CHAPTER 3: Removing the Threat of Diclofenac to Critically Endangered Asian Vultures

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REMOVING THE THREAT OF DICLOFENAC TO CRITICALLY ENDANGERED ASIAN VULTURES

Gerry Swan[1], Vinasan Naidoo[1], Richard Cuthbert[2], Rhys E. Green[2,3], Deborah J. Pain[2], Devendra Swarup[4], Vibhu Prakash[5], Mark Taggart[6], Lizette Bekker[1], Devojit Das[5], Jörg Diekmann[7], Maria Diekmann[7], Elmarié Killian[1], Andy Meharg[6], Ramesh Chandra Patra[4], Mohini Saini[4], Kerri Wolter[8]

1 Department of Paraclinical Sciences and Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, **2** Royal Society for the Protection of Birds, The Lodge, Sandy, Bedfordshire, United Kingdom, **3** Conservation Biology Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge, United Kingdom, **4** Indian Veterinary Research Institute, Izatnagar 243122, Uttar Pradesh, India, **5** Bombay Natural History Society, Hornbill House, S.B. Singh Road, Mumbai, India, **6** School of Biological Sciences, Department of Plant and Soil Science, University of Aberdeen, St Machar Drive, Aberdeen, United Kingdom, **7** Rare and Endangered Species Trust, PO Box 178, Otjiwarongo, Namibia, **8** Vulture Unit, De Wildt Cheetah and Wildlife Trust, PO Box 1756, Hartbeespoort, South Africa

3.1 Abstract

Veterinary use of the non-steroidal anti-inflammatory (NSAID) drug diclofenac in South Asia has resulted in the collapse of populations of three vulture species of the genus *Gyps* to the most severe category of risk of global extinction. Vultures are exposed to diclofenac when scavenging on livestock treated with the drug shortly before death. Diclofenac causes kidney damage, increased serum uric acid concentrations, visceral gout and death. Concern about this issue led the Indian Government to announce its intention to ban the veterinary use of diclofenac by September 2005. Implementation of a ban is still in progress late in 2005, and to facilitate this we sought potential alternative NSAIDs by obtaining information from captive bird collections worldwide. We found that the NSAID meloxicam had been administered to 35 captive *Gyps* vultures with no apparent ill effects. We then undertook a phased programme of safety testing of meloxicam on the African white-backed vulture *Gyps africanus*, which we had previously established to be as susceptible to diclofenac poisoning as the endangered Asian *Gyps* vultures. We estimated the likely maximum level of exposure (MLE) of wild vultures and dosed birds by gavage (oral administration) with increasing quantities of the drug until the likely MLE was exceeded in a sample of 40 *G. africanus*. Subsequently, six *G. africanus* were fed tissues from cattle which had been treated with a higher than standard veterinary course of

meloxicam prior to death. In the final phase, ten Asian vultures of two of the endangered species (*G. bengalensis*, *G. indicus*) were dosed with meloxicam by gavage; five of them at more than the likely MLE dosage. All meloxicam-treated birds survived all treatments, and none suffered any obvious clinical effects. Serum uric acid concentrations remained within the normal limits throughout, and were significantly lower than those from birds treated with diclofenac in other studies. We conclude that meloxicam is of low toxicity to *Gyps* vultures and its use in place of diclofenac would reduce vulture mortality substantially in the Indian subcontinent. Meloxicam is already available for veterinary use in India.

3.2 Introduction

Veterinary use of the non-steroidal anti-inflammatory drug diclofenac is a major cause of the catastrophic collapse of *Gyps* vulture populations in the Indian sub-continent [1-3]. Three species of vultures endemic to South Asia, which together used to number tens of millions, are now at high risk of global extinction and are listed as Critically Endangered [4]. Populations of Oriental white-backed (*Gyps bengalensis*), long-billed (*G. indicus*) and slender-billed vultures (*G. tenuirostris*) have declined by more than 95% since the early 1990s [5,6], and continue to decline at an annual rate of 22% to 48% [3].

Diclofenac is a widely available veterinary drug in the Indian sub-continent, where it is used for the symptomatic treatment and management of inflammation, fever and/or pain associated with disease or injury in domestic livestock. Vultures are exposed to the drug when they consume carcasses of cattle that were treated with diclofenac shortly before death. Following experimental exposure to diclofenac or diclofenac-contaminated tissues, *Gyps* vultures die within days from kidney failure with clinical signs of extensive visceral gout (formation of uric acid crystals within tissue) [1,7]. These clinical signs and diclofenac residues in vulture tissues have been found in carcasses of wild *Gyps* vultures from across India, Pakistan and Nepal [1,2], and the proportion of vulture carcasses with signs of diclofenac poisoning is consistent with this being the main, and possibly the only, cause of the vulture decline [3].

The loss of tens of millions of vultures over the last decade has had major ecological consequences across the Indian subcontinent that pose a potential threat to human health.

In many places, populations of feral dogs (*Canis familiaris*) have benefited from the disappearance of *Gyps* vultures as the main scavenger of wild and domestic ungulate carcasses [8]. Associated with the rise in dog numbers [9] is an increased risk of human cases of rabies. If rat (*Rattus* spp.) populations also increase at carcass dumps in and near settlements, the risk of transmission of diseases including bubonic plague to humans may also increase. Vultures probably also helped to control livestock diseases, such as brucellosis, tuberculosis and anthrax by disposing of infected carcasses [10, 11]. The loss of vultures has had a social impact on the Indian Zoroastrian Parsi community, who have traditionally utilized vultures to dispose of human corpses in "sky burials" [12] and are now having to seek alternative disposal methods [13]. As a consequence of the collapse of vulture populations, national and international conservation organisations have concluded that it is essential to ban the use of diclofenac in livestock so as to remove it as a contaminant of the food of wild vultures [14]. At a Meeting of the National Wildlife Board in March 2005, the Government of India announced that they intended phasing out the veterinary use of diclofenac [15].

