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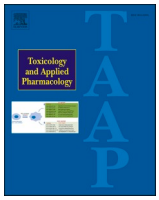

A premature stop codon in the CYP2C19 gene may explain the unexpected sensitivity of vultures to diclofenac toxicity

Emmanuel Oluwasegun Adawaren [a], Christiaan Labuschagne [b], Aron Abera [b], Vinny Naidoo [a,*]

[a] Department of Paraclinical Science, Faculty of Veterinary Science, University of Pretoria, Gauteng, South Africa
[b] Iqaba Biotechnical Industries (Pty), 525 Justice Mahomed St, Muckleneuk, 0002 Pretoria, Gauteng, South Africa

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ABSTRACT

The unintended environmental exposure of vultures to diclofenac has resulted in the deaths of millions of old-world vultures on the Asian subcontinent. While toxicity has been since associated with a long half-life of elimination and zero order metabolism, the actual constraint in biotransformation is yet to be clarified. For this study we evaluated if the evident zero order metabolism could be due to defects in the CYP2C9/2C19 enzyme system. For this, using whole genome sequencing and de-novo transcriptome alignment, the vulture CYP2C19 open reading frame was identified through Splign analysis. The result sequence analysis revealed the presence of a premature stop codon on intron 7 of the identified open reading frame. Even if the stop codon was not present, amino acid residue analysis tended to suggest that the enzyme would be lower in activity than the equivalent human enzyme, with differences present at sites 105, 286 and 289. The defect was also conserved across the eight non-related vultures tested. From these results, we conclude that the sensitivity of the old-world vultures to diclofenac is due to the non-expression of a viable CYP2C19 enzyme system. This is not too dissimilar to the effects seen in certain people with a similar defective enzyme.

1. Introduction

Vultures are obligate scavengers that play a role in the recycling of dead carcasses in the environment (Morales-Reyes et al., 2017; Markandya et al., 2008). Unfortunately, due to their dependence on carrion for their nutrient requirements, they tend to be inadvertently exposed to chemicals in these carcasses, some of which can be extremely toxic. Of the numerous toxins known to be dangerous for vultures, the most widely noted agent was reported in South Asia in the late 1990s when three populations of Gyps vulture species were devastated (>99% population deaths reported) following their exposure to diclofenac (Oaks et al., 2004; Gilbert et al., 2007; Naidoo et al., 2007). While diclofenac was never used in vultures directly, the birds were exposed to this veterinary drug as residue following the wide scale use of the product in livestock. Subsequent to the discovery of the toxicity of diclofenac, other non-steroidal anti-inflammatory drugs (NSAIDs) such as carprofen and ketoprofen have also been shown to be toxic to old world vultures (Naidoo et al., 2010a, 2010b).

Despite the unprecedented population declines following exposure to diclofenac, the reason for the sensitivity of the species to this group of drugs remains unknown. However, based on trends in the toxicokinetic of the various NSAIDs showing the presence of zero order metabolisms in birds that succumbversus those that survived (Table 1), constraints in metabolism are the likely reason for the population sensitivity. This latter supposition is also supported by evidence from toxicity studies in other bird species, with diclofenac toxicity being associated with prolonged half-lives of elimination above 12 h while species with low to no susceptibility had more rapid metabolism and half-lives of elimination at 6 h or lower (Hassan et al., 2018; Naidoo et al., 2011). Constraints in metabolisms are also evident when the level of exposure at the low dose of 0.8 mg/kg is compared between species, vulture are exposed (area under the curve) to a much higher diclofenac concentration than other species thus far studied,with a range of 4 to 250 hold higher exposure respectively from cattle to the domestic dog (Table 2). To further understand the metabolic constraints associated with diclofenac toxicity a substantial body of literature exists in humans for comparison, as a subpopulation of people have been reported as poor metabolisers of the drug. Under normal circumstances in humans, diclofenac is preferentially metabolised (>40%) by the cytochrome P450 2C family of enzymes, specifically CYP2C9 and CYP2C18, to form 4-hydroxydiclofenac (4-OH), 3-hydroxydiclofenac (3-OH) and 5-hydroxydiclofenac (5-OH) (Davies and Anderson, 1997). Diclofenac is also metabolised in a minor

* Corresponding author at: Faculty of Veterinary Science, Department of Paraclinical Science, University of Pretoria, Pretoria, South Africa.
E-mail address: vinny.naidoo@up.ac.za (V. Naidoo).

