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Untargeted DNA-demethylation therapy neither prevents nor attenuates ischemia-reperfusion-induced renal fibrosis

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Running head: Decitabine treatment does not attenuate renal fibrosis.

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Abstract

Background: Current treatment options for chronic kidney disease (CKD) are limited and focussed on slowing progression by addressing co-morbidities. Fibrosis, the common histopathological process in CKD, is a major therapeutic research target. In CKD, fibroblasts are terminally activated due to alterations in their DNA-methylation pattern, particularly hypermethylation. Preventing the copying of pathological DNA-methylation patterns in proliferating fibroblasts could be a new effective therapeutic strategy for treating CKD.

Methods: To evaluate the therapeutic effect of short-term treatment with the DNA-methyltransferase (DNMT)-inhibitor decitabine on fibrosis (either developing or already established), male C57Bl/6 mice underwent warm unilateral ischemia-reperfusion injury. Respectively 3 days, 3 and 6 weeks after surgery, decitabine treatment (0.25 mg/kg) was initiated for 10 days after which animals were followed up to 12 weeks after ischemia. Efficacy of therapy on fibrosis was evaluated by *collagen I* and *tgfb* gene expression and histological quantification of collagen I staining. In addition, the effect of decitabine treatment on tubular injury (*Kim-1*, *Ngal*), inflammation (*TNF α* , *IL6*), DNA-methyltransferases (*Dnmt1*, *3a* and *3b*) and global methylation status was determined.

Results: Following ischemia there was a significant increase in fibrotic, injury and inflammatory markers as well as of the various *dnmts*. Although decitabine treatment transiently increased renal injury, had a moderately decreasing effect on *dnmt* expression and on global DNA-methylation upon immediate treatment, none of the treatment regimens succeeded in preventing, attenuating or diminishing fibrosis on the long run.

Conclusion: Administration of untargeted nucleoside analogues seems unsuited as a first-line treatment in developing or established CKD.

Introduction

Existing strategies for treatment of chronic kidney disease (CKD) in essence are systemic in nature and focus on slowing progression in order to improve survival and quality of life [1]. Patients with end-stage renal disease (ESRD) have to rely on dialysis treatment or transplantation, both of which still are temporary solutions with a high rate of complications and premature death: the 5-year survival of dialysis patients is 40.6% in Europe [2] and 40.2% in the United States [3] whilst the average 10-year survival after transplantation is 56% in Europe and 43% in the United States [4]. As in CKD, surviving transplanted kidneys cannot escape development of fibrosis, a detrimental accumulation of extracellular matrix (ECM) and the histopathological hallmark of progressive renal decay [5]. Currently, therapeutic approaches directly targeting cell biological mechanisms underlying fibrosis, are intensively investigated [6]. It is believed that effective therapeutic prevention, attenuation or reversal of the fibrotic process will significantly slow down the progressive decline of both chronically injured and transplanted kidney function [7].

Fibroblasts, independent of their origin [8], are considered to be the main effector cells that produce ECM. Furthermore, in concert with cytokines and growth factors, they sustain and further promote the fibrotic process and attract inflammatory cells [9]. In normal physiological wound repair, fibroblast activation is transient, and fibroblasts return to their resting state after resolution of the primary insult. In chronic fibrosis, fibroblasts are unable to return to quiescence and, as a consequence, continue to proliferate and produce excess ECM [10]. It was hypothesized by Bechtel et al. that fibroblast activation in fibrotic kidney disease is the result of epigenetic alterations, more precisely DNA-methylation, which renders them terminally activated [11]. It is becoming clear that DNA-methylation, in addition to cancer pathologies, also plays an important role in renal pathologies since independent investigators

found that aberrant DNA-methylation is associated with inflammation [12,13], development of renal fibrosis [11,14-16] and occurs during ischemia in transplantation [17,18].

Importantly, aberrant DNA-methylation is thought to influence allograft survival since advanced donor age, differentiation and polarization of immune cells as well as ischemia are associated with alterations in the DNA-methylation pattern [5,19,20]. Targeting DNA-methylation has thus become a new hopeful avenue in development of CKD therapeutics.

