University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: Simpson Forename: Lorna

   E-mail address: 2063052S@student.gla.ac.uk

2. Supervisor:

   Surname: Bulleid Forename: Neil

   E-mail address: neil.bulleid@glasgow.ac.uk

3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Measuring the level of reactive oxygen species in cells

   3.2 Project Lay Summary (copied from application):

   It is now well established that hydrogen peroxide, nitric oxide and hydrogen sulfide can act as signalling molecules within the body. These gases are important regulators of cell function. However, excessive production of these highly reactive molecules can cause a variety of different diseases including cancer, neurodegeneration leading to Alzheimer's or Parkinson's disease, diabetes or cardiovascular disease. Considering their important biological role it is somewhat surprising that we have little idea of their cellular levels and how these levels change. Here we will evaluate a novel approach to quantify the levels of hydrogen peroxide in mammalian cells with both temporal and spatial resolution.

3.3 **Start Date**: 27th July 2015 **Finish Date**: 4th September 2015

3.4 Original project aims and objectives (100 words max):

Objectives

(i) Overall, to develop a novel approach to quantify the level of various gasotransmitters with both spatial and temporal resolution

(ii) To quantify the chemoselective conversion of the conjugate of the purified SNAP-tag into product SNAP-tag conjugate by hydrogen peroxide, quantifying this transformation by using antibody selective binding to reaction product.

(iii) To validate the use of the chemical probes in cells using detection by antibody selective binding to reaction products after treating cells with reagents known to increase intracellular level of hydrogen peroxide

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Prior to the project beginning, the following materials were prepared:

1. Cell lines expressing SNAP-tag localised to the cytosol and endoplasmic reticulum.
2. Chemoselective benzylguanine derivatives B1 and B2 which react highly selectively with H_2O_2 and A which reacts with HNaS converting a boronate grouping to a phenolic product.
3. Anti SNAP P antibody raised against the phenolic product which has been shown to react specifically with the modified SNAP-tag.

Cell-lines expressing SNAP-tag were modified with 5µM of the boronate probes for 30 minutes, providing a means to follow its conversion to the phenolic product. The cells were subsequently lysed and spun for 10 minutes. The supernatant containing the cellular proteins was reacted with 120µl of either H_2O_2 or HNaS for 10 minutes. Samples were run on 15% SDS page gels alongside negative controls which hadn't been reacted with the gasotransmitters. The levels of reacted probe present were assessed by western blotting with the anti-SNAP P antibody. Total levels of SNAP-tag within the cell were assessed using an anti-Flag antibody. Differences between band intensities of reacted and unreacted probes were measured to determine the effectiveness of each probe at quantifying intracellular levels of gasotransmitters.

A time course was run for both the B2 and A probes with the cells being incubated with the probe for either 30, 45 or 60 minutes.

An additional experiment was run in which the gasotransmitters were added prior to lysing the cell.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

The ER cell line was shown to not be over-expressing SNAP at detectable levels therefore; we were unable to determine whether the probes could measure intracellular hydrogen peroxide with spatial resolution.

Within the cytosolic cells:

The B1 probe only showed an average increase in band intensity of 2.8% between unreacted and reacted samples.

The B2 probe in contrast showed a clear difference in band intensity between the unreacted and reacted samples with an average increase of 98% over two experiments.

The anti-SNAP P antibody was also shown to be able to differentiate between the boronate and phenolic products of the A probe when reacted with HNaS. There was an average increase of 161.9% over three experiments between unreacted and reacted samples.

Results for the time course with probe A showed that over the 60 minute period, the intensity of the anti-SNAP P signal increased from 0.45 at 30 minutes to 1.12 after an hour. However, the percentage difference between the anti-Flag and anti-SNAP P signals decreased drastically from 435.88% to 39.15 % over the same period.

Similarly, results from the B2 time course showed an increase in band intensity for anti-SNAP P from 0.34 at 30 minutes to 0.82 at 45 minutes before decreasing slightly to 0.67 after 60 minutes. This coincided with a large decrease in the percentage difference between unreacted and reacted SNAP from 1264.43% to 12.38% over the hour.

Treating the cells with H_2O_2 or HNaS pre-lysis appeared to decrease both the signal intensity of the bands and the percentage increase between the boronate and phenolic products. A percentage increase of -19.37% was measured for B1, 29.62% for B2 and 12.41% for A – much lower than those measured when the gasotransmitters were added post-lysis.

3.7 Discussion (500 words max):

The two experiments run using B1 only showed an increase of 4.63% and 1.04% respectively after the addition of H_2O_2. The similarity in band intensity between the unreacted and reacted samples over the two experiments suggests that the anti-SNAP P antibody is binding to both the boronate and phenolic forms of the probe. An explanation of this observation is

that both the reactant and product forms are too structurally similar and that consequently the antibody is unable to distinguish between the two. Therefore, use of the B1 probe would not be suitable as a method of detecting the presence of H_2O_2 within cells.

