Identifying lead compounds for S100P in pancreatic cancer therapy using *in vitro* biophysical techniques.

By

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Being a project submitted in partial fulfilment of the requirements of the University of Hertfordshire for the degree of Masters of Science with Honours in Biotechnology

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University of Hertfordshire

Date

DECLARATION

I declare that:

(a) all the work described in this report have carried out by me – and all the results (including any survey findings, etc.) given herein were first obtained by me – except where I may have given due acknowledgement to others;

(b) all the prose in this report have written by me in my own words, except where I may have given due acknowledgement to others and used quotation marks, and except also for occasional brief phrases of no special significance which may be taken from other people's work without such acknowledgement and use of quotation marks;

(c) all the figures and diagrams in this report have been devised and produced by me, except where I may have given due acknowledgement to others.

I understand that if I have not complied with the above statements, I may be deemed to have failed the project assessment, and/or I may have some other penalty imposed up-on me by the Board of Examiners.

Signed …… Date ……..

Name: Thamarasi Vithana Kekule-Vithanage Programme code:

Abstract

The protein S100P has been associated with the aggressiveness of several neoplastic disease, including pancreatic ductal adenocarcinoma (PDAC). S100 proteins are a family of calcium binding proteins that are small dimeric members of the EF-hand superfamily of Ca^{2+} binding proteins. In pancreatic ductal adenocarcinoma, protein S100P is overexpressed, and thereby meditate tumour growth, drug resistance and metastasis. Extracellular S100P binding to the cell surface receptor for advanced glycation end products (RAGE), triggers downstream signalling pathways and mediates cell growth and differentiation, cell cycle regulation and metabolic control. An effective treatment strategy would be to disrupt the formation of this S100P-RAGE complex by blocking the active binding site of S100P to the RAGE receptor. The aim of the present study was to investigate the interactions of drug compounds that are designed to block the S100P-RAGE complex formation. In order to perform this S100P protein was expressed in *Escherichia. coli* using a recombinant plasmid for wild type S100P and purified by affinity chromatography using a HisTrap column. The purity of the protein was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified protein was determined by either the Bradford assay or bicinchoninic acid assay (BCA). In this study, the interactions sites of the recombinant S100P with each of the compounds was assessed by nuclear magnetic resonance (NMR) spectroscopy. The interaction sites were modelled by a HADDOCK approach, using the interacting residues as constraints to model the structure. In conclusion the ligands C72, IBS409, IBS798 and IBS863 were identified to interact with S100P mainly in the hydrophobic pockets of the protein S100P. Binding sites of the ligands were identified via NMR, and the binding was found to be dominated by hydrophobic interactions. Further ITC experimentation may be required in order to gain an understanding of the stoichiometry and the binding affinity for these ligands.

Acknowledgements

I would like to thank my principal supervisor, Dr Pryank Patel and second supervisor Dr Louise Mackenzie for their constant support, guidance and encouragement throughout this project.
I would also like to extend my gratitude to Dr.Sharon Rossiter and her team, through whose work has led to the development of 70 compounds having the potential of binding to S100P.
I would also like to thank Dr. Igor Barsukov from the University of Liverpool for providing the plasmid encoding WT S100P in pET28 vector, and also Dr. Alain Oregioni and Dr.Tom Frenkiel from the Medical Research Council (MRC) Biomedical NMR Centre: The Francis Crick Institute, for their support in collecting and processing the NMR data. I am thankful for Dr Tina Daviter of Institute of Structural and Molecular Biology, Birkbeck for demonstration of using the ITC equipment.
Last, but not least I thank all the technical staff within the Division of Biosciences for their knowledge, advice and for being very helpful in the practical aspect of the project.

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List of Abbreviations

5-FU: 5-fluorouracil
ASA: accessible surface area
BCA: bicinchoninic acid assay
BMRB: Biological Magnetic Resonance Bank
BSA: bovine serum albumin
CEA: carcinoembryonic antigen
CSP: chemical shift pertubation
cv: column volume
DTT: dithiothreitol
EB 1: Elution Buffer 1
EB 2: Elution Buffer 2
HSP: heat shock proteins
HSQC: heteronuclear single quantum coherence
IMAC: immobilized metal ion affinity chromatography
IPTG: Isopropyl β-D-1-thiogalactopyranoside
ITC: isothermal titration calorimetric assay
MAPK: mitogen-activated protein kinase
MUC: associated mucins
MWM: molecular weight markers
NF-κB: nuclear factor-kappa B
NMR: nuclear magnetic resonance
PanIN: preneoplastical epithelial neoplasms
PDAC: Pancreatic ductal adenocarcinoma
PDB: Protein Data Bank
RAGE: Receptor for advanced glycation end products
RSA: relative solvent accessibility
SDS PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
wt: wild-type

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1 Introduction

1.1 Pancreatic ductal adenocarcinoma (PDAC)

PDAC is one of the most common types of pancreatic cancer, where 85% of the cancerous tumours of the pancreas are adenocarcinomas(Dunne and Hezel 2015). There are approximately 8000 people in the UK diagnosed with pancreatic cancer in 2011 while approximately equal amount of deaths in 2012 and an equal amount of deaths in 2012 (CRUK, 2015). It is understood that the cause of cancer is due to the mutation of a type of gene known as an oncogene, which gives the abnormal growth potential to cells. In more than 90% of PDAC, there is a mutationally activated oncogene known as K-*ras.* K-*ras* is a member of the RAS family of GTP-binding proteins mediating a range of cellular functions such as proliferation, differentiation, and cell survival through the various effector pathways, notably the mitogen-activated protein kinase (MAPK) (Dunne and Hezel 2015). Another type of genes known as tumour suppressor genes; the type of genes prevent the cancer growth, *p53* gets inactivated in more than 50% of PDAC. The loss of activity of *p53* can cause the *p53* dependent DNA damage checkpoint response to be skipped and thereby serves to enable the growth and survival of cells, with damaged DNA(Dunne and Hezel 2015). It is believed that more than one gene is responsible for the occurrence of cancers however the exact molecular mechanism of the development and the progression of PDAC by these genetic mutations is still incompletely understood(Dunne and Hezel 2015).

PDAC arises in the exocrine tissues of the pancreas, specifically in the cells of the ducts that carry digestive enzymes to the small intestine. PDAC is distinct from other cancers due to the biological barrier that it builds around itself as shown in the schematic representation in Figure 1(NCI, 2013). It has been demonstrated using mouse models that PDAC tumours are surrounded by a dense cellular matrix or stroma that shields the tumour mass (Figure 1). This increases the fluid pressure within the tumour microenvironment compressing existing blood vessels and limits the blood supply to the tumour (Grippo and Munshi, 2012).Consequently, on administration of a chemotherapy drug, the restricted blood flow prevents sufficient amounts of the drug from reaching the tumour. Inefficient drug delivery is one of the reasons for why PDAC does not respond to chemotherapy (NCI, 2013). In

addition, PDAC aggressively invades regional lymph nodes and the liver, and less often the lungs and visceral organs (Grippo and Munshi, 2012)

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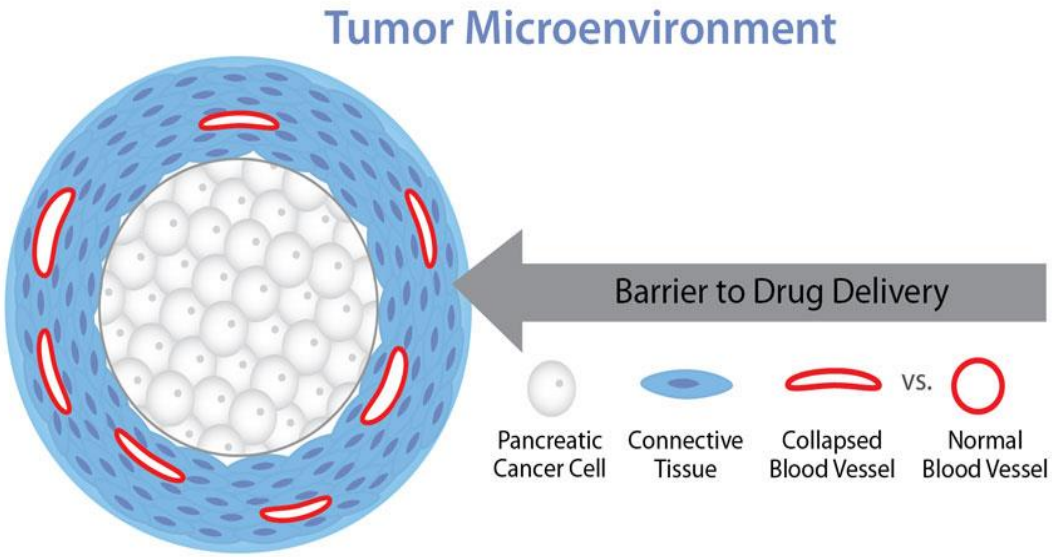

Figure 1: The schematic representation of a pancreatic ductal adenocarcinoma. The figure illustrates the appearance of the collapsed blood vessels in the connective tissue surrounding the tumour. This causes restriction in the blood flow and inefficient drug delivery (National Cancer Institute, 2014)

1.2 PDAC Screening and Treatment

Presently there is no agreed screening test to identify or early diagnose pancreatic cancer. A physical examination of the signs of the disease such as lumps or anything unusual, together with a history of the patient's health habits, past illnesses and treatments is the initial procedure. However the deep anatomic location of the pancreas makes it unlikely to detect small localised tumours by routine abdominal examination (NCI, 2015). The two main functions of the pancreas is to produce digestive juices (exocrine pancreas cells) and hormones (endocrine pancreas cells) such as insulin and glucagon, that aid in the control of blood sugar levels. Therefore unusual levels of substances such as blood sugar can be a sign of disease (NCI, 2015).

The best known blood marker is CA19-9, a “carbohydrate associated antigen” also known sialylated Lewis (a) antigen, which is elevated in pancreatic cancer. The principal limitation of using this biomarker is its frequent elevation in non-malignant conditions like pancreatitis and obstructive jaundice, and its inability to be detected at early stages of the malignancy. It is also unsuitable for about 5-10% of patients who lack the Lewis antigen (NCI, 2015)

There are also specific DNA mutations that are found in pancreatic cancer in the well-known genes K-*ras*, *p16, p21, p53*, *DPC4*, and *BRCA2* (Dowen *et al*. 2005). Recent studies using Affymetrix and cDNA micro-arrays have identified a number of differentially expressed genes in PDAC (Grutzmann, Pilarsky et al. 2004). These assays however have not yet been translated into screening tests or diagnostic markers. There are several other agents such as cell surface associated mucins (MUC), carcinoembryonic antigen (CEA), and heat shock proteins (HSP) which are studied as screening and diagnostic markers, however without much success (NCI, 2015).

Generally, the diagnosis of pancreatic cancer is difficult at an early stage due to non-specific symptoms such as loss-of-appetite, weight loss, abdominal discomfort and nausea. One of the common physical signs is jaundice with associated itching. Non-specific symptoms of pancreatic cancer causes delay of diagnosis at an early stage and thus the majority of patients (>80%) have tumours that are already locally at an advanced stage or metastatic upon diagnosis(He and Yuan 2014). At this stage of the disease, surgical resection of the tumour is not effective leaving such patients with a particularly bleak prognosis. Moreover, patients who undergo surgical treatment are still at risk due to the high rate of recurrence of the disease (He and Yuan 2014).

There are no universally agreed guidelines for the medical treatment of patients with pancreatic cancer who are not candidates for surgery or who have recurrence of the cancer after surgical re-section (NCI, 2015). Surgical resection is currently the only potentially curative option for localised tumours. However, only 15-20 of individuals of the individuals with pancreatic cancer are eligible for surgery, as surgical resection of the tumour is only available to patients with localised tumours (He and Yuan 2014). The metastatic nature of PDAC along with its chemoresistance, leads to the disease being one of the most lethal human cancers having a 5-year survival of less than 5% (Dakhel, Padilla et al. 2014).

Treatment of PDAC therefore remains a major challenge in oncology, as the PDAC has a general resistance to chemotherapeutic approaches. This indicates the importance of finding new biological targets that chemotherapeutic treatment is effective. The chemotherapy agent 5-FU (fluorouracil) has been used in the medical treatment of pancreatic cancer patients. Fluorouracil acts as an inhibitor of thymidylate synthase, which is an enzyme that is critical factor in the synthesis of pyrimidine (a building block of the DNA replication) thus affecting cell cycle progression (Giovannetti, Mey et al. 2006). The underlying effect of most treatment agents is by interfering normal cell cycle progression. These chemotherapeutic agents tend to affect most cells in the body, although the effect is severe on unstable rapidly growing cancer cells. Gemcitabine has been used as a drug for treatment of pancreatic cancer, however it offers meagre therapeutic benefit (Sedov and Poplin 2010). This has led to the search for novel therapeutic approaches for PDAC.

1.3 S100P: A Potential Therapeutic Target for Cancer

S100P is a 95 amino acid member of the S100 subfamily that share the Ca^{2+} binding structural motif, EF-hand (Zhang, Wang et al. 2003). The protein was first purified from placenta, (Becker, Gerke et al. 1992) hence it was designated with a 'p'. The Figure 2 A below demonstrates the secondary structure of the S100P protein indicating the two α-helices that provide the Ca^{2+}-binding site. The conformational changes that are introduced by calcium in the S100P protein, exposes a hydrophobic surface responsible for the binding of target proteins (Arumugam and Logsdon 2011). S100P monomer structure contains four helices: residues 3-18 (Helix-1), 30-40 (Helix-2), 53-61 (Helix-3) and 71-92 (Helix-4) (Zhang, Wang et al. 2003). (Figure 2 B)

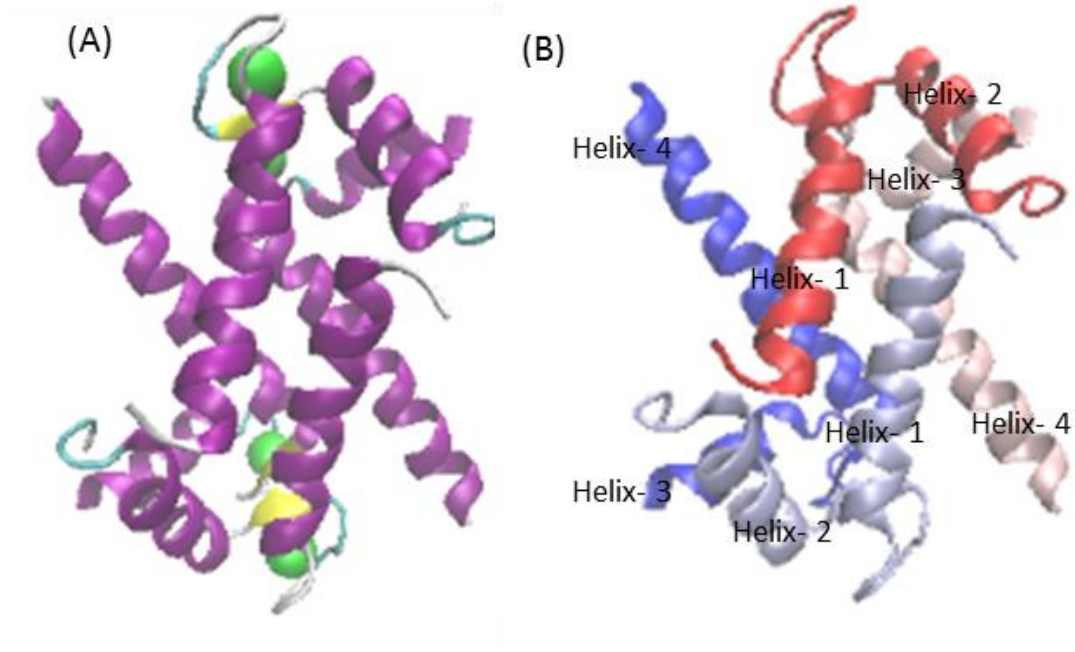

Figure 2: (A) The secondary structure of Ca^{2+} bound human S100P protein dimer, indicating the Ca^{2+} atoms in green. The alpha helices are in purple and the beta strands in yellow. (B) A representation of the S100P structure showing the two domains of the dimer. The helices are named as 1, 2 3 and 4 for each domain.

A study by Logsdon *et al.* 2003 showed that the protein S100P is overexpressed in PDAC. Expression of S100P was indicated to be specific for pancreatic cancer cells and not found in chronic pancreatitis samples. The specificity of the expression of S100P in cancer cells suggests that it is a useful histological marker for cancer (Logsdon *et al*. 2003). A study by Crnogorac Jurcevic *et al*, 2003 also reported that the overexpression of S100P observed in several carcinomas including PDAC (Crnogorac-Jurcevic *et al.* 2003) Furthermore, S100P prompted

the progression of these cancer types and have specific roles in survival, cell proliferation, angiogenesis and metastasis. As reviewed here S100P, has been found to have dysregulated expression in PDAC; evidence suggest that it promotes tumour growth and metastasis. Therefore blocking S100P function might be a useful in improving the therapeutic responses to treatment

1.4 Role of S100P in Pancreatic Ductal Adenocarcinoma

One of the key molecular targets of S100 proteins in pancreatic cancer is the receptor for advanced glycation end products (RAGE) (Arumugam, Simeone *et al*. 2004). RAGE is a member of the immunoglobulin protein family of cell surface molecules. The RAGE receptor is composed of an N-terminal V-type (variable) domain and two C-type (constant) domains. The ligand binding site of RAGE is reported to be on the V-type domain. RAGE binds to multiple proteins that are implicated in several diseases; including S100 calcium binding proteins such as: S100A12, S100A1, S100B, and S100P (Arumugam, Simeone *et al*. 2004). S100P acts through binding of the extracellular domain of RAGE, which triggers the cytoplasmic domain of RAGE to homodimerise and activate the downstream pathways involved in cell proliferation and survival as the MAPK and nuclear factor-kappa B (NF-κB). The intracellular signalling mechanisms activated by RAGE is not completely understood.

Figure 3 is a schematic representation of the signalling pathway proposed by Penumutchu, Chou and Yu, 2014b for the calcium-bound S100P-RAGE. The figure also demonstrates the extracellular, V domain and C domains of the RAGE receptor and also the cytoplasmic domains. In pancreatic cancer these pathways are constitutively active (Arumugam, Simeone *et al*. 2004). The overexpression of the S100P seen in PDAC, may meditate tumour growth metastasis. These effects can be blocked via agents that interfere with RAGE, indicating that the S100P can act in an autocrine manner with RAGE (Arumugam, Simeone *et al*. 2005). An effective strategy in the treatment of diseases caused by the overexpression of the S100P could be to prevent the formation of this S100P-RAGE complex.

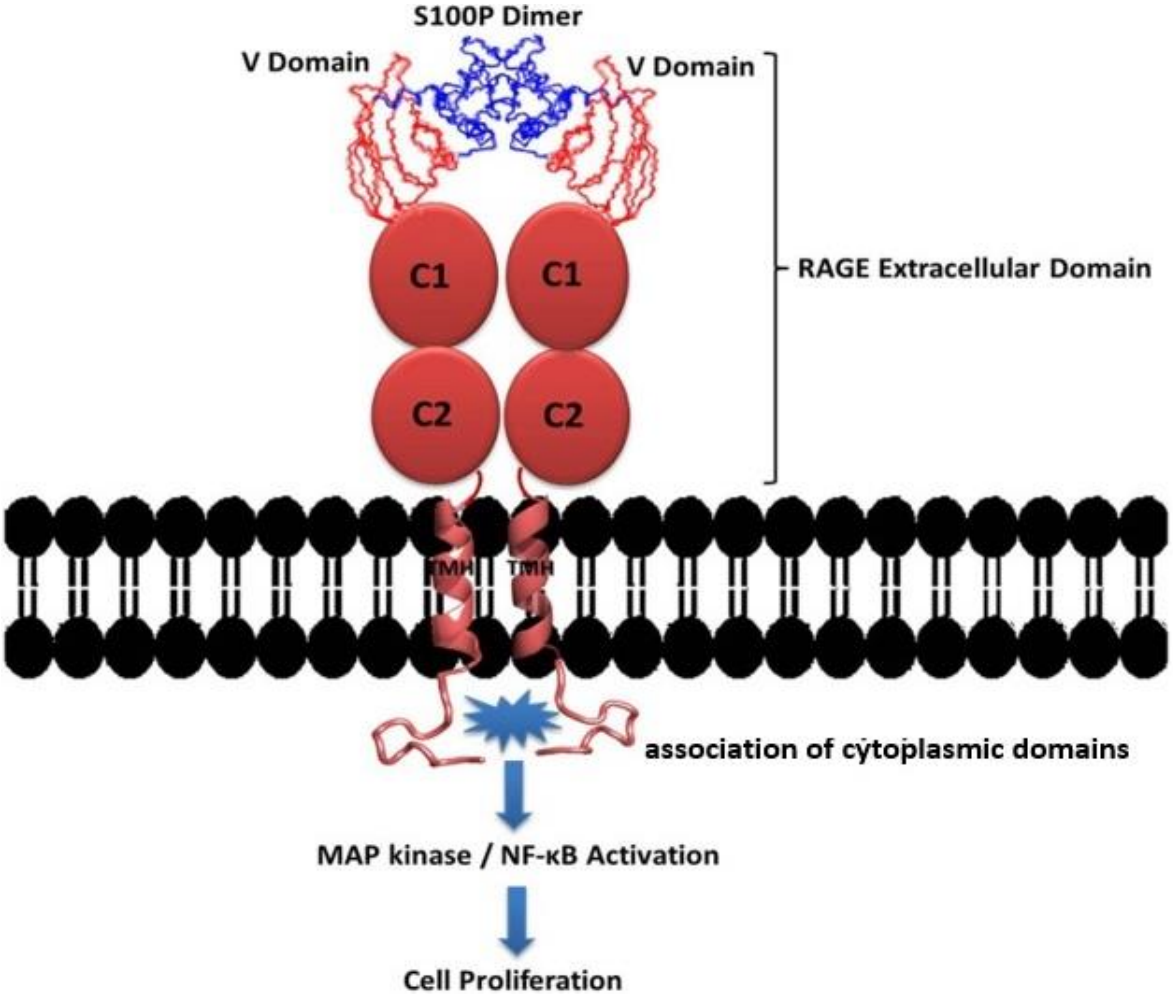

Figure 3: Schematic representation of the signalling pathway proposed by Penumutchu, Chou and Yu, 2014b for the calcium-bound S100P-RAGE. The figure also demonstrates the extracellular, V domain and C domains of the RAGE receptor and also the cytoplasmic domains.

Efforts to inhibit the interaction of S100P and RAGE had led to the identification of a number of specific molecules that interact with RAGE and block activation of the RAGE receptor. Several molecules have shown binding affinity towards S100P, including cytoskeletal ezrin. This protein interacts with S100P in a Ca^{2+} dependent manner and influences its ability to bind actin (Koltzscher, Neumann *et al*. 2003). S100P has shown interactions with CacyBP/SIP. This is a component of the novel ubiquitinylation pathway leading to β catenin degradation (Filipek, Jastrzebska *et al*. 2002). Although these molecules are shown to interact with S100P *in vitro*, it is not known whether there is an interaction *in vivo* or the effect the interactions have on cell function. Studies using mouse models have shown that anti-allergic drug cromolyn is able to interact with S100P and prevent the activation of RAGE. However, despite cromolyn being able to inhibit RAGE, it has been found that the

inhibition of RAGE is weak in human mast-cell secretion and cromolyn is also is poorly absorbed orally. Therefore, cromolyn has not been clinically tested for its benefits for use as a cancer drug. (Arumugam, Ramachandran *et al*. 2013)

1.5 Aim and Objectives

The purpose of this project was to identify lead compounds that can inhibit the interaction of the S100P with RAGE and may be beneficial as a cancer therapy. The potential candidate compounds developed by Dr. Rossiter and her team were tested for other effects on cellular function including proliferation and migration (Camara, Rossiter, Kholi, and Kirton, 2013). In order to perform this S100P protein was produced in *Escherichia. coli* by using a recombinant plasmid for wild type S100P (provided by the University of Liverpool). The S100P protein was then expressed and purified by affinity chromatography using a HisTrap column (GE Life Sciences). The purity of the protein was assessed using SDS PAGE and the yield of the purified protein was determined by either the Bradford or the BCA assay. The interactions sites of the recombinant S100P with each of the compounds were assessed by NMR spectroscopy and the interaction sites were modelled by a HADDOCK approach using the interaction sites as constraints to model the structure. Isothermal Titration Calorimetry (ITC) was used to study the binding affinity and the stoichiometry of the ligands that interacted with S100P.

2 Materials and Method

2.1 Gene sequence and vector used for expression of S100P

The S100P protein was expressed using a plasmid encoding wild type (WT) human S100P in pET28 vector including a hexa-histidine tag and a kanamycin resistance gene. This plasmid was provided by the University of Liverpool.
A ProtParam analysis of the sequence of the plasmid (MGSSHHHHHH SSGLVPRGSH MTELETAMGM IIDVFSRYSG SEGSTQTLTK GELKVLMEKE LPGFLQSGKD KDAVDKLLKD LDANGDAQVD FSEFIVFVAA ITSACHKYFE KAGLK) indicated the molecular weight of the S100P protein to be 12,563.2 Da

2.2 Transformation of plasmid to bacterial cells

Transformation by heat shock is a basic technique which involves the insertion of a foreign plasmid into bacteria, in this case plasmid encoding WT S100P pET28 vector into *Escherichia coli* strain BL21 Star. Initially the chemically competent bacterial cell mixture and plasmid DNA were defrosted on ice. 1µL of plasmid DNA was added to the competent bacterial cell vial and mixed by gentle rotating action of the pipette tip, and incubated for 15 minutes on ice. The vial was then placed on a heat block set at 42°C for 30 seconds and then re-placed on ice (heat shock). Approximately 600µL of sterile Super Optimal broth with catabolite repression (Tryptone 2% w/v, Yeast extract 0.5% w/v, NaCl 10mM, KCl 2.5mM, MgCl2 10mM, Glucose 20mM) was added to these transferred cells and were incubated at 37°C for 45 min with agitation.

The transformed bacteria were plated on to Luria Bertani agar plates containing 50µg/mL kanamycin (LB agar tablets by Sigma Aldrich) and incubated at 37°C overnight. A single colony was used to prepare a starter culture of *E. coli* bearing the S100P plasmid.