The identification of alternative non-steroidal anti-inflammatory drugs (NSAIDs) that are effective for the treatment of livestock, but also relatively non-toxic to vultures, would facilitate the removal of diclofenac from the food of vultures. NSAIDs are characterised by their ability to inhibit cyclo-oxygenase enzymes, which are involved in the formation of prostaglandins. However, there are marked differences between drugs in their selective inhibition of the two sub-types of cyclo-oxygenase COX-1 and COX-2, with the latter being involved with the modulation of inflammatory responses and pain, while the former modulates blood flow to the kidneys. The ability of NSAIDs to inhibit both these subtypes has been implicated as a cause of the severe side effects occasionally associated with the use of some NSAIDs [16]. Toxic effects on the kidneys of birds have been observed following treatment with a number of NSAIDs [1,17]. However, there are marked inter-specific differences in toxicity [18-20] and it is necessary to establish the safety of individual NSAIDs to *Gyps* vultures. To identify candidate alternative drugs, we contacted veterinarians at zoos and wildlife rehabilitation centres worldwide, and requested information on the clinical use of NSAIDs on captive *Gyps* vultures, including the outcome of such treatment. Preliminary results suggested that the NSAID meloxicam is a potential alternative for diclofenac, because 35 individuals from six *Gyps* species (including five

Oriental white-backed vultures) treated with meloxicam, typically at doses of 0.2-0.5 mg kg^{-1}, showed no ill effects; whilst the use of several other NSAIDs was associated with renal failure (RSPB and NBPT unpublished data).

As all three of the resident Asian *Gyps* vultures are Critically Endangered, we considered it unacceptable to use these species for safety testing without first evaluating the safety of meloxicam on a suitable surrogate. The African white-backed vulture (*G. africanus*) was chosen as a surrogate because it has a favourable global conservation status (category Least Concern) [4] and diclofenac has been shown experimentally to be as toxic to it as it is to the endangered *G. bengalensis* [7]. Clinical signs at post-mortem examination of experimentally dosed birds indicate a similar mechanism of toxicity in both species. Diclofenac-dosed *G. africanus* showed significant increases in serum uric acid concentrations 12-24 hours after dosing and exhibited lethargy and neck-drooping behaviour before death [7].

In this paper, we report tests on the safety of meloxicam to *Gyps* vultures, which we dosed with meloxicam by gavage (oral administration) and by feeding them with tissue from meloxicam-dosed cattle. With both routes of drug administration, the range of dose levels we used exceeded our estimated likely maximum level of exposure (MLE) of meloxicam to wild vultures. To minimise the risk of suffering and death of experimental animals, safety testing was undertaken in six phases (summarised in Table 1). During the first three phases, the dose rate of meloxicam administered by gavage to *G. africanus* was progressively increased from 0.5 mg kg^{-1} vulture body weight to 1 mg kg^{-1} and then to the highest dose of 2 mg kg^{-1}, which exceeds our estimate of the MLE (Protocol S1). At the conclusion of each phase the results were evaluated and the study only proceeded to the next phase if all of the dosed birds were healthy and had clinically normal serum concentrations of uric acid and alanine transferase (ALT), both of which are known to be elevated beyond the normal range in *G. africanus* after treatment with diclofenac [7]. In the fourth phase, meloxicam was administered at 2 mg kg^{-1} to captive *G. africanus* in South Africa and wild vultures in Namibia, thereby exposing a larger number of vultures from two distinct populations to the estimated MLE of meloxicam in the wild. The fifth phase of the study simulated the natural route of NSAID exposure, by feeding vultures with liver and muscle tissue from cattle that had received a higher than standard veterinary course of

meloxicam treatment, with daily injections over five days. The final phase of testing was to assess the safety of meloxicam to two of the three critically endangered Asian vultures, by administering meloxicam by gavage to captive *G. bengalensis* and *G. indicus* in India.

3.3 Results and Discussion

3.3.1 *Phases I-III: Safety testing using captive G. africanus*

In each of the first three phases of our study, we administered a single dose of meloxicam to five vultures by gavage (oral administration into the crop via a five mm tube) and gave sterilised water to three control birds by the same method. The birds' apparent health and serum parameters were then assessed for seven days after treatment. Dose rates in Phases I to III were 0.5, 1 or 2 mg kg^{-1} respectively, and were set so that the highest dose just exceeded the likely MLE of wild vultures (estimated as 1.83 mg kg^{-1} vulture body weight; Protocol S1). No ill-health was observed in any of the 15 vultures treated with meloxicam at these three dose levels and all birds were alive and healthy at the end of the experimental period (Table 3-2). There was a significant loss of body mass during the experimental period in Phases I, II and III (matched pairs *t* test; Phase I $t_7 = 7.28$, $p < 0.001$; Phase II $t_7 = 2.97$, $p < 0.05$; Phase III $t_7 = 2.96$, $p < 0.05$). However, there was no significant difference between the meloxicam dosed and control birds in body mass change as a percentage of initial mass in any of the three Phases (2-sample *t* test; Phase I $t_6 = 0.13$, $p > 0.89$; Phase II $t_6 = 0.46$, $p > 0.66$; Phase III $t_6 = 0.61$, $p > 0.56$). Because of this, and because no significant loss of body mass was observed in later phases of the experiment, when birds were handled for sampling on fewer occasions and not moved from their normal holding aviaries (see below), we believe that the loss of body mass was most likely due to the stress caused by handling and sampling, rather than by meloxicam.

We compared the survival of vultures in these experiments with that of two *G. africanus* treated with comparable doses of diclofenac using the same methods [7]. In each phase, all five meloxicam-treated vultures survived the experimental period, whereas both diclofenac-treated birds died with extensive visceral gout. This represents a statistically significant difference in death rate between the two drugs (2-tailed Fisher exact test; 0/5 deaths versus 2/2 deaths, $p = 0.0476$ in each phase). However, because of the small sample sizes, these results do not exclude the possibility that, in a worst-case scenario, meloxicam

might have caused appreciable mortality if used on a larger sample. For example, with a total sample of 15 treated birds there still could statistically be a 5% chance of no birds dying, even if the true probability of death per trial was as high as 18% ($(1-0.18)^{15} = 0.05$). If only the five birds treated in Phase III with more than the MLE are considered, the failure to observe any deaths implies that there could be a 5% probability that the true risk of death per trial might be as high as 45% ($(1-0.45)^5 = 0.05$), which led us to test a larger sample of birds in Phases IV and V (see below).

Although the survival of all of the meloxicam-treated vultures in Phases I-III is not robust evidence of safety on its own, it can be combined with information obtained by sampling the blood of experimental and control birds. There were no significant differences in serum concentrations of uric acid, ALT, albumin and creatinine kinase between treated and control groups in any of the three phases and for any of the sampling times after dosing (Table 3-2). Inspection of the magnitude of average differences in serum concentrations between treated and untreated birds showed no indication of a systematic trend for any of the serum constituents in relation to dose (Figure 3-1, Table 3-2). Since the serum concentration of uric acid has been shown to be elevated well beyond the normal range in *G. africanus*, *G.bengalensis* and *G. fulvus* treated with comparable, fatal doses of diclofenac [1,7], these observations provide substantial further evidence of safety.