Since DNA-methylation patterns are propagated through cell division by the “maintenance” DNA-methyltransferase (DNMT1) [21,22], and findings of Bechtel et al. demonstrated that DNMT1 suppression in heterozygous knockout mice ameliorates folic acid induced fibrosis [11], prevention of the copying of the pathological DNA-methylation pattern in proliferating fibroblasts, by inhibition of DNMT1, could be a new valuable therapeutic strategy for treatment of CKD. Therefore, we hypothesized that by interfering with the DNMTs, copying of the DNA-methylation pattern during proliferation of terminally activated fibroblasts could be prevented, thereby breaking the vicious circle of terminal fibroblast activation, and thus resulting in the attenuation of progressive fibrosis. Hereto, we evaluated the long-term therapeutic effect of short-term treatment (10 days) with the DNMT-inhibitor decitabine in a murine model of fibrosis induced by unilateral ischemia reperfusion injury (UIRI). To verify the effectiveness of treatment initiated at different stages along the natural course of fibrosis development in this AKI-to-CKD model [23], the 10-day decitabine treatment period was initiated either 3 days, 3 weeks or 6 weeks after UIRI.

Methods

Animals and experimental design

All animal procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics

Committee (approval number 2011-51). Animals had free access to standard chow and tap water.

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously [23] (see supporting information). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent 30 minutes of warm (37°C body temperature) UIRI, with continuously monitoring of body temperature. The right kidney was left undisturbed. Sham-operated animals received the same surgical procedure except placement of the clamp.

Treatment regimen: After surgery, animals were randomly allocated to 8 treatment groups (Figure 1). 1) Sham (n=12); 2) UIRI + untreated (n=12); 3) UIRI + vehicle starting 3 days after UIRI (n=12); 4) UIRI + decitabine starting 3 days after UIRI (n=16); 5) UIRI + vehicle starting 3 weeks after UIRI (n=12), 6) UIRI + decitabine starting 3 weeks after UIRI (n=16); 7) UIRI + vehicle starting 6 weeks after UIRI (n=12) and 8) UIRI + decitabine starting 6 weeks after UIRI (n=16). For the remainder of this manuscript, we termed groups 3 and 4 as “immediate treatment”, groups 5 and 6 as “delayed treatment” and group 7 and 8 as “late treatment”. The 3day delay in the “immediate” group was included to allow the animals to recover from surgery first. Decitabine was diluted in phosphate buffered saline (vehicle) in a concentration of 0.25 mg/kg and administered for 10 days through subcutaneous injection, as this route of administration has been proven superior [24,25]. Optimal decitabine dosage (0.25mg/kg/day) was determined in a dose-finding experiment (see supporting information).

Euthanasia: Half of the animals of the groups receiving decitabine (4, 6 and 8) and vehicle treatment groups (3, 5 and 7) were euthanized 12 days after treatment (designated as subgroups “a”) whilst the remaining animals were euthanized 12 weeks after UIRI (subgroups “b”) (Figure 1). Animals were euthanized through exsanguination via the abdominal vena cava under ketamine-xylazine anesthesia. Kidneys were excised, renal pole fractions were

snap frozen in liquid nitrogen and transversal slices of renal tissue were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and 10% neutral buffered formalin for 4 hours and 24 hours, resp., rinsed with 70% isopropanol and embedded in paraffin for histology.

Analysis of genomic DNA-methylation in ischemic kidney tissue

The efficacy of a DNA-methyltransferase inhibitor is generally determined by its ability to induce demethylation and reactivation of one or a few marker genes [26]. In the current study, the reactivation of marker genes was not evaluated. Instead, the amount of genomic DNA-methylation in the ischemic kidney was determined by Ultra-Performance Liquid Chromatography (UPLC), as this is one of the most significant and experimentally straightforward parameters for determining the efficacy of a DNA-methyltransferase inhibitor [26]. Here, DNA is extracted from a pole section of the ischemic kidney (Allprep Mini Kit; Qiagen, Belgium) and global DNA-methylation is determined through UPLC (see supporting information) as reported previously [27]. Briefly, isolated genomic DNA samples were enzymatically hydrolysed to individual deoxyribonucleosides and dissolved in water. Using reference standards for 5'-methyl-2'-deoxycytidine (5-mdC; N-1044, Jena Bioscience, Germany) and 2'-deoxycytidine (dC; D3897-1G, Sigma-Aldrich), a series of calibration solutions was prepared in a range of 0.1-10 ng/mL and 1–100 ng/mL respectively. The same calibration standards were used in all of the experiments. Global DNA-methylation was obtained by quantifying 5-mdC and dC using UPLC, in combination with tandem mass spectrometry (MS-MS; Waters® Micromass Quattro Premier™ Mass Spectrometer) with injections performed on a Waters® UPLC column (BEH C18, 50 mm x 2.1 mm, 1.7 µm) at 40°C.

