

MOAC PhD-project proposal

Project proposal: Using Proteomic Methods to Investigate the Toxicity-related, Non-specific Binding of Light Activated Cisplatin Analogs with the Cancer-related Proteins cMyc and p53.

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1. Track Records:

Prof. Peter B. O'Connor has recently moved to the University of Warwick from Boston University School of Medicine and is currently supervising four PhD students: Xiaojuan Li, Konstantin Aizikov (staying in Boston), Nadezhda Sargaeva (visiting PhD student), and Tzu-yung Lin (transferring). His former PhD students have completed PhD theses in Chemistry (Dr. Jason Cournoyer, Dr. Vera Ivleva) and Electrical Engineering (Dr. Parminder Kaur, Dr. Raman Mathur). These PhD theses have resulted in ~35 peer reviewed publications, with 19 of them as first author publications by one of these students. Prof. O'Connor has ~70 publications total.

Prof. Peter J. Sadler, FRS, is the head of the department of chemistry and has been working at the interface between inorganic chemistry, biology, and medicine for 30+ years. He has won numerous awards and medals for his work on cisplatin and other drug molecules of potential use in cancer therapy. He has published over 400 papers in refereed international journals and filed 20 patent applications, and his laboratory is well funded by the Wellcome trust, RCUK, MRC, EC, and EPSRC.

Dr. Michael Kahn the head of Molecular Medicine and director of the Lipid and Cardiovascular Risk Clinic at the University Hospitals of Coventry and Warwickshire. He has recently (2008) graduated one MOAC PhD, Sam Robson, and is currently supervising another, Liam Jones, who is in his 2nd year. His laboratory is well funded from Leverhulm trust, Wellcome trust, BBSRC, EU, and EPSRC funding as well as from numerous small trusts and industrial funding. He's well published in the biomedical literature.

2. Research Proposal

A. Aims

- To develop a biotin-linked-photoactivatable cisplatin analog which, when bound to proteins, can be pulled out of solution by an avidin column.
- To test how this molecule binds to cMyc and p53.
- To test avidin/biotin cleanup procedures to determine best methods for separation of bound from unbound proteins and peptides.
- To test these cleanup procedures in cell culture mixtures reacted with the cisplatin analog.
- To determine methods to identify the linking positions between the cisplatin analog and proteins of interest using high resolution mass spectrometry.

B. Background

CMyc and p53.

The two proteins cMyc and p53 are implicated in many cancers. cMyc is a 11 kDa DNA binding protein that appears to signal cell growth and is upregulated in many types of cancer cells. While it is not known whether cMyc is a "cause" of cancer, it's clearly important. Disrupting cMyc's growth function, or activating it's apoptotic signalling function are methods that are being considered for cancer treatments.^{1,2}

p53 has an opposite role in cells. p53 is a 44 kDa protein involved in DNA repair and has an apoptotic signalling function when DNA repair isn't possible. cMyc's proapoptotic signal is believed to function by activating p53. Additionally, the cervical cancer-causing human papillomavirus functions by creating a protein that interferes with p53.

Both cMyc and p53 are known to be highly post-translationally modified. Particularly interesting, p53 has ~40 known phosphorylation sites, 6 known ubiquitination sites, 7 acetylation sites, 2 SUMOylation sites, several O-glcNAcylation sites, and many more. Very little is known about the occupancy or mutual interaction of these many PTMs.

Photoactivatable Cisplatin Analogs.

Cisplatin, $\text{PtCl}_2(\text{NH}_3)_2$, is a chemotherapy drug used to treat some kinds of cancers and works by binding and crosslinking DNA in a manner that activates apoptosis. A primary problem with cisplatin, and the other drugs of its class, is toxicity caused by non-specific reactions of cisplatin with many molecules, including proteins such as CMyc and p53. Two such cases in the literature show reactions of cisplatin with the proteins superoxide dismutase and albumin.^{3,4} These nonspecific reactions are the cause of the side-effects that make cisplatin a very dangerous drug which is only worth using in cancer patients who would otherwise die, nevertheless, dosage must be carefully controlled. Despite the risks, platinum-based drugs like cisplatin are the worlds-best selling anticancer drugs, accounting for 6% of the world anticancer drug

market. Ideally, one wants cisplatin to only be active in the vicinity of a cancer cell, and nowhere else. If this can be done, cisplatin will be far safer and more effective, and also more profitable.