The phenolic forms of both the B1 and B2 probes have the same structure; however the boronate forms differ in that the B2 probe has an additional phenol group. This addition is significant as it appears to be substantial enough to allow the anti-SNAP P antibody to be able to differentiate between the unreacted and reacted forms of the probe. Thus it will only bind to the probe if it has reacted with hydrogen peroxide rather than with both the reactant and product as is the case for the B1 probe. Consequently the use of the B2 probe is a much more effective method for detecting the presence of hydrogen peroxide within cells. This experiment would however, would need to be repeated in order to ascertain whether these results are statistically significant.

Similarly over three experiments, probe A consistently showed a large increase in band intensity upon the addition of HNaS. The calculated p value of 0.039135 is less than the 0.05 significance level therefore the null hypothesis, that there is no significant difference between quantity of reacted and unreacted probe, can be rejected. This confirms the molecule's suitability for detecting the presence of HNaS in cells.

A time course was run for the B2 and A probes in which probes were incubated in the cells for either 30, 45 or 60 minutes before lysis. Out of times tested, 30 minutes appears to be optimum time for probe incubation. Over time the quantity of reacted probe increases in both the reacted and unreacted samples and the percentage difference between the two decreases. This suggests that the probes are reacting with gasotransmitters already present within the cells. This theory could be tested by inhibiting the production of H_2O_2 and HNaS in the cell and repeating the experiment. An additional time course experiment could be set up to test times lower than 30 minutes.

When cells were treated with the gasotransmitters prior to lysis, the percentage increase in band intensity decreased significantly, suggesting this method is not as effective as that used previously.

Initial results appear to indicate that the method developed could be a viable means of quantifying intracellular levels of H_2O_2 and HNaS. We were unable however, to ascertain whether we could quantify the level of the gasotransmitters with spatial resolution due to the failure of ER cells to over-express SNAP.

4. Reflection by the student on the experience and value of the studentship (300 words max):

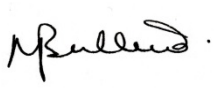
Overall I found the studentship to be an incredibly valuable experience. I was taught a wide variety of lab techniques such as cell culture, making SDS gels, western blotting, buffer preparation and protein purification. All of which will be very useful throughout the rest of my undergraduate degree and my future career. I was continually encouraged to ask questions and to interpret my own results. I increased in confidence over the six weeks and was increasingly given greater responsibility for

my own experiments as the project progressed. Everyone in the lab was very helpful and gave up a lot of their time to assist me. They were also glad to talk to me about their projects and career paths. This enabled me to gain a greater insight into what it would be like to work in an academic research environment and what a PhD would involve. Completing this placement has confirmed my interest in a career in research and in perhaps completing a PhD after finishing my undergraduate degree.

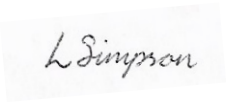
5. **Dissemination: (note any presentations/publications submitted/planned from the work):**

   The results obtained ill form the basis of a future publication. The work is a continuation of a past PhD student. The work will be presented at our lab meetings and to other vacation students on the programme.

6. Signatures: Supervisor Date Student Date



23/09/2015



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Appendix:

