A glycomics approach to discover novel renal biomarkers in birds by administration of cisplatin and diclofenac to chickens

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ABSTRACT

Avian species have a unique renal structure and abundant blood flow into the kidneys. Although many birds die due to nephrotoxicity caused by chemicals, there are no early biomarkers for renal lesions. Uric acid level in blood, which is generally used as a renal biomarker, is altered when the kidney function is damaged by over 70%. Therefore, early biomarkers for kidney injury in birds are needed. In humans, glycomics has been at the forefront of biological and medical sciences, and glycans are used as biomarkers of diseases, such as carcinoma. In this study, a glycomics approach was used to screen for renal biomarkers in chicken. First, a chicken model of kidney damage was generated by injection of diclofenac or cisplatin, which cause acute interstitial nephritis (AIN) and acute tubular necrosis (ATN), respectively. The nephrotoxicity levels were determined by blood chemical test and histopathological analysis. The plasma *N*-glycans were then analyzed to discover renal biomarkers in birds. Levels of 14 glycans increased between pre- and post-administration in kidney-damaged chickens in the diclofenac group, and some of these glycans had the same presumptive composition as those in human renal carcinoma patients. Glycan levels did not change remarkably in the cisplatin group. It is possible that there are changes in glycan expression due to AIN but they do not reflect ATN. Although further research is needed in other species of birds, glycans are potentially useful biomarkers for AIN in avian species.

57 Key words: acute interstitial nephritis (AIN), acute tubular necrosis (ATN), bird, glycan,
58 renal biomarker
59

INTRODUCTION

Avian species have a unique kidney structure with abundant blood flow into the kidneys (Harr, 2002) because of the renal portal veins. This system does not exist in mammals (Lierz, 2003), and the avian kidney is vulnerable to various chemicals from the blood.

Indeed, many birds die due to nephrotoxicity caused by chemicals. In the Indian subcontinent, over 90% of three vulture species were killed by the **non-steroidal anti-inflammatory drug (NSAID)**, diclofenac (Green et al., 2004; Swan et al., 2006). Diclofenac was used for the medical treatment of cattle, and the drug was accidentally ingested by vultures when they consumed cattle carcasses (Oaks et al., 2004). It has been reported that primary cultures of avian kidney cells were much more susceptible to diclofenac than mammalian cell cultures (Naidoo and Swan, 2009). In addition, other NSAIDs, such as ketoprofen, cause renal lesions in birds as a side effect (Mohan et al., 2012). Furthermore, **lead (Pb)**, **mercury (Hg)**, and other therapeutic agents, such as anticancer drugs and antifungal agents, cause renal toxicity in birds (Johnson, 1998; Wolfe et al., 1998; Joseph, 2000; Filippich et al., 2001). In the case of humans, drug-induced kidney injury is a serious problem in clinical practice and account for 19% – 26% of cases of **acute kidney injury (AKI)** among hospitalized patients (Hosohata, 2016). In avian species, there have been many reports of renal damage in both wild birds and companion birds.

The diagnosis of kidney disease in birds is challenging. Generally, **uric acid (UA)** level in blood is used as a renal biomarker in birds. However, UA is not an early

biomarker because its levels can be altered when the kidney function is damaged by > 70% (Lierz, 2003). The end product of protein metabolism in birds is UA, and because most of the UA in the urine is in an insoluble form, it does not have an osmotic effect (Styles and Phalen, 1998). Furthermore, as most UA is secreted from the proximal tubules and not filtered, blood UA levels will not be affected by moderate changes in the **glomerular filtration rate (GFR)** (Styles and Phalen, 1998). Even in the event of extensive tubular disease, polyuria and resultant polydipsia and the resulting increase in glomerular filtration can maintain UA levels within the normal range. Therefore, UA concentrations may not reflect glomerular disease and widespread tubular disease may also be present long before UA levels rise above normal. Diagnosis of kidney disease is further complicated in that it is difficult to obtain urine samples from birds because the ureter opens into the cloaca, and the urine is stored in the cloaca or intestine until defecation of a semisolid mixture of urine and feces (Skadhauge, 1968). The level of phosphorus is not changed commonly in all species of birds although the concentration increases due to renal lesions in some avian species (Tully et al., 2009). Therefore, discovery and identification of novel biomarkers for kidney injury in birds are required.

Genomics and proteomics approaches are generally used for the discovery of biomarkers. Glycomics is also a useful tool to identify biomarkers (Adamczyk et al., 2012). Glycosylation is a frequent co-/posttranslational modification of proteins, which modulates a variety of biological functions (Dall'Olio et al., 2013). Glycan structures on newly synthesized glycoproteins are crucial for protein secretion (Moremen et al., 2012). Over 50% of proteins are glycosylated in humans, and the effects of disease states on

glycan biosynthesis can be more evident than those on proteins (Adamczyk et al., 2012). It has been reported that glycans in humans may potentially be used as biomarkers of renal carcinoma (Hatakeyama et al., 2014). Therefore, it is possible that glycans would be useful as biomarkers in birds.

The present study was performed to identify novel renal biomarkers in avian species for the conservation and to develop cures for wild birds as well as companion birds. First, a model of kidney damage was generated in chickens by injection of diclofenac or cisplatin, and renal biomarkers were examined. Acute kidney injury includes **acute interstitial nephritis (AIN)** and **acute tubular necrosis (ATN)** (Hosohata, 2016); diclofenac causes AIN, whereas cisplatin causes ATN. Diclofenac caused severe nephrotoxicity in birds as mentioned above, and the anticancer drug, cisplatin, induces renal lesions as a side effect and is used to make models of kidney injury in rats (Pinches et al., 2012). The kidney shows greater accumulation of cisplatin than other organs, and the kidney is the major route for its excretion (Yao, X., Panichpisal, K., Kurtzman, 2007). To our knowledge, this is the first study to use glycomics to discover biomarkers in birds. Plasma *N*-glycans in chicken were analyzed by glycoblotting, which can be used for high-throughput analysis of biological samples (Hirose et al., 2011), along with **matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF MS)**, and they were profiled to discover novel biomarkers for kidney injury in avian species.

MATERIALS AND METHODS

Animal Experiments

All experimental protocols were approved by the Laboratory Animal Care and Use Committee of Graduate School of Veterinary Medicine, Hokkaido University, Japan. The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, which conforms to **the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC)** (approval number: 14-0119). The endpoints were weight loss > 20% compared to pre-injection, or severe clinical symptoms. Euthanasia was carried out by carbon dioxide inhalation under anesthesia by an overdose of isoflurane (Abbott Laboratories, Chicago, IL) after fasting for over 12 hours. The animals were monitored twice per day during the administration period to check their health. Their body weight was measured from pre-administration to the final day of the experiment. A 27 G needle was used for injection to reduce pain and stress.