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Table 1
Half-life of elimination of various NSAIDs between vultures that survived or died.

Drug	Dose (mg/kg)	Half-life of elimination		Reference
		Died	Survived	
Diclofenac	0.8	12.24		Naidoo and Swan, 2009
Ketoprofen	5	7.38	3.24	Naidoo et al., 2010a, 2010b
Carprofen	64	37.75	8.74	Naidoo et al., 2018
Nimesulide	17.58	14.06		Galligan et al., 2022
Tolfenamic Acid*	3.5	65	14	Prakash et al

* Calculated from mean data presented in the paper using the same methodology that was used for Ketoprofen. The animals that died showed evidence of a two compartmental and the half-life is based on two points and thus would require further confirmation.

manner (<40%) by the CYP3A4 enzyme as well as undergoing direct glucuronidation (Rollason et al., 2014). In persons with absence or mutations in the cytochrome enzymes, constraints in metabolism could result in longer half-lives of elimination and toxicity, leading to the supposition that a deficiency of the CYP2C9 enzyme as a major metabolic system, could play a role in diclofenac metabolism in the vulture. While no metabolic information is available for diclofenac in vultures, a study evaluating the plasma metabolomics of nimesulide in vultures succumbing to exposure (Mathesh et al., 2023) showed the absence of the plasma hydroxynimesulide metabolites, a well-defined CYP2C9 metabolite in humans.

In contrast to the toxicity of diclofenac, the safety of meloxicam has been demonstrated in various vulture species together with a substantial shorter half-life of elimination. This was an unexpected finding as both these NSAIDS are meant to metabolised by CYP2C9 and CYP3A4, with CYP2C9 being the major enzyme system. To better understand this difference Adawaran was able to demonstrate, using vulture liver slice cultures, that the difference was linked to a trend of faster metabolism of meloxicam by hepatocytes (Adawaren et al., 2018). In a second study Adawaren et al. (2019) was also able to demonstrate the presence of a second hydroxy metabolites of meloxicam, which was not only the predominate metabolite but also a metabolite that was yet to be identified in another species. The latter led to the speculation that metabolism of meloxicam was due to a third yet to be identified CYP enzyme. In addition, the same study showed that the concentration of the 5-hydroxymethyl metabolite of meloxicam was very low, supporting lower CYP2C9 activity as this enzyme system accounts for more the 40% formation of the specific metabolite in mammals as a primary degradation pathway. A second NSAID, tolfenamic acid, has since also been shown to be safe with rapid plasma decline (Chandramohan et al., 2022). While the metabolites to tolfenamic acid were not identified in this study, this NSAIDs has been demonstrated to be predominantly metabolised by CYP1A2 instead of CYP2C9 in humans (Venkataraman et al., 2014).

Experimental support for the vulture analogue to CYP2C9 being defective or deficient, can also be found in nearest susceptible species, the domestic chicken. In the chicken, Kawalek et al., 2006, was able to demonstrate that the birds metabolised diclofenac into a 5-OH and 4-OH metabolites through the activity of CYP2C9/8 enzymes. In a model validation study, Naidoo et al., 2007 was able to demonstrate that the domestic chicken was an adequate model for the study of the pathophysiology of diclofenac toxicity, with the domestic chicken showing an LD_{50} of 10 mg/kg, only ten-fold higher than the vulture. Further at the low dose the half-life of elimination for diclofenac was 0.89 h at 0.8 mg/kg, but escalated to 14.34 h at 10 mg/kg when 50% of the dosed chickedn died. In another LD_{50} study in chickens, Locke et al., 2022, was able to confirm that toxicity and death in chickens was associated with higher diclofenac plasma concentration with corresponding lower concentrations of metabolites in comparison to the birds that survived, as would be expected with zero order metabolism due to a lower hepatic metabolic capacity. For the same study, when a second group of birds were simultaneously treated with the CYP2C9/CYP3A4 inhibitor fluconazole with diclofenac, an even more marked decrease in the metabolism of diclofenac was evident in the birds that died in conjunction with an overall lower concentration of the three metabolites peak areas.

With a similar enzyme system likely being involved in toxicity in both the vulture and chicken, for this study we attempt to identify the CYP2C9 equivalent genes and mRNA sequences present in Cape vultures and potential sequence defects using DNA and mRNA sequencing.

