**Report on BST research visit**

**Martina Mušković**

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| **Name of the grantee** | Martina Mušković |
| **Home institution** | Department of biotechnology, University of Rijeka, Croatia |
| **Host institution**  | Faculty of Science and Engineering and PET Research Centre, University of Hull, UK |
| **Home Supervisor** | Dr Nela Malatesti |
| **Host Supervisor** | Prof Ross W Boyle  |
| **Visit period** | August 17th – November 17th  |

**Introduction to the research field**

Photodynamic therapy (PDT) is a relatively novel, non-invasive type of cancer treatment that consists of photosensitizer (PS), light and oxygen. When combined, they can produce reactive oxygen species (ROS), which leads to an oxidative stress in tumor tissue and consequently to its destruction.

Porphyrins are widely used as photosensitizers due to their preferential physicochemical properties for use in PDT, like high stability, production of reactive oxygen species, negligible toxicity without irradiation and good absorption in the red region of the spectra. Another property connected to porphyrins is relatively easy chelation of the core with various metals. This ability allows them to chelate metal radiotracers for use in positron emission tomography (PET) imaging.

PET imaging is a nuclear imaging technique based on the use of biologically active molecules labeled with positron-emitting radionuclides, such as the widely used 18F. The emitted positrons undergo rapid annihilation after encountering an electron resulting in two gamma rays of opposite directions, detected by a PET scanner. In addition to 18F for PET tumour imaging, 68Ga is gaining popularity due to its half-life of $\~$68 minutes, compatible with the biodistribution time of many small molecules, high positron abundance and easy radionuclide generation from 68Ge/68Ga generators.

So far, there are only a few papers in the literature that show radiolabeling of porphyrins using [68Ga]gallium. In all the studies mentioned, scientists managed to radiolabel porphyrins easily, however biodistribution was done poorly or not at all.

**Connection of the research visit with my PhD studies**

In our laboratory in Rijeka, we are interested in the synthesis of amphiphilic porphyrins and investigation of different hydrophobicity/hydrophilicity ratios on biological activity of the

compounds. Conjugation with an alkyl chain of different lengths changes lipophilicity of the molecule, which is important for cellular uptake studies, while water solubility is achieved by *N*-methylation of pyridyl groups.

Since we have shown some preliminary differences in cytotoxicity and cellular uptake based on alkyl chain length in our study so far, we wanted to test porphyrins with 1C-, 9C-, 13C-, and 17C-atom chain as potential radiotracers for PET imaging. In addition, there are only three papers related to radiolabeling of cationic porphyrins with [68Ga]gallium without showing any PET/CT scans, so these were the first attempts to analyze biodistribution and tumour uptake of cationic porphyrins by PET imaging.

**Aim of the research**

Based on the literature, we decided that our aim was to investigate a group of four tricationic pyridylporphyrins conjugated with alkyl chains of different lengths (1C-, 9C-, 13C- and 17C-atoms) as potential agents for PET imaging and to investigate their biodistribution using PET imaging.

Here are the tasks we planned to accomplish to achieve our goal:

1. Radiolabeling of porphyrins
2. Investigate their physicochemical properties (logD, serum stability, metal chelate and formulation stability)
3. Investigate the biodistribution of compounds using PET imaging
4. Analysis of metabolites after PET imaging

**Research plan in action**

Upon arrival in the UK, my first task was to quaternise pyridylporphyrins I brought from Croatia and to characterize them and analyze their purity. These porphyrins were then used at the PET center for radiolabeling. In PET Research Center, I worked under the supervision of Professor Steve Archibald, and my trainer was Dr Juozas Domarkas.

To radiolabel my group of compounds, we combined previously published methods for cationic porphyrins. All four porphyrins showed > 80% radiochemical efficiency (determined using HPLC) within 30 minutes of heating at 100°C.

However, the specific activity of porphyrins was very low, and only a small amount of porphyrin was chelated. In order to achieve high specific activity, and have 100% pure porphyrin, we performed semi preparative HPLC using methanol / water mixture as solvent. Due to the high stickiness to the 18C column, porphyrin with 17C-atom alkyl chain could not be purified. Further experiments with this porphyrin should include optimization of purification using C5 or C8 column. On the other hand, porphyrin with 1C-atom chain was too hydrophilic, so we could not capture enough activity on SepPak for further experiments, so we continued with this porphyrin busing it with only low specific activity.

