Protein arginine methyltransferases as modulators of lipid metabolism and inflammation and the relevance for atherosclerosis

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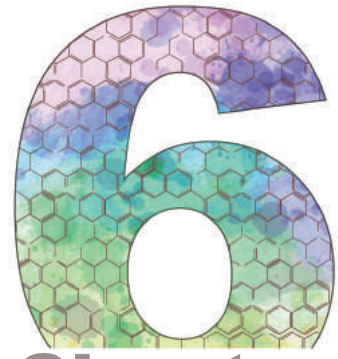

Summary, General discussion and Perspectives

SUMMARY

Acute cardiovascular clinical events such as myocardial infarction and cerebral stroke represent the major cause of death in Western societies. These pathologies are primarily resulting from atherosclerosis, a progressive condition characterized by the accumulation of lipids, immune cells, and fibrous elements in large arteries [1][2]. The pathogenesis of atherosclerosis involves complex interactions between a wide variety of cells, including monocytes, macrophages, neutrophils, and lymphocytes (as reviewed in **Chapter 1**). Since hyperlipidemia is an established risk factor for the development of atherosclerotic lesions, current therapeutic approaches are primarily focused on lowering plasma lipid levels, i.e. through statin treatment. Unfortunately, statins reduce atherosclerosis-associated mortality burden by only 25% [3]. It is therefore essential to identify novel targets for therapeutic application in order to reduce the residual atherosclerotic cardiovascular disease risk in current and future patients.

Importantly, recent studies have suggested that members of the protein arginine methyltransferase (PRMT) family can potentially serve as novel therapeutic targets for atherosclerosis because of their regulatory role in inflammation and metabolism [4][5][6][7][8]. To validate the contribution of PRMTs in the progression of atherosclerosis, in the studies presented in this thesis we have investigated the effect of inhibition of PRMT functionality on atherosclerosis susceptibility in established atherosclerotic mouse models. Of note, previous studies have indicated that mice with a genetic deletion of PRMTs display severely inhibited growth and survival issues. For example, Shibata et al showed that genetic deletion of PRMT1 is embryonically lethal in mice [9]. Similarly, deletion of PRMT4 in mice leads to developmental defects in the respiratory system and neonatal death [10]. As such, it was anticipated that generating hypercholester-

olemic mice with a genetic defect in one or more PRMTs would be very difficult or even impossible. To address the role of PRMTs in atherosclerosis, we therefore made use of specific PRMT inhibitors, i.e. TC-E 5003 for PRMT1 inhibition, TP-064 for PRMT4 inhibition, and GSK3326595 for PRMT5 inhibition, that thus far have primarily been applied in vivo in the context of cancer treatment. For a global overview of the findings obtained with the research, see table 1.

Table 1: *Overview of the effects on atherosclerosis, inflammation and metabolism induced by treatment with different PRMT inhibitors used in the studies presented in this thesis*

PRMT inhibitors	Mouse model	Atherosclerosis	Inflammation	Metabolism
TC-E 5003 (PRMT1)	·Male LDL receptor knockout mice	·Lesional macrophages ↓	·Inflammatory Ly6C^{hi} monocytes ↓ ·Effector memory T cells ↑	·Body weight gain = ·Liver TG level ↓ ·Hepatic lipogenesis genes ↓ ·Plasma CHOL & TG ↓
TP-064 (PRMT4)	·Male APOE knockout mice	·No obvious changes	·LPS-induced M1 macrophage polarization & cytokine production ↓ ·Pro-inflammatory neutrophilia ↑	·Body weight gain ↓ ·Adipogenesis genes ↓ ·Insulin secretion & signaling ↓ ·Plasma CHOL = ·Plasma TG ↓
GSK3326595 (PRMT5)	·Female LDL receptor knockout mice	·No obvious changes	·IFNγ-induced M1 macrophage polarization & cytokine production ↑	·Body weight gain = ·Liver TG level ↑ ·Hepatic lipogenesis genes ↑ ·Hyperlipidemia =