2. Materials and methods

2.1. Sample collection

The following study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (project numbers V093–15 and V097–17). In all cases samples were collected from adult Cape vultures (*Gyps coprotheres*), ten in total: A single liver sample was removed aseptically from bird 1, by opening the abdominal cavity under a laminar hood and transferred into a sample bottle containing cooled William Medium E (WME) solution and stored in liquid nitrogen. A single skin sample was excised with a scalpel blade from bird 2, and collected into cryotubes and dropped immediately into liquid nitrogen after which the cryotubes were stored at −80 °C until a more appropriate time for DNA extraction. Blood was obtained venepuncture into plain blood tubes from birds 3 to 10.

2.2. In silico identification of the enzyme responsible for diclofenac metabolism in the chicken

The chicken CYP2C9-like enzyme cDNA sequence (XP_015144012.2) was extracted from the NCBI database and evaluated against the Golden

Table 2
Half-life of elimination and area under curve to the last time point (AUClast) of diclofenac in various species.

Species	Species	Dose[!]	AUClast (ug/ml*h)	Adjusted AUC[#]	T1/2	Ref
CGV	*G coprotheres*	0.8		100.5	12.24	
AWBV	*G africanus*	0.8		77.4	16.78	
Duck	*Anas platyrhynchos*	0.8		0.65	1.58	
Quail	*Coturnix coturnix*	0.8		0.19	3.41	Hassan et al., 2018.
Pigeon	*Columba livia*	0.8		0.33	3.42	
Turkey Vulture	*Cathartes aura*	0.8		1.73	6.43	
Pied Crow	*Corvus albus*	0.8		0.05	2.33	
Dog	*Canis lupus familiaris*	50	25	0.4	2.1	Al-Jenoobi, 2010
Cow	*Bos taurus*	2.2	67.57	24.57091	9	Yang et al., 2019
Pig	*Sus scrofa*	50	46	0.736		Tse et al., 2012
Rabbit	*Oryctolagus cuniculus*	50	29	0.464	1.554	Ahmad et al., 2009
Rat	*Rattus norvegicus*	2	2.448	0.9792	1.12	Yuan et al., 2017.
Sheep	*Ovis aries*	1	12.17	9.736	1	Rahal et al., 2008

Table 3
Primers sequences used for the amplification of cytochrome 2C19 gene.

NAME	FORWARD PRIMERS	REVERSE PRIMERS	PCR PRODUCT SIZE
CAPEVULTURE-1	GGAGAGCACAGAGCTGGTAGGCAGG	CCTTCTCAGCAGGTAAAGCACAGGC	6299 bp
CAPEVULTURE-2	GCCTGTGCTTTACCTGCTGAGAAGG	CTGCTGGCCACTGCCAGAGGCAGG	6299 bp
CAPEVULTURE-3	CCTGCCTCTGGCAGTGGCCAGCAG	CAGATGCCCTACACAGATGC	6298 bp
CAPEVULTURE-4	GAACTGCAAGACCAGACTGC	CTGTCTCCAATGTCATATGCTCC	6299 bp
CAPEVULTURE-5	GGATGTGCTGGGTGTGAGATG	CAGAAGCAAATGGASYTCCTGGG	6299 bp
CAPEVULTURE-6	GGCAGTTGGACTGCAGGACCTCTAG	CTGCGATATGGCTACTCAATGTACC	6299 bp
CAPEVULTURE-7	GTGCAGTGGGAAGACACCAATG	CTTGAGGAAGCTTGCCCACTAAC	6297 bp
CAPEVULTURE-8	GCTTGAGGCTATGTTTGCATTCAAG	CAGTGAGTCCCAAGGAAGCACC	6299 bp
CAPEVULTURE-9	CACAGGCWCTGCRGACCTACTCTGC	CACTTCCAGGGAAGCGAATATGCC	6298 bp
CAPEVULTURE-10	CATCATGTCCGTGCAGCACCACCAC	GTAATATTGACTAACTCAGCCACGTG	6299 bp

