Published in final edited form as:

Org Biomol Chem. 2018 August 08; 16(31): 5575–5579. doi:10.1039/c8ob01098f.

Rhodol-based Thallium Sensors for Cellular Imaging of Potassium Channel Activity

Brendan F. Dutter^a, Anna Ender^b, Gary A. Sulikowski^{a,c,d}, and C. David Weaver^{a,c,d}

^aDepartment of Pharmacology, Vanderbilt University, Nashville, TN 37232.

^bInstitute of Biochemistry, Leipzig University, Leipzig, Germany

^cDepartment of Chemistry, Vanderbilt University, Nashville, TN 37232.

^dInstitute of Chemical Biology, Vanderbilt University, Nashville, TN 37232.

Abstract

Thallium (Tl⁺) flux assays enable imaging of potassium (K⁺) channel activity in cells and tissues by exploiting the permeability of K⁺ channels to Tl⁺ coupled with a fluorescent Tl⁺ sensitive dye. Common Tl⁺ sensing dyes utilize fluorescein as the fluorophore though fluorescein exhibits certain undesirable properties in these assays including short excitation wavelengths and pH sensitivity. To overcome these drawbacks, the replacement of fluorescein with rhodols was investigated. A library of 13 rhodol-based Tl⁺ sensors was synthesized and their properties and performance in Tl⁺ flux assays evaluated. The dimethyl rhodol Tl⁺ sensor emerged as the best of the series and performed comparably to fluorescein-based sensors while demonstrating greater pH tolerance in the physiological range and excitation and emission spectra 30 nm red-shifted from fluorescein.

Abstract

Conflicts of Interest

CDW is an owner of WaveFront Biosciences, the manufacturer of Panoptic, and he receives royalties from Vanderbilt University from the sale of Thallos.

A rhodol-based thallium sensor improves upon currently used fluorescein-based sensors for imaging potassium channel activity.

Ion channels are pore-forming membrane proteins which promote the passage of ions across cellular membranes. Within the superfamily of ion channels are subfamilies with different ionic selectivity.¹ The largest subclass is potassium (K^+) channels which are expressed in every tissue in the body where they participate in control of excitability of neurons, muscles, endocrine glands, and the heart^{2–4} while in organs like the kidney they play critical roles in the formation of urine and maintenance of proper Na^+/K^+ homeostasis.⁵ K^+ channel activity can be modulated by changes in transmembrane potential as well as a wide variety of extracellular and intracellular factors including Ca^{2+} , membrane lipids, G-protein-coupled receptors, protein kinases, and ATP.⁶ A rapidly expanding number of mutations are being identified in K^+ channels which can result in neurological, cardiovascular, renal, neoplastic, and metabolic diseases.^{7,8,9} The physiological roles of many of these K^+ channels remain uncharacterized underscoring the need for robust chemical tools to study them.

Electrophysiology is the most sensitive technique for assaying ion channel activity though it can be time consuming and technically challenging. An alternate approach is the use of ion flux assays where channel activity is monitored by changes in fluorescence of an ion-sensing dye as a measure of passage of that ion through the channel of interest. In particular, the thallium (Tl^+) flux assay¹⁰ (Figure 1) is extensively used for imaging K^+ channel activity in cells and tissue samples. Tl^+ flux assays are highly compatible with kinetic imaging-based high-throughput screening (HTS) assays. HTS is an important method for the discovery of K^+ channel modulators by enabling the rapid testing of thousands-to-millions of molecules to identify those with activity against the channel of interest. Tl^+ flux has been used in numerous HTS campaigns to identify modulators of K^+ channels implicated in disease^{11–13} and have also been used to screen compounds for activity against K^+ transporters, Na^+ channels, and non-selective cation channels.^{14,15}

Tl⁺ flux assays exploit the ability of Tl⁺ to pass through K⁺ channels coupled with a cell-permeable fluorescent Tl⁺ indicator. Several fluorescent Tl⁺ indicators have been reported with the most robust, such as Thallos (**1**), consisting of a fluorescein moiety coupled to an amino dicarboxylic acid metal-binding unit.¹⁶ The fluorescence pathway is partially quenched in the non-Tl⁺ bound form by a presumed photoinduced electron transfer (PET) between the metal-binding unit and fluorophore. When Tl⁺ is bound, the PET-pathway is inhibited resulting in a higher fluorescence quantum yield for the complex which is observed as a Tl⁺ concentration-dependent increase in fluorescence.