3.3.2 *Phase IV: Safety testing using larger numbers of captive and wild-caught G. africanus*

Our objective in the next phase of the study was to narrow the range of possible values of the true rate of meloxicam-induced mortality that would be consistent with our data by testing larger numbers of vultures with more than the likely MLE. In this phase, we treated two groups of *G. africanus*. In Phase IV.1 we used 14 long-term captive birds that had been used more than six weeks previously in Phases I to III (11 as experimental birds and three as controls). We treated all 14 birds with meloxicam. In Phase IV.2 we captured 25 wild *G. africanus* in Namibia and held them temporarily. Of these birds, 21 were treated with meloxicam and four received sterilised water and acted as controls. All treated birds in Phase IV were given 2 mg kg^{-1} of meloxicam by gavage (Table 3-1).

All 35 meloxicam-treated birds survived the seven-day experimental period and the wild-caught vultures used in Phase IV.2 were all successfully released after the experiment. There was no significant change in the body mass of meloxicam-treated birds between the beginning and end of the seven-day period for either captive (matched pairs *t* test; t_{13} = 0.29, $p > 0.77$) or wild caught birds (matched pairs *t* test; t_{24} = 1.68, $p > 0.10$). For the wild-caught birds there was also no significant difference in the percentage mass change of meloxicam dosed and control birds (2-sample *t* test; Phase I t_{23} = 0.30, $p > 0.77$). Serum uric acid concentrations did not differ significantly between experimental and control groups and showed no trend during the experimental period (Table 3-2, Figure 3-1). Neck-drooping behaviour, similar to that seen in diclofenac dosed birds [7], was observed in the Phase IV.2 birds soon after the collection of the second blood sample at 48 hours following treatment, and two birds lay on the ground. However, neck-drooping was observed in both meloxicam-dosed and control birds, and occurred during the heat of the day. Hence, we consider that the neck-drooping we observed was most likely to be a thermoregulatory activity [21] in response to high ambient temperature and an elevation of body temperature caused by the stress of handling and sampling, rather than a response to meloxicam treatment. By the end of the day, all birds (including the two recumbent birds) had resumed a normal body posture. Neck-drooping was not observed over the remaining five days of the trial. Hence, we consider it to be a non-specific response to stress caused by heat or handling and not a specific response to NSAID poisoning.

When the results from Phases III and IV of the study are combined, 40 *G. africanus* were treated by gavage with more than the likely MLE of meloxicam and all survived with no ill-effects observed that were attributable to the drug. These data indicate a 95% probability that the true probability of death per trial consistent with these data was no higher than 7% ($(1-0.07)^{40} = 0.05$). Taken together with the evidence of lack of an effect of meloxicam on serum uric acid concentrations, these results indicate that meloxicam administered by gavage does not cause appreciable mortality in *G. africanus*.

3.3.3 *Phase V: Safety testing by feeding G. africanus on tissues of meloxicam-treated cattle*

We wished to assess the possibility that, although meloxicam itself appears safe when administered to vultures at the MLE, metabolites produced by treated cattle might be toxic. To test this, we gave daily injections of 1.0 mg kg^{-1} of meloxicam to three cattle (*Bos taurus*) for five days. This is a higher dose level than the two standard veterinary doses recommended in India (0.5 to 0.7 mg kg^{-1} daily for five consecutive days). We slaughtered the three cattle eight hours after the last injection and fed liver or muscle to six captive *G. africanus*. An experiment by EMEA on *Bos taurus* found that tissue meloxicam concentrations in treated animals were higher in liver than other tissues tested, and peaked at the 8 hour sampling period (Protocol S1) [20]. In our experiment, concentrations of parent meloxicam in cattle tissues at slaughter averaged 0.50 ± 0.13 (± 1 standard deviation) mg kg^{-1} for muscle and 8.12 ± 1.10 mg kg^{-1} for liver. Vultures consumed an average of 0.59 ± 0.21 (± 1 standard deviation) kg of liver and 0.67 ± 0.32 kg of muscle tissue, of the 1 kg with which they were each provided, within the 48 hour feeding period. On one occasion, a bird ate all of the liver provided and on two occasions, birds ate the entire portion of muscle. The dose of parent meloxicam ingested ranged from 0.03-0.15 mg kg^{-1} vulture body weight for muscle, and from 0.57-1.98 mg kg^{-1} body weight for birds feeding on liver. Because we administered meloxicam for five days at a higher dose (1.0 mg kg^{-1}) than in the EMEA study (0.7 mg kg^{-1}) [22], the maximum dose ingested by a vulture (1.98 mg kg^{-1} bw) and the maximum cattle liver tissue concentrations (8.91 mg kg^{-1}) are somewhat higher than those predicted from the EMEA work (Protocol S1). For comparison, we also administered meloxicam by gavage at doses (1.18 to 2.45 mg kg^{-1} vulture body weight) intended to be similar to those ingested by birds feeding on liver. All six birds survived the treatments and no ill-effects or altered feeding behaviour was observed. There was no significant change in body mass between the start and end of the five-day experimental period for any of the three treatment types (matched pairs *t* test; muscle $t_5 = 1.00$, $p > 0.36$; liver $t_5 = 2.44$, $p > 0.05$; gavage $t_5 = 1.46$, $p > 0.20$). Serum uric acid concentrations remained within the 95% range observed in these individuals before treatment at both sampling times and also within the similar 95% ranges for uric acid for

wild *G. africanus* captured in Namibia and reported for *G. africanus* captured in Kenya [23] (Figure 3-2). There was no significant relationship between uric acid concentration and meloxicam dose at 48 h or 96 h (OLS regressions of log uric acid concentration on log meloxicam dose for each of the three administration routes; $p > 0.05$ in all cases). This was also the case when the log of the ratio of the uric acid concentration after treatment to that before treatment was used as the dependent variable. A more elaborate analysis of variance in which log uric acid concentration was modelled as a function of treatment method, time period and log meloxicam dose, with pre-treatment log uric acid concentration as a covariate, also gave no indication of any significant effect on serum uric concentration of treatment with meloxicam by any of the three routes (Protocol S2). The absence of mortality or elevation of serum uric acid levels indicates that tissues of cattle treated with meloxicam shortly before death are unlikely to be toxic to *G. africanus*. The experiments using liver tissue are particularly informative, because the quantity of liver eaten by one bird approached the maximum meal size likely to be consumed by a wild vulture and this bird received a dose of parent meloxicam in excess of the likely MLE.