Eagle (*Aquila chrysaetos chrysaetos*) by Blast for the equivalent protein sequence in the species. The identified CYP gene, the chicken 2C9, Eagle 2C19 (XP_029887105.1), human 2C9 (NP_000762.2) and dog 2C21 (NP_001183973.1) were subsequently aligned to identify the six substrate recognition sites and to ascertain if it contained the amino acid resides reported as being necessary for diclofenac metabolism. The sequences were also evaluated for the presence of the two signature sequence (FxxGx(H/R)xCxG and (A/G)Gx(E/D)T) also reported as being conserved in the cytochrome P450 enzyme family, with the threonine in the second signature being important for catalysis (Gotoh, 1992). Lastly sequences were evaluated for the presence of following amino acids residues reported as being key in diclofenac metabolism: Asparagine at location 289; serine at 286; arginine at locations 97, 105, 108; phenylalanine at location 114 and aspartic acid at location 293 (Klose et al., 1998; Tang, 2003; Dickmann et al., 2004).

2.3. Cape vulture transcriptome analysis

The transcriptome analysis was undertaken on the same sample used in the liver slice cultures as by Adawaren et al. (2018). The sample was previously demonstrated to have a trend for the lower clearance of diclofenac than meloxicam, as well as having an average microsomal protein content of 3325 mg/g liver, which was 25% of the predicted protein content for a mammalian species the same size. Samples were analysed at the genomic platform of the Agricultural Research Council of South Africa. For extraction, the liver sample from bird 1 was finely minced and 150 mg of the material was used to extract total RNA following the protocol of Quick RNA Miniprep kit (Zymo Research, Irvine, CA). First strand cDNA was synthesized using LunaScript RT Super mix kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions in a total volume of 20ul containing 500 ng total RNA. Transcriptome next generation sequencing was undertaken using Illumina technology at the Biotechnology platform of the Agricultural Research Council (ARC), South Africa. Truseq stranded-mRNA kit were used to remove rRNA according to manufacturer instructions. The kit uses poly-A beads to specifically capture mRNA and exclude rRNA. Hiseq 2500 Illumina sequencer using v4 chemistry (2x125bp reads) was used to generate 50 million short read nucleotide bases.

The transcriptome NGS sequence quality was evaluated using the FastQC software (Andrews, 2011). and trimmed using the Trimmomatic program (version 0.39) (Bolger et al., 2014). The de-novo assembly was performed using the Trinity software (version 2.8.4) on the default settings using the Galaxy platform (Grabherr et al., 2011). The CYP2C19 enzyme (XP_029887105.1) identified in the Golden Eagle, determined as being equivalent to the chicken CYP2C9 (XP_015144012.2) by Blast analysis, was used for identification of the equivalent enzyme sequence in the Cape vulture in the Trinity assembled local BLAST database.

2.4. Whole Genome sequencing and CYP2C19 gene sequence confirmation

DNA from skin sample of bird 2 was extracted using approximately 25 mg of tissue using the ZR Genomic DNA Isolation kit (Zymo Research) according to manufacturer's instructions at the Inqaba laboratory. DNA samples from the skin were sent to the Uppsala University Genomic Centre, Sweden for 200 bp ION S5 analysis (Thermofischer, 2015). The genome sequencing was performed according to manufacturer's instruction. The run was based on 200 bp read length chemistry on an ION-540 chip. Sequence reads were subsequently aligned in HISAT2 to the reference genome of the golden Eagle (PRJEB33202) (Mead et al., 2021) using the Galaxy platform. The Golden Eagle genome was used as part of the analysis, as complete mitochondrial genome analysis showing the Eagle to be closely related to the Cape vulture (Adawaren et al., 2020). The sequence tentatively corresponding to the CYP2C19 gene (NC_044014.1) on the Eagle chromosome 11 was identified using the Integrative Genomics Viewer (IGV)(Broad institute).

Following the tentative identification of the CYP2C19 gene, DNA extracted from blood sampled intravenously from 9 Adult Cape vultures (Bird 3 to 10) were subjected to Sanger sequencing using ten pairs of primers based on the Eagle CYP2C19 sequence (Table 3). Quick-DNA Miniprep Plus Kit (Zymo Research) was used to extract DNA from the samples according to manufacturer's instructions. The quantity of the extracted DNA was evaluated using a NanoDrop spectrophotometer. Furthermore, PCR amplification was achieved using OneTaq Quick load 2 x Master mix from NEB, following manufacturer guidelines in 25ul reaction. The thermal cycling program was the same for all 10 pieces of the complete gene fragment: Initial denature 94 °C for 30 s (*sec*), 40 cycles of (denature 94 °C for 30 s; annealing 55 °C for 30 s and elongation 68 °C for 2 min) and final elongation 68 °C for 5 min. In addition, The PCR products were evaluated to determine the DNA fragment length using 1% agarose gel electrophoresis at 150 Volts for 1 h. The purified PCR products were sequenced on the ABI 3130 Genetic Analyser (Applied Biosystems) using the BigDye Terminator sequencer Cycle Sequencing Kit version 3.1.

