# CHAPTER 7. Progress Toward Small-Molecule Activators of Voltage-Gated Ion Channels for Treatment of Visual Impairment Resulting from Photoreceptor Loss*

## 7.1 ABSTRACT

Vision loss in patients with age-related macular degeneration (AMD) and retinitis pigmentosa (RP) results from a loss of photoreceptors in the retina. While the photoreceptor neurons are lost, the retinal infrastructure of ganglion cells and other neurons remain intact. Previous work has shown that electrical stimulation of these remaining neurons by a microelectrode array implant can restore vision in RP patients. A collaboration was formed between laboratories at Caltech and USC to develop a small molecule alternative to the microelectrode arrays. This chapter describes our initial efforts to reach this goal by using functionalized $Ru^{2+}(bpy)_3$ complexes to activate a voltage-gated ion channel in a *Xenopus* ooctye model system.

## 7.2 INTRODUCTION

Age-related macular degeneration (AMD) is an aging-associated disease that affects vision in the center of the visual field, leaving patients with only peripheral vision. This affects the patient's ability to see objects clearly, complicating common daily tasks such as reading and driving. AMD is the leading cause of irreversible severe vision loss in people over 50 years of age in the United States.[1] Retinitis pigmentosa (RP) refers to a

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* *This work was done in collaboration with the labs of Dr. Mark Humayun (USC), Prof. Robert Chow (USC), Prof. Harry Gray (Caltech), and Prof. Robert H. Grubbs (Caltech).*

group of genetic eye conditions that lead to incurable blindness. AMD and RP affect millions of people worldwide and both result from loss of photoreceptors in the macula.[2]

The macula is located at the center of the retina, the light-sensitive tissue that lines the back of the eye.[3] The retina is comprised of several layers of neurons that are interconnected by synapses. Light sensitivity in the retina is conferred by specialized neurons called photoreceptors, which are further classified as rods or cones. Rods are more sensitive than cones, responding to fewer photons of light, thereby enabling vision in dim lighting. Cones facilitate daytime vision and color perception. In humans, three types of cone cells are responsible for color vision, each responding to a different wavelength of light. In total, the human retina contains ~120 million rods and ~5 million cones. The outer segment of each photoreceptor is lined with membranes that are stacked with a light-sensitive protein called opsin (a G-protein coupled receptor) that contains the pigment molecule retinal. Upon irradiation, the retinal pigment undergoes a photoisomerization that induces a conformational change in the opsin protein, which ultimately culminates in the hyperpolarization of the photoreceptors (they are depolarized in the dark) and a halt in the release of neurotransmitter glutamate, which either activates or deactivates other neurons in the retina. These neurons process the information received and ultimately transmit it to the retinal ganglion cells whose axons form the optic nerve. The optic nerve transmits information directly to the brain for final processing.[3]

In AMD, photoreceptors are lost due to abnormal blood vessel growth in the macula ("wet" AMD)[4] or atrophy of the pigment epithelial layer ("dry" AMD).[5] Retinal degeneration in RP diseases also results in photoreceptor loss, but from genetic causes. A

loss of photoreceptors means that the affected tissue is no longer sensitive to photons, accounting for vision loss in these patients.

While the photoreceptors are lost in these diseases, the underlying bipolar and ganglion cells remain intact and capable of transmitting information to the brain. Many studies have shown that electrical stimulation of the retina can induce percepts in visually impaired animals and human subjects, presumably by activation of ganglion or other retinal neurons.[6-9]

A team led by Mark Humayun, a physician/scientist at USC, has developed a retinal prosthesis system (now in production by Second Sight Medical Products, Sylmar, CA) that is based on these findings.[7] This prosthesis is comprised of a surgically implanted microelectrode array consisting of 60 electrodes, an inductive coil for transmitting power/data to the implant, a video processing unit (VPU) worn externally on a belt, and a small camera mounted externally on a pair of glasses. The device is designed to capture visual input from the camera, which then relays this information to the VPU that in turn digitizes and filters the signal to create a series of electrical stimulus pulses. These pulses are then delivered to the retina via the microelectrode array. In test studies, 96% (26/27) of RP patients implanted with these retinal prostheses show improvements in accuracy in spatial motor tasks and 93% (25/27) show improvements in the repeatability of these tasks, proving that electrical stimulation of the retinal neurons can restore vision.[7] In some cases, formerly blind patients are given enough visual acuity to shoot a basketball or retrieve a cup from a table.