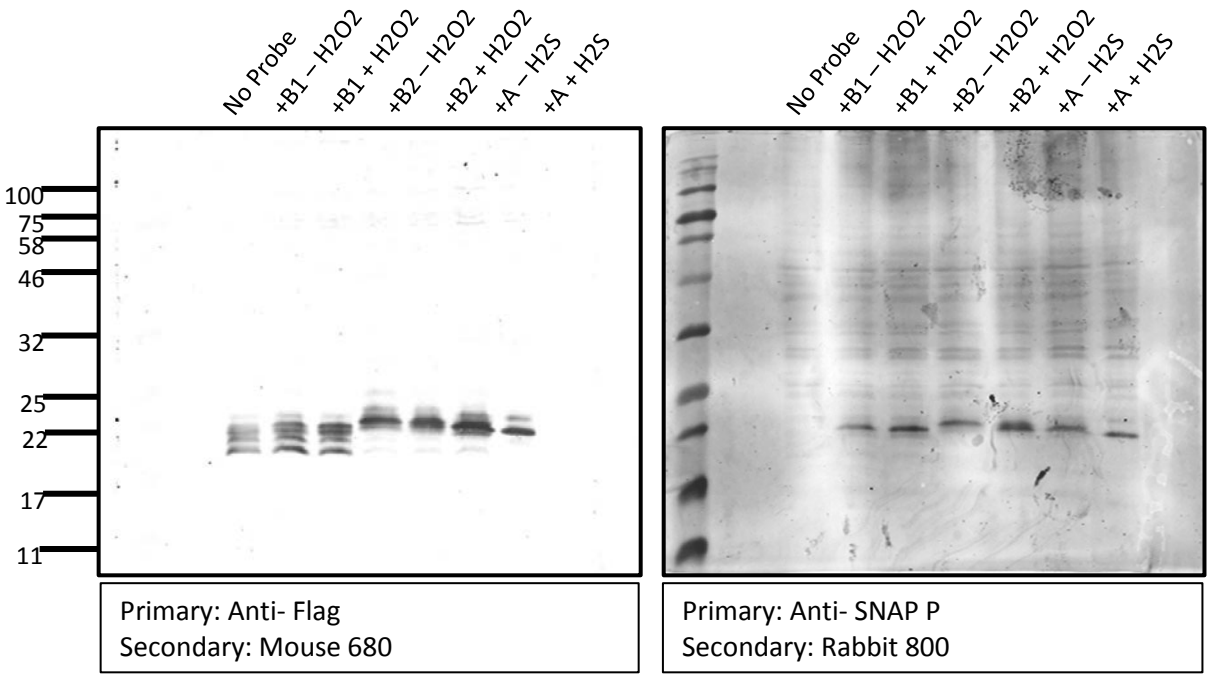

Figure 1: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivatives B1, B2 or A and treatment with gasotransmitters.

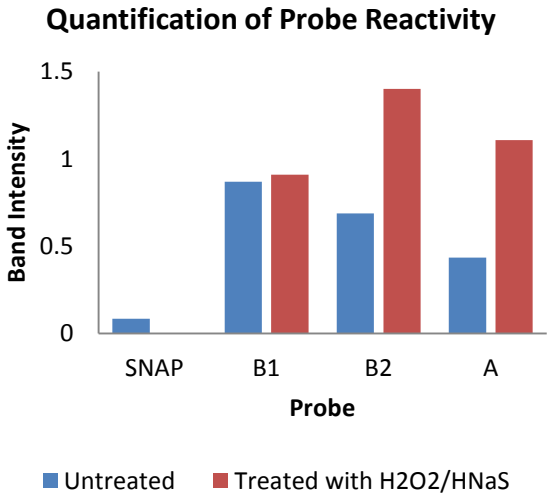

Figure 2: Comparison between western blot band intensity of cells which had been reacted with gasotransmitters and negative controls.

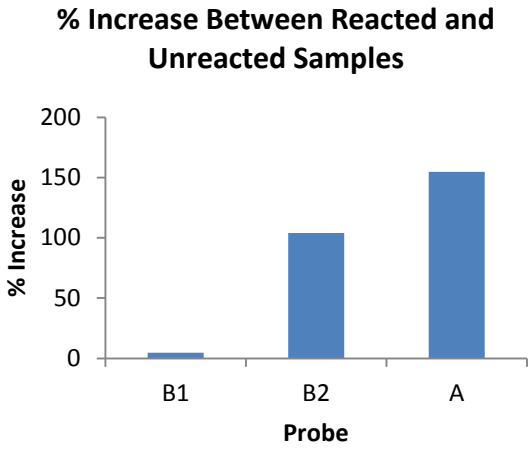

Figure 3: Analysis of percentage increase in western blot band intensity between reacted and unreacted cells.