The effect of PRMT inhibitor treatment on atherosclerosis susceptibility

Previous clinical studies have demonstrated that higher plasma levels of the PRMT methylation products, asymmetric dimethylarginine (ADMA) and monomethylarginine (MMA), independently correlate with an increased risk for

cardiovascular disease, including coronary heart disease and stroke [11][12][13][14]. This suggests that PRMT activities can potentially modulate the risk for atherosclerotic cardiovascular disease. Among the PRMTs, 90% of the ADMA generating methylation activity is executed by the type I methyltransferase PRMT1 [15]. In support, Dhar et al. showed that cellular loss of PRMT1 activity through genetic deletion significantly reduces ADMA amino acid levels [16]. In addition to its role in ADMA production, PRMT1 has also been found to be overexpressed in myocardial tissue of patients with coronary heart disease [17]. Furthermore, PRMT1 is upregulated in livers from mice fed a high-fat diet [18] and has been identified as an essential modulator of hepatic lipid metabolism [19][20]. Based upon the aforementioned findings illustrating its involvement with multiple risk factors, we hypothesized that PRMT1 may be a promoter of atherosclerosis development. In **Chapter 2**, we have shown that pharmacological inhibition of PRMT1 by TC-E 5003 administration in mice was associated with a variety of systemic effects, including reduced hepatic triglyceride accumulation, diminished hyperlipidemia, and a reduced monocyte activation in the context of increased T cell activation. Furthermore, we found that the aortic macrophage content was reduced in TC-E 5003-treated mice, suggesting a lower atherosclerosis susceptibility in response to PRMT1 inhibition. It can thus be concluded that PRMT1 (1) plays a role in regulating both inflammatory and metabolic activities in vivo and (2) stimulates the development of atherosclerosis.

Similar to PRMT1, PRMT4 (CARM1) is a type I PRMT found to be overexpressed in white blood cells from patients with coronary heart disease [21]. PRMT4 is a co-activator of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor that regulates inflammatory activities [60]. Since augmented inflammation can theoretically promote atherosclerotic lesion development, we hypothesized that inhibition of PRMT4 by its specific inhibitor TP-064 may dampen the chronic inflammatory response dur-

ing the development of atherosclerosis and thereby reduce plaque burden. However, in **Chapter 4**, we have shown that TP-064-mediated PRMT4 inhibition did not significantly change the susceptibility for early atherosclerotic lesion development in male hyperlipidemic APOE knockout mice, while it did significantly impact both inflammatory and metabolic processes in vivo.

PRMT5 is a type II PRMT family member that has also been linked to inflammatory and metabolic activities [7][22]. Upregulation of PRMT5 in blood cells or downregulation of PRMT5 in cardiomyocytes were found to be correlated with increased development of cardiovascular disease [23][24]. As such, it is currently not clear what the exact impact of PRMT5 on atherosclerosis susceptibility is. For this purpose, in the study presented in **Chapter 5**, we treated Western-type diet-fed LDL receptor knockout mice with the PRMT5 inhibitor. Unfortunately, we found that pharmacological PRMT5 inhibition did not alter the general inflammatory state or atherosclerosis susceptibility in our experimental setting. However, PRMT5 inhibition was associated with an unexpected increase in atherogenic diet-induced hepatic triglyceride accumulation.

Overall, these combined findings show that pharmacological inhibition of PRMT1, PRMT4, or PRMT5 does not have a major impact on atherosclerosis susceptibility in hypercholesterolemic mice. However, it should be noted that in all our different experiments we studied the effect of PRMT inhibition on early atherosclerosis development as highlighted by the small lesions found in our experimental mice (lesion sizes $<200 \times 10^3\ \mu m^2$). Importantly, the suggestion of PRMTs as potential targets for atherosclerosis in the human clinical setting was derived from patients who already displayed advanced stages of atherosclerotic lesions [17][25][21][23][24]. Thus, it cannot be excluded that PRMTs rather contribute to atherosclerosis in later stages of the disease pathology. It is therefore still considered worthwhile to further study the role of these specific PRMTs in more advanced atherosclerotic disease settings and experimental models for atherothrombosis.