In recent years the Humayun and Chow labs from USC and the Gray, Grubbs and Dougherty labs from Caltech formed a collaboration to develop a small-molecule

alternative to the retinal prosthesis developed by Humayun. Our strategy is to use a light-sensitive small molecule to stimulate retinal neurons by activating the voltage-gated ion channels (VGICs) that are contained within their cellular membranes. VGICs are a class of ion channels that are activated by local changes in membrane potential. Upon opening their ion-conducting pore, VGICs allow a rapid and coordinated depolarization of the cell (or hyperpolarization depending on the specific channel and cell). In the case of a ganglion cell, this would ultimately lead to the propagation of an electrical signal to the optic nerve, much like Humayun's microelectronics-based retinal implants.

We envisioned that a membrane-imbedded small molecule capable of photo-induced electron transfer could be used to produce a small local change in membrane potential that could activate a nearby VGIC, ultimately leading to the productive cascade of electrical signaling that results in vision. In this scenario, the lost photoreceptors are essentially replaced with a light-sensitive small molecule.

We first sought to test this theory in a model system. In this chapter, the Shaker IR (ShIR) $K^+$ channel heterologously expressed in *Xenopus* oocytes is used as our testbed. Several $Ru^{2+}(bpy)_3$ -based small molecules were tested for their ability to activate ShIR upon irradiation. A useful assay was developed for testing the effectiveness of these compounds. Future work, conducted by Erin C. Lamb of the Dougherty lab, will use this assay to screen promising small molecule candidates.

## 7.3 PROGRESS

The ShIR $K^+$ channel is a voltage-gated potassium channel that is readily expressed in *Xenopus oocytes*. ShIR is a truncated version of the Shaker B channel discussed in

Chapter 6, where IR stands for "inactivation domain removed" because a large portion of the N-terminal tail is removed to prevent N-type inactivation.[10-12] Like its parent channel, ShIR is a tetramer composed of four identical subunits, each consisting of six transmembrane segments (labeled S1-S6). The fourth membrane-spanning segment (S4) of the Shaker proteins contains several Arg and Lys residues that are likely to carry a positive charge at physiological pH. The preceding segments (S1-S3) contain several negatively charged residues. Collectively, S1-S4 segments form a voltage-sensing domain that, by virtue of the location of their many charged residues, sense local changes in membrane potential. Upon sensing these changes, it is thought that the S4 domain moves, resulting in a conformational change in the Shaker protein that culminates in pore opening, allowing ions to flow across the membrane to restore the resting membrane potential.[13]

Our goal was to place a small molecule that is capable of light-induced electron-transfer in the vicinity of the voltage-sensing domain of the membrane embedded ShIR protein expressed in *Xenopus oocytes* (**Figure 7.1**). The first systems we chose to work with were based on $Ru^{2+}(bpy)_3$ as shown in **Figure 7.2**. The Gray lab had previously prepared these complexes for other chemical biology applications. These systems contain long alkyl chains, which could potentially associate with cellular membrane of the oocytes, hopefully positioning the $Ru^{2+}(bpy)_3$ in the vicinity of ShIR channels. We were particularly optimistic about **[Ru] 3** (**Figure 7.2**) because it contained two carboxylate groups, which should help with the water solubility of the complex, facilitate the orientation of the alkyl chains into the membrane (and the negatively charged carboxylates away from the phosphate head groups), and also give the overall complex a

net formal charge of 0. The theory is that upon irradiation, the $Ru^{2+}$ is reduced to $Ru^{0}$ by a small molecule reductive quencher, which would create a local change in electron density of the media that could potentially be sensed by the S1-S4 voltage sensor of the ShIR protein. This process would culminate in receptor activation (ion channel opening), which we planned to measure by standard two-electrode voltage-clamp electrophysiology.

