Prologue

The aim of this project was to investigate the roles of hydrogen sulfide in inflammation, especially in MPO-ANCA associated vasculitis. During the 4 years we reached the overall aim of this project and now we have a drug candidate in our hand that may advance to human studies to treat this deadly disease. **To aid these endeavors we are in the process of filing multiple patents on the results of this project and therefore we request to please keep this summary strictly confidential!**

We obtained strong evidence that H_2S can protect against neutrophil induced inflammatory damage both in the latent and in the acute phases of the disease. In order to better understand our observations, we dissected the underlying molecular mechanisms of protection. Moreover, we obtained evidence that phagocytosis, a pivotal innate immune function of neutrophils in bacterial clearance is not affected by H_2S treatment. We corroborated the results of our cellular experiments in an MPO-ANCA mouse model that we set up in our laboratory. Furthermore, we designed and synthesized a new molecule, which will be further investigated on drug development grounds to follow up our results. The most exciting element of our study was the significant decrease of inflammation we detected in our MPO-ANCA mouse model upon treatment with our drug candidate. The number of affected glomeruli in the kidneys was significantly lower and we observed a diminished number of inflamed vessels and significantly lower levels of leukocyte infiltration in the kidneys and also in the lungs. Since the start of the project we produced 15 publications and numerous conference abstracts. The 15 publications consist of 9 original articles, 2 clinical studies, 2 invited book chapters and 2 invited reviews with the overall impact factor of 129.32. Most of these results emerged from national and international collaborations. The PI of this project serves as corresponding author in 8 of these articles.

The citations depicted with red below are outputs of this project.

Major findings

The roles of hydrogen sulfide in oxidative stress and inflammation has been studied for almost two decades (Kimura and Kimura 2004), (Wen et al. 2013). Several original research (Wallace 2007), (Wallace, Ferraz, and Muscara 2012) and review articles (Whiteman and Winyard 2011), (Lo Faro et al. 2014) investigated the effects of hydrogen sulfide in the inflammatory processes of multiple diseases and injuries. It was previously demonstrated by our group that hydrogen sulfide efficiently and reversibly inhibits the halogenation and peroxidase activities of the myeloperoxidase enzyme (MPO) (Palinkas et al. 2015). MPO is one of the most abundant protein of human neutrophil granulocytes (Klebanoff 1999) that can serve as an autoantigen in ANCA associated vasculitis (Gross, Schmitt, and Csernok 1993) and the inflammatory effects of MPO has also been investigated in a number of pathologic conditions (Klebanoff 2005).

In our project we developed a novel method to measure the inhibitory effect of sulfide on the Reactive Oxygen Species generating capacity of neutrophil granulocytes. Using this new method, we showed that sulfide inhibits MPO activity in low concentrations in the supernatants of 12-phorbol 13-myristate acetate (PMA) activated neutrophils (Garai et al. 2019). Furthermore, we developed a hydrogen sulfide detection method, that can be used for sulfide measurements in various biological samples, including blood plasma, blood serum, cell lysates and supernatants (Ditroi et al. 2019). Applying this method we confirmed that the IC_{50} for MPO inhibition of free sulfide in activated neutrophil supernatants were similar to the IC_{50} that was measured in isolated MPO enzymatic activity assays (Palinkas et al. 2015). **Therefore, sulfide is likely to have a strong inhibitory effect on MPO-induced oxidative tissue damage during the acute phase of MPO-ANCA vasculitis.**