3.3.4 *Phase VI: Safety testing of meloxicam on Endangered Asian Gyps*

Although the experiments we have reported so far indicate that meloxicam appears safe for *G. africanus*, this does not exclude the possibility that it might be toxic to Asian *Gyps* species, though this seems unlikely in view of the close phylogenetic relationships within the genus [24] and the similarity of the response to diclofenac of *G. africanus* and *G. bengalensis*. We therefore administered meloxicam doses of 0.5 mg kg^{-1} by gavage to three captive *G. bengalensis* and two *G. indicus* and the MLE of 2.0 mg kg^{-1} to three *G. bengalensis* and two *G. indicus*. All 10 meloxicam-treated birds survived the seven-day experimental period and they remain alive and healthy four months afterwards. None showed signs of ill-health or abnormal behaviour. There was no significant change in body mass during the experimental period (paired t-test; $t_5 = 2.07$, $p > 0.09$).

Hence, although the number of birds tested was small, there is no indication of adverse effects of meloxicam on these two species of Asian *Gyps* vultures.

3.4 Conclusions

The results of this study demonstrate that meloxicam is much less toxic than diclofenac in at least three *Gyps* species, including two of the Critically Endangered Asian species. Indeed, we found no evidence that meloxicam administered at doses exceeding our estimated likely maximum level of exposure caused any deaths or even elevation of serum uric acid concentrations. Combining the results of this study with those from the questionnaire to zoo veterinarians, a total of at least 88 individual birds from seven *Gyps* species are known to have received meloxicam at various doses with no recognized adverse effects. Hence, with this total of treated birds there is a 95% chance that the per trial probability of mortality caused by meloxicam is no higher than 3.5%. The observation that serum concentrations of uric acid remain within the normal range for all meloxicam dose rates adds substantially to the evidence that meloxicam has low toxicity to *G. africanus*, given that uric acid concentrations in this and two other *Gyps* species were markedly elevated by lethal treatment with diclofenac [1,7]. Preliminary results from the NSAID questionnaires indicate the safety of meloxicam to a wide range of other vultures, raptors and scavenging bird species, and to date we know of over 700 individuals from more than 30 species that have been treated with no apparent adverse affects (RSPB and NBPC unpublished data). This demonstrates that, at recommended clinical dose levels, meloxicam is not toxic to a wide range of avian species.

Any replacement for diclofenac must be effective for the treatment of livestock as well as safe for vultures. Meloxicam is one of the newer NSAIDs with preferential COX-2 inhibition, having analgesic, antipyretic and anti-inflammatory properties and a reduced risk of adverse effect on renal function [16,25]. It is used to treat a variety of veterinary ailments [26-30], and it is rated as a highly effective NSAID [30-32]. Meloxicam is approved for human use in more than 80 countries including India [33, 34]. It is used and licensed as a veterinary drug in India, Europe and North America [35,36] and is already manufactured in India, where, like diclofenac, it is available as both an injectable solution and oral bolus. We hope that efforts to prevent diclofenac being used to treat domestic livestock in the Indian subcontinent and in other *Gyps* vulture range states will continue as a matter of urgency. Where the availability of alternative drugs is seen as a barrier to achieving this objective, we recommend that governments consider advocating the use of

meloxicam as an alternative to diclofenac. Because vulture populations are now very low and contamination of even a small proportion of livestock carcasses is sufficient to cause adverse impacts on vulture populations [3] we also advocate immediate intensification of efforts to establish viable captive populations of all three Critically Endangered species.

3.5 Materials and Methods

Trial Animals: Non-releasable captive vultures held at the De Wildt Cheetah and Wildlife Trust (South Africa) were used for Phases I-III, Phase IV.1 and Phase V. All birds at De Wildt were habituated to captivity and eating regularly. In Phase IV.2 wild *G. africanus* (*n*=25) were captured using a walk-in-trap located at a feeding site for vultures in Namibia [37], run by the Rare and Endangered Species Trust. Captive *G. bengalensis* and *G. indicus* for Phase VI of the trials were held at the Bombay Natural History Society/Haryana State Vulture Conservation Breeding Centre, Pinjore, Haryana State, India. All birds used in Phase I to VI were adults and sub-adults. Ethical issues relating to the experimental protocols were considered and approved by the Animal Use and Care Committee and the Research Committee of the Faculty of Veterinary Science of the University of Pretoria, the Research Council of the Indian Veterinary Research Institute and the Board of the Bombay Natural History Society.

Housing and Management: Birds used for Phases I-III were transported from De Wildt to the University of Pretoria Biomedical Research Centre (UPBRC) seven days prior to the start of Phases I through III. At UPBRC vultures were housed individually in primate cages (1.2 x 0.87 x 0.78 m) in an environmentally controlled room in which the room temperature (19-22 °C) and light cycle were kept constant and humidity was allowed to vary with that outside (between 19% and 50% humidity). Vultures used for Phase IV.1 and Phase V were kept at De Wildt, either within their normal holding aviaries (IV.1), or within smaller isolation cages (V). Birds captured in Namibia (Phase IV.2) were kept in the walk-in-trap (11 x 5.5 x 5.5 m) [37], which doubled as a holding aviary for the seven-day trial. Birds in India were captured from their flight aviaries six days before the start of the trials. Five birds with pre-existing healed wing or leg injuries were held in three small aviaries (4 x 3 x 2.5 m), the remaining two groups of five birds were kept in two large holding aviaries (15 x 10 x 5 m). The vultures were not fed for 24 hours prior to treatment with meloxicam and for up to four hours afterwards. Thereafter birds were fed according to

their normal feeding regime (200 g of meat daily at De Wildt and 1.0 kg of meat every third or fourth day at Pinjore), with the exception of the wild birds in Namibia, which were free to feed from the remains of an adult donkey (*Equus asinus*) placed in the aviary. All meat was from known sources, which were selected because we were confident that they did not use any NSAIDs on their livestock.

