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# Small Molecule Inhibitors of Regulators of G Protein Signaling (RGS) Proteins

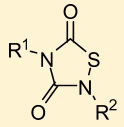
Emma M. Turner,[†] Levi L. Blazer,[‡] Richard R. Neubig,[‡] and Stephen M. Husbands*,[†]

[†]Department of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, United Kingdom

[‡]Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48109, United States

Supporting Information

**ABSTRACT:** Recently, regulators of G protein signaling (RGS) proteins have emerged as potential therapeutic targets since they provide an alternative method of modulating the activity of G protein-coupled receptors, the target of so many drugs. Inhibitors of RGS proteins must block a protein−protein interaction (RGS-G$\alpha$) but also be cell and, depending on the therapeutic target, blood−brain barrier permeable. A lead compound (**1a**) was identified as an inhibitor of RGS4 in a screening assay, and this has now been optimized for activity, selectivity, and solubility. The newly developed ligands (**11b** and **13**) display substantial selectivity over the closely related RGS8 protein, lack the off-target calcium mobilization activity of the lead **1a**, and have excellent aqueous solubility. They are currently being evaluated in vivo in rodent models of depression.



Selective RGS4 Inhibitors

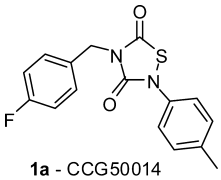
**KEYWORDS:** *RGS4, RGS protein, thiadiazolidinone, GPCR, protein−protein interaction*

G protein-coupled receptors (GPCRs) are widely distributed throughout the body and are an important class of therapeutic targets for drug discovery.[1] The majority of GPCR-targeted drugs act as orthosteric agonists or antagonists at the canonical ligand binding site, but recent attention has focused on drugs acting allosterically to modulate receptor activity.[2] One advantage of allosteric agents is their ability to modulate ongoing physiological signaling. Other approaches to modulate GPCR-mediated signal transduction may also provide significant therapeutic benefit. To this end, we have focused on targeting a class of proteins that negatively regulates GPCR signaling.

Regulators of G protein signaling (RGS) proteins are potent negative modulators of GPCR signaling. They accelerate the rate of GTP hydrolysis by G$\alpha$ subunits of heterotrimeric G proteins, shortening the duration and decreasing the magnitude of signal after receptor activation.[3] The important role of RGS proteins in GPCR signaling has generated, in recent years, significant interest in RGS proteins as therapeutic targets in their own right.[4,5] One of the attractions is that while many receptors are expressed throughout the body, modulators of the signaling pathway, including RGS proteins, are expressed in a more tissue-specific manner. This may be of particular importance for the development of centrally acting agonist therapeutics. Of over 20 mammalian RGS proteins identified, RGS4 is one of the most extensively characterized. It is broadly, but heterogeneously, expressed in the central nervous system (CNS),[6,7] less so in peripheral tissues, and has been shown to modulate activity of the $\mu$ opioid (MOP), $\delta$ opioid (DOP), and M3 muscarinic receptors among others.[8,9] The selectivity in regional distribution, coupled with the finding that RGS4 can selectively suppress signaling through DOP receptors as compared to MOP receptors,[10] suggests that a level of specificity for RGS4 inhibitory action will be possible.

The RGS-G$\alpha$ site is a large, relatively featureless protein−protein interaction (PPI) interface.[11] Inhibitors of RGS proteins must act by disrupting this interaction either directly or through an allosteric mechanism. While a number of PPI inhibitors are known against a variety of targets, most of these compounds are large molecules that lack access to the CNS.[5] The development of cell- and blood−brain barrier-permeable small molecules that can act as selective PPI inhibitors is a nontrivial task but one that could ultimately provide significant clinical benefit.

Our group recently discovered small molecule inhibitors of RGS4[12−15] using a high-throughput flow cytometry protein interaction assay (FCPIA).[16] As part of this screening process, CCG-50014 (**1a**, Figure 1) was identified as a selective inhibitor of



**1a** - CCG50014
RGS4 $IC_{50}$ 30.1 nM
RGS8 $IC_{50}$ 11.0 μM

**Figure 1.** Structure and activity of the lead compound, CCG50014.

RGS4 that acted by forming a covalent adduct to cysteine residues in the RGS protein.[15,17] With an $IC_{50}$ of 30 nM, it is the most potent RGS inhibitor reported to date. In an attempt to further define the structural requirements for high-potency inhibition of this protein, analogues of **1a** have been synthesized with variation in both the N2 and the N4 side chains. In addition, a set of these newly synthesized RGS4 inhibitors have been evaluated for their effects on calcium mobilization, an off-target activity displayed by **1a**.

