TRPswitch — a step function chemo-optogenetic ligand for the vertebrate TRPA1 channel

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ABSTRACT: Chemo-optogenetics has produced powerful tools for optical control of cell activity, but current tools suffer from a variety of limitations including low unitary conductance, the need to modify the target channel, or the inability to control both on and off switching. Using a zebrafish behavior-based screening strategy, we discovered “TRPswitch”, a photoswitchable non-electrophilic ligand scaffold for the transient receptor potential ankyrin 1 (TRPA1) channel. TRPA1 exhibits high unitary channel conductance, making it an ideal target for chemo-optogenetic tool development. Key molecular determinants for the activity of TRPswitch were elucidated and allowed for replacement of the TRPswitch azobenzene with a next-generation azoheteroarene. The TRPswitch compounds enable reversible, repeatable, and nearly quantitative light-induced activation and deactivation of the vertebrate TRPA1 channel with violet and green light, respectively. The utility of TRPswitch compounds was demonstrated in larval zebrafish hearts exogenously expressing zebrafish Trpa1b, where heartbeat could be controlled using TRPswitch and light. Therefore, TRPA1/TRPswitch represents a novel step-function chemo-optogenetic system with a unique combination of high conductance, high efficiency, activity against an unmodified vertebrate channel, and capacity for bidirectional optical switching. This chemo-optogenetic system will be particularly applicable in systems where a large depolarization current is needed or sustained channel activation is desirable.

Introduction

Optogenetics has proven to be a transformative technology for various fields of basic research, particularly in neuroscience. It allows for a non-invasive, localized, and temporally selective optical modulation of selected cells within an animal. Optogenetic technologies hold great promise for clinical applications. For example, preclinical animal models have demonstrated potential utility in treating retinitis pigmentosa with AAV-delivered channelrhodopsin2 (ChR2).¹ However, high expression of the light-gated ion channel ChR2, originating from green algae, has cytotoxic effects.² It remains unclear whether or not expression of ChR2 in humans will result in immune rejection or inflammation. Therefore, to advance the utility of optogenetics in clinical applications, development of optogenetic actuators that are vertebrate in origin or endogenous to humans, as well as those based on ion channels with high unitary conductance, might be advantageous. The use of vertebrate protein actuators might reduce the risk of immunological reactions due to long-term expression of exogenous proteins. High

conductance actuators should also decrease the amount of channel expression needed for sufficient light-controlled activity. Some progress has been made toward these goals, including the development of an endogenous protein-targeting photoswitch that confers light-sensitivity on endogenous neuronal ion channels, proposed to have the potential to restore vision to the blind.³⁻⁵

Discovery of new optogenetic actuators that possess novel and unique properties will undoubtedly enhance our ability to dissect biological systems such as the complex neuronal networks of the brain. For example, optogenetic experiments that require long activation periods would benefit greatly from “step function” optogenetic tools that allow stable, bi-directional on and off switching of channels. Step function opsins (SFOs) are engineered versions of ChR2 that persist in their open state after illumination ceases.⁶⁻⁸ As such, they are useful in modifying the spontaneous firing rate of neurons, as well as in applications where behavioral analysis without continuous optic fiber tethering is desired.⁹⁻¹⁰ However, as

mentioned above, the non-vertebrate origin of these opsins present potential challenges in clinical applications. As a complementary approach, chemo-optogenetic tools that combine chemicals and optogenetics have been under rapid development. Using a chemical design approach, photochromic soluble ligands (PCLs) for various wild-type ion channels have been synthesized to allow for light-controlled channel activity.¹¹⁻¹⁵ These photoswitchable PCLs block and unblock their

corresponding ion channel either in their *E* or *Z* configuration. Examples include PCLs for the TRPV1 channel,¹¹ NMDA receptor,¹⁶⁻¹⁷ and kainate receptor.¹⁸⁻¹⁹

TRPA1 is a member of the Transient Receptor Potential (TRP) channel family. It is a non-selective cation channel that plays an important role in inflammatory and neuropathic pain, itch, and respiratory diseases.²⁰⁻²² We have previously reported

Figure 1. Photoswitching of TRPswitch-A activates the Trpa1b channel. (a) Schematic diagram of the behavioral screening assay setup. Screening was performed using a 96-well plate format. 3 wild-type zebrafish larvae at 3 days post fertilization (dpf) were placed in each well. Individual small molecules were added to each well. A series of different wavelengths of light from WL1 – WL4 were applied to each well and the motion activity from the well was recorded. WL1, 450 nm – 500 nm; WL2, 415 nm – 455 nm; WL3, 352 nm – 402 nm; WL4, white light. The screening assay was performed on a motorized inverted compound microscope. A hit is defined as having motion in the well after photoactivation. (b) Image of one well on the 96-well plate with 3 zebrafish larvae. (c) Line graph showing the zebrafish behavioral response of TRPswitch-A treated animals. The vertical bars indicate the timing of a 1 s light stimulus at the indicated wavelength. (d) Electrophysiology analysis of TRPswitch-A in HEK293T cells expressing zebrafish Trpa1b. Current density-Voltage relationships of Trpa1b current without treatment (black), 10 μ M TRPswitch-A with violet light stimulus (magenta), and then followed by green light stimulus (green). (e) Isomerization of TRPswitch-A as indicated by the absorbance profile of TRPswitch before irradiation (black dotted line), after violet light (352 - 402 nm) irradiation (magenta line), and subsequently after green light (500 - 600 nm) irradiation (green line). (f) Basal zTrpa1b currents (black) are activated (blue trace) and subsequently desensitize (orange trace) with AITC.