Fluorescein-based metal sensors have found great utility in biology as imaging agents and components of activity assays.¹⁷ However, there are drawbacks to their use including relatively short excitation wavelengths and an undesirable pH sensitivity in the physiological range leading to decreased brightness at lower pH.¹⁸ The short excitation wavelength is problematic in HTS assays since many compound libraries contain molecules with comparable excitation and emission spectra which interfere with the assay. The number of compounds in a library generating this kind of interference tends to decrease as excitation wavelength increases.¹⁹ In addition, autofluorescence in tissues tends to decrease when longer wavelengths are used for imaging using fluorescence microscopy.²⁰ Rhodamines, which are red-shifted ~50 nm relative to fluorescein and exhibit excellent pH tolerance, have also been used as the fluorescent components of metal sensors¹⁷, but have also been shown to accumulate in the mitochondria²¹ which is generally undesirable in flux assays that are focused on plasma membrane channels. Rhodols are a hybrid of fluorescein and rhodamines exhibiting excitation and emission spectra ~30 nm red-shifted from fluorescein, excellent pH tolerance in the physiological range²², and predominantly cytoplasmic accumulation.²³ PET-based metal sensors utilizing rhodol fluorophores have been reported for imaging Cu⁺²⁴, Na⁺²⁵, and Ca²⁺²⁶. Here we report the synthesis and evaluation of a library of rhodol-based Tl⁺ sensors for Tl⁺ flux assays of K⁺ channels and other Tl⁺ conducting systems.

A variable route to the rhodol component of the Tl⁺ sensor was envisioned utilizing a similar strategy as previously described²⁷ but incorporating protecting groups to allow for orthogonal deprotection of the phenol and distal carboxylic acid functional groups (Scheme 1). Starting from 5- carboxyfluorescein (**2**), mono-phenol protection as the benzyl ether was accomplished by treatment with benzyl chloride and potassium carbonate in DMF under microwave heating followed by benzyl ester saponification with lithium hydroxide to afford **3**. Carboxylic acid **3** was then protected as the *t*-butyl ester and converted to triflate **4**. From this common intermediate, rhodols **5a – m** were prepared by Buchwald-Hartwig coupling with the corresponding amine. These compounds were converted to *O*-acetyl rhodols **6a – m** by sequential hydrogenolysis of the benzyl ether, removal of the *t*-butyl ester by treatment with TFA, and *O*-acetylation. **6a** and **6b** were prepared from a slightly modified route (Supplemental Information). Carboxylic acids **6a – m** were coupled to **7** to provide rhodol Tl⁺ sensors **8a – m**. (Table 1)

To determine the *in vitro* photochemical properties of the active Tl⁺ sensing species, **8a – m** were treated with base to remove the acetoxymethyl esters and *O*-aryl acetate and then diluted into 10 mM HEPES buffered 150 mM KCl with 50 μM EDTA at pH = 7.22.

Saponified **8a** – **m** exhibited photochemical properties comparable to previously reported rhodols.^{27,28} (Table 1).

The performance of rhodol Ti^+ sensors in the Ti^+ flux assay was assessed by incubating HEK-293 cells co-expressing G-protein-gated inwardly rectifying K^+ (GIRK) channel subunits GIRK1 and GIRK2 with **8a** – **m** in dye loading buffer for 1 hour prior to addition of 1 mM (final concentration) of Ti^+ stimulus and the fold increase in brightness calculated. Dimethyl rhodol **8c** demonstrated a 4.21-fold increase in brightness in the presence of Ti^+ in HEK-293 cells. A notable decrease in ability to sense Ti^+ among the dialkyl series **8c** – **8f** as the alkyl chain length increases was observed. Monoalkyl rhodols **8g** and **8h** as well as cyclic amine containing rhodols **8j**, **8l**, and **8m** exhibit >3 fold increases in brightness upon addition of Ti^+ stimulus. Gemdifluoro **8k** exhibited almost half the response to Ti^+ compared with the non-fluorinated analog **8j**. Phenyl rhodol **8i** exhibited very low initial fluorescence in HEK-293 cells requiring a longer exposure time than the other dyes and a minor increase in fluorescence upon addition of Ti^+ . Fluorescence was not detected in HEK-293 cells incubated with acetamide **8b** before or after the addition of Ti^+ . Considering photochemical properties and response to Ti^+ in HEK-293 cells, dimethyl rhodol **8c** was identified as the best of the series and further evaluated for its utility as a reagent for Ti^+ flux assays.

Cytoplasmic localization of Ti^+ indicators is desirable for Ti^+ flux assays of ion channels and transporters localized to the plasma membrane. The cellular localization of **8c** was compared to Thallo by treating GIRK1 and GIRK2 co-expressing HEK-293 cells with Thallo, **8c**, or the mitochondrial stain Rhodamine 123 for 1 h and all counterstained with the nuclear stain Hoechst. **8c** exhibited diffuse cytoplasmic staining comparable to Thallo, in contrast to the punctate mitochondrial staining observed with Rhodamine 123 (Figure 2).