Experimental Design

Male white leghorn chickens (*Gallus gallus domesticus*) (n = 15, 10 weeks old, body weight: 1.2 – 1.4 kg) were purchased from Hokudo Co., Ltd. (Tokyo, Japan) and were housed under conditions of constant temperature (20°C ± 2°C) and humidity (40% ± 10%), with a 12:12 hour light:dark cycle and given food and water *ad libitum*. They were allowed to acclimatize to the environment for 1 week before commencement of the experiment. Animals were divided into the following four groups: (1) control group

148 (injection of 20% DMSO, $n = 3$: Cont. -1, -2, -3); (2) diclofenac sodium group A (1.5
149 mg/kg body weight, $n = 4$: A-1, -2, -3, -4); (3) diclofenac sodium group B (2.0 mg/kg
150 body weight, $n = 4$: B-1, -2, -3, -4); and (4) cisplatin group (3.5 mg/kg body weight, $n =$
151 4: C-1,-2, -3, -4). Injection doses were considered according to reference papers of
152 diclofenac treatments (Naidoo et al., 2007; Jain et al., 2009; Mohan et al., 2012) or
153 cisplatin administrations (Cacini and Fink, 1995; Filippich et al., 2001).
154 Pre-administration plasma was collected 1 week after arrival. Administration
155 was started after a further 1 week. The control group received injection of 20% dimethyl
156 sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) diluted in saline once daily in the
157 morning for four consecutive days. The treatment groups received administration of
158 diclofenac sodium diluted in 20% DMSO into the pectoral muscle once daily in the
159 morning for four consecutive days, or a single dose of cisplatin (Wako Pure Chemical
160 Industries, Osaka, Japan) diluted in saline into the basilic vein. Control and diclofenac
161 groups were euthanized on day 5 after the first administration, whereas the cisplatin
162 group was euthanized on day 3 because of the endpoint of clinical signs.
163
164 ***Blood Collection***
165 Blood collection (6 mL) from the basilic vein was carried out pre- and
166 post-injection in the morning (from 9:00 a.m.) using a 23 G or 24 G needle and
167 heparin-containing syringe. Regarding the post-injection, administration was started just
168 after the blood collection. Whole blood was stored on ice after collection and plasma
169 was prepared by centrifugation within 2 hours after collection. Centrifugation was

170 performed at 1630 × *g* for 20 minutes at 4°C. Plasma specimens were immediately
171 frozen in liquid nitrogen and stored at –80°C.
172
173 ***Tissue Sample Collection***
174 Chickens were euthanized with an overdose of isoflurane (Abbott Laboratories)
175 and carbon dioxide. After euthanasia, the kidneys, liver, lungs, and heart were collected.
176 The weights of the whole body, liver, and kidney were measured. The excised tissues
177 were cut into small pieces and stored in 10% neutral buffered formalin for
178 histopathological analysis.
179
180 ***Blood Tests***
181 **Aspartate aminotransferase (AST), total plasma protein (TPP), lactate**
182 **dehydrogenase (LDH), creatine phosphokinase (CK), inorganic phosphate (P), and**
183 **calcium (Ca)** levels were determined using Cobas Ready® (Roche Diagnostics K.K.,
184 Basel, Switzerland). The upper detection limit of UA by COBAS is 20 mg/dL, and UA
185 in birds with high degrees of kidney damage exceeds this level. Therefore, UA level was
186 analyzed by **high-performance liquid chromatography separation and ultraviolet**
187 **detection (HPLC-UV,** 20A series; Shimadzu, Kyoto, Japan). The significance of each
188 test is shown in the Supporting Information (Text S1 in Supporting Information).
189 For measurement of **hematocrit (Ht)**, 200 μL of whole blood was collected
190 from the basilic vein before dissection using a 24 G needle without heparin and
191 immediately moved into tubes containing **ethylenediaminetetraacetic acid (EDTA)**.

Ht level was measured using a microhematocrit centrifuge (Kubota 3220; Kubota Corporation, Tokyo, Japan) at 15000 × *g* for 5 minutes.

Uric Acid Analysis by HPLC

Analyses were performed by HPLC-UV (20A series; Shimadzu) and the improved HPLC method of the Japan Society of Clinical Chemistry (JSCC) was used for measurement of UA. Briefly, 25-μL aliquots of plasma specimens or standard solution were mixed with 225 μL of 0.3 mol/L perchloric acid and cooled on ice for 30 minutes. The samples were mixed again by vortexing and centrifuged at 750 × *g* for 10 minutes at 4°C. Aliquots of 150 μL of the supernatants were collected and centrifuged again at 750 × *g* for 10 minutes at 4°C. Then, 50-μL aliquots of the supernatants were collected and moved to HPLC vials, followed by addition of 50 μL of 10 mM ammonium acetate. For HPLC calibration, 100.3 mg of uric acid (Wako Pure Chemical Industries) was dissolved in 0.01 mol/L lithium carbonate and made up to 100 mL (1 g/L). Standard solutions (1.25, 2.5, 5, 10, 25 and 50 mg/L) were diluted with deionized distilled water. A UV detector set at 284 nm was used to monitor the effluent. Mobile phase A consisted of 10 mM ammonium acetate (pH 4.8) and phase B consisted of 100% methanol. An Inertsil ODS-3 column (2.1 mm × 150 mm: GL Sciences, Inc., Tokyo, Japan) was used for separation at a flow rate of 0.2 mL/min and the injection volume was 5 μL. The R^2 value of the linear regression line was 0.998.

Histopathological Analysis

214 Paraffin-embedded kidney sections were stained with periodic acid-Schiff, and
215 liver, heart, and lung sections were stained with hematoxylin and eosin.
216
217 ***Glycoblotting-based Plasma Glycomics***
218 Analyses of *N*-glycans were performed according to the methods of Kamiyama
219 et al. (Kamiyama et al., 2013). Plasma specimens were pretreated for release of
220 *N*-glycans and subjected to glycoblotting for enrichment and quantification of
221 *N*-glycans prior to MALDI-TOF/MS. Briefly, pretreatment of plasma glycoproteins was
222 performed to release whole *N*-glycans using PNGase F. Glycans were selectively
223 captured by glycoblotting using BlotGlyco® beads. Methyl esterification of sialic acid
224 residues and transiminization reaction to tag *N*-glycans with **benzyloxyamine (BOA)**
225 were carried out on the beads. BOA-tagged *N*-glycans were subjected to
226 MALDI-TOF/MS analysis. More details regarding pretreatment, glycoblotting, mass
227 spectrometry, and data analysis are presented in S2 Text. Expression levels of each
228 glycan were expressed by the ratio with the plasma mixture of healthy chickens.
229
230 ***Statistical Analysis***
231 To compare the results of biochemical analyses between pre- and
232 post-administration, the weight of the body and tissues between controls and treatment
233 groups were analyzed by Steel's test. For comparison of renal damage scores and glycan
234 levels among chickens, data were analyzed using Spearman's rank correlation

