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1 **Revised manuscript**

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

4
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16
17 *Running head:* Notch inhibition in kidney diseases

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22 *Contribution of authors:* LJJ and DG designed the project, evaluated the results and wrote the
23 manuscript; JDA participated in the design of the project, designed and prepared the
24 compounds and participated in the evaluation of the results and the writing of the manuscript;
25 MS designed, performed and evaluated the pharmacokinetics data and participated in the
26 writing of the manuscript; JCW, RK and JM performed the experiments, evaluated the results
27 and participated in the writing of the manuscript.

28

29 **Abstract**

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

46

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

49

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51 **Introduction**

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

70

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

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

93

94 **Materials and Methods**

95 *Animal models of induced kidney injury*

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

119 ***Immunohistochemistry***

120 OCT-embedded frozen kidneys were cut at 7 μ m. The sections were air-dried, fixed for 10
121 min in cold (-20 °C) methanol, rinsed in PBS 0.1% Triton X-100 (PBS/Triton), and blocked
122 for 30 min with PBS/Triton containing 5% bovine serum albumin (BSA). Endogenous
123 peroxidase and biotin were blocked using 3% H₂O₂ and avidin/biotin blocking kit (Vector
124 Laboratories, Burlingame, CA 94010, USA), respectively. The rabbit anti-Notch1 antibody
125 (clone D1E11, Cell Signaling Technology, Leiden, The Netherland; diluted 1/50 in PBS/5%
126 BSA) or the rabbit anti-cleaved Notch1 antibody (clone Val1744, Cell Signaling; diluted 1/50
127 in PBS/5% BSA) were added to the sections for 1 h. The slides were rinsed with PBS/Triton
128 three times, incubated for 1 h with biotinylated anti-rabbit secondary antibody (Vector
129 Laboratories, diluted 1/500 in PBS/5% BSA), washed with PBS, incubated with
130 streptavidin/horse radish peroxidase (HRP) (Dako, Bollschweil, Germany; diluted 1/500) for
131 1 h, followed by 15 min with 3,3'-diaminobenzidine (DAB, Dako). The slides were washed in
132 distilled water, mounted in Aquamount, (Immu-mount, Thermo Shandon Pittsburgh, PA,
133 USA) and analyzed.

134 For the staining of α -smooth muscle actin (α -SMA), paraffin-embedded kidney sections were
135 used. Slides were deparaffined following standard procedures and endogenous peroxidase was
136 blocked using 1% H₂O₂ in methanol. Slides were then rehydrated by washing in decreasing
137 gradients of ethanol (100% twice, 95% twice, 80% once) followed by tap water, then blocked
138 10 min in PBS/10% goat serum before adding the primary antibody for 1 h (rabbit anti- α -
139 SMA, Abcam; diluted 1/200 in PBS-0.1% BSA), followed by anti-rabbit HRP and DAB. The
140 slides were rinsed in tap water and briefly counterstained with Harris hematoxyline. For NF κ B
141 p65, the same protocol was applied with an added antigen retrieval step using citrate buffer
142 pH 6 and heating in microwave, before adding the primary antibody (rabbit anti-NF κ B p65,
143 GeneTex; diluted 1/1000 in PBS/0,1% BSA).

144 ***Real-time quantitative PCR (qRT-PCR)***

145 Total RNA was extracted from frozen kidney fragments of either untreated mice or mice
146 treated with the various drugs (n=5-7 mice per experimental group), using the TRIzol reagent
147 (Life Technologies, USA) as per the manufacturer's instructions. Briefly, 10mg of kidney
148 sample was homogenized using a polytron (VWR International). The nucleic acids were
149 purified by chloroform/isopropanol extraction, quantified with the NanoDrop-ND2000
150 (Thermo Scientific, USA) and treated by DNase (Promega, USA). DNase-treated RNA
151 samples (260/280 nm absorbance ratio of 1.9-2.0) were subjected to cDNA synthesis with the
152 iScriptTM cDNA Synthesis Reverse Transcription (RT) kit (Bio-rad Laboratories, USA) as per
153 the manufacturer's instructions. For gene expression profiling, SYBR Green (SensiMixTM
154 SYBR kit, Quantace)-based qPCRs were performed for quantification of a particular
155 transcript using specific primers with Rotor-Gene 6000 instrument (Corbett Research,
156 Australia). Intron spanning and exon-specific primers were designed and synthesized by
157 Microsynth, Switzerland. The sequences of the primers used are provided in **Table 1**.
158 Standard curve analysis (>80% efficiency with single melting curve) was performed to
159 validate the primers and PCR amplicons checked on ethidium bromide-containing agarose
160 gels. To calculate the relative changes in mRNA expression, the ddCt method (**25**) was used.
161 Gene expression levels were normalized to *Gapdh* and the control (vehicle-treated) animal
162 group was assigned 100%. The levels of expression of interleukin (IL)-1 β , IL-6, nuclear
163 factor-kappa (NF κ)B1 and NF κ B2, Notch1, hairy and enhancer of split-1 (Hes1), Neph1n1,
164 Snail, cyclin-dependent kinase (CDK)2, angiotensinogen (AGT), renin, APA and angiotensin
165 receptor 1 (AT1) mRNAs were quantified by qRT-PCR and averaged for all animals.