The pK_a s of **8c** and Thallo were determined to be 5.23 and 6.52, respectively (Supplemental Figure 1) indicating that **8c** may be superior to Thallo when conducting assays at the lower range of physiological pH.

The performance of **8c** was evaluated in a Ti^+ flux assay to generate a concentration-response curve (CRC) of the GIRK activator ML297.²⁹ HEK-293 cells co-expressing GIRK1 and GIRK2 were loaded with 5 μM of **8c** for 1 h, treated with 0.01 – 10 μM of ML297, and then subjected to Ti^+ stimulus **8c** exhibited a 3.09 ± 0.004 -fold increase in fluorescence at the highest ML297 concentration (Figure 3a). Normalized, control subtracted fluorescence intensity values were sampled along the curves following Ti^+ addition at each concentration and a CRC was fit using GraphPad Prism (Figure 3b). The EC_{50} was determined to be 257 ± 3.7 nM while the same procedure utilizing Thallo produced an EC_{50} of 272 ± 23 nM, which was not statistically significantly different.

Using the same cell line and GIRK activator, **8c** was also compared to the commercially available, red-shifted Ti^+ sensor FluxOR Red (Molecular Probes). Following the manufacturer's procedure, which requires supplementation of assay and stimulus buffers with a proprietary background suppressor, FluxOR Red exhibited a 5.37 ± 0.001 -fold increase in fluorescence at the highest concentration of ML297 compared to Thallo (4.30

± 0.008 -fold increase) and **8c** (3.09 ± 0.004 -fold increase). However, omission of the FluxOR Red background suppressor resulted in a significantly diminished increase in fluorescence of 1.17 ± 0.007 at the highest ML297 concentration for FluxOR Red while Thallos and **8c** only showed modest decreases in the absence of background suppressor (2.80 ± 0.009 and 2.87 ± 0.004 -fold increases in fluorescence, respectively, Supplemental Figure 2). In addition, **8c** exhibited a comparable dynamic range and EC₅₀ results using 544/24 nm excitation and 593/40 nm emission filters indicating **8c** can also be used very effectively with longer wavelength, off-peak filters (Supplemental Figure 3).

Strict adherence to the FluxOR Red protocol may not be compatible with or convenient for assaying certain channels or transporters. For example, many buffers for TI⁺ flux assays, including those in most commercial kits, utilize gluconate as the primary anion due to the insolubility of TI⁺ in Cl⁻ containing buffers. However, gluconate-based buffers may not be optimal for all targets and assay systems.³⁰ In addition, the components of extracellular fluorescence masking dyes may affect the pharmacology of targets of interest. Tolerance of the TI⁺ sensing dye towards varied assay conditions would allow for greater flexibility. The performance of **8c**, Thallos, and FluxOR Red were compared using a bicarbonate-based stimulus buffer with 500 μ M of TI⁺ under two conditions. First, an automation compatible “no-wash” procedure was tested where the wash step after dye loading was omitted and all buffers were supplemented with 1 mM of Allura Red AC to suppress extracellular fluorescence. Under these conditions, **8c** and Thallos exhibited 3.40 ± 0.009 and 3.83 ± 0.007 -fold increases in fluorescence and ML297 EC₅₀s of 204 ± 5.2 nM and 219 ± 23 nM, respectively, were calculated. In contrast, FluxOR Red exhibited no change in fluorescence upon TI⁺ addition and the data could not be fit to a CRC. Second, using similar conditions omitting Allura Red AC while employing a wash step after dye loading produced comparable results (Supplemental Figure 2, Supplemental Table 1). These data demonstrate the utility of **8c** in assays where extracellular fluorescence masking dyes are not compatible or where “no wash” procedures are desirable.

Conclusions

In conclusion, dimethyl rhodol **8c** has been identified from a series of rhodol-based TI⁺ sensors as a versatile reagent for TI⁺ flux assays. It localizes to the cytoplasm similarly to Thallos while exhibiting greater pH tolerance and excitation and emission spectra red-shifted ~30 nm. **8c** exhibits an excellent dynamic range using both on-peak and longer off-peak filter sets and is compatible with a variety of assay conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge the Vanderbilt High Throughput Screening Facility and members of the Weaver laboratory for critical reading of this manuscript. Confocal microscopy was performed through the use of the Vanderbilt Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, DK59637, and EY08126) and with the assistance of Dr. Jenny Schafer. The Zeiss LSM880 AiryScan was acquired with an NIH S10 instrumentation grant: 1S10OD021630–01. B.F.D. was funded by a Vanderbilt University Discovery Grant. A.E.

was funded by the Max Kade Foundation Vanderbilt-Leipzig Exchange Program. The WaveFront Biosciences Panoptic 2 was funded by NIH S10-OD021734-01.