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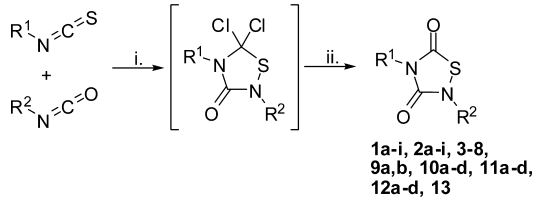
The synthesis of CCG-50014 (**1a**) and its thiadiazolidinone (TDZD) analogues is shown in Scheme 1. Commercially available isothiocyanates were reacted with isocyanates in the presence of sulfuryl chloride (Scheme 1).[18] This allowed a range of $R^1$ and $R^2$ substituents, having varying lipophilic, electronic, and steric properties, to be evaluated. While chlorine gas[19] or *N*-chlorosuccinimide[20] are also used in literature procedures for making TDZDs, we found the use of sulfuryl chloride straightforward and consistent. The resulting *S*-chloroisothiocarbamoyl chloride, proposed by Slomczyńska and Barany,[18] was subsequently oxidized in atmospheric oxygen to the desired products. This reaction was easily carried out in parallel, with typically 11 different reactions running simultaneously. In total, 75 TDZD analogues were synthesized, with 39 (**1a−i**, **2a−i**, **3−8**, **9a,b**, **10a−d**, **11a−d**, **12a−d**, and **13**) reported in Table 1. Data for all compounds are reported in the Supporting Information.

**Scheme 1. Synthesis of CCG50014 and Analogues**[a]




[a]Conditions: (i) $SO_2Cl_2$, THF, 0 °C−room temperature, 18 h. (ii) Air, 30 min.

**Table 1. Inhibition of Gαo Binding to RGS4 and RGS8**

| | | | RGS4[a] | RGS8[a] | selectivity |
|---|---|---|---|---|---|
| | $R^1$ | $R^2$ | $IC_{50}$ (nM) | $IC_{50}$ (μM) | RGS4/RGS8 |
| **1a** | 4-FBn | 4-MePh | 30.1 | 11.0 | 366 |
| **1b** | 4-FBn | Ph | 16.3 | 6.20 | 380 |
| **1c** | 4-FBn | 4-ClPh | 13.5 | 7.60 | 564 |
| **1d** | 4-FBn | 4-MeOPh | 10.9 | 11.4 | 1050 |
| **1e** | 4-FBn | 3,4-diClPh | 35.7 | 17.2 | 481 |
| **1f** | 4-FBn | 3-$CF_3$Ph | 79.3 | 16.2 | 204 |
| **1g** | 4-FBn | 3-MePh | 121 | 7.10 | 59 |
| **1h** | 4-FBn | 3-ClPh | 52.3 | 12.8 | 245 |
| **1i** | 4-FBn | 4-MeBn | 12.9 | 39.8 | 3090 |
| **2a** | Bn | 4-MePh | 14.4 | 7.5 | 519 |
| **2b** | Bn | Ph | 23.5 | 5.60 | 239 |
| **2c** | Bn | 4-ClPh | 28.7 | 5.20 | 183 |
| **2d** | Bn | 4-MeOPh | 23.9 | 12.3 | 515 |
| **2e** | Bn | 3,4-diClPh | 88.9 | 13.2 | 149 |
| **2f** | Bn | 3-$CF_3$Ph | 57.4 | 16.4 | 286 |
| **2g** | Bn | 3-MePh | 38.2 | 21.3 | 558 |
| **2h** | Bn | 3-ClPh | 32.5 | 10.9 | 335 |
| **2i** | Bn | 4-MeBn | 7.20 | 20.4 | 2840 |
| **3** | 4-ClBn | 4-MePh | 5.40 | 11.8 | 2170 |
| **4** | 4-MeBn | 4-MePh | 8.60 | 11.6 | 1340 |
| **5** | 3-ClBn | 4-MePh | 17.4 | 17.5 | 1005 |
| **6** | 3-MeBn | 4-MePh | 14.5 | 9.90 | 679 |
| **7** | 4-MeOBn | 4-MePh | 176 | 312 | 1780 |
| **8** | 3,4-diClBn | 4-MePh | 34.2 | 15.7 | 460 |
| **9a** | 4-FBn | *n*-Bu | 15.6 | 31.6 | 2020 |
| **9b** | 4-FBn | Et | 22.3 | 18.7 | 842 |
| **10a** | Me | 4-MePh | 18.9 | 8.40 | 445 |
| **10b** | Me | Et | 22.3 | 37.0 | 1660 |
| **10c** | Me | *n*-Bu | 23.5 | 28.4 | 1210 |
| **10d** | Me | *t*-Bu | 27.8 | 56.0 | 2020 |
| **11a** | *n*-Bu | 4-MePh | 19.7 | 9.50 | 483 |
| **11b** | *n*-Bu | Et | 14.4 | 83.5 | 5810 |
| **11c** | *n*-Bu | *n*-Bu | 29.8 | 122 | 4110 |
| **11d** | *n*-Bu | *t*-Bu | 53.6 | 119 | 2220 |
| **12a** | *i*-Bu | 4-MePh | 14.0 | 7.70 | 550 |
| **12b** | *i*-Bu | Et | 26.3 | 70.6 | 2680 |
| **12c** | *i*-Bu | *n*-Bu | 38.6 | 98.0 | 2540 |
| **12d** | *i*-Bu | *t*-Bu | 29.1 | 194 | 6660 |
| **13** | $MeOCH_2CH_2$ | Et | 54.3 | 36.1 | 665 |
| **14** | 4-MeBn[b] | 4-MePh[b] | >100000 | >100 | N/A |
| **15** | Bn[c] | 4-MePh[c] | 93300 | 0.0 | 0 |