166

167 ***Western blots***

168 Frozen mice kidneys were homogenized using a polytron in RIPA lysis buffer (150mM
169 sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0,
170 complete-EDTA free protease inhibitor cocktail (Roche, Germany)) and centrifuged for 20
171 min (13000g) at 4°C. Tissue lysates were separated by SDS-PAGE and proteins transferred to
172 PVDF membrane (Bio-Rad, USA). Following transfer, the membranes were probed with
173 rabbit anti-cleaved Notch1 antibody (clone Val1744; diluted 1:1000) overnight at 4°C. After
174 washing, blots were incubated for 1 h with a secondary anti-rabbit HRP antibody (dilution
175 1:1000, Cell Signaling Technology) at room temperature. Blots were probed with an anti-
176 GAPDH antibody (clone 14C10, Cell Signaling Technology; diluted 1:1000) as a loading
177 control. Membranes were developed using Pierce ECL Plus (Thermo Scientific, USA). Bands
178 intensities were quantified using Image J and presented as relative expression to the loading
179 control (GAPDH).

180

181 *Statistical analysis*

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

186 **Results**

187 *Effects of the functionalized GSIs in the aristolochic acid-induced murine model of acute* 188 *tubulointerstitial injury*

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

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

214

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

234

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

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

254

255 ***Evaluation of downstream pathways associated with the inhibition of Notch1 cleavage***

256 At the end of the treatments (day 7), the animals were sacrificed and kidney sections were
257 stored snap frozen for the determination by qRT-PCR of the mRNA levels of Notch1-
258 responsive genes (**Figure 5A**, *Notch1*, *Nephrin1*, *HES1*, *Snail* and *CDK2*) and Notch1-
259 inducible inflammatory genes (**Figure 5B**, *IL-1 β* , *IL-6*, *NF κ B1* and *NF κ B2*). By itself, the N-
260 Ac- γ -Glu-GSI did not induce the expression of genes of the Notch-dependent pathways.

261 Interestingly, compared to control animals, in animals exposed to AA, a significant induction
262 of genes of the Notch downstream signaling pathway (*Notch1*, *Nephrin1*, *HES1*, *Snail*,
263 *CDK2*) and of pro-inflammatory cytokines (*IL-1 β* and *IL-6*) was observed, as well as an
264 increased expression of genes encoding the transcription factors NF κ B1 and NF κ B2; all of
265 which could be significantly reduced by concurrent treatment with N-Ac- γ -Glu-GSI.
266 Comparable information was obtained when analyzing kidneys of mice administered AA and
267 the amine-GSI (data not shown). To further confirm these gene expression data on Notch-
268 responsive genes and related inflammatory pathways, we investigated the expression of NF κ B
269 at the protein level; NF κ B being one of the main regulators of cellular stress and inflammatory
270 responses. As shown by immunohistochemistry on kidney sections (**Figure 5C**), while there
271 was a high expression of NF κ B p65 (active subunit of the NF κ B transcription complex) in AA-
272 injured kidneys, this expression was limited if the mice had also received the N-Ac- γ -Glu-GSI
273 treatment.

