Cláudia Rodrigues, PhD student
Faculty of Pharmacy, University of Lisbon
Av. Prof. Gama Pinto 1649-003, Lisboa Portugal
crodrigues@ff.ulisboa.pt

To:
COST MC Chair: Dr Florence Velge-Roussel
Tel. +33247366058
Fax +33247366147
velge@univ-tours.fr

Short Term Scientific Mission (STSM)

COST Action BM1406 – Ion Channels and Immune Response toward a global understanding of immune cell physiology and for new therapeutic approaches

SCIENTIFIC REPORT

Reference code: ECOST-STSM-BM1406-151116-081219
STSM title: Role of aquaporin in resistance to chronic oxidative stress of breast and colon cancer cells
Host: Ana Čipak Gašparović, Institut Ruđer Bošković, Zagreb, Croatia
Period: from 15/11/2016 to 15/12/2016

Contents of the report:
1- Purpose of the visit STSM
2- Description of the work carried out during the STSM
3- Description of the main results obtained
4- Future collaboration with host institution (if applicable)
5- Projected publications/articles resulting or to result from the STSM (if applicable)

1. Purpose of the visit STSM

Aquaporins (AQPs) are a family of small transmembrane proteins (≈30kDa) expressed in all kingdoms. The AQP family members have high homology (25%–60%) in protein sequence and so far, 13 isoforms (AQP0-12) were identified in humans showing specific organ, tissue and cellular localization thus suggesting a relation between its expression and its function in each organ. AQPs mediate rapid movement of water fluxes across plasma membrane in response to osmotic gradients which is extremely important for cells adaptation to the external environment. In addition to passive transport of water, a subset of the AQP family is also permeable to small-uncharged solutes like glycerol, hydrogen peroxide (H_2O_2) urea, CO_2, among others. AQPs have an essential role in a broadly range of pshysiological functions, such as water/salt homeostasis, exocrine fluid secretion, brain oedema, adipocyte metabolism. Their dysfunction or abnormal expression has been associated with several pathologies related water and energy homeostasis, like kidney disorders, brain edema, obesity and cancer.

Aquaporins are also shown to be involved in tumor angiogenesis, invasion, metastasis and growth. Moreover, transport of H_2O_2 by some AQP isoforms has been considered as a possible mechanism explaining their involvement in cell redox status and tumorigenesis, although the mechanism of their implication in oxidative stress is still uncertain.

Actually it is well known that lipids are not merely a barrier but also contribute directly or indirectly, through peroxidation end-products like 4-hydroxynonenal (HNE) to numerous cellular processes. HNE activates most of the pathways triggered by oxidative stress by binding to redox sensitive molecules. HNE-protein adducts were detected in numerous pathologies like cancer. A recent study reported that HNE was find bound to AQP4 in epilepsy and suggested it could be a new player in oxidative neuronal damage.

The main aim of the proposed project is to investigate the relation between oxidative stress and collagen in the extracellular space on breast cancer stem cell (BSCs) lines in inducing conditions. Also, HNE will be applied to collagen and thus prepared growth surface will present oxidatively modified extracellular matrix. For this purpose MTT assay will be used to characterize cell growth on different growth surfaces, different stressors and type of stress. After the establishment of concentrations of stressor (by MTT assay) and modifications of collagen that cause adaptation, components of cellular antioxidative defense, glutathione, glutathione-S-transferase and catalase will be tested in response to selected conditions acute and chronic stress on unmodified and modified collagen. At same time, the effect of hydrogen peroxide on aquaporin expression will be tested in breast cancer and colon cell lines, as well as in sphere-inducing conditions, and RNA will be isolated from control and treated cell lines to analize AQP expression by qPCR.

This work also aim at study possible involvement of HNE as a signaling molecule and aquaporins as modulators of H_2O_2 flux. To accomplish, dot-blot analysis of HNE protein adducts, Nrf2 and AQP5 will be performed in our test model. Finally, these results would indicate if AQP5 play a role in the process of adaptation to chronic stress of extracellular matrix. Understanding the relation between aquaporins and oxidative modification of extracellular matrix could indicate possible mechanism that lead to therapy resistance and lead to new model that mimic cellular environment during tumor development.

2- Description of the work carried out during the STSM

Breast cancer stem cell line SUM159 and colon cancer stem cell line HT-29 were cultivated for 7 to 10 days at 37°C, 5% CO_2 in sphere inducing media DMEM supplemented with EGF 20ng/mL, bFGF 20ng/mL, heparin 20ng/mL and B27 2% (v/v). For colonspheres, sphere inducing media was also supplemented with insulin 4U/L.