[a]Values are an average from two independent experiments. The calculated $\Delta pIC_{50}$ gave a mean error of 0.2 for RGS4 and 0.14 for RGS8. [b]Scheme 2 for the structure of **14**. [c]Scheme 3 for the structure of **15**.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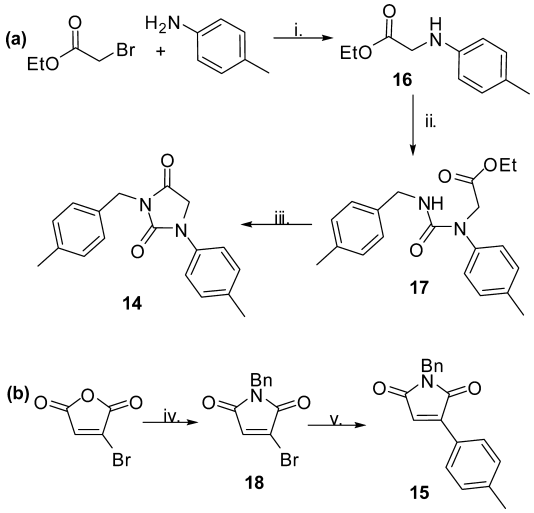
In addition to the large series of TDZD compounds synthesized, an imidazolidine-2,4-dione (**14**) and a maleimide (**15**) were both prepared to compare their activity to the TDZDs. The synthesis of **14** was carried out in three steps, starting from ethyl bromoacetate and *p*-toluidine (Scheme 2a).

**Scheme 2. Synthesis of the non-TDZD Analogues**[a]




[a]Conditions: (i) NaOAc, EtOH, 80 °C, 1 h. (ii) Methyl benzyl isocyanate, toluene, reflux, 5 h. (iii) NaH, THF, 0 °C−room temperature, 18 h. (iv) Benzylamine, AcOH, 50 °C, 18 h. (v) *p*-Tolylboronic acid, CsF, $Cl_2Pd(dppf)\cdot CH_2Cl_2$, dioxane, 40 °C, 1 h.

