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3 **1 Role of eprinomectin as inhibitor of the ruminant ABCG2 transporter: Effects on**
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5 **2 plasma distribution of danofloxacin and meloxicam in sheep**
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65 18 **Abstract**
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68 19 Therapeutic outcome results of the coadministration of several drugs in veterinary
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70 20 medicine is affected by, among others, the relationship between drugs and ATP-binding
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72 21 cassette (ABC) transporters, such as ABCG2. ABCG2 is an efflux protein involved in
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74 22 the bioavailability and milk secretion of drugs. The aim of this work was to determine
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76 23 the role of eprinomectin, a macrocyclic lactone (ML) member of avermectin class, as
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78 24 inhibitor of ABCG2. The experiments were carried out through in vitro inhibition
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80 25 assays based on mitoxantrone accumulation and transport assays in ovine ABCG2
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82 26 transduced cells using the antimicrobial drug danofloxacin and the anti-inflammatory
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84 27 drug meloxicam, both widely used in veterinary medicine and well known ABCG2
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86 28 substrates.

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89 29 The inhibition results obtained showed that eprinomectin was an efficient in vitro
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91 30 ABCG2 inhibitor, tested in mitoxantrone accumulation assays. In addition, this ML
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93 31 decreased ovine ABCG2-mediated transport of danofloxacin and meloxicam. To
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95 32 evaluate the role of eprinomectin in systemic exposure of drugs, pharmacokinetic assays
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97 33 based on subcutaneous coadministration of eprinomectin with danofloxacin (1.25
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99 34 mg/kg) or meloxicam (0.5 mg/kg) in sheep were performed obtaining a significant
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101 35 increase of systemic exposure of these drugs. Especially relevant was the increase of the
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103 36 systemic concentration of meloxicam, since coadministration with eprinomectin
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105 37 increased significantly the plasma concentration of meloxicam, obtaining an increase of
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107 38 AUC (0-72 h) value of more than 40%.

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110 39 **Keywords**
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113 40 ABCG2; danofloxacin; eprinomectin; macrocyclic lactone; meloxicam; sheep.
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1. Introduction

Anthelmintic macrocyclic lactones (MLs) are antiparasitic drugs with notable broad-spectrum activity widely used for treatment of both internal and external parasites in animals and humans. Due to their lipophilicity, they are distributed throughout the body in the blood and lymph circulations, which produces long persistence in the host organism and consequently a long period of protection against parasite infection. Of special relevance is the semisynthetic ML eprinomectin, which is a drug derived from the natural product avermectin B1 (abamectin), whose efficacy in goats and sheep is very high and, additionally, it is not transported into milk (Rostang et al., 2020).

Among other factors, the interactions between MLs and ATP-binding cassette (ABC) transporters control the systemic exposition of these drugs (Lespine et al., 2012; Virkel et al., 2018). MLs, including eprinomectin, strongly interact with P-glycoprotein (P-gp), the multidrug resistance 1 (MDR1) transporter (Lespine et al., 2012) which has been clearly identified as the main factor that controls the body concentration of MLs, such as ivermectin (Lespine et al., 2012; Merola and Eubig, 2018). Furthermore, milbemycins (such as moxidectin) and avermectins (such as ivermectin and doramectin) interact with the ABC transporter ABCG2 (Mealey, 2012; Real et al., 2011) which affects in vivo absorption, distribution and elimination of MLs. The coadministration of different drugs may promote alteration in disposition mediated by ABC transporters (Ballent et al., 2012; Mahnke et al., 2016; Virkel et al., 2018). ABCG2 expression in mammary gland is induced during lactation, and so plays an important role in the active secretion of many drugs into the milk of ruminants (García-Lino et al., 2019). The combination of drugs that interact with the ABCG2 transporter, including MLs, may affect their systemic exposure and their secretion into milk (Ballent et al., 2012).

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179
180 66 The aim of the present study, therefore, was to characterize eprinomectin as an inhibitor
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182 67 of the ABCG2 transporter using two different ABCG2 substrates, the antimicrobial
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184 68 drug, danofloxacin (Real et al., 2011) and an anti-inflammatory drug, meloxicam
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186 69 (Garcia-Lino et al., 2020). The assays were performed in vitro using ABCG2 transduced
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188 70 ovine cells in a transepithelial transport and mitoxantrone accumulation assay, and in
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190 71 vivo using lactating Assaf sheep in a pharmacokinetics assay.