2.5. PCR analysis

Analysis was undertaken at the commercial Inqaba laboratory in Pretoria, South Africa. The same liver sample as for the transcriptome analysis was used to extract total RNA following the protocol of Quick RNA Miniprep kit (Zymo Research, Irvine, CA). First strand cDNA was synthesized using LunaScript RT Super mix kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions in a total volume of 20ul containing 500 ng total RNA. In addition, PCR was performed using One taq polymerase kit from NEB and AWB liver cDNA as a template. (Primers were F: GTTTGCATTGCTTGCCTGCTATC and_R: GCTATCCATCTGCAAAGCGTG). The amplification protocol was as follows: Initial denature 94 °C for 30 s (*sec*), 40 cycles of (denature 94 °C for 30 s; annealing 55 °C for 30 s and elongation 68 °C for 2 min) and final elongation 68 °C for 5 min. The PCR products was run on 1% agarose gel (qPCR method reported in the supplementary material).

Table 4
An example of the nine introns identified by Splign anlysis following the alignment of a single Vulture CYP2C19-like genome sequence to the Eagle CYP2C19-like mRNA sequence.

Query	Subject	Idty	Len	Q.Start	Q.Fin	S.Start	S.Fin	Type	Details
Eagle	Vulture	0.956	180	1	180	45	224	<exon>GT	M5R2M8RM19RM27RM10RM
Eagle	Vulture	0.957	163	181	343	566	728	AG<exon>GT	M36RM67RM2R2M18RM7RM
Eagle	Vulture	0.98	150	344	493	1220	1369	AG<exon>GT	M32RM92RMRM22
Eagle	Vulture	0.975	161	494	654	1672	1832	AG<exon>GT	M62RM45RM20RM7RM23
Eagle	Vulture	0.95	180	655	831	2226	2405	AG<exon>GT	M15RM8RM14I3M19RM9RM
Eagle	Vulture	0.972	142	832	973	2930	3071	AG<exon>GT	M71R2M37RM26RM4
Eagle	Vulture	0.963	188	974	1161	3501	3688	AG<exon>GT	M16RM18RM31RM53R2M38
Eagle	Vulture	0.993	142	1162	1303	4894	5035	AG<exon>GT	M113RM28
Eagle	Vulture	0.967	601	1304	1904	5690	6290	AG<exon>	M50RM72RM13RM49RM15R

2.6. Analysis of CYP2C19 gene and predicted transcript

The complete gene structure of CYP2C19 from the Sanger sequencing was further investigated to predict the exon-intron boundaries. The open reading frame (ORF) was investigated using NCBI Splign alignment tools (Burge and Karlin, 1997; Kapustin et al., 2008). For the Splign alignment, the partial sequence from the transcriptome of the Cape vulture and the complete golden Eagle CYP2C19 mRNA sequence were evaluated. Following identification of the ORF on the Cape vulture CYP2C19 gene, the predicted CYP2C19 transcript sequence which could have resulted if the premature stop codon was not present, was also evaluated for the presence of the amino acid residues mentioned above.

3. Results

3.1. CYP2C9 equivalent gene in the eagle

Following literature suggesting the role of the CYP2C9 enzyme in the metabolism of diclofenac in the chicken, the sequence in the Eagle corresponding to the enzyme was identified as CYP2C19-like (XM_030031245.2/XP_029887105.1,) in the NCBI database. The sequence has 80,2% identity and 100% query coverage. The Eagle enzyme was predicted to be found on Chromosome 11 (LR606191.1) and more specifically at location NC_044014.1. The Chicken CYP2C9 like enzyme was also equivalent to the chicken CYP2C18 (NP_001001752.2; also referred to as CYP2C45) at 99.39% identity and 100% query coverage.