The global DNA methylation is expressed as a percentage of 5-mdC versus the sum of 5-mdC and dC $[5\text{-mdC}/(5\text{-mdC} + \text{dC})]$ %. Samples were measured in duplicate. The average

methylation value of both measurements was calculated and used for further statistical analysis.

Real-time PCR

Total mRNA is extracted from a pole section of the ischemic kidney (PureLink RNA Mini Kit; Life Technologies, Merelbeke, Belgium) and converted to cDNA (High Capacity cDNA archive kit; Life Technologies). To quantify gene expression, qPCR, based on the Taqman fluorescence method (ABI Prism 7000 sequence detection system; Life Technologies), was used. Taqman probes and primers for *gapdh* (Mm99999915_g1), *collagen I α 1* (Mm00801666_g1), *tgfb1* (Mm01178820_m1), *dnmt1* (Mm00599763_m1), *dnmt3a* (Mm00432881_m1) and *dnmt3b* (Mm01240113_m1), *havcr1* (Mm00506686_m1), *lcn2* (Mm01324470_m1), *tnfa* (Mm00443258_m1) and *il-6* (Mm00446190_m1) were purchased from Life Technologies. Each gene was analyzed in triplicate and the expression was normalized to the reference gene *gapdh*. Calculations were made conform the comparative Cq-method.

Histology

For collagen I immunostaining, paraffin embedded 4 μ m thick sections of ischemic kidney tissue were blocked with normal goat serum and incubated overnight with the primary polyclonal rabbit-anti-mouse collagen I antibody (Biodesign International, Maine). After washing, sections were incubated with a biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, California) and subsequently incubated with avidin and biotinylated horseradish peroxidase (AB-complex, Vector Laboratories). A dark brown color was developed with diaminobenzidine in the presence of 0.03% H₂O₂. Sections were counterstained with methyl green to visualize nuclei. Collagen I immunostaining was quantified using the Axiovision image analysis software (Carl Zeiss, Jena, Germany). Data are presented as area % positive

stain, calculated as the ratio of the absolute area of positive staining versus the total surface of the tissue section.

Statistics

For statistical analysis, IBM SPSS Statistics 23 software was used. Data are presented as mean $\pm$ standard deviation (SD), or as individual values. Outliers were discarded based on SPSS' internal stringent Tukey rule. Driven by the number of animals per treatment group non-parametric statistics is preferred over parametric. Comparisons between groups were therefore made using a Kruskal-Wallis test, followed by a Mann-Whitney U test when significant. The data presented in this manuscript originates from 2 separate experimental runs, one for “delayed” and “late” decitabine treatment and one for “immediate” decitabine treatment analysis, each with their own sham, vehicle and untreated groups. Not affecting the conclusions, the data were merged for reason of clear visual presentation. Values of $p < 0.05$ are considered significant.

Results

Unilateral ischemia-reperfusion induces atrophy and fibrosis

The sham, untreated and vehicle groups clearly demonstrate a progressive loss of renal mass of the ischemically injured kidney (Figure 2 A). Already 3 weeks after UIRI renal mass of the vehicle treated group decreased with ca. 60% as compared to sham (0.069 ± 0.003 mg vs. 0.163 ± 0.005 mg, $p < 0.05$). Vehicle groups sacrificed at week 6, week 9 and week 12 (“endpoint analysis”) corroborate the progressive nature of this renal atrophy as well as the consistency of the UIRI model. Steady state is considered to be reached from week 6 on and ranges between 25 and 30% of sham kidney mass. The weights of the contralateral uninjured kidneys show a tendency to increase in untreated and vehicle-treated animals as compared to sham, which is in accordance with the physiological fact that the contralateral kidney

becomes hypertrophic to compensate for the loss of function of the ischemic kidney (Figure 2B).