274

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

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

291

292 *Effects of the functionalized GSIs in the adriamycin-induced murine model of glomerular* 293 *injury*

294 The effect of the functionalized GSIs was also determined in another experimental model of
295 renal disease induced by the administration of adriamycin (ADR). As opposed to AA which
296 mainly induces acute tubulopathy, ADR administration is followed by progressive podocyte
297 injury leading to glomerulosclerosis. Using human and mouse samples of diseased kidneys,
298 we have previously shown that the enzyme γ -GT is preferentially expressed in the proximal
299 tubules of injured kidneys and only marginally in the glomerulus (17). Therefore, in the ADR
300 model, we used the already active amine-GSI metabolite which is not dependant on γ -GT
301 enzymatic activation within the target organ. In the ADR experimental model as in the AA
302 model, concomitant treatment with the amine-GSI metabolite was able to control proteinuria,
303 but not weight loss (**Figure 7A**). ADR administration also induced Notch1 expression and
304 genes of the RAS in the injured kidneys, which could to some extent be down-regulated by
305 concomitant administration of the amine-GSI (**Figure 7B**). As compared to the AA model of
306 severe tubulopathy, acute inflammatory pathway genes were not all consistently upregulated
307 in this setting, which corresponded to a less acutely toxic and destructive effect of ADR on
308 renal tissues; ADR mainly inducing progressive glomerular damage. **Figure 7C** indeed shows
309 that ADR-mediated lesions are discrete on normal histology (light microscopy HE staining) at
310 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

317 Discussion

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

333

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

342 in the present study, the potential beneficial nephroprotective effects of targeting Notch with
343 these functionalized GSI-based prodrugs were investigated using two *in vivo* murine models.
344 AA is a natural herbal component which is toxic to the renal tubular epithelial cells, leading to
345 a dose-dependent rapidly progressive interstitial nephropathy and renal failure (5). AA acute
346 tubular toxicity is a result of mitochondrial injury with defective activation of antioxidative
347 enzymes leading to impaired regeneration, apoptosis and defective autophagy of proximal
348 tubular epithelial cells, thus progressive tubular atrophy and interstitial fibrosis (37,50). ADR
349 is an anticancer chemotherapeutic agent widely used in the clinic. ADR-induced nephropathy
350 (10,23,29,32) is a well described rodent model of progressive glomerular disease, mediated by
351 an oxidative stress and characterized by massive proteinuria due to podocyte injury, followed
352 by glomerulosclerosis, tubulo-interstitial inflammation and fibrosis. ADR-induced renal
353 injury has been shown in numerous studies to be modulated both by non-immune and immune
354 factors. In the present study, we show that treatment with functionalized GSIs could alleviate
355 proteinuria and slightly limit acute kidney dysfunction of mice exposed to AA or ADR. Gene
356 expression profiles analysis of kidney sections demonstrated the induction of Notch1 and its
357 downstream signalling, as well as a very high expression of the active cleaved form of Notch1
358 by immunohistochemistry and western blot analysis of injured kidneys, which was reduced by
359 concomitant treatment with the functionalized GSIs. However, although the administration of
360 these GSIs could inhibit the activation of Notch and its downstream pathways, selectively
361 blocking Notch activation in the kidney proved insufficient to prevent acute renal failure due
362 to severe tubulointerstitial or glomerular injury, such as induced in our models.

363

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

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

388

389 The renin-angiotensin system (RAS) is a main contributor in the regulation of kidney function
390 in homeostatic and disease conditions, acting independently in the blood and the kidney
391 (12,42). All the RAS components have been found in the kidney, differentially expressed in

392 various renal compartments. Conversion by APA, a membrane-bound zinc-dependent
393 aminopeptidase expressed in renal proximal tubules and in glomerular cells and which is
394 upregulated upon tissue injury (17), of Ang II to Ang III was shown to be critical for
395 angiotensin-mediated effects in the kidney (19,27,28,33). The AT1 and AT2 receptors display
396 opposing functions and selectivity for Ang II and Ang III (4,33). In the intact kidney the Ang
397 II/AT1R axis is the more highly expressed, whereas in diseased conditions the Ang III/AT2R
398 axis may represent a physiological response to renal tissue stress. Previous data suggest a
399 crosstalk between Ang II and the activation of the Notch pathway in the development of renal
400 diseases. Ang II was shown to induce the synthesis by murine podocytes of extracellular
401 matrix components and transforming growth factor (TGF)- β 1 that could be inhibited by GSIs
402 (48). In isolated perfused rat kidneys and cultured human podocytes, Ang II down-regulated
403 Nephlin expression via Notch1 activation and nuclear translocation of Snail. HES1 is a
404 Notch1-downstream transcription factor that was shown to activate Snail in cultured
405 podocytes. Changes of the Snail/Nephlin axis in patients with advanced diabetic nephropathy
406 were normalized by pharmacological inhibition of the RAS. Overall, these data point to the
407 relevant role of Ang II in promoting glomerular injury via activation of Notch1/Snail
408 signaling in podocytes, resulting in the down-regulation of Nephlin expression, the integrity
409 of which is crucial for the glomerular filtration barrier (9). Therefore, our observation in the
410 present report of a link between targeted blockade of the Notch pathway, inflammatory stress
411 responses and the RAS opens new therapeutic implications for the treatment of kidney
412 diseases, suggesting that the addition of drugs able to control Notch activation such as
413 functionalized GSIs may be of therapeutic value.