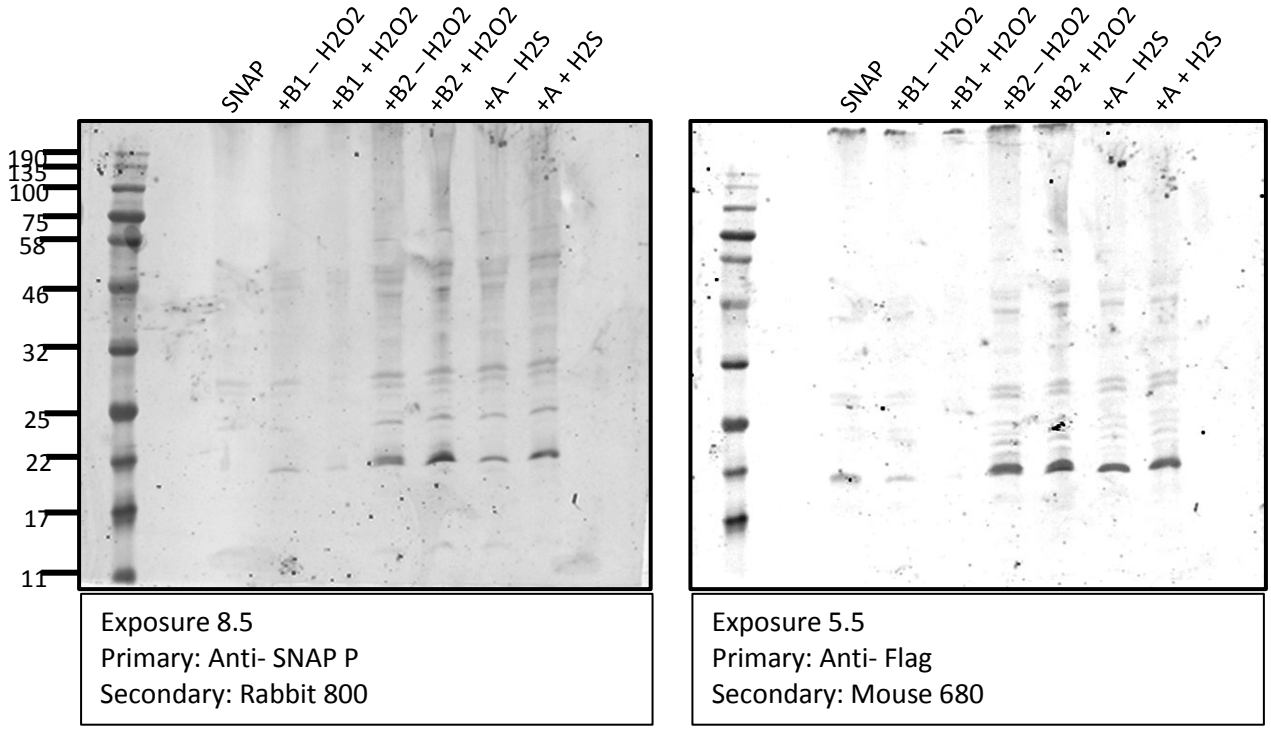

Figure 4: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivatives B1, B2 or A and treatment with gasotransmitters.

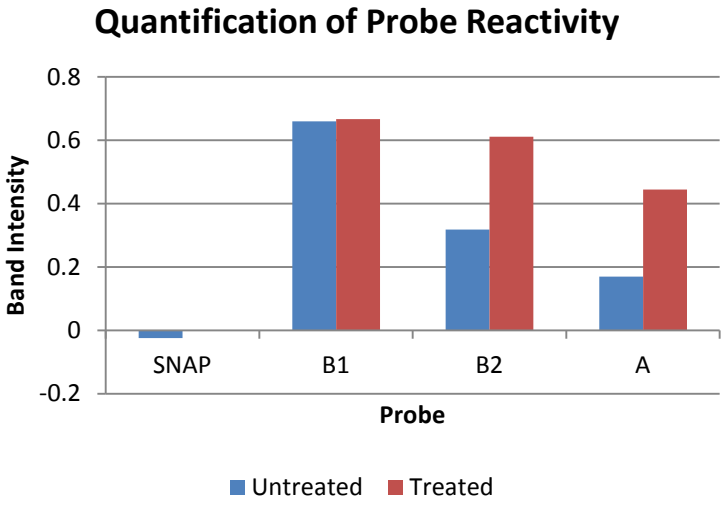

Figure 5: Comparison between western blot band intensity of cells which had been reacted with gasotransmitters and negative controls.

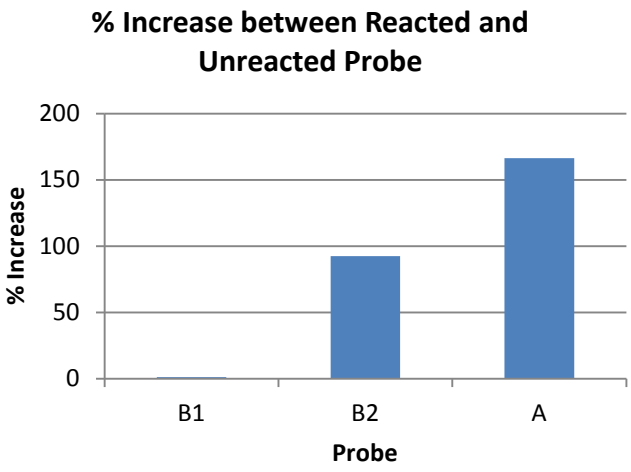

Figure 6: Analysis of percentage increase in western blot band intensity between reacted and unreacted cells.