Treatment and study design for oral gavage experiments: Phases I-III followed a randomised, two-treatment group, parallel study design with 24 non-releasable captive *G. africanus*. In each phase (I to III), vultures were randomly allocated to a meloxicam-treated group (n=5) and a control group (n=3). In Phase IV.1, we treated 14 captive vultures (no controls) and in Phase IV.2 we treated 21 wild vultures and there were four control birds (Table 3-1). The vultures used in Phase IV.1 had also been used in Phases I-III. To minimise the chance of any effect of earlier treatment we ensured that the interval between the end of one treatment and the beginning of the next was at least six weeks. To minimise the risk to captive *G. bengalensis* and *G. indicus* in India, Phase VI of the meloxicam testing was staggered. Two injured non-releasable birds were first treated by gavage with 0.5 mg kg^{-1} and one control bird was sham-dosed with sterilised water. After 48 hours no apparent ill-effects of the treatment were observed, so a further three birds were dosed with 0.5 mg kg^{-1}, two injured non-releasable birds were dosed with 2 mg kg^{-1}, and a further two control birds were sham-dosed. After another 48 hours, three more birds were dosed with 2 mg kg^{-1} along with two final control birds. All birds (with the exception of birds fed muscle and liver tissue in Phase V) were administered meloxicam as a single dose by oral gavage, with the gavage tube flushed with 2ml of water. Control birds were sham-treated by gavage with sterilised water. Birds were observed following dosing for any regurgitation, but none occurred. The meloxicam used came from >20 bottles of the product purchased from several pharmacies in India. Meloxicam used in all phases of the study was "Melonex", manufactured by Intas Pharmaceuticals Ltd, Ahmedebad, India. The stated concentration of meloxicam (500 mg l^{-1}) within two bottles was verified against pure meloxicam sodium salt (M-3935, Sigma-Aldrich, St Louis, MO, USA), through the HPLC analysis method described below and found to be within the accepted 10% limits for pharmaceutical products (450 mg l^{-1} and 460 mg l^{-1}).

Phase V treatment and design: Phase V used a randomised three period, three treatment crossover design with a washout period of two weeks between repeat dosing.

Pharamacokinetic studies indicate that meloxicam is rapidly metabolised in five other bird species (elimination half-life ($t_{½\ el}$) of 0.5 to 2.4 hours [20]) and eliminated within 12 hours in *G. africanus* and the two-week washout period was chosen to ensure that no meloxicam residues were likely to be present on repeat dosing. It was intended that each bird should receive all three treatments in turn with a two-week washout period between treatments. The three treatments were (1) feeding with muscle from a meloxicam-treated cow, (2) feeding with liver from a meloxicam-treated cow and (3) oral gavage with a dose of meloxicam intended to be similar to that taken in treatment (2). In each of the three treatment periods, all three treatments were administered to two birds. Hence, two birds were allotted at random to receive the sequence 1,2,3, two to receive 2,3,1 and two to receive 3,1,2. In each treatment period the muscle and liver was taken from one cow. In practice, an error was made so that two birds received the wrong treatment in the final period and instead received 2,3,2 and 3,1,1. Hence, although all three treatments were each administered on six occasions, and to two birds in each of the three periods, two birds received the same type of treatment in two periods. All six birds had previously been trained to consume food from bowls. On the day of dosing two birds were presented with 1 kg of muscle, two birds with 1kg of liver tissue and two birds were dosed by oral gavage. Any food remaining after 48hours was removed and weighed. Doses of meloxicam per kg vulture bw were estimated from the mass of tissue consumed and the concentration of meloxicam within cattle tissues (see below). In the first part of this experiment, neither of the two birds given liver ate much of it, so all six birds were routinely fed liver (between testing sessions) to habituate them to eating liver in the trials.

Treatment of meloxicam dosed cattle for Phase V: Three *Bos taurus* steers of around 18 months of age and weighing 300-400 kg were housed at the UPBRC. Each animal received an intramuscular injection of meloxicam at a dose of 1 mg kg^{-1} on each of five days prior to slaughter. To avoid unnecessary pain the drug volume injected into any one site never exceeded 20 ml, with all injections placed in the neck on the left and right side on alternating days. This dose is twice the lower of the two standard doses (0.5 and 0.7 mg kg^{-1}) recommended for veterinary medicine in India. It is also higher than the dose (0.7 mg kg^{-1}) administered in the EMEA study [22] that we used to calculate the likely MLE of vultures to meloxicam in the wild (Protocol S1). Cattle were slaughtered at the Veterinary Pathology Department, University of Pretoria, by means of captive bolt to the brain

followed by the transection of the spinal cord at the level of the atlanto-occipital junction, without subsequent exsanguination. Each animal was slaughtered eight hours after the last meloxicam dose and on the day prior to vulture feeding. The entire liver and quadriceps femoris muscle were collected (sufficient to supply liver and muscle for two vultures) and refrigerated until feeding on the following day.

Measuring meloxicam in tissues: Meloxicam concentrations in liver and muscle tissues were measured through standard HPLC methods calibrated against a known standard concentration of the drug. Two 1kg pieces of liver and muscle were cut from each slaughtered animal. Five sub-samples of tissue weighing 3-5 g (four from the surface and one from the centre) were taken from each 1 kg block and homogenised. Meloxicam was extracted from a 0.5 g sample of the homogenised tissue, through homogenisation with 2 ml of HPLC grade acetonitrile, which was then centrifuged at 1200 rpm for 10 minutes and subsequently dried at 60 °C under a flow of nitrogen. This was followed up by a clean up process using Waters Oasis (Milford, Ma) HLB solid phase extraction cartridges [38]. The dried eluate was reconstituted in 50 µl MeOH and 100 µl 0.4% acetylacetate and analysed in duplicate by HPLC. For each homogenised sample, the mean of the four values was used as the final estimate of meloxicam concentrations. Meloxicam sodium salt (M-3935, Sigma-Aldrich, St Louis, MO, USA) was used for calibration, with nine standards ranging from 100 to 50,000 µg l^{-1}. The HPLC apparatus comprised a model 126 dual solvent pump, model 168 diode array detector and a 508 autosampler (Beckman Coulter, Fullerton CA, USA). Chromatographic separation was achieved using a Synergi MAX-RP C18 column (2.1 mm x 150 mm, 5 µm; Phenomex, Torrance CA, USA) with UV detection at 275 nm e.g. Quantification was done with peak areas acquired from UV detection at 275 nm.

Observations on vultures: For all birds and all phases, body mass was measured on the day of treatment (day 0) and at the end of each trial period or when birds were returned to their normal aviaries. For Phase I, II, III and V, birds were weighed 12, 8, 12 and 5 days after treatment respectively. Birds from Phase IV.1, IV.2 and VI were weighed on day seven. Body mass was measured to the nearest 0.5 kg (South Africa and Namibia) and 0.1 kg (India). Observations for signs of toxicity and abnormal feeding behaviour were undertaken daily. In Phases I-III, blood (2.5 ml) was taken at 0 h (prior to dosing) and at 4, 12, 24, 48, 96 and 148 h after meloxicam treatment to quantify serum uric acid and albumin concentrations, and creatinine kinase (CK) and ALT activity. In Phase IV blood

(5ml) was taken just prior to dosing and 48 and 168 hours afterwards to determine serum uric acid concentrations. Blood sampling for Phase V was undertaken 24 hours before feeding or dosing by oral gavage, and at 48 and 96 hours after dosing or the start of feeding.