192 72 **2. Materials and methods**

193 73 *2.1. Reagents and drugs*

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198 74 Mitoxantrone (MXR), eprinomectin, danofloxacin, meloxicam and difloxacin were
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200 75 purchased from Sigma-aldrich (St. Louis, MO). Isoflurane Isovet® was purchased from
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202 76 Braun (Barcelona, Spain). For the pharmacokinetic studies, eprinomectin solution
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204 77 (Eprecis®) was purchased from Ceva Salud Animal (Barcelona, Spain), danofloxacin
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206 78 solution (Advocin® 2.5 %) was purchased from Zoetis (Madrid, Spain) and meloxicam
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208 79 solution (Metacam® 20 mg/ml) was purchased from Boehringer Ingelheim.

209 80 *2.2. Cell cultures*

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214 81 Madin-Darby Canine Kidney (MDCKII) cells were kindly provided by A. H. Schinkel
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216 82 (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Stably-transduced
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218 83 MDCKII cells with ovine variant of ABCG2 were generated and characterized by our
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220 84 research group in previous studies (González-Lobato et al., 2014). Culture conditions
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222 85 were as previously described (González-Lobato et al., 2014).

223 86 *2.3. Accumulation assay*

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228 87 In vitro accumulation assays were carried out as previously described (Pavek et al.,
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230 88 2005) using MXR as a fluorescent substrate.

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239 89 Relative cellular accumulation of MXR of at least 5000 cells was determined by flow
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241 90 cytometry using a CyAn cytometer (Beckam Coulter, Fullerton, CA). Flow cytometry
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243 91 data were processed and analyzed using SUMMIT version 4.3 software (Innovation
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245 92 Drive, For Collins, CO). ABCG2 inhibition increases accumulation of MXR and thus
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247 93 increases MF. Inhibitory potencies of eprinomectin were calculated as previously
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249 94 described (Pavek et al., 2005) in MDKCII and oABCG2 cells according to the
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251 95 following equation: inhibitory potency = (MF with eprinomectin – MF without
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253 96 eprinomectin) / (MF with Ko143 – MF without eprinomectin).

254 255 256 257 97 *2.4. Transport assays*

258
259 98 Transepithelial transport assays using Transwell plates were carried out as described
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261 99 elsewhere (Perez et al., 2013) with minor modifications. The effect of eprinomectin as
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263 100 inhibitor in transport assay was tested using two ABCG2 substrates at concentrations
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265 101 used in previous in vitro studies, danofloxacin (10 μ M) (Real et al., 2011) and
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267 102 meloxicam (30 μ M) (Garcia-Lino et al., 2020). Parental MDCKII and ABCG2 ovine
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269 103 transduced subclones cells were grown for 3 days after seeding on microporous
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271 104 polycarbonate membrane filters at a density of 1.0×10^6 cells per well. To check the
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273 105 tightness of the monolayer, transepithelial resistance was measured in each well using a
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275 106 Millicell ERS ohmmeter (Millipore). Two hours before the start of the experiment,
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277 107 medium at both the apical and basolateral sides of the monolayer was replaced with 2
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279 108 ml of OptiMEM medium, and either with or without the eprinomectin as inhibitor. The
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281 109 experiment was started ($t=0$) by replacing the medium in either the apical or basolateral
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283 110 compartment with fresh OptiMEM medium, either with or without eprinomectin and
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285 111 containing meloxicam or danofloxacin. Cells were incubated at 37 °C in 5% CO₂ and
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287 112 100 μ L aliquots of culture media were taken at 2 h and 4 h in the opposite compartment
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289 113 and this volume was replaced with fresh medium. The presence of danofloxacin or
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298 114 meloxicam in the opposite compartment was presented as the fraction of total substrate
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300 115 added at the beginning of the experiment. Active transport across MDCKII monolayers
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302 116 was expressed by the relative transport ratio (R), defined as the percentage apically
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304 117 directed transport percentage divided by the percentage basolaterally directed
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306 118 translocation percentage, after 4 h.
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309 119 *2.5. Animals*

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312 120 Animals were housed on the Experimental Farm of the University of León, Spain, and
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314 121 handled according to institutional guidelines complying with European legislation.
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316 122 (2010/63/EU). Experimental procedures were approved by the Animal Care and Use
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318 123 Committee of the University of León and the Junta de Castilla y León ULE_011_2016
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320 124 and ULE_008_2016.
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323 125 *2.6. Pharmacokinetic studies*