photoactivable ligands for TRPA1, such as optovin, and demonstrated their ability to act as chemo-optogenetic tools.²³⁻²⁴ TRPA1 has a channel conductance of approximately 100 pS,²⁵ 1000 times greater than ChR2, making it ideal for applications where high conductance or low expression levels are desired. Importantly however, while photochemical stimulation of optovin-class chemo-optogenetic ligands activate TRPA1 rapidly (in low millisecond time scales), channel deactivation depends upon spontaneous (i.e. non-photochemical) reversal of ligand action, which occurs on the time scale of seconds. In other words, the approach is somewhat akin to photodecaging, where a 'protected' ligand is activated ('deprotected') following a light stimulus,²⁶ but where deactivation requires spontaneous and often slow dissipation of the ligand. It would be a great advancement to develop a chemo-optogenetic system that preserves the high conductance of TRPA1 and the rapid activation of optovin-class ligands, but enables rapid, light-controlled channel deactivation: a photoreversible/photoswitchable system.

In this study, we used a customized, light-responsive chemical library coupled with a behavior-based screening assay with zebrafish larvae to discover 'TRPswitch' azoarene photoswitchable ligands for the TRPA1 channel. Our TRPswitch molecules allow for optical control of both the activation and deactivation of the TRPA1 channel. Our analysis suggests that the zebrafish Trpa1b channel is necessary and sufficient for TRPswitch light-induced activity. Channel activation and deactivation can be controlled by violet light and green light illumination, respectively. The TRPswitch/light-induced TRPA1 channel activity is reversible and repeatable *in vivo*, and sustained channel activation is achieved after only a short pulse of light illumination. To our knowledge, this is the first time a photoswitchable TRPA1 system has been described. Our data show that this TRPA1/TRPswitch system is a robust chemo-optogenetic tool that can be applied to both neuronal and non-neuronal cells. The TRPA1/TRPswitch system's step function properties, along with its high

unitary conductance, make it a complementary alternative to existing chemo-optogenetic tools.

Result

Zebrafish behavior-based chemical screening identifies TRPswitch-A

With the discovery of photochromic soluble ligands (PCLs) as a means to control ion channel function using light,¹¹⁻¹⁵ we reasoned that molecular photoswitches for TRPA1 could be discovered using small molecule screening. To achieve this goal, we developed a modified version of the behavioral assay that we previously utilized to identify photoactivable, but non-photoreversible, ligands of TRPA1.²³ This medium-throughput, semi-automated screening assay was performed using a 96-well plate format where three larvae per well were incubated with small molecules. We screened a library of 1,000 structurally diverse small molecules enriched for molecular photoswitch moieties such as acylhydrazone,²⁷ azobenzene,²⁸⁻³¹ azoheteroaryl³²⁻³³ and stilbene.^{31, 34} We particularly focused on molecular photoswitches that operate via a reversible *E/Z* isomerization using different wavelengths of light. In this new assay, each well of a 96-well plate is illuminated with a series of different wavelengths of light (450-500 nm, 415 – 455 nm, 352-402 nm and white light) for one second to induce isomerization of the photoswitchable compounds (Fig. 1a). Each illumination event is separated by a dark period of 5 s and the motion of larvae during this light illumination sequence is recorded and analyzed (Fig. 1b). Wild-type 3-days-post-fertilization larvae (dpf) were used, as they have a relatively developed central nervous system and show no motion response to light exposure at this stage of development. Zebrafish larvae express Trpa1b in a subset of trigeminal and Rohon-Beard sensory neurons.³⁵ Activation of Trpa1b induces a reproducible and robust motion response.^{23-24, 35-36} A light-induced motion response, due to the presence of a photoactivated ligand for ion channels such as Trpa1b, is used as the readout for the assay. DMSO treated larvae on each screening plate served as negative controls. Using this screening assay, we identified TRPswitch-A, an azobenzene containing

small molecule with no previously annotated biological activity. The presence of TRPswitch-A led to a light-induced motion response across multiple wavelengths, with the most activity in a bandwidth between 415 nm - 455 nm (WL2 in Fig. 1c). Using whole-cell patch-clamp recordings of HEK293T cells expressing zebrafish Trpa1b, we characterized the photocurrents elicited by the Trpa1b/TRPswitch-A pair upon light stimulation. Violet light stimulation of TRPswitch-A-primed Trpa1b channels generated high amplitude currents when compared to baseline measurements (Fig. 1d). When the same cell was subsequently stimulated with green light, Trpa1b photocurrent recovered to its baseline current magnitude (Fig. 1d). This reversible light response corresponds to the reversible *E/Z* isomerization of TRPswitch-A observed upon illumination with violet and green light, as judged by UV-Vis absorbance measurements (Fig. 1e). Expression of functional zTrpa1b channels in HEK293T cells was confirmed by the observance of allyl isothiocyanate (AITC)-activated currents (Fig. 1f), as AITC is a potent TRPA1 agonist. We also observed desensitization of AITC-induced current over time, further confirming the activity of zTrpa1b channels (Fig. 1f, S1). Overall, these data indicate that TRPswitch-A is a light-dependent and light-reversible activator of Trpa1b.

