Chapter 8
Summary, general discussion, and future perspectives
Summary

Vascular redox disturbances are a common factor in highly prevalent diseases including diabetes mellitus, kidney disease and atherosclerosis. ROS-induced vascular dysfunction also plays a role in less prevalent but no less severe settings such as surgical kidney injury, kidney transplantation, pulmonary arterial hypertension, and dysregulated sulfur amino acid metabolism. The severity and global burden of vascular diseases reflects a gap in targeted pharmacotherapy. The work in this thesis therefore aimed to advance pre-clinical development of novel treatment for redox disturbances in vascular disease. In part I, an existing library of novel 6-chromanol based compounds with mitochondrial effects was investigated in a phenotypic screening pipeline. Subsequently, hit compounds were validated for efficacy in the treatment of vascular disease in experimental models of kidney injury, diabetes, kidney transplantation, and pulmonary hypertension. In part II, new treatment targets were identified and validated in vascular disease: perivascular adipose tissue in experimental atherosclerosis in rats, and cystathionine-β-synthase in a novel mouse model of inducible hyperhomocysteinemia.

General discussion and future perspectives

Phenotypic screening in modern-day drug discovery

Compared to modern reverse drug discovery where large numbers of compounds are screened for hits on a specific (protein) target with a known structure, the strategy selected for SUL compounds was a paradigm return to phenotypic screening, i.e., forward pharmacology. The desired effect in this campaign was a phenotype observed in seasonal hibernators, in which vascular disease is absent despite conditions which greatly tilt homeostasis towards potential for injury. The novel class of 6-chromanols was designed to mimic mitochondrial organ protection and studied for the purpose of vascular therapy. In chapter 2, a phenotypic screening of a library of 6-chromanols in a model of cell hypothermia resulted in the identification of the protective compounds SUL-121 and SUL-138. Aside from cell protection, these compounds exhibited favorable pharmacokinetic properties and effects in mitochondria. The efficacy in treatment of experimental vascular disease associated with redox disturbances was subsequently demonstrated in several in-vivo models, validating the phenotypic screening approach to discover effective SUL compounds.
Chemical structure-function relationships in the context of redox vascular disease

An intriguing property of SUL compounds is their efficacy in different disease models, also called multipotency. For example, SUL-121 demonstrated direct ROS-scavenging effects, protected cells from cooling-induced death, prevented vascular disease and stopped the progression of kidney damage in a mouse model of diabetes, demonstrated anti-inflammatory effects in COPD, and halted vascular dysfunction in PAH (chapter 5). The vascular diseases investigated in this thesis shared oxidative stress as an underlying factor; and elevated ROS are typically a result of mitochondrial dysfunction. As shown in adipose tissue-derived stem cells and in chapter 5, the effect of SUL compounds on the mitochondria presents a plausible explanation of their multipotency in redox-based vascular disease. Although a specific target of SUL compounds in the mitochondria was not yet confirmed, 6-chromanols share a common feature that provides a promising cue, i.e. the opening of the 6-chromanol ring and formation of a redox quinol/quinone system (Figure 1). Theoretically, 6-chromanol ring opening leads to an increase in the number of rotatable bonds (Figure 1), thereby creating conformational isomers, and potentially increasing the number of interactions with different receptors. In addition, the formation of quinones, which are known electron acceptors for complexes I and II, can also shuttle electrons from the cytosol to complex III. This is conceivable as their small size enables SUL compounds to pass through the outer mitochondrial membrane freely via porins, and subsequently be exposed to the enzymatic ensemble and ROS of the intermembrane space and inner mitochondrial membrane (IMM). It is yet unknown if the putative oxidative ring opening of SUL compounds occurs spontaneously in aqueous solutions or if enzymatic catalysis is required. Theoretically, SUL compounds can donate a hydrogen from the 6-OH group in a single-electron oxidation reaction (Figure 1) as is the case for the chromanol moiety in α-tocopherol. In a second-electron oxidation, the chromanol ring opens to yield a redox-capable quinone moiety with increased water solubility. The quinol/quinone moiety is also the functional core of ubiquinol (Coenzyme Q10, CoQ10), which carries electrons within the IMM from complexes I and II to complex III. Unlike SUL compounds, ubiquinol is hydrophobic and shuttles electrons while remaining in the IMM. Together, evidence accumulated of mitochondrial redox effects of SUL compounds in various disease models. The theoretical structural considerations of chromanol ring opening suggest that SUL compounds may in fact be prodrugs that should be tested separately alongside their parent drugs.