Recently, Prof. Sadler introduced the concept of a photoactivatable cisplatin analog which would be completely inert until activated by light, at which time the molecule would decompose into an active and aggressive platinumated chemotherapy drug. Since light can be focused and can penetrate tissue to a depth of 1-2 centimeters (particularly in the infrared), it is possible to conceive of an experiment where a patient is treated with a high dose of this drug, but it is only activated in the regions in and around the tumors using focused light of the appropriate wavelengths. Deeper tissues could be treated using endoscopic probes and fiber optics.⁵⁻⁷

Prof. Sadler's group have made a number of different platinum and ruthenium based photoactivatable complexes which have the potential for the appropriate activities. However, like cisplatin, these molecules also react with proteins, binding covalently, and little is understood about the specificity or positions of this binding. Modern mass spectrometric methods can assess these binding sites.

Proteomics methodologies.

Mass spectrometry of proteins can be used to sequence (partially or fully) proteins which allows both assignment of the identity of an unknown protein (by comparison with protein sequence databases) and determination of sites of modification. There are a wide variety of methods to achieve this, but they are roughly classified into "top-down" and "bottom-up" methodologies, which differentiates between different methods of generation of the protein fragments whose masses are compared with the protein sequence to determine amino acid composition and order. The "bottom-up" methods use proteolytic

enzymes, such as trypsin, which degrade proteins into smaller peptides in solution prior to use of mass spectrometry and/or liquid chromatography to separate these fragments and measure their masses. "Top-down" methods refer to use of mass spectrometry to measure the mass of the whole protein, prior to degradation in the mass spectrometry using a wide variety of (so-called tandem mass spectrometry or MS/MS) methods such as collisionally activated dissociation, electron capture dissociation, or photodissociation. These MS/MS methods are also used to dissociate the peptides generated after "bottom-up" proteolysis to generate more fragments, and hence more sequence information.

"Proteomics" refers to any method that allows bulk study of proteins in a system, cell, tissue, or etc, and these "bottom-up" and "top-down" methods are the primary proteomics tools, when the resulting measured masses are compared to protein sequence database theoretical fragment masses in order to assign protein identity. One problem that occurs frequently using these methods is that the samples (from cells, tissues, etc) are remarkably heterogeneous in terms of the proteins, modifications, and variants that occur, so that the data density can be overwhelming. One method for simplifying the mixture is a technique referred to as the Isotopically-Coded Affinity Tag (ICAT), in which a thiol reactive species with a biotin moiety is reacted with a protein or peptide mixture, and the resulting peptides are then washed over an avidin column so that only the thiol containing peptides are retained.

This project involves design of a modified cisplatin analog which contains a long flexible linker (probably a poly-ethylene glycol or such) which is attached to a biotin. This reagent can then be reacted with cMyc and p53 (*in vitro*, or in cells), and the biotin tether can be used to pull out the bound proteins or peptides which can then be easily cleaned up and analyzed for crosslinks or other platinum or ruthenium related modifications.

C. Preliminary Data

Prof. Sadler's group has previously prepared and tested a number of different cisplatin analogs, and in one case (Figure 4) has tested them against bovine superoxide dismutase (beSOD) showing that up to

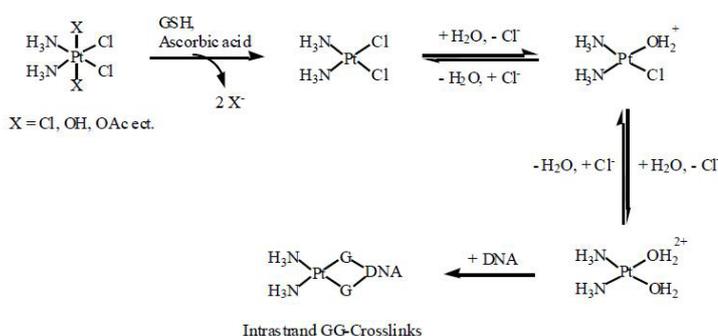


Figure 1. Typical reaction of cisplatin with DNA.