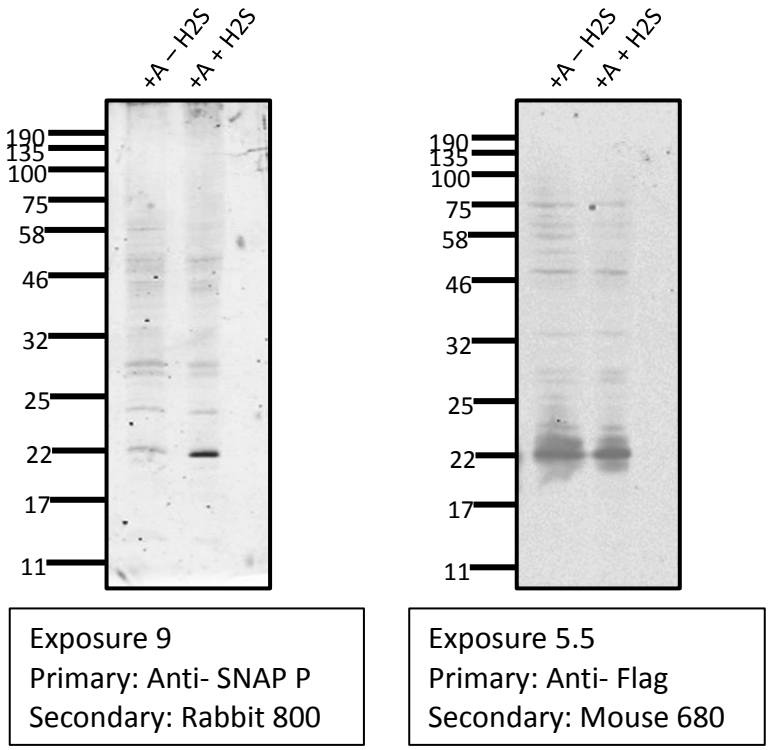

Figure 7: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivative A and treatment with HNaS.

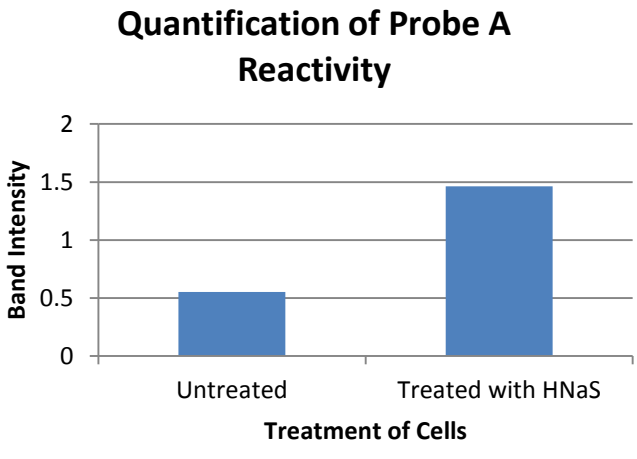

Figure 8: Comparison between western blot band intensity of cells which had been reacted with HNaS and negative controls.

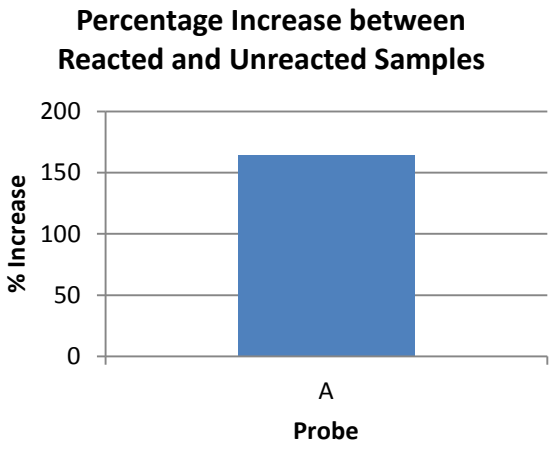

Figure 9: Analysis of percentage increase in western blot band intensity between reacted and unreacted cells.