2.3 Expression of recombinant S100P

Initially a starter culture of *E.coli* strain BL21 Star cells (100mL) bearing the S100P plasmid was grown overnight at 37ºC in Luria broth medium containing 50µg/mL of

kanamycin. The overnight culture was pelleted at 5,000 rpm for 15 min, the pellets were then gently re-suspended in ~2ml of minimal media.

Table 1: Recipe for 500mL of minimal growth medium and 500mL of Solution A (autoclaved).

Minimal growth medium	
10X Solution A	50mL
1 M $MgSO_4$ (autoclaved)	500µL
1 M $CaCl_2$(autoclaved)	68 µL
Thiamine solution (filter sterilized 0.22µm filter)	25 µL
Glucose*	2g
NH_4Cl*	0.5g
10X Solution A (autoclaved)	
KH_2PO_4	62.5g
Na_2HPO_4	37.52g

*For isotopic labelling $^{15}NH_4Cl$ was substituted for NH_4Cl. The glucose and NH_4Cl was dissolved thoroughly using a small amount of the solution and filter sterilised back in to the media using a 0.22 µL filter. Kanamycin (50µg/mL) was added in order to make sure that only bacterial cells with kanamycin resistance gene (i.e. bacterial cells containing the plasmid) will survive in the minimal growth media. The protein is produced by the expression of the plasmid which are grown on minimal medium supplemented with $^{15}NH_4Cl$ and wild-type (wt) glucose. This enables the protein to be labelled with ^{15}N. In order to perform NMR, it is necessary that the S100P protein produced has a ^{15}N label.

About 1mL of the dissolved pellet was added to the minimal medium prepared (500mL) by starting with an OD^{600} (optical density at 600nm) of ~0.100 for re-suspended pellet. Transformed bacteria were grown to an OD600 of 0.6, and recombinant protein expression was then induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cell culture was incubated overnight at 18°C in an agitating incubator at 200 rpm. After incubation, cells were harvested by centrifugation (4500 rpm; 20 min) and re-suspended in ~ 30mL of lysis buffer (25 mM imidazole, 20 mM Tris, 500 mM NaCl, 2 mM dithiothreitol (DTT); pH 6.5) and stored at -20ºC until purification.

2.4 Purification of recombinant S100P

The cell suspension was defrosted and, to prevent proteolysis, an EDTA-free protease inhibitor (1 tablet dissolved in 2mL of water) was added. The cell suspension was then sonicated on ice (six 30 second periods at 25 W, with 30 second breaks between the pulses) using a Bransson sonifier and centrifuged at high speed (15,000 rpm for 45 minutes a Beckman centrifuge). The supernatant obtained after high-speed centrifugation was filtered through a 0.22 µL filter. A sample of filtered lysate was taken for SDS PAGE analysis. The S100P protein encoded has a hexa-histidine tag. Histidine-tagged proteins have a high selective affinity for Ni^{2+} and several other metal ions that can be immobilized on a chromatographic media using chelating ligands. This chromatographic technique is known as *immobilized* metal ion affinity chromatography (IMAC) (Gelifesciences, 2015). The protein purification was performed using a HisTrap High Performance 5mL column volume (cv) (GE Healthcare). This Nickel Sepharose column contained 20% ethanol (storage conditions), therefore when setting up the column, it was washed out with 10 cv water then equilibrated with 10 cv of lysis buffer. The filtered lysate was then applied and the flow-through collected. The column was washed again with 10 volumes of lysis buffer and flow-through collected. A sample of the flow-through collected was taken for SDS PAGE analysis. Wash buffer (100 mM imidazole, 20 mM Tris, 500 mM NaCl, 2 mM DTT; pH 6.5) containing a higher concentration of imidazole than the lysis buffer was used to wash the column (10 cv).

For the elution step, 5 cv of Elution Buffer 1(EB 1) (350 mM imidazole, 20 mM Tris, 500 mM NaCl, 2 mM DTT; pH 6.5) was applied while collecting the flow-through in fractions. Fractions where collected until their absorbance at 280nm wavelength reached the baseline. A sample of each of the fractions was taken for SDS PAGE analysis.

Finally 5 cv of Elution Buffer 2 (EB 2) (500 mM imidazole, 20 mM Tris, 500 mM NaCl, 2 mM DTT; pH 6.5) was applied. A concentration of 500 mM imidazole in the EB 2 ensures complete elution of the histidine-tagged protein. The flow-through was collected and a sample was taken for SDS PAGE analysis. The fractions with increasing absorbance readings where dialysed overnight at +4ºC in an appropriate buffer, depending on the method that the S100P expressed would be used for. The Figure 4 is a schematic representation of the general steps involved in the purification of histidine tagged proteins.

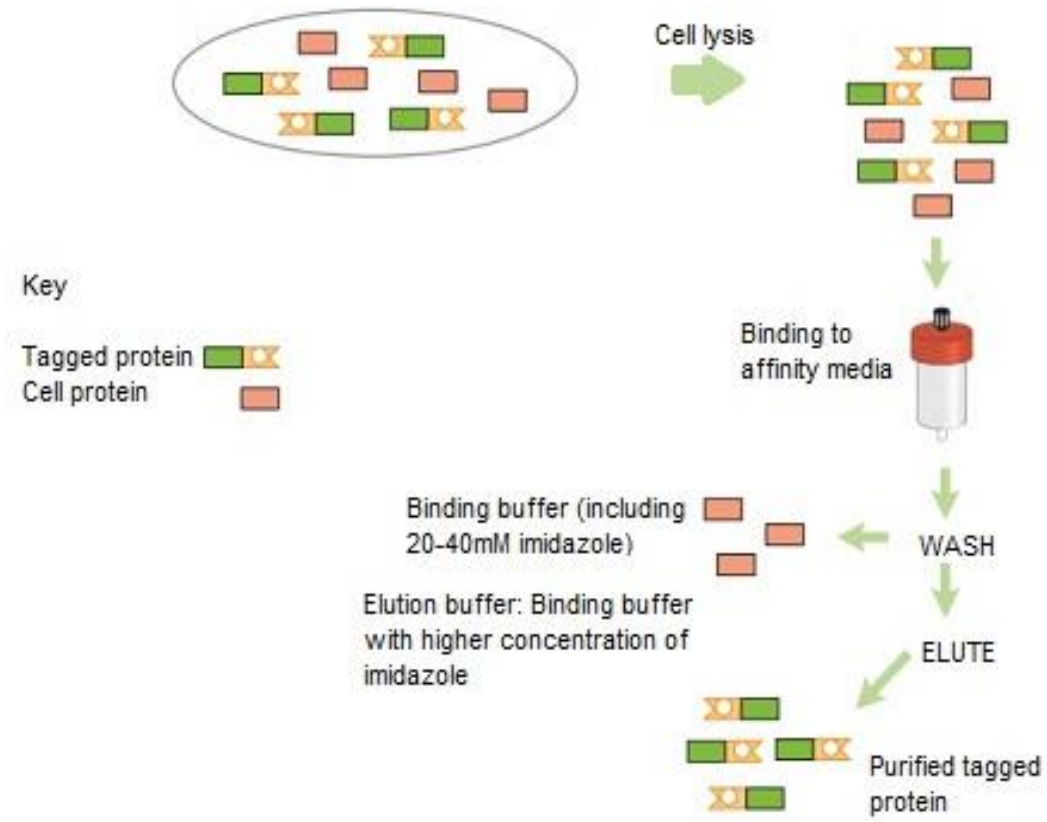

Figure 4: Schematic representation of the general purification of histidine-tagged proteins (adapted from Gelifesciences, 2015)

2.5 SDS-PAGE

Samples were obtained from each stage of the purification for SDS PAGE analysis in order determine if the protein was expressed in the bacteria and also to assess the purity of the sample. SDS-PAGE is one of the most common methods of PAGE for separation of proteins. In SDS-PAGE the samples are heated in SDS (anionic detergent) in the presence of a reducing agent such as DTT, which cleaves the di-sulphide bonds and aid in the unfolding of the protein. SDS also coats the linear protein with a negative charge. Therefore during electrophoresis the separation of the protein depends on the molecular weight of the protein (Walker, 2002). A set of reference proteins known as molecular weight markers are run alongside the samples in order to provide a reference to which the molecular weight of the sample proteins could be compared to (Lifetechnologies, 2015).

The samples collected from each of the stages of the protein purification was analysed by SDS PAGE. The molecular weight marker (BIO-RAD broad range 161-0317) used was also provided by the technical staff diluted in 2× SDS-PAGE sample buffer (supplied by technical staff-125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.04% bromophenol blue, 20 mM DTT) at a ratio 1:20. The samples were diluted in a 1:1 ratio with 2 × SDS-PAGE sample buffers and denatured at 95°C for 5 minutes along with the molecular weight markers. The analytes were separated alongside the molecular weight markers (MWM) on 15% polyacrylamide resolving gels.

The SDS analysis will also show the fractions containing the most amount of protein, these fractions were dialysed overnight at +4ºC in relevant buffers (depending on the assays the protein will be used for). The S100P protein sample was then stored at +4ºC. The unused samples were snap frozen using liquid nitrogen and stored at -20ºC until used.

2.6 Bradford Assay

The quantity of protein purified was determined by a standard chromogenic method known as the Bradford assay. This is a colorimetric assay based on the shift of the absorbance of the Coomassie Brilliant Blue G-250 dye. Under acidic conditions the dye changes from the red form to the blue form when protein bound. The bound form of the dye has a maximum absorbance of 595nm. Bovine serum albumin (BSA) standard solutions of concentrations ranging from 0-1.0 mg/mL were prepared and 100 µL of each standard solution was loaded into test tubes in duplicates. 100 µL of the purified protein solution was also added to test tubes in duplicates. 5 mL of Bradford Reagent (prepared by technical staff) was added to the 100 µL of each of the standard solutions and the purified protein solutions. The test tubes were incubated at room temperature for 5 minutes before their optical density was measured at 595nm using a spectrophotometer (Walker, 2002). The dialysate used in the dialysis of the protein in PBS was used as the blank of the assay. A calibration curve of optical density at 595nm against the concentration of BSA standard solutions was constructed. The calibration curve was then used to determine the concentration of the purified protein solution.

2.7 Bicinchoninic Acid Protein assay

The BCA assay is a biochemical assay performed to determine the concentration of protein in a solution (0.5 µg/mL to 1.5 mg/mL). Similar to the Bradford assay, BCA assay is a colorimetric assay where the total protein concentration exhibited by a colour change of the sample solution from green to purple in proportion to protein concentration. The BCA assay primarily relies on two reactions. Initially, peptide bonds in protein reduce Cu^{2+} ions from the copper (II) sulphate to Cu^{+}. Two molecules of bicinchoninic acid then chelate with each Cu^{+} ion, forming a purple-coloured product absorbs light at a wavelength of 562 nm. The amount of protein present in a solution is quantified by measuring the absorption spectra and comparing with protein solutions of known concentration. A calibration curve of optical density against the concentration of BSA standard solutions was constructed. The calibration curve is used to determine the concentration of the purified protein solution.

The BCA protein assay was performed for samples that contained DTT by using a BCA kit which was compatible with reducing agents as DTT. This kit contained a compatible dye known as compatibility reagent, which enables samples containing up to 5 mM DTT to be measured. BSA standard solutions of concentrations ranging from 0-2.0 mg/mL were prepared and 9 µL of each standard solution was loaded into the 96 well plates in duplicates. Similarly, 9 µL of the samples to be tested was loaded into the 96 well plates in duplicates. This was followed by the addition of 4µl of the compatibility reagent to each of the wells. Finally the prepared BCA reagent (50 parts of BCA Reagent A with 1 part of BCA Reagent B) to each well and the plate then incubated for 30minutes at 37°C, cooled and read at 590nm using a plate reader. Concentrated samples were stored at +4°C before use and remainder of the sample was snap frozen using liquid nitrogen and stored at -20°C.

2.8 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is one of the most important methods used in the pharmaceutical industry as a tool to examine protein drug interactions (Skinner and Laurence 2008). For the purpose of this project heteronuclear single quantum coherence (HSQC) spectroscopy experiments were performed on the target protein S100P in the presence and the absence of ligand, correlating ^{1}H and ^{15}N resonances in two-dimensional NMR spectrum. The experiment allows monitoring of the binding events in a residue specific manner as it observes the amide groups that become chemically unique in the context of the protein's tertiary structure. The changes in the protein chemical shift in the presence of a drug indicate binding, and the epitope to which the ligand binds of the protein mapped by protein resonance assignments. Chemical shift changes are strongly dependent on the electronic environment of the nucleus observed. Ligand association with the protein moiety will therefore influence the chemical shifts of the nearby protein moieties, which measured by NMR by using isotopically labelled protein (Dias and Ciulli 2014). The chemical shift perturbations of the S100P observed can be mapped to protein resonance assignment of S100P which are available on the Biological Magnetic resonance Bank (BRMB)(Dias and Ciulli 2014). The protein ligand binding sited highlighted by this. Although this is a very effective method in determining the protein ligand interactions, the disadvantages of this method is that the isotope labelling of the protein is expensive and prohibitive for certain proteins.

NMR HSQC titrations were monitored by recording 2D ^{1}H-^{15}N HSQC spectra at 298.2K on a Burker 600 MHz spectrometer equipped with a cryogenic probes. For HSQC characterisation, all protein samples were prepared at a concentration of 0.3 mM in 20mM MES ((pH 6.5, 10% D_2O), 50mM NaCl, 4 mM $CaCl_2$. The ligands were added to uniformly ^{15}N-labeled calcium-bound S100P at an excess molar ratios of 5:1 and 2:1. The data was collected and processed by Dr. Alain Oregioni and Dr.Tom Freinkel from the Francis Crick NMR lab.

2.9 CCPNMR Analysis

The data was analysed using a software known as CCPNMR Analysis v.2.4.1. (Vranken, Vuister *et al*. 2015). This spectrum was compared with the standard list of chemical shifts obtained from the BRMB library (accession code 17866) and the peaks that matched were assigned with their respective residue name and sequence number (Markley, Ulrich *et al*. 2008). This spectrum was then used for as the standard spectrum in the ligand titration experiments that followed.

2.9.1 Defining significant chemical shift perturbations

In order to determine the active residues the ^{15}N-^{1}H HSQC spectra of 0.3 mM 15N-labeled S100P and in the complex form with each drug at different concentration ratios were overlaid, and any changes chemical shifts of the crosspeaks were observed. Chemical shift perturbations (CSP) can be calculated using the following equation.

$$\Delta_{\text{ppm}} = \sqrt{(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}} * \alpha_{\text{N}})^2},$$

where $^{\Delta\delta}HN$ and $^{\Delta\delta}N$ are the chemical shift changes of amide protons and nitrogens, respectively and a scaling factor (α_N) of 0.15.(Tochio, Hung *et al*. 2000)

This is also the same as the '`shift sum`' value in CCPNMR Analysis. The residues that showed a chemical shift perturbations larger than or equal to the average was defined as 'significantly' perturbed.

2.9.2 Defining active and passive residues

One of the important parameters in determining the active residues that take part in the protein ligand interaction is the relative solvent accessibility (RSA). In order to determine this, the accessible surface area (ASA) was calculated for each residue in S100P protein using the Helixweb server (http://helixweb.nih.gov/structbio/.)
The RSA was calculated using equation 2:

$$\mathrm{RSA} = \mathrm{ASA} / \mathrm{MaximumASA} ,$$

where “Maximum ASA” corresponds to the maximum ASA value, as determined by the normalisation scale used, for the focal amino acid (Tien *et al*. 2013). The maximum ASA was obtained from Miller *et al*. 1987. The Maximum ASA values and the RSA values for each residue can be found in the Appendix (1)
The residues with a relative accessibility of larger than 50% of the main chain were selected. The experimental data (chemical shift perturbation) and the solvent accessibility were combined and the active residues were defined precisely to use in HADDOCK docking software.(De Vries *et al*. 2010). Passive residues were automatically defined by the HADDOCK server, where all solvent accessible surface neighbours of the active residues were used.

2.10 Molecular Docking

The model of the S100P-ligand complex was generated using the HADDOCK2.0 web server: (De Vries *et al*. 2010). The atomic coordinates for calcium-bound S100P were obtained from the RCSB Protein Data Bank (PDB), entries 1J55. The active residues were defined as residues that exhibited a maximum increase in the chemical shift perturbation, as described in section.2.10. The docking trials were performed using the standard HADDOCK protocol. Visualisation and structural representations were rendered using VMD.v.1.9.2. (Humphrey *et al.* 1996). LigPlot+ software was used as a tool for producing schematic representations of the Ligand protein binding sites. (Wallace *et al*. 1995)

2.11 Isothermal Titration Calorimetry

ITC measures heat generated or absorbed upon ligand binding. This is a technique for precisely determining binding constants. ITC is also the only biophysical technique that take into account the contributions of enthalpy and entropy to ligand binding. In this method a biomolecule is placed in the syringe and a ligand solution is injected into the cell. The ITC instrument (VP-ITC Microcalorimeter) detects the heat generated or absorbed as a result of an interaction. This is calculated as the changes in the power required to maintain isothermal conditions between the reference and the sample cell. Repeated injections of ligand are carried out until the peak sizes become constant. At this point the biomolecule is saturated with ligand. On completion of the titration the individual peaks are integrated and presented as a Wiseman Plot. This is not a suitable method for primary screening, as it requires large amounts of protein and is a low throughput technique (Williams and Daviter, 2013).

For the preparation of protein samples, purified S100P protein was dialysed in 20 mM MES (pH 6.5), 50 mM NaCl and 2 mM EDTA overnight and then dialysed into a fresh buffer of 20 mM MES (pH 6.5), 50 mM NaCl overnight using a dialysis membrane with a molecular weight cut-off of approximately 3500 Da. All of the protein samples were degassed thoroughly under vacuum for 10 minutes in order to remove bubbles prior to the titration experiments ITC was performed using a MicroCal VP-ITC calorimeter.

A 0.015mM solution of $CaCl_2$ solution was placed in the syringe as the ligand and titrated into a sample of 0.2 mM S100P protein in the sample cell. All titrations were performed at 30°C. A volume of 3 µL was used in the initial injection and 10 µL per injection for all subsequent titrations. The initial delay of the injection was 60-s and a delay in the intervals between the injections was120-s.

The data was analysed using the Origin software by fitting models to calculate: reaction stoichiometry (n) binding constant (K), enthalpy (ΔH), and entropy (ΔS). (Williams and Daviter, 2013)

3 Results

3.1 Protein purification analysis by SDS PAGE

In order to identify the potential candidate compounds that would inhibit the interaction of S100P with RAGE, the protein S100P was expressed and purified in *Escherichia coli* using a recombinant plasmid for wild type S100P. The S100P protein produced was purified using affinity chromatography by using a HisTrap column. At each stage of the purification a sample was obtained for SDS PAGE analysis.

Figure 5 shows the SDS PAGE analysis of one of the purifications performed. All the SDS PAGE gel profiles for the purifications were similar. The majority of the S100P protein was detected in the sample obtained during the preparation of the bacterial cell lysate (Lane 2) indicated by the intense band in Lane 2 (~12KDa). This band was faint in Lane 3 which indicates that the protein S100P was depleted in the filtered lysate flow-through, as the protein was bound to the HisTrap column at this stage. However there were other protein bands detected in the filtered lysate flow-through. The Wash 1 (Lane 4) and Wash 2 (Lane 5) flow-through samples did not produce any bands.

The EB 1 had a higher concentration of imidazole than the wash buffer. However in the SDS PAGE analysis of the EB 1 fractions the protein bands were faint. The abundance of the protein S100P was detected in the EB 2 flow-through, indicated by the intense band at ~12kDa on Lane 8.

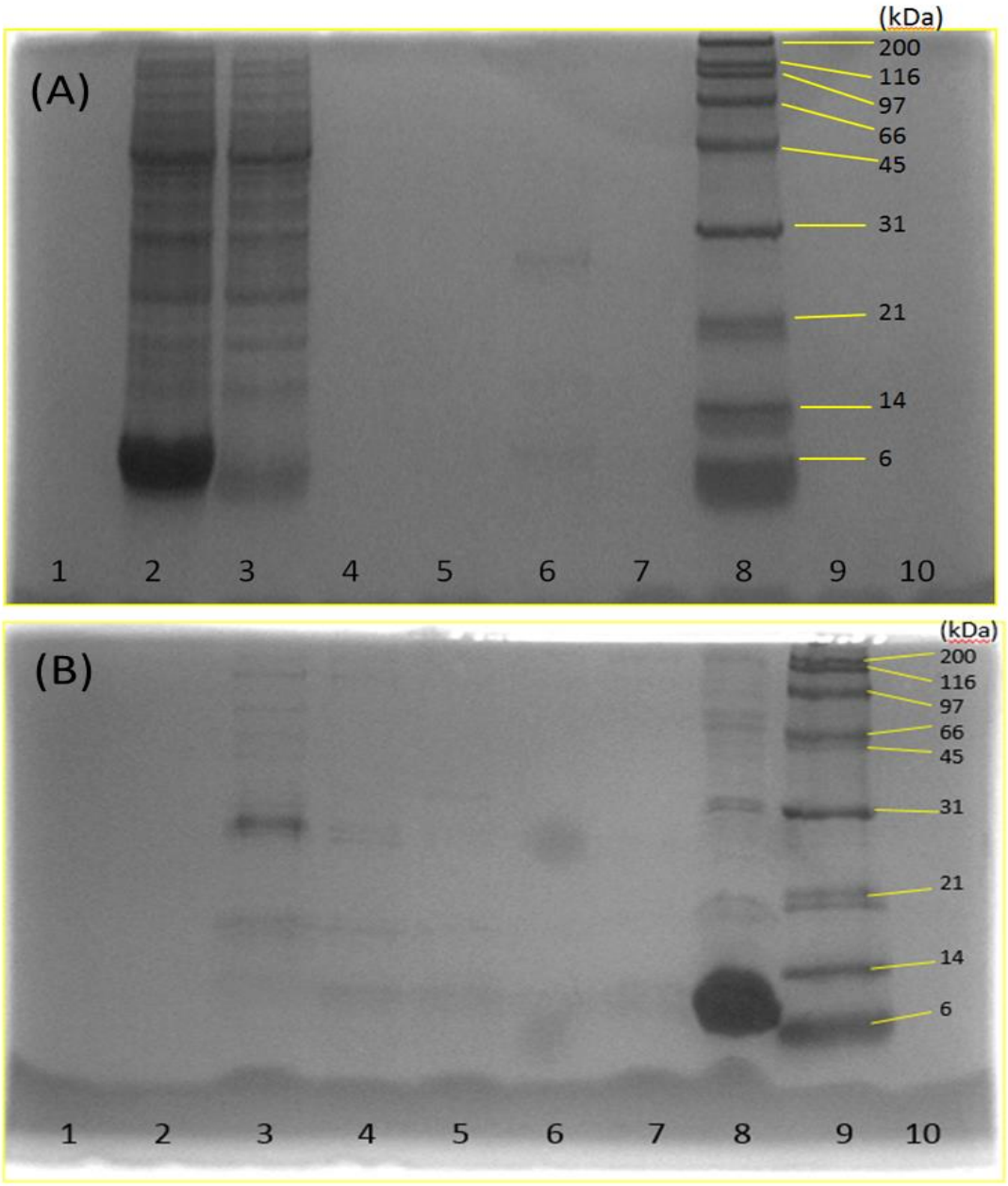

Figure 5: **Protein purification analysis by SDS PAGE**. (A) Lane 2 indicates the filtered lysate sample lane, and lane 3 indicates the flow through of the filtered lysate. The S100P protein band (~12KDa) was absent in this lane. Lanes 4 and 5 were the wash buffer 1 and wash buffer 2 respectively. Lane 6 had had the S100P sample dialysed in buffer(EB 1 fractions containing most amount of protein pooled together and dialysed in appropriate buffer) (B) The lanes 2, 3, 4,5 ,6 and 7 had the EB 1 fractions 1 to 6 respectively. Lane had the EB 2. The molecular weight markers of gel A and Gel B were loaded at Lane 8 and 9 respectively. Sample buffer was added to the empty lanes.

3.2 Protein Concentration Assay

The protein concentration was initially determined by performing the Bradford assay. However this method used 200 µL of sample, therefore the BCA assay with compatibility for reducing agents was performed in subsequent purifications. A sample volume of only 9 µL was required for the BCA assay.

Both NMR and ITC were done in two sessions, and therefore fresh protein was purified for each session for each technique. The protein concentration of the S100P samples used on the first session of NMR data collection was calculated to be 295µM and second session calculated to be 430µM. The protein concentration of the S100P samples used on the first session of ITC was calculated to be 301.8 µM and second session was calculated to be 356 µM

3.3 NMR Spectroscopy for the characterisation of the binding interface between S100P and the ligands

NMR is an invaluable tool for the drug discovery, particularly in the characterisation and optimisation of lead compounds. In this project NMR was used for examining the protein–ligand interactions emphasising identification of the sites to which the drug compounds bind on the protein. The binding interface was mapped as a perturbation of the chemical shifts of the cross peaks in ^{15}N-^{1}H HSQC spectra of S100P protein in the ligand-protein complex. A series of ^{15}N-^{1}H HSQC titrations was performed where the ligand (cromolyn, C72, IBS409, IBS798, and IBS863) was added in excess to S100P, at a ratio of 2:1 and increased further to 5:1 excess of ligand to protein respectively. Changes in the ^{15}N-^{1}H backbone resonance of S100P were detected on addition of the ligand, and these were analysed relative to the spectra of S100P.

3.3.1 NMR characterisation of the binding interface between cromolyn and S100P

The initial titration performed was with cromolyn as the ligand molecule at ligand to protein concentrations ratios 2:1 and 5:1 respectively. The chemical shift perturbations for the peaks at each concentration of cromolyn were obtained using CCPNMR Analysis `shift sum` value. The average CSP for each titration was used as a cut off point for determining the significantly shifted residues.

