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Targeted gamma-secretase inhibition of Notch signaling activation in acute renal injury

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Running head: Notch inhibition in kidney diseases

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Abstract

The Notch pathway has been reported to control tissue damage in acute kidney diseases. To investigate potential beneficial nephroprotective effects of targeting Notch, we developed chemically functionalized γ-secretase inhibitors (GSIs) targeting γ-glutamyltranspeptidase (γ-GT) and/or γ-glutamylcyclotransfase (γ-GCT), two enzymes overexpressed in the injured kidney, and evaluated them in *in vivo* murine models of acute tubular and glomerular damage. Exposure of the animals to disease-inducing drugs together with the functionalized GSIs improved proteinuria and, to some extent, kidney dysfunction. The expression of genes involved in the Notch pathway, acute inflammatory stress responses and the renin-angiotensin system was enhanced in injured kidneys, which could be downregulated upon administration of functionalized GSIs. Immunohistochemistry staining and western blots demonstrated enhanced activation of Notch1 as detected by its cleaved active intracellular domain during acute kidney injury, and this was down-regulated by concomitant treatment with the functionalized GSIs. Thus, targeted γ-secretase-based prodrugs developed as substrates for γ-GT/γ-GCT have the potential to selectively control Notch activation in kidney diseases with subsequent regulation of the inflammatory stress response and the renin-angiotensin pathways.

***Key words**:* γ-secretase inhibitors / Notch / γ-glutamyltranspeptidase (γ-GT) / aminopeptidase A / kidney diseases / renin-angiotensin / drug-targeting

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Introduction

Recent advanced knowledge on acute and chronic renal injury has yielded several common candidate pathways for designing targeted therapeutics, which include the Notch pathway, the oxidative stress response and the renin-angiotensin system (RAS) (**3,20,21,31,42**). The Notch pathway is a target for therapeutic intervention, not only in kidney diseases but also in several other disorders (**20,36**). Notch is a membrane inserted protein with its active part directed toward the intracellular space and which needs hydrolysis by the γ-secretase complex to become active (**20**). The enzyme γ-secretase is a large protease complex composed of a catalytic aspartyl protease subunit (presenilin-1 or -2) and three support subunits (presenilin enhancer protein (pen)-2, aph-1 and nicastrin), all being membrane-inserted proteins. The γ-secretase complex activates Notch by hydrolyzing a peptide bond of the Notch protein at an intra-membrane site, allowing cleaved Notch, also referred to as Notch intracellular domain (NICD), to migrate to the nucleus where it activates responsive genes (**45**). The intra-membrane activity of the γ-secretase has also been involved in the release from the membrane of other biologically relevant membrane proteins involved in physiological and pathological processes, including amyloid precursor protein, LDL-receptors, insulin-like growth factor or CD44. Therefore, in order to develop selective therapies for kidney diseases involving the control of Notch activation, it is desired to achieve only localized γ-secretase inhibition, thus protecting the other functions of this enzyme and of the Notch pathway in non-target organs.

Toward this goal and based on our previous knowledge that the activities of the peptidases γ-glutamyltranspeptidase (γ-GT) and aminopeptidase A (APA) are increased in various compartments of injured kidneys in rodent experimental models as well as in human samples, we have designed, synthesized and evaluated targeted γ-secretase inhibitors (GSIs) as prodrug substrates for theses enzymes (**17**). Preliminary *in vivo* results suggested the possibility of the

approach of using targeted GSIs in an experimental model of acute kidney injury. However, in our previous report, the biological consequences of exposing the animals to these targeted compounds and the *in vivo* effects on the expression of the components of the Notch pathway and other associated cellular responses were not studied. Thus in the present report, these functionalized γ-secretase-based prodrugs were evaluated in the experimental murine model of severe acute tubulointerstitial injury induced by aristolochic acid (AA) and the more progressive model of glomerular damage after exposure to adriamycin (ADR). Control and treated animals were monitored throughout the experiments for weight, proteinuria and relevant serum chemistry values. The toxicological profile of the N-acetyl-γ-Glu-γ-secretase-inhibitor (N-Ac-γ-Glu-GSI) prodrug and its metabolite amine-GSI was determined in the plasma. To investigate the potential biological consequences of exposing the animals to these various compounds, we used real-time quantitative PCRs performed on mRNA extracted from the kidneys of the experimental animals as well as immunohistology and western blotting. The results demonstrated an activation of Notch1 with upregulation of the expression of genes involved in the Notch pathway, inflammatory stress response and the RAS, which could be selectively down-regulated upon administration to the mice of the N-Ac-γ-Glu-GSI and/or its active metabolite amine-GSI, together with selective inhibition of Notch cleavage.

Materials and Methods

Animal models of induced kidney injury

All experiments were conducted in accordance with federal and local regulations, according to a protocol approved by the animal ethics committee of the Canton de Vaud, Switzerland (permit No 2655.0). Kidney injury was induced by intraperitoneal (i.p.) injection of aristolochic acid (AA, Sigma-Aldrich, Buchs, Switzerland, 1x5 mg/kg) or of adriamycin (Adriblastin, Pfizer, Zürich, Switzerland, 1x10 mg/kg) in 10 weeks old BALB/c male mice (n=5-7 mice/experimental group). The γ-secretase inhibitors (GSIs) compounds were diluted in 0.9% NaCl and administered i.p., starting one day before the disease-inducing drugs (day -1) at a dose of 10 mg/kg for amine-GSI or 30 mg/kg for N-Ac-γ-Glu-GSI, and then twice daily until day 6 evening. A control group received the GSIs prodrugs without induction of kidney injury. The animals were weighted at days 0, 3 and 6, and sacrificed at day 7 morning. Proteinuria was assessed semi-quantitatively using Albustix reagent strips (Bayer, Basel, Switzerland). At the end of the treatment period, the mice were sacrificed, and the liver and both kidneys were removed. The kidneys were spliced in four equal fragments containing equivalent amounts of cortex and medulla. One fragment was snap-frozen in liquid nitrogen for qRT-PCR and western blot experiments, one fragment was included in OCT (Tissue-Tek, VWR International, Dietikon, Switzerland) and frozen for histoenzymography and immunohistochemistry experiments, one fragment was frozen at -80°C and was used to quantify drugs, and one fragment was fixed in 4% paraformaldehyde and included in paraffin for histology. Hematoxylin/eosin (HE) and Masson's trichrome blue (MTB) stainings of paraffin-embedded mouse kidney sections were performed using standard routine procedures. Blood samples were collected in tubes containing EDTA, plasma was separated by centrifugation and stored at -80°C. The clinical blood chemistry evaluation (kidney and liver function tests) in mouse plasma was performed according to standard procedures.

Immunohistochemistry

OCT-embedded frozen kidneys were cut at 7 μm. The sections were air-dried, fixed for 10 min in cold (-20 °C) methanol, rinsed in PBS 0.1% Triton X-100 (PBS/Triton), and blocked for 30 min with PBS/Triton containing 5% bovine serum albumin (BSA). Endogenous peroxidase and biotin were blocked using 3% H_2O_2 and avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA 94010, USA), respectively. The rabbit anti-Notch1 antibody (clone D1E11, Cell Signaling Technology, Leiden, The Netherland; diluted 1/50 in PBS/5% BSA) or the rabbit anti-cleaved Notch1 antibody (clone Val1744, Cell Signaling; diluted 1/50 in PBS/5% BSA) were added to the sections for 1 h. The slides were rinsed with PBS/Triton three times, incubated for 1 h with biotinylated anti-rabbit secondary antibody (Vector Laboratories, diluted 1/500 in PBS/5% BSA), washed with PBS, incubated with streptavidin/horse radish peroxidase (HRP) (Dako, Bollschweil, Germany; diluted 1/500) for 1 h, followed by 15 min with 3,3′diaminobenzidine (DAB, Dako). The slides were washed in distilled water, mounted in Aquamount, (Immu-mount, Thermo Shandon Pittsburgh, PA, USA) and analyzed.