The cytotoxic effect of $AgNO_3$ was tested by using a colorimetric method based on the tetrazolium salt MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cells were seeded in 96-well microplate ($1x10^5$ cells/mL) and plate 200 µL per well and cultivated for 24h at 37°C, 5% CO_2. Next day, supernatant was discard and replaced for 100 µL prewarmed (37°C) medium. Cells were treated with 1µM, 2.5µM and 5µM $AgNO_3$ (final concentration) for 10min and 60min. During this period, it was prepared a 5 mg/mL stock solution of MTT in PBS and filtered using 0.2-µm filter. After treatment, medium was discard and replaced for 200 µL Hank's solution. Cells were treated with 0.5mg/mL MTT dye and incubated at 37°C, 5% CO_2 for 90min. After treatment, the absorbance was determined at wavelength of 620nm and 450nm.

Cellular ROS level was also examined. Cells were seeded in white 96-microwell plates ($1x10^5$ cells/mL) and plated 100 µL per well and incubated with 10 µM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) at 37°C for 45 min, centrifuged at 202xg for 5 min. Cells were treated with optimized conditions determined by MTT assay (1µM, 2.5µM and 5µM $AgNO_3$) at 37ºC for 10min and centrifuged at 202xg for 5 min. After discard the supernatant, cells were treated with 50µM, 100µM and 200µM H_2O_2. DCF fluorescence was measured before treatment and every 10 minutes after treatment for 60 minutes using a multiwell plate reader at excitation and emission wavelengths of 500 and 530 nm, respectively.

6-well tissue culture plates were coated with 1) collagen (17µg/mL); 2) collagen (17µg/mL) and 1µM HNE; 3) collagen (17µg/mL) and 10µM HNE. This allowed to investigate not only the relation between oxidative stress and collagen in the extracellular space but also the oxidatively modified extracellular matrix by addition of HNE. Breast cancer stem cell line SUM159 was seeded in coated and non-coated (control) plates ($1x10^5$ cells/mL) and plated 2.5mL per well. Cells were treated with 10µM HNE and cultivated for 10 days at 37°C, 5% CO_2. At every 2 days, medium was discard and replaced for fresh prewarmed medium and treated with 10µM HNE. Cells seeded in non-coated plates needed to be centrifuged at 202xg for 5 min to replace for fresh medium. After 10 days of incubation, cells were harvested and stored at -80°C. Then, cells were ressuspended in PBS and lysed by 4 freeze/zhaw cycles with wortexing inbetween for cellular antioxidative defense system.

The protein concentration were measured by Bradford's assay (Coomassie Brilliant Blue G-250). We analysed the catalase activity either of lysed cells and supernatant by treating with 65mM H_2O_2 and stopping the catalase reaction after 5 minutes with 32,4mM ammonium molybdate. This compound reacts with hydrogen peroxide and changes its colour to yellow, allowing to calculate the H_2O_2 concentration by measure the absorbance at 405nm. The catalase activity was calculated using the next expression:

$$Activity\ (U) = \frac{(65mM - [conc\ H2O2\ calculated]) \times V\ (65mM\ on\ sample\ mL)}{minutes}$$

For GSH level, samples were diluted to 0.03 mg/ml protein and reaction was started by addition of fresh reaction mix (0.6mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 0.4U GSH reductase (GR) and 0.1mM NADPH in phosphate buffer 100 mM NaH_2PO_4, 5 mM mM EDTA pH 7.4) to standards and samples. Fresh reaction mix was prepared and added. The formation of of 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically in a plate reader at 405 nm. Antioxidant defense status assays were performed in duplicates.

For RNA isolation, either breast as colon cancer cell lines were seeded in 6-well plates ($3.33x10^5$ cells/mL), plated 3mL per well and cultivated at 37°C, 5% CO_2, overnight. Cell lines were treated with 100 µM H_2O_2 for 24h at 37°C, 5% CO_2 and sample were stored in Trizol® at -80ºC. RNA extraction and cDNA synthesis will be performed in Lisbon to analyse AQP profile expression in cell lines.