235 coefficient. Statistical analyses were performed using JMP Pro 13 (SAS Institute, Cary,
236 NC). In all analyses, $P < 0.05$ was taken to indicate statistical significance.
237
238
239 **RESULTS**
240 ***Clinical Signs***
241 In the diclofenac-treated group, chicken B-1 showed depressed activity and
242 polyuria from the 2nd day of administration, and was dead on the 3rd day. Therefore,
243 regarding chicken B-1, blood samples were collected until the 3rd day. Chicken A-1 also
244 showed weakness from the 4th day. All four cisplatin-treated chickens had polyuria and
245 depressed activity from the 2nd day. The other chickens generally appeared normal. The
246 control group did not show any symptoms.
247
248 ***Biochemical Analysis***
249 The plasma concentrations of UA, P, Ca, AST, LDH, CK, and TPP as indicators
250 of various tissues are shown in Table 1. The normal UA concentration in chickens
251 ranges from 2.5 to 8.1 mg/dL (Miller and Fowler, 2014). Although there were no
252 significant differences between pre- and post-administration in any of the groups,
253 several chickens in the treatment groups showed high levels of UA. In the diclofenac
254 group, chicken A-1 exceeded the normal UA level 72 hours after injection. Chickens
255 B-1 and B-2 showed high levels of UA after 24 hours, and the level in chicken B-4
256 increased after 72 hours. However, the UA level in chickens B-2 and B-4 recovered

after 96 hours. In the cisplatin group, UA level was high in all chickens 48 hours after the injection, and markedly exceeded the reference level. As other indicators of renal lesions, P concentration was also elevated in some of these chickens; the reference level in chicken is 6.2 – 7.9 mg/dL (Miller and Fowler, 2014). Although AST level of chickens in the treatment group increased, the levels were within the normal range for turkeys of 255 – 499 IU/L, and the LDH level exceeded the reference level for turkeys of 420 – 1338 IU/L (Miller and Fowler, 2014).

Gross Pathology

All cisplatin-treated chickens, and chickens A-1, B-1, and B-2 in the diclofenac group showed pale kidneys compared with the controls (Fig. 1). Kidneys in these chickens were enlarged although there were no significant differences in kidney weight between controls and each exposure group (Table S1). The control group and the other chickens in the treatment groups did not show any gross pathological changes. The liver specimens in all chickens had no lesions and there were no significant differences in tissue weight (Table S1).

Histopathological Analysis

All cisplatin-treated chickens and four diclofenac-treated chickens showed renal damage (Fig. 2). Although they commonly showed degenerative and necrotic lesions in the proximal and distal tubules, and sometimes glomeruli, and the shape of nuclei in the proximal tubules became unclear, the histology was different between

279 diclofenac- and cisplatin group. Diclofenac-treated chickens showed the infiltration of
280 leukocytes such as heterophils in interstitium. In the cisplatin group, necrosis of tubules
281 was shown and many proteinaceous casts in the tubular lumen were also found. In
282 addition, tubular epithelial cells were detached from the basement membrane of some
283 tubules in chickens A-1 and B-1 in the diclofenac group and all cisplatin-treated
284 chickens. Chickens B-2 and B-4 in the diclofenac group showed mild degenerative
285 lesions, such as slight dilation of proximal and distal tubular lumens. Chickens A-2, A-3,
286 A-4, and B-3 appeared normal.

287 According to the histopathological changes, renal lesions were given scores
288 from K0 (no lesions) to K5 (most severe). For scoring, the ratio of outer/lumen area at
289 cross section of tubules was measured using Axiovision Rel 4.8 software (Zeiss,
290 Germany). In addition, three stages of histopathological alterations of kidney (Salamat
291 et al., 2014) were used as a reference. Damage scores are as follows: K0, no lesions (the
292 median of outer/lumen area was > 10); K1, mild damage, such as infiltration of
293 heterophils and cells in the proximal tubular lumens in a very limited area (the median
294 of outer/lumen area was 8-9); K2, moderate damage, such as infiltration of heterophils
295 and cells in proximal tubular lumens, and dilation of distal tubular lumens in a large
296 area (the median of outer/lumen area was 5-7); K3, severe damage, such as infiltration
297 of heterophils and cells in the proximal tubular lumens, dilation of tubular lumens, and
298 proteinaceous casts (the median of outer/lumen area was approximately 4); K4, severe
299 damage with unclear structure of renal tubules and glomeruli, and several proteinaceous
300 casts (the median of outer/lumen area was approximately 3); or K5, severe damage with

301 many necrotic cells, and proteinaceous casts (the median of outer/lumen area was 1-2
302 and most of tubules were disintegrated). The damage levels of each chicken were as
303 follows: K0 (control); K1 (B-4); K2 (B-2); K3 (C-2); K4 (A-1, C-4); K5 (B-1, C-1, C-3).
304 In the exposure group, chickens A-2, A-3, A-4, and B-3 did not show histopathological
305 changes, and they were ranked K0.
306 The livers in chicken B-1 and all cisplatin-treated chickens showed mild
307 hyperemia (Fig. S1). There were no lesions in the heart or lung in any of the chickens,
308 and the control group did not show histopathological changes in any tissues.
309
310 ***Glycomics Analysis***
311 For glycomics analysis, 10 chickens (Cont.-1, -2, A-1, B-1, B-2, B-4, C-1, -2,
312 -3, and -4) were selected according to the results of histopathological analysis and
313 biochemical analysis, and plasma *N*-glycans were measured. A total of 40 plasma
314 *N*-glycans were detected, and the levels of each glycan are shown by the ratio with
315 glycan expression of plasma mix from the controls and pre-injection chickens (Table
316 S2). The control group did not show any significant differences due to injection.
317 Fourteen glycans were increased in the diclofenac-treated kidney damaged chickens
318 (Fig. 3, Table S3).
319 In the cisplatin group, glycan levels did not change significantly according to
320 kidney injury, although the renal lesions were severe and UA concentrations were high.
321
322

DISCUSSION

In the present study, we succeeded to cause various stages of nephrotoxicity in chickens after cisplatin (3.5 mg/kg) or two doses of diclofenac (1.5 – 2.0 mg/kg) treatments. The results of biochemical analyses indicated that UA, P, and LDH levels exceeded the respective normal ranges in several chickens. Both UA and P indicate severe renal lesions in chickens, but UA level was altered earlier than P level. According to the UA level, kidney function in the cisplatin group (C-1, -2, -3, and -4) would be markedly damaged 48 hours after injection. In the diclofenac group, chicken A-1 had severe renal injury from 72 hours after injection. The kidneys of chickens B-1 and B-2 were impaired with heavy renal failure after 24 hours, and chicken B-1 died due to nephrotoxicity. Although LDH level increased in the treatment groups, it was difficult to identify the cause, because elevation of LDH is nonspecific, and is found in skeletal and cardiac muscle, liver, kidney, bone, and erythrocytes (Tully et al., 2009).