For the staining of α-smooth muscle actin (α-SMA), paraffin-embedded kidney sections were used. Slides were deparaffined following standard procedures and endogenous peroxidase was blocked using 1% H_2O_2 in methanol. Slides were then rehydrated by washing in decreasing gradients of ethanol (100% twice, 95% twice, 80% once) followed by tap water, then blocked 10 min in PBS/10% goat serum before adding the primary antibody for 1 h (rabbit anti-α-SMA, Abcam; diluted 1/200 in PBS-0.1% BSA), followed by anti-rabbit HRP and DAB. The slides were rinsed in tap water and briefly counterstained with Harris hematoxyline. For NF_kB p65, the same protocol was applied with an added antigen retrieval step using citrate buffer pH 6 and heating in microwave, before adding the primary antibody (rabbit anti-NF_kB p65, GeneTex; diluted 1/1000 in PBS/0,1% BSA).

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from frozen kidney fragments of either untreated mice or mice treated with the various drugs (n=5-7 mice per experimental group), using the TRIzol reagent (Life Technologies, USA) as per the manufacturer's instructions. Briefly, 10mg of kidney sample was homogenized using a polytron (VWR International). The nucleic acids were purified by chloroform/isopropanol extraction, quantified with the NanoDrop-ND2000 (Thermo Scientific, USA) and treated by DNase (Promega, USA). DNase-treated RNA samples (260/280 nm absorbance ratio of 1.9-2.0) were subjected to cDNA synthesis with the iScript^{TM} cDNA Synthesis Reverse Transcription (RT) kit (Bio-rad Laboratories, USA) as per the manufacturer's instructions. For gene expression profiling, SYBR Green (SensiMixTM SYBR kit, Quantace)-based qPCRs were performed for quantification of a particular transcript using specific primers with Rotor-Gene 6000 instrument (Corbett Research, Australia). Intron spanning and exon-specific primers were designed and synthesized by Microsynth, Switzerland. The sequences of the primers used are provided in **Table 1.** Standard curve analysis (>80% efficiency with single melting curve) was performed to validate the primers and PCR amplicons checked on ethidium bromide-containing agarose gels. To calculate the relative changes in mRNA expression, the ddCt method (**25**) was used. Gene expression levels were normalized to *Gapdh* and the control (vehicle-treated) animal group was assigned 100%. The levels of expression of interleukin (IL)-1β, IL-6, nuclear factor-kappa (NF_k)B1 and NF_kB2, Notch1, hairy and enhancer of split-1 (Hes1), Nephrin1, Snail, cyclin-dependent kinase (CDK)2, angiotensinogen (AGT), renin, APA and angiotensin receptor 1 (AT1) mRNAs were quantified by qRT-PCR and averaged for all animals.

Western blots

Frozen mice kidneys were homogenized using a polytron in RIPA lysis buffer (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0, complete-EDTA free protease inhibitor cocktail (Roche, Germany)) and centrifuged for 20 min (13000g) at 4°C. Tissue lysates were separated by SDS-PAGE and proteins transferred to PVDF membrane (Bio-Rad, USA). Following transfer, the membranes were probed with rabbit anti-cleaved Notch1 antibody (clone Val1744; diluted 1:1000) overnight at 4°C. After washing, blots were incubated for 1 h with a secondary anti-rabbit HRP antibody (dilution 1:1000, Cell Signaling Technology) at room temperature. Blots were probed with an anti-GAPDH antibody (clone 14C10, Cell Signaling Technology; diluted 1:1000) as a loading control. Membranes were developed using Pierce ECL Plus (Thermo Scientific, USA). Bands intensities were quantified using Image J and presented as relative expression to the loading control (GAPDH).

Statistical analysis

The level of statistical significance between multiple experimental groups was assessed using one-way analysis of variance (ANOVA) along with Tukey's post-test for multiple comparisons (GraphPad Prism version 6, California). P values<0.05 were considered significant (*p<0.05, **p<0.01, ***p <0.001).

Results

Effects of the functionalized GSIs in the aristolochic acid-induced murine model of acute tubulointerstitial injury

The chemical structures of the compounds used here are shown in **Figure 1**. Previous *in vitro* and *ex vivo* experiments (**17**) had demonstrated the cleavage of the inactive prodrug N-Ac-γ-Glu-GSI resulting in the release of the active amine-GSI in the presence of the enzymes γ-GT/γ-GCT, while no further hydrolysis to the free inhibitor occurred. Pharmacokinetic experiments, measuring the distribution of the N-Ac-*γ*-Glu-GSI prodrug and its metabolite amine-GSI following i.p. administration in mice, had determined the optimal dose and mode of administration for these compounds (**17**). Using a preliminary *in vivo* setting, we could also demonstrate that the potent *γ*-secretase inhibitor amine-GSI was selectively liberated from the prodrug N-Ac-γ-Glu-GSI in injured kidneys (**17**). Thus, the N-Ac-γ-Glu-GSI prodrug and its amine-GSI metabolite were chosen to test selective kidney protection in the experiments described hereafter. To further investigate for potential beneficial nephroprotective effects of the compounds, firstly, the aristolochic acid (AA)-induced *in vivo* murine model of acute tubulointerstitial renal damage was selected. The efficacy and toxicity profile of the N-Ac-*γ*-Glu-GSI prodrug and its metabolite amine-GSI in control and diseased animals was analyzed using standard clinical chemistry markers at day 7 after administration in our *in vivo* model (**Table 2**). The results showed that control mice treated with the N-Ac-*γ*-Glu-GSI prodrug alone experienced no obvious toxicity other than a slight increase in liver enzymes values (mainly alanine aminotransferase, ALAT and aspartate aminotransferase, ASAT). AA treatment induced acute renal failure as evidenced by significant elevation of serum creatinine and urea levels. The addition of the N-Ac-*γ*-Glu-GSI ameliorated kidney dysfuntion, but did not allow complete prevention of acute tubulopathy induced by AA. Interestingly, AA had a moderate hepatotoxic effect (mainly cytolysis) which was also limited by the adminisration of

the prodrug, possibly due to upregulation of *γ*-GT in acutely injured hepatocytes. Administration of the amine-GSI i.e. the N-Ac-*γ*-Glu-GSI prodrug metabolite directly yielded similar results.

Mice exposed to AA alone or treated with either the N-Ac-γ-Glu-GSI or the metabolite amine-GSI were evaluated clinically (behavior, feeding) and their weight and level of proteinuria was measured at baseline (day 0), then at day 3 and 6 after administration of the various compounds (**Figure 2**). AA exposure induced severe weight loss, most probably due to decreased food and liquid intake (as determined by monitoring daily the food and drink stocks as well as cages beddings). While there was no significant beneficial effect of the GSIs analogs on weight loss during the 7 days follow-up, both drugs resulted in a remarkable improvement of proteinuria, already by day 3. Histological evaluation of the kidneys of the experimental mice confirmed the development of severe acute tubulointerstitial lesions after AA exposure (**Figure 3A**), which affected mainly the proximal tubules as previously described (**2**). The prodrug N-Ac-γ-Glu-GSI given alone had no obvious deleterious effects on the liver and kidney architectures. In our experimental setting, the N-Ac-γ-Glu-GSI treatment regimen could only partially prevent the severe tubulopathy induced by AA, corroborating the kidney function data. Despite the observed slight elevation of liver enzymes, the liver structure was mostly preserved in all experimental groups (**Figure 3B**). In our experimental model, mice were sacrificed on day 7, and besides the protective effect of the N-Ac-γ-Glu-GSI prodrug on acute tubulointerstitial lesions, we also observed a decreased expression of α-smooth muscle actin (α-SMA) at this early time-point, suggesting a protective effect on the development of a profibrotic response following acute injury.