Analysis of pro-fibrotic gene expression showed significant upregulation of *collagen I* and *tgfb* (21.9 ± 9.9 and 10.2 ± 5.1 -fold resp.) in vehicle treated UIRI groups from week 3 on (Figure 3A and B), with elevated expression persisting throughout the full study duration ($p < 0.05$ vs. sham group). Additionally, renal fibrosis was quantified on collagen I immunostaining (Figure 3C and 4). Significantly more positivity was present in the ischemic kidneys of vehicle-treated animals ($7.9 \pm 2.2\%$) as compared to sham ($2.8 \pm 0.8\%$, $p < 0.05$) from week 3 on and up to endpoint analysis at week 12 ($p < 0.05$ vs. sham group). These observations were confirmed by quantitative analysis of Masson stained sections (data not shown).

Neither immediate, delayed, nor late treatment with decitabine could attenuate renal fibrosis

In this study, 16% mortality was observed upon decitabine treatment, whilst no mortality was seen in vehicle-treated animals. A short-term treatment with decitabine either immediately, delayed or late after UIRI did not influence the loss of renal mass/ of the ischemic kidney 12 days after the end of treatment (week 3, 6 and 9 after UIRI respectively) as compared to vehicle treatment (Figure 2A). The contralateral kidney of decitabine-treated animals showed a delayed increase in renal mass as compared to vehicle-treated animals. This was statistically significant at intermediate analysis when treatment was initiated immediately as well as late after UIRI (Figure 2 B). However, after additional follow-up until week 12 after UIRI, this effect was no longer seen in any of the decitabine treatment groups (Figure 2 B).

A short-term treatment with decitabine either immediately, delayed or late after UIRI did not significantly influence the expression of the fibrosis-related genes *collagen I* and *tgfb* as compared to vehicle treatment (Figure 3A and B), nor at intermediate nor at endpoint

analysis. Only a small trend towards lower expression of *tgfb* in the group with delayed decitabine treatment was noticeable. Furthermore quantification of renal fibrosis on collagen I immunostaining showed no effect of decitabine treatment on the amount of staining in the ischemic kidneys (Figure 3C). These observations were also confirmed by quantification of Masson's stain (data not shown).

Ischemia-reperfusion induces severe long-term tubular injury and inflammation

Expression of tubular injury markers *havr1* (KIM-1) and *lcn2* (NGAL) was highly upregulated in vehicle treated mice at week 3 (6.9 ± 0.8 and 14.5 ± 4.5 -fold resp., $p < 0.05$), week 6 (14.8 ± 3.6 and 37.4 ± 13.9 -fold resp.) ($p < 0.05$) and week 9 (17.0 ± 8.2 and 29.2 ± 5.1 fold resp.) (Figure 5A and B). A tendency towards decreased expression of *havr1* (and to a lesser extent *lcn2*), was observed after the follow-up period in vehicle treated and untreated animals, with the lowest upregulation reached at the 12 week endpoint for the immediately treated groups.

Gene expression of the inflammatory cytokines *tnfa* and *il-6* was significantly upregulated in vehicle treated animals from week 3 on (8.6 ± 3.6 and 3.2 ± 0.9 fold resp.) and remained stably elevated throughout the study (Figure 5C and D).

Neither immediate, delayed, nor late treatment with decitabine could attenuate tubular injury or inflammation

Decitabine-treatment immediately after UIRI induced significant higher expression of *havr1* and *lcn2* as compared to vehicle treatment (12.6 ± 2.17 vs. 6.9 ± 0.8 -fold and 52.4 ± 24.8 vs. 14.5 ± 4.5 fold resp.) ($p < 0.05$) at week 3 (Figure 5A and B). This difference persisted for *lcn2* for delayed and late decitabine treatment with analysis at the intermediate time points week 6 and 9 after UIRI respectively. For *Havr1* this effect of delayed decitabine treatment was only true at week 6 after UIRI. After the additional follow-up period, differences in expression induced by decitabine could no longer be measured.

Treatment with decitabine did not have an effect on the expression of the inflammatory cytokines *tnfa* and *il-6*, although *il-6* tended to be higher than vehicles at all but one condition, i.e. the endpoint analysis of late decitabine treatment (Figure 5C and D).