Blood collection from vultures: In Phase I, blood samples were taken by use of an indwelling catheter, placed under anaesthesia in the jugular vein. This procedure was considered to be unsatisfactory, and rapidly abandoned. Subsequently blood samples in all phases of the study were collected by direct veno-puncture from the brachial or tarsal veins. A total of approximately 15 ml of blood (c. 3% of estimated blood volume) was collected from each vulture over a seven-day period.

Measurement of serum constituents: Blood samples were spun at 1200 rpm for 15 minutes in a refrigerated centrifuge (4°C) to separate serum. Uric acid concentration was measured using ACE TM Uric Acid Reagent, albumin concentration using the NExT TM Albumin reagent, ALT activity using the Alfa Wasserman ALT, and CK using the Alfa Wasserman CK Reagent e ACE TM clinical chemistry system (Alfa Wassermann, Bayer Health). The analyses were performed by means of the ACE TM and NExT TM Clinical Chemistry Systems (Alfa Wassermann, Bayer Health Care, SA).

3.6 Acknowledgements

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Table 3-1: Summary of results and experimental schedule for the testing of the NSAIDs diclofenac and meloxicam on *Gyps bengalensis* and *G. indicus* vultures, and on the non-threatened *G. africanus*. There was no mortality in any of the control birds.

Gyps species	NSAID	Phase	Dose mg kg^{-1}	Route	*N* dosed	*N* died	% Mortality	*N* control	Status and source of birds
G. bengalensis	Diclofenac	-	0.007 to 0.940	Fed treated tissue	20	13	65	-	Captive birds (Pakistan) [1]
G. bengalensis	Diclofenac	-	0.25 and 2.5	Gavage	4	3	75	2	Captive birds (Pakistan) [1]
G. africanus	Diclofenac	-	0.8	Gavage	2	2	100	2	Captive birds (South Africa) [2]
G. africanus	Meloxicam	I	0.5	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	II	1.0	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	III	2.0	Gavage	5	0	0	3	Captive birds (South Africa)
G. africanus	Meloxicam	IV.1	2.0	Gavage	14 [3]	0	0	-	Captive birds (South Africa)
G. africanus	Meloxicam	IV.2	2.0	Gavage	21	0	0	4	Wild-caught birds (Namibia)
G. africanus	Meloxicam	V	0.03 to 1.98	Fed treated tissue	6 [4]	0	0	-	Captive birds (South Africa)
G. africanus	Meloxicam	V	1.18 to 2.45	Gavage	6 [4]	0	0	-	Captive birds (South Africa)
G. bengalensis	Meloxicam	VI	0.5	Gavage	3	0	0	1	Captive birds (India)
G. bengalensis	Meloxicam	VI	2.0	Gavage	3	0	0	1	Captive birds (India)
G. indicus	Meloxicam	VI	0.5	Gavage	2	0	0	2	Captive birds (India)
G. indicus	Meloxicam	VI	2.0	Gavage	2	0	0	1	Captive birds (India)

[1] Experimental results from reference [1]

[2] Experimental results from reference [7]

[3] Experimental and control birds from phases I to III (including 3 control birds not previously dosed with meloxicam)

[4] 5 of the 6 birds were experimental birds from Phase III and IV.1. The same birds were used for feeding tissue and oral gavage, with a two week washout period between treatments (see Materials and Methods)

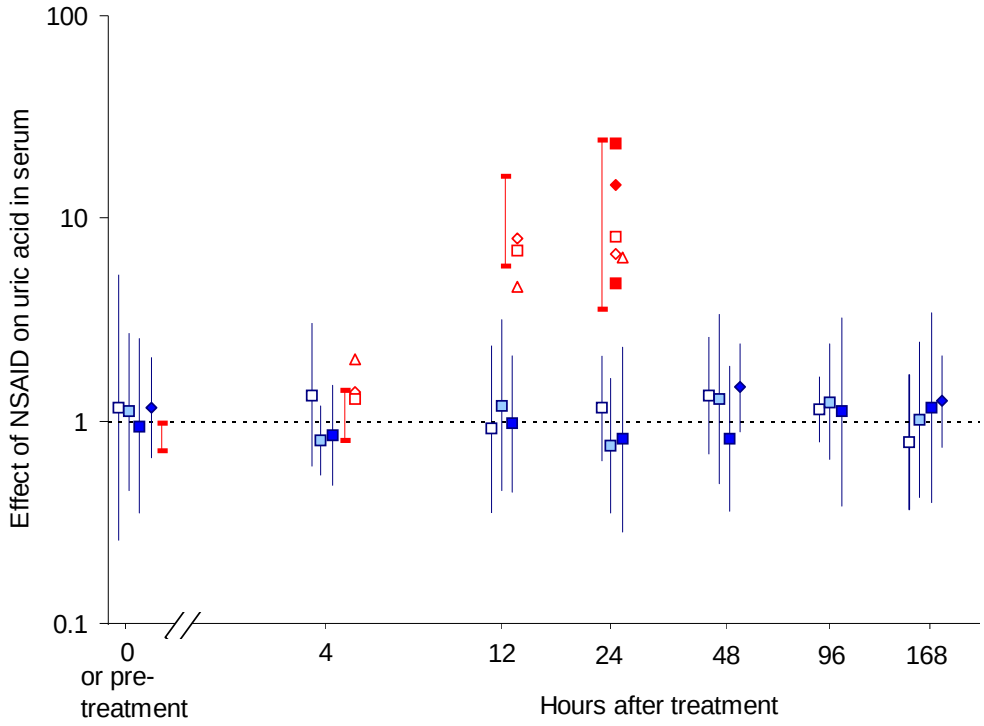

Figure 3-1: Effect of Administration of Meloxicam and Diclofenac by Gavage on Uric Acid in the Serum of Vultures

