circuit is indicated by an audiovisual alarm, and as the instrument continuously records the total Coulombs delivered, a controlled and calibrated delivery of the drug is ensured.

Figure 5.



Coulomb-Controlled Iontophoresis System: Battery operated programmable control unit connected to the ocular applicator with fluidic lines connected to a syringe and to the drug reservoir.

3.2. Retinoblastoma Model

Several DNA viruses, including simian virus 40 (SV40), have the potential to oncogenically transform infected cells and are, therefore, known as DNA tumor viruses. For each of these viruses, the ability to transform cells is mediated by specific viral oncoproteins that function by binding and inactivating critical cellular proteins. In the case of the SV40 this related to the pRB suppressor protein [195-197]. Thus, these viruses functionally mimic a situation in which a cell has sustained inactivating mutations in these critical cell cycle regulators, leading again to unregulated proliferation. The expression of the viral genes (and transformation of the cell) depends on the promoters and suppressors within the control region adjacent to the viral oncogene, which are capable of turning gene expression on and off in response to specific molecular signals. Tissue-specific promoters are capable of turning on the gene only within certain tissues in the body [119].

The first transgenic model of retinoblastoma was the result of an attempt to produce a transgenic model of pituitary adenoma. A genetic construct, consisting of the viral oncogene, SV40 Tag, linked to the promoter for human luteinizing hormone beta subunit, LHbeta, was used to produce

transgenic mice in which Tag expression would be restricted to pituitary gonadotrophic cells. Several independent lines of LHbeta-Tag transgenic mice were created, and most expressed Tag specifically in the pituitary, resulting in late-onset pituitary adenomas. However, a single line of mice developed bilateral retinal tumors, resulting from high levels of expression of Tag within the eye. [198] In this line of mice, the tumors arise beginning at 1 to 2 months of age and are multifocal in origin.

Microscopically, the intraocular tumors produced in the LHbeta-Tag transgenic mouse were identical to human retinoblastoma [198-200].

Two types of multicellular structures common to human retinoblastoma are also seen within the LHbeta- Tag retinal tumors. The Flexner- Wintersteiner rosettes consist of small, hyperchromatic cells with large nuclei. The Homer-Wright rosettes are single-layered rows of tumor cells surrounding a central lumen that is filled with neurofibrils, which are characteristic for human retinoblastoma. As in human retinoblastoma, the mouse tumors were locally invasive into the choroid, vitreous, and optic nerve, and necrosis was frequently prominent with perivascular preservation of viability [199]. Ultrastructural studies of the LHbeta-Tag retinal tumor cells have demonstrated characteristic features of human retinoblastoma. The most distinguishing characteristic is the presence of microtubules in a “9-1-0” arrangement, which are seen in normal photoreceptor cells of the retina as well as differentiated human retinoblastoma. Other ultrastructural features include lamellar nuclear membranes, neurosecretory granules, and cytoplasmic microtubules, which are characteristically present in differentiated human retinoblastoma cells. Tumor cells involved in Flexner-Wintersteiner rosette formation demonstrate early photoreceptor structural differentiation [198-200]. Immunohistopathologic studies of LHbeta-Tag model retinal tumors showed a similar pattern of antigenic reactivity in this model to the antigenic profile of human retinoblastoma [149]. Neuron- specific enolase and synaptophysin are positively stained, whereas other antibodies including vimentin, glial fibrillary protein, and S-100 are not absorbed by tumor cells, and they stain only in adjacent areas of glial reactivity [199, 201-203].

This neuronal pattern of differentiation is typical of human retinoblastoma [148-149]. However, glial differentiation may also occur in human retinoblastoma, [148] a pattern of antigenicity that is not observed in the SV-Tag models. Furthermore the LHbeta-Tag model also phenotypically resembles human retinoblastoma in the development of midline intracranial tumors, although the cell of origin of the intracranial tumors is different [200].

The cause of the retinal and midbrain tumors in this single line of LHbeta-Tag mice is not yet known. In chromosomal analysis of the transgenic mice the site of transgene integration was

identified to be on mouse chromosome 4, [198] which does not correspond to the location of the mouse RB1 gene (chromosome 14) [204]. Thus, direct inactivation of the RB1 gene is not responsible for the retinoblastoma phenotype. The most likely explanation is that the integration site of the LHbeta-Tag transgene on chromosome 4 lies within or near a retina-specific gene in such a way that its expression pattern is now dictated by the regulatory elements of this putative gene. However, no retina specific genes have thus far been identified in the region of the transgene integration site. Regardless of the mechanism by which Tag expression has become directed to the retina, it is likely that Tag-mediated inactivation of pRB in these cells is at least in part responsible for the formation of tumors so closely resembling human retinoblastoma. Tag protein is expressed in the LHbeta-Tag tumors and has been shown to bind pRB within the tumor cells [198]. However, as Tag is also interacting with additional cellular proteins, like p53, it is possible that some of these other interactions may also contribute to the tumorgenesis in this model.