We next investigated specific local inhibition of the cleavage of Notch in the kidneys of

animals exposed to AA alone or in the presence of N-Ac-γ-Glu-GSI (**Figure 4**). Immunohistochemistry stainings (**Figure 4A**) showed that while exposure to AA induced Notch1 expression and Notch1 cleavage reflecting Notch activation, treatment with the functionalized N-Ac-γ-Glu-GSI significantly prevented the expression of cleaved Notch1. By itself and in the absence of AA-mediated injury, N-Ac-γ-Glu-GSI had no effect on the cleavage of Notch in the kidney. These results were futher confirmed by western blot analysis of cleaved Notch1 (also referred to as NICD) expression levels in control, AA-diseased and AA-diseased-N-Ac-γ-Glu-GSI-treated kidney samples (**Figure 4B**). Finally, using histoenzymography, we further evaluated the effects of AA and the N-Ac-γ-Glu-GSI on the activity of the target enzyme, γ-GT. At day 7 after AA exposure, kidneys of diseased mice were severely damaged so that we could not analyze any γ-GT activity at this late time-point nor illustrate, directly on tissue sections, the specific local activation of the GSI prodrug as substrate for this enzyme. However, in previous *ex-vivo* experiments, we were able to demonstrate early upregulation of the enzyme γ-GT mainly in proximal tubules of diseased kidneys and targeted activation of our prodrug allowing local inhibition of the hydrolytic cleavage of Notch (**17**). Overall, these data highlighted the protective effects but also the limitation of functionalized GSIs analogs, when used alone, in preventing severe acute tubulointerstitial injury such as in our *in vivo* model.

Evaluation of downstream pathways associated with the inhibition of Notch1 cleavage

At the end of the treatments (day 7), the animals were sacrificed and kidney sections were stored snap frozen for the determination by qRT-PCR of the mRNA levels of Notch1-responsive genes (**Figure 5A**, *Notch1, Nephrin1, HES1, Snail and CDK2*) and Notch1-inducible inflammatory genes (**Figure 5B,** *IL-1β, IL-6, NFκB1 and NFκB2*). By itself, the N-Ac-γ-Glu-GSI did not induce the expression of genes of the Notch-dependent pathways.

Interestingly, compared to control animals, in animals exposed to AA, a significant induction of genes of the Notch downstream signaling pathway (*Notch1, Nephrin1, HES1, Snail, CDK2)* and of pro-inflammatory cytokines (*IL-1β* and *IL-6*) was observed, as well as an increased expression of genes encoding the transcription factors NFκB1and NFκB2; all of which could be significantly reduced by concurrent treatment with N-Ac-γ-Glu-GSI. Comparable information was obtained when analyzing kidneys of mice administered AA and the amine-GSI (data not shown). To further confirm these gene expression data on Notch-responsive genes and related inflammatory pathways, we investigated the expression of NF_kB at the protein level; NF_kB being one of the main regulators of cellular stress and inflammatory responses. As shown by immunohistochemistry on kidney sections **(Figure 5C),** while there was a high expression of NF_kB p65 (active subunit of the NF_kB transcription complex) in AA-injured kidneys, this expression was limited if the mice had also received the N-Ac-γ-Glu-GSI treatment.

As dysfunction of the RAS has been involved in the development and/or progression of inflammatory disorders of the kidney, we also determined by qRT-PCR if modulation of the Notch pathway by GSIs, either the N-Ac-γ-Glu-GSI or the amine-GSI, may also induce kidney-specific modifications in the expression of components of the RAS (**Figure 6**). In the RAS, the enzymes renin and angiotensin converting enzyme (ACE) sequentially hydrolyze the substrate angiotensinogen (AGT) to release the active octapeptide angiotensin (Ang) II able to bind to two functional receptors, AT1, which is the main receptor in the kidney, and AT2. Then the enzyme APA can hydrolyze the N-terminal Asp of Ang II, releasing Ang III, with different functions than Ang II. Thus, as APA is the main peptidase initiating the degradation of Ang II, we also evaluated this gene. Following AA administration to the mice, up-regulation of all the mRNAs evaluated for the RAS was observed, including the

expression of *APA* mRNA, suggesting a feed-back mechanism. However, the induction of *AT1* gene was low, and not always consistent between the experiments. Overall, these experiments demonstrated that the functionalized GSI prodrug was able to control, in part, the tissue stress response *in vivo* after severe acute kidney tubular injury by controlling the activation of the Notch pathway and its responsive genes downstream.

Effects of the functionalized GSIs in the adriamycin-induced murine model of glomerular injury

The effect of the functionalized GSIs was also determined in another experimental model of renal disease induced by the administration of adriamycin (ADR). As opposed to AA which mainly induces acute tubulopathy, ADR administration is followed by progressive podocyte injury leading to glomerulosclerosis. Using human and mouse samples of diseased kidneys, we have previously shown that the enzyme γ-GT is preferentially expressed in the proximal tubules of injured kidneys and only marginally in the glomerulus (**17**). Therefore, in the ADR model, we used the already active amine-GSI metabolite which is not dependant on γ-GT enzymatic activation within the target organ. In the ADR experimental model as in the AA model, concomitant treatment with the amine-GSI metabolite was able to control proteinuria, but not weight loss (**Figure 7A**). ADR administration also induced Notch1 expression and genes of the RAS in the injured kidneys, which could to some extent be down-regulated by concomitant administration of the amine-GSI (**Figure 7B**). As compared to the AA model of severe tubulopathy, acute inflammatory pathway genes were not all consistently upregulated in this setting, which corresponded to a less acutely toxic and destructive effect of ADR on renal tissues; ADR mainly inducing progressive glomerular damage. **Figure 7C** indeed shows that ADR-mediated lesions are discrete on normal histology (light microscopy HE staining) at an early time-point, as ADR affects the glomerulus with no acute tubulointerstitial injury.

311 There is however a certain degree of glomerulosclerosis (fibrotic lesions are stained in blue in
312 MTB sections, **Figure 7C lower panels**) in the absence of amine-GSI treatment. Finally,
313 western blot analysis of kidney samples confirmed the modulation of Notch1 activation (as
314 detected by its active cleaved form) upon administration of amine-GSI to ADR-treated mice
315 (**Figure 7D**).
316

Discussion

Within the kidney, injury to tubular or glomerular cells is the initiating cause of acute and chronic diseases, leading to progressive dysfunction and end-stage renal failure. Inflammatory and non-inflammatory stresses affect the tubulointerstitial tissue and/or the glomerulus and lead to alterations in their structure, permeability and function. However, irrespective to the initial insult, disease progression ultimately leads to irreversible glomerulosclerosis, interstitial fibrosis and tubular atrophy. Recent studies of various renal diseases in humans and rodent experimental models have yielded several candidate pathways for therapy, which include the Notch pathway (**3,6,7,14,22,30,31,40,43,47**). In experimental mouse models, conditional overexpression of the active Notch1 protein in podocytes results in massive proteinuria and glomerulosclerosis, leading to renal failure and death of the animals. Genetic deletion of Notch transcriptional binding partners or treatment with γ-secretase inhibitors, preventing Notch activation and translocation to the nucleus, protected the animals from nephrotic syndrome. Thus, current data strongly suggest that targeted pharmacologic inhibition of the Notch signaling pathway may prevent kidney damage and improve organ survival.

