Author's response to reviews

Title: Safety, tolerability, pharmacokinetics and pharmacodynamics of GSK233633, A CC-chemokine receptor 4 antagonist, in healthy male subjects: results from an open-label and from a randomised study

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Author's response to reviews: see over
DATE

To: Professor François Marceau
Associate Editor
BMC Pharmacology and Toxicology

RE: Resubmission of revised manuscript MS: 1120050917945349

Dear Professor Marceau,

We write to acknowledge the safe receipt of your email dated 14 January that included comments from two Reviewers. We have taken the opportunity to fully review the manuscript and we also provide responses to the Reviewers’ comments (see below). We enclose a revised version of the manuscript.

I hope you will find our responses satisfactory and will find the manuscript acceptable for publication in BMC Pharmacology and Toxicology.

Please do not hesitate to contact me if you require any further information.

Yours sincerely,

Dr Anthony Cahn
RESPONSE TO COMMENTS FROM REVIEWER 1

Comment 1: The phrase "determined using an internally validated analytical method" is used more than once in the manuscript which makes it hard to review properly. Either the authors should provide full details of methods used or provide references upon which the methods are based.

Response from authors: The following text with full details of the assays performed has been added as Supplemental material:

Microdose Intravenous Study. Aliquots (0.06 mL) of human plasma were analysed for total radioactivity by accelerator mass spectrometry (AMS). Samples were first prepared so that the carbon within the samples was harvested and converted to graphite. This involved a two stage process of sample combustion (oxidation) followed by graphitisation (reduction) as previously published [1]. The carbon analysis was performed using a Costech Elemental Combustion System (Model 4010) CHNS-O analyser (Valencia, CA, USA; supplied by Pelican Scientific Ltd, Chester, UK). The AMS used was a 250 kV single stage accelerator mass spectrometer (National Electrostatics Corp., Middleton, Wisconsin, USA). The instrument was operated via NEC proprietary “AccelNET” software on a Linux operating system. Post-acquisition data processing was performed using the NEC software “abc”. Further details about the operating conditions have been previously published [1].

Plasma concentrations of GSK2239633 were determined using a validated analytical method based on extraction by protein precipitation of 1 mL of human plasma with three volumes of acetonitrile. Both GSK2239633 and [14C]-GSK2239633, as a tracer, were extracted from 1 mL of human plasma using three volumes of acetonitrile containing non-labelled GSK2239633 at a concentration of 10 µg/mL, as an internal standard. The tubes were mixed by vortex and centrifuged for 10 minutes at approximately 3000 g prior to transfer of the supernatant to clean tubes. The dried extracts were reconstituted with 200 µL of water:acetonitrile (95:5, v:v) containing 0.1% formic acid, mixed and centrifuged at 3000 g for 30 minutes. The extract was
then injected (100 µL) into a high performance liquid chromatography system utilising an
Phenomenex Luna C18 (3 µ packing, 150 x 4.6 mm) column (Phenomenex Ltd., Macclesfield,
UK) and eluted with a gradient of aqueous 0.1% formic acid and 0.1% formic acid in
acetonitrile. GSK2239633 had a retention time of approximately 16.5 minutes and this was
collected as discrete fractions into quartz tubes for further processing to harvest graphite and
subsequent analysis by AMS as described above. The assay had a linear dynamic range of 1–
500 pg/mL and quantification was performed against GSK2239633 spiked recovery standards
based on demonstrated linearity with non-weighted regression.

**Single Oral Dose Study.** Blood concentrations of GSK2239633 were determined using a validated
analytical method based on extraction from a dried blood spot, with a 3 mm disc punched from a
0.015 mL sample on Ahlstrom 226™ card (PerkinElmer, Greenville, SC, USA). GSK2239633 was
extracted using methanol (0.1 mL) containing an isotopically labelled \[^2\text{H}_2, ^{15}\text{N}_2, ^{13}\text{C}_1\] \[\text{GSK2239633}\] at a concentration of 10 ng/mL as an internal standard. The extraction tubes were
shaken for 1 hour at room temperature before transferring the supernatant into clean tubes. The
supernatant was injected (5 µL) into a high performance liquid chromatography system with an
Acquity BEH C18 (1.7 µ packing 50 x 2.1 mm) column (Waters, Boston, Mass, USA) and eluted
using a gradient of aqueous 0.1% formic acid and acetonitrile. GSK2239633 had a retention time
of approximately 1 minute, with detection performed by tandem mass spectrometry on a Sciex
4000 (Applied Biosystems, Warrington, UK) using TurboIonSpray in positive polarity mode.
Mass transitions from precursor ions were monitored for 368 m/z>374 m/z for GSK239633 and
549 m/z>555 m/z for internal standard. The assay had a linear dynamic range of 10–
10,000 ng/mL and quantification was performed using peak area ratios with \(1/x^2\) weighted linear
regression.