414

415

416

417 **Abbreviations**

418 Ac: acetyl

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

420 ADR: Adriamycin

421 Ang: angiotensin

422 APA: glutamyl aminopeptidase (EC 3.4.11.7.)

423 AT1/2: angiotensin receptor type 1 or type 2

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

425 RAS: renin-angiotensin system

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

427

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436

437 **Conflicts of interest**

438 MS and JDA are employees of F. Hoffmann-La Roche but declare no conflict of interest.

439 JCW, RK, JM, LJJ and DG declare no conflict of interest.

440

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628 but alleviated nonapoptotic cell death in mouse acute aristolochic acid nephropathy model.
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634

635 **Tables**

636

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

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

		<i>Creat</i>	<i>Urea</i>	<i>Prot</i>	<i>Alb</i>	<i>ASAT</i>	<i>ALAT</i>	<i>AlkP</i>
		$\mu\text{mol/l}$	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
	\pm 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
AA		4.0	0.5	2.5	1.3	212.1	26.3	18.1
		251.4	112.3	52.2	29.4	459.4	179.8	108.7
AA +		69.0	15.6	4.7	3.0	204.6	73.8	27.8
		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).

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

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

686

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

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

699

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

701 *mice administered aristolochic acid and treated with functionalized GSIs.*

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

708

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

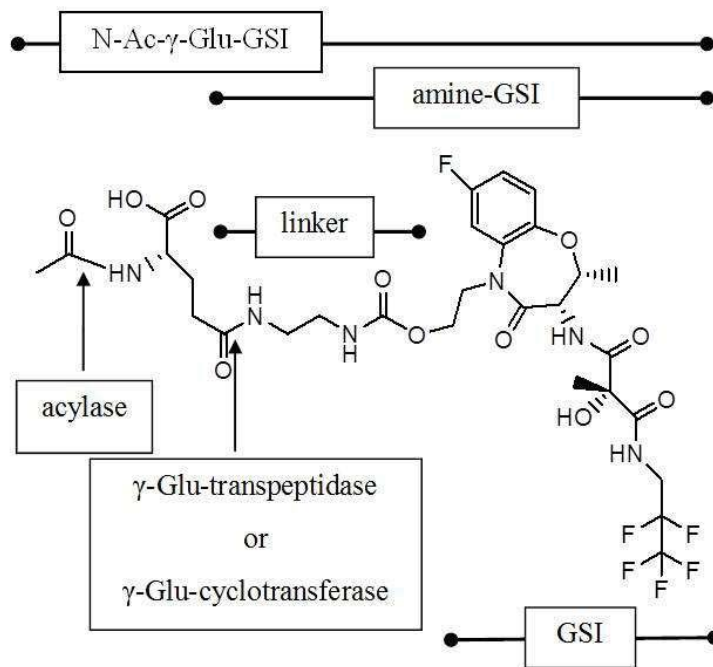
711 Glomerular injury was induced in 10 weeks old BALB/c male mice by i.p. injection of
712 adriamycin (ADR). The amine-GSI metabolite was administered i.p., starting one day before
713 injection of ADR (day -1) and then twice daily until day 6 evening. (A) Mice were monitored
714 clinically daily, weighted and the level of proteinuria was semi-quantitatively assessed at day
715 0, day 3 and day 6. Results were averaged for all mice per experimental group and means \pm sd
716 are shown. (B) After 7 days of treatment, with either ADR alone or together with amine-GSI,
717 the animals were sacrificed and mRNAs were extracted from the snap-frozen kidneys. The
718 levels of expression of the mRNAs for the Notch1-inducible genes and genes of the renin-
719 angiotensin system (RAS) were quantified by qRT-PCR. Results were averaged for all
720 animals per experimental group and are presented as % of changes in the mRNA levels in the
721 treated animals *versus* the control animals \pm sem. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (C)
722 Kidney sections histology with hematoxylin/eosin (HE) and Masson's trichrome blue (MTB)
723 stainings after ADR administration, alone or with amine-GSI. (D) Western blot analysis of
724 cleaved Notch1 expression in kidney samples from control and ADR-treated mice, without or
725 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