SUL compounds are not the only multipotent compounds with mitochondrial activity. Other 6-chromanols are also under development for the treatment of diseases that are rooted in mitochondrial dysfunction. One of these, the compound sonlicromanol (KH176) owned by the Nijmegen-based company Khondrion, is currently investigated in phase 2 clinical trials for a rare mitochondrial disease caused by the mitochondrial
DNA mutation tRNA\text{Leu}(UUR) m.3243A>G\textsuperscript{12}. In preclinical screening of KH176, the same principle of 6-chromanol ring opening as in SUL compounds was proposed\textsuperscript{13}. The parent drug KH176 was found almost fully converted to the quinone metabolite KH176m in plasma of dogs, and KH176m was also detected in brain, heart, muscle, and liver. The metabolite KH176m demonstrated increased potency over its parent drug KH176 in protection from cell death induced by buthionine sulfoximine (BSO) via inhibition of glutathione synthesis. The discovery of higher potency of the metabolite KH176m over its parent drug KH176 consequently led to studies dedicated to the effects of KH176m on prostaglandin biosynthesis\textsuperscript{14}. Importantly, by substituting the 6-hydroxy group on the chromanol moiety of KH176 by a methoxyl, the resulting compound KH176i could no longer form a quinol and lost all ROS activity. Together, the structure-function information obtained for KH176 indicated that the phenolic 6-hydroxy group in 6-chromanols is the active redox moiety. In closed form, 6-chromanols may owe antioxidant properties to a single 6-phenolic group, while opening of the chromane ring creates a quinol/quinone system with higher redox capacity.

Although the 6-chromanol ring opening is also possible in Trolox, SUL compounds are much more potent in protecting HEK293 cells from hypothermia-induced death. Unlike Trolox which is a carboxylic acid, SUL compounds are amides. The simplest compound SUL-125 is a hydroxamide of Trolox and demonstrated over 100-fold higher potency over Trolox\textsuperscript{7}. The derivative of propofol SUL-131 which has potential for ring opening and formation of quinol/quinone and is also a hydroxamide demonstrated over 1000-fold higher potency over Trolox\textsuperscript{7}. Other SUL compounds are generally piperazinecarboxamides of Trolox or propofol. Together, these structure-activity relationship data suggest that the switch on the 6-chromanol chiral atom from carboxylic acid to carboxamide contributes to an increased potency in cell protection from hypothermia.

![Figure 1](image1.png)

**Figure 1** Oxidative opening of 6-chromanol heterocycle forms a quinone. \textit{R1} indicates different substituents on SUL compounds, and * indicates chiral carbon.

While chromanol ring opening and mitochondrial effects could contribute to the multipotency of SUL compounds in redox vascular diseases, the anti-adrenergic effect observed in chapter 4 is likely independent of mitochondria. The chemical property that lends SUL-121 the binding specificity for adrenergic receptors is chirality, i.e., existence of molecules with the same atoms but different orientation of substituents on the chiral carbon. Some SUL compounds were synthesized from a mixture of two enantiomers...
(racemate) of Trolox, therefore the product was also a racemate. Stereochemistry can cause differences in receptor interactions and for this reason the racemate SUL-121 was characterized in chapter 4, specifically the mechanisms of action of the individual enantiomers (designated SUL-150 and SUL-151). The \((R)\)-enantiomer SUL-150 was found to be an antagonist of \(\alpha_1\)-adrenergic receptors, resulting in improved machine perfusion parameters of cold stored porcine kidneys during rewarming\textsuperscript{15}. These enantio-specific effects of SUL-150 were later utilized for a specific application in pulmonary arterial hypertension (PAH, chapter 5). Chirality of SUL compounds is therefore considered a source of additional undiscovered mechanisms of action with therapeutic benefit.

Adrenergic antagonism of \((R)\)-enantiomers may represent an undesired effect in treatment of other diseases, which is why \((S)\)-enantiomer SUL-138 was selected for advancement to clinical trials for Alzheimer’s disease\textsuperscript{16}. In the study of 2-disubstituted 6-chromanols, enantiospecific effects should be either characterized or eliminated by stereopure synthesis.