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326 126 Twenty-four lactating Assaf sheep (3–4 months in lactation) and weighing 70 to 85 kg
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328 127 were used. The animals were parasite-free and drinking water was available ad libitum.
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330 128 The experimental design was performed with animals divided into four groups: (1) the
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332 129 first group (n=6) received a single SC injection at a therapeutic dose of (1.25 mg/kg)
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334 130 Advocin® (2.5%); (2) the second group (n=6) was injected SC with 1.25 mg/kg of
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336 131 Advocin® (2.5%) and co-administrated with a single SC dose of eprinomectin
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338 132 (Eprecis®) at 0.5 mg/kg based on previous studies (Rostang et al., 2020); (3) the third
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340 133 group (n=6) received a single SC injection at therapeutic dose (0.5 mg/kg meloxicam)
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342 134 of Metacam® (20 mg/mL); (4) and the fourth group (n=6) was injected SC with 0.5
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344 135 mg/kg of Metacam® 20 mg/kg and co-administrated with a single SC dose of
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346 136 eprinomectin (Eprecis®) at 1 mg/kg based on previous studies (Lifschitz et al., 2008;
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348 137 Rehbein et al., 2014).
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357 138 Blood samples were collected from the jugular vein and milk samples were collected
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359 139 after complete milking of the gland before each treatment at 0.25, 1, 2, 3.75, 6.25, 8,
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361 140 9.5, 12, 24, 32 and 48 h after danofloxacin administration and at 0.5, 1, 2, 4, 6, 8, 10,
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363 141 12, 24, 36, 48 and 72 h after meloxicam administration. Plasma was separated by
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365 142 centrifugation at 3000 x g for 15 min. Plasma and milk samples were stored at -20 °C
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367 143 until HPLC analysis.

370 144 *2.7. High Performance Liquid Chromatography*

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373 145 The chromatographic system used in samples analysis consisted of a Waters 2695
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375 146 separation module and a Waters 2998 UV photodiode array detector.

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378 147 Conditions for HPLC analysis of danofloxacin were modified in accordance with Perez
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380 148 et al. (2011). Standard samples in the appropriate drug-free matrix were prepared
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382 149 yielding a concentration range from 0.019 to 5 µg/ml, with coefficients of correlation >
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384 150 0.99. The Limits of Quantification (LOQs) were 0.039 µg/ml for transport samples,
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386 151 0.006 for plasma samples and 0.1 µg/ml for milk samples. The Limits of Detection
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388 152 (LODs) were 0.012 µg/ml for transport samples, 0.002 µg/ml for plasma samples and
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390 153 0.042 µg/ml for milk samples. The extraction recovery levels for concentration in the
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392 154 standard curve were 82% for plasma and 85% for milk samples.

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395 155 Conditions for HPLC analysis of meloxicam have been described previously (Garcia-
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397 156 Lino et al., 2020). Standard samples in the appropriate drug-free matrix were prepared
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399 157 yielding a concentration range from 0.019 to 15 µg/ml, with coefficients of correlation
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401 158 > 0.99. The LOQ was 0.01 µg/ml and the LOD was 0.005 µg/ml for transport samples;
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403 159 the LOQ was 0.02 µg/ml and the limit of LOD was 0.008 µg/ml for plasma samples;
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405 160 LOQ 0.02 µg/ml and LOD 0.007 µg/ml for milk samples. The extraction recovery
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407 161 levels for concentration in the standard curve were 88% for plasma and 90% for milk
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409 162 samples.

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416 163 2.8. *Pharmacokinetic calculations*
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419 164 The peak concentration (C_{\max}) and time-peak concentration (T_{\max}) were read from the
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421 165 plotted concentration-time curve for each animal. The area under concentration-time
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423 166 curves (AUC) from time zero to time of last sampling and to infinity were calculated
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425 167 using the trapezoidal method. Mean residence time (MRT) was calculated by the linear
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427 168 trapezoidal rule without extrapolation to infinity, using the formula: $MRT =$
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429 169 $AUMC/AUC$, where AUMC was the area under the momentum curve. These
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431 170 calculations were made using the PK Solutions computer program (Farrier, 1997) and
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433 171 determined by non-compartmental analyses.
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437 172 2.9. *Statistical analysis*
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439 173 Comparisons between groups were performed by the Student's t-test (normal variables)
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441 174 and the Mann-Whitney U test (not normally distributed variables). All analyses were
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443 175 carried out on the assumed significance level of $p \leq 0.05$ using SPSS Statistics software
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445 176 (v. 24.0; IBM, Armonk, New York, NY, USA). The results are shown as mean \pm
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447 177 standard deviation (SD).
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450 178 **3. Results**