Figure 6 (A) demonstrated the overlaid spectral images of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 0.55 mM of the cromolyn (green). Figure 6 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.004. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 3Glu, 10Met, 4Leu, 16Ser, 17Arg, 26Gln, 43Gly, 86His, 89Phe, 90Glu, 91Lys, 92Ala, 93Gly as labelled in Figure 6 (A)

Figures 7 (A) demonstrated the overlaid spectral images of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 1.35 mM of the cromolyn (green). Figure 7 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.009. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 10Met, 17Arg, 23Gly, 43Gly, 56Lys' 86His' 89Phe' 90Glu, 91Lys, 92Ala, 93Gly, 94Leu as labelled in Figure 7 (A)

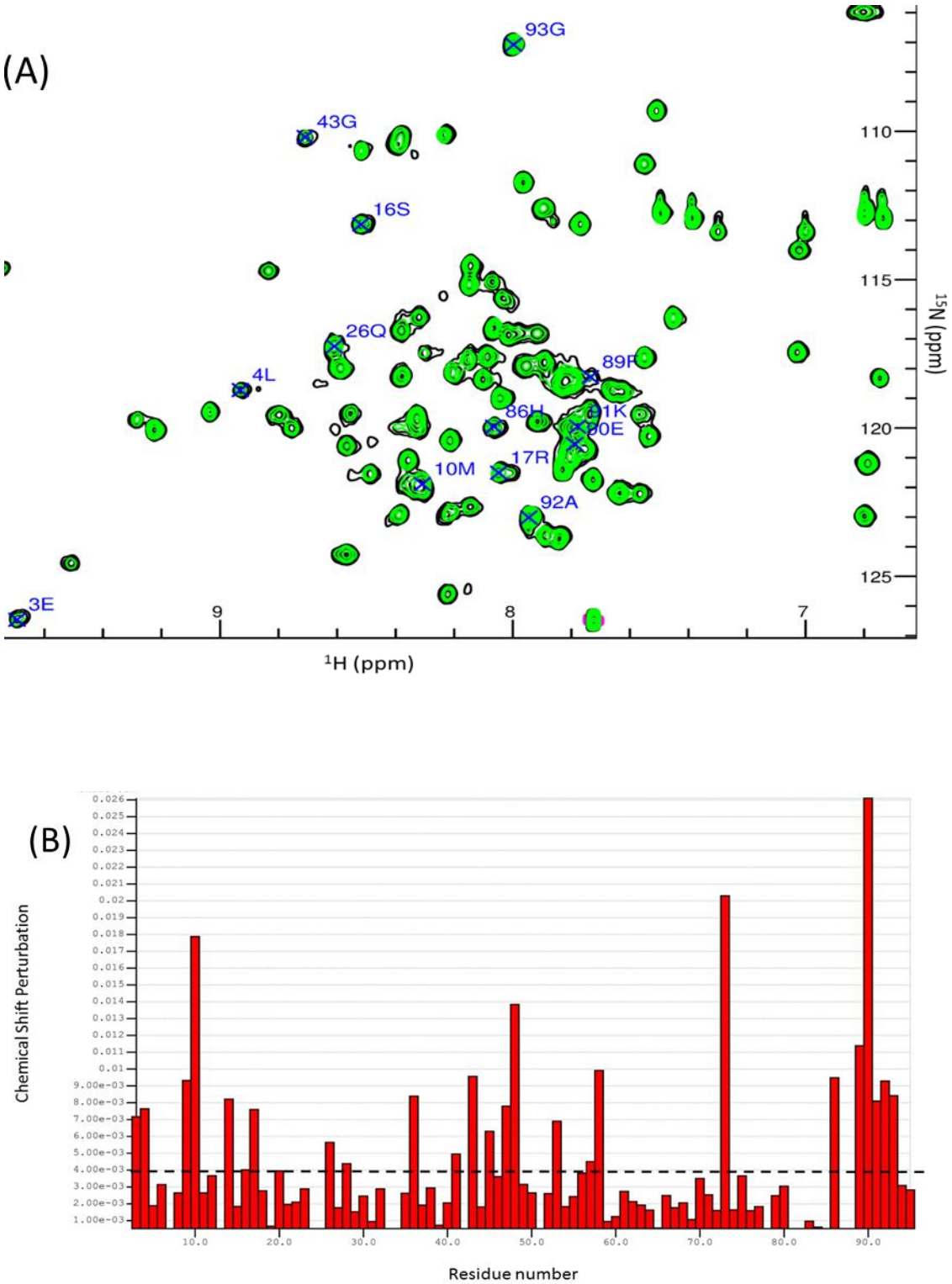

Figure 6*:* (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 0.55 mM of the cromolyn (green), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–cromolyn adduct at concentration ratio 2:1, cromolyn to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.004 ppm).

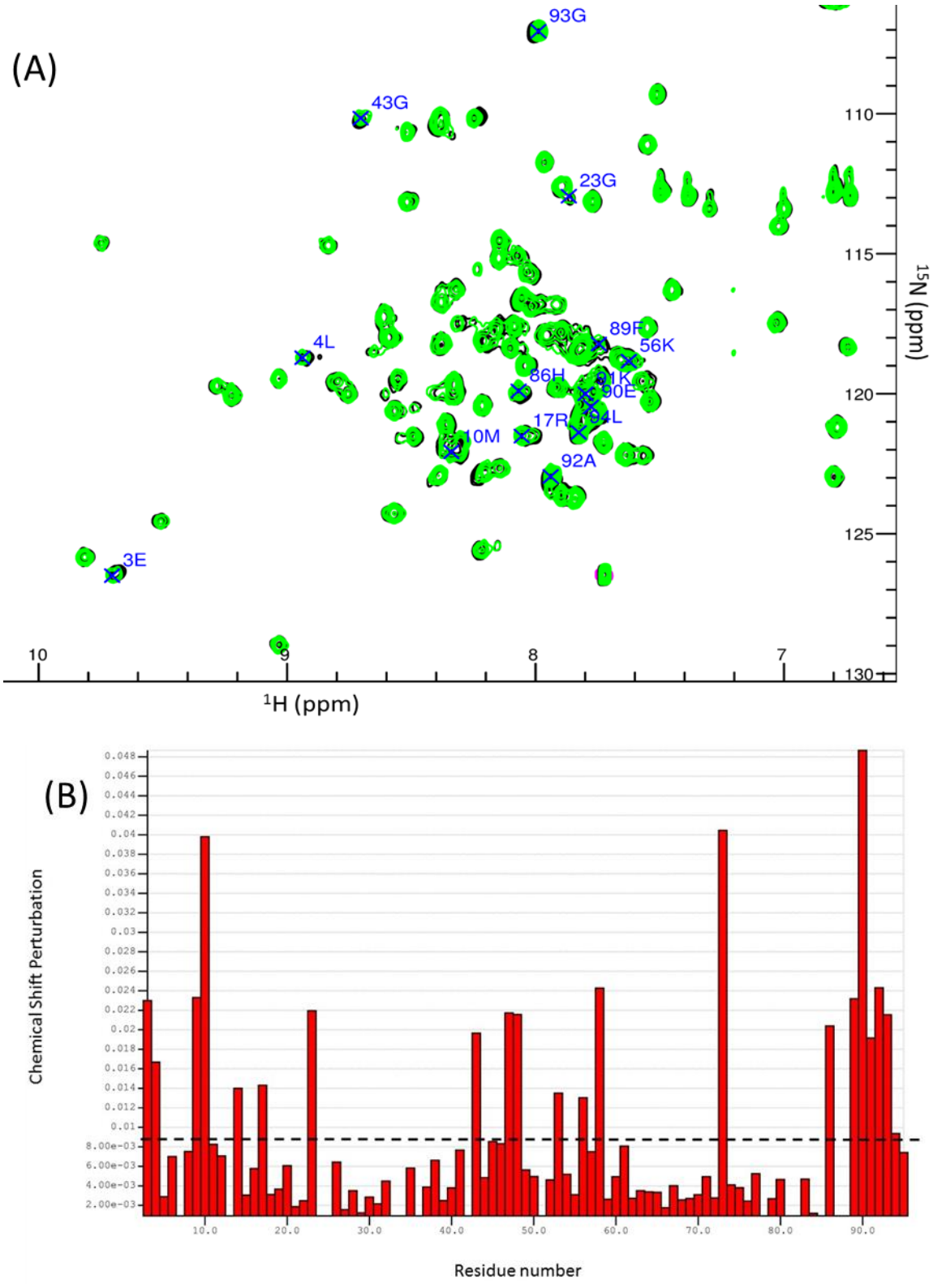

Figure 7*:* (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 1.35 mM of the cromolyn (green), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–cromolyn adduct at concentration ratio 5:1, cromolyn to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.009 ppm).

3.3.2 Modelling of the S100P-cromolyn complex when the concentration of cromolyn to S100P was 2:1

The molecular docking web server HADDOCK was used with the NMR-derived restraints to model the S100P–cromolyn complex. There were 200 structures of solvent refinement from HADOCK that were separated into 2 clusters when the cromolyn to S100P was at a concentration ratio 2:1. When the lowest energy conformations of the clusters were analysed it showed that the cromolyn moiety was situated in the hydrophobic pocket of S100P. The residues that involved in the interaction are shown in the LigPlot analysis in Figure 8. There were hydrophobic interactions within cromolyn and the S100P at residues 5Glu, 8Met, 9Gly, 12Ile, 26Glu, 86His, 89Phe and 90Glu. There were some hydrogen bonds that were observed between the residues formed between the hydroxyl group of cromolyn and 6Thr, 9Met, 91Lys, 92Alaand 93Gly. The interaction between the cromolyn and S100P (at a concentration ratio of 2:1 respectively) dominated by hydrophobic interactions.

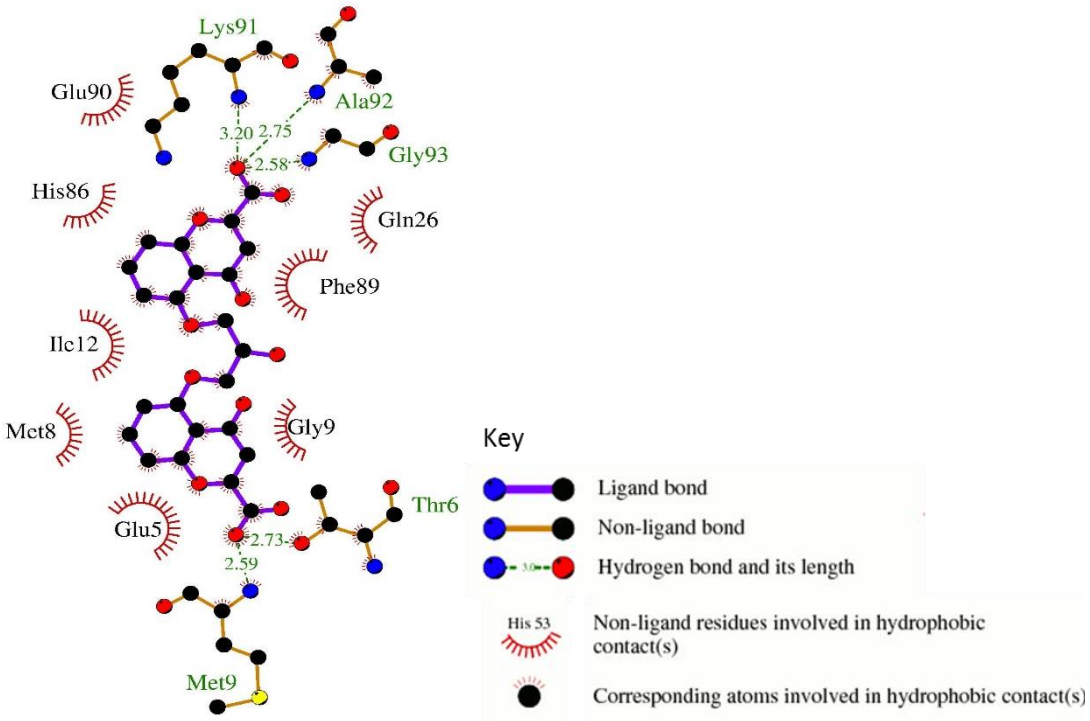

Figure 8 A schematic representation of the residues that involved in the interaction within cromolyn and the S100P at a concentration ratio 2:1

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Residues that involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located mainly in the Helix-1 and Helix- 4 as demonstrated in Figure 9 (A).This was a hydrophobic pocket of the S100P protein (Figure 9 (B)).

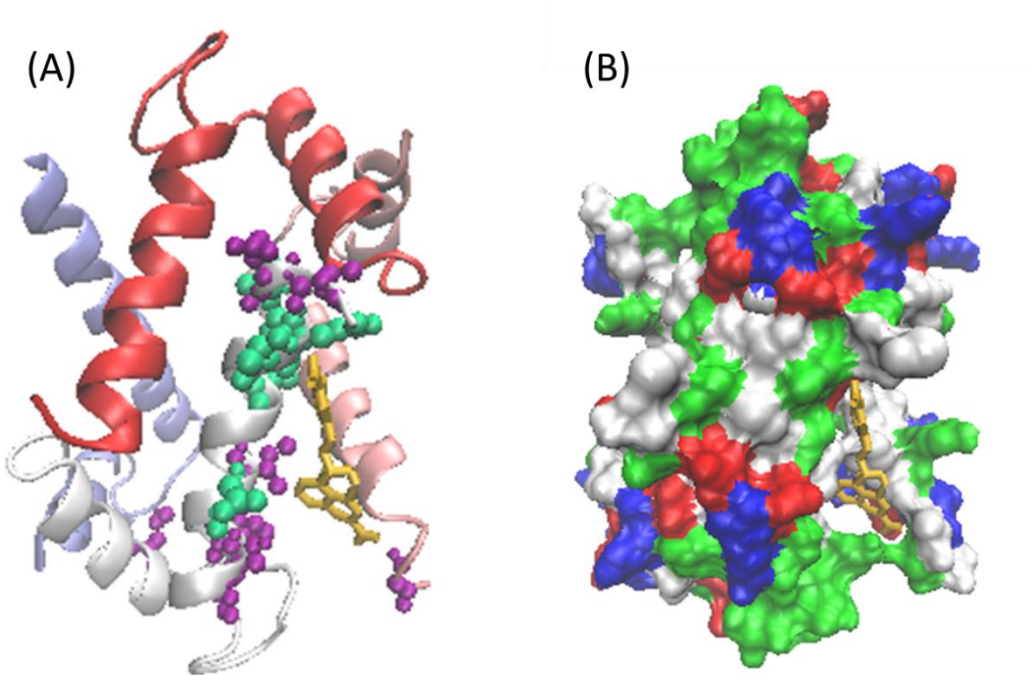

Figure 9 **HADDOCK structure of the S100P−cromolyn complex with 2:1 cromolyn.** (A) Ribbon-stick representation of the overlap of the S100P−cromolyn complex structure. Cromolyn is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−cromolyn complex coloured by charged residue type. Cromolyn was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively.

3.3.1 Modelling of the S100P-cromolyn complex when the concentration of cromolyn to S100P was 5:1.

The molecular docking web server HADDOCK was used with the NMR-derived restraints to model the S100P–cromolyn complex. There were 199 structures of solvent refinement from HADOCK that were separated into 2 clusters when the cromolyn to S100P was at a concentration ratio 5:1. The lowest energy conformations of the clusters were further analysed, and it demonstrated that the cromolyn molecule moiety was situated at the hydrophobic pocket of the S100P. The residues that are involved in the interaction are shown in the LigPlot analysis in Figure 10. There were hydrophobic interactions within cromolyn and the S100P; at a concentration ratio 5:1 respectively, were 1Met, 5Glu, 8Met, 9Gly, 88Try, 90Glu, 89Phe, 91Lys and 93Gly. Hydrogen bonds between were formed between 6Thr, 26Gln and 94Leu and the hydroxyl group of cromolyn, except for one hydrogen bond with 26Gln, where the hydrogen bond was with the oxygen in the phenyl ring. The number of interactions between the cromolyn and S100P (at a concentration ratio of 5:1 respectively) is dominated by hydrophobic interactions.

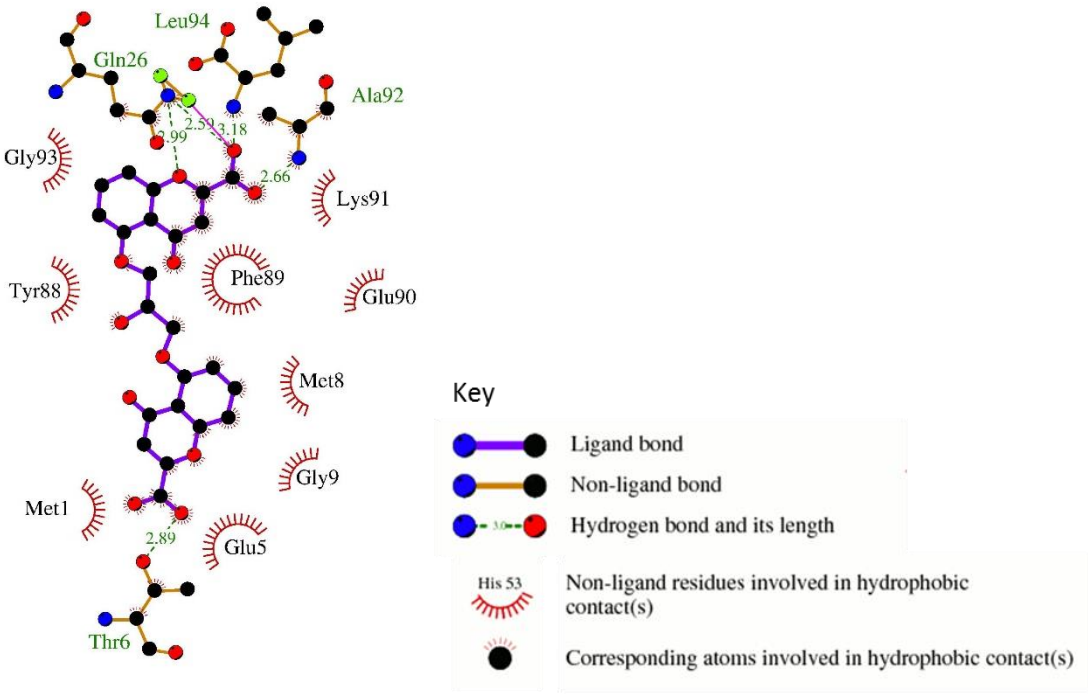

Figure 10 A schematic representation of the residues that are involved in the interaction within cromolyn and the S100P at a concentration ratio 5:1 respectively. The atoms shown in lime green are hydrogen atoms of the NH_2 group of 26Gln.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located mainly in the Helix-1 and Helix-4 as demonstrated in

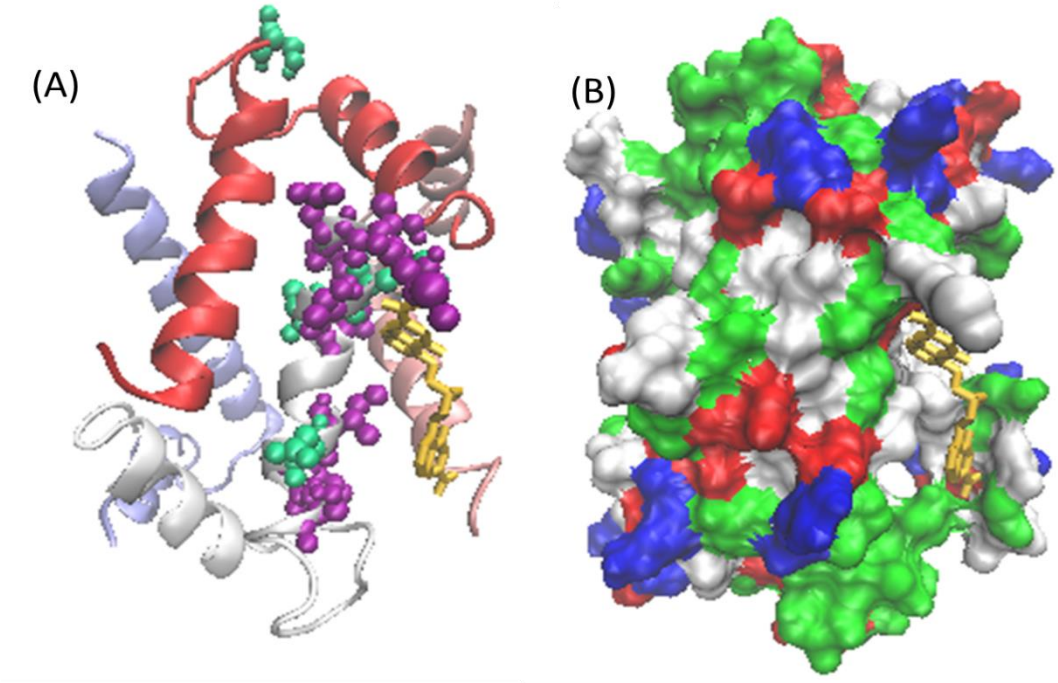

Figure 11(A).This was a hydrophobic pocket of the S100P protein (

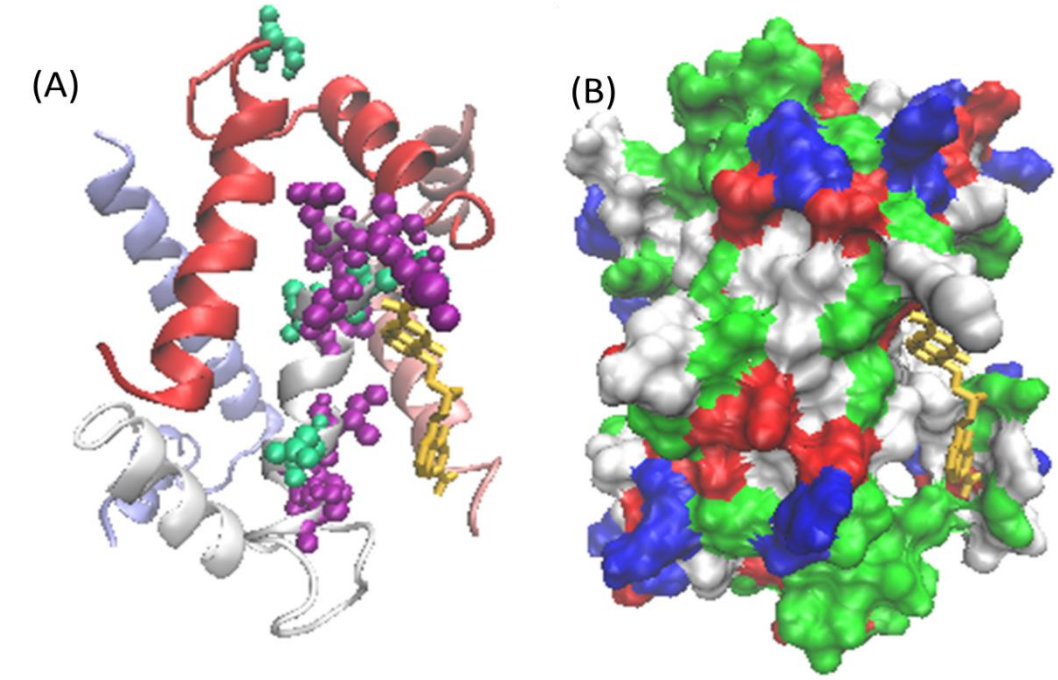

Figure 11(B)).

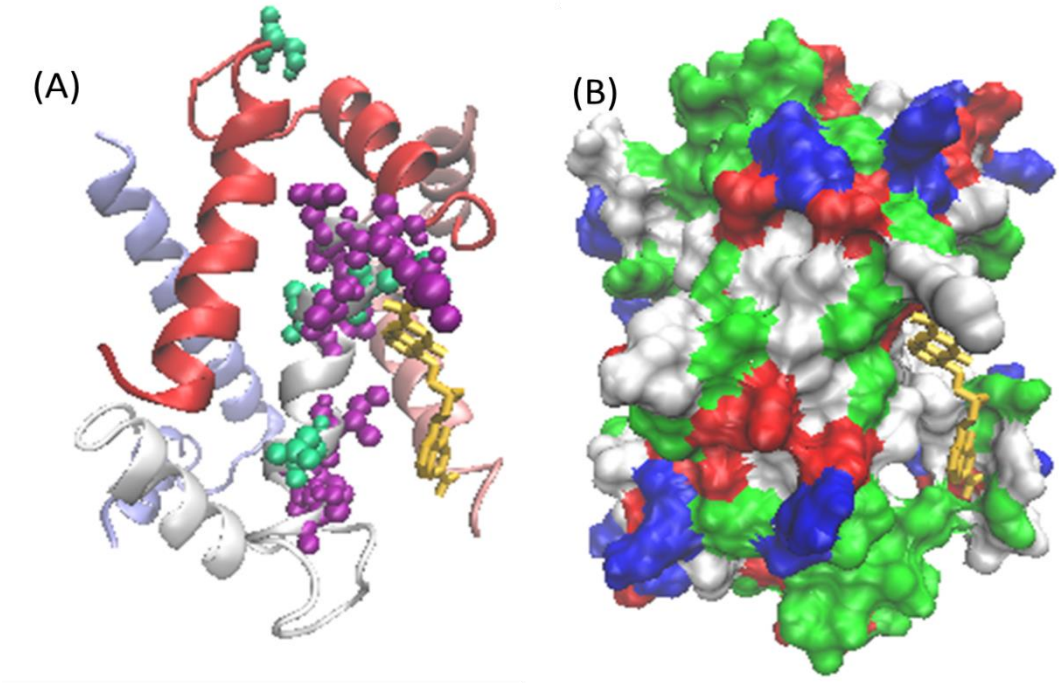

Figure 11 **HADDOCK structure of the S100P−cromolyn complex with 5:1 cromolyn**. (A) Ribbon-stick representation of the overlap of the S100P−cromolyn complex structure. Cromolyn is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B Representation of the S100P−cromolyn complex coloured by charged residue type. Cromolyn was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively.

3.3.1 NMR characterisation of the binding interface between C72 and S100P

The next titration was the drug C72 at concentrations ratios 2:1 and 5:1 with the 15N labelled S100P. The combined chemical shift perturbations for the peaks at each concentration of C72 were calculated using in analysis as the shift sum.

Figures 12 (A) shows the overlaid spectra of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 0.55 mM of the C72 (cyan). Figure 12 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.03. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 4Leu, 10Met, 17Arg, 20Gly, 26Gln, 43Gly, 60Asp, 72Ser, 76Val, 79Ala, 86His, 90Glu, 91Lys, 92Ala, 93Gly, 94Leu as labelled in Figure 10 (A).