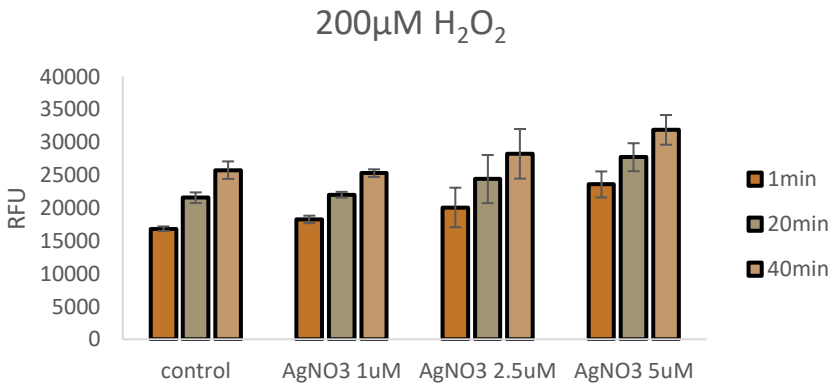
For dot-blot analysis, protein extracts (10µg each) were spotted onto nitrocellulose membranes (Amersham). The membranes were incubated in blocking solution (1% nonfat milk powder in PBS) at room temperature for 60 min and subsequently incubated overnight with rabbit polyclonal antibodies directed against Nrf2 protein (1:100 in 1% BSA in PBS). Blots were then washed and incubated for 1h with goat anti-rabbit antibody DAKKO Flexi system (dilution 1:25 in PBS/1%BSA). Immunocomplexes were visualized using the DAB DAKKO substrate kit.

During our STSM visit, I have learned:

1. Cultivation techniques for breast cancer and colon stem cancer cell lines (spheres)
2. MTT assay
3. ROS production
4. Quantification of glutathione level and catalase activity assay (Antioxidant defense status)
5. Modification of extracellular matrix
6. RNA isolation
7. Dot-blot analysis

3- Description of the main results obtained

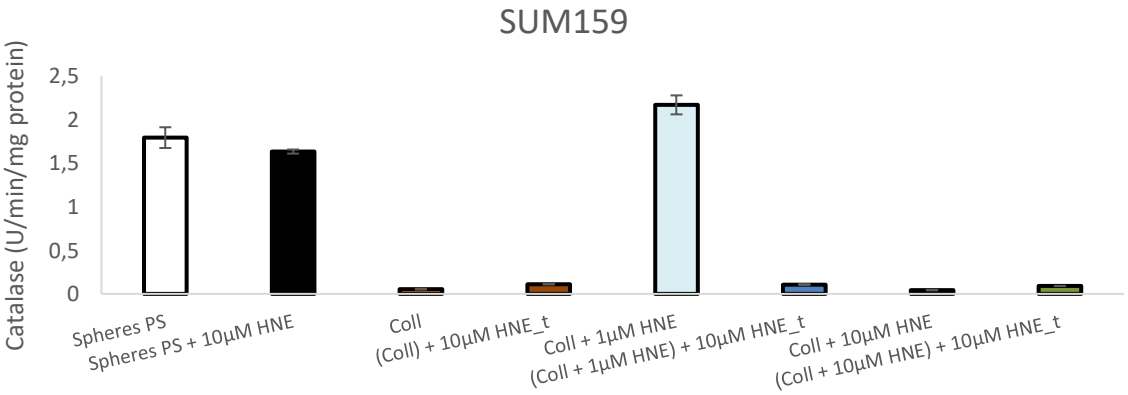
1. *ROS production*



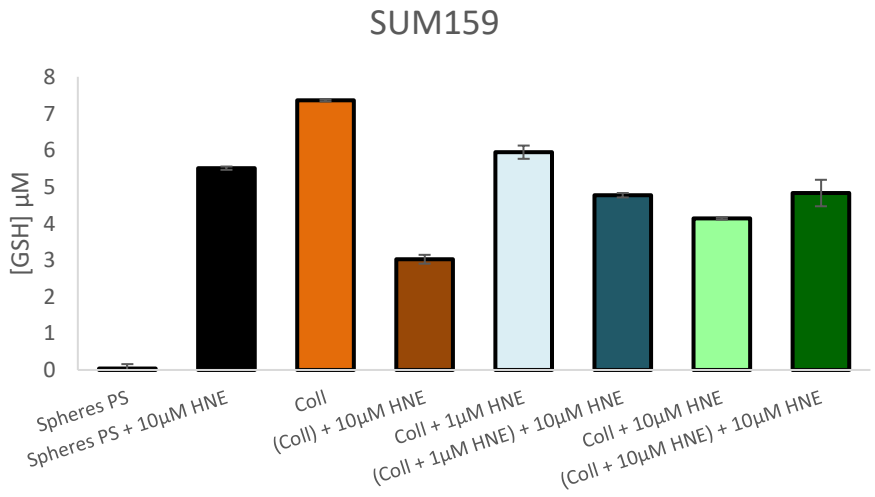
Cells were incubated with 50µM-200µM H_2O_2 concentration range and cellular ROS production was analyzed at every 10min until 60min. The representative ROS measurement after incubation with 200µM H_2O_2 indicates a significant fluorescence increase over time in control as result of H_2O_2 uptake. To determine if the uptake of H_2O_2 was through AQPs expressed in plasma membrane, cells were incubated with 1µM-5µM $AgNO_3$ concentration range, previously described as a potential AQP inhibitor. Although we didn't observe reduction of H_2O_2 uptake by cells after treatment with $AgNO_3$ it is worth mentioning that discovery of specific AQP modulators has been slow and inhibitors used for AQP inhibition can be unspecific or change membrane properties and therefore this result need complement experiments.