In the next phase of the project we successfully established a method to activate neutrophil granulocytes (isolated from blood of healthy volunteers) with isolated IgG fractions of ANCA vasculitis patient blood samples upon priming the neutrophils with TNF-α. We next investigated the effects of the sulfide donor molecule GYY4137 on different functions of ANCA-activated neutrophil granulocytes. First, with already published reliable assays we demonstrated, that GYY4137 inhibited the oxidative burst of autoimmune activated neutrophils in a dose dependent manner. We then confirmed that this inhibitory activity was

not due to inhibition of neutrophil NADPH oxidase activity, which is an essential component of the oxidative burst mechanism, but a result of diminished antibody-antigen recognition. Inhibition was even greater when the sulfide donor was added before neutrophil priming, which suggested that sulfide may also interfere with the priming mechanism, during which the autoantigen MPO is transferred to the cell surface. We confirmed this effect using STED microscopy, which showed that low concentrations of GYY4137 inhibited neutrophil priming by TNF-α, which is an important step in ANCA-induced activation of neutrophils. **Hence, we have evidence that sulfide diminishes ANCA-induced neutrophil oxidative burst via inhibiting both antibody-antigen recognition and neutrophil priming.**

Our next exciting data showed that sulfide inhibited neutrophil degranulation upon TNF-α treatment followed by ANCA activation. Using a published assay for neutrophil degranulation we determined the IC_{50} concentration of GYY4137 on this process, which was found to be lower than the IC_{50} concentrations for the inhibition of the oxidative burst. This difference also suggests additional involvement of sulfide with ANCA activation other than the inhibition of the initial activation by the autoantibodies. The decrease in excreted granular enzyme concentrations was not observed upon PMA activation, which was confirmed with SDS-PAGE analysis followed by silver staining of the neutrophil supernatants. **This experiment corroborates the strong inhibitory potential of sulfide on MPO-ANCA-antibody-induced neutrophil activation. The alleviating effects of sulfide on the autoimmune activation of neutrophils and the production and release of tissue damaging molecules could serve as a basis for new therapeutic interventions during the latent and the acute phases of ANCA vasculitis.**

Furthermore, we obtained strong evidence that sulfide diminishes the formation of neutrophil extracellular traps (NETs) upon activation with PMA. Induction of NET formation with ANCA showed great variability, however in experiments where NETosis was detected in untreated samples, treatment with a sulfide donor showed inhibitory effects. **Since NET formation was also associated with the severity of MPO-ANCA vasculitis we have further proof that sulfide could protect against tissue damage during the acute phase of the disease.**

The primary task of neutrophil granulocytes is destruction of pathogens via phagocytosis. Therefore, we investigated the influence of sulfide on this host protecting function of neutrophil white blood cells. Our data showed that the presence of sulfide did not influence

the ability of neutrophil granulocytes to engulf bacteria. **These finding suggest that despite the diminishing effects of H_2S on ANCA-induced activation, neutrophils retain their antimicrobial functions in the presence of sulfide.**

After the experiments with isolated cells, **we set up an animal model for ANCA vasculitis to test the validity of our results *in vivo*** as described in the original proposal. We optimized the immunization protocol to achieve high levels of anti-MPO antibodies in MPO knockout mice. Immune deficient mice (Rag2 knockout) were injected with isolated splenocytes of the immunized mice to induce the symptoms of ANCA vasculitis. Tissue samples were prepared for histological analyses. In collaboration with clinicians, the characteristic pathologies were identified. After the establishment of this animal model we tested the effects of several sulfide donors with different protocols of treatment on ANCA vasculitis symptoms presented by the animals. **Our results showed that: 1) Control animals did not show any signs of inflammation in the kidneys and lungs. 2) The untreated group showed significant signs of kidney and lung inflammation including necrotizing crescentic glomerulonephritis and small vessel vasculitis in the kidneys and lungs. 3) These symptoms were significantly diminished in the groups that were treated with the sulfide donor GYY4137.**

The animal experiments were also carried out using ATB-346, another sulfide donor molecule. With our collaborators we demonstrated that ATB-346 had substantial sulfide releasing potential in human subjects as part of a phase II clinical trial of the drug to treat osteoarthritis. (Wallace et al. 2020). Unfortunately, ATB-345 was not that efficient in our MPO-ANCA model systems as a protecting molecule.