Quantitatively, SUL compounds are more potent in hypothermic cell preservation than adrenergic antagonism. Specifically, the \(EC_{50}\) of SUL-121 and SUL-138 in protecting HEK293 cells from hypothermia-induced death was approximately 3nM\textsuperscript{7}, while the \(A_2\) value of SUL-121 in \(\alpha_1\)-adrenergic receptor antagonism was in the 10µM magnitude\textsuperscript{15}. Therefore, the mitochondrial action remains the primary mechanism of SUL compounds, though a specific mitochondrial receptor target(s) still remain unidentified.

In the initial library of SUL compounds, there were several 6-chromanols that protected cells from hypothermic death with an \(EC_{50}\) of 10-30nM but were not selected for further development because SUL-121 and SUL-138 were more potent options\textsuperscript{7}. This set of compounds has structural diversity that represents an opportunity for the discovery of secondary targets similarly to \(\alpha_1\)-adrenergic receptors and SUL-121. The benefit of developing new multipotent 6-chromanols may be the targeting of specific organs, tissues, leveraging different pharmacokinetic properties, and ultimately diversification of treatment.

**Subcellular trafficking of 6-chromanols**

In addition to understanding the structure-function relationships of SUL compounds, appraisal of their full pharmacodynamic effects necessitates an understanding of the compounds’ distribution within the cell. This knowledge will help direct the search for molecular targets, but also identify potential bottlenecks in subcellular transport in disease conditions, such as, active transporters. The 6-chromanol core structure resembles small mitochondrial redox molecules including tocopherols and ubiquinol, which have a long hydrophobic chain that causes incorporation into the inner mitochondrial membrane. Here, tocopherols convey protection from lipid peroxidation by rapid ROS scavenging\textsuperscript{17}. It is not known exactly how tocopherols are trafficked into the inner mi-
tochondrial membrane\textsuperscript{17,18}, though it was suggested that their transport corresponds to those of lipids\textsuperscript{19}. Similarly, synthetic triphenylphosphonium-based compounds such as Mitoquinol (MitoQ), also contain a lipophilic chain which targets them to the IMM\textsuperscript{20}. SUL compounds lack such long aliphatic chains and remain hydrophilic. It is yet unclear whether and why hydrophilic 6-chromanols such as SUL compounds or sonicromanol would specifically target mitochondria. The hydrophilicity and size of SUL compounds supports passive diffusion across the outer mitochondrial membrane, as opposed to active transport. Nevertheless, it is currently unknown whether SUL compounds accumulate in mitochondria. If the subcellular distribution of 6-chromanols favors mitochondria, it is also unclear whether they accumulate in the intermembrane space, or in the matrix. Thus far, there are no active mitochondrial transporters of 6-chromanols identified. A good starting point for the search of transporters might be the similarity of the core scaffold to α-tocopherol, and the similarity of the open-ring quinone metabolite to Coenzyme Q\textsubscript{10}. Moreover, the fact that 6-chromanols with different substituents on C2 carry mitochondrial effects supports the involvement of the core scaffold in subcellular distribution. Subcellular distribution of SUL compounds is currently under investigation by the means of separating subcellular fractions, lysis, and quantification using chromatography. Another approach would be to prepare radiolabeled SUL compounds and measure radioactive counts in isolated subcellular fractions. Whether SUL compounds are actively transported can be experimentally tested by cooling cells to halt ATP-dependent transport and measuring the concentrations of SUL compounds in isolated mitochondria.

A curated database of the mitochondrial proteome, the MitoCarta3.0\textsuperscript{21}, currently contains 1140 mouse proteins, of which 52 were found localized in the intermembrane space. Of these, most are factors involved in the assembly of OXPHOS, in protein turnover and transport, in autophagy and apoptosis, and 9 have unknown ontology. There were two proteins in the intermembrane space with ontology that suggested a potential for targeting by SUL compounds. Stard7, which was flagged in MitoCarta3.0 for small molecule transport, specifically that of phosphatidylcholines, may also be involved in the transport of SUL compounds. Catalase, which is responsible for the breakdown of H\textsubscript{2}O\textsubscript{2}, may be facilitating the antioxidant effects of SUL compounds. The binding of SUL compounds to these two candidate receptors could be verified by measuring a shift in thermal denaturation peaks of purified protein in the absence and presence of SUL-compounds.