Blue symbols show the ratio of the geometric mean plasma concentration of uric acid for a group of *Gyps africanus* treated with meloxicam by gavage to that for a control group treated with water and sampled at the same time. Vertical lines show 95% confidence limits for the ratio. The dashed horizontal line indicates a ratio of 1; i.e. no effect of treatment. At each of six times of sampling after treatment, results are shown for experiments in which different doses of drug were used. The fill colour of the blue symbols indicates the meloxicam dose for the treated group: white = 0.5 mg kg^{-1} (Phase I); light blue = 1.0 mg kg^{-1} (Phase II); dark blue = 2.0 mg kg^{-1} (squares = Phase III, diamonds = Phase IV-2). Red vertical bars show the maximum and minimum values of the equivalent ratio for two groups of *G. africanus*, one group treated with 0.8 mg kg^{-1} of diclofenac by gavage and another group treated with water and sampled at the same time. Open red symbols show the ratio of the plasma concentration after treatment to that at the time of treatment for three individual *G. fulvus* given 0.8 mg kg^{-1} of diclofenac by gavage. Filled red symbols show the ratio of the plasma concentration 24 hours post-treatment to that 1 hour post-treatment for three individual *G. bengalensis* given 0.25 mg kg^{-1} (squares) and 2.5 mg kg^{-1} (diamond) of diclofenac by gavage. Data from diclofenac experiments were taken from references 1 and 7.

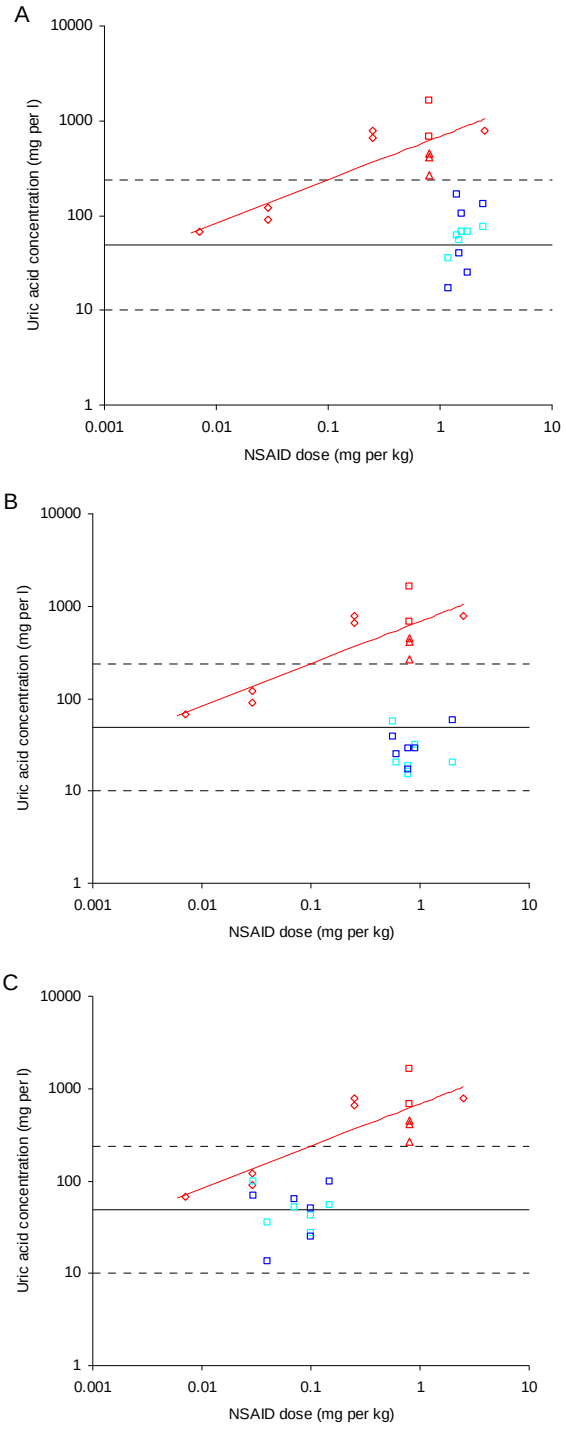

Figure 3-2: Relationship between Uric Acid in Serum the Dose of Meloxicam and Diclofenac Administered and Administration Method

Serum concentration of uric acid in *Gyps africanus* 48 hours (turquoise) and 96 hours (blue) after treatment, in relation to the dose of meloxicam administered per kg of vulture body weight. For comparison, the geometric mean uric acid level (horizontal solid line) and 95% range (horizontal dashed lines) of the experimental birds 24 hours before treatment are shown. Also shown are serum concentrations of uric acid 24 hours after treatment in *G. africanus* (red squares), *G. bengalensis* (red diamonds) and *G. fulvus* (red triangles) to which diclofenac was administered by various methods. The red line shows the regression model fitted to these data. Panels show results for different methods of administration of meloxicam to *G. africanus*: (A) gavage, (B) by feeding liver from meloxicam-treated cattle, (C) by feeding muscle from meloxicam-treated cattle. Data from diclofenac experiments were taken from references 1 and 7.

3.8 Electronic Addendum

3.8.1 *Protocol S2*

Analysis of Phase V data

In addition to the regression analyses reported in the main text, we also examined the Phase V data for subtle effects of feeding *G. africanus* with liver or muscle from meloxicam-treated cattle and dosing with meloxicam by gavage. We carried out analyses of covariance with the log of serum uric acid concentration as the dependent variable. We fitted regression models by ordinary least squares. Independent variables included in the full model were bird identity (BIRD a 6-level factor: 6 birds), stage of the experimental sequence (SEQ a 3-level factor: first, second or third set of trials), time of blood sampling (T a 2-level factor: 48 h or 96 h after treatment began), route by which meloxicam was administered (TREAT a 3-level factor: by feeding liver, by feeding muscle, by gavage), log dose of meloxicam administered (LDOSE a continuous variable) and the log uric acid concentration in the serum each bird before meloxicam was administered in a given trial (LURP a continuous variable). We tested the effect of meloxicam treatment by comparing the following models: (A) BIRD + SEQ + T + LURP + TREAT + LDOSE + TREAT.LDOSE and (B) BIRD + SEQ + T + LURP. This comparison indicated that there was no significant effect of the route of meloxicam dosing and meloxicam dose on serum uric acid level ($F_{5,21} = 0.58$, $p > 0.50$). This was also the case when the equivalent analysis was performed separately for data collected 48 h after dosing and 96 h after dosing and when the tissue feeding and gavage routes of administration were subject to two separate analyses. We also fitted models to the full dataset with all possible combinations of the variables listed above, including the TREAT.LDOSE interaction. We then used the results to select the Minimal Adequate Model (MAM; Crawley 1993). The MAM was BIRD + LURP. In this model, both of these two variables had highly significant effects (BIRD: $F_{5,29} = 5.34$, $p < 0.005$; LURP: $F_{1,29} = 17.26$, $p < 0.001$), but no other variable or combination of variables had a significant further effect ($p > 0.10$) when added to the MAM.

