Small Molecule Inhibitors of Regulator of G Protein Signalling (RGS) Proteins

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Supporting Information Placeholder

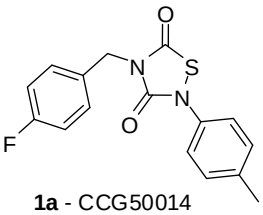
ABSTRACT: Recently regulators of G protein signalling (RGS) proteins have emerged as potential therapeutic targets since they provide an alternative method of modulating the activity of GPCRs, the target of so many drugs. Inhibitors of RGS proteins must block a protein-protein interaction (RGS-Gα), but 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 optimised for activity, selectivity and solubility. The newly developed ligands (**11b**, **13**) display substantial selectivity over the closely related RGS8 protein, lack the off-target calcium mobilisation activity of the lead **1a** and have excellent aqueous solubility. They are currently being evaluated *in vivo* in rodent models of depression.

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 signalling. Other approaches to modulate GPCR-mediated signal transduction may also provide significant therapeutic benefit. To this end, we have focused upon targeting a class of proteins that negatively regulates GPCR signalling.

Regulators of G protein signalling (RGS) proteins are potent negative modulators of GPCR signalling. They accelerate the rate of GTP hydrolysis by Gα 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 signalling 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 signalling 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 characterised. 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 amongst others.[8,9] The selectivity in regional distribution, coupled with the finding that RGS4 can selectively suppress signalling through DOP receptors compared to MOP receptors,[10] suggests a level of specificity for RGS4 inhibitory action will be possible.

The RGS-Gα 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. Whilst a number of PPI inhibitors are known against a variety of targets, most of these compounds are large molecules which 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 non-trivial 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 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 synthesised with variation in both the N2 and N4 side chains. In addition, a set of these newly synthesised RGS4 inhibitors have been evaluated for their effects on calcium mobilisation, an off-target activity displayed by **1a**.



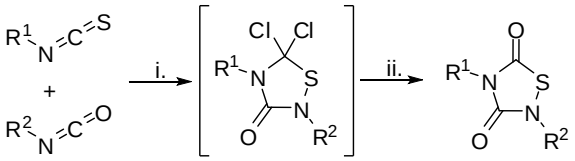
1a - CCG50014
RGS4 IC_{50} 30.1 nM
RGS8 IC_{50} 11.0 mM

Figure 1: Structure and activity of lead compound, CCG50014

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 oxidised 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 synthesised, with 39 (**1a-i**, **2a-i**, **3-8**, **9a,b**, **10a-d**, **11a-d**, **12a-d, 13**) reported in Table 1. Data for all compounds is reported in the supplementary information.

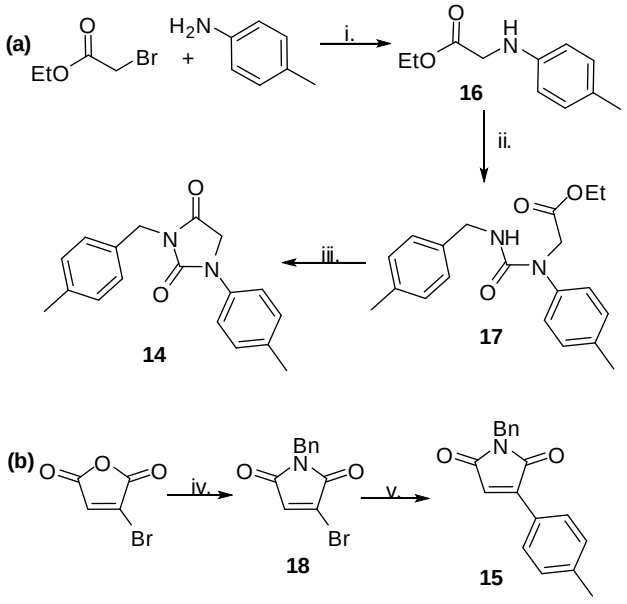
Scheme 1: Synthesis of CCG50014 and analogues



Conditions: i. SO_2Cl_2, THF, 0 °C – rt, 18 h ii. air, 30 min

In addition to the large series of TDZD compounds synthesised, an imidazolidine-2,4-dione (**14**) and a maleimide (**15**) were both prepared to compare their activity to the TDZDs. Synthesis of **14** was carried out in three steps, starting from ethyl bromoacetate and *p*-toluidine (Scheme 2a). 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 cyclised using sodium hydride, providing **14**. Compound **15** was synthesised 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**.