As the concentration of C72 was titrated until a ligand to protein concentration was 5:1, the spectrum produced was very unclear, and it was impossible to assign any peaks for this spectrum.

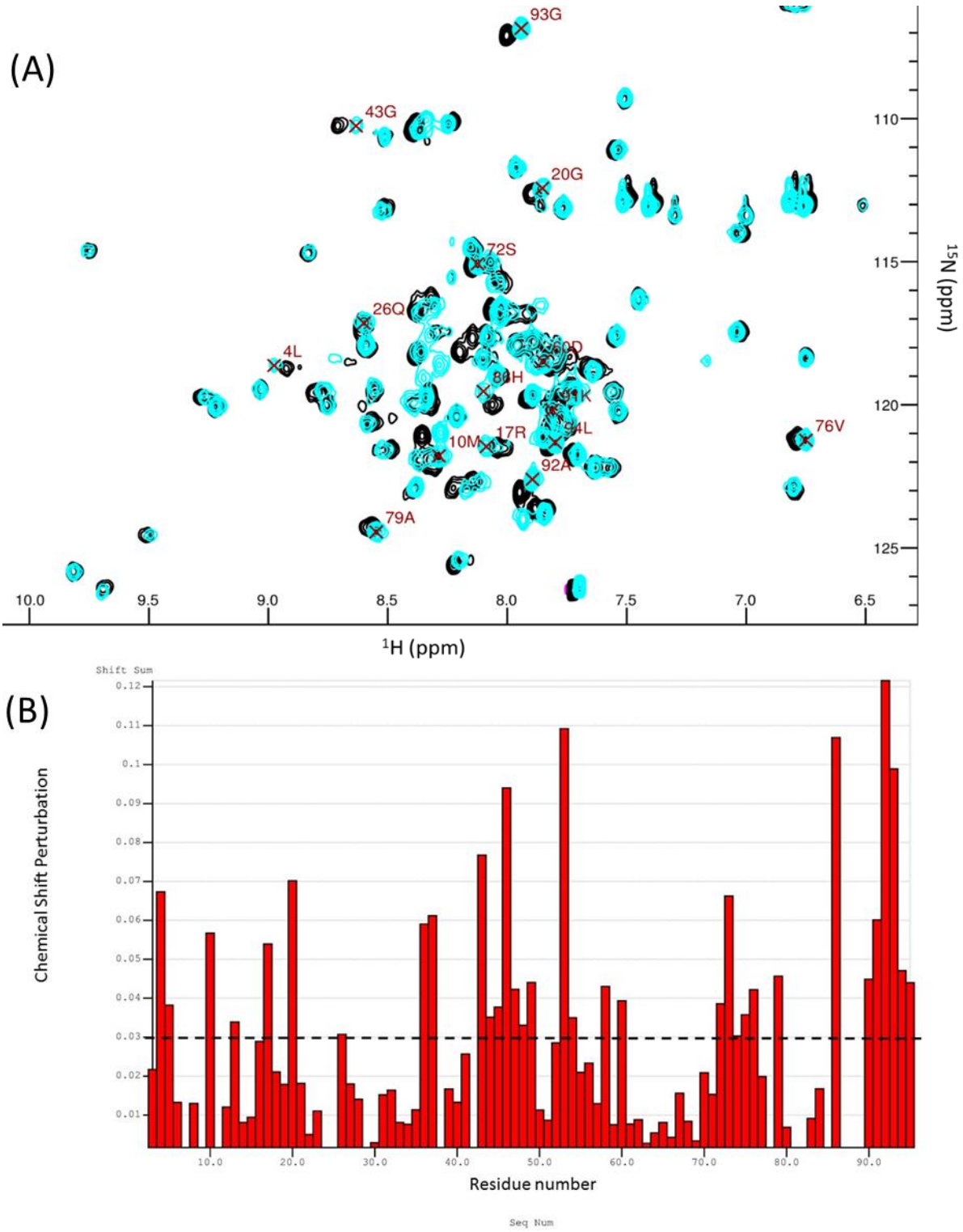

Figure 12(A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.28 mM ^{15}N-labeled S100P (black) and in the complex form with 0.55 mM of the C72 (cyan), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–C72 adduct at concentration ratio 2:1, C72 to S100P respectively. The range of shifts is double that of cromolyn. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.03 ppm).

3.3.2 Modelling of the S100P–C72 complex

Similar to cromolyn the modelling of the S100P-C72 complex was by using the molecular docking web server HADDOCK. The active residues were defined as significantly perturbed residues there was considerable shifts differences observed in the ^{15}N-^{1}H HSQC backbone upon S100P-C72 complex formation. In total, 200 structures of solvent refinement from HADDOCK resulted in a single cluster for the initial titration of C72 to S100P concentration ratio 2:1. The lowest energy conformations of the cluster were further analysed, and it demonstrated that the C72 molecule moiety was also situated at the hydrophobic pocket of the S100P.

Ligplot+ software did not create a schematic representation of the C72-S100P binding sites due to complexity of the arrangement. Therefore the interactions were assessed based on the residues that are at close proximity to the ligand. The Figure 13 below shows the surface view of the residues that are involved in the interaction. The 44Phe, 92Ala, 93Gly contain hydrophobic regions that are at close proximity to the drug, indicating that the hydrophobic bonds in this region.

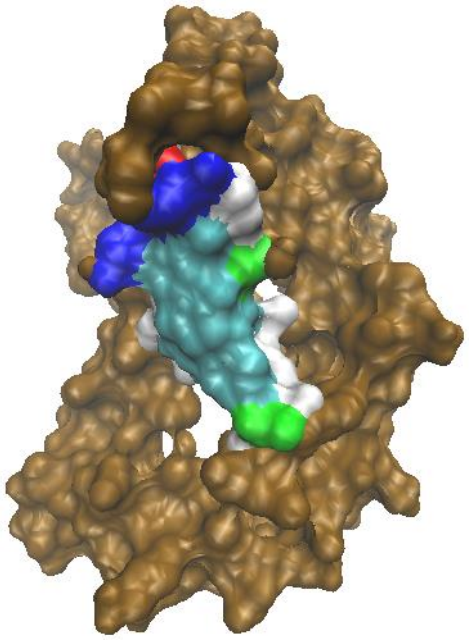

Figure 13 **The surface view of the residues that are involved in the C72-S100P interaction**. The ligand C72 is coloured light blue, and the positively charged, negatively charges polar and non- polar residues are shown in blue, red, lime green and white respectively.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located in the Helix-4 as demonstrated in Figure 14

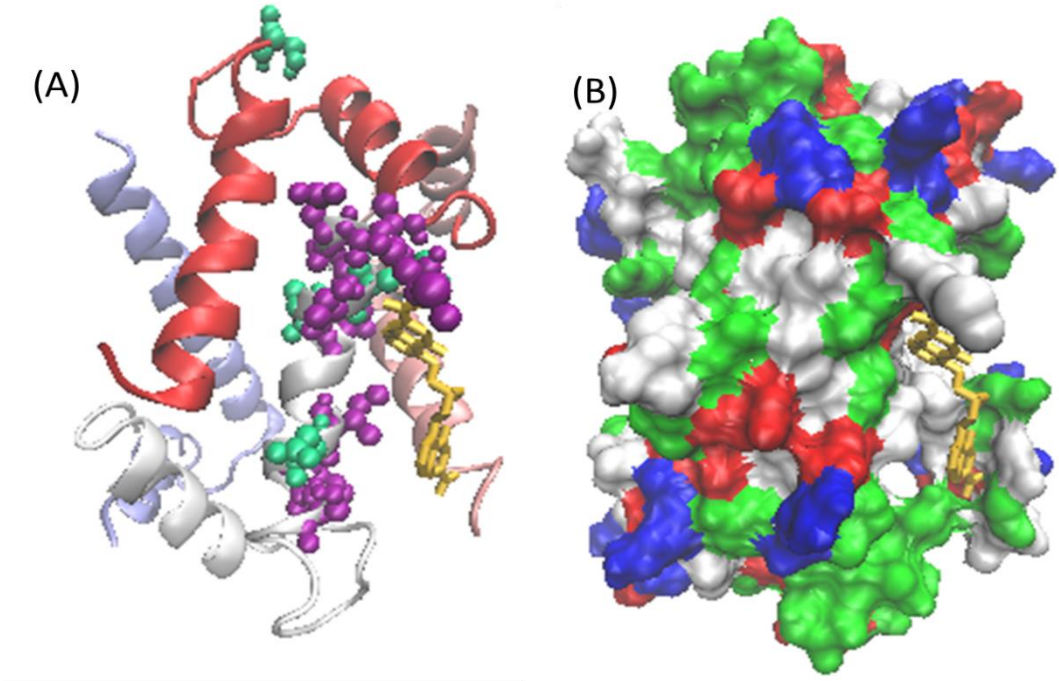

Figure 11(A).This was a hydrophobic pocket of the S100P protein (Figure 14 (B)).

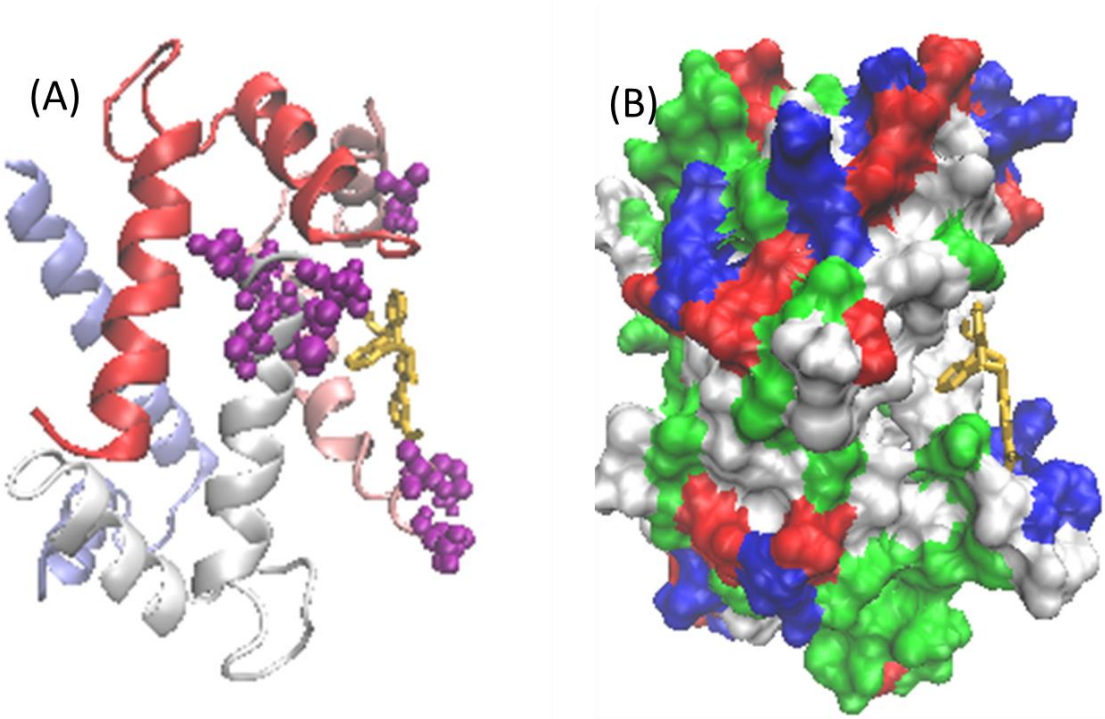

Figure 14 HADDOCK structure of the S100P−C72 complex with 5:1 C72. (A) Ribbon-stick representation of the overlap of the S100P−C72 complex structure. C72 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−C72 complex coloured by charged residue type. C72 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.1 NMR characterisation of the binding interface between IBS409 and S100P

The next titration were performed on the drug compound IBS409 at concentrations ratios 2:1 and 5:1 with the ^{15}N labelled S100P. The combined chemical shift perturbations for the peaks at each concentration of IBS409 were calculated using in analysis as the shift sum. The figures are included in the appendix 2.

Figure 28 (A) shows the overlaid spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM IBS409 (magenta). Figure 28 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.020. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that 3Glu, 4Leu, 10Met, 17Arg, 52Asp, 56Lys, 59Lys. 63Ala, 76Val, 79Ala, 86His. 90Glu, 92Ala were accessible to the solvent were determined to be as labelled in Figure 28 (A)

Figures 29 (A) shows the overlaid spectra of 0.42 mM 15N-labeled S100P (black) and in the complex form with 2.02 mM IBS409 (magenta). Figure 29 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.029. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 10Met, 17Arg, 26Gln, 38Glu, 76Val, 79Ala, 86His, 91Lys, 92Ala, 93Gly as labelled in Figure 29 (A)

.

3.3.2 Modelling of the S100P-IBS409 complex when the concentration of IBS409 to S100P was 2:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the titration of IBS409 to S100P at a concentration ratio 2:1. The schematic representation in Figure 15 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 57Leu, 60Asp, 61Leu, 76Val, 77Phe, 79Ala, 80Ala and the ligand IBS409 at a concentration ratio 2:1. Hydrogen were formed between 83Se, 84Ala and 87Lys and the hydroxyl group of IBS409, The number of interaction between the IBS409 and S100P (at a concentration ratio of 2:1 respectively is dominated by hydrophobic interactions.

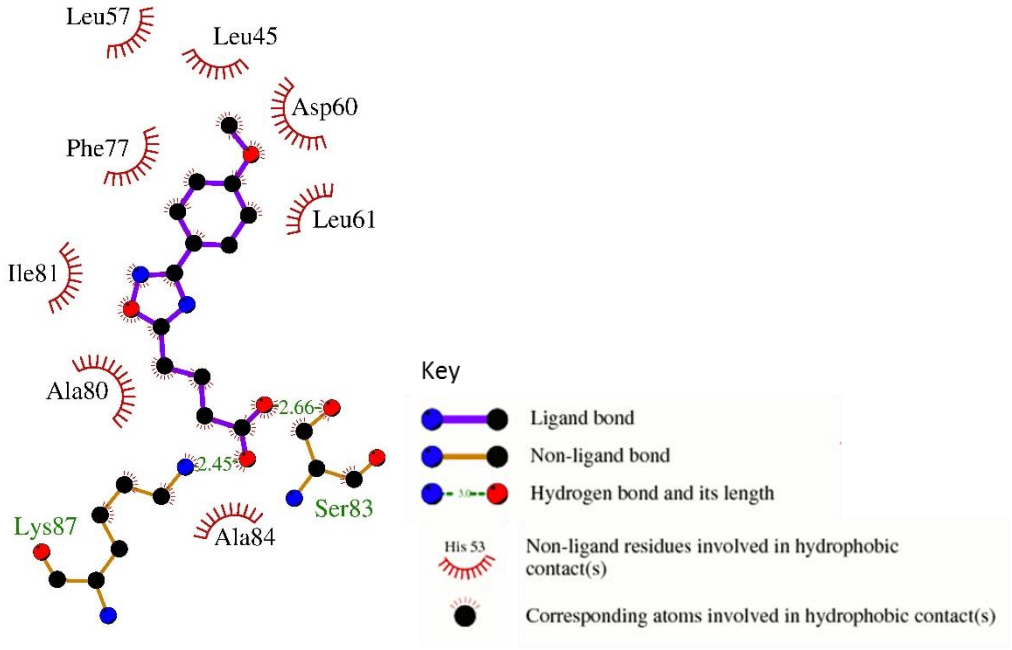

Figure 15 A schematic representation of the residues that are involved in the interaction within IBS409 and the S100P at a concentration ratio 2:1.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located in the Helix- 3, and Helix-4 as demonstrated in Figure 15(A).This was a hydrophobic pocket of the S100P protein (Figure 15 (B)).

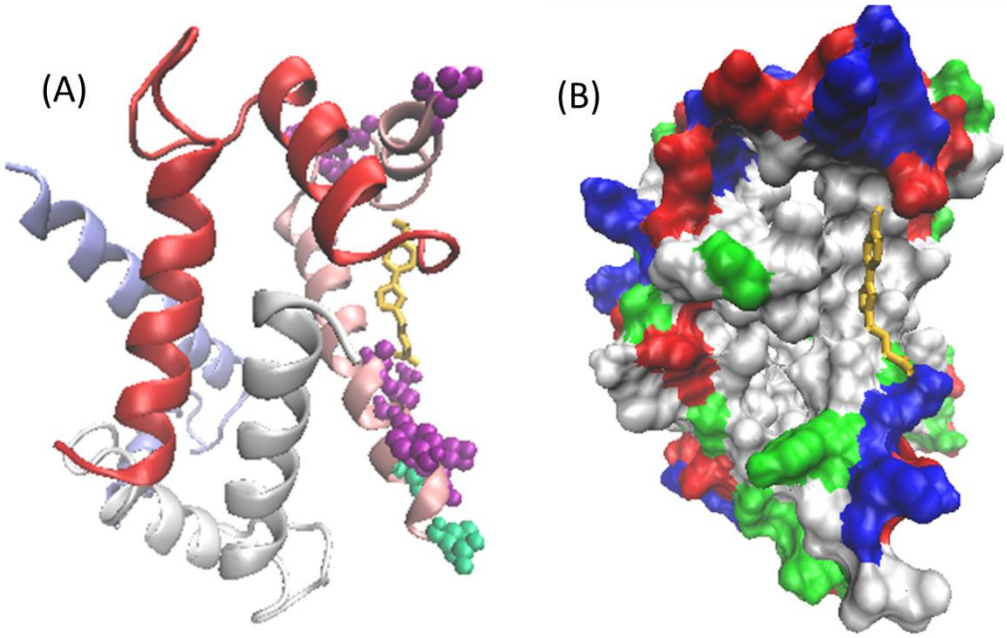

Figure 16 **HADDOCK structure of the S100P- IBS409 complex with 2:1 IBS409** . (A) Ribbon-stick representation of the overlap of the S100P−IBS409 complex structure. IBS409 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS409 complex coloured by charged residue type. IBS409 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.3 Modelling of the S100P-IBS409 complex when the concentration of IBS409 to S100P was 5:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the complex of IBS409 to S100P at a concentration ratio 5:1. The schematic representation in Figure 15 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 9Phe, 79Ala, 80Ala, 83Ser, 84Ala, 87Lys, 88Tyr and 92 Ala and the ligand IBS409 at a concentration ratio 5:1. Hydrogen were formed between 90Glu, 91Gly, 93Gly and the hydroxyl group of IBS409, The interaction between the IBS409 and S100P (at a concentration ratio of 5:1 respectively) is dominated by hydrophobic interactions.

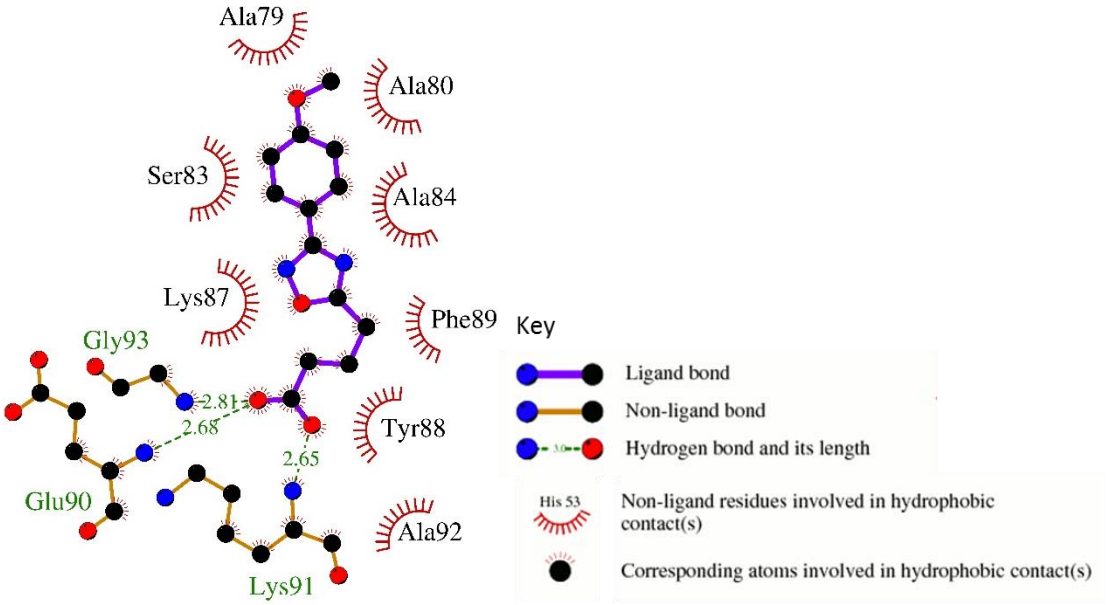

Figure 17 A schematic representation of the residues that are involved in the interaction within IBS409 and the S100P at a concentration ratio 5:1.

Residues that are involved in the interaction was mapped on S100P dimer in VMD. It was observed that the residues that are involved in the interaction were located in the Helix-1 and Helix-4 as demonstrated in Figure 18(A).This was a hydrophobic pocket of the S100P protein (Figure 18 (B)).

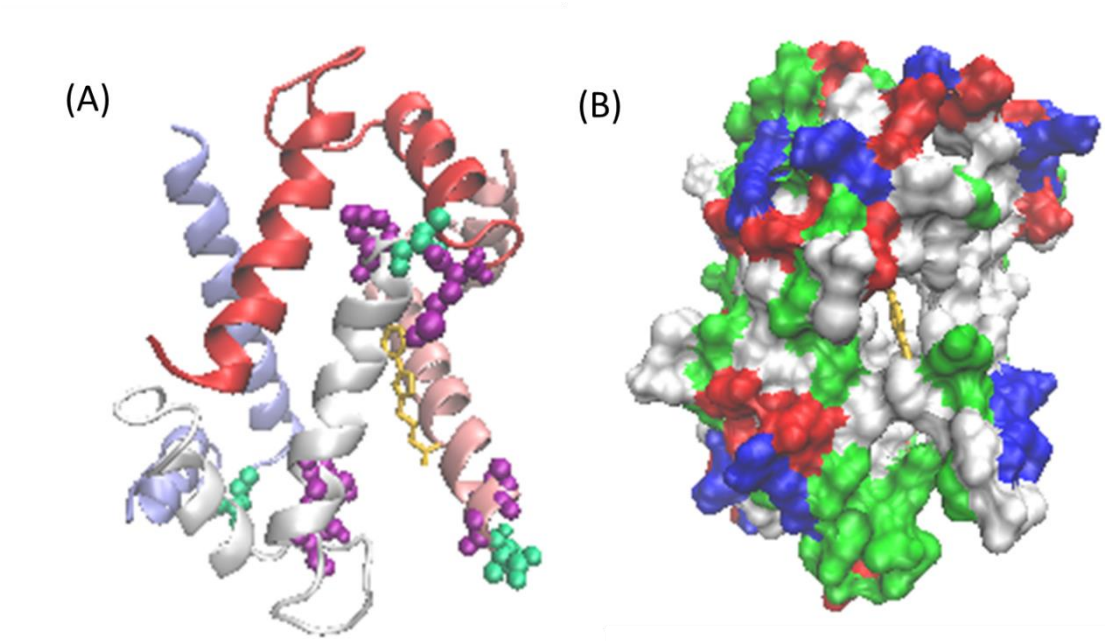

Figure 18 **HADDOCK structure of the S100P- IBS409 complex with 5:1 IBS409** . (A) Ribbon-stick representation of the overlap of the S100P−IBS409 complex structure. IBS409 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS409 complex coloured by charged residue type. IBS409 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.4 NMR characterisation of the binding interface between IBS798 and S100P

The next titration were performed on the drug compound IBS798 at concentrations ratios 2:1 and 5:1 with the ^{15}N labelled S100P. The combined chemical shift perturbations for the peaks at each concentration of IBS798 were calculated using in analysis as the shift sum. The figures are included in the appendix 3.

Figure 30 (A) shows the overlaid spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM of the IBS798 (orange). Figure 30 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.018. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 8Met, 17Arg, 38Glu, 39Lys, 52Asp, 59Lys, 63Ala, 76Val, 90Glu, 92Ala as labelled in Figure 30 (A)

Figures 31 (A) shows the overlaid spectra of 0.42 mM 15N-labeled S100P (black) and in the complex form with 2.02 mM of the IBS798 (orange). Figure 31 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.018. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be as 3Glu, 4Leu, 17Arg, 65Gly, 76Val, 79Ala, 84Ala, 90Glu, 92Ala, 93Gly, 94Leu labelled in Figure 31 (A)

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3.3.5 Modelling of the S100P-IBS798 complex when the concentration of IBS798 to S100P was 2:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the complex of IBS798 to S100P at a concentration ratio 2:1. The schematic representation in Figure 19 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 44Phe, 57Leu, 60Asp, 61Leu, 63Ala, 76Val, 77Phe, 81Ile, and the ligand IBS798 at a concentration ratio 2:1. The interaction between the IBS798 and S100P (at a concentration ratio of 2:1 respectively) is dominated by hydrophobic interactions.

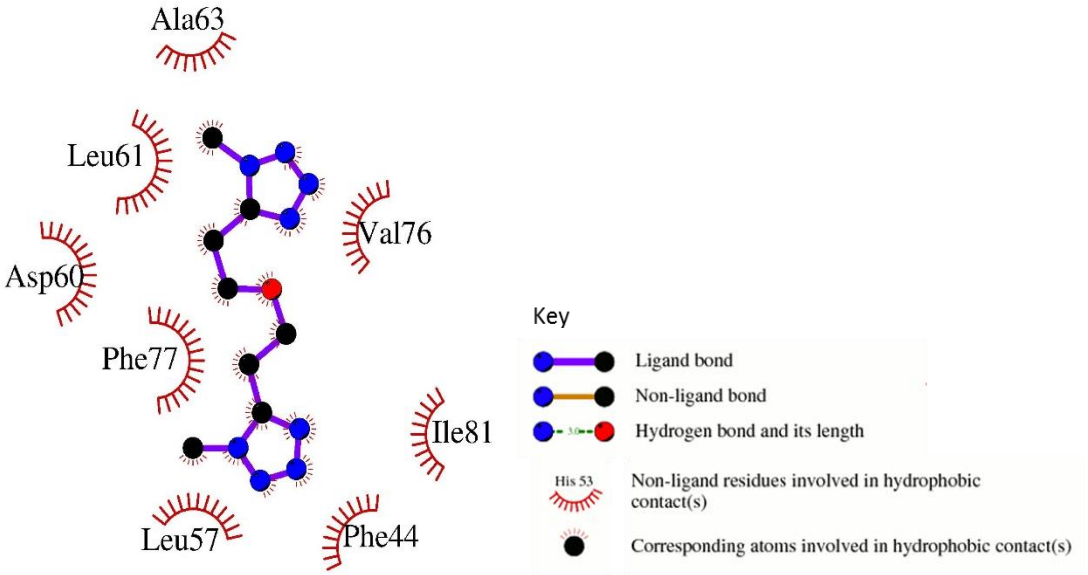

Figure 19 A schematic representation of the residues that are involved in the interaction within IBS798 and the S100P at a concentration ratio 2:1.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located mainly in the Helix-4 and the linker regions as demonstrated in Figure 20 (A).This was a hydrophobic pocket of the S100P protein (Figure 20(B)).