Ischemia-reperfusion induces upregulation of DNA-methyltransferases

As evidenced by the vehicle groups, UIRI induced a significant upregulation of *dnmt1*, *dnmt3a* and *dnmt3b* gene expression as compared to sham from week 3 on (4.8 ± 0.4 , 4.0 ± 1.0 and 6.4 ± 2.5 -fold resp.) ($p<0.05$) and remained rather stably elevated throughout the study (Figure 6A, B and C). Genomic DNA-methylation in the ischemic kidneys, as measured by UPLC, showed no significantly increased or decreased levels of methylated cytosines in vehicle-treated animals after UIRI in comparison to sham group within the time frame of this study (Figure 6D).

Delayed, but neither immediate nor late treatment with decitabine affects dnmt gene expression

Decitabine-treatment either initiated immediately or late after UIRI did not influence expression of *dnmt1*, *dnmt3a* and *dnmt3b* as compared to vehicle treatment, neither at intermediate analysis (week 6 and 9 resp.), nor at endpoint analysis (week 12) (Figure 6A, B and C). In contrast, delayed treatment resulted in a consistent trend ($p>0.05$) of decreased *dnmt1*, *dnmt3a* and *dnmt3b* expression both at intermediate and endpoint analysis. As compared to vehicle treatment, this decrease was only significant for *dnmt3b* at week 6 of intermediate analysis (Figure 6C). As compared to the untreated group, both *dnmt1* and *dnmt3a* were also significantly decreased at week 12 ($p=0.036$ and $p=0.017$ resp.). At the level of genomic DNA-methylation only an immediate treatment with decitabine appeared to have an effect within the currently analyzed time points. Both at intermediate analysis at week 3 and persisting up to endpoint analysis at week 12, decitabine significantly lowered the methylated cytosine content as compared to sham (5.13 ± 0.20 and 5.04 ± 0.18 vs. 5.50 ± 0.4) and

demonstrated a decreased trend vs the respective vehicle groups ($p=0.053$ and $p=0.082$) (Figure 6D).

Discussion

The (myo)fibroblast, independent of its origin, is the principal effector cell for the development of fibrosis in organs. Bechtel et al. have demonstrated that in folic acid induced renal fibrosis, fibroblasts become terminally activated due to hypermethylation of the *rasal1* gene, rendering fibroblasts unable to return to their resting state after the resolution of the primary insult [11]. This permanent activation is particularly characterized by proliferation and excess production of extracellular matrix. Importantly, during proliferation, established DNA-methylation patterns of CpG island promoters are copied to the daughter cells [28], providing them with a terminally activated phenotype as well. Since the maintenance DNA-methyltransferase 1 (DNMT1) is responsible for this, one may hypothesize that by inhibiting DNMT1 activity, copying of the DNA-methylation pattern of proliferating, terminally activated fibroblasts to daughter cells might be prevented. As in clinical practice most patients already present with chronic fibrosis at the moment of CKD diagnosis, we investigated whether treatment with the DNMT inhibitor decitabine is able to attenuate progression of established renal fibrosis. In addition, we investigated whether decitabine is also able to delay development of fibrosis upon immediate treatment after an acute insult and whether such treatment would have long-term benefit.

Decitabine is a 5-aza substituted nucleoside analogue [29,30] that is incorporated into the DNA, serves as a substrate for DNMTs [30] and induces depletion of genomic DNMT stores [26,29]. Since decitabine is neither cell type- nor DNA-sequence specific, we opted for a short-term treatment (10 days) to minimize adverse effects, whilst ensuring to cover multiple fibroblast cell divisions.

This study was conducted in a mouse model of severe unilateral renal ischemia-reperfusion injury (UIRI) without contralateral nephrectomy, which we recently demonstrated to result in development of long-term renal fibrosis [23]. Ischemia-reperfusion is a major obligate injurious factor in renal transplant pathology and a common clinical cause of AKI for which a role for pathologic DNA-methylation has been suggested [17,31]. To the best of our knowledge in the present study we report for the first time persistent long-term upregulation of *dnmt1*, *3a* and *3b* following UIRI (Figures 7 A and C), thereby complementing reports from experimental studies using models of folic acid-induced renal fibrosis [11] and hyperhomocysteinemia nephropathy [32]. Furthermore, we found that genomic DNA-methylation was significantly increased 14 days after UIRI (supplemental Figure S2) and noted a tendency towards higher DNA-methylation at week 12 after UIRI in vehicle treated groups with delayed and late vehicle treatment (Figure 6D). In contrast, DNA-methylation measured 12 days after the end of a 10-day vehicle treatment period that was initiated immediately after UIRI demonstrated a tendency to decrease (Figure 6D). These observations, in combination with reports of altered DNA-methylation of specific genes in CKD patients [11,14,16,18,33], further corroborate that aberrant DNA-methylation is a pathological entity also in ischemia-reperfusion induced AKI/CKD, supporting the use of this model in a tentative therapeutic intervention study based on DNA-demethylation.