3.2. Transcriptome analysis

The short-read sequence obtained were deposited in GenBank (PRJNA912371). De Novo alignment in Trinity revealed a partial sequence, that was not deposited due to the sequence being incomplete (Supplementary Fig. 1). The sequence consisted of 416 amino acids and contained an abnormal stop codon. Real-time PCR was unable to confirm the whole sequence despite four primer pairs being used. Of these only two short sequences were amplified (Supplementary material).

3.3. Genome sequencing

The 200 bp next generation ION S5 sequencing had 85,525,205 reads, 15.2 G total bases with a mean, median and mode read length of 178 bp, 194 bp and 201 bp respectively (Supplementary fig. 2). Raw sequence reads were deposited into GenBank (Accession: PRJNA389655). Alignment against the Golden Eagle genome chromosome 11 in HISAT2, revealed a good coverage at 81.46% for CYP2C19 in the vulture with numerous gaps identified. In total the identified sequence covered 5755 bp and had 954 gaps (Supplementary fig. 3). Based on this result, the full amplification of Cape vulture CYP2C19 gene from eight birds were successfully attempted. The resulting CYP2C19 genes had a nucleotide base size ranging between 6297 and 6299 base pairs (OP758364-OP758371).

3.4. Cape vulture ORF

The Cape vulture genomic CYP2C19 sequences aligned to the golden Eagle CYP2C19 mRNA using NCBI Splign, revealed a single open reading frame with nine introns (Table 4). In intron 7, the sequencing investigation led to the finding of a premature stop codon "TGA" at position 3535–3537 (Fig. 1). The location of the stop codon was conserved across all eight vultures evaluated and corresponded with the stop codon evident on the transcriptome analysis.

3.5. Cytochrome enzymology

Six substrate recognition sites were identified (Fig. 2). All the sequences evaluated has the signature motif FSAGKRICVG present. In contrast, the second motif was only highly conserved in the human, dog and chicken CYP2C9 being characterised as AGTET. The Eagle and vulture had TGTET and TRTET respectively, indicating that there may be some deficiency in the enzyme, although all species had the high conserved T in the second motif which was described as an important feature of the enzymes' catalytic activity. For the other amino acid residues previously reported as being specifically important for the metabolism of diclofenac, all the species had Arg87, Arg 108, Phe115 and Asp295 present, while Arg105 was present only in human and the dog. Apart from the human enzyme which had Asn289 and Ser286 present, the other sequence were defined by Asn286 and Ile289 for dogs and Thr289 for the avian species.

4. Discussion

The unintended exposure of vultures to diclofenac resulted in an unprecedented toxicity in the species with 99% mortality and millions of birds dying. While it has been well accepted that death was associated with renal failure and gout, the reason behind the unprecedented species susceptibility is yet to be unexplained. With toxicity being associated

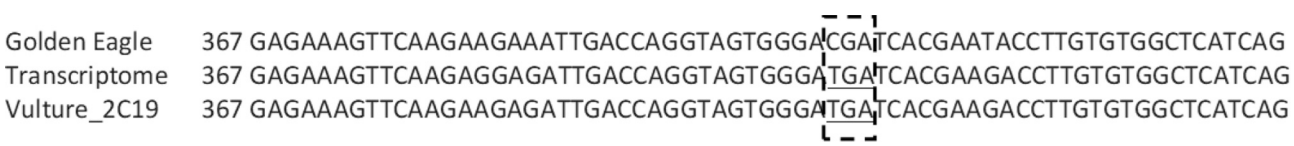

Fig. 1. The premature stop Codon was identified in intron 7.

