STSM REPORT

STSM Application number: CA 21111-3df1871c

STSM Grantee Name: Narimantas Čėnas

Young Researcher and Innovator? (Yes or No - Young Researcher and Innovator= <40 years old)

STSM title: Redox reactions of plasmodione with oxyhemoglobin or heme (Fe^{2+})

Home Institution: Institute of Biochemistry of Vilnius University, Vilnius, Lithuania

Host Institution: Laboratoire d'Innovation Moleculaire et Applications (LIMA), UMR7042 CNRS-Unistra-UHA, Strasbourg, France

STSM start and end date: 24/04/2024 to 02/05/2024

Working Group: *(Within which Working Group WG1-4) does this research fit best?)* WG2,3

Purposes of the STSM (max 200 words):

The high antiplasmodial activity of 3-[4-(trifluoromethyl)benzyl]menadione (plasmodione, PD) was discovered in the host laboratory [1]. Subsequent studies expanded the range of PD derivatives, their studies in various parasite strains, and detailed the mechanism of their action ([2], and refs. therein). The latter direction partly involving the applicant's team, highlighted the possibility of formation of PD benzhydrol metabolite (PD-bzol), and, subsequently, highly active PD 3-benzoyl metabolite (PDO) by reactive oxygen species (ROS) during the redox cycling of PD radicals formed after its reduction by *P. falciparum* flavoenzymes, e.g. ferredoxin:$NADP^+$ oxidoreductase (*Pf*FNR) [2]. It has been found that certain hemoproteins, e.g. cytochrome *c*, stimulates this reaction, but this aspect has not been studied in detail.

In order to elucidate the additional pathways of PD transformation, we intended to investigate the possibility of PD oxidation by replacing cytochrome *c* with hemoglobin (Hb). This is linked to the high content of Hb in erythrocytes, and the fact that various redox forms of Hb catalyze the hydroxylation of aromatic compounds [3,4].

Description of the work carried out during the STSM (max 500 words):

The redox cycling of 50 µM plasmodione (PD) and ^{13}C-enriched PD (1:1) by 2.0 µM *Pf*FNR in the absence or presence of 150 µM methemoglobin was performed in 0.05 M K-phosphate buffer solution (pH 7.0) in a total volume 200 µl in a glass tube. The 5 mM stock solutions of PD and $^{13}C_{18}$-enriched-PD were prepared in DMSO. As the reductant, 100 µM NADPH and a system for its regeneration based on 1.0 U/ml glucose-6-phosphate dehydrogenase and 10 mM glucose-6-phosphate, were prepared.
Preliminary assays were performed to adjust the optimized conditions (data not shown). The final conditions are presented in Table1.

The reaction was started by the introduction of *Pf*FNR, and lasted 2 h and 15 min at 37 °C. All reactions were performed in duplicate. After 2 h15 min, the four reaction mixtures "**1**$^{-}$" and "**2**$^{-}$" (without Hb), "**1**$^{+}$" and "**2**$^{+}$" (with Hb) were centrifugated and then,100 µl aliquotes were collected and added to 30 µl DMSO. Samples were analyzed by LC-MS. Human Hb was also analyzed as control. LC/MS analyses were performed using an Agilent 1100 series LC coupled to a maXis II Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated with a capillary voltage of 4500 V in positive mode. Acquisitions were performed on the mass range m/z 200−1850. Calibration was performed using the singly charged ions produced by a solution of Tune mix (G1969−85000, Agilent, U.S.A.). Compounds were separated on a XBridge Peptide BEH C18 column (300 Å, 3.5 µm, 2.1 mm × 250 mm). The gradient was generated at a flow rate of 250 µL/min at 60 °C by mixing two mobile phases. Phase A

consisted of 0.1% trifluoroacetic acid (TFA) in water and phase B, of 0.08% TFA in ACN. Phase B was increased from 5% to 85% in 45 min. Data analysis was performed using Compass Data Analysis 4.3 (Bruker Daltonics).

Table 1. Composition of assay mixture.