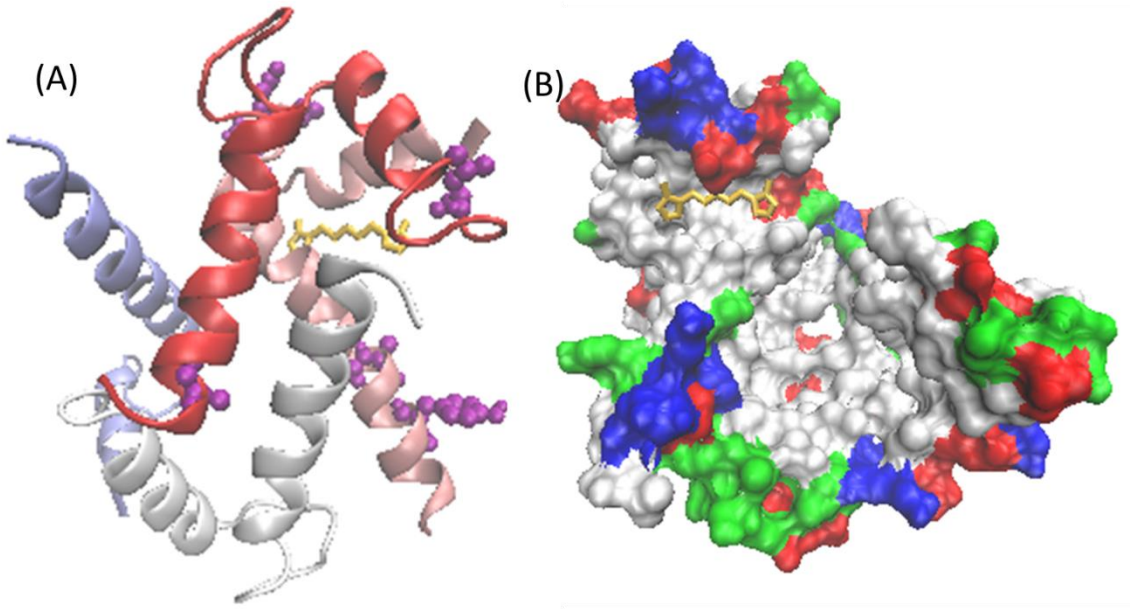

Figure 20 **HADDOCK structure of the S100P- IBS798 complex with 2:1 IBS798** . (A) Ribbon-stick representation of the overlap of the S100P−IBS798 complex structure. IBS798 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS798 complex coloured by charged residue type. IBS798 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.6 Modelling of the S100P-cromolyn complex when the concentration of IBS798 to S100P was 5:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the complex of IBS798 to S100P at a concentration ratio 5:1. The schematic representation in Figure 21 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 8Met, 44Phe, 84Ala, 85Cys, 88Tyr, 89Phe, 91Lys, 92 Ala, and the ligand IBS798 at a concentration ratio 5:1 Hydrogen were formed between 87Lys, 90Glu and 93Gly and the hydroxyl group of IBS798, The interaction between the IBS798 and S100P (at a concentration ratio of 5:1 respectively) is dominated by hydrophobic interactions.

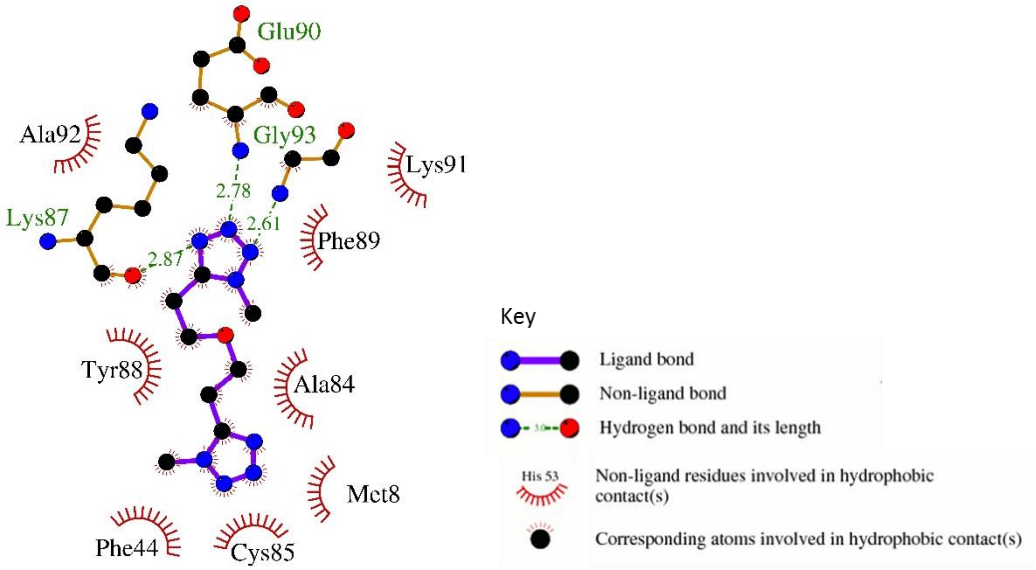

Figure 21 A schematic representation of the residues that are involved in the interaction within IBS798 and the S100P at a concentration ratio 2:1.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located mainly in the Helix-1 and Helix 4 and also in the linker region as demonstrated in Figure 22(A). This was partly in the hydrophobic pocket, of the S100P protein, and partly exposed to the polar and basic residues in the structure as demonstrated in Figure 22(B).

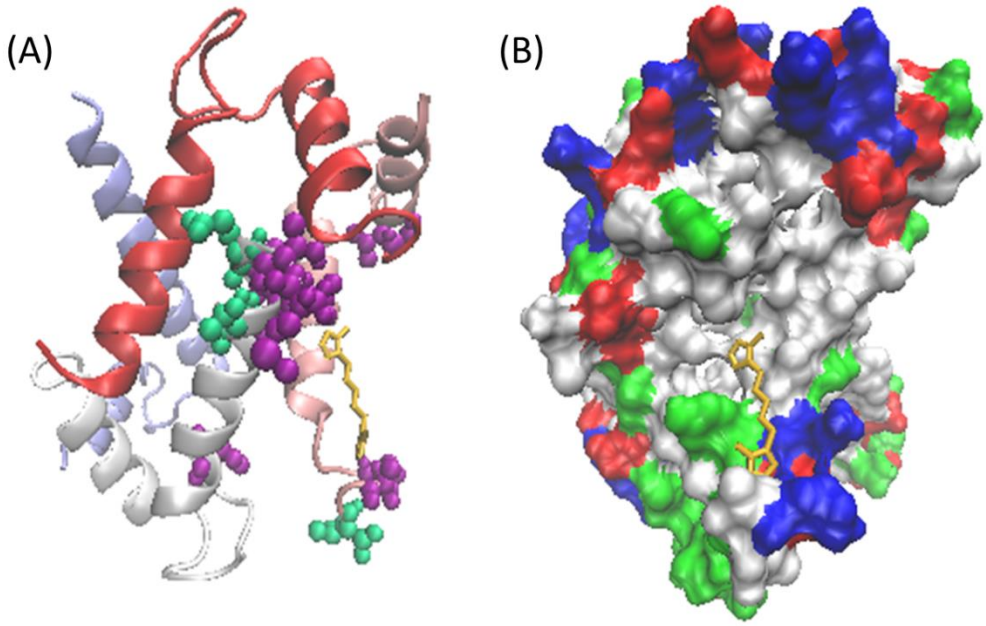

Figure 22 **HADDOCK structure of the S100P−IBS798 complex with 5:1IBS798**.
(A) Ribbon-stick representation of the overlap of the S100P−IBS798 complex structure. IBS798 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS798 complex coloured by charged residue type. IBS798 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.7 NMR characterisation of the binding interface between IBS863 and S100P

The next titration were performed on the drug compound IBS863 at concentrations ratios 2:1 and 5:1 with the ^{15}N labelled S100P. The combined chemical shift perturbations for the peaks at each concentration of IBS863 were calculated using in analysis as the shift sum. The figures are included in the appendix 4.

Figure 32 (A) shows the overlaid spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM of the (red). Figure 32 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.008. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 10Met, 12Ile, 17Arg, 39Lys, 43Gly, 63Ala, 76Val, 86His, 92Ala as labelled in Figure 32 (A)

Figure 33 (A) shows the overlaid spectra of 0.42 mM 15N-labeled S100P (black) and in the complex form with 2.02 mM of the IBS863 (red). Figure 33 (B) shows a bar graph demonstrating the chemical shift perturbations of each residue, where the significantly perturbed residues were determined as residues with CSP greater than or equal to an average CSP of 0.08. The solvent accessibility for the residues were calculated using equation 2 and the most perturbed residues that were accessible to the solvent were determined to be as 3Glu, 10Met, 17Arg, 26Gln, 38Glu, 39Lys, 56Lys, 60Asp, 63Ala, 65Gly, 76Val, 84Ala, 86His, 89Phe, 92Ala, 94Leu as labelled in Figure 33 (A)

3.3.8 Modelling of the S100P-IBS863 complex when the concentration of IBS863 to S100P was 2:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the complex of IBS863 to S100P concentration ratio 2:1. The schematic representation in Figure 23 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 4Leu, 7Ala, 8Met, 9Gly, 11Ile, 81Ile and 85Cys. and the ligand IBS863 at a concentration ratio 2:1 Hydrogen bonds were formed between residues 7Ala, 10Met, 12Ile and the hydroxyl group of IBS863, The interaction between the IBS863 and S100P (at a concentration ratio of 2:1 respectively is dominated by hydrophobic interactions.

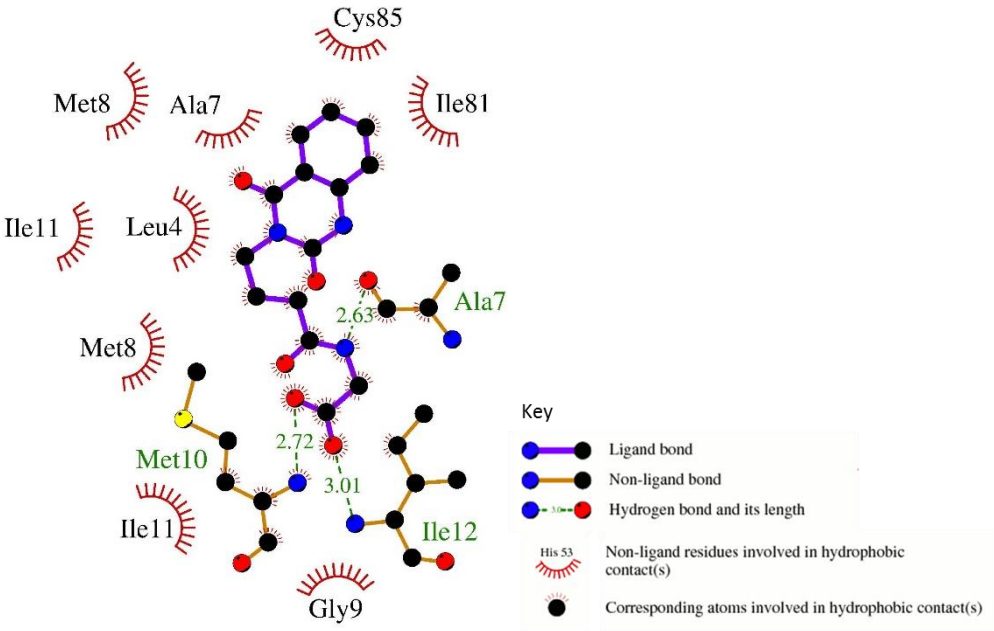

Figure 23 A schematic representation of the residues that are involved in the interaction within IBS863 and the S100P at a concentration ratio 2:1.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located in the Helix-1 Helix-4 as demonstrated in Figure 24 (A). This was in the hydrophobic pocket, of the S100P protein as demonstrated in Figure 24(B).

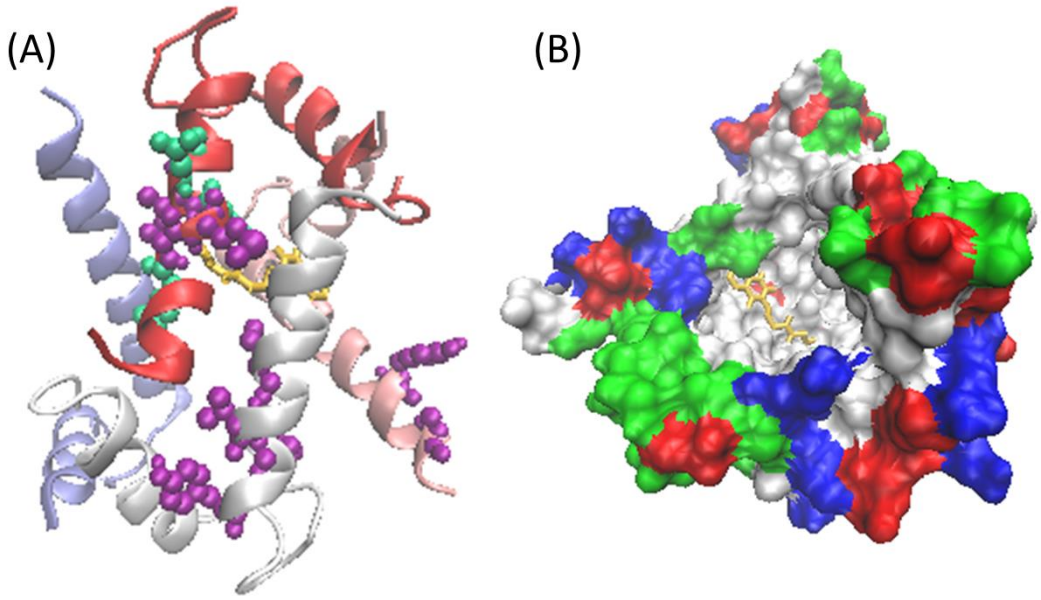

Figure 24 **HADDOCK structure of the S100P−IBS863 complex with IBS863**
(A) Ribbon-stick representation of the overlap of the S100P−IBS863 complex structure. IBS863 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS863 complex coloured by charged residue type. IBS863 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.3.9 Modelling of the S100P-IBS863 complex when the concentration of IBS863 to S100P was 5:1

HADDOCK clustered 200 structures in 1 cluster, which represents 100.0 % of the water-refined models for the titration of IBS863 to S100P concentration ratio 5:1. The schematic representation in Figure 25 shows the residues that are involved in the interaction. There were hydrophobic interactions between the S100P residues 4Leu, 7Ala, 8Met, 9Gly, 11Ile, 85Cys and 81Ile and the ligand IBS863 at a concentration ratio 2:1. Hydrogen bond was present between the SH group of 85Cys and the NH. The hydrogen bonding occurred between the cyclic nitrogen group of the drug and a hydroxyl group of 87Lys, and amine groups in 89Phe and 91Lys. The interaction between the IBS863 and S100P (at a concentration ratio of 5:1 respectively is dominated by hydrophobic interactions.

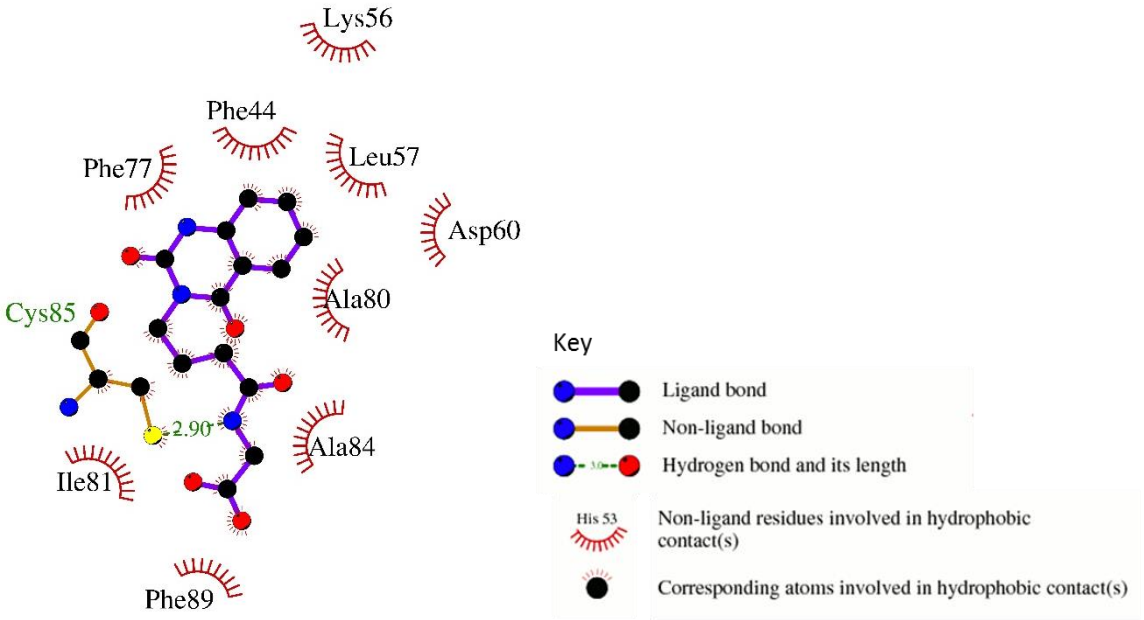

Figure 25 A schematic representation of the residues that are involved in the interaction within IB863 and the S100P at a concentration ratio 5:1.

Residues that are involved in the interaction was mapped on S100P dimer. It was observed that the residues that are involved in the interaction were located in mainly the Helix-1 and 4 as demonstrated in Figure 26 (A). This was in the hydrophobic pocket of the S100P protein structure as demonstrated in Figure 26 (B).

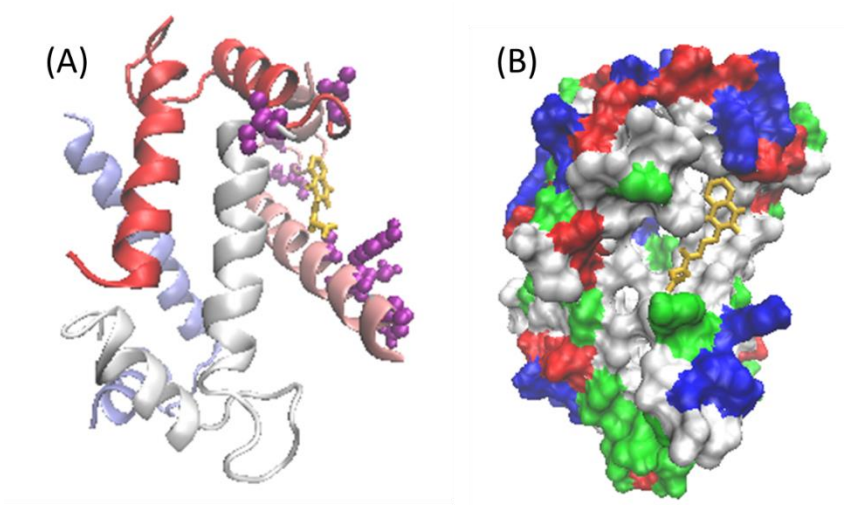

Figure 26 **HADDOCK structure of the S100P−IBS863 complex with IBS863**
(A) Ribbon-stick representation of the overlap of the S100P−IBS863 complex structure. IBS863 is shown in yellow of the S100P homodimer and the domains 1 and 2 of the S100P dimer are coloured in red and blue respectively. The residues that are involved in hydrogen bonding are shown in blue-green colour and the residues taking part in hydrophobic interactions are in purple. (B) Representation of the S100P−IBS863 complex coloured by charged residue type. IBS863 was displayed in yellow. The positively charged, negatively charged, nonpolar and polar residue surfaces are represented in blue, red and white, and lime green respectively

3.4 Isothermal titration Calorimetry

The ITC assay was performed in order to assess the binding affinity and the stoichiometry of the S100P-ligand complexes, by determining the heat changes during the interaction. The initial assay was performed with the ligand cromolyn and C72. [The Wilmans plot for these experiments are in Appendix 5, Figure 34 (B) and Figure 35 (B).] However from these experiments very little or no binding was observed, and therefore binding parameters could not be determined. ITC was performed by using $CaCl_2$ as a ligand as a positive control for the assay. This titration was successful giving a stoichiometry of binding interaction is 1:4. The binding isotherm for the interaction between S100P and the $CaCl_2$ is presented in Figure 27.

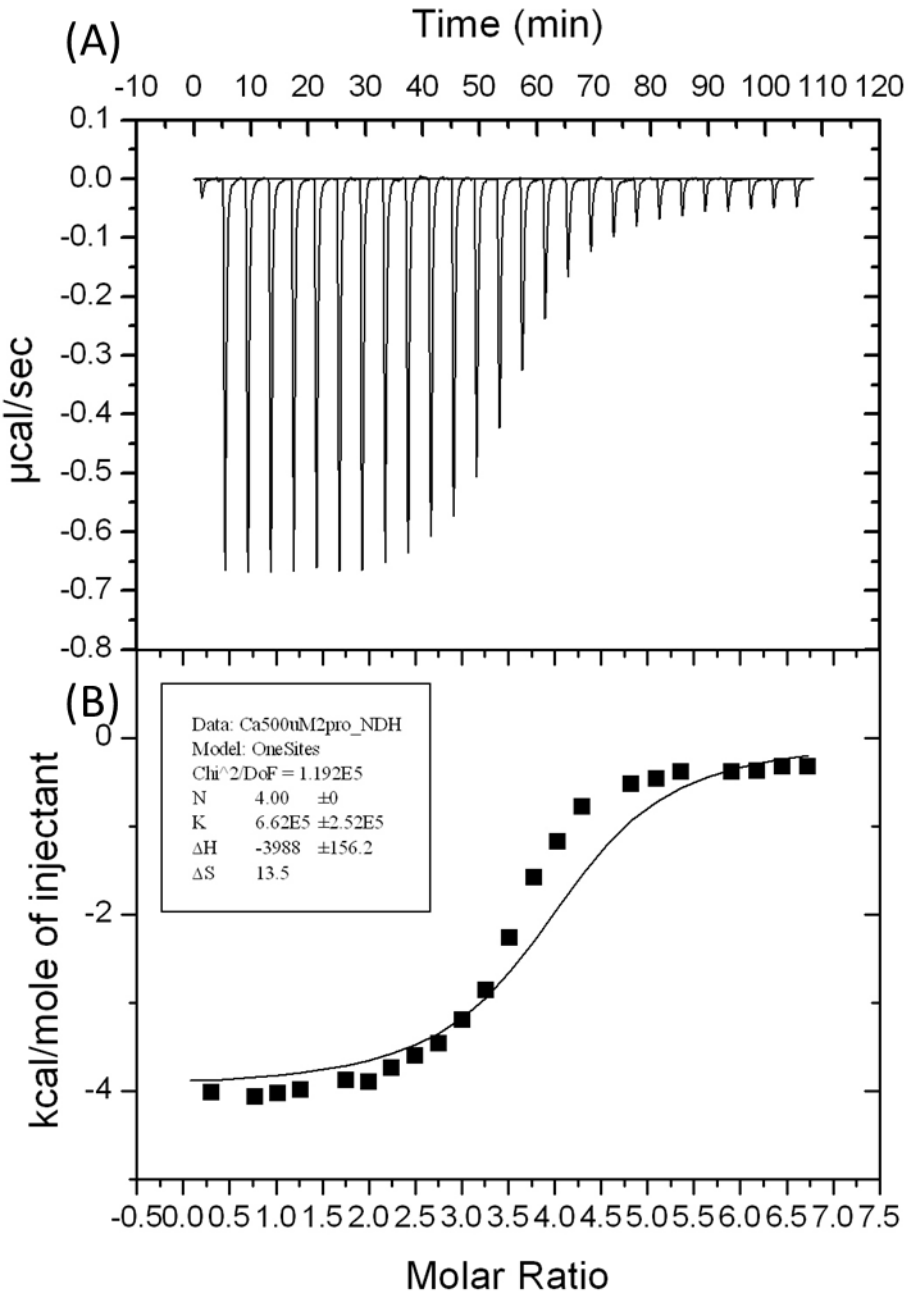

Figure **27 The ITC titration of calcium chloride to S100P.** (A) Raw data of the ITC titration with $CaCl_2$. (B) The Wilmans plot of $CaCl_2$ titration, where data was fitted a non-linear least square approach to the 'one set of sets' site model. This yielded a dissociation constant of K_d=6.62E5±0.52E5 µM with additional parameters of N=4±0, ΔH=-3988± 156.2 kcal/mol, and ΔS=13.6 cal/mol/deg. The stoichiometry of binding interaction is 1:4.

4 Discussion

Earlier studies have associated the aggressiveness of PDAC to the dysregulated expression of the calcium binding protein S100P. S100P is secreted from cancer cells and acts via interaction with the RAGE receptor. The activation of the RAGE receptor through the binding of S100P leads to the key signalling events as activation of the MAP kinase and NF-κB.(Arumugam *et al.* 2004). Blocking S100P function has been shown to reduce tumour growth and metastasis (Arumugam *et al*. 2004). This data suggests that S100P is a promising therapeutic target. An effective strategy in the treatment of diseases caused by the overexpression of the S100P could be to prevent the formation of this S100P-RAGE complex. Dr. Sharon Rossiter and her team have developed some new chemotherapy drug compounds that are specifically aimed at attaching to and inhibiting RAGE from binding to S100P. In the present study the interactions between the drug compounds and the S100P protein were investigated, using biophysical techniques including NMR and ITC. In order to perform these assays, the recombinant plasmid bearing the wildtype human S100P protein in was expressed in *E. coli* Star bacterial cells. The S100P protein expressed was purified by affinity chromatography using a HisTrap column and the purity of the protein was assessed using SDS PAGE.

