PROJECT CATMAN: Catalytic Manganese-based anti-oxidants: an integrated approach from chemical design to bio-activity in cells

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Aim of the project and context:

Inflammatory Bowels Diseases (IBDs) are very disabling life-long diseases, with flares and remissions, characterized by a chronic inflammation of the gut associated to an overproduction of intracellular reactive oxygen species (ROS), including superoxide (O_2^{-}). Superoxide dismutases (SODs) are part of cellular anti-oxidant defense arsenal and they act by catalyzing the dismutation of superoxide into dioxygen and hydrogen peroxide, to which cells are more tolerant than superoxide. A deficiency of the anti-superoxide defenses in intestinal epithelial cells (IEC) has been shown to play a crucial role in IBD, the mitochondrial MnSOD (Mn-superoxide dismutase) being overexpressed but in an inactive form, and the cytosolic CuSOD being down-regulated.[1] Most patients require treatments involving immunosuppressants, which can induce detrimental secondary effects, such as increased susceptibility to infections and cancers.[2] Therefore, new approaches are clearly needed for managing these diseases and slow down the irreversible gut damage due to flares during disease course long life.

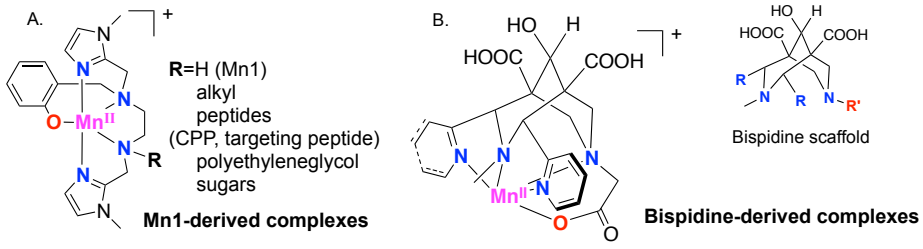
Targeting oxidative stress and specifically superoxide is an attractive approach that we have been exploring with a series of promising experiments in collaboration between the two groups, “metals in biology” from LBM and MI2 from Centre de Recherche de Saint-Antoine.[3] SOD mimics (SODm) are low-molecular weight complexes reproducing the activity of SODs.[4] SODm belong to the emerging class of catalytic drugs[5] as they react with superoxide in a catalytic manner and not as mere stœchiometric scavengers. Catalysis has many advantages such as the opportunity to lower dosage, which is important for a therapeutic use. Few SODm have been assayed so far in the context of IBD, but with some success.[3, 6]

The catalytic anti-oxidant we develop are manganese-based and bio-inspired by SODs active sites and efficient in a cellular context.[4c] Mn was selected as the leading cation as it does not lead to Fenton chemistry –associated with oxidative stress exaltation.[4a, c, 7] The usage of Mn-based anti-oxidants in a biological context foster new challenges in inorganic chemical biology, both for the characterization of the active species in cells and for the understanding of their bio-activity: as biological environments abound with Lewis bases and metal ion coordinating agents, the nature of the coordination sphere (or *speciation* of the complex) can be modified in cells, with the coordination of additional Lewis base(s), partial or complete decoordination of the original ligand (see scheme with coordinative atoms in blue or red). Apart from intrinsic catalytic activity, other parameters are essential to high biological efficacy:[8] cellular uptake, intracellular location(s), and concentration(s), or *speciation*. This question of the speciation is in line with issues associated with the lability of transition metal complexes: we will look for solutions to improve inertness, and thus limit decoordination in biological environments. It is thus important to investigate the activity of SOD mimics directly in the cellular environment under oxidative stress.[4c, 9] The thesis project “Catalytic Manganese-based anti-oxidants: an integrated approach from chemical design to bio-activity in cells” will be organized in three work-packages, including chemical design of SOD mimics, studies in a cellular model (bio-activities, intracellular quantification and distribution) and metabolic screening.