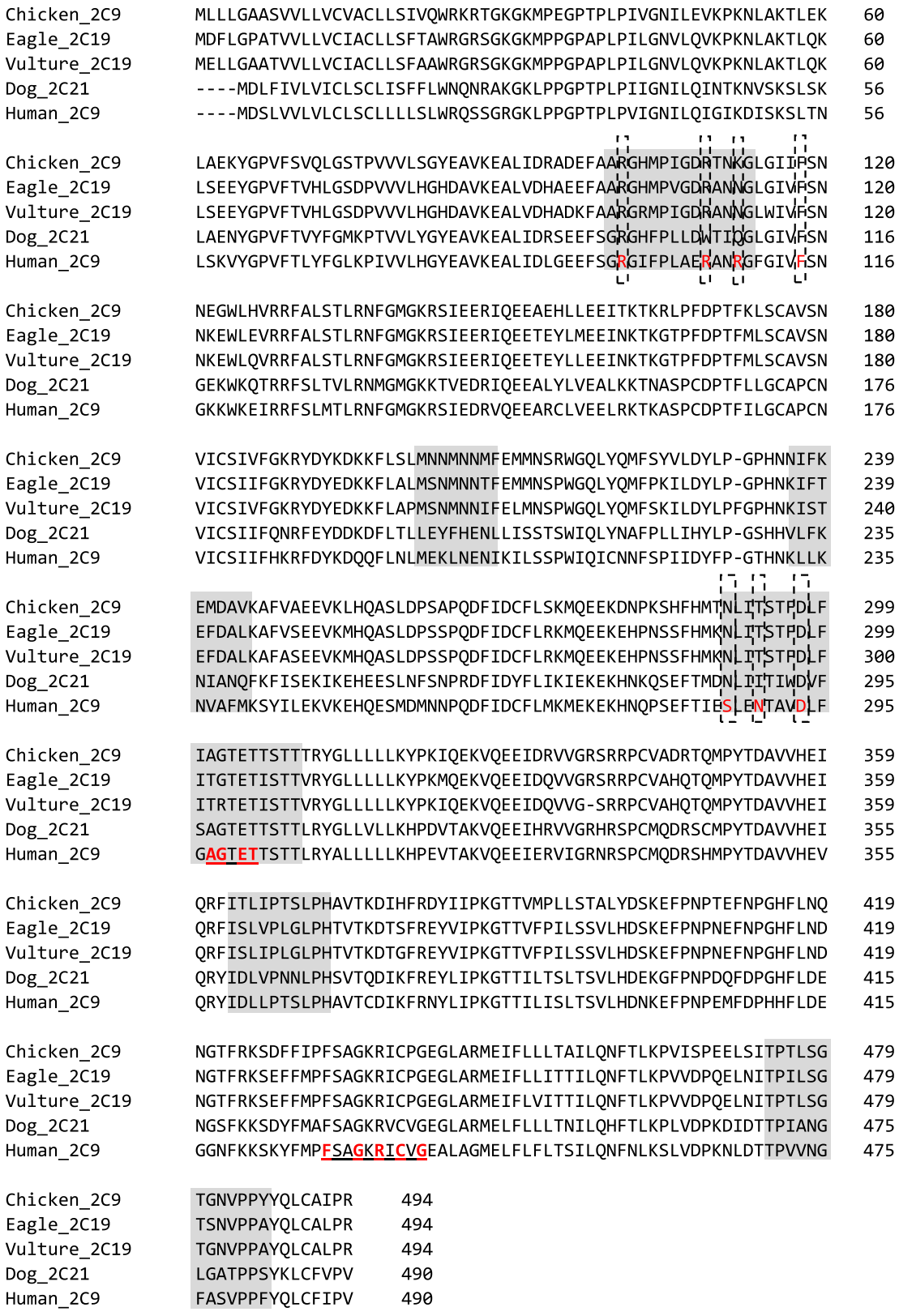

Fig. 2. Comparison of the translated sequence for the applicable CYP enzyme in the chicken, Eagle, vulture, dog and human, expected to be involved in diclofenac metabolism (Court, 2013). The highlighted areas represent the six substrate recognition sites. The underlined sections are the two conserved sequence motifs, and the broken lines are the amino acid residues reported as being important for diclofenac binding and metabolism for Human CYP2C9.

with long half-life of elimination, and the toxicity of other NSAIDs being associated with zero order metabolism, constraints in biotransformation has been suggested as an underlying mechanism. With the metabolism of diclofenac being linked to the CYP2C9 and CYP2C18 enzymes in humans, a similar metabolic pathway is likely in avian. Support for the latter is evident from studies in chickens where the two common metabolites were associated with CYP2C8/9 activity (Davies and Anderson, 1997).