Histopathological analysis of the kidney, liver, heart, and lung showed that chickens A-1, B-1, B-2, and B-4 and all cisplatin-treated chickens had damage almost specific to the kidney. Furthermore, the histology of kidney might indicate that diclofenac caused AIN and cisplatin induced ATN in chicken, although the infiltration of leukocytes in interstitial in diclofenac-treated chickens was not so severe compared to AIN in human. In the case of diclofenac exposure, renal damage levels were completely different depending on the individual, and this tendency was also described in the reports mentioned above. There may be large differences in sensitivity even within a

344 species. Therefore, the renal damage was shown by scores for comparison with other
345 analyses.

346 Glycomics analysis showed that a high degree of kidney damage was
347 associated with increased levels of both sialylated and non-fucosylated glycans in
348 diclofenac-treated chickens. Although not applicable to six glycans (No. 9, 22, 24, 26,
349 34, and 37), this tendency was seen for the remaining 34 glycans. The synthesis
350 pathway of *N*-linked glycans starts from the cytosolic surface of the endoplasmic
351 reticulum membrane by addition of sugars, and in the early secretory pathway, the
352 glycans have important roles in protein folding, oligomerization, quality control, sorting,
353 and transport (Helenius and Aebi, 2001). The glycans acquire more complex structures
354 and new functions in the Golgi complex (Helenius and Aebi, 2001). The *trans*
355 compartment elaborates additional branching and capping reactions on complex
356 *N*-glycans, and capping reactions are continued in the *trans*-Golgi network (Moremen et
357 al., 2012). Therefore, the final synthesis pathway, where sialic acids are attached, could
358 be disturbed, or glycoproteins that have these glycans may be susceptible to the effects
359 of kidney injury.

360 In cisplatin-treated chickens, there is no relation between glycan expression
361 and kidney damage. Therefore, the glycans that increased in the diclofenac group may
362 indicate the effects of diclofenac injection, rather than kidney injury itself. Another
363 possibility is that glycans may have reflected parts of kidney injury, and not all types of
364 renal damage.

With regard to AIN and ATN, although the mechanism underlying AIN is underestimated even in human, it is generally accepted that the pathogenesis is based on an immunologic reaction against endogenous nephritogenic antigens or exogenous antigens processed by tubular cells, with cell-mediated immunity having a major pathogenic role (Praga and González, 2010). In avian species, there is less information. However, it is reported that diclofenac causes nephrotoxicity due to reduction of UA transport by interfering with p-aminohippuric acid (PAH) channels in the chicken (Naidoo and Swan, 2009). The mechanism of diclofenac-induced renal failure in oriental white backed vultures has been proposed to be through inhibition of the modulating effect of prostaglandin on angiotensin II-mediated adrenergic stimulation (Jain et al., 2009). Cisplatin induces DNA damage, either necrotic or apoptotic cell death, formation of reactive oxygen species, mitochondrial dysfunction, and caspase activation (Ramesh and Reeves, 2002). These events cause both acute and chronic kidney injury, and the nephrotoxicity is characterized by activation of both proinflammatory cytokines and chemokines (Havasi and Borkan, 2016).

The mechanisms of metabolism of diclofenac and cisplatin are different. Therefore, it is possible that glycans reflect the limited pathway of kidney injury, and glycan expression profiles change due to AIN but do not reflect ATN. Although further studies including investigation of species differences are needed, glycans have the potential to be useful biomarkers for AIN in avian species. The glycan expression profile may reflect some types of kidney injury, and these molecules have the potential for use as biomarkers for the evaluation of functional disorders in birds.

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Table 1. Plasma concentrations of UA (uric acid), P (inorganic phosphate), Ca (calcium), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), CK (creatine phosphokinase), and TPP (total protein) in diclofenac- or cisplatin-treated and control chickens

		pre-injection	24 h	48 h	72 h	96 h
	UA (mg/dL)	5.0 ± 0.5	3.2 ± 0.3	2.4 ± 1.0	2.7 ± 0.6	2.9 ± 0.6
	P (mg/dL)	7.1 ± 0.5	6.2 ± 0.4	6.4 ± 0.6	6.1 ± 0.0	6.1 ± 0.9
	Ca (mg/dL)	9.7 ± 0.5	9.6 ± 0.5	9.8 ± 0.5	10.1 ± 0.2	10.3 ± 0.5
Cont. (n = 3)	AST (IU/L)	132 ± 8	142 ± 5	168 ± 18	164 ± 17	149 ± 18
	LDH (IU/L)	706 ± 358	575 ± 164	639 ± 99	497 ± 48	514 ± 171
	CK (IU/I)	1110 ± 468	844 ± 107 *2	1290 *1	1437 ± 191 *2	514 ± 171
	TPP (g/dL)	2.9 ± 0.1	2.7 ± 0.3	2.9 ± 0.3	2.9 ± 0.3	2.6 ± 0.5
	UA (mg/dL)	4.4 ± 1.0	3.7 ± 1.0	3.8 ± 1.2	72.9 ± 117.8	48.8 ± 78.6
	P (mg/dL)	7.5 ± 0.3	6.8 ± 0.7	7.0 ± 0.4	8.1 ± 1.4	8.8 ± 3.2
Diclofenac A	Ca (mg/dL)	9.9 ± 0.2	9.7 ± 0.2	9.3 ± 0.1	9.3 ± 0.2	9.1 ± 1.2
(1.5 mg/kg, n =	AST (IU/L)	132 ± 5.9	198 ± 10.1	250 ± 16.1	304 ± 69.6	302 ± 88.1
4)	LDH (IU/L)	433 ± 43	1490 ± 234	1967 ± 639	1710 ± 1002	941 ± 202 *3
	CK (IU/I)	1134 ± 284	>2000	>2000	>2000	>2000
	TPP (g/dL)	3.0 ± 0.3	2.9 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	2.8 ± 0.3
Diclofenac B	UA (mg/dL)	3.7 ± 0.5	52.6 ± 64.5	74.5 ± 103.9	12.0 ± 6.6 *3	6.7 ± 3.3 *3
(2.0 mg/kg, n =	P (mg/dL)	7.9 ± 0.7	7.2 ± 0.8	7.3 ± 0.9 *3	7.9 ± 1.2 *3	6.1 ± 1.1 *3
4)	Ca (mg/dL)	9.8 ± 0.3	9.7 ± 0.1	9.2 ± 0.6	9.6 ± 0.4 *3	9.5 ± 0.2 *3