4.1 Analysis of the purity of the protein purified by SDS PAGE

SDS PAGE is the most common polyacrylamide gel electrophoresis method used for assessing the purity of a protein sample. In SDS PAGE the SDS shields the respective charge of the proteins in a mixture giving them a negative charge and DTT is a reducing agent that cleaves di-sulphide bonds and converts protein to their secondary structure. This allows the proteins to be separated based on their molecular weight. The target protein band can be identified using the apparent molecular weight obtained by including standard molecular weight markers in the analysis (Walker, 2002).

There were several purifications performed during this project and an SDS PAGE assay was performed for the samples collected at each stage, for each of the purifications. In all of these purification gels bands of a similar profile were observed. The most abundant protein detected in the bacterial lysate sample was

at a molecular weight of ~12KDa, Post application of filtered lysate sample to the column the intensity of the band at ~12kDa faded, this implies that the protein bound to the HisTrap column at this stage. The S100P protein prepared includes of a hexa-histidine-tag, therefore it has a high selective affinity for Ni^{2+} ions in the Nickel Sepharose column. Other host cell protein bands were detected in the filtered lysate flow-through, this indicates that the histidine-tagged S100P binding to the column is highly selective (Gelifesciences, 2015). The filtered lysate sample has a low concentration (25 mM) of imidazole. Imidazole competes with the proteins to bind to the Nickel Sepharose column. The low concentration of imidazole in the filtered lysate sample prevents unwanted host cell proteins from binding to the column.

The Wash 1 and Wash 2 flow-through sample lanes usually had no bands or very faint bands. The initial wash step with the lysis buffer was performed in order to facilitate the filtered lysate sample passing through the column. The second step was with a buffer that had a slightly higher concentration (100 mM) of imidazole than the lysis buffer which ensures the removal of contaminants that may otherwise be co-purified with the histidine-tagged S100P. At higher concentrations, imidazole decreases the binding of the histidine-tagged protein to the Ni Sepharose column (Gelifesciences, 2015).

The EB 1 fraction lanes usually contained a protein band at ~12kDa. The EB 1 contained a higher concentration of imidazole (350mM) than the wash buffer, causing the elution of histidine-tagged S100P protein. However, most of the protein S100P was detected in the EB 2 fractions, indicated by an intense band at ~12kDa. The intensity of the S100P protein band indicated that the most abundant protein present in the sample was S100P and the purification was successful. However the level of purity of the protein sample could be obtained through an additional purification step by gel filtration.

4.2 NMR characterisation of the binding interface between the ligands and S100P

Two-dimensional NMR ^{1}H-^{15}N HSQC experiment is a useful method for the identification of ligand binding sites on proteins. The spectra provide information on the connections between the amide protons (NH) and the nitrogen atoms connected. The peaks in these spectra correspond to the NH group of each residue, and act as a 'fingerprint' for the protein.

In order to obtain a greater understanding of the interaction between the S100P protein and the drug compounds, a series of 2-D HSQC NMR experiments performed. The initial experiment was performed with 0.3mM S100P protein in 20mM MES (pH 6.5), 50mM NaCl, 4 mM $CaCl_2$ and 10% D_2O. This initial spectrum overlaid with calcium bound S100P spectrum previously deposited in the BRMB (accession code 17866) and their peaks were compared. Most of the peaks in the BRMB spectrum (reference) matched with the peaks of the spectrum of expressed and purified for this studies. This confirmed that the purified protein was S100P with minimal impurities. This spectrum was used as the reference spectrum for the rest of the assessments with that preparation of S100P. A Bruker 600Hz spectrometer equipped with cryogenic probes were used for the purpose of these experiments.

Previous studies have demonstrated that in mouse models, cromolyn has the ability to block S100P interaction with its receptor RAGE in and thereby reduce growth and invasion of pancreatic cancer. (Arumugam *et al*. 2006) Another study also demonstrated binding affinity using NMR as an analysis tool. (Penumutchu *et al.* 2014b). Since cromolyn has been studied in relation to being an S100P inhibitor, in this study cromolyn was used as a positive control. The next set of experiments included the S100P protein in the presence of excess anti-allergy drug cromolyn at ratios of 2:1 and 5:1. The experiment of ligand addition was performed as titration NMR by increasing the ligand ratio to the S100P. Upon the addition of the ligand (cromolyn) there were changes in the ^{15}N-^{1}H that were observed in the spectra. The ^{15}N-1H backbone resonance of the S100P relative to that of the protein spectra demonstrated residues that shifted significantly upon the addition of the ligand. The chemical shifts are very sensitive to the electronic environment of the nuclei. Therefore, perturbations in the chemical shift may not only be due to changes in the covalent molecular structure but also through non-covalent interaction with solvent

molecules and ligands. Chemical shifts are therefore a sensitive probe for the identification of the interaction surfaces of protein complexes. The chemical shifts that are perturbed usually belong to the residues that are closest to the interaction surface.

It is important to understand that changes in the chemical shift merely imply the magnetic environment of the nuclei has changed, and are not necessarily due to a direct interaction with the ligand. If substantial structural rearrangement takes place in the protein through complex formation, wide spread chemical shift perturbations, may occur including residues that are distant from the actual site of interaction, but does experience a change in their structural environment. Therefore, it is important to take into account the accessible surface area of the protein in order to determine the interaction sites (Tien *et al.* 2013). The solvent accessibility of the residues was determined for the S100P molecule, and found that from the residues that significantly shifted, are also the ones that were accessible to the ligand.

The most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 10Met, 16Ser, 17Arg, 26Gln, 43Gly, 86His, 89Phe, 90Glu, 91Lys, 92Ala, 93Gly when the concentration of cromolyn to S100P was 2:1. The most perturbed residues that were accessible to the solvent were determined to be 3Glu, 4Leu, 10Met, 17Arg, 23Gly, 43Gly, 56Lys, 86His, 89Phe, 90Glu, 91Lys, 92Ala, 93Gly, and 94Leu when the concentration of cromolyn to S100P was 5:1. These residues were used as active residues in HADDOCK modelling analysis. The passive residues were defined by the HADDOCK program automatically, where all solvent accessible surface neighbours of the active residues were used. The lowest energy conformations of the clusters generated by HADDOCK were analysed, and it showed that the cromolyn molecule was situated in the hydrophobic pocket of S100P. LigPlot was used to obtain a schematic representation of the residues that were involved in the interaction. There were hydrophobic interactions within cromolyn and the S100P at residues 5Glu, 8Met, 9Gly, 12Ile, 26Glu, 86His, 89Phe and 90Glu. There were some hydrogen bonds that were observed between the residues formed between the hydroxyl group of cromolyn and 6Thr, 9Met, 91Lys, 92Ala, and 93Gly. The interaction between the cromolyn and S100P (at a concentration ratio of 2:1 respectively) dominated by hydrophobic interactions. Similarly hydrophobic interactions within cromolyn and the S100P; at a concentration ratio 5:1 respectively, were 1Met, 5Glu, 8Met, 9Gly, 88Try, 89Phe, 90Glu, 91Lys and 93Gly. Hydrogen bonds between were

formed between 6Thr, 26Gln and 94Leu and the hydroxyl group of cromolyn, except for one hydrogen bond with 26Gln, where the hydrogen bond was with the oxygen in the phenyl ring. The interaction between the cromolyn and S100P (at a concentration ratio of 5:1 respectively) is dominated by hydrophobic interactions.

Similarly the other drug compounds assessed also had hydrophobic interactions dominating the interaction between S100P and ligand. A study that investigated the interactions between the calcium-bound S100P and the V domain of RAGE, reported that the binding of the S100P to the V domain of RAGE is stabilised by hydrophobic residues in the interface between S100P and the RAGE-V domain (Penumutchu, Chou *et al.* 2014b). In the present study it was found that the hydrophobic bonds dominate in the binding of the S100P to the ligands. This suggest that the residues involved in the binding of ligand to S100P are related to the residues in the binding interface of S100P to RAGE V domain. If the hydrophobic bonds between S100P and the ligand is stronger than hydrophobic bonds involved in S100P and V domain of RAGE receptor, those drugs compounds may serve a therapeutic use for treating S100P induced cancer.

A study by Penumutchu *et al*., 2014a, mapped the interfacial residues on S100P and cromolyn contact surface by ^{1}H-^{15}N HSQC, therefore cromolyn was used as a positive control for studying the interaction sites by NMR. NMR characterisation of the binding interface between cromolyn and S100P demonstrated that the residues with significantly perturbed chemical shifts were the 3Glu, 4Leu, 5Glu, 10Met, 11Ile, 12Ile, 14Val and 15Phe in the Helix-1 and 43Gly, 44Phe from the linker region and 84Ala, 89Phe, and 93Gly from Helix-4. This study modelled the S100P-cromolyn complex, and the area to which cromolyn showed to have interactions in the present study was similar to the region with the cromolyn interacted in the study by Penumutchu *et al* 2014a. (Figures 9 and 11). Residues that are involved in the interaction were mapped onto the S100P dimer. It was observed that the residues that are involved in the interaction were located mainly in Helix-1 and Helix 4 at both 2:1 and 5:1 cromolyn to S100P concentration. This suggests that cromolyn has a binding affinity towards the S100P. The structural characterisation of the complex S100P-RAGE using NMR, mutagenesis and functional assays reported that the RAGE V binding site is situated in the in the linker region and the Helix-1 and Helix-4. (Penumutchu, Chou *et al.* 2014a). The study also shows that the binding region of cromolyn (44Phe, 89Phe and 5Glu) is involved in S100P RAGE binding. This

suggests that if a ligand such as cromolyn binds to the S100P RAGE binding interface, S100P RAGE interaction could be blocked and therefore prevent the activation of the RAGE receptor via S100P. The present study also shows that the residues that are involved in the interaction with cromolyn to S100P includes the binding region residues (5Glu, 44Phe and 89Phe) mentioned in Penumutchu, Chou *et al.* 2014a. However since the binding constant of S100P-cromolyn was weak compared to S100P-RAGE, the blocking of the S100P through cromolyn might not be effective.

The other ligands that were analysed by the NMR titration analysis also revealed interactions to S100P. The drug compound C72 interacted with the residues 44Phe, 84Ala, 92Ala, 88Tyr, 93Gly, 85Cys, 87Lys, 91Lys and 90Glu. Residues that involved in the interaction were located to be at mainly in Helix-4 as demonstrated in Figure 14 (A). This was a hydrophobic pocket of the S100P protein (Figure 14 (B)). According to the structural characterisation of the complex S100P-RAGE using NMR the RAGE V binding site is situated in the linker region and the Helix-1 and Helix-4. (Penumutchu, Chou *et al.* 2014b). This suggests that ligand C72 which has its binding site in Helix-4 is able to bind to the S100P and thereby block S100P from binding to RAGE receptor.

Similarly binding regions of the other three ligand molecules analysed (IBS409, IBS798 and IBS863) also had binding sites in Helix 1, 4 and the linker region. Although all the ligands show interaction toward the S100P molecule, the strength of the binding would determine if these compounds are efficient to be used as drugs.

4.3 Isothermal titration Calorimetry

The ITC assay was performed in order to assess the binding affinity and the stoichiometry of the S100P-ligand complexes, by determining the heat changes during the interaction. The initial experiments were performed with the ligands cromolyn and C72, but from these experiments showed very little or no binding was observed, and therefore binding parameters could not be determined. The ITC performed by using $CaCl_2$ as a ligand, gave a protein to ligand binding stoichiometry of 1:4. This experiment with calcium titration was performed in order to use it as a reference. Figure 27 (B) shows the Wilmans plot of $CaCl_2$ titration, where data was fitted a non-linear least square approach to the 'one set of sites' site model. This yielded a dissociation constant of K_d=6.62E5±0.52E5 µM with additional parameters of N=4±0,

ΔH=-3988± 156.2 kcal/mol, and ΔS=13.6 cal/mol/deg. The negative enthalpy of ΔH=-3988 kcal/mol suggests that the reaction is exothermic (releasing heat), this is also shown by the negative peaks in Figure 27 (A). The stoichiometry of binding interaction was 1:4. This information is evident as there are 2 EF hands in the S100P protein. Each EF hand binds two Ca^{2+} ions, therefore four Ca^{2+} ions can bind to one molecule of S100P.

4.4 Limitations

It is important that the protein that is expressed and purified contains minimal impurities when used for NMR experimentation as traces of impurities in the sample may produce peaks in the NMR spectra. If these peaks are not identified as caused by impurity the spectra can be misinterpreted. This can lead to the assignment of an incorrect structure to the compound being studies. The protein expressed also contained a hexa-histidine tag, in order to use the HisTrap column in purification of protein by affinity chromatography. This histidine tag could be removed by thrombin cleavage of the tag (Koltzscher, Neumann *et al.* 2003). Additional purification of the sample may be achieved by size-exclusion chromatography (gel filtration) of the sample and if necessary a final 'polishing' step would be to use ion exchange chromatography. Gel filtration also has the ability of removing reducing agents as DTT from the sample that can interfere with the BCA protein concentration assay.

The method of protein concentration calculation for this protein was by using the BCA assay. This assay had a compatible dye for reducing agents for reducing agents enabling it to be used with protein samples containing up to 5 mM DTT (Johnson, 2015). In the BCA assay, the protein concentration is determined by creating a standard curve from a standard protein (e. g: BSA), however if S100P does not interact with the BCA dye in a similar way as the standard protein, the concentration could be underestimated or overestimated. More accurate and quicker methods as measuring absorbance at 280nm cannot be performed as the S100P protein does not contain tryptophan residues (Johnson, 2015). The analysis of the stoichiometry by ITC highly depends on the accuracy of the concentration of the protein used. Therefore any inaccuracies in the protein concentration will be

affect the ITC data interpretation. One other limitations of ITC is that it requires a large amount of protein.

4.5 Conclusion

Previous studies by Arumugam *et al* 2006, that the anti-allergy drug cromolyn binds to S100P blocking it's interaction with the RAGE receptor, and in this manner reduce the tumour growth and invasion of pancreatic cancer in mouse models. Therefore this suggested that ligand molecules that block S100P from binding and activating RAGE, to be effective as a therapeutic agent for pancreatic cancer. All the ligands, C72, IBS409, IBS798 and IBS863 showed interactions with S100P. Binding sites of the ligands were identified via NMR, and the binding was found to be dominated by hydrophobic interactions. Further ITC experimentation may be required in order to gain an understanding of the stoichiometry and the binding affinity for these ligands.

4.6 Further work

More compounds could be analysed and if strong binding indicated with one or more lead compounds, crystallisation trials of S100P in complex with these compounds may be conducted in order to obtain the three-dimensional structure of S100P in complex. The data that collected from this project may have implications for cancer drug discovery and ultimately lead on to further research on the effects *in vivo*, a potential patent and collaborations with other national/international research groups.

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6 Appendix 1

Table 2 Proposed values for ASA normalization (in Å), compared to previously used scales defined by Miller et al.(Tien, Meyer et al. 2013)

Residue	Maximum Accessibility (A°)
Alanine	113.0
Arginine	241.0
Asparagine	158.0
Aspartate	151.0
Cysteine	140.0
Glutamate	183.0
Glutamine	189.0
Glycine	85.0
Histidine	194.0
Isoleucine	182.0
Leucine	180.0
Lysine	211.0
Methionine	204.0
Phenylalanine	218.0
Proline	143.0
Serine	122.0
Threonine	146.0
Tryptophan	259.0
Tyrosine	229.0
Valine	160.0

Table 3 Relative solvent accessibility calculated for each residue by using equation 2. The values highlighted in yellow are the relative solvent accessibility of ≥50% of the main chain were selected

Residue	Solvent accessible Surface area (Å2)	Relative Solvent Accessibility (Å2)	Relative Solvent Accessibility (%)
1	184.33	0.904	90
2	78.67	0.539	54
3	161.53	0.883	88
4	126.67	0.704	70
5	82.13	0.449	45
6	53.91	0.369	37
7	42.5	0.376	38
8	105.14	0.515	52
9	28.58	0.336	34
10	105.23	0.516	52
11	44.28	0.243	24
12	97	0.533	53
13	59.46	0.394	39
14	29.32	0.183	18
15	12.29	0.056	6
16	66.58	0.546	55
17	167.56	0.695	70
18	31.62	0.138	14
19	1.58	0.013	1
20	47.24	0.556	56
21	99.26	0.814	81
22	91.02	0.497	50
23	65.1	0.766	77
24	51.32	0.421	42
25	92.53	0.634	63
26	132.32	0.700	70
27	14.26	0.098	10
28	0	0.000	0
29	27.17	0.186	19
30	100.02	0.474	47
31	35.58	0.419	42
32	5.28	0.029	3
33	0.76	0.004	0
34	94.06	0.446	45
35	68.65	0.429	43
36	1.39	0.008	1
37	2.55	0.013	1
38	95.69	0.523	52

Residue	Solvent accessible Surface area (Å2)	Relative Solvent Accessibility (Å2)	Relative Solvent Accessibility (%)
39	145.18	0.688	69
40	68.77	0.376	38
41	50.33	0.280	28
42	94.18	0.659	66
43	78.68	0.926	93
44	96.61	0.443	44
45	86.83	0.482	48
52	170.48	1.129	113
53	44.22	0.391	39
54	22.76	0.142	14
55	44.49	0.295	29
56	141.57	0.671	67
57	13.12	0.073	7
58	10.59	0.059	6
59	138.99	0.659	66
60	119.32	0.790	79
61	26.74	0.149	15
62	24.68	0.163	16
63	91.94	0.814	81
64	95.92	0.607	61
65	65.29	0.768	77
66	56.77	0.376	38
67	65.09	0.576	58
68	46.58	0.246	25
69	0	0.000	0
70	41.01	0.272	27
71	63.96	0.293	29
72	77.16	0.632	63
73	14.66	0.080	8
74	5.82	0.027	3
75	59.78	0.328	33
76	103.2	0.645	65
77	13.95	0.064	6
78	30.7	0.192	19
79	56.78	0.502	50
80	55.28	0.489	49
81	40.91	0.225	22
82	81.6	0.559	56
83	58.61	0.480	48
84	57.98	0.513	51
85	67.2	0.480	48

Residue	Solvent accessible Surface area (Å2)	Relative Solvent Accessibility (Å2)	Relative Solvent Accessibility (%)
86	131.07	0.676	68
87	121.16	0.574	57
88	148.55	0.649	65
89	140.76	0.646	65
90	103.72	0.567	57
91	129.92	0.616	62
92	75.87	0.671	67
93	58.25	0.685	69
94	208.03	1.156	116

7 Appendix 2

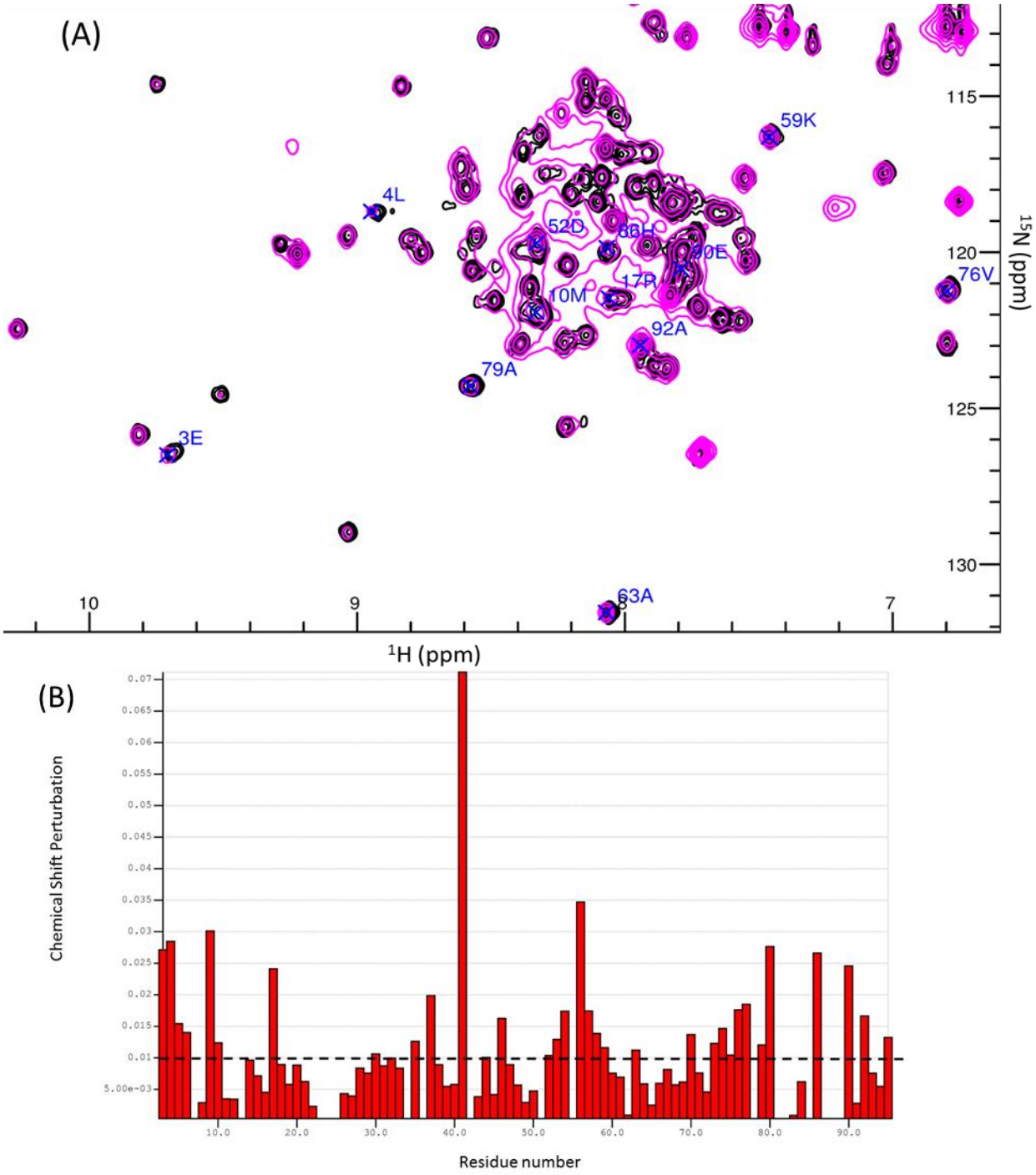

Figure 28(A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM of the IBS409 (magenta), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS409 adduct at concentration ratio 2:1, IBS409 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.02 ppm)

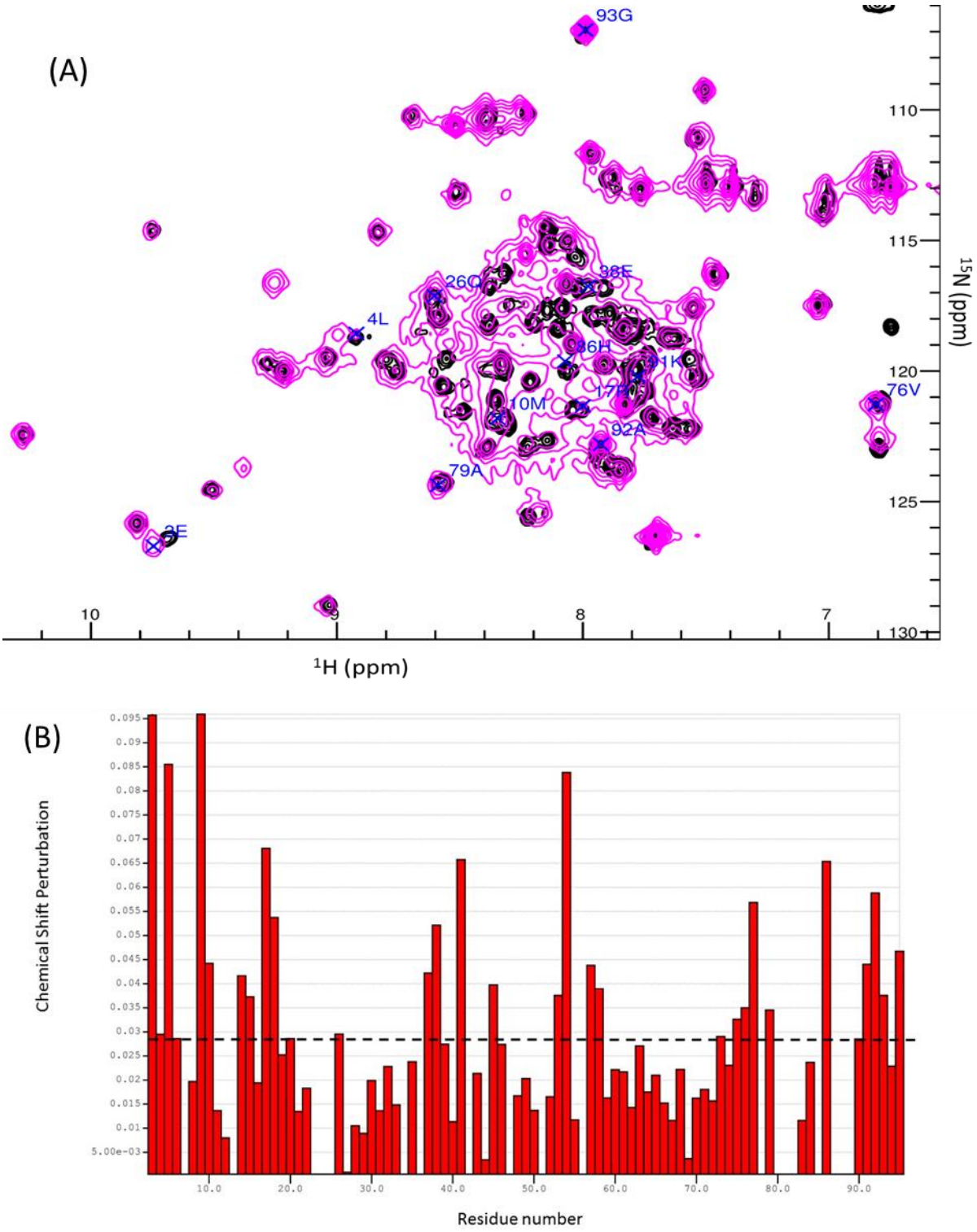

Figure 29: (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 2.02 mM of the IBS409 (magenta), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS409 adduct at concentration ratio 5:1, IBS409 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.029 ppm)