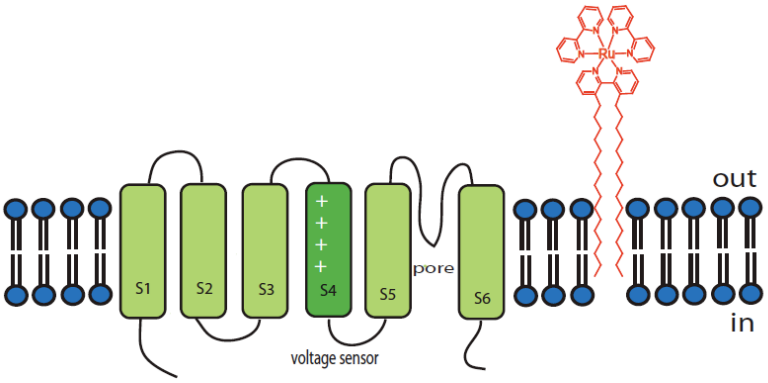
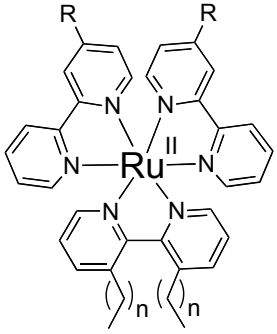



**Figure 7.1.** Depiction of experimental design for studies with **[Ru] 1**, **2,** and **3**. The alkyl chains of [Ru] embed into the ooctye membrane in the vicinity of the ShIR protein.




R = H; n = 11 **[Ru] 1**
R = H; n = 15 **[Ru] 2**
R = $CO_2^-$ n = 11 **[Ru] 3**

**Figure 7.2.** Structure of $Ru^{2+}(bpy)_3$ complexes with alkyl chains (**[Ru] 1**, **2**, and **3**).

To test this theory, *Xenopus* oocytes expressing ShIR were incubated with micromolar concentrations of $Ru^{2+}(bpy)_3$ complexes for various time periods (15 min–24 hrs). Voltage-clamp recordings were then conducted on an electrophysiology rig fitted

with an appropriately filtered Hg/Xe arc lamp. During recordings, the cells were exposed to buffered solutions containing small molecule reductive quenchers (4-methoxy-*N*,*N*-dimethylaniline, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, ascorbate or a combination of ascorbate with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) in the presence or absence of appropriately filtered UV light (~460 nm to excite $Ru^{2+}(bpy)_3$). Importantly, the ooctyes survived multiple hours of manipulation in the presence of the $Ru^{2+}(bpy)_3$ complexes (they maintained their shape and healthy resting potentials). Under the conditions tested, however, no current from the ShIR channel was observed in the presence of the $Ru^{2+}(bpy)_3$, quencher and filtered light, suggesting that these systems did not activate ShIR. Similar experiments were simultaneous conducted at USC in Prof. Robert Chow's lab. In these experiments, several types of mammalian cells (CHO and HEK cells) were used in place of the *Xenopus* oocytes. Unfortunately, these cells were not as tolerant to the $Ru^{2+}(bpy)_3$ complexes as were our oocytes, which made voltage-clamp recordings difficult. The membranes of these cells did stain with **[Ru] 1** in confocal studies, suggesting that the alkyl chain was successfully imbedding into the cell membrane.