Reagent	Final concentration (µM)	Added volume (µl)
1 mM human Hb (or buffer in the Hb-free reactions)	150	30
5 mM stock solution of PD	50	2
5 mM stock solution of $^{13}C_{18}$-PD	50	2
10 mM NADPH	100	2
10 mM glucose-6-phosphate	1000	20
G6PD 10 U/ml	1 U/ml	20
266.7 µM *Pf*FNR	2	1.5
0.05 M K-phosphate buffer solution (pH 7.0)		122.5
Total		200

Description of the main results obtained:

The four reaction mixtures "**1**$^{-}$" and "**2**$^{-}$" (without Hb), "**1**$^{+}$" and "**2**$^{+}$" (with Hb) were analyzed by LC-MS. Calculated exact masses of the expected molecular and protonated species analyzed by ESI-MS are given in Scheme 1 (Appendix).

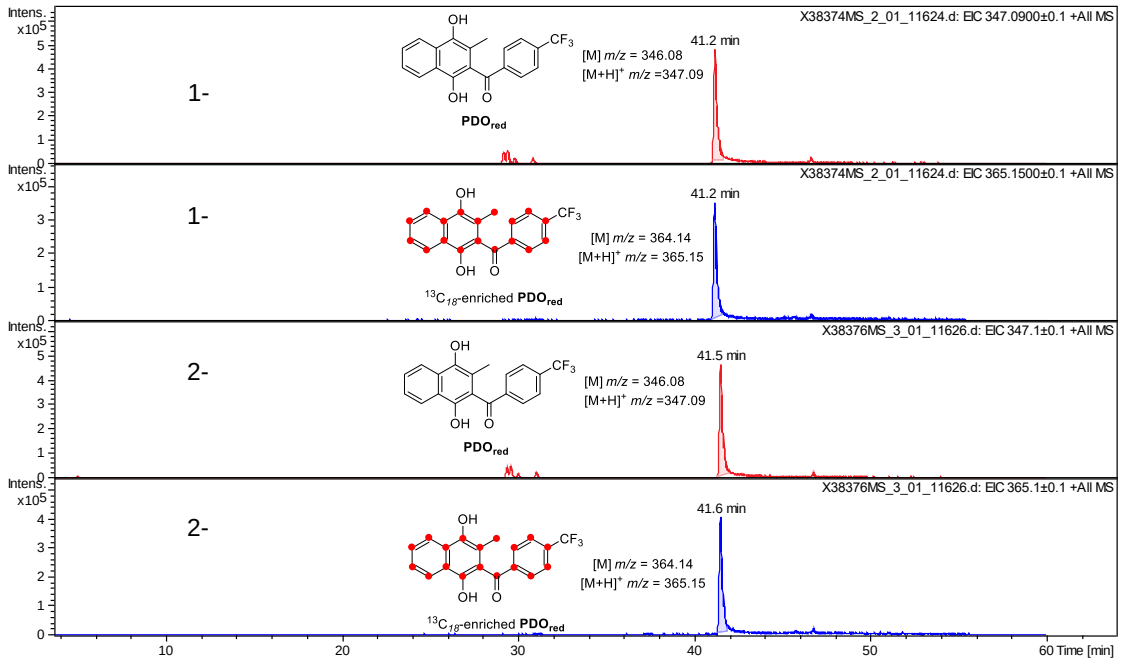


Figure 1. Mass spectra of reaction mixtures "1-" (A,B) and "2-" (C,D) with the observed extracted ion currents *m/z* for reduced PDO (A,C), ^{13}C-enriched reduced PDO (B,D), at the end of the reactions (RT=41.2 min) in accordance with calculated $[M+H]^+$.

The MS spectra of reaction mixtures (without Hb, **Figures 1, 2A,B**) demonstrate the presence of reduced form of PDO (m/z = 347.09, retention time (RT) = 41.4 min, and m/z = 365.15, RT = 41.5 min for its ^{13}C-enriched derivative (**Figures 1A-D**), remaining PD (m/z = 331.09, RT = 47.6 min, **Figure 2A**), reduced form of PD-bzol (m/z = 349.15 for its ^{13}C-enriched form (M – H_2O + H^+) , RT = 42.2 min, mixture "**1-**", **Figure 2B**). The retention times of compounds were similar to those obtained by Cichocki *et al*., 2021 [2].