The effect of PRMT inhibitor treatment on inflammation

Inflammation is a key process involved in the pathogenesis of atherosclerosis. This process is mainly driven by activation of toll-like receptors (TLRs) located in a variety of immune cells [26]. TLRs work through recruitment of many downstream signaling proteins that activate the transcription factors NF-kB, activating protein-1 (AP-1), and interferon regulatory factors (IRFs) [27]. Interestingly, PRMTs are considered target molecules for modulating inflammatory responses via NF-kB signaling [7]. Lipopolysaccharide (LPS) is widely used to study TLR-mediated NF-kB activation and downstream cytokine production [28]. Therefore, we investigated the effect of the different PRMT inhibitors on LPS-induced NF-kB-driven macrophage polarization and pro-inflammatory cytokine production. In **Chapter 3**, we showed that pharmacological PRMT4 inhibition using TP-064 significantly reduced the LPS-induced macrophage polarization and pro-inflammatory cytokine production in both thioglycollate-elicited peritoneal macrophages and the RAW264.7 mouse macrophage cell line. Both in **Chapter 3** and **Chapter 4**, our ex vivo studies also showed that the production of the inflammatory cytokine TNF-alpha was reduced in a dose dependent manner in response to in vivo TP-064 treatment of LPS-stimulated monocytes. It can thus be suggested that PRMT4 is indeed a regulatory target for inflammatory monocyte/macrophage polarization and activities. This is in line with the finding of Covic et al. showing that PRMT4 acts as a transcriptional coactivator of NF-kB [29].

Figure 1: *Schematic overview of the relevance of different PRMTs in tissues and associated diseases*

PRMT inhibitors	Cell type	In vitro/ ex vivo/in vivo	Stimulus	Pro-inflammatory effects
TC-E 5003 (PRMT1)	Splenic monocyte	In vivo	/	↓ inflammatory polarization
TP-064 (PRMT4)	RAW264.7 cell line	In vitro	LPS	↓ inflammatory polarization & IL-6 and IL12p40 expression/secretion
	Blood monocyte cytokine production	Ex vivo	LPS	↓ cytokine production
	Thioglycolate-elicited peritoneal monocytes/ macrophages	In vivo	/	↑ inflammatory polarization
		Ex vivo	LPS	↓ inflammatory polarization & TNF-α, IL-1β, IL-6, and IL12p40 expression or secretion
GSK3326595 (PRMT5)	Thioglycolate-elicited peritoneal macrophage	In vitro	LPS	No change
			IFNγ	↑ inflammatory polarization & IP-10 expression and secretion ↓MHCII expression
	Peritoneal macrophages	Ex vivo	IFNγ	↑ inflammatory polarization & IP-10 expression

Tumorigenesis-related studies have indicated that PRMT5 is also an activator of the NF-kB-dependent inflammatory response [30]. However, as can be seen from the inflammation-related results overview in Table 2, no obvious effects on macrophage polarization were observed upon treatment with the PRMT5 inhibitor GSK3326595, neither in the basal state nor after LPS stimulation (see **Chapter 5**). Interestingly, inhibition of PRMT5 did enhance polarization of macrophages to a pro-inflammatory state when IFN-gamma was added to the cells as judged by the augmented secretion of the pro-inflammatory cytokine IP-10. This is in line with the suggestion of Fan et al. that PRMT5 is a cellular sensor that connects stress stimuli (such as IFN-gamma) to nuclear transcriptional events [31]. From our studies it thus appears that PRMT5 inhibition-in-

duced changes in inflammatory macrophage polarization rely more on a change in IFN-gamma signaling rather than on a difference in LPS-induced NF-kB signaling. Moreover, these findings stress that each of the PRMT family members affect specific regulatory pathways.

Although the in vitro studies described in this thesis clearly showed that PRMTs are regulators of macrophage polarization and their inflammatory responses, the outcome of the in vivo studies was complex and showed that PRMT inhibitors induce both pro- and anti-inflammatory effects in different inflammatory cells. In **Chapter 2**, a less pro-inflammatory monocyte phenotype, presented by a shift from a $Ly6C^{hi}$ to a more $Ly6C^{low}$ monocyte population, was found in spleens of mice treated with the PRMT1 inhibitor TC-E 5003. In contrast, PRMT1 inhibition led to a more inflammatory T cell phenotype, as judged from the shifted polarization of $CD4^+$ and $CD8^+$ T cells from the naïve phenotype towards the pro-inflammatory effector memory phenotype. In **Chapter 3** and **Chapter 4**, we showed that also PRMT4 inhibition was associated with both pro-and anti-inflammatory effects. More specifically, we found that PRMT4 inhibition did not affect overall monocyte numbers in vivo, but did reduce the LPS-stimulated secretion of the pro-inflammatory cytokine TNF-alpha by blood monocytes. In addition, neutrophilia was induced by PRMT4 inhibitor treatment in both WT and APOE knockout mouse models, in a dose-dependent manner.