**What is the mode of treatment of SUL compounds?**

For the development of SUL compounds for therapeutic purposes, it is important to distinguish whether they have prophylactic effects, halt the progression of disease, or cure disease. In studies in rats in chapter 2, SUL-138 was administered prior to ischemia/
reperfusion-induced acute kidney injury and demonstrated prophylaxis in terms of normalized NGAL, urine volume, creatinine, urea, potassium and magnesium. In cell models of hypothermia in **chapter 2**, SUL compounds needed to be administered before the inception of cooling to be effective in preventing cell death (unpublished data). This limitation in administration time may be specific to the HEK293 cell model where ATP levels start decreasing after 2 hours of cooling,

\(^{22}\)

, are significantly deficient after 12 hours of cooling,

\(^{23}\)

, and any resulting cell death is not reversible. In the mouse diabetic db/db model studied in **chapter 3**, treatment was started at an early stage of diabetes with mild albuminuria. Progression of albuminuria was halted upon SUL-121 treatment, but albuminuria did not decrease in the subsequent weeks. In **chapter 5**, treatment with SUL-150 was initiated 7 days after administration of monocrotaline that induced the development of pulmonary arterial hypertension (PAH). The timing of administration was synchronized with the placement of the aortocaval shunt and was designed to capture the effects of SUL-150 during a reversible stage of PAH. SUL-150 halted the remodeling or pulmonary arteries and the increase in \(\alpha_1\)-adrenergic vasoconstriction but did not normalize these parameters to the levels of sham groups. Therefore, the identified modes of treatment of SUL compounds were prophylaxis and halting of disease progression.

**Further development of SUL compounds**

Although the SUL compounds that emerged from the initial library branched into different development paths, collectively they underwent a battery of early preclinical tests detailed in **chapter 2**. From the initial library of SUL compounds, the compound SUL-138 is currently the furthest in development (Figure 2). Clinical trials for Alzheimer’s disease are already planned by the Turkish pharmaceutical company Gen\(^{16}\) (Figure 2). However, to advance the compound SUL-138 to first-in-man clinical trials, several prerequisites must still be met. These include animal testing in GLP facilities to demonstrate the absence of organ damage using histology, dose escalation, repeat-dose toxicity, reproductive and development toxicology, photosafety, and local tolerance. Specific requirements may depend on whether the trial will be regulated by European or Asian authorities. Also, parameters need to be identified for clinical monitoring of potential adverse effects. Based on the intended indication of SUL-138 detailed in **chapter 2**, these parameters may include hemodynamic and respiratory parameters, markers of kidney damage and systemic ROS. The multipotency of SUL compounds is also a benefit for efficient advancement of clinical development. For instance, specific data generated in safety testing of SUL-138 prior to trials in Alzheimer’s disease may be applicable to development of SUL-138 for the prevention of surgical acute kidney injury. Simultaneous development of one drug for multiple applications can therefore reduce costs and accelerate the process.
Ongoing research in SUL compounds lends from the original library of newly designed and synthesized 6-chromanols that were processed by a phenotypic characterization pipeline. Due to the absence of a known target protein, hits were directly advanced to testing in animals without prior optimization of the lead chemical structure. Though the compound SUL-109 achieved potency in the low micromolar range in protecting cells from hypothermia, the potency of SUL-150 in inhibiting $\alpha_1$-adrenoceptors was in the 10-100µM range. Compared to the potency of prazosin, which is effective in nanomolar concentration, the therapeutic concentrations of SUL-150 may not achieve the effects desired for adrenergic antagonism. Therefore, lead optimization of SUL-150 is warranted for applications requiring both mitochondrial protection and $\alpha_1$-adrenoceptor antagonism.