	AST (IU/L)	130 ± 14	228 ± 67	287 ± 22	282 ± 12 *[3]	258 ± 12 *[3]
	LDH (IU/L)	492 ± 132	1596 ± 672	2437 ± 978 *[3]	2032 ± 984 *[3]	985 ± 313 *[3]
	CK (IU/I)	1340 ± 423	>2000	>2000	>2000	>2000
	TPP (g/dL)	2.9 ± 0.2	2.9 ± 0.3	2.9 ± 0.3	3.1 ± 0.6 *[3]	2.8 ± 0.4 *[3]
	UA (mg/dL)	3.5 ± 0.3	8.0 ± 1.0	77.0 ± 16.2	—	—
	P (mg/dL)	4.7 ± 0.4	6.3 ± 0.2	8.0 ± 1.0	—	—
	Ca (mg/dL)	10.5 ± 0.5	10.9 ± 0.3	11.6 ± 1.5	—	—
Cisplatin (3.5 mg/kg, n = 4)	AST (IU/L)	137 ± 8	170 ± 8	246 ± 19	—	—
	LDH (IU/L)	425 ± 74	370 ± 71	870 ± 302	—	—
	CK (IU/I)	870 ± 69	1069 ± 70 *[3]	1020 ± 169	—	—
	TPP (g/dL)	3.4 ± 0.2	3.8 ± 0.0	3.3 ± 0.6	—	—

* The values were calculated from one chicken*[1], two chickens*[2] or three chickens*[3], because the others exceeded the detection limit (LDH: 3937 IU/L, CK: 2000 IU/L).
In diclofenac group B, one chicken died on the 3rd day of administration.
There were no significant differences between pre- and post-administration (Steel's test, $P < 0.05$).

506 **Figure legends**

507

508 **Fig. 1. Gross pathological features of kidneys in diclofenac- or cisplatin-treated and**
509 **control chickens**

510 The chickens treated with diclofenac (a) or cisplatin (b) showed pale and enlarged kidneys
511 compared with the controls (c). The kidney weights are shown in Table S2.

512

513 **Fig. 2. Histopathological features of the kidneys in diclofenac- or cisplatin-treated and**
514 **control chickens**

515 Chickens treated with diclofenac (a) or cisplatin (b) showed severe renal lesions compared
516 with controls (c). Briefly, there were degenerative and necrotic lesions, such as proteinaceous
517 casts (arrowheads), heterophil infiltration into the tubulointerstitium and dead cells in the
518 proximal tubular lumens (white arrows), and dilation of proximal and distal tubular lumens
519 (black arrows). Diclofenac-treated chickens showed much infiltration of heterophils and
520 cisplatin-treated chickens had a large number of proteinaceous casts. The controls showed
521 normal renal structures.

522

523 **Fig. 3. Spearman's rank correlation coefficients of expression levels for 14 increased**
524 ***N*-glycans (μM) on the final day and renal damage score in the diclofenac-treated group.**
525 No. 16, 29 and 38 had significant correlation and lines showed the linear approximation.

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Fig. 1. Gross pathological features of kidneys in diclofenac- or cisplatin-treated and control chickens

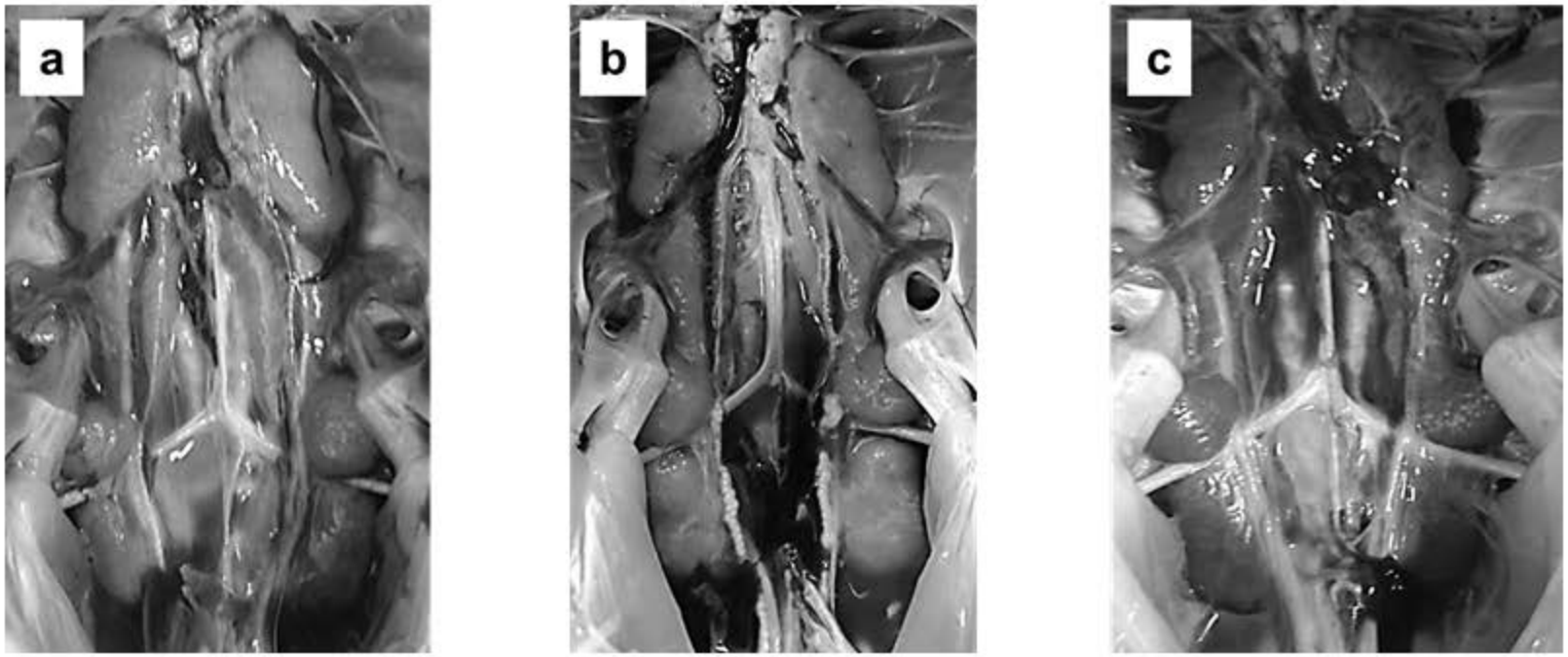


Fig. 2. Histopathological features of the kidneys in diclofenac- or cisplatin-treated and control chickens