References

1. Ackerman MJ and Clapham DE, *N. Engl. J. Med.*, 1997, 336, 1575–1586. [PubMed: 9164815]
2. Lüscher C and Slesinger PA, *Nat. Rev. Neurosci.*, 2010, 11, 301–315. [PubMed: 20389305]
3. Quayle JM, Nelson MT and Standen NB, *Physiol. Rev.*, 1997, 77, 1165–232. [PubMed: 9354814]
4. De León DD and Stanley CA, *Nat. Clin. Pract. Endocrinol. Metab.*, 2007, 3, 57–68. [PubMed: 17179930]
5. Welling PA and Ho K, *Am. J. Physiol. Physiol.*, 2009, 297, F849–F863.
6. Choe S, *Nat. Rev. Neurosci.*, 2002, 3, 115–121. [PubMed: 11836519]
7. Schmitt N, Grunnet M and Olesen S-P, *Physiol. Rev.*, 2014, 94, 609–653. [PubMed: 24692356]
8. Maljevic S and Lerche H, *J. Neurol.*, 2013, 260, 2201–2211. [PubMed: 23142946]
9. FM A., *J. Physiol. Sci.*, 2009, 59, 8–8.
10. Weaver CD, Harden D, Dworetzky SI, Robertson B and Knox RJ, *J. Biomol. Screen.*, 2004, 9, 671–677. [PubMed: 15634793]
11. Li Q, Rottländer M, Xu M, Christoffersen CT, Frederiksen K, Wang M-W and Jensen HS, *Anal. Biochem.*, 2011, 418, 66–72. [PubMed: 21782781]
12. Yue J, Qiao G, Liu N, Nan F and Gao Z, *Acta Pharmacol. Sin.*, 2016, 37, 105–110. [PubMed: 26725738]
13. Raphemot R, Kadakia RJ, Olsen ML, Banerjee S, Days E, Smith SS, Weaver CD and Denton JS, *Assay Drug Dev. Technol.*, 2013, 11, 532–43. [PubMed: 24266659]
14. Gill S, Gill R, Wen Y, Enderle T, Roth D and Liang D, *Assay Drug Dev. Technol.*, 2017, 15, 167–177. [PubMed: 28631939]
15. Weaver CD, in *Methods in molecular biology* (Clifton, N.J.), 2018, vol. 1684, pp. 105–114.
16. US9,103,791 B1, 2015.
17. Carter KP, Young AM and Palmer AE, *Chem. Rev.*, 2014, 114, 4564–4601. [PubMed: 24588137]
18. Chang CJ, Nolan EM, Jaworski J, Burdette SC, Sheng M and Lippard SJ, *Chem. Biol.*, 2004, 11, 203–210. [PubMed: 15123282]
19. Simeonov A, Jadhav A, Thomas CJ, Wang Y, Huang R, Southall NT, Shinn P, Smith J, Austin CP, Auld DS and Inglese J, *J. Med. Chem.*, 2008, 51, 2363–2371. [PubMed: 18363325]
20. Minta A, Kao JP and Tsien RY, *J. Biol. Chem.*, 1989, 264, 8171–8. [PubMed: 2498308]
21. Sensi SL, Ton-That D, Weiss JH, Rothe A and Gee KR, *Cell Calcium*, 2003, 34, 281–284. [PubMed: 12887975]
22. Whitaker JE, Haugland RP, Ryan D, Hewitt PC, Haugland RP and Prendergast FG, *Anal. Biochem.*, 1992, 207, 267–279. [PubMed: 1481981]
23. Kamino S, Ichikawa H, Wada S-I, Horio Y, Usami Y, Yamaguchi T, Koda T, Harada A, Shimanuki K, Arimoto M, Doi M and Fujita Y, *Bioorg. Med. Chem. Lett.*, 2008, 18, 4380–4384. [PubMed: 18621526]
24. Dodani SC, Firl A, Chan J, Nam CI, Aron AT, Onak CS, Ramos-Torres KM, Paek J, Webster CM, Feller MB and Chang CJ, *Proc. Natl. Acad. Sci.*, 2014, 111, 16280–16285. [PubMed: 25378701]
25. Poronik YM, Clermont G, Blanchard-Desce M and Gryko DT, *J. Org. Chem.*, 2013, 78, 11721–11732. [PubMed: 24245744]
26. Contractor AA and Miller EW, *Biochemistry*, 2017, 57, 237–240. [PubMed: 29182306]
27. Peng T and Yang D, *Org. Lett.*, 2010, 12, 496–499. [PubMed: 20067265]
28. Grimm JB, Muthusamy AK, Liang Y, Brown TA, Lemon WC, Patel R, Lu R, Macklin JJ, Keller PJ, Ji N and Lavis LD, *Nat. Methods*, 2017, 14, 987–994. [PubMed: 28869757]
29. Kaufmann K, Romaine I, Days E, Pascual C, Malik A, Yang L, Zou B, Du Y, Sliwoski G, Morrison RD, Denton J, Niswender CM, Daniels JS, Sulikowski GA, (Simon) Xie X, Lindsley CW and Weaver CD, *ACS Chem. Neurosci.*, 2013, 4, 1278–1286. [PubMed: 23730969]