As shown in chapter 5, targeting mitochondria and redox mechanisms, as well as $\alpha_1$-AR represents a novel strategy for the treatment of vascular disease. The multipotency of SUL-121 or SUL-150 might also be fitting for the treatment of Raynaud’s disease which is characterized by cold-induced, ROS- and adrenergic-driven vasoconstriction in the extremities, especially in the fingers and toes. Antagonists of $\alpha_1$ adrenergic receptors may be suitable for countering adrenergic vasospasms in the extremities but should not adversely affect systemic blood pressure. Rather, novel drugs should act locally only during episodes of vasospasm. We found that the $\alpha_1$ adrenergic receptor antagonists prazosin and SUL-150 had increased potency when porcine intrarenal arteries were cooled from 37°C to 24°C (unpublished data). This feature could be leveraged to apply dosing regimens that are ineffective in controlling systemic blood pressure but effective in countering adrenergic constriction in cooled extremities. The advantage of SUL compounds over established $\alpha_1$-AR antagonists would be the additional protection from cold-induced ischemia and from ROS damage. The proposed treatment of Raynaud’s disease with SUL compounds can be tested in the rat tail artery, with the tail being considered an extremity, or in mouse cutaneous plantar arteries, which were previously
used to demonstrate the involvement of α2C- and α1-adrenoceptors (ARs) in cold-induced vasoconstriction24.

Another application of SUL derivatives may be in benign prostate hyperplasia, which is rather successfully treated with selective α1-blockers but often accompanied by unwanted sexual and neurological side effects. SUL-150 demonstrated a larger affinity towards the α1A-AR subtype than to α1B or α1D, and α1A-ARs are abundant in the prostate as compared to the vascular α1B-receptor. Therefore, the selectivity of SUL-150 could be used to develop novel therapy for benign prostate hyperplasia with minimized adverse effects.

**Application of 6-chromanols to newly identified pathophysiology**

In chapter 3, the compound SUL-121 was used to treat diabetic db/db mice with elevated ROS, endothelial dysfunction, and kidney damage, resulting in halted disease progression. The endothelium-protective effects of SUL-121 that were seen in diabetic mice are of therapeutic interest and can be extended to other diseases. Specifically, endothelial dysfunction is present throughout the development of atherosclerosis, and is associated with elevated ROS and inflammation of the vascular wall including PVAT (chapter 6). The compound SUL-121 is an interesting candidate for the treatment of atherosclerosis for its combined efficacy in disease with endothelial dysfunction, elevated ROS, and inflammation6,8. It would be interesting to investigate whether SUL compounds inhibit infiltration of inflammatory cells into the vascular wall, specifically in the intima in atherosclerosis, but also in PVAT, especially when considering the high abundance of macrophages found in chapter 6. Consistent with previous findings of the ability to inhibit mitochondrial ROS production, SUL-121 reduced oxidative lipid breakdown into MDA induced by LPS in guinea pig lungs. This effect may also be responsible for stabilizing the endothelial cell membrane in vascular disease models treated with SUL compounds (chapters 2, 3). Furthermore, in airway smooth muscle cells challenged with cigarette smoke extract, SUL-121 reduced the release of the inflammatory cytokine IL-86, which is known to be responsible for neutrophil recruitment25. Decreased levels of p65 were found in the nucleus, indicating an inhibition of NF-κB by SUL-121. SUL-121 also decreased nuclear translocation of the anti-oxidant and anti-inflammatory transcription factor Nrf2 in airway smooth muscle cells (ASMCs) after stimulation with cigarette smoke extract or LPS6. Together, several observations from these studies suggest that SUL-121 exerts anti-inflammatory effects, possibly via a decreased requirement for the translocation of nuclear transcription factors that boost ROS defense. However, it is unclear whether SUL-121 binds specifically to inflammatory mediators or transcription factors, or whether this apparent mechanism is the sole result of ROS inhibition or preservation of ATP levels.
Chronic treatment with SUL-121 also restored the sensitivity of vascular smooth muscle (VSM) to nitric oxide (NO) by a yet unknown mechanism. In *chapter 6*, we found that in atherosclerosis, perivascular adipose tissue (PVAT) can take over the role of impaired endothelium by releasing NO. Sensitizing the VSM to NO by SUL-121 would improve the responsiveness of the vascular wall to PVAT-released NO. Furthermore, the discovery of PVAT-specific compensation of NO in *ApoE*−/− rats was made in isolated tissue on a functional level. It would be worth investigating whether SUL compounds and PVAT itself suppress or promote the development of atherosclerotic lesions in this model.