1. Young GC, Corless S, Felgate CC, Colthup PV. Comparison of a 250kV single-stage
accelerator mass spectrometer with a 5MV tandem accelerator mass spectrometer - fitness for
Comment 2: Please explain the criteria used for the investigator to determine whether an adverse event was related to the study drug.

Response from authors: For both studies, a possible causal relationship between the study drug and the adverse event was determined by the Investigator using his/her clinical judgment and in accordance with current standards. For increased clarity, the sentence below has been added to the Methods:

Adverse events were recorded throughout both studies. For each event, the potential causal relationship with the study drug was assessed by the investigator. Other safety assessments in both studies included clinical laboratory tests (chemistry, haematology, urinalysis), vital signs, 12-lead electrocardiogram (ECG) and continuous cardiac telemetry.

Comment 3: The Ro experiments and data raise a number of issues, not least whether the drug is really binding to the receptor as there appears to be very little change in occupancy as the dose of study drug increases. The authors should show that the drug is actually effective in this assay rather than reporting as unpublished observations in the introduction. It would also be useful for the authors to display the data for the placebo group in Table 5 to give an idea of what the Ro is in this untreated group to allow evaluation of the noise in this system. In addition when comparing the Ro between placebo and treated groups (Figure 1B), the 900 mg dose appears most effective, but there is little discussion of the shifts in EC50 as a result of drug treatment. Was there any difference in the fed cohort?
Response from authors: Regarding the comment of the reviewer as to whether the drug is really binding to the receptor, we have modified the sentence (shown below) in the Methods to make it clearer that receptor occupancy was estimated and not calculated:

The fractional occupancy of CCR4 (Ro) was then estimated by determining the dose-ratio (DR) from the change in effective concentration giving 50% of the maximal response (EC$_{50}$) of the TARC concentration-response curve before and after dosing with GSK2239633 and using the formula Ro = (DR – 1)/DR [32].

We have added a figure to the manuscript (Figure 2) containing results obtained in *in vitro* studies. This figure shows TARC-induced increases in filamentous actin content of CD4$^+$ CCR4$^+$ T-cells in whole human blood samples. The results section was updated accordingly. To address the point made by the reviewer regarding the presentation of placebo data in Table 5, we have added the text below at the end of the Results section. In addition, Ro was estimated using data from the same subject where pre-dose data acted as a control. If this is done for the placebo data then all the derived values are negative or in other words, there is no indication of inhibition. To clarify this, we added the following text to the Methods:

Results

**Blood from placebo subjects, analysed in the same way and at the same time-points, did not show any shifts in the response curves, which would indicate CCR4 inhibition, when the post-dose curves were compared with the pre-dose curves. The placebo data also revealed the inherent variability in the technique as shown by the approximately 3-fold range in variability for the derived EC$_{50}$ of TARC (pre-dose = 0.34 nM; all data = 0.1–0.34 nM).**
Methods

Analysis of the entire individual pharmacodynamic and pharmacokinetic datasets was conducted to derive mean EC\textsubscript{50} estimates pre-dose and in the presence of GSK2239633 (each subject acted as their own control as their pre-dose data was compared with their post-dose data). Although not a direct method for formal calculation of Ro, this DR was used to give an estimate of Ro as described above.

The reviewer also questions the comparisons between placebo and treated groups for Ro (Figure 1B). We would like to clarify that the figure was used for a visual checking of all the curves and not for comparisons between placebo and active or for representing effect in a quantitative manner. This figure just shows that we have generated dose response curves and that no more drug effect is observed. Due to the nature of the model and sources of variability, the analysis was done within subjects (ie pre-dose was used as baseline/placebo control and not the placebo data), as previously explained. Taking this into consideration, the reason for the 900 mg dose appearing to be the most effective stems from an incorrect interpretation of Figure 1B as this figure shows only a drug effect from placebo. The EC\textsubscript{50} was more variable than DR due to inter-subject variability. By using each subject’s pre-dose value as a control, we hoped to reduce this source of variability. The range of the EC\textsubscript{50} for active treatment is not mentioned because we felt that in the context of the manuscript it will not be appropriate since we are explaining the effect of the drug and not TARC pharmacodynamics. Nonetheless, as shown above, we have added this information for placebo at the end of the Results. Also, to clarify that the assay/analysis was not performed for the fed cohort, we have added the following sentence to the Methods:

For pharmacodynamic analysis, blood was collected pre-dose and at 1, 4 and 24 hours post-dose.

The pharmacodynamic analysis was only conducted for subjects in the fasted condition; no
analysis was performed for the fed cohort. An aliquot of urine was collected pre-dose; after
dosing, all urine was collected and pooled during a 24 hour interval.