Scheme 2: Synthesis of the non-TDZD analogues



Conditions: i. NaOAc, EtOH, 80 °C, 1 h ii. methyl benzyl isocyanate, toluene, reflux, 5 h iii. NaH, THF, 0 °C – rt, 18 h iv. benzylamine, AcOH, 50 °C, 18 h v. *p*-tolylboronic acid, CsF, $Cl_2Pd(dppf).CH_2Cl_2$, dioxane, 40 °C, 1 h

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^{++} responses to GPCRs in a manner unrelated to its activity at RGS proteins. The most interesting of the new ligands were also assessed for this off-target effect.

Table 1: Inhibition of Gαo binding to RGS4 and RGS8

	R^1	R^2	RGS4[a]	RGS8[a]	Selectivity
			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_3Ph	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_3Ph	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	311.6	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*	4-MePh*	>100000	>100	N/A
15	Bn**	4-MePh**	93300	0.0	0

[a]Values are an average from two independent experiments. The calculated ΔpIC_{50} gave a mean error of 0.2 for RGS4 and 0.14 for RGS8. * Scheme 2 for structure of **14** ** Scheme 3 for structure of **15**

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 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**, **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 three orders of magnitude selectivity. In fact, of the compounds discussed so far, i.e. 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 (ClogP 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**, **12a**), potency and selectivity at RGS4 was retained. Making both R groups short alkyl chains (**10b-d**, **11b-d**, **12b-d**) substantially improved solubility (complete solubility at 500 μM) whilst 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** means 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** prepared. This compound (**13**) retained good potency (56 nM) and excellent selectivity (>600-fold).

The effect of **1a**, **11b**, and **13** were tested on the Ca^{2+} transient induced by M3 muscarinic receptors in HEK293T cells. **1a** at 10 μM 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]

We have previously published our studies which 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 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]

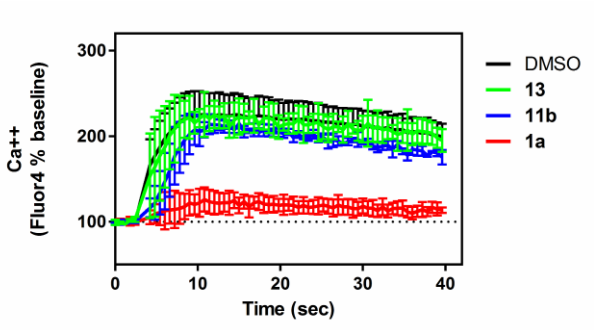
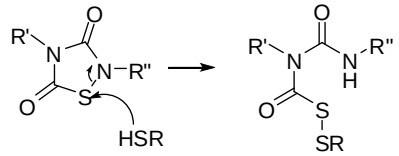


Figure 2: Effect of compounds on carbachol-simulated Ca++ responses. HEK-293 cells stably transfected with the human M3 muscarinic receptor were plated in black, clear-bottomed, 96-well plates overnight. They were loaded with Fluo4-NW according to the manufacturer's instructions. After 30 minutes of loading at 37 °C, the indicated compounds were added at a concentration of 10 uM (with 1% DMSO). After 30-45 minutes, 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).

Scheme 3: Proposed mechanism of reaction of a thiol with **1a**



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

Previously thiadiazolidine-3,5-diones have been reported as having a number of biological effects,[22-24] including being glycogen synthase kinase 3β (GSK-3β) inhibitors with activities in the μM 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-3β IC_{50} 70 μM) meaning that it has significant selectivity (almost 5000-fold) for RGS4 over GSK-3β. 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 has been synthesised with improved selectivity over RGS8 and lacking the off-target calcium mobilisation activity of the lead **1a**. One compound, **11b** combines potency (RGS4 IC50 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 whilst 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

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

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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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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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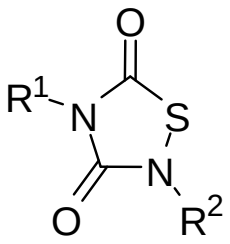

Selective RGS4 Inhibitors