TRPswitch-A structure activity relationship analysis

The TRPswitch-A chemical structure contains an azobenzene and a 2-furamide group in both the *ortho* and *para* positions of one of the benzene rings (Fig. 2, compound **1**). It is clear that while TRPswitch-A undergoes reversible *E/Z* isomerization upon illumination with violet and green light, the *Z-E* photoswitching event

is incomplete (Fig. 1e). We sought to determine the chemical features responsible for TRPswitch-A's biological activity and how photoswitch performance correlates to the biological effects observed. We therefore designed, synthesized and analyzed the structure activity relationships of key derivatives of TRPswitch-A. We found that the *para* amide is needed for Trpa1b activation as suggested by the reduced activity of compound **2** (Fig. 2). Substituting the furan for thiophene was tolerated (Fig. 2, compound **3**), albeit with a slightly lower photoresponse which might be due to the overall chemical structural change. Derivatives bearing *para* electron-withdrawing groups – to increase the “push-pull” character³⁷⁻³⁸ – were prepared (compounds **4**, **5**, **6**, **7**) and found to be far less active in the assay (Fig. 2). This poor activity may, in part, be ascribed to the incomplete *E-Z* photoswitching of these compounds (Fig. S2 and Supp. Table 1). We have previously found that azoarene performance can be improved/tuned by substituting one of the benzene rings in a conventional azobenzene for a 5-membered heteroaromatic ring.^{32, 39-40} Specifically, we have found the azopyrazoles show near quantitative photoswitching in both directions and exhibit long *Z*-isomer thermal half-lives. Replacement of the phenyl ring in compound **1** (TRPswitch-A) and compound **3** with a trimethylpyrazole generates compounds (**8** and **9**) that undergo quantitative photoswitching in both directions (Fig. 3f) and that have long thermal half-lives. The thermal *Z*-isomer half-life of compound **9** (TRPswitch-B), in DMSO-*d*₆, is 17 hours (Fig. 3a). While we observed some aqueous solubility issues for compound **8**, which likely limits its biological response, compound **9** (TRPswitch-B) has a comparable biological response to TRPswitch-A.

Figure 2. TRPswitch structure activity relationship analysis. The structure of individual compounds and their *in vivo* activity are shown in the bar graph side by side for comparison. The assay was performed as described in Fig. 1. Each bar in the graph represents the motion response after photoactivation using individual wavelength sets as indicated in the lower x-axis. WL1, 450 nm – 500 nm; WL2, 415 nm – 455 nm; WL3, 352 nm – 402 nm; WL4, white light. 1% DMSO was used as a control. Values are means $\pm$ SEM from at least 3 experimental setups. Parenthetical values represent the number biological replicates. Compounds with robust biological activity are labeled in red.

Table 1. Photostationary state (PSS) of TRPswitches at 365 nm and 495 nm determined by UV-Vis spectroscopy in 30% water:DMSO.

Compound	Estimated 365 nm PSS (<i>E-Z</i>)	Estimated 495 nm PSS (<i>Z-E</i>)
TRPswitch-A	86% <i>Z</i>	73% <i>E</i>
TRPswitch-B	92% <i>Z</i>	100% <i>E</i>

To further characterize the thermal half-lives of TRPswitches in conditions that more closely resemble those used in biological experiments, we measured their thermal half-lives at higher water contents (30% water:DMSO solutions). We found that the thermal half-lives of TRPswitch-A and -B are 43 min and 1 hour, respectively. When measurements were made in DMSO-

d_6 , the half-lives for TRPswitch-A and -B were 11 and 17 hours, respectively.

To further characterize the photochemical properties of TRPswitches, the photostationary state (PSS) of TRPswitches in 30% water:DMSO solutions were examined via UV-Vis. By extrapolating a pure *Z* spectrum, using methodology outlined in previous publications,^{32, 39, 41} we approximate the PSS compositions to be 86% *Z* at 365 nm

and 73% *E* at 495 nm for TRPswitch-A; and 92% *Z* at 365 nm and 100% *E* at 495 nm for TRPswitch-B (Table 1). In addition, we also examined the PSS of TRPswitches in DMSO- d_6 at 365, 420 and 495 nm (Supp. Table 2, Figs. S11-16). From our analysis, TRPswitch-B demonstrates

superior, near quantitative photoswitching compared to the azobenzene analogue, at least under the conditions tested. These data suggest that photoswitch performance, particularly *Z*-isomer half-life, contributes to the activity and that both azobenzene

Figure 3. Properties of TRPswitch. (a) Thermal kinetics of TRPswitch-A and TRPswitch-B at room temperature. The natural log of the *Z*-isomer percentage is plotted against time. (b) Activity of TRPswitch requires Trpa1b. Bar graph showing the average light-induced motion response of the different treatment groups. WT, Wild-type; Trpa1b^{-/-}, Trpa1b mutant. Values are means $\pm$ SEM from 3 experiments. (c) Line graph indicating the positive correlation between the percentage of wild-type larvae that were pretreated with TRPswitch and showed light-induced motion response with increasing duration of light stimulus. Values are means $\pm$ SEM from 3 experiments. (d) Bar graph showing the response latency (time from the beginning of light pulse to the first motion response) with various intensities of light stimulation as indicated. Values are means $\pm$ SEM 3 experiments. (e) Dose response curve showing TRPswitch's effects on animal behavior ($n = 3$). (f) Isomerization of TRPswitch-B as indicated by the absorbance profile of TRPswitch before irradiation (black dotted line), after violet light (352 - 402 nm) irradiation (magenta line), and subsequently after green light (500 - 600 nm) irradiation (green line). (g-h) Electrophysiology analysis of TRPswitch. Representative peak whole cell Trpa1b current densities measured at -100 mV (blue trace) and +100 mV (black trace) with TRPswitch-A (g) and TRPswitch-B (h) treatment. Violet light and green light illumination were applied at the time indicated by the

Table 2. Activity of TRPswitch-B on Trpa1b ohnolog and orthologs.