Notch1-4 are transmembrane proteins that interact with ligands of the Jagged and Delta family. This interaction triggers a series of proteolytic cleavages within the cell. The final γ-secretase complex-mediated cleavage releases the NICD, which is a transcription factor. The function of Notch is context-dependent, regulating tissue homeostasis, cell differentiation and stem cell maintenance in adult life. Regulation of Notch pathway signaling mainly occurs at the levels of ligand binding and γ-secretase complex-mediated cleavage (**20**). We have previously shown that an N-Ac-γ-Glu-GSI prodrug was selectively metabolized in the kidney after i.p. application to an amine-GSI metabolite displaying high Notch antagonism (**17**). Thus

in the present study, the potential beneficial nephroprotective effects of targeting Notch with these functionalized GSI-based prodrugs were investigated using two *in vivo* murine models. AA is a natural herbal component which is toxic to the renal tubular epithelial cells, leading to a dose-dependent rapidly progressive interstitial nephropathy and renal failure (**5**). AA acute tubular toxicity is a result of mitochondrial injury with defective activation of antioxidative enzymes leading to impaired regeneration, apoptosis and defective autophagy of proximal tubular epithelial cells, thus progressive tubular atrophy and interstitial fibrosis (**37,50**). ADR is an anticancer chemotherapeutic agent widely used in the clinic. ADR-induced nephropathy (**10,23,29,32**) is a well described rodent model of progressive glomerular disease, mediated by an oxidative stress and characterized by massive proteinuria due to podocyte injury, followed by glomerulosclerosis, tubulo-interstitial inflammation and fibrosis. ADR-induced renal injury has been shown in numerous studies to be modulated both by non-immune and immune factors. In the present study, we show that treatment with functionalized GSIs could alleviate proteinuria and slightly limit acute kidney dysfunction of mice exposed to AA or ADR. Gene expression profiles analysis of kidney sections demonstrated the induction of Notch1 and its downstream signalling, as well as a very high expression of the active cleaved form of Notch1 by immunohistochemistry and western blot analysis of injured kidneys, which was reduced by concomitant treatment with the functionalized GSIs. However, although the administration of these GSIs could inhibit the activation of Notch and its downstream pathways, selectively blocking Notch activation in the kidney proved insufficient to prevent acute renal failure due to severe tubulointerstitial or glomerular injury, such as induced in our models.

Following Notch activation mediated by the transmembrane γ-secretase complex, the released NICD translocates to the nucleus where it interacts with transcription factors and histone acyltransferases (**8,35,46**). Nuclear localization of the NICD promotes the transcription of

Notch-dependent target genes in a context- and cell-dependent manner. Several studies conducted to identify genes regulated by Notch have demonstrated that these responsive genes include *Notch* itself, *HES1*, *Snail*, *Nephrin*, *CDK2* and genes involved in pro-inflammatory pathways (**13,18,24,26,34,38,39,41,49**). In mice, a loss of the slit diaphragm protein Nephrin was observed exclusively in podocytes expressing activated Notch. Overexpression of activated Notch decreased cell surface Nephrin and increased cytoplasmic Nephrin in transfected HEK 293 cells. Thus, Notch signaling induces endocytosis of Nephrin, thereby triggering the onset of proteinuria (**44**). Notch signaling has been shown to be associated with inflammatory diseases (**1,11,14**). The pro-inflammatory cytokine IL-6 has also been shown to be regulated by Notch signaling and controlled by p53 and the NFκB pathway (**11,15**). A complex signaling crosstalk has also been described in cardiovascular diseases where inflammatory responses regulate Notch signaling and reciprocally Notch has a functional role on inflammatory processes (**36**). Overall, Notch signaling has a role in controlling the cell cycle via CDK2, cell differentiation and transcription via HES1, cell adhesion and epithelial-to-mesemchymal transdifferentiation (EMT) via Snail and Nephrin, as well as the immune response via the cytokines IL-1β, IL-6 and the NFκB pathway. In the present report, quantitative real-time PCRs performed on mRNAs extracted from the acutely injured kidneys demonstrated an upregulation of the expression of down-stream genes of the Notch pathway, including *Notch1*, *HES1*, *Snail* and *CDK2*, and of *IL-1β* and *IL-6* likely mediated by the NFκB pathway, which could all be selectively down-regulated upon administration to the AA and ADR-exposed animals of the functionalized GSIs.

The renin-angiotensin system (RAS) is a main contributor in the regulation of kidney function in homeostatic and disease conditions, acting independently in the blood and the kidney (**12**,**42**). All the RAS components have been found in the kidney, differentially expressed in

various renal compartments. Conversion by APA, a membrane-bound zinc-dependent aminopeptidase expressed in renal proximal tubules and in glomerular cells and which is upregulated upon tissue injury (**17**), of Ang II to Ang III was shown to be critical for angiotensin-mediated effects in the kidney (**19,27,28,33**). The AT1 and AT2 receptors display opposing functions and selectivity for Ang II and Ang III (**4,33**). In the intact kidney the Ang II/AT1R axis is the more highly expressed, whereas in diseased conditions the Ang III/AT2R axis may represent a physiological response to renal tissue stress. Previous data suggest a crosstalk between Ang II and the activation of the Notch pathway in the development of renal diseases. Ang II was shown to induce the synthesis by murine podocytes of extracellular matrix components and transforming growth factor (TGF)-β1 that could be inhibited by GSIs (**48**). In isolated perfused rat kidneys and cultured human podocytes, Ang II down-regulated Nephrin expression via Notch1 activation and nuclear translocation of Snail. HES1 is a Notch1-downstream transcription factor that was shown to activate Snail in cultured podocytes. Changes of the Snail/Nephrin axis in patients with advanced diabetic nephropathy were normalized by pharmacological inhibition of the RAS. Overall, these data point to the relevant role of Ang II in promoting glomerular injury via activation of Notch1/Snail signaling in podocytes, resulting in the down-regulation of Nephrin expression, the integrity of which is crucial for the glomerular filtration barrier (**9**). Therefore, our observation in the present report of a link between targeted blockade of the Notch pathway, inflammatory stress responses and the RAS opens new therapeutic implications for the treatment of kidney diseases, suggesting that the addition of drugs able to control Notch activation such as functionalized GSIs may be of therapeutic value.

Abbreviations

Ac: acetyl

AA: aristolochic acid; 8-methoxy-6-nitrophenanthro[3,4-*d*][1,3]dioxole-5-carboxylic acid

ADR: Adriamycin

Ang: angiotensin

APA: glutamyl aminopeptidase (EC 3.4.11.7.)

AT1/2: angiotensin receptor type 1 or type 2

γ-GT: γ-glutamyl-transpeptidase (EC 2.3.2.2.)

RAS: renin-angiotensin system

γ-GCT: γ-glutamylcyclotransferase (EC 2.3.2.4.)

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Conflicts of interest

MS and JDA are employees of F. Hoffmann-La Roche but declare no conflict of interest. JCW, RK, JM, LJJ and DG declare no conflict of interest.