8 Appendix 3

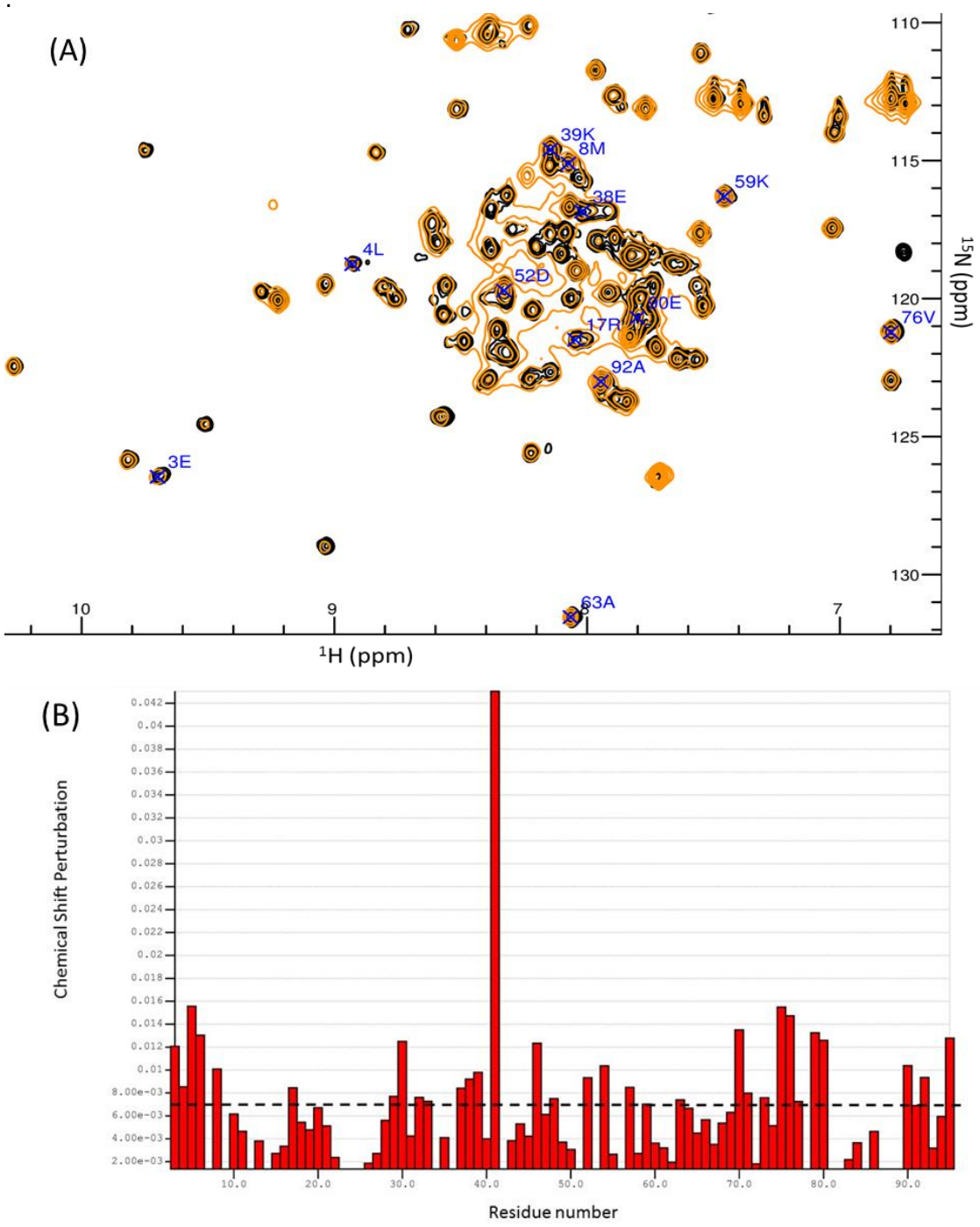

Figure 30: (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM of the IBS798 (orange), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS798 adduct at concentration ratio 2:1, IBS798 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥ 0.018 ppm).

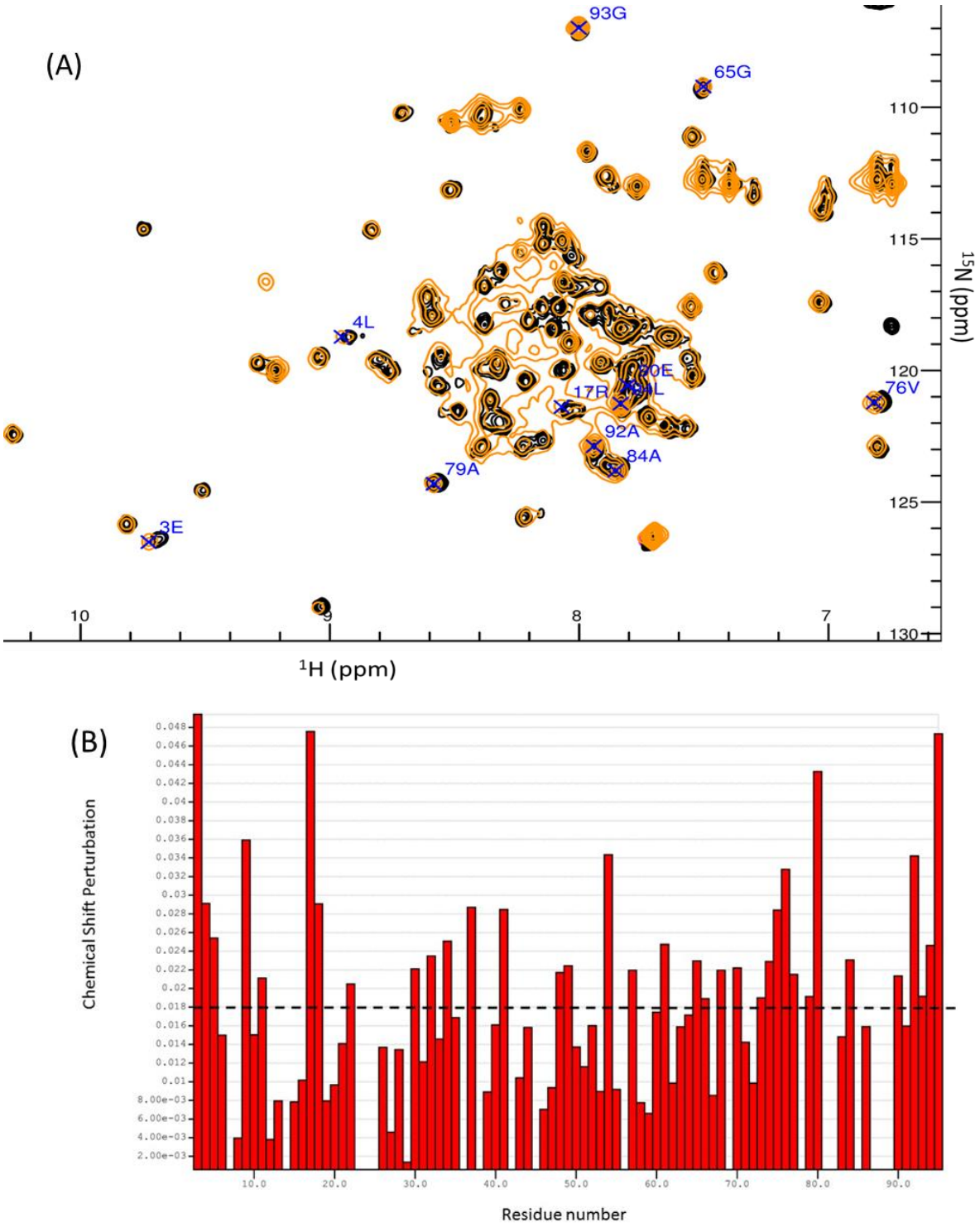

Figure 31: (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 2.02 mM of the IBS798 (orange), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS798 adduct at concentration ratio 5:1, IBS798 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm>0.018 ppm).

9 Appendix 4

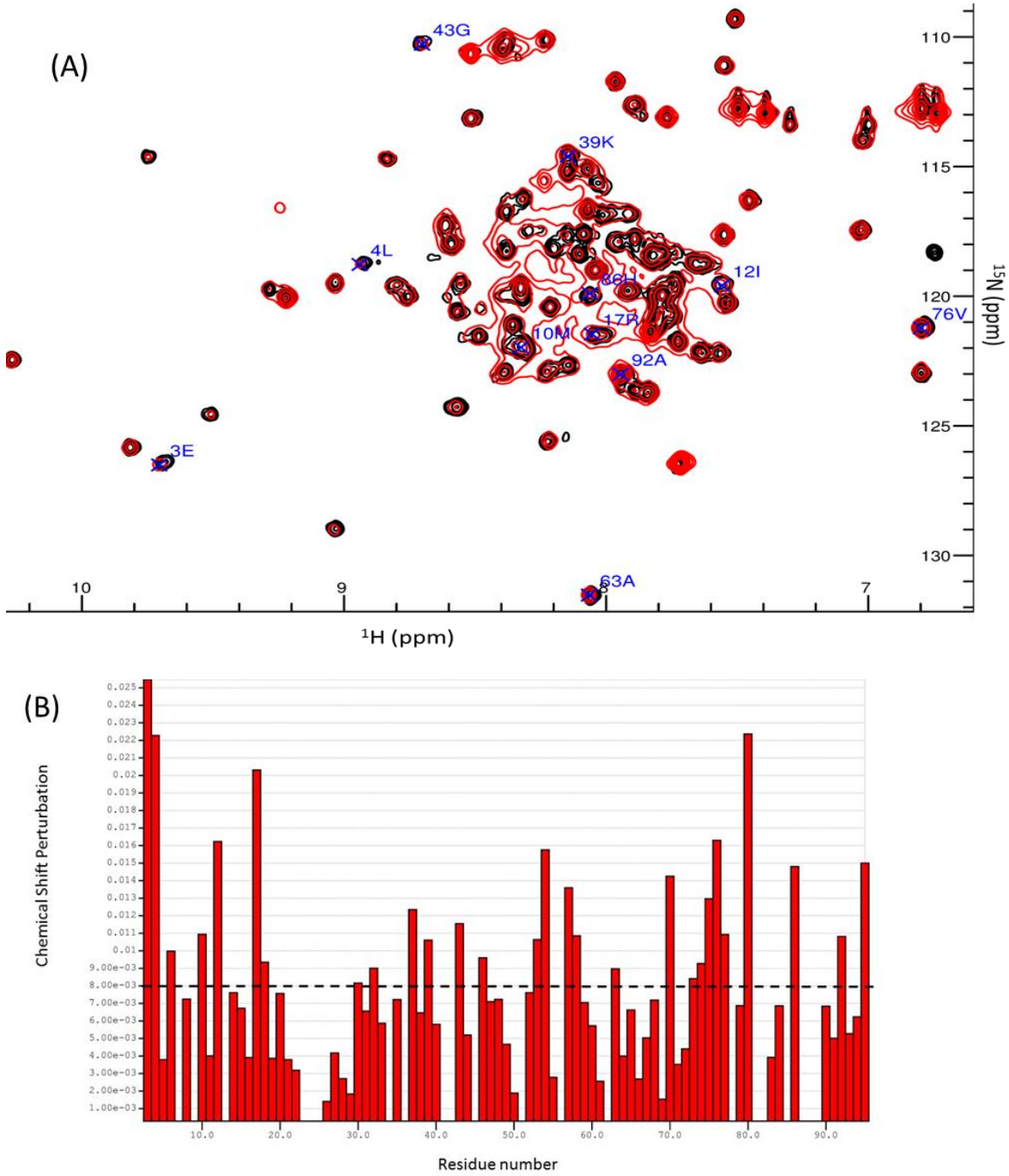

Figure 32: (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 0.84 mM of the IBS863 (red), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS863 adduct at concentration ratio 2:1, IBS863 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm ≥0.008 ppm).

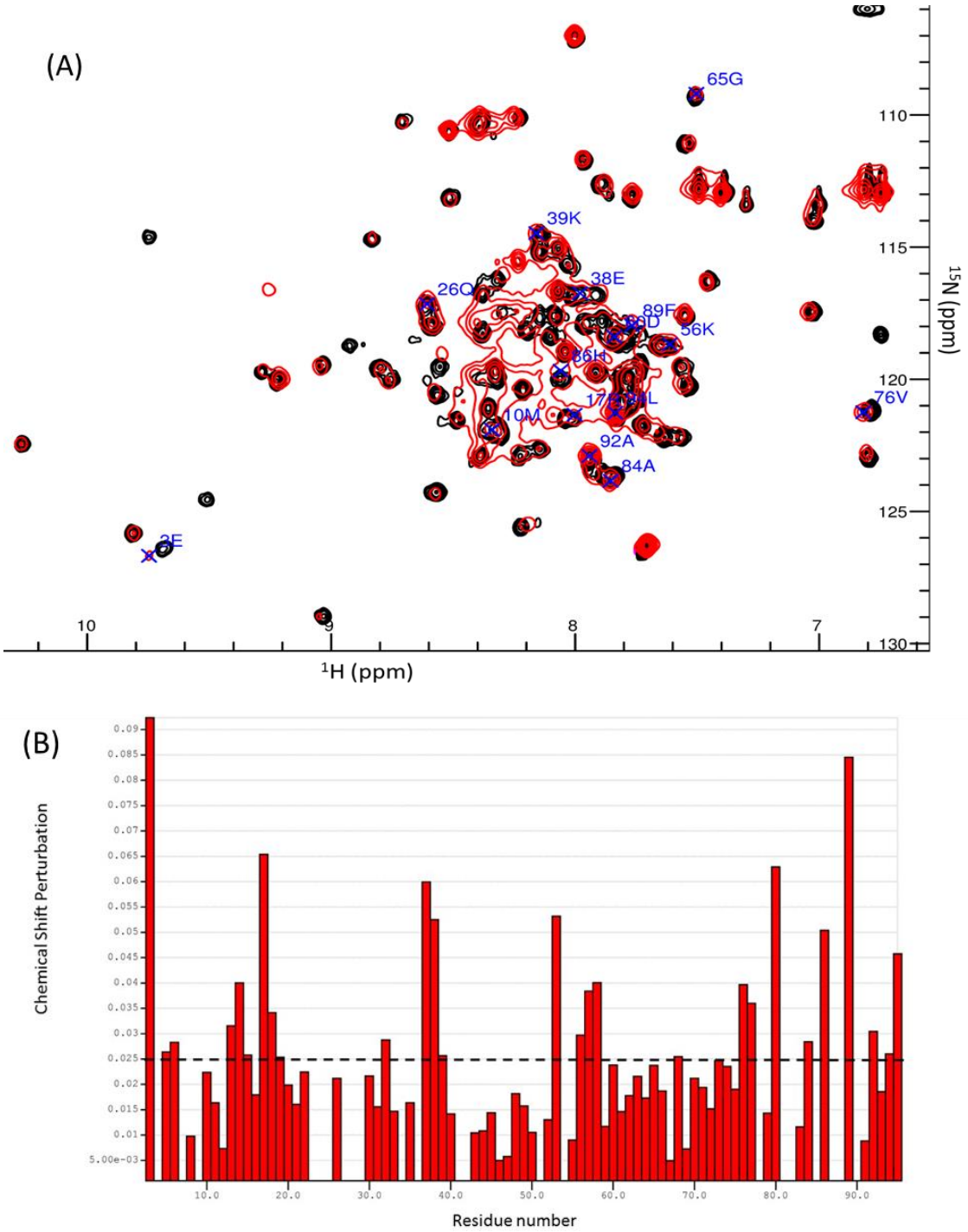

Figure 33: (A) Overlay of the ^{15}N-^{1}H HSQC spectra of 0.42 mM ^{15}N-labeled S100P (black) and in the complex form with 2.02 mM of the IBS863 (red), are shown. (B) Bar graph of the weighted average of the chemical shift (^{1}H and ^{15}N) variations of the amino acid residues in the S100P–IBS863 adduct at concentration ratio 5:1, IBS863 to S100P respectively. The black dashed line represents the average chemical shift perturbation (Δppm > 0.008 ppm)

10 Appendix 5

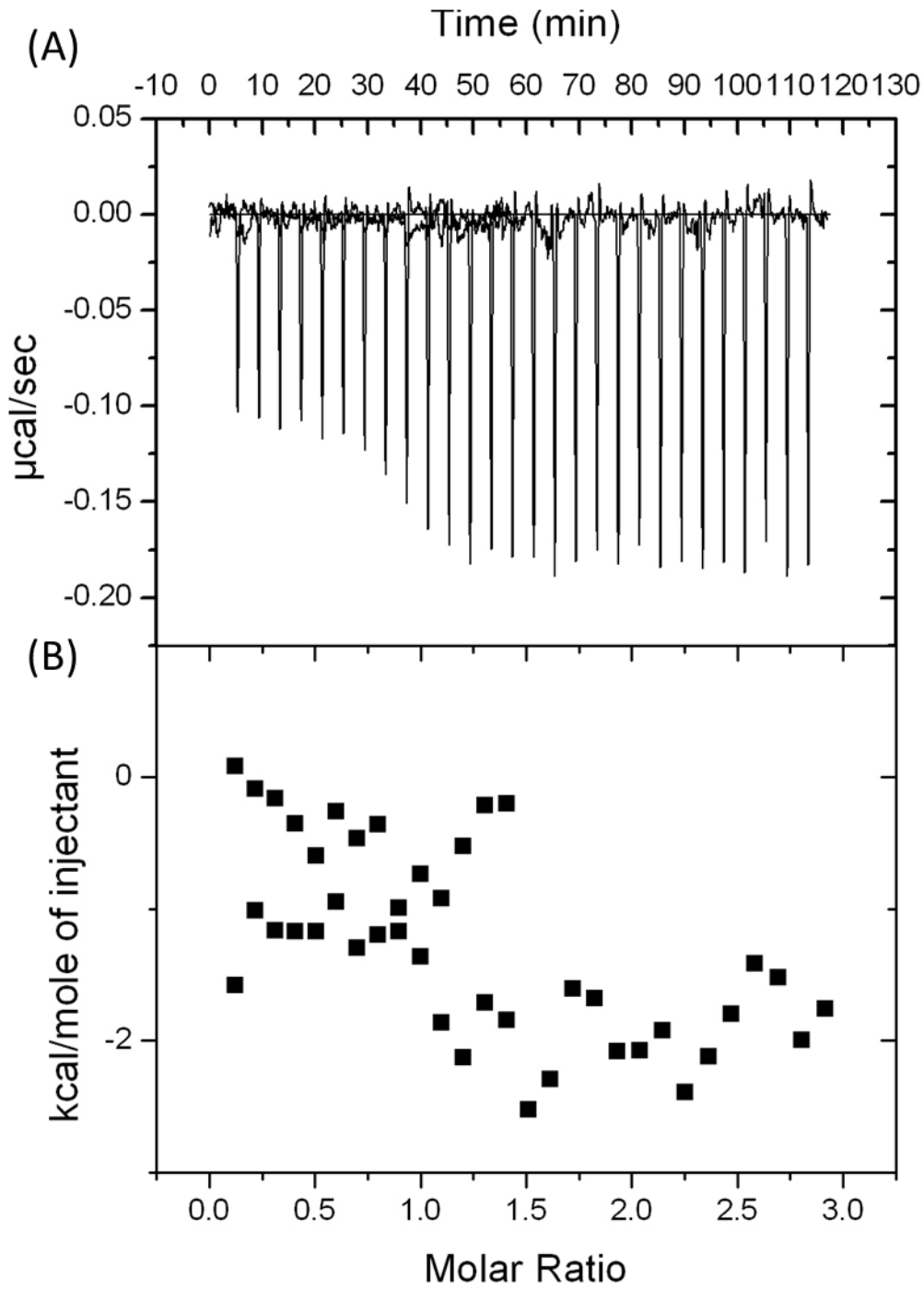

Figure 34 **The ITC titration of cromolyn to S100P**. (A) Raw data of the ITC titration with cromolyn. (B) The Wilmans plot of cromolyn titration, Very little or no binding was observed, and therefore binding parameters could not be determined.

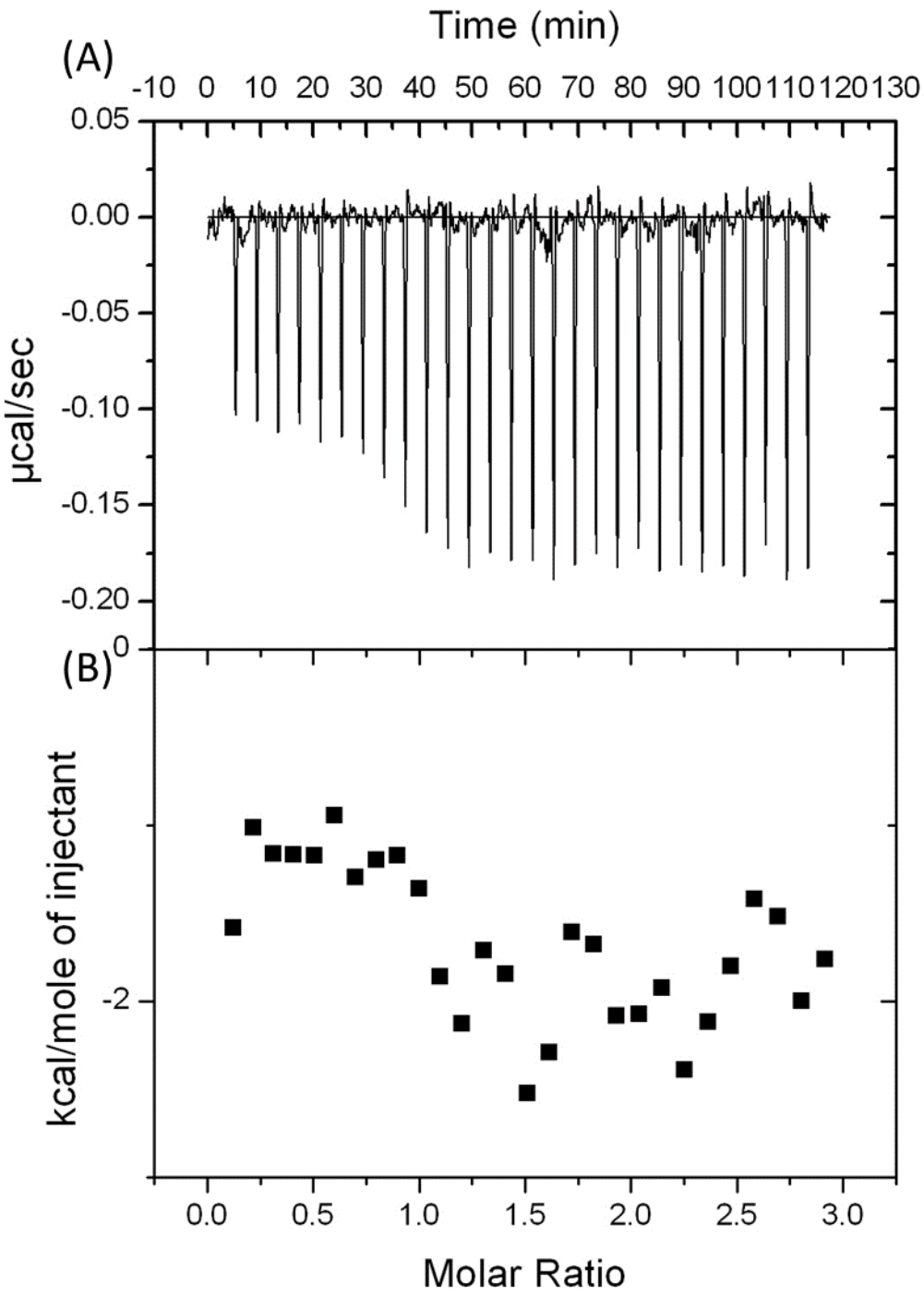

Figure 35 **The ITC titration of C72 to S100P** (A) Raw data of the ITC titration with C72. (B) The Wilmans plot of C72 titration, Very little or no binding was observed, and therefore binding parameters could not be determined.

11 Appendix 6

11.1 Control of Substances Hazardous to Health (COSHH) Assessment

Ref No:	
Date:	
Review Date:	

ACTIVITY INFORMATION	
Name of Assessor/Contact details	Name:Thamarasi Kekule Vithanage Email address: thamarasivithana@ymail.com Ext no:
Title of Activity	Protein Purification
Location of Activity	CP Snow Building
Description of Activity	Preparation of cell lysate by sonication. Protein purification by Ni-affinity chromatography.
Personnel Involved	Staff members, Students

HAZARD CLASSIFICATION

Hazards involved in project.

□ Harmful/Irritant 	□ Corrosive 	□ Radioactive 	□ Biological 
□ Toxic 	□ Health Hazard 	□ Explosive 	□ Oxidising 
□ Flammable	□ Hazardous to the Environment	□ Gas under pressure	

			

Severity Classification

Harmful/Irritant 2	Corrosive 3	Radioactive *	Biological **
Toxic 3	Health Hazard 4	Explosive 5	Oxidising 5
Flammable 3	Hazardous to the Environment 4	Gas under pressure 5	

* Radioactive – If any work will involve use of Radioactivity contact the Radiation Protection Supervisor, Janet Evans (ext 4379, j.s.evans@herts.ac.uk) for an accurate severity classification *before* work begins.

** Biological – If any work will involve using biological samples or suspected biohazards contact the Biological Safety Advisor.
For Department of Human and Environmental Sciences contact Di Francis (ext 4527, d.l.francis@herts.ac.uk).
For Department of Pharmacy contact James Stanley (ext 4599, j.k.stanley@herts.ac.uk).