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Table 3-2: Blood serum constituents summary statistics

Results of analyses of measurements of uric acid and albumin concentrations, and alanine transferase (ALT) and creatinine kinase (CK) activity in blood serum before dosing and at 0, 4, 12, 23, 48, 96 and 168 hours after dosing for birds from Phase I (meloxicam at 0.5 mg kg^{-1}), Phase II (1.0 mg kg^{-1}) and Phase III and Phase IV (2.0 mg kg^{-1}). Blood was only collected at 0, 48 and 168 hours in Phase IV. The geometric mean of meloxicam-dosed and control (sham-treated) birds is presented along with the sample size and *P* values from two-tailed t-tests between meloxicam-dosed and control groups.

		Uric acid mg l^{-1}			Albumin mg l^{-1}			ALT U l^{-1} (37° C)			CK U l^{-1} (37° C)		
Phase	Time h	Control Mean (n)	Dosed Mean (n)	*p*	Control Mean (n)	Dosed Mean (n)	*P*	Control Mean (n)	Dosed Mean (n)	*p*	Control Mean (n)	Dosed Mean (n)	*p*
Phase I dose 0.5 mg kg^{-1}	0	34.5 (3)	40.2 (5)	> 0.80	11.3 (3)	13.1 (5)	> 0.10	21.0 (3)	33.1 (5)	> 0.10	432.2 (3)	235.1 (5)	> 0.05
	4	23.9 (3)	32.1 (5)	> 0.40	11.3 (3)	13.8 (5)	> 0.20	45.7 (3)	38.4 (5)	> 0.70	591.1 (3)	459.7 (5)	> 0.30
	12	116.9 (3)	106.5 (5)	> 0.80	11.7 (3)	13.3 (5)	> 0.30	31.9 (3)	31.0 (5)	> 0.90	506.7 (3)	472.6 (5)	> 0.80
	24	44.7 (3)	51.7 (5)	> 0.50	12.2 (3)	14.2 (5)	> 0.20	43.0 (3)	40.0 (5)	> 0.80	440.1 (3)	443.5 (5)	> 0.95
	48	48.7 (2)	64.8 (4)	> 0.30	13.1 (2)	15.2 (4)	> 0.20	69.8 (2)	39.5 (4)	> 0.10	386.4 (2)	347.1 (4)	> 0.80
	96	55.6 (3)	63.2 (5)	> 0.40	11.8 (3)	13.9 (5)	> 0.05	51.3 (3)	43.3 (5)	> 0.05	461.7 (3)	603.2 (5)	> 0.30
	168	99.8 (3)	79.1 (5)	> 0.40	12.5 (3)	14.0 (5)	> 0.10	51.3 (3)	46.0 (5)	> 0.50	570.6 (3)	539.0 (5)	> 0.80
Phase II dose 1.0 mg kg^{-1}	0	72.0 (3)	80.2 (5)	> 0.70	11.1 (3)	11.5 (5)	> 0.50	32.5 (3)	19.0 (5)	> 0.20	178.0 (3)	223.9 (5)	> 0.20
	4	39.8 (3)	32.0 (5)	> 0.20	12.1 (3)	11.3 (5)	> 0.40	28.5 (3)	23.1 (5)	> 0.50	442.2 (3)	603.2 (5)	> 0.50
	12	125.8 (3)	150.4 (5)	> 0.60	17.7 (3)	11.1 (5)	> 0.20	72.4 (3)	37.5 (5)	> 0.30	842.4 (3)	397.2 (5)	> 0.40
	24	73.9 (3)	55.6 (5)	> 0.30	10.1 (3)	10.9 (5)	> 0.20	33.8 (3)	44.4 (5)	> 0.20	327.8 (3)	492.8 (5)	> 0.30
	48	73.0 (3)	93.5 (5)	> 0.50	10.4 (3)	11.2 (5)	> 0.10	43.0 (3)	44.9 (5)	> 0.80	278.7 (3)	405.2 (5)	> 0.30
	96	124.6 (3)	154.5 (5)	> 0.40	11.8 (3)	12.9 (5)	> 0.20	39.8 (3)	42.5 (5)	> 0.70	316.4 (3)	234.6 (5)	> 0.30
	168	114.5 (3)	116.3 (5)	> 0.95	10.6 (3)	11.6 (5)	> 0.05	54.9 (3)	50.8 (5)	> 0.60	327.1 (3)	327.2 (5)	> 0.95
Phase III dose 2.0 mg kg^{-1}	0	60.1 (3)	56.8 (5)	> 0.80	12.6 (3)	11.6 (5)	> 0.05	76.6 (3)	74.0 (5)	> 0.70	243.0 (3)	318.1 (5)	> 0.40
	4	21.2 (3)	18.1 (5)	> 0.50	11.8 (3)	10.6 (5)	> 0.10	37.2 (3)	46.5 (5)	> 0.50	431.4 (3)	459.3 (5)	> 0.70
	12	96.3 (3)	93.1 (5)	> 0.90	11.0 (3)	10.0 (5)	> 0.10	36.6 (3)	40.7 (5)	> 0.70	328.3 (3)	506.9 (5)	> 0.05
	24	63.8 (3)	51.8 (5)	> 0.60	10.8 (3)	10.1 (5)	> 0.40	40.6 (3)	46.8 (5)	> 0.40	278.7 (3)	438.9 (5)	> 0.10
	48	65.4 (3)	53.2 (5)	> 0.50	10.5 (3)	9.7 (5)	> 0.30	47.9 (3)	40.4 (5)	> 0.60	220.4 (3)	357.6 (5)	> 0.10
	96	76.5 (3)	84.7 (5)	> 0.80	11.5 (3)	10.2 (5)	> 0.10	55.2 (3)	52.7 (5)	> 0.90	236.1 (3)	429.3 (5)	> 0.20
	168	73.3 (3)	84.8 (5)	> 0.70	12.8 (3)	11.7 (5)	> 0.20	44.3 (3)	54.2 (5)	> 0.60	531.7 (3)	647.2 (5)	> 0.60
Phase IV dose 2.0 mg kg^{-1}	0	95.4 (4)	111.7 (21)	> 0.50	-	-	-	-	-	-	-	-	-
	48	70.5 (4)	102.9 (21)	> 0.10	-	-	-	-	-	-	-	-	-
	168	100.2 (4)	125.4 (21)	> 0.30	-	-	-	-	-	-	-	-	-