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635 **Tables**

636

637 ***Table 1. Sequences of the primers used for the qPCR experiments.***

gene symbol	*forward sequence (5'–3')*	*reverse sequence (5'–3')*
AGT	GGCAAATCTGAACAACATTGG	TTCCTCCTCTCCTGCTTTGA
APA	TGGACTCCAAAGCTGATCCT	TCAGCCCATCTGACTGGAAT
AT1	ACTCACAGCAACCCTCCAAG	CTCAGACACTGTTCAAAATGCAC
CDK2	TTCCTCTTCCCCTCATCAAG	ACGGTGAGAATGGCAGAAAG
Gapdh	GTCGGTGTGAACGGATTTG	AAGATGGTGATGGGCTTCC
HES1	TGCCAGCTGATATAATGGAGAA	CCATGATAGGCTTTGATGACTTT
IL-1β	GGGCCTCAAAGGAAAGAATC	CTCTGCTTGTGAGGTGCTGA
IL-6	AGAAGGGCCTGGAATGAAAC	AAGACCCTGCTGGAACAAGA
Nephrin1	GGATATAGTCTGCACCGTCGAT	TCAGTTCCTCCTCGTCTTCC
NFκB1	GGGTCTGGGGATACTGAACA	GCCTCCATCAGCTCTTTGAT
NFκB2	TGGAACAGCCCAAACAGC	CACCTGGCAAACCTCCAT
Notch1	CTGGACCCCATGGACATC	AGGATGACTGCACACATTGC
Renin	GGAGGAAGTGTTCTCTGTCTACTACA	GCTACCTCCTAGCACCACCTC
Snail	CTTGTGTCTGCACGACCTGT	CAGGAGAATGGCTTCTCACC

638

639

640 ***Table 2. Serum chemistry profile of mice exposed to aristolochic acid (AA) alone, or with***

641 ***the N-Ac-γ-Glu-GSI or the amine-GSI.***

642

		Creat	***Urea***	***Prot***	***Alb***	***ASAT***	***ALAT***	***AlkP***
		µmol/l	*mmol/l*	***g/l***	*g/l*	*U/l*	*U/l*	*U/l*
Control	mean	43.4	8.8	53.4	30.7	372.0	55.6	141.5
	± sd	4.8	1.1	3.2	2.5	77.6	4.2	11.5
N-Ac-γ-Glu-GSI		37.4	7.7	54.0	30.8	516.0	92.4	136.1
		4.0	0.5	2.5	1.3	212.1	26.3	18.1
AA		251.4	112.3	52.2	29.4	459.4	179.8	108.7
		69.0	15.6	4.7	3.0	204.6	73.8	27.8
AA +		160.3	85.2	46.8	26.3	375.9	43.4	94.6
N-Ac-γ-Glu-GSI		69.3	20.5	15.1	8.1	120.4	16.8	25.6
AA +		169.0	90.0	57.6	31.0	550.6	208.8	140.0
amine-GSI		29.3	9.8	2.4	2.8	219.6	83.5	20.3

643

644 *Abbreviations*: Creat: creatinine; Prot: proteins; Alb: albumin; ASAT: aspartate aminotransferase; ALAT:
645 alanine aminotransferase; AlkP: alkaline phosphatase.
646

647

648

649

650

651 **Figures Legends**

652 **Figure 1.** ***Chemical structure of the N-Ac-γ-Glu-GSI prodrug and its proteolytic activation.***

653 The prodrug is composed of the active compound (γ-secretase inhibitor, GSI), a linker
654 (amine) and the targeting N-acetyl (N-Ac)-γ-Glu-moiety as substrate for the releasing acylase
655 and γ-Glu-transpeptidase (γ-GT) and/or γ-Glu-cyclotransferase peptidases γ-GCT (arrows).

656

657 **Figure 2.** ***Body weight (A) and proteinuria (B) of mice exposed to aristolochic acid, N-Ac-γ-***
658 ***Glu-GSI, aristolochic acid together with N-Ac-γ-Glu-GSI and aristolochic acid with amine-***
659 ***GSI.***

660 Acute tubular injury was induced in 10 weeks old BALB/c male mice by i.p. injection of
661 aristolochic acid (AA). The GSI analogs were administered i.p., starting one day before
662 injection of AA (day -1) and then twice daily until day 6 evening. The animals were
663 monitored clinically daily, weighted and the level of albuminuria was semi-quantitatively
664 assessed at day 0, day 3 and day 6. Results are presented as means $\pm$ sd for all mice in each
665 experimental group, with comparisons between treated *versus* control animals or between
666 treatments. (**$p<0.01$; ***$p<0.001$; NS not statistically significant).

667

668 **Figure 3.** ***Histology of the kidneys and livers of mice treated with aristolochic acid and the***
669 ***N-Ac-γ-Glu-GSI.***

670 Acute kidney injury was induced by i.p. injection of aristolochic acid (AA) (1x5mg/kg) in 10
671 weeks old BALB/c male mice. Hematoxylin/eosin (HE), Masson's trichrome blue (MTB) and
672 α-smooth muscle actin (α-SMA) staining of mouse kidney sections (**A**) and MTB staining of
673 liver sections (**B**) of untreated (controls), mice treated with N-Ac-γ-Glu-GSI, or after
674 exposure to AA without or with N-Ac-γ-Glu-GSI treatment. Representative
675 images/experimental groups are shown (40x).

Figure 4. ***Expression of Notch1 and cleaved Notch1 in the kidneys of mice treated with aristolochic acid and N-Ac-γ-Glu-GSI.***

(A) Frozen kidneys sections (7µm) of control and aristolochic acid **(**AA)-treated mice, without or with the N-Ac-γ-Glu-GSI, were exposed to either anti-Notch1 or anti-cleaved Notch1 antibodies, followed by the alkaline phosphatase-fast-red chromogen staining and hematoxylin counterstaining. Immunostaining is visualized as a red-brown precipitate. **(B)** Western blot analysis of cleaved Notch1 expression in kidney samples from control and AA-treated mice, without or with the N-Ac-γ-Glu-GSI. Results of 3 representative mice/experimental group are shown. GAPDH was used as loading control and the intensities of the bands were quantified relative to GAPDH.

Figure 5. ***Notch1 and inflammatory pathway-responsive genes in the kidneys of mice exposed to aristolochic acid and the N-Ac-γ-Glu-GSI.***

Acute kidney injury was induced by i.p. injection of aristolochic acid (AA) in 10 weeks old BALB/c male mice. At the end of the experiment (day 7 morning), the animals were sacrificed and mRNAs were extracted from the snap-frozen kidneys. The levels of expression of the mRNAs for (**A**) Notch1-responsive genes and (**B**) Notch1-inducible inflammatory markers were quantified by qRT-PCR and results were averaged for all animals in each experimental group. Results are presented as % of changes in the mRNA levels in the treated animals *versus* control animals $\pm$ sem. (*p<0.05; **p<0.01; ***p<0.001). **C.** Immunohistochemistry staining of NF_kB p65 on kidney sections of untreated (controls), control mice treated with N-Ac-γ-Glu-GSI, or after exposure to AA without or with N-Ac-γ-Glu-GSI treatment.

Figure 6. ***Modulation of genes of the renin-angiotensin system (RAS) in the kidneys of***

mice administered aristolochic acid and treated with functionalized GSIs.

After 7 days of treatment, with either (**A**) aristolochic acid (AA) alone or together with N-Ac-γ-Glu-GSI or (**B**) AA alone or with amine-GSI, the animals were sacrificed and their kidneys extracted for the determination by qRT-PCR of the mRNA levels of genes of the components of the RAS. Results were averaged for all animals per experimental group and are presented as % of changes in the mRNA levels in the treated animals *versus* control animals ± sem. (*p<0.05; **p< 0.01; ***p<0.001).