Substance Used or Produced.	Concentration or amount used.	Hazard Words and Pictograms	Route of Entry	Severity
DTT (Dithiothreitol)	Stock 1M -> 1-10mm final	 Harmful/Irrtant	Ingestion Inhalation Skin Eye	2
Human S100P gene in PET28 plasmid		Not Hazardous	Ingestion Inhalation Skin Eye	1
Na_2HPO_4 (Disodium Hydrogen Phosphate)		Not Hazardous	Ingestion Inhalation Skin Eye	1
NaH_2PO_4 (Sodium Dihydrogen Phosphate / Orthophosphate)		Not Hazardous	Ingestion Inhalation Skin Eye	1
NaCl (Sodium Chloride)	Stock 5M-> 50- 500mm	Not hazadrous	Ingestion Inhalation Skin Eye	1

Tris	Stock: 1.5/2M -> 20nm	Not Hazardous	Ingestion Inhalation Skin Eye	1
HCl (Hydrochloric Acid)	<10ml	 Harmful/Irritant  Corrosive	Ingestion Inhalation Skin Eye	3
EDTA (Ethylenediaminetetraacetic acid)	Stock : 0.5M -> 5-50mM	 Harmful/Irritant	Eye	2
MES 2-(N-Morpholino)ethanesulfonic acid sodium salt		Not Hazardous	Ingestion Inhalation Skin Eye	1
HisTrap™ HP, 5 ml, 5 x 5 ml (Catalogue number : 17-5248-02)		 Flammable	Ingestion Inhalation Skin Eye	3
NaOH (Sodium Hydroxide)		 Corrosive	Skin Eye	3
Imidazole	Stock 2M -> 20- 50mm	 Harmful/Irrtant  Corrosive  Health hazard	Ingestion Inhalation Skin Eye	4
Triton X-100	1%	 Harmful/Irrtant  Corrosive	Ingestion Inhalation Skin Eye	3
$NiCl_2$ (Nickel Chloride)		 Health hazard   Harmful/Irrtant Hazardous to the Environment	Ingestion Inhalation Skin Eye	4
Information sources (eg MSDS)	MSDS (SIGMA – ALDRICH) and GE HealthCare			

CONTROL MEASURES
Additional measures to be used over and above local codes of practice and local rules.
Keep away from heat/sparks/open flames/hot surfaces. – No smoking. Avoid exposure – obtain special instructions before use. Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation at places where dust is formed.
Are alternative less hazardous substances available? If so please list below and state why they cannot be used.
NO

EMERGENCY AND DISPOSAL PROCEDURES
Measures to be taken in case of Spillage or Uncontrolled release
Solid spill: Avoid dust formation. Avoid breathing vapours, mist or gas. Sweep up and shovel. Keep in suitable, closed containers for disposal. Liquid Spill: Soak up with inert absorbent material and dispose of as hazardous waste. Keep in suitable closed containers for disposal. Contain spillage, and then collect an electrically protected vacuum cleaner or wet –brushing and place in container for disposal according to local regulations
Disposal Measures
Burn in a chemical incinerator equipped with an afterburner and scrubber but exert extra care in igniting, as this material is highly flammable. Offer surplus and non-recyclable solutions to a licensed disposal company $NiCl_2$; Offer surplus and non-recyclable solutions to a licensed disposal company. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.
Measures to be taken in case of Fire
Types of fire extinguisher to be used: Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide Do not use water jet. Emergency procedure in case of fire: Use personal protective equipment. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Remove all sources of ignition. Evacuate personal in areas. Beware of vapours accumulating to form explosive concentration. Vapours can accumulate in low areas. Toxic fumes emitted under fire conditions: Carbon oxides, Iron oxides, Oxides of phosphorus, Sodium oxides, Nitrogen oxides, Hydrogen cyanide
Measures to be taken for First Aid

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.
In contact with eyes:
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
In contact with skin:
Wash off soap and plenty of water. Consult a physician.
If inhaled:
If breathed in, move person into fresh air. If not breathing give artificial respiration. Consult a physician.
If ingested:
Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician. Immediately call a POISON CENTER or doctor/ physician

Signs and symptoms of exposure:
HCL; Burning sensation, cough, wheezing, laryngitis, short of breath, spasm, inflammation and oedema of larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema, material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes and skin.
$NiCl_2$; Substances that can cause occupational asthma (also known as asthmagens and respiratory sensitisers) can induce a state of specific airway hyper-responsiveness via an immunological, irritant
or other mechanisms. These symptoms can range in severity from a runny nose to asthma.

Is hospitalisation required? Yes

Persons at Special Risk: Pregnancy, Asthma patients

Ref No:	
Date:	
Review Date:	

ACTIVITY INFORMATION	
Name of Assessor/Contact details	Name:Thamarasi Kekule Vithanage Email address:thamarasivithana@ymail.com Ext no:
Title of Activity	Bacterial transformation and protein expression
Location of Activity	C.P Snow Building
Description of Activity	Transformation of competent E.coli with plasmid constructs for S100P protein formation. Induction of protein expression using IPTG
Personnel Involved	Staff members, students

HAZARD CLASSIFICATION

Hazards involved in project.

□ Harmful/Irritant 	□ Corrosive 	□ Radioactive 	□ Biological 
□ Toxic 	□ Health Hazard 	□ Explosive 	□ Oxidising 
□ Flammable 	□ Hazardous to the Environment 	□ Gas under pressure 	

Severity Classification

Harmful/Irritant 2	Corrosive 3	Radioactive *	Biological **
Toxic 3	Health Hazard 4	Explosive 5	Oxidising 5
Flammable 3	Hazardous to the Environment 4	Gas under pressure 5	

* Radioactive – If any work will involve use of Radioactivity contact the Radiation Protection Supervisor, Janet Evans (ext 4379, j.s.evans@herts.ac.uk) for an accurate severity classification *before* work begins.

** Biological – If any work will involve using biological samples or suspected biohazards contact the Biological Safety Advisor.
For Department of Human and Environmental Sciences contact Di Francis (ext 4527, d.l.francis@herts.ac.uk).
For Department of Pharmacy contact James Stanley (ext 4599, j.k.stanley@herts.ac.uk).

Substance Used or Produced.	Concentration or amount used.	Hazard Words and Pictograms	Route of Entry	Severity
Escherichia coli		 Biological	Ingestion Inhalation Skin Eye	2
Human S100P gene in PET28 plasmid		Not Hazardous	Ingestion Inhalation Skin Eye	1
LB (Luria Bertani Broth)		Not Hazardous	Ingestion Inhalation Skin Eye	1
Tryptone		Not Hazardous	Ingestion Inhalation Skin Eye	1
Yeast Extract		Not Hazardous	Ingestion Inhalation Skin Eye	1
NaCl		Not Hazardous	Ingestion Inhalation Skin Eye	1
Agar		Not Hazardous	Ingestion Inhalation Skin Eye	1
Kanamycin	Stock: 34mg/ml -> 34 µg/ml final concentration	Not Hazardous	Ingestion Inhalation Skin Eye	1

Ampicillin	Stock: 150mg/ml -> 100 µg/ml final concentration	 Harmful/Irrtant hazard  Health	Ingestion Inhalation Skin Eye	4
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Stock: 1M -> <2mM final concentration	 Flammable  Toxic  Health hazard	Ingestion Inhalation Skin Eye	4
Information sources (eg MSDS)	MSDS (SIGMA – ALDRICH)			

CONTROL MEASURES
Additional measures to be used over and above local codes of practice and local rules.
Provide appropriate exhaust ventilation at place where dust is formed. Keep away from heat/sparks/open flames/ hot surfaces. – No smoking. Do not breathe dust/ fume/ gas/ mist/ vapours/ spray.
Are alternative less hazardous substances available? If so please list below and state why they cannot be used.
NO

EMERGENCY AND DISPOSAL PROCEDURES
Measures to be taken in case of Spillage or Uncontrolled release
Solid spill: Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal Liquid Spill: Soak up with inert absorbent material and dispose of a hazardous waste. Keep in suitable, closed containers for disposal
Disposal Measures
Offer surplus and non-recyclable solutions to a licenced disposal company. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.
Measures to be taken in case of Fire
Types of fire extinguisher to be used: Use water spray, alcohol- resistant foam, dry chemical or Carbon dioxide Emergency procedure in case of fire: Use personal protective equipment. Avoid dust formation. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Evacuate personal to safe areas. Toxic fumes emitted under fire conditions: Carbon oxides, Nitrogen oxides, Hydrogen chloride gas, Sulphur oxides, Sodium oxides

Measures to be taken for First Aid
General advice Consult a physician. Show this safety data sheet to the doctor in attendance. In contact with eyes: Rinse thoroughly with plenty of water at least 15minutes and consult a physician. In contact with skin: Take off contaminated clothes and shoes immediately. Wash off with soap and plenty of water. If inhaled: If breathed in, move person to fresh air. If not breathing, give artificial respiration. Consult a physician. If ingested: Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician. If experiencing respiratory symptoms: Call a POISON CENTER or doctor/ physician. Signs and symptoms of exposure: IPTG; Nausea, dizziness, gastrointestinal disturbance, weakness, confusion, drowsiness, unconsciousness. Is hospitalisation required? Yes Persons at Special Risk: No data available.

Ref No:	
Date:	
Review Date:	

ACTIVITY INFORMATION	
Name of Assessor/Contact details	Name:Thamarasi Kekule Vithanage Email address:thamarasivithana@ymail.com Ext no:
Title of Activity	SDS- PAGE
Location of Activity	C.P Snow Building
Description of Activity	SDS-PAGE analysis of recombinant proteins
Personnel Involved	Staff members, students

HAZARD CLASSIFICATION

Hazards involved in project.

□ Harmful/Irritant 	□ Corrosive 	□ Radioactive 	□ Biological 
□ Toxic 	□ Health Hazard 	□ Explosive 	□ Oxidising 
□ Flammable 	□ Hazardous to the Environment 	□ Gas under pressure 	

Severity Classification

Harmful/Irritant 2	Corrosive 3	Radioactive *	Biological **
Toxic 3	Health Hazard 4	Explosive 5	Oxidising 5
Flammable 3	Hazardous to the Environment 4	Gas under pressure 5	

* Radioactive – If any work will involve use of Radioactivity contact the Radiation Protection Supervisor, Janet Evans (ext 4379, j.s.evans@herts.ac.uk) for an accurate severity classification *before* work begins.

** Biological – If any work will involve using biological samples or suspected biohazards contact the Biological Safety Advisor.
For Department of Human and Environmental Sciences contact Di Francis (ext 4527, d.l.francis@herts.ac.uk).
For Department of Pharmacy contact James Stanley (ext 4599, j.k.stanley@herts.ac.uk).

Substance Used or Produced.	Concentration or amount used.	Hazard Words and Pictograms	Route of Entry	Severity
Tris		Not Hazardous	Ingestion Inhalation Skin Eye	1
HCl (Hydrochloric acid)		 Harmful/Irritant  Corrosive	Ingestion Inhalation Skin Eye	3
Acrylamide		 Toxic  Health Hazard	Ingestion Inhalation Skin Eye	4
TEMED (1,2-Bis(dimethylamino)ethan)		 Flammable  Harmful/Irritant  Corrosive	Ingestion Inhalation Skin Eye	3
APS (Ammonium persulfate)		 Flammable  Harmful/Irritant  Health Hazard	Ingestion Inhalation Skin Eye	4
SDS (Sodium Dodecyl Sulphate)		 Flammable  Harmful/Irritant	Ingestion Inhalation Skin Eye	3
Glycine		Not Hazardous	Ingestion Inhalation	1

			Skin Eye	
Glycerol		Not Hazardous	Ingestion Inhalation Skin Eye	1
Bromophenol blue		Not Hazardous	Ingestion Inhalation Skin Eye	1
DTT (Dithiothreitol)		 Harmful/Irritant	Ingestion Inhalation Skin Eye	2
Coomassie brilliant blue		Not Hazardous	Ingestion Inhalation Skin Eye	1
Methanol		 Flammable  Toxic  Health Hazard	Ingestion Inhalation Skin Eye	4
Glacial acetic acid		 Flammable  Corrosive	Ingestion Inhalation Skin Eye	3
Information sources (eg MSDS)	MSDS (SIGMA – ALDRICH)			

CONTROL MEASURES
Additional measures to be used over and above local codes of practice and local rules.
Keep away from heat/sparks/open flames/ hot surfaces. – No smoking. Keep/Store away from clothing combustible materials Provide appropriate exhaust ventilation at place where dust is formed. Avoid breathe dust/ fume/ gas/ mist/ vapours/ spray.
Are alternative less hazardous substances available? If so please list below and state why they cannot be used.
NO

EMERGENCY AND DISPOSAL PROCEDURES
Measures to be taken in case of Spillage or Uncontrolled release
Avoid dust formation. Avoid breathing vapours, mist or gas Sweep up and shovel. Keep in suitable, closed containers for disposal Prevent further leakage or spillage if safe to do so. Do not let product enter drains Contain spillage, and then collect with an electrically protected vacuum cleaner or by wet-brushing and place in container for disposal according to local regulations
Disposal Measures

Burn in a chemical incinerator equipped with an after burner and scrubber but exert extra care in igniting as this material is highly flammable. Offer surplus and non-recyclable solutions to a licensed disposal company.

Measures to be taken in case of Fire

Types of fire extinguisher to be used:
Use water spray, alcohol- resistant foam, dry chemical or Carbon dioxide

Emergency procedure in case of fire:
Wear self-contained breathing apparatus for firefighting if necessary
Use personal protective equipment. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Remove all sources of ignition. Evacuate personal to safe areas. Beware of vapours accumulating to form explosive concentrations. Vapours can accumulate in low areas

Toxic fumes emitted under fire conditions:
Carbon oxides, Nitrogen oxides, Hydrogen bromide gas, Sulphur oxides

Measures to be taken for First Aid

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.
In contact with eyes:
Rinse thoroughly with plenty of water at least 15minutes and consult a physician.
In contact with skin:
Take off contaminated clothes and shoes immediately. Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
If inhaled:
If breathed in, move person to fresh air. If not breathing, give artificial respiration. Consult a physician.
If ingested:
Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

Signs and symptoms of exposure:
TEMED; Material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes and skin, spasm, inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema
SDS; Sneezing, the sodium salt of dodecyl sulphate has been reported to cause pulmonary sensitization resulting in hyperactive airway dysfunction and pulmonary allergy accompanied by fatigue, malaise, and aching.
DTT; Nausea, Headache, Vomiting, Central nervous system depression.
Methanol; Headache, Dizziness, Drowsiness, Metabolic acidosis, Coma, Seizures.
Glacial acetic acid; Spasm, inflammation and oedema, burning sensation, cough, wheezing, laryngitis, shortness of breath, headache, nausea, vomiting , ingestion or inhalation of concentrated acetic acid causes damage to tissues of respiratory and digestive tracts
Symptoms include: haematuria, anuria, uraemia, albuminuria, haemolysis, convulsions, bronchitis, pulmonary oedema, pneumonia, cardiovascular collapse, shock, and death. Direct contact or exposure to high concentration of vapour with skin or eyes can cause: erythema, blisters, tissue destruction with slow healing, skin blackening, hyperkeratosis, fissures, corneal erosion, opacification, iritis, conjunctivitis, and possible blindness
Is hospitalisation required? Yes

Persons at Special Risk: Pregnancy, Asthma, Allergy.

Ref No:	
Date:	
Review Date:	

ACTIVITY INFORMATION	
Name of Assessor/Contact details	Name:Thamarasi Kekule Vithanage Email address:thamarasivithana@ymail.com Ext no:
Title of Activity	Bradford assay or BCA assay
Location of Activity	C.P Snow Building
Description of Activity	Protein quantification
Personnel Involved	Staff members, students

HAZARD CLASSIFICATION

Hazards involved in project.

□ Harmful/Irritant 	□ Corrosive 	□ Radioactive 	□ Biological 
□ Toxic 	□ Health Hazard 	□ Explosive 	□ Oxidising 
□ Flammable 	□ Hazardous to the Environment 	□ Gas under pressure 	

Severity Classification

Harmful/Irritant	2	Corrosive	3	Radioactive	*	Biological	**
Toxic	3	Health Hazard	4	Explosive	5	Oxidising	5
Flammable	3	Hazardous to the Environment	4	Gas under pressure	5		

* Radioactive – If any work will involve use of Radioactivity contact the Radiation Protection Supervisor, Janet Evans (ext 4379, j.s.evans@herts.ac.uk) for an accurate severity classification *before* work begins.

** Biological – If any work will involve using biological samples or suspected biohazards contact the Biological Safety Advisor.
For Department of Human and Environmental Sciences contact Di Francis (ext 4527, d.l.francis@herts.ac.uk).
For Department of Pharmacy contact James Stanley (ext 4599, j.k.stanley@herts.ac.uk).

Substance Used or Produced.	Concentration or amount used.	Hazard Words and Pictograms	Route of Entry	Severity
Bradford reagent		 Corrosive  Flammable  Health Hazard  Harmful/Irritant	Ingestion Inhalation Skin Eye	4
BCA Reagent A Sodium carbonate [2,2'Biquinoline]-4,4'dicarboxylic acid, sodium salt (2:1)	~	 May cause irritation to respiratory system	Ingestion Inhalation Skin Eye	2
BCA : Reagent B Copper (II) sulphate, Pentahydrate (1:1:5)		 May cause irritation to respiratory system	Ingestion Inhalation Skin Eye	2
BCA Reconstitution buffer		Not Hazardous	Ingestion Inhalation Skin Eye	1

BCA Assay Compatibility Reagent		 Health Hazard  Toxic	Ingestion Inhalation Skin Eye	4
BSA		Not Hazardous	Ingestion Inhalation Skin Eye	1
Information sources (eg MSDS)	MSDS (SIGMA – ALDRICH)			

CONTROL MEASURES
Additional measures to be used over and above local codes of practice and local rules.
Do not breathe dust/ fume/ gas/ mist/ vapours/ spray. Remove all sources of ignition. Evacuate personnel to safe areas. Beware of vapours accumulating to form explosive concentrations. Vapours can accumulate in low areas.
Are alternative less hazardous substances available? If so please list below and state why they cannot be used.
NO

EMERGENCY AND DISPOSAL PROCEDURES
Measures to be taken in case of Spillage or Uncontrolled release
Soak up with inert absorbent material and dispose of a hazardous waste. Keep in suitable, closed containers for disposal
Disposal Measures
Burn in a chemical incinerator equipped with an afterburner and scrubber but exert extra care in igniting, as this material is highly flammable. Other surplus and non-recyclable solutions to be licenced disposal company.
Measures to be taken in case of Fire
Types of fire extinguisher to be used: Use water spray, alcohol- resistant foam, dry chemical or Carbon dioxide Emergency procedure in case of fire: Wear respiratory protection. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Remove all sources of ignition. Evacuate personnel to safe areas. Beware of vapours accumulating to form explosive concentrations. Vapours accumulate in low areas. Toxic fumes emitted under fire conditions: Toxic fumes of phosphorus oxides and/or phosphine, Carbon oxides, Oxides of phosphorus
Measures to be taken for First Aid

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.
In contact with eyes:
Rinse thoroughly with plenty of water at least 15minutes and consult a physician.
In contact with skin:
Take off contaminated clothes and shoes immediately. Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
If inhaled:
If breathed in, move person to fresh air. If not breathing, give artificial respiration. Consult a physician.
If ingested:
Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

Signs and symptoms of exposure:
Methanol in Bradford Reagent; Headache, Dizziness, Drowsiness, Metabolic acidosis, Coma, Seizures.
Acetic acid; Spasm, inflammation and oedema, burning sensation, cough, wheezing, laryngitis, shortness of breath, headache, nausea, vomiting , ingestion or inhalation of concentrated acetic acid causes damage to tissues of respiratory and digestive tracts
Symptoms include: hematemesis, bloody diarrhoea, oedema and or perforation of the oesophagus and pylorus, pancreatitis, haematuria, anuria, uraemia, albuminuria, haemolysis, convulsions, bronchitis, pulmonary oedema, pneumonia, cardiovascular collapse, shock, and death. Direct contact or exposure to high concentration of vapour with skin or eyes can cause: erythema, blisters, tissue destruction with slow healing, skin blackening, hyperkeratosis, fissures, corneal erosion, opacification, iritis, conjunctivitis, and possible blindness
H_3PO_4 in Bradford Reagent; Material is extremely destructive to tissue of the mucous membranes and the upper respiratory tract, eyes, and skin, spasm inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema, burning sensation, cough, wheezing, laryngitis, shortness of breath, headache, nausea
Is hospitalisation required? Yes

Persons at Special Risk: No data available.

	SIGNATURES			
	Staff/PhD student/MSc student/Undergraduate	Name (Print)	Signature	Date
Assessor				
Supervisor (if Assessor is a student)				
Local health and safety advisor / Laboratory Manager				

11.2 Risk Assessment

Ref No:	
Date:	
Review Date:	

ACTIVITY INFORMATION	
Name of Assessor/ Contact details	Name: Thamarasi Kekule Vithanage Email address: thamarasivithana@ymail.com Ext no:
Title of Activity	To follow a drug discovery cascade to identify the lead compound for S100P binding.
Location of Activity	CP Snow Building
Description of Activity	The competent *E.coli* cells will be transformed with plasmid constructs for the S100P protein formation. Then they grow in LB medium and induce protein expression using IPTG, protein purification using Ni affinity chromatography. Identification and characterisation of the proteins will be achieved using SDS-PAGE.
Personnel Involved	Staff Members, students

TYPES OF HAZARD LIKELY TO BE ENCOUNTERED		
☐ Animal Allergens ☐ .Biological Agents (see COSHH) ☐ .Chemical Compounds (see CoSHH) ☐ .Compressed/liquefied gases ☐ Computers ☐ .Electricity ☐ Falling Objects ☐ Farm Machinery ☐ .Fire ☐ .Glassware Handling	☐ .Hand Tools ☐ Ionising Radiation ☐ Office Equipment ☐ .Laboratory Equipment ☐ Ladders ☐ Manual Handling ☐ Non-ionising Radiation ☐ .Hot or cold extremes ☐ Repetitive Handling ☐ Severe Weather	☐ Sharps ☐ .Slips/trips/falls ☐ Stress ☐ Travel ☐ Vacuum systems ☐ Pressure systems ☐ Vehicles ☐ Violence, physical or verbal abuse ☐ Workshop Machinery
The above is not an exhaustive list – all other hazards should be listed here.		

HAZARD ASSESSMENT						
Severity of Consequences	Factor	Risk Classification				
No or minor injury/ health disorder Minor Damage or Loss Insignificant Environmental Impact Group 1 Biological agents	1	Trivial (1)	Trivial (2)	Trivial (3)	Trivial (4)	Tolerable (5)
Injury or Health Disorder – resulting in absence up to 3 days Moderate Damage or Loss Moderate Environmental Impact Group 2 Biological agents	2	Trivial (2)	Trivial (4)	Tolerable (6)	Tolerable (8)	Moderate (10)
Injury or Health Disorder – resulting in absence over 3 days Substantial Damage or Loss Serious Environmental Impact Group 3 Biological agents	3	Trivial (3)	Tolerable (6)	Moderate (9)	Moderate (12)	Substantial (15)
Long Term Injury or Sickness – resulting in permanent incapacity Extensive Damage or Loss Major Long Term Environmental Impact	4	Trivial (4)	Tolerable (8)	Moderate (12)	Substantial (16)	Intolerable (20)
Death Serious Structural Damage Environmental Catastrophe Group 4 Biological agents	5	Tolerable (5)	Moderate (10)	Substantial (15)	Intolerable (20)	Intolerable (25)
	Likelihood	1	2	3	4	5

Note on Risk Classification: 1-4 Trivial 5-7 Tolerable 8-12 Moderate 13-16 Substantial >20 Intolerable		Almost Impossible	Unlikely – possible exposure every 1-3 years	Harm is possible	Harm is likely to occur	Harm will occur or is very likely to occur.

ASSESSMENT OF RISK CLASSIFICATION			
Hazard	Likelihood Score	Severity Score	Risk Classification
Chemical compunds	2	4	8
Biological agents	2	2	4
Laboratory equipment esp. Bunsen burners, glassware	2	3	6
Electric Hazard (electrophoresis)	2	1	2
Burn hazard	2	1	2
Escherichia coli	2	2	4
Ampicillin	1	4	4
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	2	4	8
DTT (Dithiothreitol)	2	2	4
HCl (Hydrochloric Acid)	2	3	6
EDTA (Ethylenediaminetetraacetic acid)	2	2	4
HisTrap™ HP, 5 ml, 5 x 5 ml (Catalogue number : 17-5248-02)	1	3	3
NaOH (Sodium Hydroxide)	2	3	6
Imidazole	2	4	8
Triton X-100	2	4	8
$NiCl_2$ (Nickel Chloride)	2	4	8
Bradford reagent	1	4	4
BCA Reagent A	2	2	4
BCA Reagent B	2	2	4
BCA Compatibility reagent	4	2	8
Acrylamide	1	4	4

TEMED (1,2-Bis(dimethylamino)ethan)	1	4	4
APS (Ammonium persulfate)	1	4	4
SDS (Sodium Dodecyl Sulphate)	2	3	6
Methanol	1	4	4
Glacial acetic acid	1	3	3
Calcium chloride $CaCl_2$	2	2	4
15N Labelled ammonium chloride	2	2	4

EFFECT OF RISK CLASSIFICATION	
Risk Classification	Action
Trivial	No further action required. Activity can begin.
Tolerable	No additional controls required. Current controls must be maintained and monitored.
Moderate	Reduce risks if cost effective. Implement new controls over an agreed period.
Substantial	Activity cannot begin without major risk reduction.
Intolerable	Activity must not begin.

RISK CONTROL MEASURES

Is the local code of practice or local rules adequate to control the risks identified? Yes/No
If no, list all additional measures required.

Additional Measures:
General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.
In contact with eyes:
Rinse thoroughly with plenty of water at least 15minutes and consult a physician.
In contact with skin:
Take off contaminated clothes and shoes immediately. Wash off with soap and plenty of water.
If inhaled:
If breathed in, move person to fresh air. If not breathing, give artificial respiration. Consult a physician.
If ingested:
Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician. If experiencing respiratory symptoms: Call a POISON CENTER or doctor/
physician.

Check for damage to leads and cables. Do not operate with wet hands.

HEALTH SURVEILLANCE ISSUES	
Persons at Special Risk	Pregnant women
Health Surveillance Measures (including symptoms and signs of exposure)	Breathing issues, skin rashes, eye irritation, burns to skin.
Exclusions	Potentially pregnant women

	SIGNATURES			
	Staff/PhD student/MSc student/Undergraduate	Name (Print)	Signature	Date
Assessor				
Supervisor (if Assessor is a student)				
Local Health and Safety Advisor / Laboratory Manager				