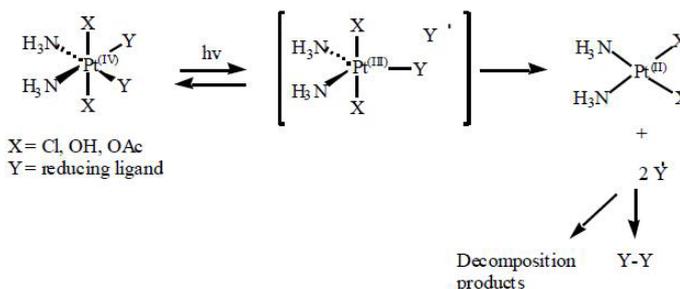


Figure 2. Photoactivation of cisplatin analogs.

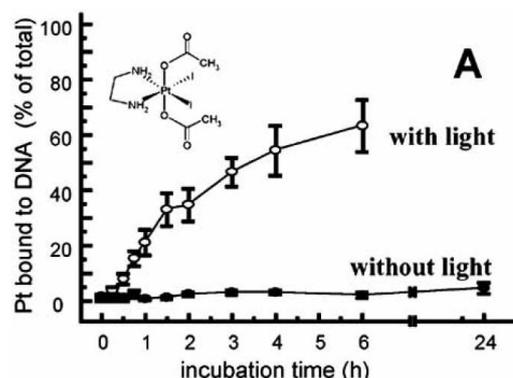


Figure 3. Typical activation profile of reaction of photoactive cisplatin analogs with DNA.

four of the cisplatin species react with the beSOD. In these experiments, the site of modification was not determined, but the masses of the adducts demonstrated were consistent with chloride loss, indicating formation of at least one covalent bond.

Prof. O'Connor's group has worked on determining identity and position of covalent modifications to proteins,⁸ and Figure 5 shows the results from top-down and bottom-up analysis of the products of peroxyinitrite oxidation of p21Ras (the protein product of the most commonly mutated oncogene).⁹ The spectra are shown in the paper. Determination of PTM's in proteins requires the sample be reasonably clean and that the reaction is reasonably homogeneous. In the case of the p21Ras, the protein was initially modified extensively so that top-down analysis was impossible since the protein was combinatorially modified at an estimated ~20 positions thus distributing signal into so many detection channels that the protein was barely detectable. Thus the direct oxidation products were detected with a bottom-up approach which destroys information about the relative abundance or importance of individual modifications. However, when the oxidation was performed in glutathione, the number of modifications dropped to only 6, which were detectable and traced using a top-down approach.

Prof. Khan's group has focused on the study of tissue mass regulation in vivo in order to help define the role of inherent tumour-suppressor activity of key growth-regulating oncogenes such as cMyc and p53 as barriers to tumourigenesis. His group is now examining the role of cMyc as a contributor to beta cell apoptosis and dysfunction in obese states and diabetes, and he has mouse models in which inducible overexpression of cMyc results in several cancers.

D. Research Design and Methods

The MOAC PhD student (PhD) in this research will first generate ESI mass spectra and MS/MS spectra of commercially available cMyc and p53 in order to gain familiarity with the instruments and top-down/bottom-up methodologies. These experiments will start with cMyc as it's the smaller of the two proteins. The experiments are expected to be done using ESI-FTICR mass spectrometry in the group of PBO, but some simpler experiments could be done using instruments in the departmental facility.

PhD will collaborate with the group of PJS to synthesize the biotin-labeled cisplatin analogs and will test their binding to native cMyc. If the binding is so heterogeneous that mass spectral signal is difficult or impossible to obtain, then PhD will either shift to studying this reaction using ubiquitin (which is only 8.5 kDa), or will use enzymatic digestion to cleave the protein into smaller, more manageable peptides. Once abundant signal is obtained, a variety of MS/MS procedures including, but not limited to, CAD and ECD will be employed to identify the site of modification. It is conceivable that ECD, in particular, may have difficulty generating ion signal nearby the modification due to the presence of the metal, but this is not yet determined to be a problem and must be tested. Once cMyc binding sites are identified, p53 will also be studied.