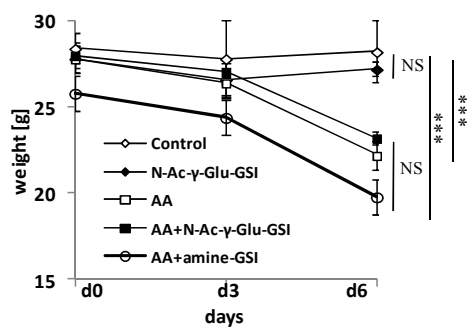
Figure 1.



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

Figure 2.

A.



B.

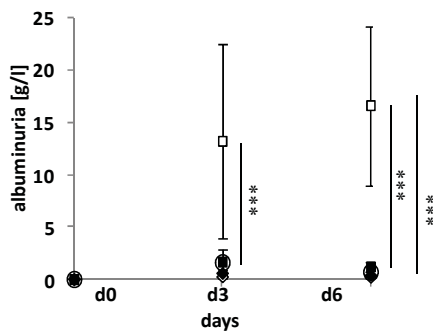


Figure 3.

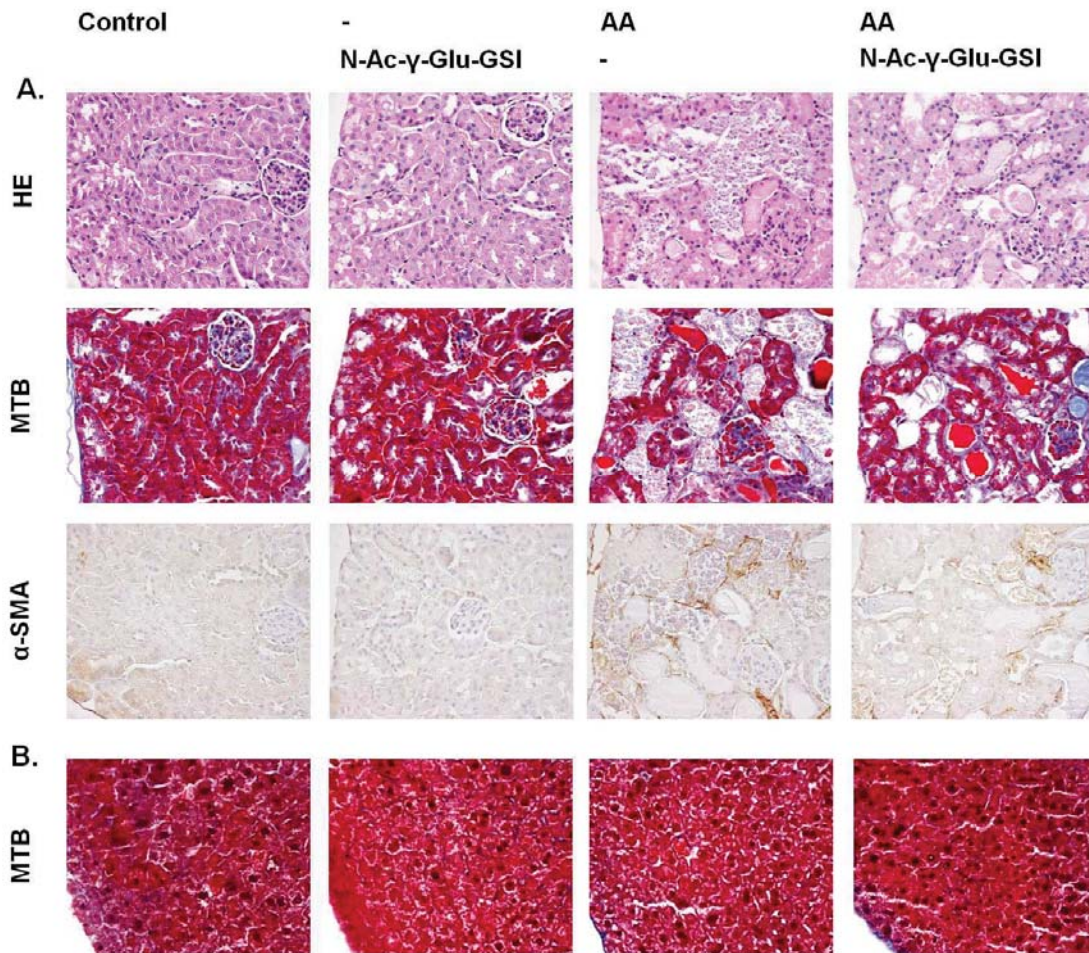


Figure 4.