On the other hand, in the reaction mixtures "**1+**" and "**2+**" (with Hb) PDO and PD-bzol were not formed (**Figure 2C,D**). This was additionally verified using extraction ion current 347.09 (reduced PDO), and 365.15 (^{13}C-enriched reduced PDO, data not shown).

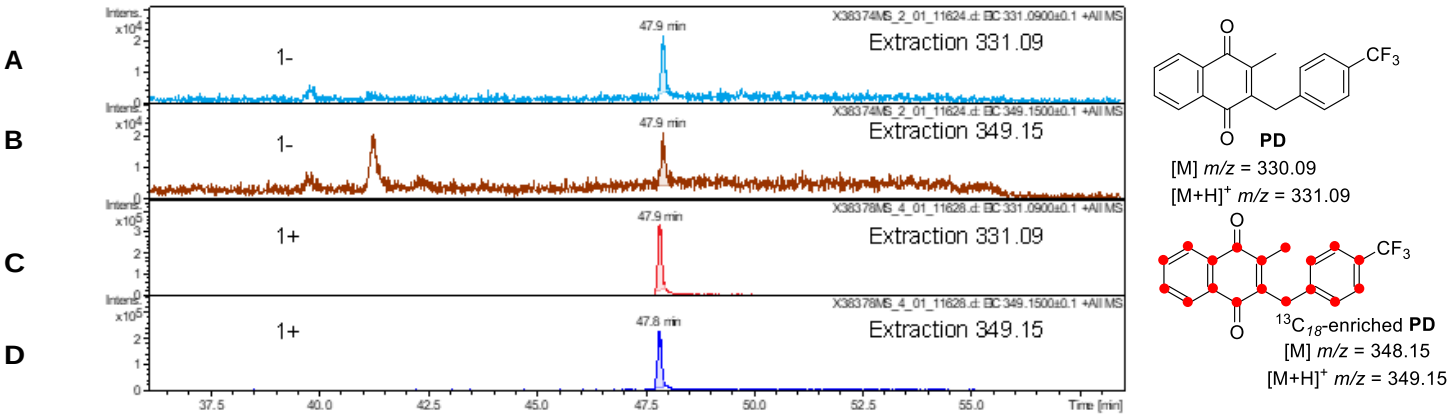


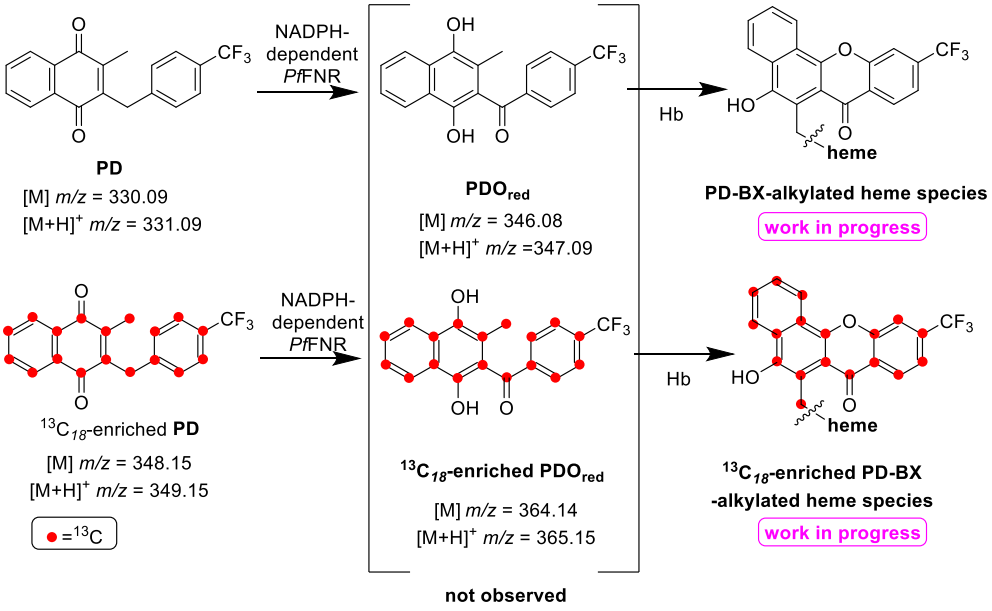
Figure 2. Mass spectra of reaction mixtures "1-" (absence of Hb, A,B) and "1+" (presence of Hb, C,D). A – before the reaction, B,C,D – at the end of reaction. The MS analysis showed both expected extracted ion currents *m/z* peaks for PD and ^{13}C-enriched PD (RT=47.8 min) in accordance with calculated $[M+H]^+$.