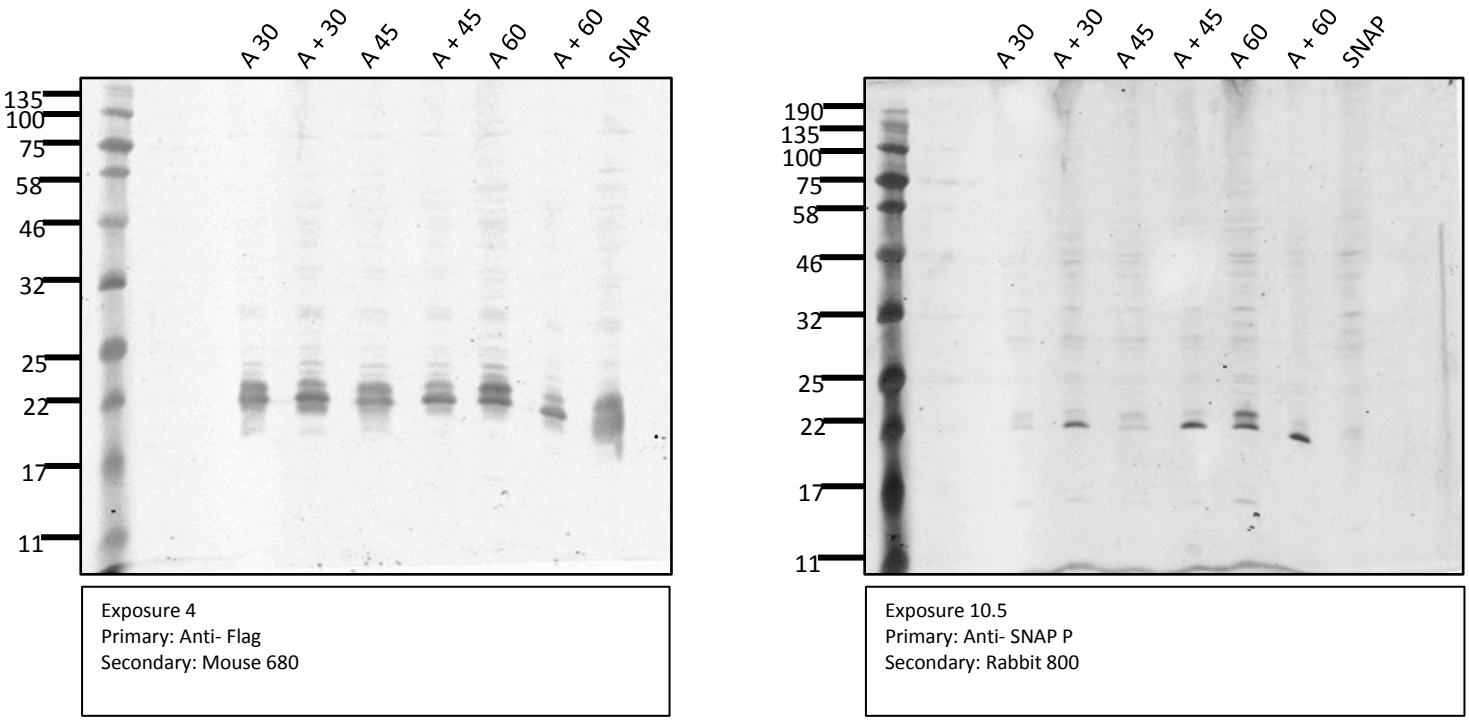

Figure 10: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivative A over 30, 45 and 60 minutes and treatment with HNaS.

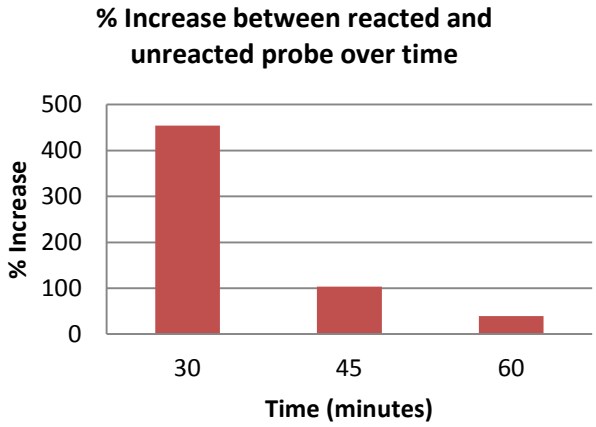

Figure 11: Analysis of percentage increase in western blot band intensity between reacted and unreacted cells after cells had been incubated with probe A for either 30, 45 or 60 minutes.

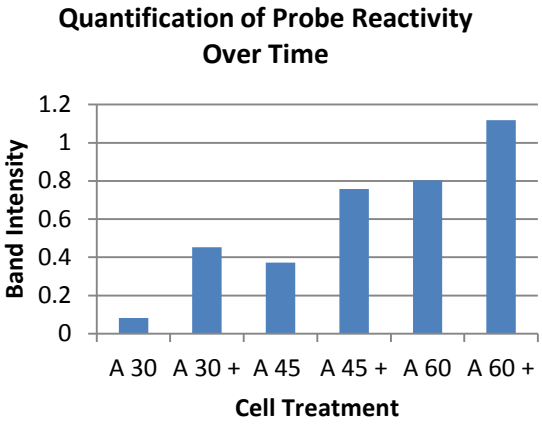

Figure 12: Comparison between western blot band intensity of cells which had been incubated with probe A for 30, 45 or 60 minutes and then reacted with HNaS and negative controls.