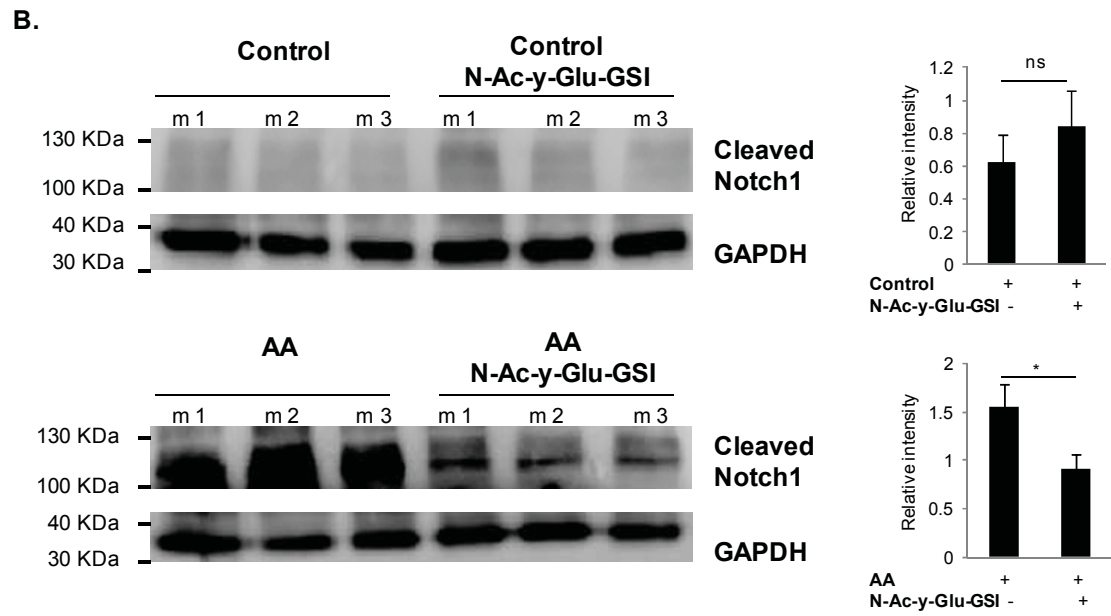
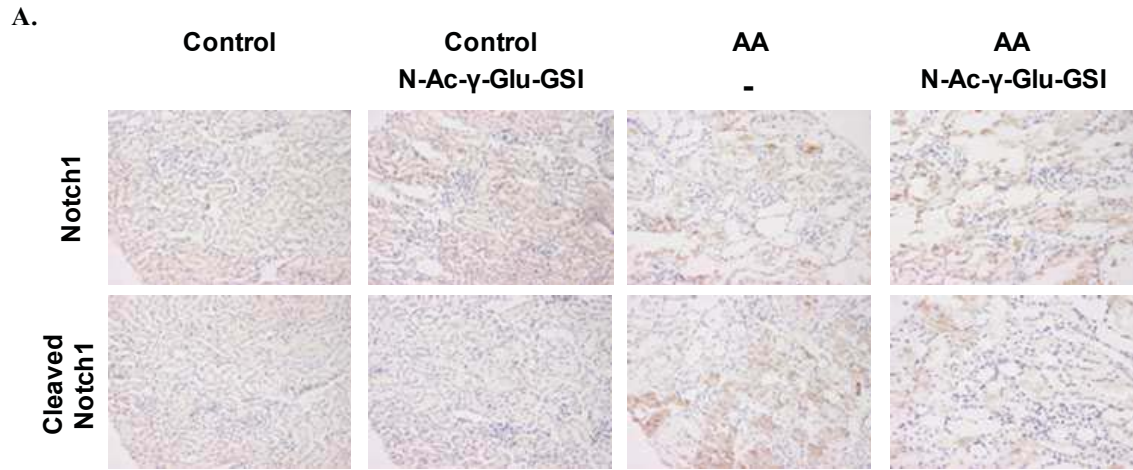
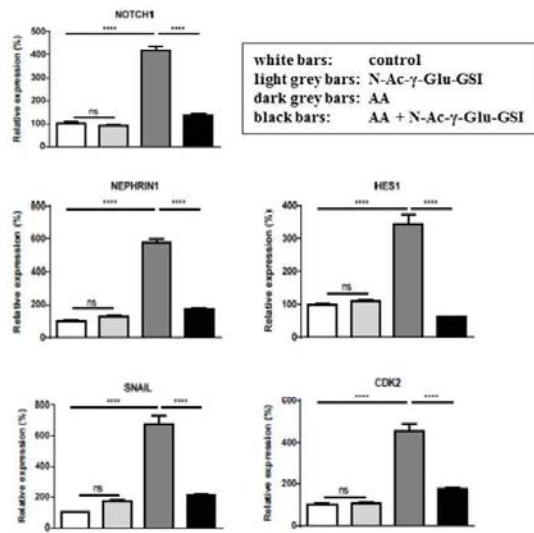
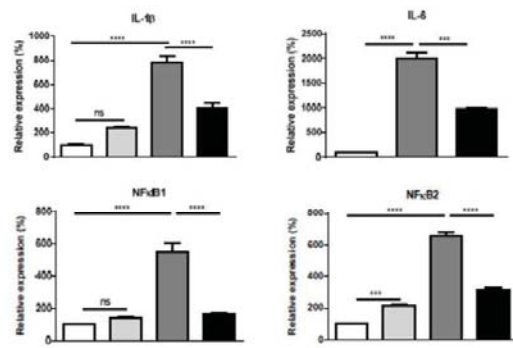