	Trpa1b ^{-/-} with transient expression of				Trpa1b ^{-/-}	WT
	zTrpa1b	zTrpa1a	mTRPA1	hTRPA1		
% response	38.5	0	0	0	0	65
N	26	16	14	42	16	20

Rescue experiments were performed in Trpa1b^{-/-} zebrafish with transient mosaic re-expression of zebrafish Trpa1b (zTrpa1b), zebrafish Trpa1a (zTrpa1a), mouse TRPA1 (mTRPA1) or human TRPA1 (hTRAP1) in Rohon-beard neurons. Embryos were pre-treated with 20 μM TRPswitch-B and decapitated right before light stimulation to elimination the light response mediated by the retina. % response is the percentage of embryos exhibited light-induced motion response within 5 s after the 1 s WL3 illumination. Only the re-expression of zTrpa1b in Rohon-beard neurons in Trpa1b^{-/-} embryos rescued the TRPswitchB-mediated light-induced motion response. N, number of embryos tested.

(TRPswitch-A) and azoheteroaryl (TRPswitch-B) moieties can be used as PCLs of Trpa1b, with the latter appearing to have an improved intrinsic photoswitch performance. Additionally, we have obtained the X-Ray crystal structure of TRPswitch-B (Fig. S3). Notably, this compound crystallizes in a chiral space group, with the azo bridge orientated to take advantage of a favorable hydrogen bonding interaction from the ortho amide.

TRPswitch is a reversible photoswitch ligand for Trpa1b

We previously identified optovin as a photoactivable ligand for the Trpa1b channel, and confirmed that its activity is abolished in Trpa1b mutant zebrafish.²³⁻²⁴ To determine if Trpa1b is necessary for TRPswitch's biological activity, we performed a behavioral assay using Trpa1b mutant larvae.³⁵ When Trpa1b mutant larvae were used, the TRPswitch light-induced motion response was abolished (Fig. 3b). This suggests that Trpa1b is required for the activity of TRPswitch *in vivo*, consistent with our electrophysiology analysis (Fig. 1d). Both TRPswitch-A- and TRPswitch-B-treated larvae showed a higher probability of light-induced motion response as the light stimulation duration increased (Fig. 3c). The probability for larvae to respond to light reached its maximum with a <1 s light-pulse length. The biological response triggered by TRPswitch/Trpa1b activation was very rapid (Supp. Movie 1). The latency to motion response from the introduction of light was in the range of milliseconds and decreased with increasing light intensity (Fig. 3d). A positive correlation was observed between the activity of TRPswitch and an increase in compound concentration, with maximum activity achieved at concentrations between 10 – 20 μM (Fig. 3e). Similar to TRPswitch-A, TRPswitch-B undergoes reversible *E/Z* isomerization upon illumination with violet and green light. However, unlike TRPswitch-A, the *Z*-to-*E* conversion by green light leads to complete return to the pre-illumination "off" state (Fig. 3f).

Next, we turned to characterize the kinetics of Trpa1b channel activity. However, in the behavioral screening

assay, the light-induced motion response does not require sustained channel activation, i.e., the duration of motion does not directly correlate with the half-life of the TRPswitch *Z* state. To characterize the stability and kinetics of Trpa1b/TRPswitch-dependent photocurrents, we recorded zTrpa1b activity in HEK293T cells. The light-induced activation and deactivation of Trpa1b/TRPswitch was triggered with violet and green light pulses, respectively (Fig. 3g and 3h). Importantly, the photocurrents were sustained after the initial 10 sec pulse of violet light illumination and did not require continuous illumination. The photocurrents could be converted back to baseline using a subsequent 10 sec pulse of green light. The current density fold increments of TRPswitch-A and TRPswitch-B upon violet light illumination were 2.4 ± 0.5 and 2.3 ± 0.6 at +100 mV and 4.4 ± 1.4 and 2.3 ± 0.4 at -100 mV, respectively. A subsequent pulse of green light reduced the current density fold increments of TRPswitch-A and TRPswitch-B to 1.2 ± 0.2 and 1.3 ± 0.3 at +100 mV and 3.7 ± 1.3 and 1.4 ± 0.4 at -100 mV, respectively. Together, these data indicate that TRPswitch-A and TRPswitch-B are novel, reversible photoswitches that act on the Trpa1b channel. Overall, the light-activated currents with TRPswitches are comparable to those induced by the canonical TRPA1 agonist AITC (Fig. 3i).

To test the potential cross activity of TRPswitch-B on the zebrafish ohnolog Trpa1a and on mammalian orthologs, we transiently re-expressed zebrafish Trpa1a, mouse TRPA1 or human TRPA1 in the Rohon-beard neurons of mutant Trpa1b^{-/-} zebrafish and performed light-induced motion response experiments in the presence of TRPswitch-B. Only re-expression of Trpa1b rescued the light-induced motion response of Trpa1b^{-/-} mutants (Table 2). Our results suggest that TRPswitch-B is specific to the zebrafish Trpa1b channel.

TRPswitch's mechanism of action

Most known activators of the TRPA1 channel are electrophilic ligands that covalently modify cysteines in TRPA1's cytoplasmic domain.⁴² Optovin, our previously identified photoactivable TRPA1 ligand, reacts with those

cysteines through a photochemical reaction involving the generation of singlet oxygen species. DABCO, a singlet oxygen quencher and triplet energy acceptor can completely suppress the optovin response.²³ To determine whether or not TRPswitch is also photoactivated in a singlet oxygen-based mechanism, we tested the ability of DABCO to suppress TRPswitch activity and found that it did not (Fig. 3j). These data suggest that light-induced generation of singlet oxygen is not necessary for TRPswitch's behavioral effect. As the TRPswitches contain either azobenzene or azopyrazole groups, it is likely that their activity is due to the *E/Z* isomerization of the compounds upon light illumination (Fig. 1e and 3f), without the production of radicals. These observations suggest that both TRPswitch-A and -B are

reversible photoswitches for the Trpa1b channel and have a distinct mechanism of activation as compared to electrophilic ligands.