We then wondered whether the tethered $Ru^{2+}(bpy)_3$ were being positioned in the membranes close enough to the ShIR voltage sensors. To circumvent this potential issue, we turned to a strategy that involved covalent attachment of the $Ru^{2+}(bpy)_3$ complex to the voltage sensor by Cys modification (**Figures 7.3** and **7.4**). Such linkages require the presence of a free Cys residue at an appropriate position in the protein. MTS labeling experiments done by Isacoff and co-workers showed that two residues, Ala359 and Met356, in the S3-S4 linker are solvent accessible, suggesting that they would be

available for covalent modification by our $Ru^{2+}(bpy)_3$ complexes.[14] These sites are just 3-6 residues from the first Arg in the S4 domain (Arg362), and so it was thought that they would be at a prime location for positioning our complexes. As such, we mutated both residues to Cys. To determine whether either mutation affected the function of the channel, current-voltage (I-V) relationships were determined at several membrane potentials (**Figure 7.5**). These experiments clearly showed that at each set membrane potential, the corresponding current responses given by the mutant voltage-gated ShIR channels were equivalent to that of the wild-type receptor.

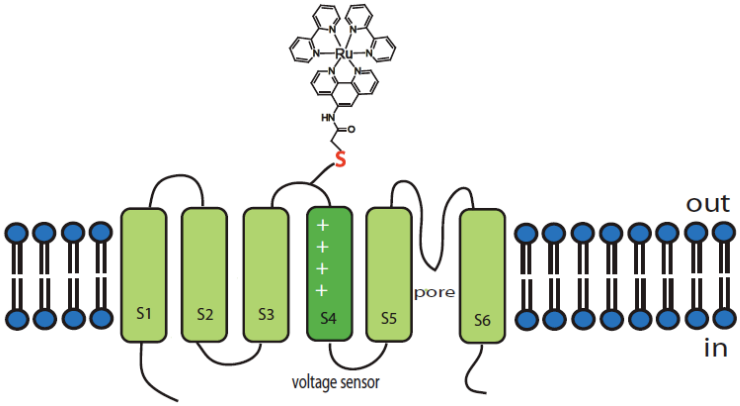
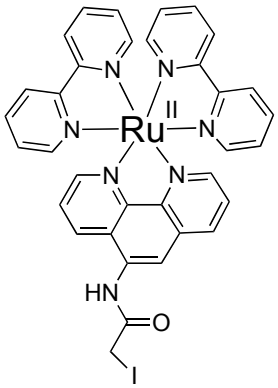



**Figure 7.3.** Depiction of experimental design for studies with **[Ru] 4**. The [Ru] complex covalently links to Ala359Cys or Met356Cys via an acetimide linkage.




**[Ru] 4**

**Figure 7.4.** Structure of **[Ru] 4**. The iodoacetimide group reacts with free cysteines.

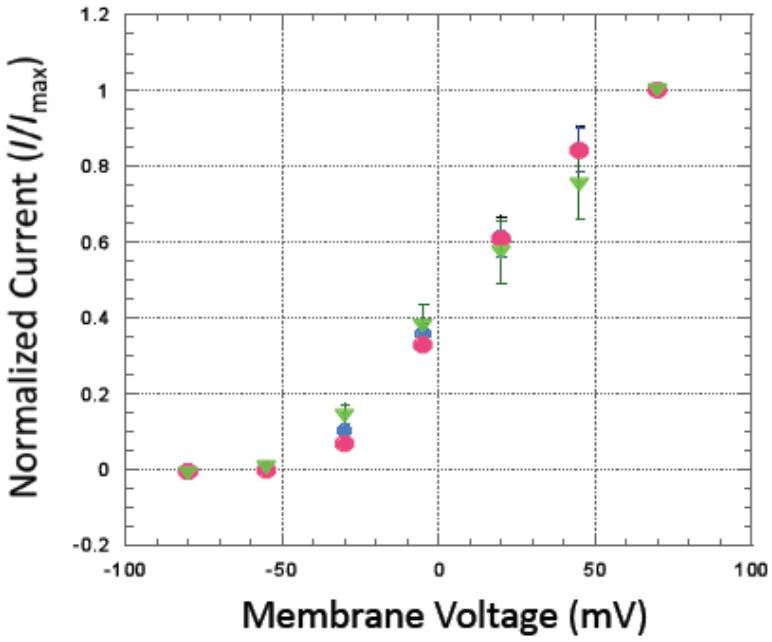



**Figure 7.5.** Current-voltage relationships of wild-type (WT) ShIR and ShIR mutants. Green triangles are WT ShIR; pink circles are the ShIR Met356Cys mutant; and blue circles are the ShIR Ala359Cys mutant.