To assess the efficacy of DNA-methyltransferase inhibition by decitabine, we investigated the DNA-methylation dynamics. In the current study, a 14-day treatment regimen with 0.25 mg/kg/day decitabine induced a significant reduction in DNA-methylation of approximately 3% in healthy animals and 11% in UIRI animals (supplemental Figure S2). Thus, upon decitabine treatment, a small yet significant reduction in genomic DNA-methylation was observed. This order of magnitude is not surprising as merely approximately 3% of CpG island promoters are methylated in normal cells [28]. However, 12 days after the end of

delayed and late decitabine treatment, there was no significant difference in genomic DNA-methylation in decitabine treated animals as compared to those receiving vehicle (Figure 6D), suggesting recovery of genomic DNA-methylation upon postponed intervention. This is in line with the finding of Kagey et al. noting that re-silencing after decitabine removal is possible due to the preservation of some repressive histone modifications upon DNA-demethylation [34], thereby rendering the effects of epigenetic drugs transient. The fact that demethylated genes can be remethylated within a few days after the end of treatment [26,28,35] may explain the observed normalisation of DNA-methylation by day 12 after treatment (Figure 6D), as was also seen in human blood samples [36]. It is of note however that immediate treatment with decitabine resulted in a persisting lower methylation level as compared to sham animals and a trend as compared to their respective vehicles (Figure 6D), thereby indicating that the moment of intervention indeed may have long term effects.

Even though the focus of this study was on the therapeutic potential of inhibition of DNA-methylation, we also investigated its effects on the expression of *dnmts*. In our experiment, gene expression of all *dnmts* was clearly upregulated following UIRI (Figure 6 A, B and C). When decitabine was administered at a delayed moment (i.e. starting at week 3 after UIRI), upregulation tended to be attenuated for all *dnmts* (Figure 6 A, B and C). Interestingly, as compared to untreated injured kidneys, decrease of both *dnmt1* and *dnmt3b* suppression reached long-lasting significance (Figure 6 A and B). The finding that Dnmt3b is also affected is interesting, particularly since overexpression experiments have revealed that the *de novo* DNA-methylation activity of DNMT3a and 3b has a broad range of targets, whereas that of DNMT1 is limited to selected genes [37,38]. Although DNMT1 is the most abundant methyltransferase in mammalian cells [39] and *in vitro* experiments suggest that it is a prominent hypermethylator in somatic cells [11,40], it remains unclear how DNMT1 would facilitate aberrant *de novo* DNA-methylation of selected genes in fibrosis [28], since the

substrate of DNMT1 is hemimethylated DNA [41]. In addition, DNMT1 knockout cells display almost normal genomic DNA-methylation levels, while cells lacking both DNMT1 and DNMT3B undergo a substantial loss of DNA-methylation in the promoter region of tumor suppressor genes [42]. These observations resulted in the hypothesis that DNMT3b is thought to be the additional activity that cooperates with DNMT1 to maintain cellular DNA-methylation patterns [30]. Although it remains a fact that DNMT1 depletion (in DNMT^{-/+} heterozygous mice) attenuates folic acid-induced fibrosis [11], the precise role of DNMT3b in fibrosis certainly warrants further investigation.