TRpa1b/TRPswitch allows cellular activation in non-neuronal cells

To demonstrate the practical utility of TRPswitch *in vivo*, we performed a heartbeat interruption experiment using a zebrafish transgenic line expressing Trpa1b in cardiomyocytes, *Tg(cmlc2:Trpa1b-2A-EGFP)*, and by applying pulses of violet and green light to the zebrafish heart. Since proper calcium handling is important for normal heartbeat, we used heartbeat as the biological readout for the activation and deactivation of Trpa1b channels. During regular

larvae *in vivo*. Experiments were performed on *Trpa1b^{-/-}* larvae expressing Trpa1b in cardiomyocytes. Larvae were pre-incubated with TRPswitch for 1 h in the dark before experimental manipulation. (a) Image of zebrafish heart showing the ventricle chamber and the position where ventricle width measurements were made and displayed in panel b. (b) Representative line graph showing the ventricle width over time in larvae treated with TRPswitch-B. The magenta and green vertical rectangles indicate photoactivation with 1 s violet light and green light, respectively (Supp. Movie 2). (c-d) Repetitive stopping and re-starting of ventricle heart contractions by violet and green light, respectively. Dotted plot showing the ventricle contraction frequency in individual larvae illuminated with a series of light flashes as indicated in the x-axis. Each dot represents measurement from one larva. Larvae were treated with 20 μ M TRPswitch-A (c, Supp. Movie 3) or TRPswitch-B (d, Supp. Movie 4). (e) TRPswitch induced a sustained heart contraction after a brief pulse of violet light. Transgenic zebrafish expressing Trpa1b in cardiomyocytes were pre-treated with 20 μ M TRPswitch. 1 s violet light was applied to the heart and the ventricle heart diastolic width ratio (comparing the diastolic width before and after photoactivation) was quantified. Values are means $\pm$ SEM from 3 experiments. In all panels, parenthetical values represent the number biological replicates.

heartbeat cycles, an increase in intracellular calcium is required for cardiomyocyte contraction as calcium binding to troponin C leads to a conformational change that displaces tropomyosin from the actin binding sites. Calcium levels must then decrease for cardiomyocyte relaxation as calcium prevents tropomyosin from returning to its original conformation. We hypothesize that a sustained elevation of intracellular calcium, such as with TRPA1 activation, will result in a sustained tetanic contraction of the heart and interrupt rhythmic beating. In transgenic larvae treated with either TRPswitch-A or TRPswitch-B, we found that the ventricle heartbeat could be stopped and would persist in a sustained systolic state (tetany) after a brief 1 s illumination with violet light (Fig. 4a-d, Supp. Movie 2). Normal ventricular rhythm was restored after briefly illuminating the heart with 1 s of green light (Fig. 4a-d, Supp. Movie 2). These data suggest that the reversible *E/Z* isomerization of the TRPswitches

Figure 4. Reversible and repeatable activity of TRPswitch. (a-d) Light-controlled heartbeat interruption in zebrafish

by violet and green light induced *in vivo* activation and deactivation of Trpa1b channels, respectively. The ability for TRPswitch/light to induce tetany depends on the number of cardiomyocytes with Trpa1b expression. In larvae with transient mosaic expression of Trpa1b in cardiomyocytes treated with TRPswitch-B, ventricle tetany can only be induced when at least around 15% of cardiomyocytes in the ventricle have Trpa1b expression (Fig. S4). Furthermore, there was no significant difference in heart rate among transgenic larvae treated with DMSO control or either TRPswitch in the dark (Fig. S5a), suggesting that the TRPswitches have no effect on Trpa1b channels without light illumination. Treating larvae that lack exogenous expression of Trpa1b in cardiomyocytes with TRPswitch-B and light illumination resulted in similar negative results (Fig. S5b), indicating that the switching off and on of heartbeat with violet and green light illumination was due to TRPswitch isomerization and the subsequent effect on Trpa1b channel activity.

Photoswitchable control of Trpa1b activity by the TRPswitches was repeatable. Cyclical rounds of illumination using violet and green light induced the stopping and restarting of the ventricle heartbeat (Fig. 4c and 4d; Supp. Movie 3 and 4). Additionally, channel activation after a brief pulse of violet light was sustained on a timescale of minutes, as measured in the gradual relaxation of ventricle width over time (Fig. 4e). As a further demonstration of the utility of the heterologously expressed zTrpa1b/TRPswitch system, we expressed zTrpa1b in human colonic fibroblast cells, CCD-18Co, which express human TRPA1 endogenously.⁴³ The expression of human TRPA1 channel in CCD-18Co cells was verified with RT-PCR (data not shown). CCD-18Co cells responded to AITC, confirming the functional expression of human TRPA1 channel (Fig. S6a). As expected, TRPswitch showed no light induced photocurrent in CCD-18Co cells without the expression of zTrpa1b (Fig. S6b). When CCD-18Co cells were transfected with zTrpa1b and incubated with TRPswitch-B, they exhibited violet light-induced photocurrent and green light-induced decrease in photocurrent (Fig. S6c). These data provide further evidence that TRPswitch-B has specific activity on zTrpa1b and does not cross react with endogenously expressed human TRPA1 channel. Taken together, these data suggest that the TRPA1/TRPswitch pair constitutes a reversible and repeatable chemo-optogenetic system that is compatible with use in zebrafish and mammalian cells.