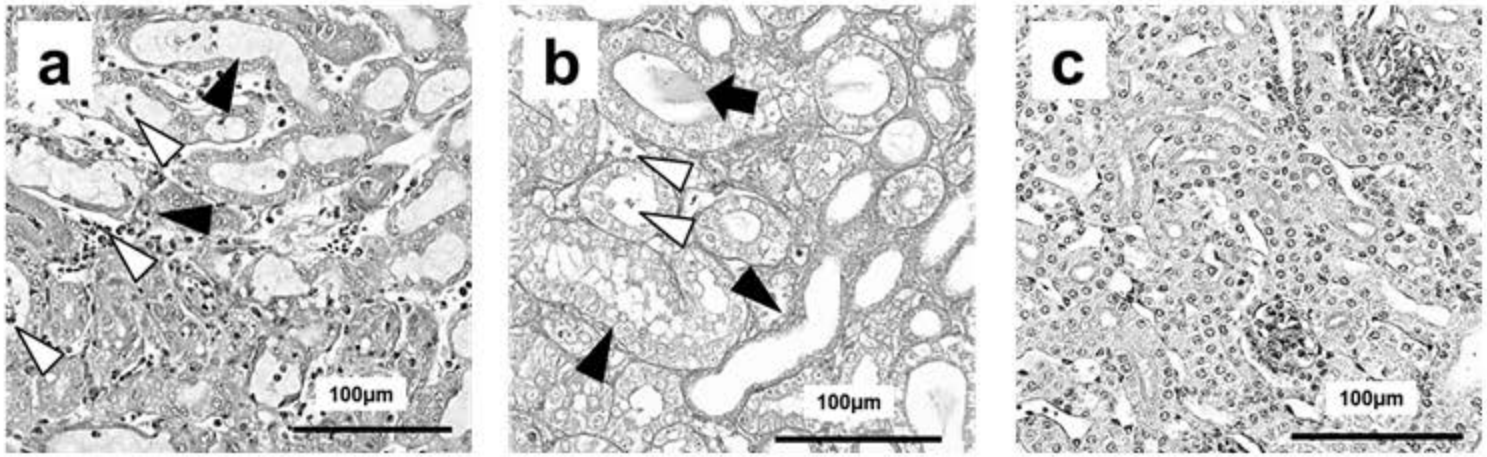
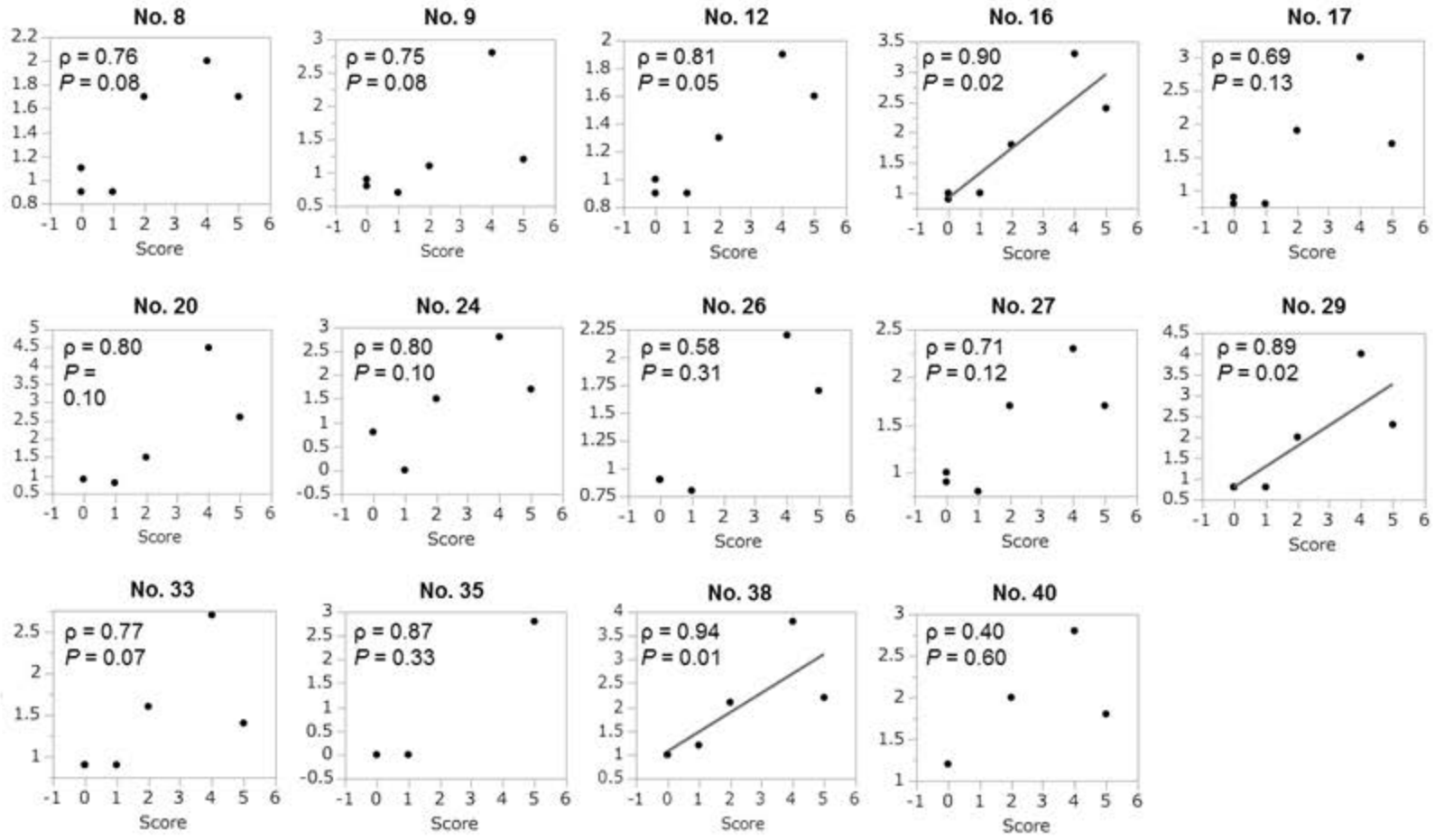


Fig. 3. Spearman's rank correlation coefficients of expression levels for 14 increased *N*-glycans (μM) on the final day and renal damage score in the diclofenac-treated group



S1 Text. Significance of each biochemical test

The plasma concentrations of UA, AST, CK, LDH, P, and Ca are indicators of various tissues as follows [1]. AST activity is considered to be a very sensitive but nonspecific indicator of hepatocellular disease because muscular damage changes AST levels in avian species. CK elevation is associated with significant disruption of skeletal muscle, cardiac muscle, and nervous tissue. Generally, increased CK levels are compared with AST and LDH levels. Elevated AST without elevation of CK is highly suggestive of liver disruption and concurrent elevation of AST and CK suggests muscle disruption or concurrent damage to the liver and muscle or liver and nervous tissue. Elevation of LDH is nonspecific because it is found in skeletal and cardiac muscle, liver, kidney, bone, and erythrocytes, but LDH levels may follow the process of liver disease and change more quickly than AST levels. Elevated P levels resulting from renal disease suggest chronicity, although this is less common in bird species, and increases are also observed in hypoparathyroidism and nutritional secondary hyperparathyroidism. Blood Ca levels are directly linked to albumin levels, and dehydration sometimes causes elevation of albumin level, leading to an increase in blood Ca. Elevated levels of Ca are also associated with vitamin D3 toxicity, osteolytic bone tumors, and renal adenocarcinoma.

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S2 Text. Glycoblotting-based plasma glycomics.

Experimental Procedures: Plasma N-Glycomics by Glycoblotting

N-glycans from plasma samples were purified by glycoblotting using BlotGlyco®. These are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite, Tokyo, Japan). All procedures used the SweetBlot automated glycan purification system containing a 96-well plate platform (System Instruments, Hachioji, Japan).