Regardless of its transient effects on DNA-methylation, inhibition of DNMTs by short-term decitabine treatment did not prevent the progression of fibrosis in this study. In particular, decitabine treatment, neither initiated immediately, delayed, nor late in fibrosis development and progression, was unable to attenuate the increased gene expression of *collagen I* or *tgfb* (Figures 3A and B). Quantitative histological analysis by collagen I immunostaining confirmed these observations (Figures 3C). In addition, analysis of gene expression of KIM-1 (*haver1*), NGAL (*lcn2*), *tnfa* and *il-6* indicated that decitabine was unable to attenuate tubular injury as well as inflammation (Figure 5A-D). In contrast, it appears that decitabine treatment adds to renal injury and inflammation, as evidenced by the transiently increased expression of KIM-1 and NGAL ($p < 0.05$ vs vehicles at intermediate analysis, Figure 5A and B) and to a lesser extent for IL6 (trend; Figure 5D). Although these upregulations were only transient, they might have had an influence on the eventual outcome of the injured kidney. An aspect which should be taken into account for future research on this matter.

Finally, we would like to point out to several limitations of the study. A first limitation is the inherent severity of renal injury in our animal model. Although not significant, when decitabine treatment was initiated with delay, a clear tendency towards decreased *tgfb* could be observed as compared to vehicle and untreated animals (Figure 3B). One could speculate

that in conditions of mild renal injury, as could reasonably be achieved with milder conditions of ischemia-reperfusion (i.e. lower body temperature, shorter ischemia times [23], or with prolonged treatment (at lower dose), decitabine might be able to attenuate renal fibrosis more clearly. Although our UIRI model was severe, one have to take into account that milder UIRI models have a higher tendency for spontaneous repair as we previously reported [23], thereby making it hard to discern an effect of treatment from a natural repair response. Since the focus in the current manuscript was on long term sequelae, we opted for rather severe ischemia conditions (30 minutes at 37°C core body temperature) in order to 1) avoid the bias of spontaneous renal recovery and 2) assure that the ischemic insult would result in development of long term chronic injury and fibrosis. A second limitation to our study is the fact that we could not assess renal function in our UIRI model. In order to assure progression to CKD of the injured kidney, we leave the unharmed kidney in the body, where it will inevitably compensate for the loss of function of the ischemic kidney. Hence, creatinine, BUN or any other parameter of renal function will remain unaffected. An approach, as performed by Aufhauser et al., where the contralateral kidneys are removed shortly (2days) before renal function measurement, would allow including such analysis [48].

Overall, we conclude that even though decitabine treatment induced a significant reduction in genomic DNA-methylation, progression of fibrosis, as evaluated by *collagen I* and *tgfb* gene expression and histological quantification of Masson stain and collagen I immunostaining, was not attenuated or prevented in this model. We would like to note that the effect of treatment with nucleoside analogues was shown valuable against fibrosis mainly in *in vitro* experiments [43-45]. However, the only evidence of prevention of fibrosis by inhibition of DNMT's *in vivo* was provided by Bechtel et al. [11] and Pushpakumar et al. [32] in the kidney and by Kim et al. [46] in cardiac fibrosis. Taken into account (i) the limited positive results of decitabine treatment on renal fibrosis in the current experiment, (ii) the overall

limited amount of positive publications on nucleoside analogues as a treatment strategy for fibrosis and (iii) the severity of adverse effects (e.g. weight loss) of this compound (even for relative short-term treatments), administration of nucleoside analogues as such seems unsuited as a first-line treatment in CKD. Evaluating the efficacy of precisely targeted DNA-demethylation strategies would therefore be invaluable to fully reveal the putative therapeutic potential of DNA-demethylation in targeting development or progression of renal fibrosis [47].

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Disclosure

The authors declare no conflicts of interest.

Ethics approval

All animal procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics Committee (approval number 2011-51).

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Figures and figure legends

Figure 1: Study set-up. After surgery, animals were randomly allocated to 8 treatment groups. 1) Sham (n=12); 2) UIRI + untreated (n=12); 3) UIRI + vehicle starting 3 days after UIRI (n=12); 4) UIRI + decitabine starting 3 days after UIRI (n=16); 5) UIRI + vehicle starting 3 weeks after UIRI (n=12), 6) UIRI + decitabine starting 3 weeks after UIRI (n=16); 7) UIRI + vehicle starting 6 weeks after UIRI (n=12) and 8) UIRI + decitabine starting 6 weeks after UIRI (n=16). Treatment regimen can thus be interpreted as being “immediate” (groups 3 and 4), “delayed” (groups 5 and 6) and “late” (groups 7 and 8).