In order to test the biotin cleanup procedures, enzymatically digested mixtures of peptides from cMyc and p53 will be washed over avidin columns and the ideal binding/elution procedures will be determined by optimization. If necessary to increase sample complexity, artificial samples with additional proteins added (such as BSA and myoglobin) will be used as well.

Finally, these methods will be tested and optimized in MK's models after induction of overexpression of cMyc. At this point, the expected peptide and modified peptide masses should be clear, and it is expected that a high quality LC-MS/MS run with the ESI-FTICR instrument should allow a high degree of coverage for the cMyc and p53 peptides to be studied. Other peptides will also be extracted from these samples, and proteomic database searches will be performed to attempt to identify the proteins, and possibly the sites of modification.

3. Quality of the Research Programme:

This project involves use of high performance mass spectrometry in the study the reaction of cisplatin on proteins of interest in cancer. These cisplatin analogs are expected to react rapidly with the proteins cMyc and p53, and it is of great interest to determine the sites and specificity of these reactions. At the very least, this information will be of use to the clinical community in giving hints about how to modify the molecules or formulations to decrease the toxicity of these drugs. In this case, the molecules in question will be modified to include a biotin tag to allow separation of the modified proteins or peptides from the unmodified ones using an avidin affinity column.

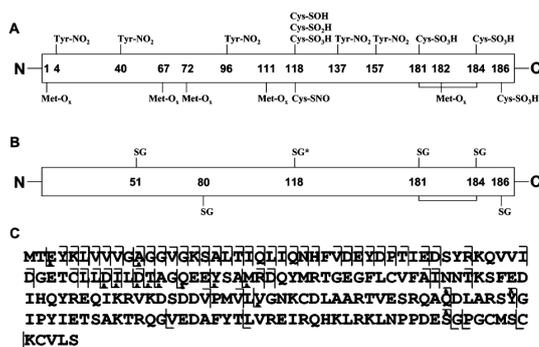


Figure 4. Oxidative PTMs on p21Ras

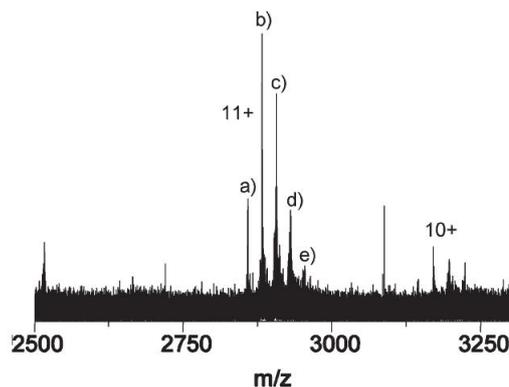


Figure 5. Mass spectrum of bovine superoxide dismutase reacted with cisplatin showing up to four covalent adducts.

The methodologies involved include electrospray FTICR mass spectrometry and tandem mass spectrometry methods such as Electron Capture Dissociation (ECD) and Infrared Multiphoton Dissociation (IRMPD). The proteins studied are cMyc (~30 kDa) and p53 (~47 kDa), which will put them within the acceptable range of the 12T FTICR mass spectrometer(s) in Prof. O'Connor's lab. 30k proteins will be relatively easy, 80k will be difficult. Final experiments with mouse model tissues will be likely to generate a range of modified proteins, while this experiment focuses on cMyc and p53, an attempt will also be made to determine the reaction of these molecules with other proteins. A combination of mild enzymatic digestions may be necessary to bring the bigger proteins down into a reasonable size range.

4. Strategic scientific development for Warwick:

Top-down methodologies are a key tool for full understanding of protein PTMs throughout the biochemical and biomedical sciences. While bottom-up methodologies can help, they frequently fail due to loss of the modified peptide or inadequate fragment coverage. Top-down methods provide another tool, and one which can not only determine the identity and local of a PTM, but can also correlate stoichiometries of multiple PTMs and their effects on each other. Warwick has a strong basis in protein chemistry and the biomedical sciences, and will need these methodologies.

5. Technical training provided:

The student will learn FTICR mass spectrometers and MS/MS methods intimately. He/She will also gain a detailed understanding of the importance and breadth of protein PTM's in general and cisplatin drugs. He/She will learn how to collaborate in a cross-disciplinary fashion and publish papers efficiently. It is hoped expected that this student will be able to provide 4-5 first author papers for their PhD Thesis.