451 179 3.1. *Inhibitory potency of eprinomectin in mitoxantrone accumulation assays*
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455 180 To demonstrate the potential inhibitory effect of eprinomectin in ovine ABCG2, the
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457 181 ability of these compound to reverse the reduced MXR accumulation in cells transduced
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459 182 with ovine variant of ABCG2 was tested in flow cytometry experiments. As expected,
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461 183 MTX accumulation was significantly lower in the ovine variant of ABCG2 transduced
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463 184 cells compared to the parental cells because ABCG2 is actively transported this
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465 185 substrate outside the cells (Fig. 1). When cells were treated with Ko143, accumulation
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467 186 of MTX in the ovine variant of ABCG2 transduced cells increased by ABCG2
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475 187 inhibition with Ko143 and thus increased the fluorescence to levels similar to those in
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477 188 the parental cells (Fig. 1). No significant differences between parental cells treated and
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480 189 non-treated with Ko143 inhibitor were observed.

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482 190 The presence of eprinomectin inhibited ovine ABCG2, thus increasing the accumulation
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484 191 of MXR in ABCG2 transduced cell in a concentration dependent manner (Fig. 1). The
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486 192 highest inhibitory potency (63%) appeared at 10 μ M for ovine ABCG2. Therefore,
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488 193 these results demonstrated that eprinomectin plays an important role as inhibitor of
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491 194 ovine ABCG2.

492 493 195 *3.2. Inhibition of in vitro transport of danofloxacin and meloxicam by eprinomectin*

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496 196 To further characterise the inhibitory properties of the eprinomectin in ovine ABCG2,
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498 197 transepithelial transport assays were carried out with MDCKII cells transduced with the
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500 198 ovine variant of ABCG2 using a model substrate of ABCG2, danofloxacin (10 μ M), and
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502 199 a new described ABCG2 substrate, meloxicam (30 μ M) (Table 1 and 2, respectively).

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504
505 200 The translocation in MDCKII parental cells treated with danofloxacin at 10 μ M in the
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507 201 apical and basolateral directions was similar, with a similar relative basal-apical:apical-
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509 202 basal transport ratio (Ratio BL-AP/ AP-BL close to 1, according with Real et al. (2011)).

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511 203 In the ovine variant of ABCG2 transduced cells, translocation in the apical direction
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513 204 was drastically decreased and translocation in the basolateral direction was increased,
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515 205 resulting in a transport ratio BL-AP/ AP-BL of higher than 12 at 4 h (Table 1). A
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517 206 significant decrease in this relative transport ratio of danofloxacin, more than 80%, was
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519 207 observed in the ovine ABCG2-transduced cells when eprinomectin was added at a
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521 208 concentration of 5 μ M (12.44 ± 6.89 vs $2.29 \pm 0.25^*$). Ovine ABCG2 mediated
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523 209 transport was almost completely reverted at a concentration of 10 μ M of eprinomectin
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525 210 with a relative transport ratio equal to that of the parental cells (1.08 ± 0.05 vs $0.86 \pm$
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534 211 0.05). These differences were not observed in the parental MDCKII cells, thus
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536 212 indicating that the observed effect is ABCG2 specific.
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539 213 Similar results were obtained in the transepithelial transport of meloxicam at 30 μ M
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541 214 (Table 2). Relative efflux transport ratio at 4 h was significantly higher in the ovine
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543 215 ABCG2-transduced cells compared to the parental cells ($24.85 \pm 4.6^*$ vs 1.06 ± 0.08).
544
545 216 These results show that meloxicam is an in vitro substrate of the ovine ABCG2 variant.
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547 217 When eprinomectin was added, the apical to basal transport in cells transduced with
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549 218 ovine ABCG2 decreased compared to the cells without eprinomectin, presenting a
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551 219 reduction of 32% in the transport ratio of meloxicam in treatment with eprinomectin at
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553 220 5 μ M and of 78% at 10 μ M. These differences were not observed in parental MDCKII
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555 221 cells.
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558 222 These results clearly show that eprinomectin is a good in vitro inhibitor of ovine
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560 223 ABCG2.