2. *Antioxidant defense status*

Antioxidant defense status (catalase activity and glutathione level) was evaluated in suspension cells (spheres) and in adherent cells, both cultivated in sphere inducing media in different extracellular conditions.

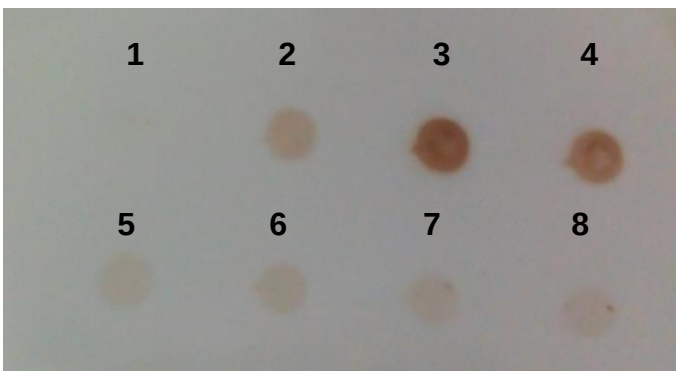


As depicted, after chronic stress induced by 10µM HNE in suspension cells we did not observe changes in catalase activity. Although, when comparing catalase activity in spheres and adherent cells, the activity was diminished in the adherent cells. This indicates that alteration in extracellular matrix causes adaptation of antioxidant defense system. Furthermore, results indicate an increase of catalase activity in oxidatively modified extracellular matrix with 1µM HNE. Chronic stress induced by 10µM HNE in this extracellular conditions reduced catalase activity to basal level as in unmodified extracellular matrix (collagen). On other hand, catalase activity in oxidatively modified extracellular matrix with 10µM HNE and after chronic stress induction is similar to unmodified extracellular matrix.



Glutathione was almost absent in spheres but after chronic stress with 10µM HNE we observed a drastic increase of glutathione as reflect as an adaption to oxidative stress. Alteration in extracellular matrix also enhance glutathione synthesis and indicates an adaption to extracellular matrix. In conditions of oxidatively modified extracellular matrix with HNE we observe an impairment of glutathione level. Chronic stress with 10µM HNE only had a significant effect in unmodified extracellular matrix in which glutathione synthesis was largely reduced.

3. *Dot-blot analysis*



Detection of Nfr2 transcription factor by dot-blot assay shows similar results when compared to glutathione level. Sample 1 and 2 correspond to spheres and spheres after chronic stress with 10µM HNE and Nfr2 was only detected in spheres after induction of chronic stress. Sample 3 and 4 correspond to unmodified extracellular matrix (collagen) and same condition after chronic stress. Dot-blot analysis showed that Nfr2 was detected in both samples. Samples 5 to 8 correspond to oxidatively modified extracellular matrix and induced chronic stress with 10µM HNE. As we expected by glutathione level, the level of detection of Nfr2 was small compared to sample 3 and 4. These results need further analysis for quantification but altogether we can observe a correlation between Nfr2 transcription factor and glutathione synthesis in adaption to extracellular matrix and chronic oxidative stress

4. *RNA extraction and cDNA synthesis*

The breast and colon cancer cell lines were grown to isolate the mRNA. We cultivated breast and colon cancer cell lines in DMEM supplemented with serum, as well as in cancer stem cell inducing conditions to generate mRNA, which will be then analysed for AQP5 expression in Lisbon.

4- Future collaboration with host institution

Our future plan is to continue collaborating together on the topic of aquaporins and cancer stem cells. We aim at unveil a possible mechanism of the effect of oxidative stress on aquaporin expression and function in mammalian cancer cells lines as a potential drug target in chronic disease as cancer.

These aims will be achieve by:

- Collaborations through specific STSM actions to be investigated further;
- Collaborations through research projects;
- Collaborations through joined publications.

5- Projected publications/articles resulting or to result from the STSM

The work done during this visit will complemented the previous results obtained by both laboratories in this area will be merged to write a paper for the effect of oxidative stress on aquaporin expression and function in cancer stem cells. Thereby, both groups will continue to closely collaborate.

14th November 2016,