After reaction in the presence of sodium acetate, the resulting amino acetate **16** was stirred at reflux in the presence of *p*-methylbenzyl isocyanate to provide the corresponding ureido acetate **17**. This was cyclized using sodium hydride, providing **14**. Compound **15** was synthesized by first reacting bromomaleic anhydride with benzyl amine in the presence of acetic acid. The desired product resulted from a Suzuki reaction coupling *p*-tolyl boronic acid to **18**.

All compounds were evaluated using the FCPIA assay as a primary screen to determine $IC_{50}$ values for inhibition of $G\alpha_o$ binding to both RGS4 and RGS8 (the closest relative to RGS4 based upon sequence homology). In preliminary studies, **1a** was also found to suppress $Ca^{2+}$ responses to GPCRs in a manner unrelated to its activity at RGS proteins. The most interesting of the new ligands was also assessed for this off-target effect.

CCG-50014 (**1a**) was confirmed as a potent inhibitor of RGS4 with excellent selectivity over RGS8. Retaining the 4-fluorobenzyl $R^1$ group and varying $R^2$ led to both more (e.g., **1d**) and less (e.g., **1g**) potent and selective compounds. It appeared that a 3-substituent on the $R^2$ aryl ring was associated with reduced RGS4 potency as compared to unsubstituted and 4-substituted analogues (e.g., **1f**, **1g**, **1h** cf. **1b**, **1c**, **1d**). While not completely consistent, this trend is repeated across a number of the series where $R^1$ is held constant and $R^2$ is varied. A 3,4-dichlorophenyl group as $R^2$ generally resulted in low potency at RGS4 and relatively low selectivity (e.g., **1e**, **2e**). In contrast, a 4-methyl substituent was more often associated with high affinity and high selectivity at RGS4, with a number displaying >1000-fold selectivity versus RGS8 (e.g., **3**, **4**, **5**, and **7**). Replacement of the phenyl group by benzyl at $R^2$ (**1i**, **2i**) did not improve activity at RGS4 but did reduce RGS8 activity, resulting in each compound having near 3 orders of magnitude selectivity. In fact, of the compounds discussed so far, that is, retaining a benzyl or substituted benzyl at $R^1$, **1i** and **2i** were the most selective.

Variation in the aryl groups of $R^1$ and $R^2$ has therefore led to the discovery of a number of ligands with high potency and excellent selectivity. However, the uniformly high lipophilicity (Clog *P* typically >4) of these ligands resulted in only moderate solubility in aqueous solution, and they were therefore less than ideal for consideration for more in-depth study. To address this problem, analogues in which one or both R groups were replaced with short alkyl chains were prepared. In the former series, where one aryl group was replaced by alkyl (**9a**,**b**, **10a**, **11a**, and **12a**), potency and selectivity at RGS4 were retained. Making both R groups short alkyl chains (**10b**−**d**, **11b**−**d**, and **12b**−**d**) substantially improved solubility (complete solubility at 500 $\mu$M) while also providing the most consistently selective group of compounds yet developed (all >1000-fold selective). The potency at RGS4 ($IC_{50}$ 14.4 nM), near 6000-fold selectivity, and high solubility of **11b** mean that it is an ideal candidate for further evaluation, including in vivo studies. As a means to even further enhance solubility of this compound, analogues containing ether side chains were considered, and the ether analogue of **11b** was prepared. This compound (**13**) retained good potency (56 nM) and excellent selectivity (>600-fold).

The effects of **1a**, **11b**, and **13** were tested on the $Ca^{2+}$ transient induced by M3 muscarinic receptors in HEK293T cells. Compound **1a** at 10 $\mu$M nearly completely abolished the carbachol-induced $Ca^{2+}$ transient (Figure 2), while **11b** and **13** had no effect. The action of **1a** on this response cannot be through effects on RGS proteins since HEK cells express minimal levels of functional RGS proteins.[21]