Figure 7. ***Clinical parameters, Notch1-related pathways activation profiles and kidney histology of mice exposed to adriamycin and the amine-GSI.***

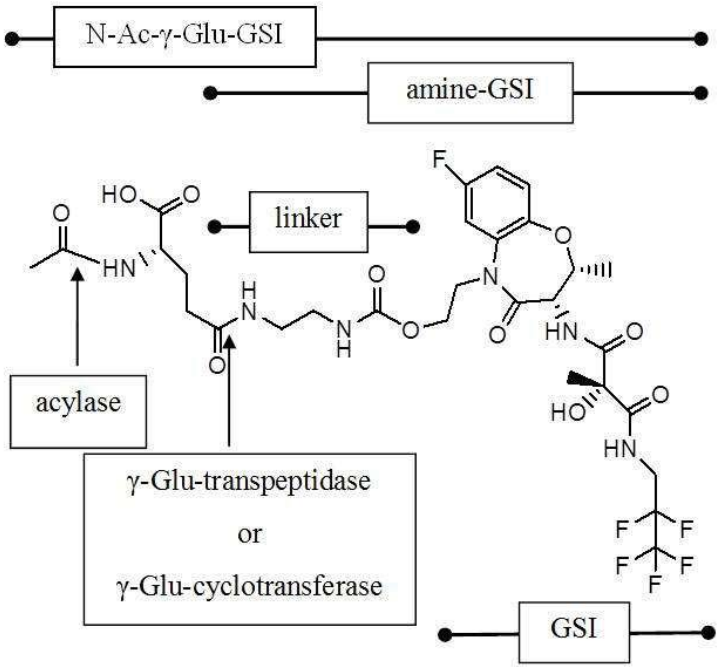
Glomerular injury was induced in 10 weeks old BALB/c male mice by i.p. injection of adriamycin (ADR). The amine-GSI metabolite was administered i.p., starting one day before injection of ADR (day -1) and then twice daily until day 6 evening. (**A**) Mice were monitored clinically daily, weighted and the level of proteinuria was semi-quantitatively assessed at day 0, day 3 and day 6. Results were averaged for all mice per experimental group and means ± sd are shown. (**B**) After 7 days of treatment, with either ADR alone or together with amine-GSI, the animals were sacrificed and mRNAs were extracted from the snap-frozen kidneys. The levels of expression of the mRNAs for the Notch1-inducible genes and genes of the renin-angiotensin system (RAS) were quantified by qRT-PCR. Results were averaged for all animals per experimental group and are presented as % of changes in the mRNA levels in the treated animals *versus* the control animals ± sem. (*p<0.05; **p<0.01; ***p<0.001). (**C**) Kidney sections histology with hematoxylin/eosin (HE) and Masson's trichrome blue (MTB) stainings after ADR administration, alone or with amine-GSI. **(D)** Western blot analysis of cleaved Notch1expression in kidney samples from control and ADR-treated mice, without or with the amine-GSI. Results of 3 representative mice/experimental group are shown. GAPDH

726 was used as loading control and the intensities of the bands were quantified relative to
727 GAPDH.

728

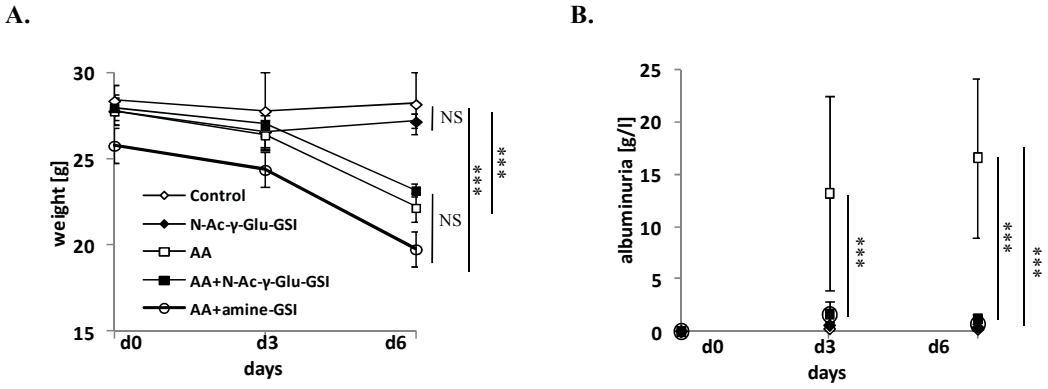
Figures

Figure 1.



Acylase and γ-Glu-cyclotransferase (γ-GCT) are cytoplasmic proteins, γ-Glu-transpeptidase (γ-GT) is a membrane-bound protein.

Figure 2.


A.
B.
30
25
20
15
weight [g]
d0
d3
d6
days
Control
N-Ac-γ-Glu-GSI
AA
AA+N-Ac-γ-Glu-GSI
AA+amine-GSI
NS
NS
***
***
25
20
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10
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0
albuminuria [g/l]
d0
d3
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days
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Figure 3.

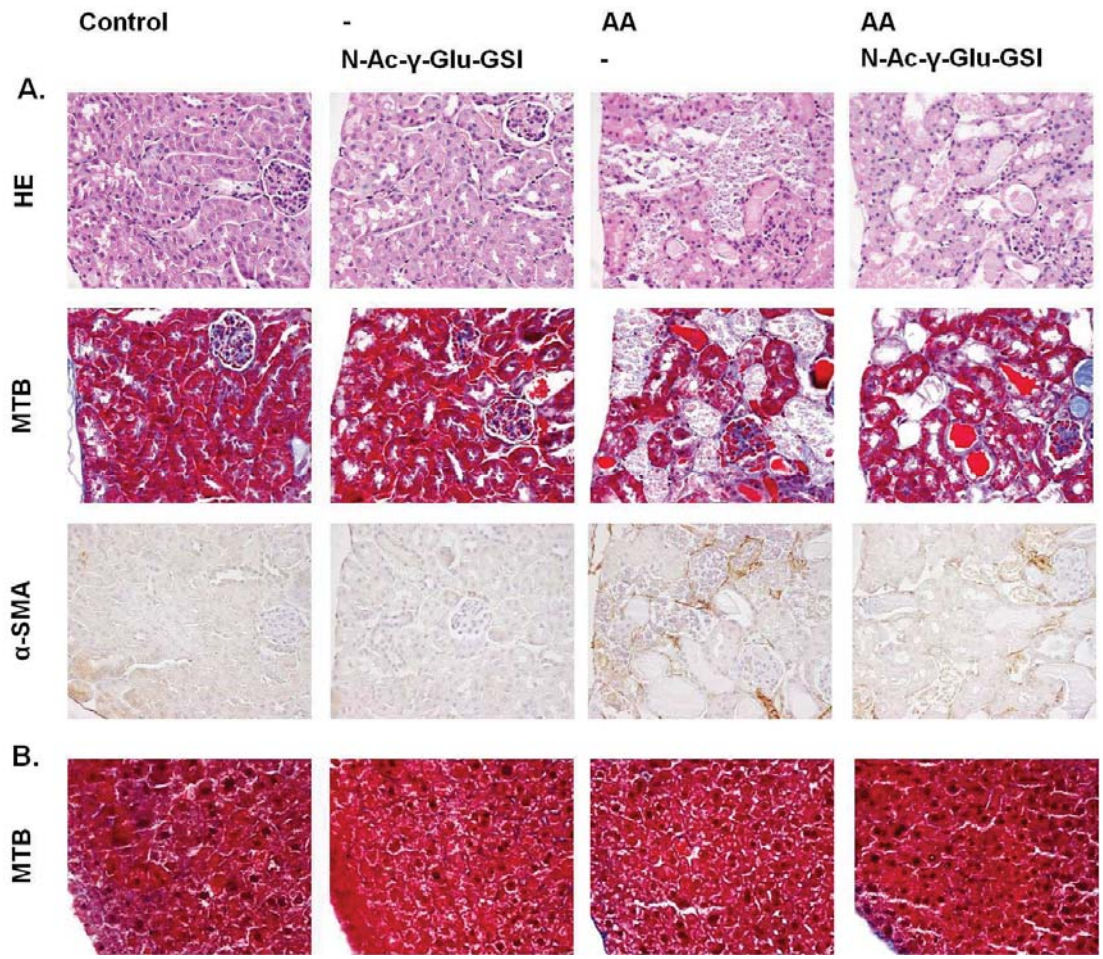


Figure 4.