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564 224 *3.3. Effect of eprinomectin on plasma pharmacokinetics and milk secretion of the*
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566 225 *antimicrobial danofloxacin and the anti-inflammatory drug meloxicam*
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569 226 To further demonstrate the in vivo ABCG2 inhibitory role of eprinomectin in clinically
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571 227 relevant drug-drug interactions, the effect of the co-administration of eprinomectin with
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573 228 danofloxacin and with meloxicam was studied in pharmacokinetic and milk secretion
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575 229 assays. A higher significant plasma concentration of both drugs, danofloxacin and
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577 230 meloxicam, was found in groups of animals coadministered with eprinomectin at
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579 231 several times: at 4 h after danofloxacin co-administration (Fig. 2) and at 6, 8, 10, 12, 24
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581 232 and 30 h after meloxicam administration (Fig. 3). In addition, differences in plasma
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583 233 pharmacokinetic parameters were observed (Table 3 and Table 4). The value of AUC
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585 234 (0-48 h) increased significantly and was almost 1.3-fold higher in animals
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593 235 coadministered with eprinomectin/danofloxacin compared with control animals. In
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595 236 sheep co-treated with meloxicam, plasma C_{max} and T_{max} were significantly higher for
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597 237 eprinomectin-treated animals compared to control animals. In addition, AUC (0-72 h)
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599 238 was almost 40% higher in animals co-administered with eprinomectin compared with
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601 239 the control group. No differences in milk concentration or pharmacokinetic parameters
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603 240 of danofloxacin and meloxicam were found (Tables 2 and 3; Figs. 2 and 3).
604
605 241 These results clearly show that the coadministration of eprinomectin influences the
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607 242 systemic distribution of danofloxacin and meloxicam without variation in milk drug
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609 243 concentration.
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615 245 **4. Discussion**

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618 246 Interaction with ABC transporters is recognized as a mechanism responsible for
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620 247 pharmacologically relevant in vivo drug-drug interactions. An unintentional result may
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622 248 be therapeutic failure or toxicity. However, a positive outcome is also possible and one
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624 249 drug may increase the systemic exposure of another one. Therefore, drug-drug
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626 250 interaction may occur after drug coadministration (Virkel et al., 2018). Interaction
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628 251 between commonly used drugs in veterinary medicine, such as fluoroquinolones
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630 252 antibacterial drugs, tyrosine kinase inhibitors and some anthelmintic benzimidazoles,
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632 253 with ABC transporters, including ABCG2, has been studied in depth (Barrera et al.,
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634 254 2013; Mealey, 2012; Virkel et al., 2018). In this study, eprinomectin is described for the
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636 255 first time as an efficient in vitro and in vivo inhibitor of ABCG2 transporter; drug-drug
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638 256 interaction mediated by ABCG2 is reported with the coadministration of this
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640 257 macrocyclic lactone and the ABCG2 substrates danofloxacin and meloxicam.
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643 258 In the MXT accumulation assays, inhibitory potency higher than 50% (IC_{50}) was
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645 259 reported at 10 μ M in ovine ABCG2, which confirms eprinomectin as a good inhibitor
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652 260 for ovine ABCG2 (Weiss et al., 2007). Other macrocyclic lactones have been described
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654 261 as in vitro ABCG2 inhibitors (Lespine et al., 2012). This role as inhibitor of
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656 262 eprinomectin was corroborated using transport assays testing interaction with
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658 263 danofloxacin, a model substrate of ABCG2, and meloxicam a novel substrate of
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660 264 ABCG2 (Garcia-Lino et al., 2020). In both cases, a reduction in the relative transport
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662 265 ratio was observed in ovine ABCG2-transduced cells versus cells in the presence of
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664 266 eprinomectin (Table 1 and 2). Eprinomectin has been previously described as a strong
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666 267 inhibitor of P-gp transporter (Lespine et al., 2012). Nevertheless, this is the first time
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668 268 that eprinomectin has been described as an in vitro ABCG2 inhibitor. Inhibition of in
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670 269 vitro transport of danofloxacin by macrocyclic lactones has been described previously
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672 270 with studies using ivermectin at 50 μ M in human ABCG2- and murine Abcg2-
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674 271 transduced cells (Real et al., 2011). However, our results show that eprinomectin
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676 272 completely inhibits transport of danofloxacin mediated by ABCG2 in ovine ABCG2-
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678 273 trasduced cells at a 5-fold lower concentration (10 μ M).
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681 274 According to our positive results obtained in the in vitro assays, the extent of in vivo
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683 275 ABCG2-mediated drug-drug interaction involving eprinomectin and veterinary ABCG2
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685 276 substrates has been determined in sheep (Figs. 2 and 3). Interaction between ABCG2
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687 277 and eprinomectin was confirmed in an in vivo setting when eprinomectin was
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689 278 coadministered to sheep together with the antimicrobial danofloxacin (Fig. 2) and with
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691 279 the anti-inflammatory drug meloxicam. The pharmacokinetics of danofloxacin and
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693 280 meloxicam reported in this study were similar to those reported previously (Real et al.,
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695 281 2011; Woodland et al., 2019). Our results show that plasma availability, and
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697 282 consequently the therapeutic potential, of danofloxacin and meloxicam increases with
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699 283 the co-administration with eprinomectin (Figs. 2 and 3). An increase in plasma
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701 284 concentration of danofloxacin was previously observed with a combined administration
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711 285 with ivermectin in sheep, causing an increase in plasma AUC values and half-life of this
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713 286 drug (Ballent et al., 2012). However, a decreased concentration of danofloxacin in milk
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715 287 was observed with the coadministration of ivermectin, a P-gp and ABCG2 inhibitor
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718 288 (Real et al., 2011). Our results after co-administration of both drugs with eprinomectin
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720 289 show no significant differences in concentration in milk compared with control animals.
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722 290 The low binding with lipoproteins that eprinomectin has compared to other MLs, such
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724 291 as ivermectin, may be among the causes of its low presence in milk (Lespine et al.,
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726 292 2012)and therefore in the mammary gland, probably reducing its ability to interact
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728 293 locally with ABCG2. It should be noted that the increase in plasma concentration of
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730 294 meloxicam produced by the co-administration of eprinomectin could have important
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732 295 therapeutic applications due to the therapeutic potential of meloxicam in small
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734 296 ruminants (Colditz et al., 2019).
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736 297 In conclusion, the role of eprinomectin as an inhibitor of ovine ABCG2, both in vitro
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738 298 and in vivo, has been demonstrated. Co-administration of eprinomectin in sheep results
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740 299 in an increase in plasma concentration of the antimicrobial drug danofloxacin and the
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742 300 anti-inflammatory drug meloxicam, producing a synergic effect of these drugs. These
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744 301 new findings establish that eprinomectin, an effective antiparasitic, with no withdrawal
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746 302 period in milk, affects systemic exposure of other drugs by inhibition of ABCG2
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748 303 transporter.
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Authorship contributions