Discussion

These studies have identified TRPswitch A and B, two photoswitchable small molecules that enable optical control of currents in Trpa1b expressing cells *in vivo*. Our data suggest that the TRPswitches specifically target Trpa1b channel and enable repeatable optical control of both neuronal and non-neuronal cells. This is the first example of TRPA1 channel activation by a

photoswitchable compound. Importantly, the TRPswitches allow for sustained channel activation after only a brief pulse of violet light illumination, but the channel can also be rapidly deactivated with green light illumination. As only short pulses of light are required to control the activity of the TRPA1 channel, cells subjected to the TRPA1/TRPswitch chemo-optogenetic system are less prone to photo-toxicity. This requirement of a short photoactivation period for target activation is a unique and advantageous feature among current PCLs for unmodified ion channels¹¹⁻¹⁵ and receptors.⁴⁴

The TRPA1 channel's high conductance combined with the step function property of the TRPA1/TRPswitch chemo-optogenetic system offers certain new opportunities for basic research. As TRPswitch activity is specific to zebrafish Trpa1b, TRPswitch can be used in heterologous applications by expressing zTrpa1b in animals or cells with endogenous mammalian TRPA1 expression, without interference from the endogenous channel. This new tool will be particularly beneficial in applications where a large depolarization current is needed, such as in large primary motor neurons, or when sustained channel activation is desirable. In addition, tools for manipulating TRPA1 activity are relevant for medical research since TRPA1 is involved in inflammatory and neuropathic pain, itch, and respiratory diseases.^{20-21, 45} TRPswitch may prove useful as a research tool to help dissect the mechanism of TRPA1-related disease, as well as to identify disease-modifying agents.

The TRPA1/TRPswitch chemo-optogenetic system offers several advantages over existing tools, and we have shown it to be robust and easy to use in cultured mammalian cells and in zebrafish. Nevertheless, its suitability for other *in vivo* and clinical applications remains unknown. TRPswitch is a freely diffusible small molecule, and exposing zebrafish larvae by incubating them in a solution containing TRPswitch is sufficient for robust activity. However, its uptake and distribution in rodents and larger mammals remains to be determined. In zebrafish larvae incubated with TRPswitch for several hours, no obvious adverse physiological phenotypes were observed. The TRPswitches show minimal activity on TRPA1 before photoactivation, highlighting the specificity of the light-induced effect of TRPswitch. On the other hand, as the TRPswitches display a wide range of spectral activity, it may be difficult to combine a TRPswitch with other optogenetic tools or fluorescence-based biosensors due to potential spectral overlap. The use of violet light for TRPswitch activation may also limit the tissue depth of its activation; however, future studies could explore the possibility for multi-photon⁴⁶ or longer near infrared (NIR) light excitation.⁴⁷

Our analysis suggests that both TRPswitch-A and TRPswitch-B have thermal half-lives in the scale of hours in DMSO. However, the half-life of the biological response to these compounds was measured to be in the

timescale of minutes during *in vivo* cardiac experiments (Fig. 4e). Several factors may account for the shorter half-life observed *in vivo*. Firstly, the thermal half-life of TRPswitch would be different under the conditions of the biological assays, for example in aqueous solution with only 1% DMSO. In fact, the thermal half-life of TRPswitches become shorter when measured in 30% water:DMSO (43 min and 1 hour for TRPswitch-A and -B, respectively). Secondly, although we found that TRPA1/TRPswitch light-induced activity is reversible and repeatable, TRPswitch does not covalently bind to TRPA1 and is therefore free to diffuse away from the channel over time. Thirdly, there might be biological adaptation during TRPA1 activation, such as receptor internalization, that leads to a shorter time of biological effect compared to the half-life measured by UV-Vis *in vitro*.

Most of the known TRPA1 ligands are electrophiles that activate TRPA1 via the covalent modification of cysteine residues present in the channel's cytoplasmic ankyrin repeat domain.⁴⁸ This includes our previously identified TRPA1 photoactivatable ligand, optovin.²³⁻²⁴ Although both optovin and TRPswitch target TRPA1 and are activated by violet light, they are unique in their mechanism of action, reversibility, and kinetics. Unlike optovin and its derivatives, which act through light-induced covalent modification, TRPswitch's photoreversible TRPA1 activity depends on differential activity of its *E* and *Z* isomers. How isomerization triggers opening and closing of the TRPA1 channel remains unknown. Mechanistically, the fifth transmembrane (TM5) domain of TRPA1 has been shown to determine channel sensitivity to non-electrophilic agonists such as menthol and anethole.⁴⁹⁻⁵⁰ Considering that menthol and anethole are structurally distinct molecules, we speculate that TRPA1's TM5 may act as a general binding site for non-electrophilic agonists and may therefore be a candidate site for the binding of TRPswitch. Alternatively, a peptidergic scorpion toxin (WaTx) was recently discovered that activates TRPA1 by binding to the same allosteric nexus that is covalently modified by electrophilic irritants.⁵¹ It is possible that TRPswitch binds to this allosteric nexus on TRPA1 channel as well.