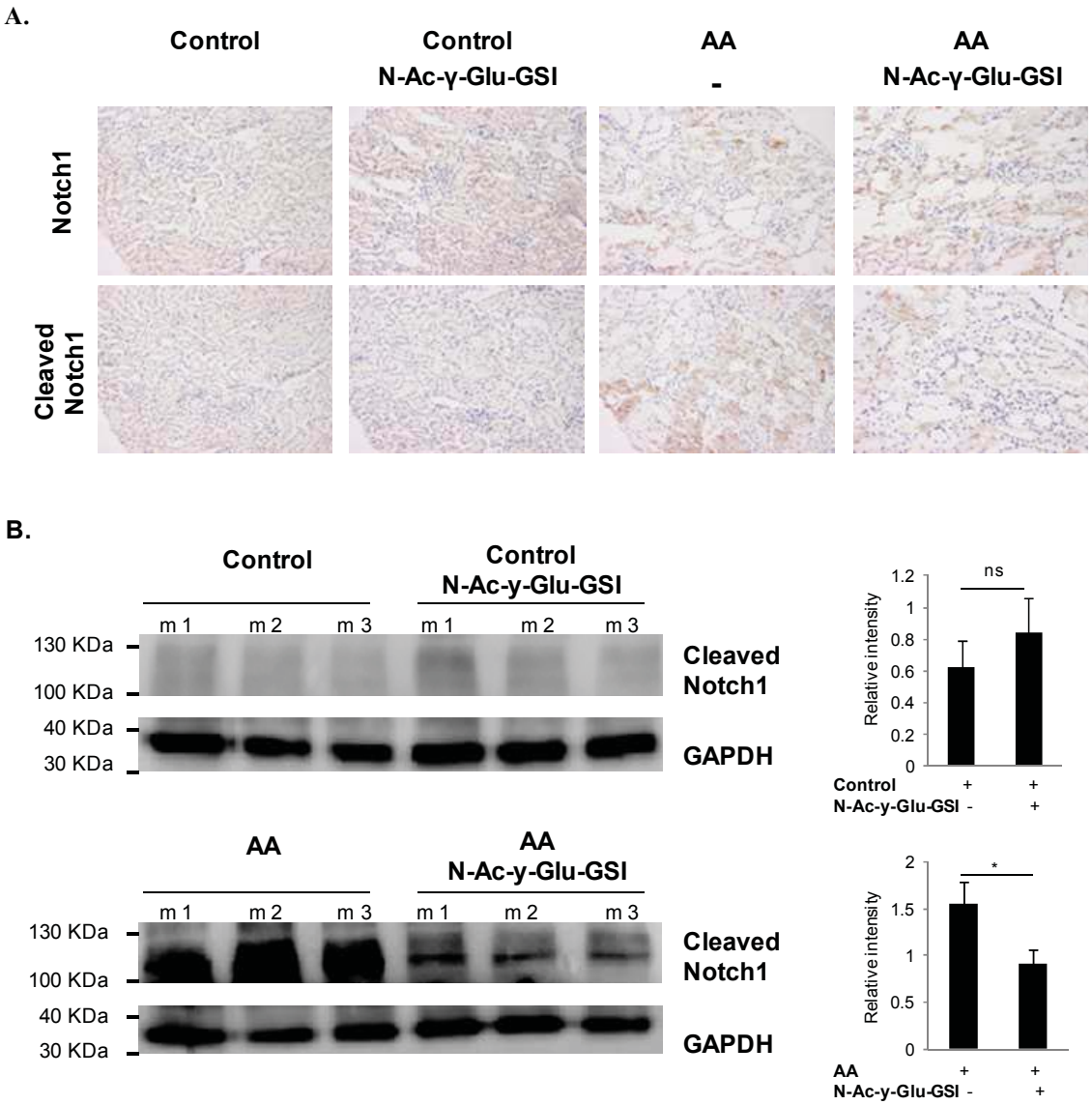

A.
Control
Control
N-Ac-γ-Glu-GSI
AA
-
AA
N-Ac-γ-Glu-GSI
Notch1
Cleaved Notch1
B.
Control
Control
N-Ac-y-Glu-GSI
m 1
m 2
m 3
m 1
m 2
m 3
130 KDa
100 KDa
40 KDa
30 KDa
Cleaved Notch1
GAPDH
ns
Relative intensity
1.2
1
0.8
0.6
0.4
0.2
0
Control + +
N-Ac-y-Glu-GSI - +
AA
AA
N-Ac-y-Glu-GSI
m 1
m 2
m 3
m 1
m 2
m 3
130 KDa
100 KDa
40 KDa
30 KDa
Cleaved Notch1
GAPDH
*
Relative intensity
2
1.5
1
0.5
0
AA + +
N-Ac-y-Glu-GSI - +

Figure 5.

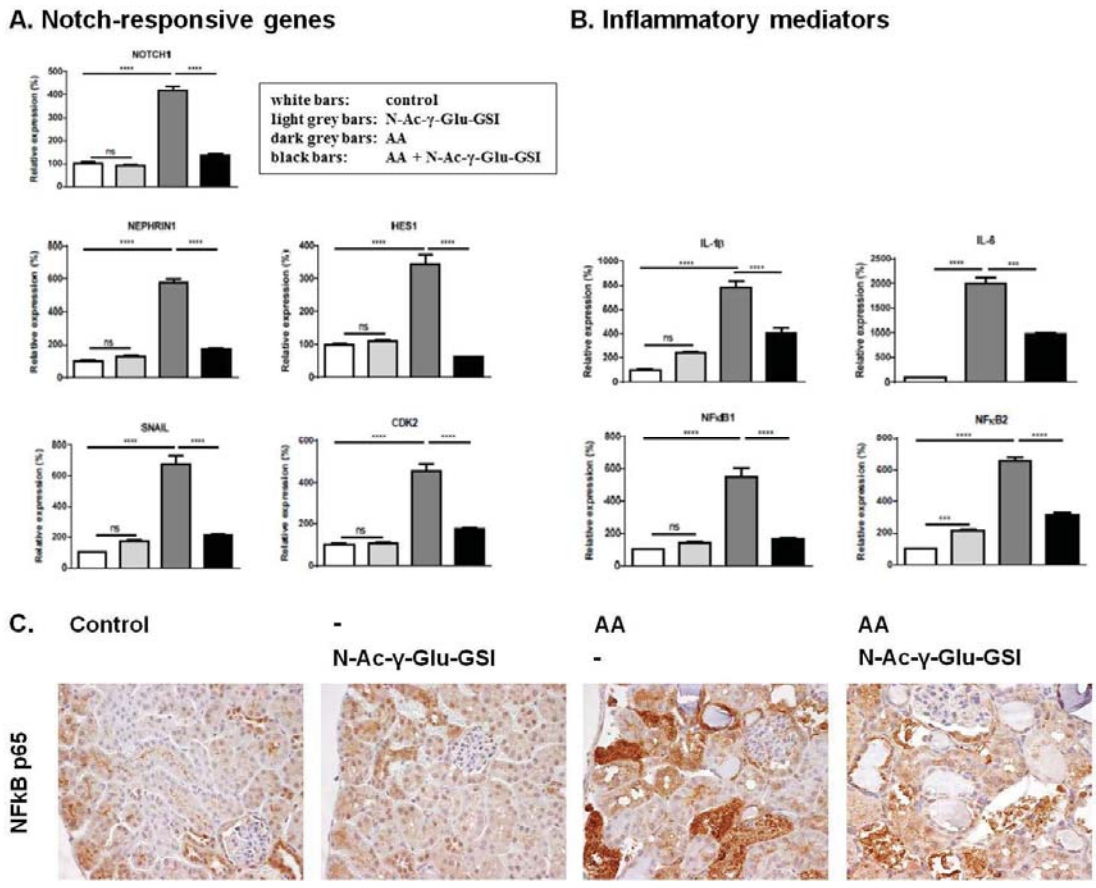

A. Notch-responsive genes
B. Inflammatory mediators
white bars: control
light grey bars: N-Ac-γ-Glu-GSI
dark grey bars: AA
black bars: AA + N-Ac-γ-Glu-GSI
NOTCH1
NEPHRIN1
HES1
IL-1β
IL-6
SNAIL
CDK2
NFκB1
NFκB2
Relative expression (%)
ns
C.
Control
-
N-Ac-γ-Glu-GSI
AA
-
AA
N-Ac-γ-Glu-GSI
NFkB p65

Figure 6.