Methodology: work plan and packages

***WP1: SOD-mimics design (LBM). Mn1** (see scheme) was designed as a SODm bio-inspired from the active site of MnSOD. **Mn1** intrinsic SOD-activity is 1% that of the enzyme MnSOD, which is quite good since the kinetics of these enzymes is only limited by diffusion. **Mn1** was shown to have an anti-inflammatory activity in HT29-MD2 cells and mice.[3a] We will vary its chemical structure to modulate its physico-chemical properties. One aspect already under investigation is the conjugation of **Mn1** by a range of targeting moieties (scheme A). In particular the thesis project will be focused on the development of Mn-complexes with an improved inertness, to limit de-coordination in biological environments, which abound with Lewis bases. Capitalizing on our previous development of using 1,2-diaminoethane based ligands (see scheme A), we will constraint the ligand structure using a bispidine central scaffold[10] (see scheme B) to limit its flexibility and favor its coordination through pre-organisation of the ligand, according to previous strategies

developed in the literature.[11] This will be performed in collaboration with Loïc Charbonnière and Alice Nonat, from Institut Pluridisciplinaire Hubert Curien who have developed bispidine-based scaffolds for the design of inert Cu complexes.[11b] We will characterize the redox potential Mn(III)/Mn(II) redox couple of the of the SODm, which is a key parameter to the intrinsic activity that will also be determined as the namely kinetics of the superoxide dismutation using the MCord and Fridovich assay.[3]



Scheme: *SODms:* ***A****. Mn1 and targeting moieties,* ***B****. Bispidine series corresponding to more rigid scaffold, with higher inertness for metal release than the* ***Mn1*** *series. Color code: in blue and red: coordinating atoms to Mn(II) in pink.*

***WP2: Evaluation of SODm with a cellular assay metal-complexes analyses**

-WP2-A: Overall effects in HT29-MD2 (MI2). HT29 is a well-known IEC model from which HT29-MD2 cell line was created in the group MI2 at the CRSA in order to induce lipopolysaccharide (LPS) responses leading to both ROS production and pro-inflammatory cytokines secretion such as Interleukin -8 (IL-8). As MD2 protein is essential for LPS signaling through its interaction with a specific receptor, namely TLR4[12], this cell-model —HT29-MD2— was constructed by stable transfection of the accessory protein MD2 gene. Most drugs that are toxic substances —meant to kill cancer cells, bacteria, parasites, or more generally pathogenic agents; in contrast, SODm are **non-toxic** redox catalysts meant to **restore the normal activity** in cells under oxidative stress: specific disrupting investigation protocols are thus required. Recently, a methodology for the study of SODm in the cellular model HT29-MD2 has been successfully set up in the case of **Mn1**[3] and will be extrapolated to other SODm with (a) evaluation of the cell viability (LDH release into the culture medium), (b) evaluation of the inflammatory response (IL-8 secretion, COX-2 expression) and (c) evaluation of the expression and activity of intracellular MnSOD and CuSOD. In the frame of this IPV project, other markers will also be investigated to provide a description of the redox status of the HT29-MD2 cells [in (a) naive cells (non-activated cells), (b) cells under LPS challenge and (c) under LPS challenge treated with SODms]: superoxide production (hydroethydin[13] and ferricytochrome[14] assays) and H_2O_2 levels (dihydrodichlorofluorescein assay), GSH/GSSG assay.[15] Expression of anti-oxidant proteins and activities (catalase, GSH-peroxydase in addition to SODs) will also be quantified. Results will be compared to negative controls (e.g. $MnCl_2$, recombinant SOD that does not enter cells; note that so far, the "empty" ligand was not assayed as it displays a too high cytotoxicity to HT29-MD2, but **Zn1**, a redox-silent analog of **Mn1**, with the same charge but no anti-superoxide activity, was found to be inactive and is a good negative control).[3]