3.3. Animals

The study protocol was approved by the University of Miami School of Medicine Animal Care and Use Review Board. All experiments in this study were conducted in accordance with the Association for Research in Vision and Ophthalmology guidelines for the use of animals in ophthalmology and vision research.

3.3.1. Rabbits

The pharmacokinetic and safety study of repetitive CCI administration was performed on 84 New Zealand White rabbits with an average weight of 3.37±0.33kg.

3.3.2. Mice

Simian Virus 40 large T antigen (SV40 Tag) transgene-bearing mice were used for the application of CCI of carboplatin on a tumor model. CCI parameters were evaluated in 8 animals. Fifty-four SV40 Tag transgene bearing mice were treated with CCI of carboplatin, beginning at 6 weeks of age. Offspring bearing the transgene were identified by polymerase chain reaction analysis of the tail DNA. Transgene positive animals develop bilateral, heritable retinoblastoma that resembles human retinoblastoma. Pathologic evidence of the tumor is noted by 4 weeks of age and is expanding to the entire globe by 16 weeks of age. Tumors in this animal model are typically small at 5 weeks, appearing with intraretinal involvement only, while at 10 weeks the tumor reaches a moderate to large size.

3.4. Treatment Modalities

Before each treatment the animals were anesthetized with 0.5–1 ml intramuscular injection of ketamine (42 mg/ml) and xylazine (8.5 mg/ml).

Euthanasia was performed with an intravenous injection of 390 mg sodium pentobarbital in rabbits, while mice received an overdose of ketamine and xylazine solution.

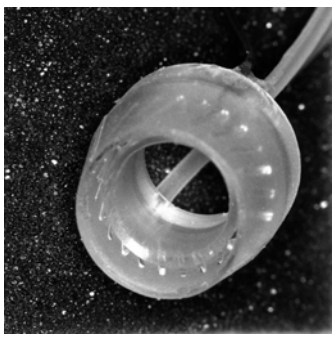
3.4.1. Subconjunctival carboplatin injections in rabbit eyes

24 New Zealand White rabbits were used for this study. carboplatin solution (Transplatin, $C_6H_{12}O_2PtO_4$, Bristol-Myers Squibb Pharmaceutical Research Institute, Hillsdale, NJ) was prepared immediately prior to administration by dissolving 5.0 mg carboplatin in 400 μl balanced salt solution (BSS, drug solution pH 5.5). carboplatin injections at doses of 5.0 mg were administered with a 33 gauge needle to the right eye. Three separate injections of 400 μl were administered into the superior, superior nasal, and inferior subconjunctival space of each eye. Left eyes were used as controls.

3.4.2. CCI delivery of carboplatin in rabbit eyes

24 NZW rabbits were used for this study. Carboplatin solution was prepared immediately prior to CCI administration by dissolving the drug powder in BSS (drug solution pH 5.5). Carboplatin was administered at a concentration of 14 mg/ml in the transscleral CCI applicator. The conical transscleral probe, custom-made for rabbits, was fabricated of silicone elastomer [MED 6033, NUSIL Inc, CA], with an annular surface of 0.5 cm^2 and an outer diameter of 17 mm. The design and size assured its location between pars plana and limbus, with a clear opening of 13 mm to avoid contact with the cornea. Before treatment, the eyes were was proptosed and CCI was applied for 20 min at a current density of 5 mA/cm^2. **(Fig. 6)** Left eyes served as controls.

Figure 6.



Autoclavable silicone rubber [MED 6033, NUSIL Inc, CA] CCI transscleral ocular applicator.

3.4.3. Intravenous delivery of carboplatin in rabbit eyes

24 New Zealand were used for this study. carboplatin solution was prepared immediately prior to CCI administration by dissolving 18.7 mg/kg of carboplatin in BSS (drug solution pH 5.5). A total volume of 60 ml was administered to the right ear vein at a flow rate of 1.0 ml per minute.

3.4.4. Repetitive CCI treatments on rabbit eyes – safety study

Two rabbits underwent six treatment sessions of transscleral CCI of carboplatin (14 mg/ml) for 20 min at a current density of 5 mA/cm^2 at 72-hour intervals. For the investigation of safety of repetitive CCI treatments, 2 animals received 6 transscleral CCI applications with BSS. Prior to the treatment, a baseline ophthalmic evaluation and electrophysiologic testing was carried out on all animals received. The pupils were dilated with topical 1% atropine and 2.5% neosynephrine. The left eye served as an internal control. ERG examinations were conducted before carboplatin treatment, after 2 treatments, and at 4 weeks after the conclusion of the treatment.