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Performed data analysis: Merino, Alvarez, Garcia-Lino.

Wrote or contributed to the writing of the manuscript: Alvarez, Merino, Garcia-Lino.

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326 **REFERENCES**

- 327 Ballent, M., Lifschitz, A., Virkel, G., Sallovitz, J., Maté, L., Lanusse, C., 2012. In vivo
328 and ex vivo assessment of the interaction between ivermectin and danofloxacin in
329 sheep. *Vet. J.* 192, 422–7. <https://doi.org/10.1016/j.tvjl.2011.09.006>
- 330 Barrera, B., González-Lobato, L., Otero, J.A., Real, R., Prieto, J.G., Álvarez, A.I.,
331 Merino, G., 2013. Effects of triclabendazole on secretion of danofloxacin and
332 moxidectin into the milk of sheep: role of triclabendazole metabolites as inhibitors
333 of the ruminant ABCG2 transporter. *Vet. J.* 198, 429–36.
334 <https://doi.org/10.1016/j.tvjl.2013.07.033>
- 335 Colditz, I., Paull, D., Lloyd, J., Johnston, L., Small, A., 2019. Efficacy of meloxicam in
336 a pain model in sheep. *Aust. Vet. J.* 97, 23–32. <https://doi.org/10.1111/avj.12779>
- 337 Farrier, D.S., 1997. PK Solutions - Easy Pharmacokinetics Software for Research and
338 Education at SummitPK.com [WWW Document]. URL
339 <http://208.73.32.70/pksolutions/pksolutions.htm> (accessed 3.31.20).
- 340 García-Lino, A.M., Álvarez-Fernández, I., Blanco-Paniagua, E., Merino, G., Álvarez,
341 A.I., 2019. Transporters in the Mammary Gland—Contribution to Presence of
342 Nutrients and Drugs into Milk. *Nutrients* 11, E2372.
343 <https://doi.org/10.3390/nu11102372>
- 344 Garcia-Lino, A.M., Blanco-Paniagua, E., Astorga-Simon, E.N., Alvarez-Fernandez, L.,
345 Garcia-Mateos, D., Alvarez-Fernandez, I., Alvarez, A.I., Merino, G., 2020. Abcg2
346 transporter affects plasma, milk and tissue levels of meloxicam. *Biochem.*
347 *Pharmacol.* 175, 113924. <https://doi.org/10.1016/j.bcp.2020.113924>
- 348 González-Lobato, L., Real, R., Herrero, D., de la Fuente, A., Prieto, J.G., Marqués,