Conclusions:

1. During the tests, the presence of PD in *m/z* the oxidized state, and the presence of PDO and PD-bzl in the reduced state, is explained by the presence of trifuoroacetic acid in the liquid chromatography media, which protonates the phenolates of dihydronaphthoquinones.
2. Differences between cytochrome *c* and Hb in PD redox cycling and oxidationare most likely related to the different structure of their hemes - the Fe atom of cytochrome *c* is fully coordinated, while the ligand can bind and dissociate in the axial position of the heme of Hb.
3. It is possible that the apparent absence of formation of PDO and PD-bzol metabolites in the presence of Hb is related to the shift of the drug metabolism to the formation of a third alkylating metabolite, a known PD metabolite called benzo[c]xanthen-7-one (PD-BX), which can covalently bind to the heme of Hb [5]. At this time, m/s analysis of alkylated heme or alkylated protein Hb is pending with distinct LC-MS analysis conditions.

APPENDIX

Scheme 1. Calculated exact masses of the expected molecular and protonated species analyzed by ESI-MS in the *Pf*FNR-catalyzed benzylic oxidation reaction mixtures in the presence of hemoglobin.



References:

1. Müller T., Johann L., Jannack B., Bruckner M., Lanfranchi D.A., Bauer H., Sanchez, C.,Yardley V., Deregnaucourt C., Schrevel J.,Lanzer M., Schirmer R.H.,Davioud-Charvet E. (2011) Glutathione reductase-catalyzed cascade of redox reactions to bioactivate potent antimalarial 1,4-naphthoquinones: a new strategy to combat malarial parasites. ***J. Am. Chem. Soc.*** 133, 11557-11571.
2. Cichocki B.A., Donzel M., Heimsch K.C., Lesanavičius M., Feng L., Montagut E.J., Becker K., Aliverti A., Elhabiri M., Čėnas N., Davioud-Charvet E. (2021) *Plasmodium falciparum* ferredoxin-NADP$^+$ reductase-catalyzed redox cycling of plasmodione generates both predicted key drug metabolites: implication for antimalarial drug development. ***ACS Infect. Dis***.7, 1996-2012.
3. Stolze K., Nohl H. (1991) Formation of methemoglobin and phenoxyl radicals from p-hydroxyanisole and oxyhemoglobin. ***Free Rad. Res. Comms.*** 11, 321-327.
4. Spolitak T., Hollenberg P.F., Ballou D.P. (2016) Oxidative hemoglobin reactions: application to drug metabolism. ***Arch. Biochem. Biophys.*** 600, 33-46.
5. Cichocki B.A., Khobragade V., Donzel M., Cotos L., Blandin S., Scaeffer-Reiss C., Cianferani, S., Strub J.-M., Elhabiri M., Davioud-Charvet E. (2021) A class of valuable (pro-)activity-based protein profiling probes: application to the redox-active antiplasmodial agent, plasmodione. ***ACS Au.*** 1, 669-689.

Mutual benefits for the Home and Host institutions:

The benefit of the Home institution was a more detailed familiarization with the LC/MS technique for analysis of low concentrations of the products of enzymatic reactions. The benefit of Host's institution was

the examination of a new type biomimetic system, potentially reflecting reactions in vivo.

Future collaboration with the Host institution (if applicable):
It is envisaged to collaborate with the host institution by developing this type of reactions at the guest institute and performing LC/MS analyzes at the Host institution.

Foreseen publications or conference presentations expected to result from the STSM (if applicable):
The obtained results may form part of a joint paper expected to be published in 2025, and/or presented at the meeting of Societe de Chimie Therapeutique.