Figure 5.

A. Notch-responsive genes



B. Inflammatory mediators



C. Control

-
N-Ac- γ -Glu-GSI

AA

-

AA

N-Ac- γ -Glu-GSI

NF κ B p65

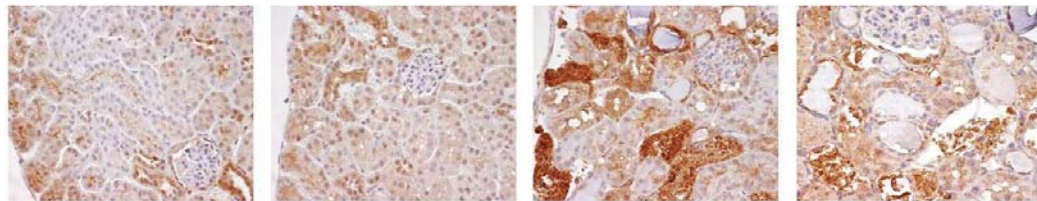
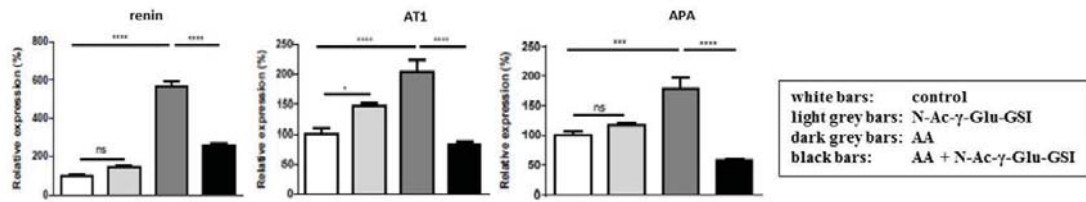


Figure 6.

A. AA and N-Ac- γ -Glu-GSI



B. AA and amine-GSI

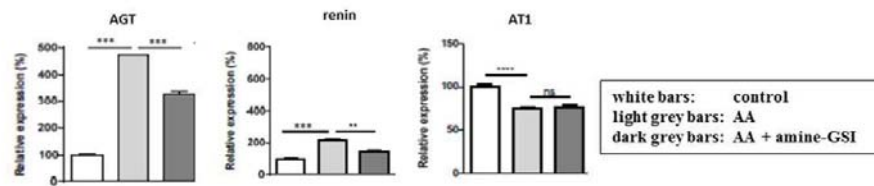


Figure 7.