Enzymatic Degradation of Plasma N-Glycans

Aliquots of 10 μL of plasma samples were dissolved in 50 μL of a 106 mM solution of ammonium bicarbonate containing 12 mM 1,4-dithiothreitol and 0.06% 1-propanesulfonic acid, 2-hydroxyl-3-myristamido (Wako Pure Chemical Industries, Osaka, Japan). After incubation at 60°C for 30 minutes, 123 mM iodoacetamide (10 μL) was added to the mixtures followed by incubation in the dark at room temperature to enable reductive alkylation. After 60 minutes, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 hours, followed by heat inactivation of the enzyme at 90°C for 10 minutes. After cooling to room temperature, the *N*-glycans were released from the tryptic glycopeptides by incubation with 325 U of PNGase F (New England BioLabs, Ipswich, MA) at 37°C for 6 hours.

N-Glycan Purification and Modification by Glycoblotting

Glycoblotting of sample mixtures containing whole plasma *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco® beads (500 μL) (10 mg/mL suspension; Sumitomo Bakelite) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic polytetrafluoroethylene (PTFE) 96-well filter plate (EMD

Millipore, Billerica, MA). After removal of the water using a vacuum pump, 20 μL of PNGase F-digested samples were applied to the wells, followed by the addition of 180 μL of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80°C for 45 minutes to capture the *N*-glycans onto the beads by a chemically stable and reversible hydrazine bond. The beads were then washed using 200 μL of 2 M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and 1% triethyl amine in methanol. Each washing step was performed twice. The *N*-glycan linked beads were next incubated with 5% acetic anhydride in ethanol for 30 minutes at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under vacuum and the beads were serially washed with 2 × 200 μL of 10 mM HCl, methanol, and dioxane as a pretreatment for sialic acid modification. On-bead methyl esterification of carboxyl groups in the sialic acids was carried out with 100 μL of 20 mM 3-methyl-1-P-tolyltriazene (Tokyo Chemical Industry, Tokyo, Japan) in dioxane at 60°C for 90 minutes to dryness. After methyl esterification of the more stable glycans, the beads were serially washed in 200 μL of dioxane, water, 1% triethylamine in methanol, and water. The captured glycans were then subjected to transiminization reaction with BOA (O-benzylhydroxylamine) (Tokyo Chemical Industry) for 45 minutes at 80°C. After this reaction, 150 μL of water was added to each well, followed by the recovery of derivatized glycans under vacuum.

Matrix-Assisted Laser Desorption Ionization, Time-of-Flight (MALDI-TOF) and TOF/TOF Analysis

The *N*-glycans purified by glycoblotting were directly diluted with α-cyano-4-hydroxycinnamic acid diethylamine salt (Sigma-Aldrich) as ionic liquid matrix and spotted onto the MALDI target plate. The analytes were then subjected to MALDI-TOF

MS analysis using an Ultraflex time-of-flight mass spectrometer III (Bruker Daltonics, Billerica, MA) in reflector, positive ion mode and typically summing 1000 shots. The *N*-glycan peaks in the MALDI-TOF MS spectra were selected using FlexAnalysis v. 3 (Bruker Daltonics). The intensity of the isotopic peak of each glycan was normalized using 40 μM internal standard (A2 amide; Tokyo Chemical Industry) for each status, and its concentration was calculated from a calibration curve using human plasma standards. The glycan structures were estimated using the GlycoMod Tool (http://br.expasy.org/tools/glycomod/), so that our system could quantitatively measure 40 *N*-glycans.

Table S1. Weights of the body, liver, and kidney after exposure in chickens

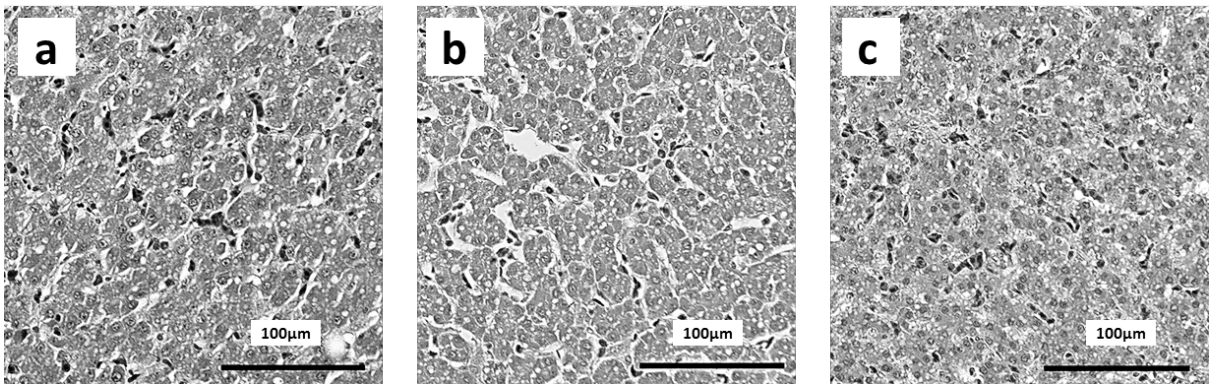
In the case of body weight, the value of the day prior to euthanasia was used because they were fasted from the night of the previous day.

Group	Body weight (g)	Liver weight (g)	Kidney weight (g)	Liver/B.W. (%)	Kidney/B.W. (%)
Control	1489 ± 121	23.9 ± 0.6	9.5 ± 0.7	1.6 ± 0.1	0.6 ± 0.1
Diclofenac A (1.5 mg/kg)	1549 ± 41	25.6 ± 3.9	13.1 ± 4.4	1.6 ± 0.2	0.8 ± 0.3
Diclofenac B (2.0 mg/kg)	1455 ± 110	26.5 ± 5.5	14.2 ± 5.9	1.8 ± 0.4	1.0 ± 0.4
Cisplatin (3.5 mg/kg)	1293 ± 86	28.1 ± 6.9	13.1 ± 2.1	2.2 ± 0.4	1.0 ± 0.1

There are no significant differences between control and treatment groups (Steel's test, $P < 0.05$).

Fig. S1. Histopathological features of the liver in diclofenac- or cisplatin-treated and control chickens.

Chickens treated with diclofenac (a) or cisplatin (b) showed mild hyperemia compared with controls (c).