Other well-known sulfide donors also did not diminish symptoms, thus using our sulfide detection method (Ditroi et al. 2019) we have measured the concentrations of sulfide from blood samples of treated animals. **We have found that treatments that effectively protected the mice against vasculitis symptoms also induced high elevation in blood sulfide levels.** Moreover, suboptimal treatment protocols did not result in significant changes in free sulfide concentrations in the blood serum of treated animals. **These results suggested that the elevation of blood sulfide levels could be an important marker of the efficacy of the treatment against MPO-ANCA vasculitis symptoms, which is important step in our future drug development endeavors.** In addition, on diagnostic grounds we have demonstrated that measurement of hydrogen sulfide levels can be a diagnostic tool for patients undergoing

vascular surgery. The results suggested that patients with higher sulfide production capacity measured from blood plasma had lower mortality after surgery during the 36 months post-surgery observation period (Longchamp et al. 2021).

After our exciting findings in the mouse ANCA model in collaboration with the University of Veterinary Medicine in Budapest **we have developed a new sulfide donor compound** that we carefully characterized on qualitative and quantitative chemical grounds. We found that the new compound was stable, and its sulfide releasing rate was not only comparable with that of GYY4137, but also more robust. We have also carried out acute toxicity and repeated dose toxicity testing in the nonlethal dose range followed by measurements of blood sulfide levels of treated animals. **Our data showed that the new molecule provoked an elongated increase in blood sulfide levels in mice without signs of toxicity.** Furthermore, we tested the new compound in the MPO-ANCA vasculitis mouse model using a similar treatment protocol that we used for GYY4137. **Our results showed that the new donor molecule strongly diminished inflammation in the kidney samples at a significantly lower dose than GYY4137.**

In our previous publications we demonstrated, that hydrogen sulfide can slowly be oxidized by MPO to generate sulfane sulfur species, which can react with cysteine residues of proteins to give cysteine per/polysulfides (Garai et al. 2017). We proposed that this process may protect proteins from irreversible oxidative damage via the formation of reducible cysteine modifications upon excessive oxidation. To be able to measure these modifications in sulfide treated samples we have developed a novel persulfide detection protocol (Doka et al. 2019; Hamid et al. 2019). In a comprehensive study we demonstrated that cysteine persulfide/polysulfide modifications of proteins can indeed be protective against oxidative damage and via this protection it can preserve protein functions, thus these reactions may also contribute to protect healthy tissue during the acute phase of MPO-ANCA vasculitis (Doka et al. 2020). In order to measure these differences in the levels of protein persulfidation of treated and untreated animals we established a new method in our laboratory that we also presented in several recent publications (Erdelyi et al. 2021; Mellis et al. 2021; Lin et al. 2019).

We have also studied the endogenous metabolic pathways of sulfide in patients with defects of sulfide producing and consuming pathways. We detected only moderately disrupted hydrogen sulfide metabolism in patients with severe inherited defects in sulfur amino acid

metabolism. These findings indicated that enzymes of the transsulfuration pathway may not be major contributors to the endogenous sulfide pool (Kozich et al. 2019).

Our studies on the reactions of hydropesulfides with myoglobin confirmed our theories that not only sulfide but its metabolic persulfide products also heavily interact with metalloproteins so we will next study their reactions with myeloperoxidase (Alvarez et al. 2020).

In addition, we also demonstrated that the biological actions of sulfide are intimately interlinked with those of nitric oxide (NO). Their chemical interactiojns produce nitrosopersulfide, which we demonstrated to be a unique cysteine persulfidating agent with reduction resistant bioactivity. It is a relatively stable molecule in biological environments and cannot be reduced by the thioredoxin and the glutathion reduction machineries, buts its decomposition products (polysulfide species) induce persulfidation on protein cystein residues which can serve as an important protecting mechanism during oxidative stress (Bogdandi et al. 2020).

Furthermore, we published two comprehensive review articles and an original research paper on the detection of reactive sulfur species and thiol oxidation states. We critically reviewed the current alkylation-based protocols, their advantages and caveats, which will serve as an important step in our further developmental endeavors to detect these important protein post-translational modifications and their functions in sulfur biology (Nagy et al. 2020; Nagy, Schwarz, and Kopriva 2019).