6. Integration of the multi-disciplinary content:

Relationships are the key to successful cross-disciplinary research progress. Specifically, both groups must have respect, confidence, and trust in their compatriots. To assist this, frequent project management meetings will be required (see below), but it is also expected that a collaborative project will produce a roughly equal number of publications from each group, and that all collaborators will be authors on all papers.

7. Justification of resources required:

This project will require a large amount of lab-bench supplies such as solvents, pipette tips, glassware/ependorf tubes, syringes, nanospray needles, reagents, etc. Thus we request the full £2k/year in supplies and the £750 for travel for the student to present his/her results at a conference.

8. Project management:

This project will require constant management. Prof. O'Connor will meet with the student weekly for at least 1 hour in the first year, and at least 30 minutes in subsequent years. Profs. Sadler, Khan, and O'Connor will have monthly meetings for the initial year (bimonthly thereafter) along with this student and relevant other students and PDRA's to discuss project direction and progress. Profs. O'Connor, Sadler, and Khan will jointly edit the student's manuscripts.

9. Initial outline plan (first 6 months):

In order to get familiar with the instrument, methods, and working with cisplatin and proteins, the student will run in vitro studies using commercially available cMyc and p53, or perhaps standards proteins such as ubiquitin. In these initial experiments, the full range of MS/MS methods will be used to determine binding to these import and cancer proteins. This should result in a publication in *Analytical Chemistry* or the *Journal of Proteome Research*.

At the same time, the student will collaborate with Prof. Sadler's group in generation of the biotin labeled cisplatin analogs and will become familiar with the literature on cancer signalling and treatment using cisplatin.

10. References:

1. Pelengaris, S.; Khan, M. The many faces of c-MYC *Arch. Biochem. Biophys.* **2003**, *416*, 129-136.
2. Pelengaris, S.; Khan, M.; Evan, G. c-MYC: More than just a matter of life and death *Nature Reviews Cancer* **2002**, *2*, 764-776.
3. Weidt, S. K.; Mackay, C. L.; Langridge-Smith, P. R. R.; Sadler, P. J. Platination of superoxide dismutase with cisplatin: tracking the ammonia ligands using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) *Chemical Communications* **2007**, 1719-1721.
4. Ivanov, A. I.; Christodoulou, J.; Parkinson, J. A.; Barnham, K. J.; Tucker, A.; Woodrow, J.; Sadler, P. J. Cisplatin binding sites on human albumin *J. Biol. Chem.* **1998**, *273*, 14721-14730.
5. Weidt, S. K.; Mackay, C. L.; Langridge-Smith, P. R. R.; Sadler, P. J. Platination of superoxide dismutase with cisplatin: tracking the ammonia ligands using Fourier-transform ion cyclotron resonance mass spectrometry (FTICR MS) *Chem. Commun.* **2007**, 1719-1721.
6. Mackay, F. S.; Moggach, S. A.; Collins, A.; Parsons, S.; Sadler, P. J. Photoactive trans ammine/amine diazido platinum(IV) complexes *Inorg. Chim. Acta* **2009**, *362*, 811-819.
7. Wang, F. Y.; Weidt, S.; Xu, J. J.; Mackay, C. L.; Langridge-Smith, P. R. R.; Sadler, P. J. Identification of clusters from reactions of ruthenium arene anticancer complex with glutathione using nanoscale liquid chromatography Fourier transform ion cyclotron mass Spectrometry combined with O-18-Labeling *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 544-549.
8. Hopkins, C. E.; O'Connor P. B.; Allen, K. N.; Costello, C. E.; Tolan, D. R. Chemical-modification rescue assessed by mass spectrometry demonstrates that gamma-thia-lysine yields the same activity as lysine in aldolase *Protein Sci* **2002**, *11*, 1591-1599.
9. Zhao, C.; Sethuraman, M.; Clavreul, N.; Kaur, P.; Cohen, R. A.; O'Connor, P. B. A Detailed Map of Oxidative Post-translational Modifications of Human p21ras using Fourier Transform Mass Spectrometry *Anal. Chem.* **2006**, *78*, 5134-5142.