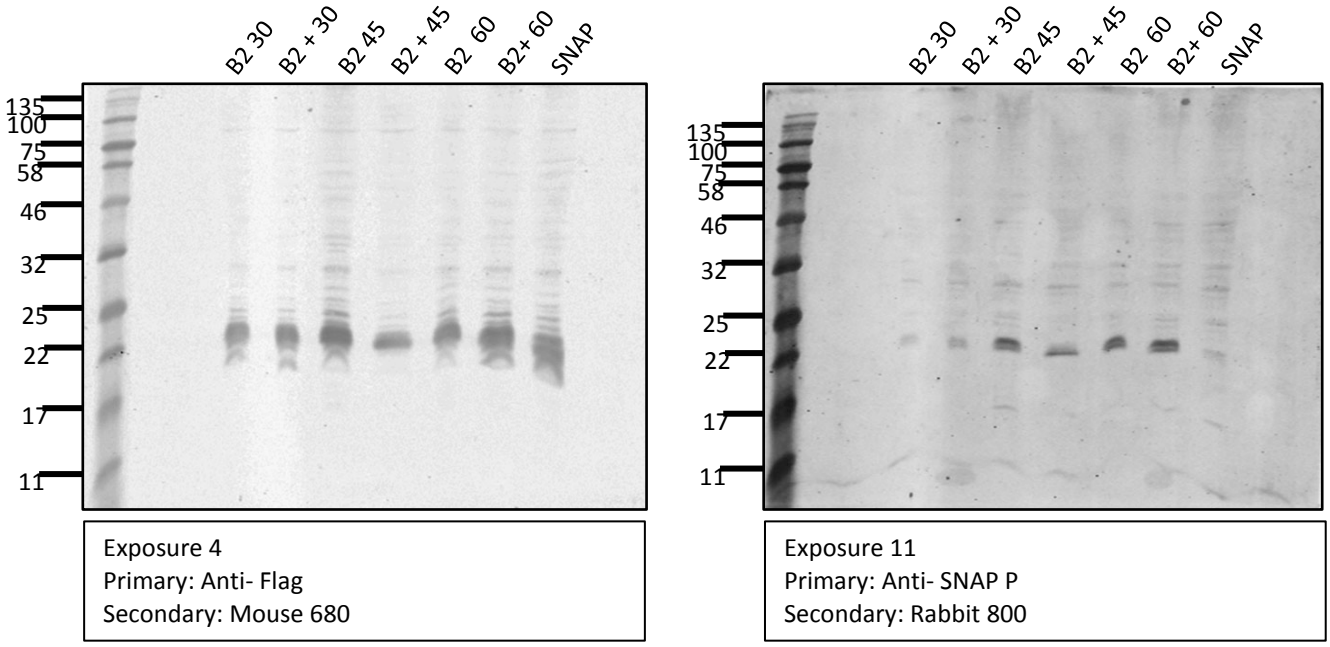

Figure 13: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivative B2 over 30, 45 and 60 minutes and treatment with H_2O_2.

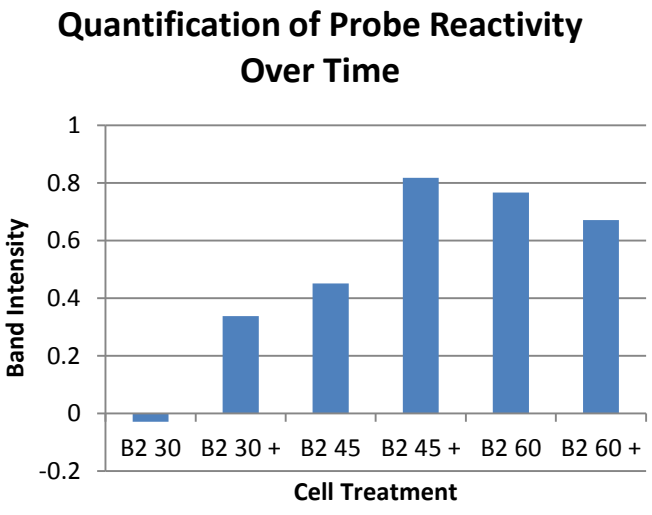

Figure 14: Comparison between western blot band intensity of cells which had been incubated with probe A for 30, 45 or 60 minutes and then reacted with HNaS and negative controls.

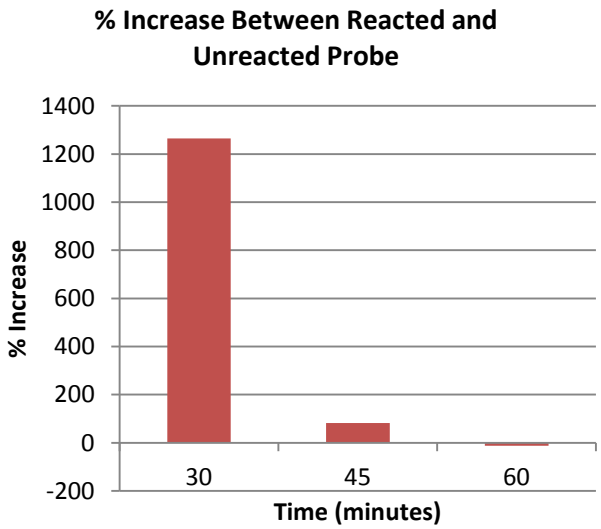

Figure 15: Analysis of percentage increase in western blot band intensity between reacted and unreacted cells after cells had been incubated with probe B2 for either 30, 45 or 60 minutes.