Comment 4: The discussion is largely a reiteration of the results with very little
attempt to explain the results in the context of the relevant literature. For example the
first statement after the summary says "The results from the Single Oral Dose Study
are consistent with the physicochemical properties of the molecule" but provides no
references for this statement. The discussion continues in this vein until the final
paragraph.

Response from authors: To address the issue concerning the physicochemical
properties of the molecule we have added the wording shown below:

The blood exposure of GSK2239633 achieved in the Single Oral Dose Study was substantially
lower than that expected based on the high oral bioavailability obtained in several pre-clinical
species; however, it may be consistent with the physicochemical properties of the molecule, high
molecular weight (549) and low solubility (0.02 mg/mL), and could explain the limited absorption
window observed in humans.

In order to explain the results in the context of the relevant literature, we have added
the following text to the Discussion:

No dose-limiting toxicity or maximum tolerated dose was identified. There was no relationship in
the frequency or severity of adverse events with increasing doses of GSK2239633. The anti-CCR4
antibody, mogamulizumab, has been tested in clinical studies, although the study population for
this was patients with relapsed CCR4+ adult T-cell lymphomas and other peripheral T-cell
lymphomas [27]. Mogamulizumab has thus far been shown to be well-tolerated in that study population and none of the adverse events reported were specific to inhibition of CCR4 [27].

A further general challenge with chemokines often speculated upon is the potential for redundancy within the chemokine system and alteration of function during evolution [2, 37]. Results from some studies [38,39,40] indicate that chemokine receptors are also a good target for adjuvant discovery, in particular CCR4, as this receptor is expressed by regulatory T cells, a subset of T cells which normally functions in the down-regulation of immune responses induced by dendritic cells [41]. One of these studies identified CCR4 antagonists acting as adjuvants for both cellular and humoral immune responses.

**Comment 5:** In the methods it states "Subjects completed a screening visit within 28 days of receiving the first dose of study medication" this is confusing as it could be read that the subjects were screened 28 days after the first dose. This should be revised for clarity.

**Response from authors:** The sentence has been modified as below:

Subjects completed a screening visit within 28 days prior to receiving the first dose of study medication.

**Comment 6:** Figure 1B is labelled as Figure 2

**Response from authors:** The labelling of Figure 1B has been corrected.
RESPONSE TO COMMENTS FROM REVIEWER 2

Comment 1: CCR4 is also expressed on regulatory T cells, the cells that play a critical role in the immune tolerance. Authors have to analyze whether CCR4 antagonists had any adverse effects on these regulatory T cells by analyzing their numbers and functions in injected subjects.

Response from authors: The intravenous study was a microdose study and we were not anticipating any pharmacological activity by the drug. The primary objective of the microdose study was to make an initial assessment of the pharmacokinetics of the study drug in man as it was not possible to make a reliable prediction of the pharmacokinetic profile based on the pre-clinical data. In line with current standards, the intravenous study used an ultra-low dose of study drug and no adverse effects on T-cells were anticipated. The single oral dose study was a safety, tolerability and pharmacokinetics, pharmacodynamics study. This study was not designed to assess efficacy in a disease setting nor the biological function of CCR4 in any of the cells on which it is expressed. Such functional studies would form part of a Phase II programme that would need to be appropriately designed to investigate these parameters. Moreover, markers and functional studies on human T-regulatory cells are particularly challenging and it is not clear at this stage whether a convincing protocol could be built into a clinical trial design or whether it would be possible to detect breakdown in immune tolerance after a single dose as given in this study. The CCR4 antagonist tested in the current study appears to be safe and well tolerated.
**Comment 2:** Are these CCR4 antagonists block Th2 cells or deplete them? This issue needs to be addressed

**Response from authors:** Unlike the monoclonal antibodies that induce cytotoxic activity that have so far been tested in clinical studies, this molecule (GSK2239633) is an allosteric inhibitor and as such, it should block the activity of Th2 cells but not deplete them.

**Comment 3:** CCR4 antagonists are recently explored to have adjuvant properties. Thus, CCR4 antagonists have broad applications. This has to be discussed and referred (Bayry et al PNAS 2008, Davies et al PLoS ONE 2009, Pere et al., Blood 2011).

**Response from authors:** To address this comment, we have added the following text to the end of the Discussion:

A further general challenge with chemokines often speculated upon is the potential for redundancy within the chemokine system and alteration of function during evolution [2, 37].

**Results from some studies** [38,39,40] **indicate that chemokine receptors are also a good target for adjuvant discovery, in particular CCR4 as this receptor is expressed by regulatory T cells, a subset of T cells which normally functions in the down-regulation of immune responses induced by dendritic cells [41]. One of these studies identified CCR4 antagonists acting as adjuvants for both cellular and humoral immune responses.**