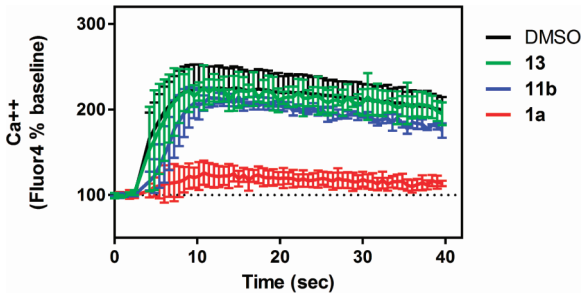



**Figure 2.** Effect of compounds on carbachol-simulated Ca++ responses. HEK-293 cells stably transfected with the human M3 muscarinic receptor were plated in black, clear-bottomed, 96-well plates overnight. They were loaded with Fluo4-NW according to the manufacturer's instructions. After 30 min of loading at 37 °C, the indicated compounds were added at a concentration of 10 $\mu$M (with 1% DMSO). After 30−45 min, the baseline fluorescence was measured in a Flex-3 plate reader (Molecular Devices). Then, carbachol (10 nM final) was injected into the wells, and the increase in intracellular Ca++ was measured and is expressed as the percentage of the baseline Ca++ level. Values are the mean ± SD of triplicate determinations (compounds) and 16 determinations (DMSO).

We have previously published our studies that indicate that the lead compound (**1a**) reacts to form an adduct with a cysteine residue on the RGS protein through disulfide bond formation.[15] The proposed mechanism (Scheme 3, **19b**) is analogous to that proposed by Nasim and Crooks for the ring-opening of TDZDs with $PPh_3$.[20] To help confirm the importance of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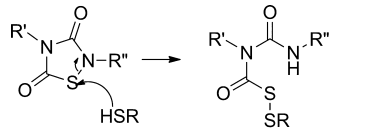
**Scheme 3. Proposed Mechanism of Reaction of a Thiol with 1a**




**19a** R = $CH_2CH_2CH_3$
**19b** R = RGS4-cys

disulfide bond formation to the activity of this series of ligands, analogues **14** and **15** were prepared. Compound **14** is the imidazolidine-2,4-dione analogue of **4**, while **15** is the maleimide analogue of **2i**; **4** and **2i** being two of the most potent inhibitors discovered. As expected, neither **14** or **15** displayed activity at RGS4. Also supporting the disulfide bond-forming mechanism, the reaction of propane thiol with **1a** appears to give efficiently and cleanly the expected adduct **19a** (Scheme 3). Importantly, **1a** is not a general cysteine alkylator, failing to inhibit the cysteine protease papain, suggesting selectivity for RGS4.[15]

Previously, thiadiazolidine-3,5-diones have been reported as having a number of biological effects,[22−24] including being glycogen synthase kinase 3$\beta$ (GSK-3$\beta$) inhibitors with activities in the micromolar range.[19] This latter activity has been suggested to account, at least in part, for the antidepressant-like effects in mice of the TDZD NP031115.[25] Interestingly, **11b** was evaluated as part of that study and was found to be one of the weaker inhibitors (GSK-3$\beta$ $IC_{50}$ 70 $\mu$M) meaning that it has significant selectivity (almost 5000-fold) for RGS4 over GSK-3$\beta$. As such, **11b** should prove to be an invaluable tool in defining the physiological role of RGS4 in vivo, including a potential role in 5-HT1A-mediated antidepressant effects.[26]

In summary, a series of RGS4 inhibitors have been synthesized with improved selectivity over RGS8 and lacking the off-target calcium mobilization activity of the lead **1a**. One compound, **11b**, combines potency (RGS4 $IC_{50}$ 14 nM) and selectivity (5800-fold over RGS8 and no calcium transient) with excellent aqueous solubility and should prove an invaluable tool for better defining the role of RGS4 and its potential as a therapeutic target. Its ether analogue (**13**) had further improved solubility while retaining good potency and selectivity. Analogues **11b** and **13** are now being evaluated in vivo with positive preliminary data, and the results of this latter work will be reported separately.

## ■ ASSOCIATED CONTENT

### Supporting Information

Tabulated pharmacological data for all compounds, representative synthetic procedures, $^1H$, $^{13}C$ NMR for all new compounds, and elemental analysis data for key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Tel: (0)1225 383103. Fax: (0)1225 386114. E-mail: s.m.husbands@bath.ac.uk.

### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

GPCR, G protein-coupled receptor; RGS, regulators of G protein signaling; CNS, central nervous system; MOP, $\mu$ opioid receptor; DOP, $\delta$ opioid receptor; PPI, protein−protein interaction; FCPIA, flow cytometry protein interaction assay; TDZD, thiadiazolidinone

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