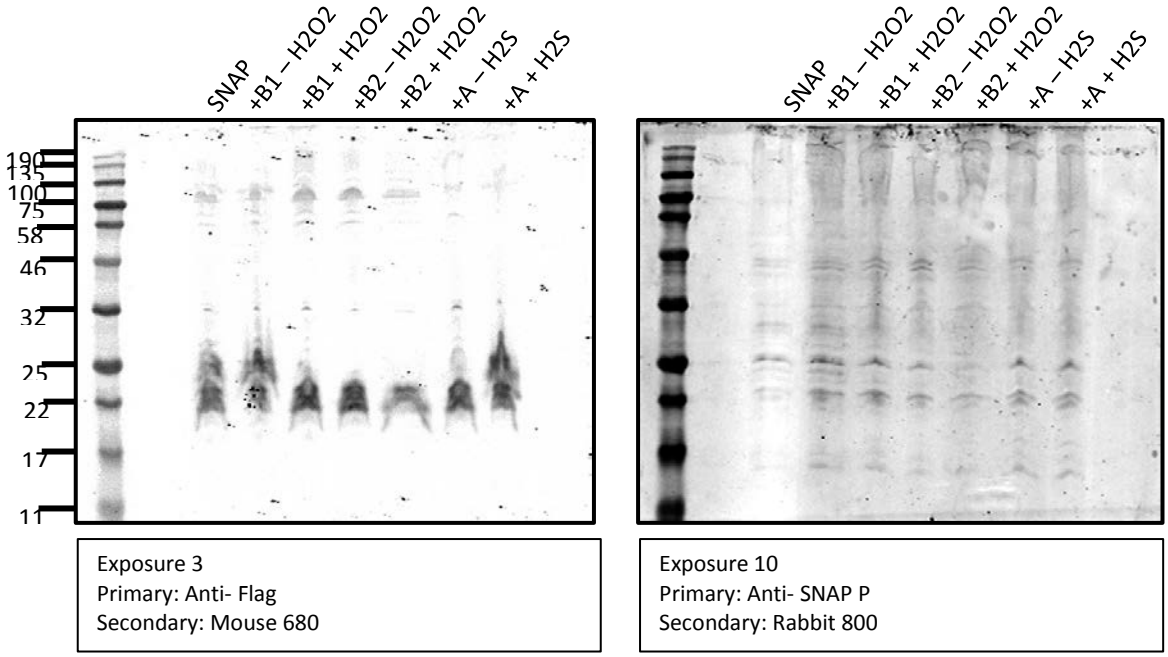

Figure 16: Western Blots of HT cells over-expressing SNAP in the cytosol after incubation with chemoselective benzylguanine derivatives B1, B2 or A and treatment with gasotransmitters pre cell lysis.

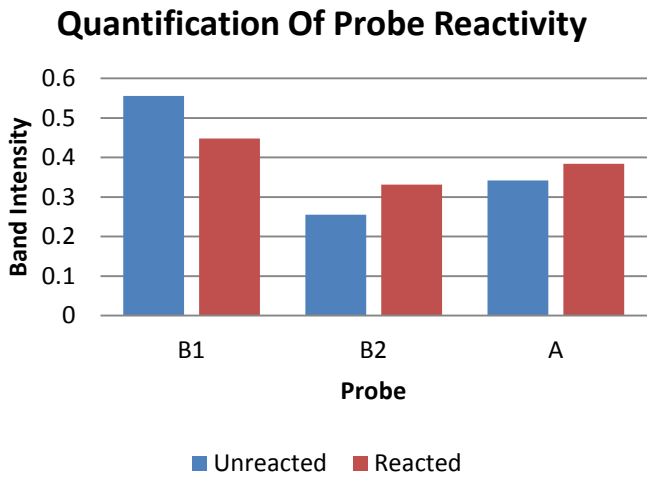

Figure 17: Comparison between western blot band intensity of cells which had been reacted with gasotransmitters pre cell lysis and negative controls.

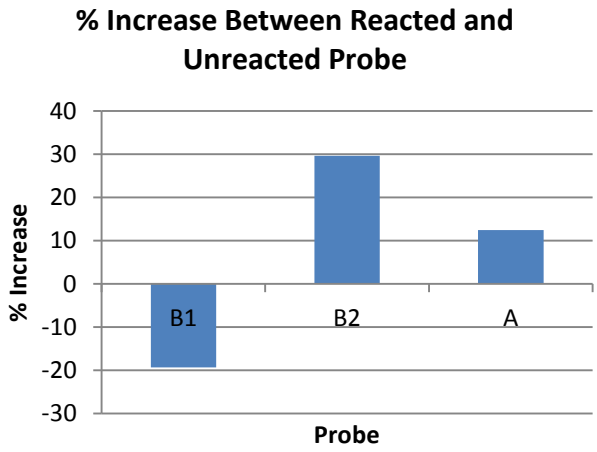

Figure 18: Analysis of percentage increase in western blot band intensity between cells which were reacted pre lysis and unreacted cells.