The basal activation state of macrophages in the peritoneum was not changed by PRMT5 inhibitor treatment, nor was the phenotype of T cell populations in several T cell-rich compartments such as blood, spleen, and peritoneum affected, as evident from **Chapter 5**. The fact that we did not observe any effect of PRMT5 inhibition on T cell activation is unexpected. In contrast to our findings, a previous study by Gerhart et al. has indicated that blocking PRMT5 function in mice severely suppresses the $CD4^+CD44^+$ memory T cell differenti-

ation [32] [33]. However, it should be noted that the differences in the response outcomes may be due to the relatively low dose of GSK3326595 that we used in our study as compared to that of Gerhart et al. [34].

Altogether, our findings imply that inhibition of PRMTs can either exert pro- or anti-inflammatory effects. The fact that some of the in vitro findings are not translated into similar in vivo effects can highly likely be explained by 1) the interaction between the different cell types that is lacking in vitro and 2) the dose administered to the mice, as low and high dosages may differentially affect the activity of certain target cells.

The effect of PRMT inhibitor treatment on fatty liver disease development

Besides their role in regulating inflammation, PRMTs are also involved in metabolic signaling during cancer therapy [6]. Therefore, it is valuable to investigate if PRMTs could also be novel targets for the treatment of metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD).

NAFLD is a high incidence metabolic syndrome defined by an increased accumulation of fat, mainly triglycerides (TGs), within hepatocytes. NAFLD is closely related to diabetes because its pathogenesis is accompanied by hepatic insulin resistance and excessive hepatic lipogenesis [35]. Since patients with NAFLD exhibit 3-fold elevated rates of hepatic de novo lipogenesis as compared to healthy individuals [3][17], it can be hypothesized that decreasing hepatic lipogenesis could be a therapeutic target for treating NAFLD. Accordingly, in **Chapter 2**, we have shown that the PRMT1 inhibitor TC-E 5003 is able to lower the hepatic triglyceride levels in Western-type diet fed LDL receptor knockout mice, likely by downregulating the expression of the hepatic lipogenesis genes SREBP1 and FASN. In line with the general assumption that

a decrease in hepatic lipogenesis will diminish the production and secretion of very-low-density lipoproteins by the liver, we also found that PRMT1 inhibitor treatment reduced the hyperlipidemia in the atherogenic diet-fed LDL receptor knockout mice. PRMT1 thus appears to be a key regulator of hepatic lipogenesis and systemic lipid metabolism. Notably, in a similar experimental model, Hoekstra et al. found that inhibition of PRMT3 by SGC707 treatment also decreased hepatic lipid levels as well as lowered plasma triglyceride levels [38][39]. The PRMT3 inhibitor-induced lowering of fatty liver disease and hyperlipidemia correlated with reduced transcription of hepatic lipogenic genes regulated by the nuclear receptor liver X receptor (LXR), a key transcriptional regulator of macrophage cholesterol metabolism and hepatic lipogenesis [40]. Considering that inhibition of either PRMT1 or PRMT3 showed similar effects in mouse models, it can be hypothesized that PRMT1 and PRMT3 are interacting with each other to modulate hepatic lipid levels. However, when critically comparing the two study outcomes it seems that PRMT1 does not do exactly the same as PRMT3, since not all LXR target genes are affected by PRMT1 inhibitor treatment. Additionally, it should be noted that a contradictory study from Xu et al., showed that deletion of hepatic PRMT1 exaggerates liver steatosis in mice fed a high-fat diet via a change in PRMT1/HNF-4α/PGC-1α signaling [41]. Further mechanistic studies are therefore needed to uncover the exact mechanisms through which PRMT1 modulates (systemic) lipid levels.