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934
935
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937
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939
940
941
942
943
944

349 M.M., Álvarez, A.I., Merino, G., 2014. Novel *in vitro* systems for prediction of
350 veterinary drug residues in ovine milk and dairy products. Food Addit. Contam.
351 Part A 31, 1026–1037. <https://doi.org/10.1080/19440049.2014.908261>

352 Lespine, A., Ménez, C., Bourguinat, C., Prichard, R.K., 2012. P-glycoproteins and other
353 multidrug resistance transporters in the pharmacology of anthelmintics: Prospects
354 for reversing transport-dependent anthelmintic resistance. Int. J. Parasitol. Drugs
355 Drug Resist. 2, 58–75. <https://doi.org/10.1016/j.ijpddr.2011.10.001>

356 Lifschitz, A., Nava, S., Guglielmone, A.A., Imperiale, F., Farias, C., Mangold, A.J.,
357 Lanusse, C., 2008. Failure of ivermectin and eprinomectin to control *Amblyomma*
358 *parvum* in goats: characterization of acaricidal activity and drug pharmacokinetic
359 disposition. Vet. Parasitol. 156, 284–92.
360 <https://doi.org/10.1016/j.vetpar.2008.05.014>

361 Mahnke, H., Ballent, M., Baumann, S., Imperiale, F., Von Bergen, M., Lanusse, C.,
362 Lifschitz, A.L., Honscha, W., Halwachs, S., 2016. The ABCG2 efflux transporter
363 in the mammary gland mediates veterinary drug secretion across the blood-milk
364 barrier into milk of dairy cows. Drug Metab. Dispos. 44, 700–708.
365 <https://doi.org/10.1124/dmd.115.068940>

366 Mealey, K.L., 2012. ABCG2 transporter: therapeutic and physiologic implications in
367 veterinary species. J. Vet. Pharmacol. Ther. 35, 105–112.
368 <https://doi.org/10.1111/j.1365-2885.2011.01313.x>

369 Merola, V.M., Eubig, P.A., 2018. Toxicology of Avermectins and Milbemycins
370 (Macrocyclic Lactones) and the Role of P-Glycoprotein in Dogs and Cats. Vet.
371 Clin. North Am. - Small Anim. Pract. <https://doi.org/10.1016/j.cvsm.2018.07.002>

372 Pavek, P., Merino, G., Wagenaar, E., Bolscher, E., Novotna, M., Jonker, J.W., Schinkel,

- 945
946
947 373 A.H., 2005. Human breast cancer resistance protein: interactions with steroid
948
949 374 drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-
950
951 375 b)pyridine, and transport of cimetidine. *J. Pharmacol. Exp. Ther.* 312, 144–52.
952
953 376 <https://doi.org/10.1124/jpet.104.073916>
954
955
956 377 Perez, M., Otero, J.A., Barrera, B., Prieto, J.G., Merino, G., Alvarez, A.I., 2013.
957
958 378 Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein:
959
960 379 Effect on plasma and milk levels of danofloxacin in sheep. *Vet. J.* 196, 203–208.
961
962 380 <https://doi.org/10.1016/j.tvjl.2012.09.012>
963
964
965 381 Real, R., Egido, E., Pérez, M., González-Lobato, L., Barrera, B., Prieto, J.G., Álvarez,
966
967 382 A.I., Merino, G., 2011. Involvement of breast cancer resistance protein
968
969 383 (BCRP/ABCG2) in the secretion of danofloxacin into milk: Interaction with
970
971 384 ivermectin. *J. Vet. Pharmacol. Ther.* 34, 313–321. <https://doi.org/10.1111/j.1365->
972
973 385 [2885.2010.01241.x](https://doi.org/10.1111/j.1365-2885.2010.01241.x)
974
975
976 386 Rehbein, S., Kellermann, M., Wehner, T.A., 2014. Pharmacokinetics and anthelmintic
977
978 387 efficacy of topical eprinomectin in goats prevented from grooming. *Parasitol. Res.*
979
980 388 113, 4039–4044. <https://doi.org/10.1007/s00436-014-4072-9>
981
982
983 389 Rostang, A., Devos, J., Chartier, C., 2020. Review of the Eprinomectin effective doses
984
985 390 required for dairy goats: Where do we go from here? *Vet. Parasitol.* 277, 108992.
986
987 391 <https://doi.org/10.1016/j.vetpar.2019.108992>
988
989
990 392 Virkel, G., Ballent, M., Lanusse, C., Lifschitz, A., 2018. Role of ABC Transporters in
991
992 393 Veterinary Medicine: Pharmaco- Toxicological Implications. *Curr. Med. Chem.*
993
994 394 26, 1251–1269. <https://doi.org/10.2174/0929867325666180201094730>
995
996
997 395 Weiss, J., Rose, J., Storch, C.H., Ketabi-Kiyanvash, N., Sauer, A., Haefeli, W.E.,
998
999 396 Efferth, T., 2007. Modulation of human BCRP (ABCG2) activity by anti-HIV
1000
1001
1002
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397 drugs. *J. Antimicrob. Chemother.* 59, 238–45. <https://doi.org/10.1093/jac/dkl474>