109 **Table S2. Ratios of all detected *N*-glycans (μM) on the final day/pre-administration**

i. High mannose type

Peak No.	*m/z*	Presumptive composition	Cont.-1	Cont.-2	A-1	B-1	B-2	B-4	C-1	C-2	C-3	C-4
1	1362.481	(Hex)2 + (Man)3 (GlcNAc)2	0.85	0.82	1.90	1.02	1.37	0.85	1.07	0.90	0.93	0.73
2	1524.534	(Hex)3 + (Man)3 (GlcNAc)2	0.85	0.84	1.20	0.91	0.83	0.66	0.98	0.88	0.94	0.64
3	1686.587	(Hex)4 + (Man)3 (GlcNAc)2	0.91	0.83	0.92	0.68	0.72	0.58	1.07	0.86	1.01	0.65
4	1848.640	(Hex)5 + (Man)3 (GlcNAc)2	0.91	0.85	0.92	0.62	0.81	0.62	1.11	0.84	1.00	0.67
5	2010.692	(Hex)6 + (Man)3 (GlcNAc)2	0.89	0.88	0.86	0.60	0.74	0.58	1.06	0.81	0.92	0.65
6	2172.745	(Hex)7 + (Man)3 (GlcNAc)2	0.84	0.91	0.73	0.51	0.55	0.46	0.94	0.65	0.75	0.55

ii. Complex type, Hybrid type

Peak No.	*m/z*	Presumptive composition	Cont.-1	Cont.-2	A-1	B-1	B-2	B-4	C-1	C-2	C-3	C-4
7	1565.560	(Hex)2 (HexNAc)1 + (Man)3 (GlcNAc)2	N. A.	0.00	N. A.	N. A.	N. A.	N. A.	1.06	1.04	1.00	0.89
8	1708.619	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	1.09	0.92	1.98	1.74	1.67	0.93	1.17	1.00	1.00	0.90
9	1727.613	(Hex)3 (HexNAc)1 + (Man)3 (GlcNAc)2	0.90	0.79	2.83	1.22	1.09	0.72	1.11	1.00	1.04	0.88
10	1793.671	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.74	0.71	0.00	0.00	0.00	0.00	0.94	0.70	0.85	0.55
11	1809.666	(Hex)1 (HexNAc)3 + (Man)3 (GlcNAc)2	0.82	0.71	1.00	0.70	0.69	0.00	0.85	0.78	0.85	0.51
12	1870.672	(Hex)2 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	0.92	1.93	1.59	1.32	0.86	1.13	0.92	1.02	0.77
13	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.97	0.87	0.00	N. A.	0.00	0.00	0.88	0.60	0.83	0.53

14	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.76	0.81	0.77	0.48	0.58	0.46	1.01	0.66	0.75	0.58
15	1971.719	(Hex)2 (HexNAc)3 + (Man)3 (GlcNAc)2	0.66	N. A.	N. A.	N. A.	0.00	N. A.	0.00	N. A.	N. A.	0.00
16	2032.724	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	0.89	3.27	2.36	1.78	0.98	1.15	0.98	0.99	0.94
17	2073.751	(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	0.87	0.81	3.01	1.69	1.95	0.84	1.14	1.11	0.96	1.07
18	2117.777	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.88	0.89	1.16	0.76	0.66	0.52	1.07	0.75	0.82	0.60
19	2219.809	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.90	0.87	0.90	0.67	0.64	0.52	0.88	0.65	0.87	0.49
20	2235.804	(Hex)3 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	N. A.	0.90	4.47	2.59	1.49	0.75	1.12	0.89	N. A.	0.86
21	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.82	0.83	0.86	0.58	0.00	0.59	1.08	0.65	0.95	0.55
22	2276.830	(Hex)2 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.80	N. A.	0.00	0.00	0.60	N. A.	0.95	0.00	0.78	0.00
23	2279.830	(Hex)3 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	1.06	0.00	0.00	N. A.
24	2304.862	(Hex)1 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	N. A.	0.81	2.77	1.69	1.54	0.00	1.23	0.67	0.88	0.00
25	2321.841	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	1.05	0.80	0.91	0.73
26	2336.851	(Hex)3 (HexNAc)4 + (Man)3 (GlcNAc)2	0.88	0.92	2.17	1.67	N. A.	0.79	1.14	0.81	0.88	0.71
27	2378.862	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)2	0.95	0.95	2.33	1.73	1.72	0.85	1.04	0.69	0.90	0.56
28	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.88	0.89	1.13	0.57	0.66	0.47	1.15	0.99	0.98	0.95
29	2438.883	(Hex)3 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.85	0.84	3.96	2.34	1.99	0.84	N. A.	N. A.	N. A.	N. A.
30	2482.909	(Hex)3 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.87	0.79	1.65	0.00	0.00	0.83	0.90	0.60	0.86	0.49
31	2524.920	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	0.75	0.99	1.29	0.80	0.51	0.56	1.07	0.63	1.06	0.00
32	2727.999	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	1.00	0.91	0.99	0.68	0.59	0.51	1.09	0.64	1.12	0.55

33	2743.994	(Hex)3 (HexNAc)3 (NeuAc)2 + (Man)3 (GlcNAc)2	0.95	0.90	2.74	1.45	1.64	0.89	1.13	0.88	1.01	0.75
34	2788.020	(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.78	0.91	0.00	0.00	0.00	0.00	N. A.	N. A.	N. A.	N. A.
35	2804.015	(Hex)4 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNAc)2	N. A.	0.00	2.4/N.	2.84	1.4/N.	0.00	1.25	0.90	1.05	0.80
36	2890.052	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.
37	3049.105	(Hex)3 (HexNAc)3 (NeuAc)3 + (Man)3 (GlcNAc)2	1.05	1.08	1.63	1.08	1.09	0.80	1.04	0.63	0.85	0.46
38	3109.126	(Hex)4 (HexNAc)4 (NeuAc)2 + (Man)3 (GlcNAc)2	0.99	1.01	3.81	2.21	2.08	1.16	1.17	0.71	0.96	0.58
39	3195.163	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 + (Man)3 (GlcNAc)2	N. A.	1.06	N. A.	N. A.	N. A.	0.00	N. A.	N. A.	N. A.	N. A.
40	3414.237	(Hex)4 (HexNAc)4 (NeuAc)3 + (Man)3 (GlcNAc)2	N. A.	1.21	2.84	1.76	2.03	N. A.	1.14	0.61	0.89	0.00

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Table S3. Ratios of increased *N*-glycans (μM) on the final day/pre-administration in diclofenac-treated group

ID	Kidney damage score	No. 8	No. 9	No. 12	No. 16	No. 17	No. 20	No. 24	No. 26	No. 27	No. 29	No. 33	No. 35	No. 38	No. 40
B-4	1	0.9	0.7	0.9	1.0	0.8	0.8	0.0	0.8	0.8	0.8	0.9	0.0	1.2	N. D./N. D.
B-2	2	1.7	1.1	1.3	1.8	1.9	1.5	1.5	1.1/N. D.	1.7	2.0	1.6	1.4/N. D.	2.1	2.0
A-1	4	2.0	2.8	1.9	3.3	3.0	4.5	2.8	2.2	2.3	4.0	2.7	2.4/N. D.	3.8	2.8
B-1	5	1.7	1.2	1.6	2.4	1.7	2.6	1.7	1.7	1.7	2.3	1.4	2.8	2.2	1.8