Six rabbits underwent 6 treatments of transscleral CCI of carboplatin (14 mg/ml) for 20 min at a current density of 5 mA/cm^2 at 72-hour intervals on the right eye with the left eyes serving as controls. Two animals received 6 transscleral CCI applications with BSS. After each treatment, the anterior segment, vitreous cavity, and fundus were examined by slit-lamp biomicroscopy and indirect ophthalmoscopy.

3.4.5. CCI treatment parameter evaluation on mice eye

Optimal current density and charge parameters were evaluated in eight, six-week-old, SV tag mice.

Treatments consisted of application of the custom CCI platinum positive electrode embedded in a polycarbonate material with silicone tubing to the mouse eye. **(Fig. 7)** The CCI eyecup placed on the eye, resting on the anterior sclera, abutting the corneal limbus (inner diameter 3.14 mm, average diameter of the mouse cornea 3.0 mm). The negatively charged electrode is attached to the 33 gauge needle inserted intramuscularly into the flank of the mouse during treatment, completing a closed circuit. carboplatin was dissolved in BSS to achieve the desired concentrations and resulted in a positively charged solution with a pH of 5.5.carboplatin solution was pumped continuously through the electrode at a rate of 0.5ml/min and a suction of 5 mmHg. Treatments were administered at current densities of 1.28, 2.57, 3.21, 3.85 and 5.14 mA/cm^2. Eyes were examined after treatment at 2, 5, and 10 minutes. Following treatment at these different intensities, eyes were examined through a slit lamp for conjunctiva, cornea and sclera

damage. At the conclusion of the study treatments, all animals underwent serial ophthalmologic examinations to evaluate for potential ocular toxicity.

Figure 7.

Autoclavable polycarbonate mice CCI eye cup for transcorneoscleral application.

3.4.6 Repetitive CCI treatments in transgenic retinoblastoma mice

Forty-four six week-old SV40 Tag mice underwent six serial CCI treatments two times per week. All treatments were administered at a controlled current of 2.57 mA/cm^2, for 5 minutes' duration. Thirty transgene–bearing mice were treated on the right eye with a total of six CCI treatments at concentrations of either 1.4, 7, 10 or 14mg/ml of carboplatin. Fourteen transgene-bearing mice were treated on the right eye with a total of six sham CCI treatments (no current) at concentrations of either 1.4, 7, 10 or 14mg/ml of carboplatin. Ten control animals received a total of 6 CCI treatments with BSS only as a safety study for repetitive treatment. Left eyes were always used as untreated controls.

3.5. Analyses Modalities

3.5.1. Rabbit ocular tissue dissection and preparation

Six animals, six eyes per time point were treated with either focal or systemically delivered carboplatin. Eyes were enucleated at 1, 2, 6 or 24 hours and placed on ice. Animals receiving focal delivery either subconjunctivally or iontophoretically, were treated in the right eye only. Left eyes were left untreated. The eyes were dissected using sterile instruments isolating the retina, choroid, and optic nerve. Samples were frozen at -80°C and weighed. Tissues were freeze-dried and dry weights recorded. All tissues were spiked with 20 µl of 16M concentrated nitric acid (Fisher Scientific Laboratories) and 180µl of 99.99% H_2O, and centrifuged at 15000 rpm for 20 minutes. At 8 hours, samples were diluted with 800 µl of 99.9% H_2O, and centrifuged again. The supernatant was extracted and placed in vials for atomic absorption spectroscopy analysis.

Vitreous was aspirated using a 1ml syringe and centrifuged for 20 minutes at 1700 rpm. Blood was drawn from an ear artery prior to enucleation. The blood was centrifuged at 1200 rpm for 10 minutes to isolate blood plasma. Samples (100 μl) were obtained, spiked with 20 μl of 16 M concentrated nitric acid (Fisher Scientific Laboratories) and 180 μl of 99.99% H_2O, and centrifuged at 15000 rpm for 20 minutes. At 8 hours, samples were diluted with 800 μl of 99.9% H_2O, and centrifuged again. The supernatant was extracted and placed in vials for atomic absorption spectroscopy analysis.