Based on our extensive cellular and animal experimental data we are working on filing a patent application on the proof of concept that is the topic of this project and have already taken steps toward the preclinical state of developing a new treatment for MPO-ANCA vasculitis. In order to avoid any novelty-destroying circumstances we strongly request not to publish our report.

References

Alvarez, L., V. Suarez Vega, C. McGinity, V. S. Khodade, J. P. Toscano, P. Nagy, J. Lin, C. Works, and J. M. Fukuto. 2020. 'The reactions of hydropersulfides (RSSH) with myoglobin', *Arch Biochem Biophys*, 687: 108391.

Bogdandi, V., T. Ditroi, I. Z. Batai, Z. Sandor, M. Minnion, A. Vasas, K. Galambos, P. Buglyo, E. Pinter, M. Feelisch, and P. Nagy. 2020. 'Nitrosopersulfide (SSNO(-)) Is a Unique Cysteine Polysulfidating Agent with Reduction-Resistant Bioactivity', *Antioxid Redox Signal*, 33: 1277-94.

Ditroi, T., A. Nagy, D. Martinelli, A. Rosta, V. Kozich, and P. Nagy. 2019. 'Comprehensive analysis of how experimental parameters affect H2S measurements by the monobromobimane method', *Free Radic Biol Med*, 136: 146-58.

Doka, E., E. S. J. Arner, E. E. Schmidt, and P. Nagy. 2019. 'ProPerDP: A Protein Persulfide Detection Protocol', *Methods Mol Biol*, 2007: 51-77.

Doka, E., T. Ida, M. Dagnell, Y. Abiko, N. C. Luong, N. Balog, T. Takata, B. Espinosa, A. Nishimura, Q. Cheng, Y. Funato, H. Miki, J. M. Fukuto, J. R. Prigge, E. E. Schmidt, E. S. J. Arner, Y. Kumagai, T. Akaike, and P. Nagy. 2020. 'Control of protein function through oxidation and reduction of persulfidated states', *Sci Adv*, 6: eaax8358.

Erdelyi, K., T. Ditroi, H. J. Johansson, A. Czikora, N. Balog, L. Silwal-Pandit, T. Ida, J. Olasz, D. Hajdu, Z. Matrai, O. Csuka, K. Uchida, J. Tovari, O. Engebraten, T. Akaike, A. L. Borresen Dale, M. Kasler, J. Lehtio, and P. Nagy. 2021. 'Reprogrammed transsulfuration promotes basal-like breast tumor progression via realigning cellular cysteine persulfidation', *Proc Natl Acad Sci U S A*, 118.

Garai, D., Z. Palinkas, J. Balla, A. J. Kettle, and P. Nagy. 2019. 'Measurements for Sulfide-Mediated Inhibition of Myeloperoxidase Activity', *Methods Mol Biol*, 2007: 179-203.

Garai, D., B. B. Rios-Gonzalez, P. G. Furtmuller, J. M. Fukuto, M. Xian, J. Lopez-Garriga, C. Obinger, and P. Nagy. 2017. 'Mechanisms of myeloperoxidase catalyzed oxidation of H2S by H2O2 or O2 to produce potent protein Cys-polysulfide-inducing species', *Free Radic Biol Med*, 113: 551-63.

Gross, W. L., W. H. Schmitt, and E. Csernok. 1993. 'Anca and Associated Diseases - Immunodiagnostic and Pathogenetic Aspects', *Clinical and Experimental Immunology*, 91: 1-12.

Hamid, H. A., A. Tanaka, T. Ida, A. Nishimura, T. Matsunaga, S. Fujii, M. Morita, T. Sawa, J. M. Fukuto, P. Nagy, R. Tsutsumi, H. Motohashi, H. Ihara, and T. Akaike. 2019. 'Polysulfide stabilization by tyrosine and hydroxyphenyl-containing derivatives that is important for a reactive sulfur metabolomics analysis', *Redox Biology*, 21.

Kimura, Y., and H. Kimura. 2004. 'Hydrogen sulfide protects neurons from oxidative stress', *Faseb j*, 18: 1165-7.