In contrast to the findings upon PRMT1 and PRMT3 inhibition, in **Chapter 5**, we showed that treatment with the PRMT5 inhibitor GSK3326595 actually upregulated the expression of genes involved in hepatic lipogenesis and increased triglyceride accumulation in liver. More specifically, our gene expression measurements in livers of the Western-type diet-fed LDL receptor knockout mice showed that GSK3326595 upregulated expression levels of CD36, involved in fatty acid uptake, and the hepatic lipogenic genes SREBF1 and FASN. We

anticipate that these effects were possibly induced via inhibition of small heterodimer partner (SHP), as a previous study has uncovered that the activity of SHP is increased by posttranslational methylation at Arg-57 by PRMT5 [42]. In further support of this hypothesis, Kanamaluru et al. have found that defective methylation of SHP leads to a higher hepatic triglyceride level as well as upregulated lipogenic gene expression [42].
Altogether, our metabolism-related findings imply that inhibition of different PRMT family members differentially affects the development of fatty liver disease. Even though more mechanistic studies are needed to further specify PRMTs' regulatory roles in hepatic lipid metabolism, our findings clearly highlight the potential of PRMT1 and PRMT3 as therapeutic targets for NAFLD-driven drug development.

The effect of PRMT inhibitor treatment on other metabolic complications
PRMTs have also been suggested to play a role in the development of other metabolic disorders, i.e. obesity and diabetes [43][44][45]. Studies from Qiao et al. have shown that PRMTs are expressed in all major metabolic tissues, including brown and white adipose tissue [46]. Importantly, the studies presented in this thesis have highlighted the importance of PRMT family members in the regulation of adipose tissue development / phenotype and the therapeutic potential of PRMTs in the treatment of obesity or diabetes. For example, in **Chapter 4**, we found that inhibition of PRMT4 by TP-064 significantly downregulated mRNA expression levels of the adipogenic target gene PPARγ in white adipose tissue of APOE knockout mice. As a possible result, the body weight gain from these Western-type diet-fed APOE knockout mice was decreased in a TP-064 dose dependent manner. Inhibition of PRMT4 can thus be a potential target for restricting the adipogenic process upon challenge with an obesogenic diet. However, we also found that inhibition of PRMT4 is associated with a decreased

insulin production and altered insulin signaling, which may cause diabetes. The development of obesity and diabetes are generally highly correlated [47][48]. Therefore, it is hard to predict whether inhibition of PRMT4 can really be of benefit for preventing metabolic disorders. It will thus be important to further investigate the specific role of PRMT4 in obesity and diabetes in more dedicated experimental disease models such as the leptin deficient obese ob/ob mice or leptin receptor mutant pre-diabetic db/db mice[49][50][51].

Previous studies have suggested that PRMT5 is an important activator of adipogenesis. More specifically, deletion of PRMT5 in adipocytes in culture decreases adipocyte differentiation and lipid droplet biogenesis [22][52]. Therefore, we also studied the effect of PRMT5 inhibitor treatment on adipose tissues from our Western-type diet fed LDL receptor knockout mice. As shown in **Chapter 5**, no differences were found in body weight development nor the morphology or triglyceride content of perigonadal white adipose tissue and subcutaneous brown fat depots. However, given that previous studies also observed that PRMT5 plays a role in maintaining beta cell function, i.e. modulates insulin production [53], and our studies suggest that PRMT5 may modulate the activity of the insulin signaling regulator SHP [54], future studies into the role of PRMT5 in the obesity and diabetes context are clearly of interest.

Future perspectives

In this thesis, we studied the effect of pharmacological inhibition of PRMT activity on atherosclerosis development, inflammation as well as metabolic consequences. Our studies highlight that the combined in vitro and vivo application of specific inhibitors of PRMTs is a good experimental approach for studying the role of these proteins in processes underlying the development of disease. However, we also showed that PRMT inhibitors may induce multiple effects

systemically that can compensate each other and that the applied treatment dosage of PRMT inhibitors in some cases was too low to establish functional effects in some of the target tissues in vivo. As such, it remains important to test the effect of these inhibitors at different dosages in disease models. Notably, special drug delivery tools, such as liposomes, may be of use to improve the effectivity of PRMT inhibitor treatment. Liposomes are the most widely investigated delivery system for phagocyte-targeted therapies (including monocytes and macrophages), given their low immunogenicity, good biocompatibility, and cell specificity [55]. For example, a recent study by our group has revealed that encapsulation of the LXR agonist GW3965 into Lyp-1-containing liposomes enhanced the delivery of the compound to macrophage foam cells located in atherosclerotic plaques without inducing unwanted systemic side effects in the liver [56]. In addition to the fact that liposomes effectively target macrophages, we also expect that liposomes can be used to target other specific cells and tissues. More specifically, for targeting drugs to metabolic tissues - such as adipocytes - liposomes can for example be functionalized with the two ligand visceral-adipose-tissue-targeting peptide and cell-penetrating peptide [58]. Encapsulation of for instance the PRMT4 inhibitor TP-064 into this specific class of drug-targeting devices could lead to a more specific delivery and efficient lowering of the inflammatory macrophage polarization, pro-inflammatory cytokine production, and adipogenesis.