The development of PCLs for endogenous targets offers theoretical advantages in clinical applications, since the introduction of exogenous gene products is not needed. The common approach for PCL discovery is to modify a known biologically active molecule with photoswitchable functionality, such as 'azologization'.⁵² Although this approach has had some success,¹¹⁻¹⁵ it is limited by the need for already identified active molecules for specific targets, which have a defined mechanism of activation and that contain a chemical structure amenable to incorporation of a photoswitch. The screening strategy described here offers an alternative, phenotypic approach for the discovery of photoswitchable ligands of novel endogenous targets.⁵³ Since screening is performed using

intact animals, hit compounds identified are, by definition, biologically active and likely to have minimal general toxicity. In the case of TRPswitches, it is noteworthy that they are specific for the zebrafish Trpa1b channel and are inactive at the mammalian channels tested. It remains to be seen whether modification of the TRPswitch scaffold can yield ligands with different species specificity, but the current specificity for zebrafish Trpa1b has significant advantages, including the ability to use the TRPswitch/Trpa1b system in mammalian cells without unwanted cross-reactivity with endogenous channels.

It should also be noted that despite having excellent and tunable switching properties, heteroaryl azo motifs are still significantly underexplored compared to their azobenzene counterparts in photoaddressable applications including as PCLs.⁵⁴ Our discovery that the azopyrazole-containing TRPswitch-B exhibits a longer half-life and more efficient photoswitching than the azobenzene-containing TRPswitch-A is a good indication that heteroaryl azo photoswitches are good alternatives for photoswitch optimization. Indeed, this is the first application of the high performance arylazopyrazoles^{32, 40} for *in vivo* photopharmacology, showcasing the potential of this scaffold for future studies.

Materials and Methods

Zebrafish

Zebrafish (*Danio rerio*) wild-type TuAB or *trpa1b* mutant³⁵ larvae were used for all experiments. Zebrafish embryos were produced using group mating of adult zebrafish. Larvae were raised in E3 media and maintained in an incubator with a 14 h light/10 h dark cycle at 28.5°C until experiments. The maintenance of adult animals, obtaining of embryos and larvae, and all experimental procedures were carried out according to protocols approved by the University of Utah's Institutional Animal Care and Use Committee (IACUC).

Chemical libraries and treatments

A total of 1,000 compounds were selected from the ChemBridge Corp. catalog. Compounds were dissolved in DMSO at a stock concentration of 1 µg/µl (~1.5 mM). The library was screened at a 1:100 dilution in E3 solution for a final concentration of ~15 µM. Negative controls were treated with an equal volume of DMSO. Groups of 3 larvae at 3 days post fertilization (dpf) were distributed into the wells of 96-well clear bottom black microplates (07-200-567; Fisher Scientific) in 150 µl E3 before the addition of small molecules. Stock solutions of compounds were added directly to zebrafish in the wells of a 96 well plate (Corning 3631), mixed, and allowed to incubate for 1 h in the dark at 28.5°C prior to behavioral evaluation in the behavioral assay. Ordering information: **1** (5533696; ChemBridge); **3** (5538408; ChemBridge). Other compound synthesis and characterization information are described in the supplementary note.

Behavioral assay

Larvae in each of the wells were exposed to four 1 second flashes of light stimulus in the following order: 450-500 nm (WL1), 415 – 455 nm (WL2), 352-402 nm (WL3), white light (WL4), with a 5 second inter-stimulus interval. Digital video recording was performed for 5 seconds before the first light stimuli and continued throughout the stimulation sequence. Band pass filters (Semrock FF02-475/50, FF02-435/40, FF01-377/50) were used to restrict the excitation light to the indicated wavelengths. Light-induced motion response was used as the assay readout as DMSO treated control larvae do not respond to light stimulus. 290 frames of digital video were recorded per well at 10 FPS using an EMCCD camera (C9100; Hamamatsu) mounted on an inverted compound microscope (AxioObserver A1; Zeiss) with a NA 0.03 1.25x objective and a barrier with a 0.7 mm diameter opening to restrict light scattering to the sample. MetaMorph software (Molecular Devices) was used to control the execution of TTL signals and camera capture using the built-in stream acquisition with trigger function. Each video was saved for review. Light stimuli were generated with an ozone free 300 Watt xenon bulb housed in a Lambda LS illuminator (Sutter Instruments). A dichroic mirror (T510LPXRT; Chroma) was used for appropriate

excitation of samples and bright field acquisition. Schott longpass absorption glass (RG610; Chroma) was added in the transmitted light path to reduce unwanted excitation to the well and to provide sufficient light for video recording. All behavioral experiments were conducted at room temperature. For Fig. 3c and 3d, experiments were performed using an inverted compound microscope (AxioObserver A1; Zeiss) equipped with an EMCCD camera (C9100; Hamamatsu), a violet LED light source (415 nm) with a CW 310 mW maximum output power source (BLS-LCS-0415-03-22; Mightex) which was controlled by a BioLED light source control module (BLS-SA02-US; Mightex), and a pulse master multi-channel stimulator (A30; World Precision Instruments). Bright field time-lapse was captured for 500 frames at ~103 FPS. A light pulse was applied at frame 20. For Fig. 3c, percentage responding was quantified based on whether there was any motion in the entire acquisition period. For Fig. 3d, response time is the duration from the beginning of the light pulse to the first movement of the larvae. Light intensity was measured using a hand-held laser meter (LaserCheck, Coherent 1098293). For *Trpa1b* ohnolog and paralog rescue experiments, *Trpa1b*^{-/-} embryos were injected with *ngn1:zTrpa1b-2A-mCherry*, *ngn1:zTrpa1a-2A-mCherry*, *ngn1:hTRPA1-2A-EGFP* or *ngn1:mTRPA1-2A-mCherry* at the 1-cell stage for mosaic Rohon-beard neuron expression. Embryos were screened at 2 dpf for fluorescent expression in Rohon-beard neurons, incubated with 20 µM TRPswitch-B for 1 hr and decapitated posterior to the eyes right before experiments. NA 0.25 5x objective and 1 s WL3 light illumination was used.