At this point it was realized that this comparison could be a useful assay for measuring the impact of the $Ru^{2+}(bpy)_3$ complexes. Should our experimental design make the channel gate more readily, the I-V relation should shift to the left (toward smaller membrane potentials). If on the, other hand, the channel became less sensitive to changes in membrane potential, then the I-V relation should shift right. This is a much more convenient and reliable assay than simply looking for current in voltage-clamp recordings, as a number of other factors including receptor function or cell health could affect this measurement.

We then attempted to covalently append **[Ru] 4** to Ala359Cys or Met356Cys via the iodoacetimide. Unfortunately, these experiments never yielded any changes in the I-V relation despite the many conditions and quenchers that were tried. Similar results were seen with the mammalian cells studied in the Chow lab.

## 7.4 FUTURE DIRECTIONS

Given these results, it seemed prudent to confirm the hypothesis that appending a molecule bearing a negative charge to the either Ala359Cys or Met356Cys could influence activation of the receptor. To do this, we envisioned using charged MTS reagents and monitoring I-V curves. This work was taken over by the capable hands of Erin C. Lamb. She has shown that covalent appendage of charged MTS reagents at Ala359Cys and Met356Cys can influence receptor activation, but not in the direction that we had anticipated. She is now looking into repeating experiments with the **[Ru] 1-3** using the new assay and is also looking into modifying the alkyl chains to include *cis* double bonds, as recent reports[15, 16] have suggested that *cis*-unsaturated fatty acids (but not saturated or *trans*-unsaturated fatty acids) can influence ShIR channel gating.

## 7.5 EXPERIMENAL SECTION

These studies used a Shaker IR cDNA in the pBSTA vector that contains a T449V mutation (to limit C-type inactivation) and a FLAG tag. Conventional mutagenesis was performed by the standard Stratagene QuickChange protocol and verified through sequencing. cDNA was linearized with the restriction enzyme NotI and mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion). Stage V-VI *Xenopus laevis* oocytes were injected with 1 ng of mRNA per oocyte in a single 75 nL injection. Oocytes were incubated at 18 °C for 15-24 hours after injection. Currents from two-electrode voltage clamp electrophysiology were recorded by an OpusXpress 6000A instrument (Axon Instruments) or Geneclamp 500 amplifier (Axon Instruments) at a holding potential of −80 mV. The latter system is equipped with a 500 W Hg/Xe arc

lamp that is connected to the rig's bath by a liquid light guide. The lamp's shutter can be opened to bathe the oocyte in light. The running buffer was a $Ca^{2+}$ free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Currents were measured during depolarizing jumps from the holding potential to +70 mV in 25 mV increments.

**[Ru] 1-3** were dissolved in DMSO and diluted into $Ca^{+}$ free ND96 containing gentamycin to a total volume of <1 % DMSO and a final concentration of [Ru] ranging from 1 nM to 100 μM. Ooctyes were incubated with these solutions for 15 min to 24 hrs prior to electrophysiological recordings. Small molecule quenchers (4-methoxy-*N*,*N*-dimethylaniline, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, and ascorbate) were dissolved in the ND96 running buffer at a concentration 10-100-fold higher than the [Ru] complexes in the original incubation media. Ooctyes were irradiated during electrophysiological recordings using the 500 W Hg/Xe arc lamp system described above and appropriate wavelength cutoff filters (focused at 460 nm). Obvious controls were run (quencher only, [Ru] only, DMSO only, with and without irradiation, etc.) to ensure the fidelity of our experiments.

## 7.6 ACKNOWLEDGEMENTS

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