For the analysis of physicochemical properties, we calculated logD by measuring activity in phosphate buffer saline (PBS) and 1-octanol layer using a γ-counter. We also performed a serum stability experiment where we incubated radiolabeled porphyrin in serum for two hours, and after precipitation with acetonitrile, supernatant was tested on HPLC. Here we have noticed that our porphyrins, especially porphyrins with 13C- and 17C-atom alkyl chain, strongly stick to the protein pellet and cannot be detected in the supernatant. Based on this finding, we decided to expand our plan and further investigate our porphyrins formulated with protein carriers that typically increase tumour uptake, human serum albumin (HSA) and low-density lipoprotein (LDL).

To avoid precipitation on the HPLC column, we proceeded with our carrier binding analysis, serum stability and metal chelate stability on size exclusion column with PBS as solvent. Our porphyrins with alkyl chain (9C-, 13C- and 17C-) were 100% bound to albumin and this formulation showed high stability for 4 h, and in serum and apo-transferrin solution for up to 2 h. However, none of the porphyrins were bound to LDL.

In parallel with my work at the PET center, in the Laboratory for Photomedicine and Photobiology I optimized the synthesis protocol for chelation of porphyrins with gallium using GaCl3. The purity of the final products, chelated porphyrins, was determined by HPLC and characterized by nuclear magnetic resonance 1H NMR and high-resolution mass spectrometry (HRMS).

The biological part of the experiments included cellular uptake assay using radiolabeled porphyrin as PBS, BSA and LDL formulation on breast cancer cell line, MDA MB 231. PET/CT scanning was performed by John D Wright, animal laboratory technician of the PET Research center. All scans were 90 min dynamic PET scans followed by CT scan on a strain type CD1 where radiotracer was administered intravenously. During my visit we performed scans using 9C- and 13C-atom porphyrins formulated in PBS and with BSA as carrier and 1C atom formulated in PBS. After the scan, they taught me to independently analyze the images and calculate biodistribution in every organ using AMIDE online software.

Some of the first results are show a high accumulation of porphyrins with 9C- and 13C- atom alkyl chains in blood and blooded organs such as the heart, kidneys and liver, while the highest activity of porphyrin with 1C-atom alkyl chain was mostly in the bladder and kidneys.

**Extra contributions**

In addition to my laboratory work, I was invited to listen to colleagues at the PET Research center group meetings every Friday afternoon. Also, I presented my work at one of the meetings, on October 15th, entitled: “Amphiphilic tricationic porphyrins as potential candidates for PET/PDT theranostic agents”.

After my arrival back to Croatia, I presented my three-month work in the UK at the 5th Symposium of the Section of Medicinal and Pharmaceutical Chemistry of the Croatian Chemical Society, on November 30th, entitled: “First *in vivo* positron emission tomography biodistribution study of [68Ga]gallium radiolabeled amphiphilic cationic porphyrins with potential applications in photodynamic therapy”.

**Overall results and impressions**

My visit to the UK and all the research I did at the University of Hull was above all my expectations. My plan was to try radiolabeling and to test if anything works, with minor expectations in comparison to final results. We did a huge amount of experiments, I developed a number of new skills in radiochemistry radioanalysis, *in vitro* radiochemical assays and formulating drugs for *in vivo* imaging and I also learned a lot of new information about my compounds. In this report you can see that we had a lot ups and downs in our project, however, many of them have been resolved and we have amazing results.

We continue with this work so that colleagues in the UK will finish with PET/CT scans and I continue to work in Croatia with synthesized natGa porphyrins to investigate these porphyrins as photosensitizers for PDT in order to obtain theranostic agents that can be used both for PET and PDT. Once we have completed all our experiments, it is planned to prepare a manuscript that will comply with the standards for high-impact journals.

In addition to working in the lab, in the UK I met a great group of scientists who were not only amazing colleagues, but also fantastic friends. I’m happy that our story is not over with this project and that we will continue to collaborate on other project(s) in the future.

This Scholarship was a step forward, not only in my PhD studies, but also in my carrier as a scientist, and I am so grateful to have been able to visit the UK, meet great scientists and do amazing and interesting experiments related to my study.