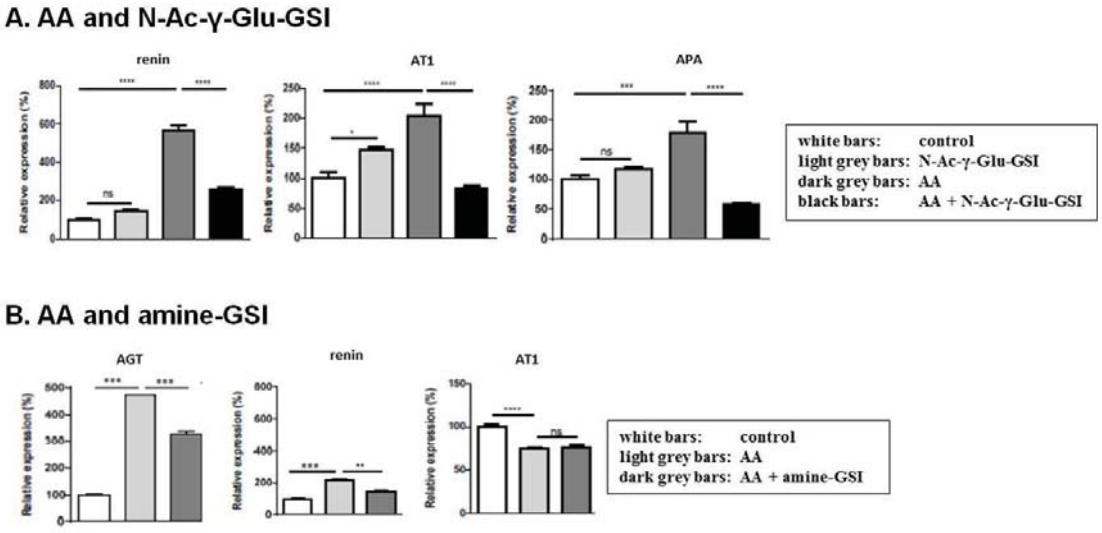
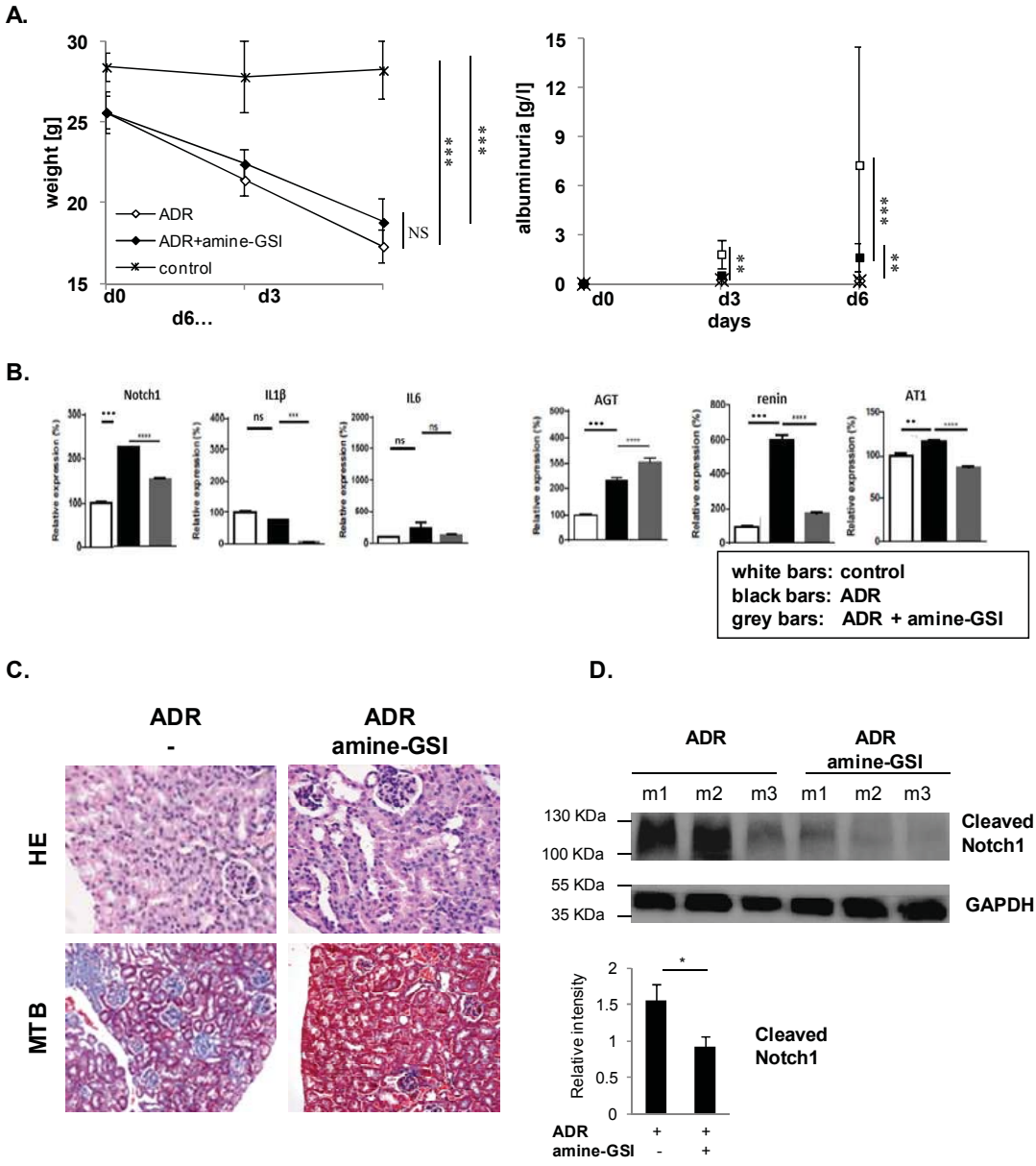

A. AA and N-Ac-γ-Glu-GSI
renin
AT1
APA
Relative expression (%)
white bars: control
light grey bars: N-Ac-γ-Glu-GSI
dark grey bars: AA
black bars: AA + N-Ac-γ-Glu-GSI
B. AA and amine-GSI
AGT
renin
AT1
Relative expression (%)
white bars: control
light grey bars: AA
dark grey bars: AA + amine-GSI

Figure 7.


A.
weight [g]
ADR
ADR+amine-GSI
control
NS
d0
d3
d6…
albuminuria [g/l]
days
B.
Notch1
IL1β
IL6
AGT
renin
AT1
white bars: control
black bars: ADR
grey bars: ADR + amine-GSI
C.
ADR
-
ADR
amine-GSI
HE
MTB
D.
ADR
ADR
amine-GSI
m1
m2
m3
130 KDa
100 KDa
55 KDa
35 KDa
Cleaved
Notch1
GAPDH
Relative intensity
Cleaved
Notch1
ADR
amine-GSI