Figure 2: Kidney weight. UIRI was performed for 30 minutes at 37°C, n=12 in sham and untreated groups, n=6 in vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of immediate treatment (wk 3 after UIRI), delayed treatment (wk6 after UIRI) and late treatment (wk 9 after UIRI), as well as 12 weeks after UIRI (endpoint of experiment) for each of the respective treatment regimens..

A: Weight of the ischemic kidneys. **B:** Weight of the contralateral kidneys

°: p<0.05 vs. Sham, *: p<0.05 vs corresponding vehicle group.

Figure 3: Effect on long-term fibrosis development in the ischemic kidney. UIRI was performed for 30 minutes at 37°C, n=12 in sham and untreated, n=6 in vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of either immediate, delayed or late treatment (i.e. week 3, 6 and 9 after UIRI respectively) and 12 weeks after UIRI (endpoint of experiment). **A:** *collagen I* gene expression (qPCR). **B:** *TGFβ* gene-expression (qPCR). **D:** Collagen I positivity on immunostained renal tissue.

°: p<0.05 vs. Sham, *: p<0.05 vs corresponding vehicle group.

Figure 4: Collagen 1 immunostaining.

Representative images of collagen I immunostained ischemic kidney tissue in the setting of “delayed” decitabine treatment. Animals designated with vehicle and decitabine started their 10-day treatment at week 3 after UIRI

and were euthanized either at week 6 or week 12 after UIRI. Sham group was euthanized 12 weeks after mock surgery. Untreated was euthanized 12 weeks after UIRI (100x).

Figure 5: Expression of tubular injury markers and inflammatory cytokines . UIRI was performed for 30 minutes at 37°C, n=12 in sham and untreated, n=6 in vehicle groups, n=8 in decitabine treatment groups.

Animals were euthanized 12 days after end of either immediate, delayed or late treatment (i.e. week 3, 6 and 9 after UIRI respectively) and 12 weeks after UIRI (endpoint of experiment). **A:** *Havcr1* (KIM-1) gene expression (qPCR). **B:** *Lcn2* (NGAL) gene-expression (qPCR). **C:** *TNFA* gene expression (qPCR). **D:** *IL-6* gene expression (qPCR).

°: p<0.05 vs. Sham, *: p<0.05 vs corresponding vehicle group

Figure 6: Parameters of DNA-methylation in the ischemic kidney. UIRI was performed for 30 minutes at 37°C, n=12 in sham and untreated, n=6 in vehicle groups, n=8 in decitabine treatment groups.. Animals were euthanized 12 days after end of either immediate, delayed or late treatment (i.e. week 3, 6 and 9 after UIRI respectively) and 12 weeks after UIRI (endpoint of experiment).

A: *Dnmt1* gene expression **B:** *Dnmt3a* gene expression **C:** *Dnmt3b* gene expression **D:** Percentage of genomic DNA-methylation in the ischemic kidney (UPLC)

°: p<0.05 vs. Sham, *: p<0.05 vs corresponding vehicle group

Description of supporting information

Additional Supporting Information may be found in the online version of this article.

Supplemental material and methods

Renal unilateral ischemia-reperfusion mouse model

Decitabine dose finding experiment

Analysis of genomic DNA-methylation in ischemic kidney tissue

Supplemental figures

Figure S1: Follow-up of body weight of mice, during treatment with different dosages of decitabine.

Weight loss was observed as a side effect of decitabine treatment. The highest dose, 0.5 mg/kg/day induced severe weight loss, and animals had to be euthanized earlier than anticipated. AdC: decitabine

Figure S2: Efficacy of inhibition of DNA-methylation in the dose-finding experiment.

A: Percentage of genomic DNA-methylation in the ischemic kidney after a treatment period with different dosages of decitabine. A significant increase in genomic DNA-methylation was observed after UIRI. Decitabine induced a dose-dependent reduction in genomic DNA-methylation in UIRI-animals. B: Gene expression analysis of *Dnmt1*, *Dnmt3a*, *Dnmt3b*. UIRI resulted in higher gene expression of all genes under investigation. As expected from literature, treatment with decitabine induced a significant reduction in *Dnmt1* gene expression.

°: $p < 0.05$ vs. Sham, *: $p < 0.05$. AdC: decitabine