30. Niswender CM, Johnson KA, Luo Q, Ayala JE, Kim C, Conn PJ and Weaver CD, Mol. Pharmacol, 2008, 73, 1213–1224. [PubMed: 18171729]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.
Overview of Tl^+ flux assay.

Figure 2. Cellular localization of Thallos (a), **8c** (b), and Rhodamine 123 (c). Confocal microscopy images were obtained in HEK-293 cells co-expressing GIRK1 and GIRK2 following incubation with Thallos (1 μM), **8c** (5 μM), or Rhodamine-123 (1 μM) for 1 h and counterstained with Hoechst 33342 (1 μg/mL). Scale bar = 10 μm.

Figure 3.

Concentration response data of ML297 in HEK-293 cells co-expressing GIRK1 and GIRK2 obtained with 8c. (a) Fluorescence intensity data from each well were normalized to the averaged fluorescence intensity of the first 6 time points of the experiment and the normalized fluorescence data for each replicate time point were averaged ($n = 12$). (b) Concentration response curve derived from data in (a) by subtracting the vehicle control from each concentration series and sampling a time point several seconds after addition of TI^+ stimulus.

Scheme 1.

Synthesis of rhodol Ti^+ sensors; a. BnCl , K_2CO_3 , DMF, 120°C , 1 h; b. 23LiOH , THF/ H_2O , 60°C , 1 h, 59 % (2 steps); c. Ac_2O pyridine, rt, 2 h, d., *O-tert*-butyldiisopropylisourea, DCM, rt, 48 h, then NaOMe, MeOH, rt, 20 min, 68 % (2 steps), e. Ti_2O , pyridine, DCM, 0°C , 1 h, 78 %; f. amine, PdOAc_2 , R-BINAP, Cs_2CO_3 , PhMe, 100°C , 20 h, 18–90 %; g. H_2 , Pd/C, EtOAc, rt, 1 h; h. TFA, DCM, rt, 12 h; i. Ac_2O , pyridine, rt, 2 h; 22–67 % (3 steps); j. HATU, Et_3N , DMSO, rt, 1 h, 33–58 %.

Table 1Photochemical properties of rhodol Ti^+ sensors **8a-m**.^{a,b}

Cmpd	X	λ_{max}	λ_{em}	ϵ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	on/off (Ti^+ flux assay)
8a	--NH ₂	496	520	57000	3.02 ± 0.08
8b	--NHAc	463	NA	24000	NA
8c	--NMe ₂	521	551	56000	4.21 ± 0.04
8d	--NEt ₂	525	551	55000	3.28 ± 0.04
8e	--NPr ₂	527	553	66000	2.94 ± 0.08
8f	--NBu ₂	528	556	58000	1.52 ± 0.01

8g		514	535	57000	4.26 ± 0.07
8h	--NHBu	511	533	48000	3.50 ± 0.07
8i	--NHPh	517	NA	52000	1.07 ± 0.01

8j		522	550	56000	3.92 ± 0.13

8k		506	530	66000	2.02 ± 0.04

8l		525	553	39000	3.54 ± 0.07

8m		526	557	48000	3.09 ± 0.04

^a Absorption maxima, fluorescence emission maxima (both in nm), and molar absorptivity were measured after saponification of **8a-m** and dilution into 150 mM KCl buffered with 10 mM HEPES and supplemented with 50 μM EDTA at pH = 7.22.

^b In cell on/off ratios were determined using HEK-293 cells co-expressing GIRK1 and GIRK2 treated with **8a - m** for 1 h and then subjected to Ti^+ stimulus. Values represent the maximum fold increase in fluorescence after Ti^+ addition (n = 6).