-WP2-B: Quantification, imaging and speciation in cells (MI2 and LBM). The SODm will be quantified in cells (EPR, ICP-MS) and their speciation will be questioned by MS/MS of cell lysates. Imaging techniques, such a microfluorescence X, will be used to determine the distribution of the SODm in cells, as previously applied in the case of **Mn1**.[3a] We will also image cells with fluorescent detectors of Mn^{II} with a range of association constants to distinguish between the SODm and "free Mn pool".[16] We will also use X-fluorescence imaging (nanoscopium, synchrotron SOLEIL) to obtain Mn distribution, as previously performed.[3]

***WP3: Metabolic screening (MI2).** Based on our previous studies using the HT29-MD2 cell line, we have already an insight into the main metabolic pathways affected by LPS and restored towards control by incubation with SODm: mitochondrial function has been shown to be affected by LPS challenge (under investigation in collaboration between the two groups). Hence, a metabolic screening with a focus on the mitochondrial membrane potential will be performed using TMRE (tetramethylrhodamine, ethyl ester). Redox status will be analyzed by high-content confocal microscopy using fluorogenic probes for measuring generalized oxidative stress in cells. The molecule 2',7'-dichlorofluorescein diacetate (DCFH-DA) freely permeates cells, and following the incorporation into cells is converted into the fluorescent 2,7-dichlorofluorescein (DCF) by oxidative substances, revealing the intracellular production of redox-active

substances.[17] In addition, we will screen the cells for the distribution of the SODm to characterize the effect with regard to the distribution, using fluorescent detectors of Mn(II) (see above) that can be implemented in a metabolic screening.

This approach of metabolic screening is really innovative in the context of SODm and goes through the comparison of (a) non-activated cells (naïve cells) as control (no induced oxidative stress, no inflammation) and (b) LPS-activated cells (c) LPS-activated cells treated with the SODm. In other terms, as in a color scale, the metabolic screening consists of questioning the proximity of (c) to (a) or (b) in a multi-dimensional space delimited by the choice of the markers. This will be performed on the screening plateforme in Institut Curie (BioPhenics high-content screening laboratory in collaboration with Dr. Elaine Del Nery).

Expected results

Metal-based drugs as coordinative complex are labile and potentially fragile in the biological environments, due to potential metal ion release, ligand and/or metal cation exchange. Our approach, by aiming at the development of Mn-complexes with improved inertness and targeting properties should lead to improved cellular activity that will potentially allow us to reduce concentration of incubation. Applying and developing an inorganic cellular chemistry approach, we will combine evaluations of the biological activity in a cellular model, with the exploration of the speciation, quantification of the intracellular content and determination of the cellular distribution through an imaging approach in the case of the study of Mn-complexes mimicking the activity of the superoxide dismutase (SOD).

From a societal point of view, the socio-economic issues are important because IBD affect young subjects and the incidence is relatively high in industrialized countries (8-15 per 100 000 inhabitants/year). Long-life risk is estimated up to 1% in Europe.[2] Thus, there is a clear and urgent need for new ways of controlling this disabling pathology. The long-term outcome of this project could be to provide a new and efficient way of managing IBD but also other intestinal pathologies associated with inflammation or oxidative stress.

In this context, the PhD student will be trained in organic synthesis of ligands, inorganic synthesis, physico-chemical studies (UV-vis, EPR, ICP-MS and MS/MS), cell biology (cell culture, biochemical and molecular biology techniques) and bio-imaging (fluorescence, X-fluorescence and metabolic screening). He/she will then benefit from an extensive training in inorganic chemical biology and cell biology.

The two groups, LBM and MI2, have a strong complementary expertise, in bio-inorganic chemistry, with an extensive expertise in SODm design and study (LBM) and cell-biology of cells under oxidative stress, with an extensive experience in IBD: the consortium, with MI2 being a group of medical doctors working on IBD, is thus an ideal context for the development of anti-oxidants efficient against IBD.

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