While cold machine perfusion is the standard preservation method for graft organs in the Netherlands, benefits of warm machine perfusion are emerging. The compound SUL-150 was investigated in *chapter 4* in graft porcine kidneys that underwent static cold storage, since that is still the most common organ preservation method globally. While SUL-150 inhibited adrenergic vasoconstriction in graft kidneys undergoing re-warming, effects on endothelial protection, organ preservation and graft function should still be investigated. SUL-150 could become an economic alternative in settings where machine perfusion is not available, and its beneficial effects in machine perfusion are by no means excluded. As SUL-150 was effective in both cooled and warm tissue, it is expected to improve preservation of organs in both hypothermic and normothermic machine perfusion. To control vascular motion and perfusion parameters, vasodilators are added to machine perfusion solution including verapamil or nitric oxide donors. In machine perfusion of graft organs, SUL compounds may be suitable for protecting endothelium-dependent NO-mediated vasorelaxation, and by sensitizing the vascular smooth muscle to NO.

The oxidative stress that accompanies vascular diseases can be caused by elevated mitochondrial ROS production or by deficient ROS defense, in fact two important antioxidant molecules glutathione and hydrogen sulfide (H2S) were found deficient in vascular diseases. Glutathione synthesis requires the amino acid cysteine which is formed in the transsulfuration pathway. Transsulfuration also forms a byproduct, the gaseous signaling molecule H2S. The entry and rate-limiting enzyme in transsulfuration is the enzyme cystathionine-β-synthase (CBS), which when deficient leads to accumulation of homocysteine, deficient synthesis of cysteine, glutathione, and H2S. Because of these properties, CBS is a major regulatory point for redox homeostasis and organ protection and is an emerging target in vascular disease. In *chapter 7*, we developed a model of global CBS knockout in adult mice that was characteristic with a mild phenotype of endothelial dysfunction, elevated ROS, and accelerated aging. Deficiency of CBS also manifested in hair loss and disruption of the morphology of hair follicles, particularly that of sebaceous glands which are known to be maintained by stem cells. It was not clear whether this pathology was due to local effect of CBS deficiency in specific tissue, or a general reduction of transsulfuration flux that leads to reduced glutathione production.
and antioxidant defense. Additionally, full body CBS knockouts did not develop severe disease, suggesting that CBS is not essential in adult healthy animals and additional antioxidant systems may cope with ROS defense. Therefore, we are currently characterizing a liver-specific CBS knockout in mice fed a high-fat diet to develop metabolic syndrome, and preliminary results confirmed signs of elevated ROS and endothelial dysfunction. In light of these facts, another follow-up study was scheduled to investigate the benefits of SUL compounds in treating liver-specific CBS deficiency in experimental metabolic syndrome.

In addition to SUL compounds, CBS deficiency could be targeted by drugs that would positively modulate CBS activity via its unique property of allosteric activation. Positive allosteric modulation of CBS was already possible using the endogenous allosteric activator S-adenosylmethionine (SAM, AdoMet)\(^\text{33}\). However, SAM is not suitable for therapeutic purpose due to its involvement in DNA methylation reactions as a universal methyl donor. Since there are no available drugs that positively modulate CBS activity in diseases with transsulfuration deficiency, we initiated a project towards the discovery of novel allosteric activators of CBS. The aim for these newly-to-be discovered drugs is to combat ROS-associated vascular dysfunction and promote organ protection in diseases with dysregulated transsulfuration such as metabolic syndrome, diabetic nephropathy, Alzheimer’s disease, inflammatory lung disease, hypertension, and edema.

**Conclusion**

In summary, ROS-associated vascular dysfunction is a highly common underlying factor in diseases including ischemic heart disease, stroke, Alzheimer’s disease, diabetes mellitus, kidney disease, and pulmonary arterial hypertension. The work in part I of this thesis advances the development of a library of 6-chromanols for the treatment of ROS-associated vascular disease. Lead compounds are characterized for their efficacy, mechanism of action, and safety. The evidence presented in this thesis constitute a background for further development of SUL compounds towards the clinic. The phenotypic screening library of SUL compounds is highlighted as a collection of compounds with unexhausted potential for future discoveries of efficacy in additional diseases and new mechanisms of action. These may include target mechanisms of atherosclerosis and transsulfuration deficiency identified in part II of the thesis.
References