Behavioral analysis

To analyze digital video recordings, custom MetaMorph software scripts were used to automatically threshold the video to identify the area of larvae in each frame. Threshold images of the frames after each light activation event were overlaid to calculate a new combined threshold area. The motion index was calculated as the percentage change between the threshold area in the frame right before light activation and the new combined threshold area. This motion index correlates with the overall amount of motion in the well.

Electrophysiology analysis

Immortalized HEK293T cells or human colonic fibroblast cells (CCD-18Co; ATCC) were used. HEK293T cells were plated on 12 mm cover slips and transiently transfected with 10 µg of *Trpa1b* plasmid (pCMV-zTrpa1b-FLAG) and 5 µg of mNeonGreen cDNA (Allele Biotechnology), then grown in a 6 cm plate for 24-48 h. CCD-18Co were cultured in Eagle's minimal essential medium with 10% FBS, 1x penicillin and streptomycin. CCD-18Co cells were plated on 12 mm cover slips and transiently transfected with 5 µg of *zTrpa1b* plasmid (pCMV-zTrpa1b-FLAG) together with 5 µg of mNeonGreen cDNA (Allele Biotechnology), then grown in a 10 cm plate for 48-72 h.

After this time the cover slips were transferred to a recording chamber containing an extracellular solution composed of (in mM): 145 NaGluconate, 4 KCl, 3 MgCl₂, 10 D-glucose, 10 HEPES; pH 7.4 adjusted with NaOH. The internal solution contained (in mM): 122 Cesium methane sulfonate, 1.8 MgCl₂, 9 EGTA, 14 creatine phosphate (Na-salt), 4 MgATP, 0.3 NaGTP, 10 HEPES, pH 7.2 adjusted with CsOH. Borosilicate glass pipettes with a resistance of 3–5 MΩ were used. Whole cell currents were measured on an Axopatch 200B amplifier (Molecular Devices) under the control of pClamp software. Signals were digitized through a Digidata1550B interface (Molecular Devices). Currents were filtered at 5 kHz (lowpass, Bessel) and sampled at 10 kHz prior to analysis with Clampfit software (Molecular Devices). All the plots and statistical tests were performed on Excel (Microsoft corp.). Basal Trpa1b whole cell currents were measured with a ramp protocol (-100 to +100 mV, at a holding potential of 0 mV). Photoactive compounds were perfused into the recording chamber and a fluorescent light generated by mercury vapor short arc (U-HGLGPS, OLYMPUS) filtered through a ET-ECFP 434/17 nm filter and through a ET-mCherry 560 nm filter (Chroma Technology) was switched ON and OFF for 10 s. All experiments were performed at room temperature. The p values were calculated using a two-tailed, paired Student's t-test.

Heart experiments

Heartbeat interruption experiments were performed *in vivo* on *Tg(cmlc2:Trpa1b-2A-EGFP)*²⁴ larvae or Tol2-cmlc2-Trpa1b-2A-EGFP plasmid²⁴ (Addgene plasmid #106405) injected larvae in a *Trpa1b*^{-/-} background. Expression of the plasmid was obtained by microinjecting 2 nL of a solution containing 7 ng/μL of DNA plasmid and 6.5 ng/μL *in vitro* transcribed (Ambion) Tol2 transposase mRNA into the cytoplasm of one-cell stage embryos. At 2 dpf, larvae were pretreated with 20 μM of TRPswitch-A or TRPswitch-B in E3 at a final concentration of 1% DMSO for 1 h in the dark at 28.5°C before experimental manipulation. Immediately prior to the onset of experiments, E3 solution was replaced with 0.2 mg/mL tricaine (Sigma, A5040) in E3 for anesthetizing the larvae. Violet light (352–402 nm) and green light (500–600 nm) were used for E to Z and Z to E isomerization, respectively. Violet and green light excitation were achieved using a band pass filter FF01-377/50 and an et500lp long pass barrier filter, respectively, together with a t600plx dichroic mirror and a 300 Watt xenon bulb light source (Sutter Instrument). Experiments were performed using a NA 0.6 40x air objective at room temperature. The zebrafish heart in the whole field of view was illuminated with 1 s violet light followed by various lengths of green light as indicated. Ventricle width measurement was performed using the ImageJ 'measure' function on the widest outer ventricle width every 100 ms.

UV-Vis measurement

Compounds were dissolved in DMSO at 250 μM and 260 μM for TRPswitch-A and TRPswitch-B, respectively

and UV-Vis absorbance was measured using a NanoDrop 1000 (Thermo Scientific).

Statistical Analyses

All results are expressed as means ± SEM. Unless otherwise indicated, a two-tailed unpaired student's t test with Mann-Whitney post-test was used to determine p values. The criterion for statistical significance was *p* < 0.05. Statistical analysis was performed using Prism (GraphPad Software).

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional experimental data (Figures S1-6, thermal isomerization kinetics (Figures S7-10), photostationary state determination (Figures S11-18), supplemental tables 1-2, general methods of compound synthesis, synthetic route of compounds, NMR spectra, and supplemental movie legends as described in the text) (PDF)

Movie 1 (AVI)

Movie 2 (AVI)

Movie 3 (AVI)

Movie 4 (AVI)

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