Klebanoff, S. J. 1999. 'Myeloperoxidase', *Proc Assoc Am Physicians*, 111: 383-9.

———. 2005. 'Myeloperoxidase: friend and foe', *J Leukoc Biol*, 77: 598-625.

Kozich, V., T. Ditroi, J. Sokolova, M. Krizkova, J. Krijt, P. Jesina, and P. Nagy. 2019. 'Metabolism of sulfur compounds in homocystinurias', *Br J Pharmacol*, 176: 594-606.

Lin, J., M. Akiyama, I. Bica, F. T. Long, C. F. Henderson, R. N. Goddu, V. Suarez, B. Baker, T. Ida, Y. Shinkai, P. Nagy, T. Akaike, J. M. Fukuto, and Y. Kumagai. 2019. 'The Uptake and Release of Polysulfur Cysteine Species by Cells: Physiological and Toxicological Implications', *Chem Res Toxicol*, 32: 447-55.

Lo Faro, M. L., B. Fox, J. L. Whatmore, P. G. Winyard, and M. Whiteman. 2014. 'Hydrogen sulfide and nitric oxide interactions in inflammation', *Nitric Oxide*, 41: 38-47.

Longchamp, A., M. R. MacArthur, K. Trocha, J. Ganahl, C. G. Mann, P. Kip, W. W. King, G. Sharma, M. Tao, S. J. Mitchell, T. Ditrói, J. Yang, P. Nagy, C. K. Ozaki, C. Hine, and J. R. Mitchell. 2021. 'Plasma Hydrogen Sulfide Is Positively Associated With Post-operative Survival in Patients Undergoing Surgical Revascularization', *Front Cardiovasc Med*, 8: 750926.

Mellis, A. T., A. L. Misko, S. Arjune, Y. Liang, K. Erdelyi, T. Ditroi, A. T. Kaczmarek, P. Nagy, and G. Schwarz. 2021. 'The role of glutamate oxaloacetate transaminases in sulfite biosynthesis and H2S metabolism', *Redox Biol*, 38: 101800.

Nagy, P., E. Doka, T. Ida, and T. Akaike. 2020. 'Measuring Reactive Sulfur Species and Thiol Oxidation States: Challenges and Cautions in Relation to Alkylation-Based Protocols', *Antioxid Redox Signal*, 33: 1174-89.

Nagy, P., G. Schwarz, and S. Kopriva. 2019. 'Highlighted mechanistic aspects in the chemical biology of reactive sulfur species', *Br J Pharmacol*, 176: 511-13.

Palinkas, Z., P. G. Furtmuller, A. Nagy, C. Jakopitsch, K. F. Pirker, M. Magierowski, K. Jasnos, J. L. Wallace, C. Obinger, and P. Nagy. 2015. 'Interactions of hydrogen sulfide with myeloperoxidase', *Br J Pharmacol*, 172: 1516-32.

Wallace, J. L. 2007. 'Hydrogen sulfide-releasing anti-inflammatory drugs', *Trends Pharmacol Sci*, 28: 501-5.

Wallace, J. L., J. G. Ferraz, and M. N. Muscara. 2012. 'Hydrogen sulfide: an endogenous mediator of resolution of inflammation and injury', *Antioxid Redox Signal*, 17: 58-67.

Wallace, J. L., P. Nagy, T. D. Feener, T. Allain, T. Ditroi, D. J. Vaughan, M. N. Muscara, G. de Nucci, and A. G. Buret. 2020. 'A proof-of-concept, Phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug', *Br J Pharmacol*, 177: 769-77.

Wen, Y. D., H. Wang, S. H. Kho, S. Rinkiko, X. Sheng, H. M. Shen, and Y. Z. Zhu. 2013. 'Hydrogen sulfide protects HUVECs against hydrogen peroxide induced mitochondrial dysfunction and oxidative stress', *PLoS One*, 8: e53147.

Whiteman, M., and P. G. Winyard. 2011. 'Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising', *Expert Rev Clin Pharmacol*, 4: 13-32.