Using the CYP2C9-like cDNA sequence from the chicken, the equivalent vulture enzyme being CYP2C19 was identified. To confirm that the identified enzyme was a functional CYP enzyme, the transcribed enzyme was compared to the human CYP enzyme and its six substrate recognition sites (Fig. 2). The chicken had both signature motifs present as described, while the Eagle differed by one amino acid in the second signature motif. Nonetheless both sequences had the highly conserved threonine present in the second signature motif which has been described as highly important for the enzyme's catalytic activity. In terms of the specific amino acid residues required for diclofenac metabolism, the Eagle and chicken enzymes had Arg87, Arg 108, Phe115 and Asp295 present, which have all described as being necessary for diclofenac binding to the enzyme for metabolism. Both the predicted avian enzymes were different to the human enzyme at amino acid 286 and 289, with the specific mutations reported in the human enzyme sequences being shown to have a positive effect with metabolic activity reported to be as much as 5-to-10-fold higher than cases with the absence of the said mutations. Also of concern was the absence of Arg105 in the chicken sequence, which from in vitro studies with the human CYP2C9 enzyme, was associated with significantly diminished metabolic activity. The various difference in sequences present, could thus explain the unexpected sensitivity of the chicken to diclofenac in a similar manner to that evident in vitro with human enzyme studies. These finding also tends to suggest that other endangered species such as the Eagle may still be susceptible to the effect of diclofenac, despite having functional CYP2C19 enzymes.

Based on the enzymology suggesting the CYP2C9-like enzyme could likely metabolise diclofenac, attempts were made to identify a similar sequence in the vulture using whole genome sequencing. With the golden Eagle shown to contain the CYP2C19 enzyme, and already known to be closely phylogenetically related to the vulture, this species was used as the reference genome for the assembly of the vulture next generation sequences. From the HISAT alignment, the corresponding CYP2C19 sequence was identified in the vulture genome, which were confirmed by subsequent Sanger sequencing. Despite the gene being present and transcriptome analysis suggesting expression, the assembly only produced a partial sequence for the CYP2C19 enzyme, and further also indicated the presence of a premature stop codon.

Using Splign and the CYP2C19 sequence from the Eagle where the full sequence was published, the Cape vulture genomic CYP2C19 sequences aligned well to the golden Eagle CYP2C19 mRNA using NCBI Splign with nine introns in the open reading frame (ORF). In intron 7, a single nucleotide mutation introduced a premature stop codon “TGA” at position 3535–3537. The location of the stop codon was conserved across all 8 vultures evaluated and corresponded with the stop codon evident on the transcriptome analysis. While attempts were made to characterise the level of expression using real-time quantitative polymerase chain reaction (RT-qPCR), repeated attempts failed to amplify the CYP2C19 cDNA. While not evaluated further, the non-amplification of the open reading frames is likely related to the premature stop codons and decay of the abnormal mRNA by a phenomenon known as nonsense mediated mRNA decay that occurs to prevent interference with other protein function (Baker and Parker, 2008). In lower organisms such decay has been associated through the action of the gene that codes for a transactive factor. In higher organisms, this is mediated by alteration in nuclear mRNA metabolism, which accelerates degredation during or before transport prior to protein synthesis (Aoufouchi et al., 1996).

Based on previous findings that the CYP450 enzymes are known to be responsible for the primary conversion of xenobiotics via phase I metabolic reaction (Bort et al., 1999), the inability of old-world vultures to metabolise NSAIDs is tentatively linked to the non-expression of CYP2C19 enzyme. This is not too dissimilar to the defects in metabolic enzyme as seen in certain people. With eight vultures, that were not directly related, all showing the same defect, it is likely that the deficiency is present across the entire old-vulture clade which would need confirmation. For the final confirmation of defect in metabolism, it would be also important to demonstrate the absence of an full length protein using blotting or a similar method.. Further it would also be important to demonstrate that a protein in which the abnormal stop codon has been corrected also has the ability to metabolise diclofenac under in vitro conditions as undertaken in studies evaluating the functional amino acid residues involved in metabolism.

5. Conclusion

In conclusion, the presence of a premature stop codon in Cape vulture CYP2C19 open reading frame could prevent the translation of the gene into a functional enzyme, thereby exposing old world vultures to NSAIDs zero order metabolism. The latter does require further confirmation through Western blotting.

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Author contributions

EA: Conceptualised the study, methodology, sample analysis and first draft; CL and AA – methodology, sample analysis, editing the manuscript; VN: Conceptualised the study, sourced funding, data analysis, substantive draft, PhD supervisor to EA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data is availablee on NCBI - Ascensions number are provided in the manuscript

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2023.116771.

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