3.5.2. Electrothermal atomic absorption spectroscopy (ET-AAS)

Carboplatin, platinum metabolites and protein conjugates were determined in ocular fluids and tissues as total acid soluble platinum (Pt) using electrothermal atomic absorption spectrometry (ET-AAS). ET-AAS has been used in previous pharmacokinetic studies of cisplatin [205] and has been demonstrated to be a valid method for the determination of platinum compounds in biological fluids [206]. This method determines total Pt in fluids or tissues, including any forms of the drug subject to hydrolytic action and subsequently inactivated by irreversible binding to protein and thus rendered inactive of cytotoxic effect [207]. Atomic Absorption Spectroscopy was performed using a Perkin Elmer Model 4100 Zeeman Graphite Furnace Atomic Absorption Spectrophotometer equipped with an autosampler and using AA Winlab software for the Pt concentration determinations. Standards and samples were injected at a volume of 10μl onto the graphite tube at a temperature of 25°C. The instrument was set for drying at 100°C for 30 seconds, ashing at 1300°C for 30 seconds, and atomization at 2200°C for 6 seconds. A platinum hollow cathode lamp was used at a detection wavelength of 265.9nm, and a low slit setting of 0.7nm. Standards were run to create a calibration curve for platinum concentration in parts per billion. A polynomial fit for absorbance versus concentration was constructed. Stock solution was 970 μg/ml diluted with 2% HNO_3 1:100 to a concentration of 9.70 μg/ml. (Single element Pt standard, Ultra Scientific Inc.) Six standards were examined; blank, 0.05, 0.1, 0.2, 0.5, and 1.0/25 ml of 2% HNO_3.

3.5.3. Electroretinography - ERG

To record the ERGs, the active electrode was an Ag:AgCl wick electrode placed on the anaesthetized cornea. A needle placed subcutaneously on the head served as the reference electrode, and the animal was grounded by a needle inserted subcutaneously in the neck region.

The electrodes were connected to a Tektronix 3A9 preamplifier (Beaverton, OR) with the half-amplitude bandpass filter set at 0.1 Hz to 10 kHz. The ERGs were displayed on an oscilloscope and fed to a computer for signal averaging.

The light for the stimulus was obtained from a 150-watt quartz halogen lamp bulb. The filament of the halogen lamp bulb was brought to focus in the plane of a Uniblitz shutter (Vincent Assoc., Rochester, NY), and a pulse generator (Grass S44) was used to control the shutter. The filament of the halogen lamp was focused by another lens onto the tip of a fiber optic bundle and the other end of the bundle was brought into the Faraday cage containing the rabbit. The tip of the fiber optic bundle was placed 1-2 mm from the cornea.

The stimulus intensity was measured with the detector of a photometer (UDT Instruments, Orlando, FL) placed on the cornea. The maximum luminance was 1.59×10^3 cd/m^2, and neutral density (ND) filters were used to reduce the maximum stimulus intensity. The stimulus intensities are designated by the nominal value of the ND filters used to attenuate the full intensity stimulus.

After the animal was set up, the eye was darkness-adapted for 30 min and ERGs were recorded from threshold to the full intensity stimulus. The stimulus intensity was increased in 0.5 log unit steps at the lower intensities (ND = 4.0 to 2.5), and in 1.0 log unit steps for the higher intensities (ND = 2.0 to 0). The stimulus duration was 2.0 sec, and the interstimulus interval was lengthened with each increase in the stimulus intensity from 1.0 to 7 min in 1 min steps.
Signal averaging was done with the Biopac MP100 program (Goleta,CA), and two responses were averaged at the low intensities (at ND = 4.0 to 2.5) and one response at the higher intensities (at ND = 2.0, 1.0 and 0). The b-wave was measured either from the baseline or the trough of the a-wave to the peak of the b-wave.

3.5.4. Histopathologic evaluation of rabbits and mice eyes

In repetitive CCI administration on rabbits the animals were euthanized after completion of the final CCI application. In the carboplatin CCI administration on transgenic retinoblastoma mice, all treated animals were euthanized at the age of 16 weeks. For all used animals, mice and rabbits both eyes were enucleated and immediately immersion-fixed in 10% formalin. The eyes were sectioned serially and stained with hematoxylin-eosin. Histopathologic evaluation was performed by a masked observer and graded for evidence of corneal, scleral, lenticular or retinal toxicity. In the tumor control study the eyes were further graded positive for tumor development if any histopathologic evidence of tumor was present. Photographic studies were performed for documentation.

3.6. Statistical Analysis

3.6.1. Pharmacokinetic study performed on rabbits

Carboplatin concentrations were analyzed at all time points and compared for each tissue utilizing repeated measures analysis of variance. Concentration and time were analyzed as independent variables. Data were expressed in means and standard deviation of means (SD) to effect normality and homogeneity of variances. Statistical evaluation was performed on a logarithmic scale. Two-tailed t-test and Anova with Duncan's multiple range and the least significant difference post-hoc test examined the relation between concentrations; time course and treatment. A p-value <0.05 was considered significant.

3.6.2. Tumor control analysis on transgenic mice

Outcomes were analyzed and a logistic regression program was used for statistical modeling to calculate dose-response curves (LogXact, Cytel Software, Cambridge, Massachusetts). Carboplatin concentration and tumor burden were analyzed as independent variables.