An important aspect that has emerged from our studies is that PRMTs may work in cooperation or via similar mechanisms. For example, inhibition of either PRMT1 or PRMT3 induces a similar effect on hepatic lipid status. Previous studies have also indicated that PRMT1 and PRMT5 both work as NF-kB coactivators in inflammation [55] [56]. It is therefore of interest to further investigate if the different family members work in cooperation or that they might have additive roles. This can be tested by administration of a cocktail of

two or more PRMT inhibitors in specific disease models. As we found that individual inhibition of PRMT1, PRMT3, or PRMT4 is associated with decreased hepatic lipid accumulation or hyperlipidemia, it can be assumed that combined inhibition of these type 1 PRMTs is even more effective. However, it should be considered that the administration of multiple inhibitors can be associated with drug-drug interactions, leading to diminished efficacy and/or toxicity. It will require extensive studies into the effects of different dosages applied for the cocktail treatment, in order to balance the therapeutic effects and unwanted side effects. Notably, since type 1 PRMT inhibitors have already been applied as anti-tumor drugs [56], it is also of interest to investigate effects of cocktail treatment in cancer settings.

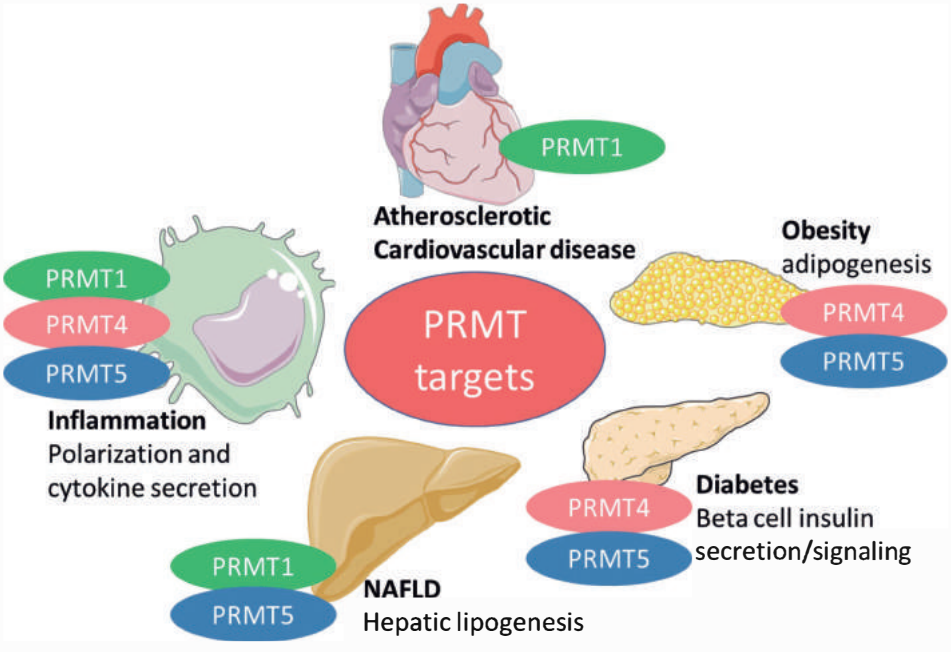

Figure1:*Schematic overview of the relevance of different PRMTs in tissues and associated diseases*

Overall conclusion

In this thesis we have studied the effect of several PRMT inhibitors in atherosclerotic mouse models. We found that PRMT1, PRMT4, and PRMT5 inhibition did not induce marked changes in atherosclerotic lesion size or composition. However, our studies have proven that each of these three PRMTs do play significant roles in different diseases involving inflammatory and/or metabolic dysregulation (highlighted in Figure 1). The pathways that PRMT inhibitors modulate depend on the specific inhibitor applied and tissues or cell types studied. Furthermore, the findings presented in this thesis highlight that drug delivery methods to achieve tissue specific targeting are needed to optimize the use of PRMT inhibitors for future disease treatment.