398 Woodland, A.N., Van der Saag, D., Kimble, B., White, P.J., Govendir, M., Lomax, S.,

399 2019. Plasma pharmacokinetic profile and efficacy of meloxicam administered

400 subcutaneously and intramuscularly to sheep. *PLoS One* 14, e0215842.

401 <https://doi.org/10.1371/journal.pone.0215842>

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1065 **Table 1.** Percentage of transport of danofloxacin (10 μ M) towards apical (BL-AP
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1067 transport) or basal (AP-BL transport) compartments in MDCK parental cells and their
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1069 ovine ABCG2-transduced cells in the absence or presence of eprinomectin at 5 μ M or
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1072 10 μ M (n= 4–7).
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		Time(h)	BL-AP (% transport)	AP-BL (% transport)	Ratio BL-AP/ AP-BL	
<i>Danofloxacin</i>	MDCKII	2	14.72 \pm 1.59	15.02 \pm 3.42		
		4	25.76 \pm 1.86	26.44 \pm 4.09	0.98 \pm 0.09	
	MDCKII ovine ABCG2	2	39.54 \pm 4.42	2.76 \pm 2.86		
		4	58.65 \pm 3.39	5.58 \pm 2.13	12.44 \pm 6.89 ^a	
	<i>Danofloxacin + Eprinomectin (5μM)</i>	MDCKII	2	12.20 \pm 1.02	9.88 \pm 1.96	
			4	20.05 \pm 1.18	22.09 \pm 0.86	0.91 \pm 0.03
MDCKII ovine ABCG2		2	21.74 \pm 0.89	5.97 \pm 2.69		
		4	32.87 \pm 3.74	14.38 \pm 0.39	2.29 \pm 0.25 ^b	
<i>Danofloxacin + Eprinomectin (10μM)</i>		MDCKII	2	11.06 \pm 1.28	13.82 \pm 3.50	
			4	21.93 \pm 4.60	25.74 \pm 5.85	0.86 \pm 0.05
	MDCKII ovine ABCG2	2	12.06 \pm 1.68	9.65 \pm 0.49		
		4	24.36 \pm 4.46	22.57 \pm 4.87	1.08 \pm 0.05 ^b	

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1111 408 Results are means \pm SDs.

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1113 409 ^a $p \leq 0.05$, significant differences from parental MDCKII cells

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1115 410 ^b $p \leq 0.05$, significant differences from MDCKII ovine ABCG2 cells without eprinomectin.

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412 **Table 2.**

413 Percentage of transport of meloxicam (30 μ M) towards apical (BL-AP transport) or basal
 414 (AP-BL transport) compartments in MDCK parental cells and their ovine-ABCG2
 415 transduced cells in the absence or presence of eprinomectin at 5 μ M or 10 μ M (n= 4–7).

		Time(h)	BL-AP (% transport)	AP-BL (% transport)	Ratio BL-AP/ AP-BL
Meloxicam	MDCKII	2	30.71 \pm 2.89	27.71 \pm 2.43	
		4	38.59 \pm 2.39	36.62 \pm 2.62	1.06 \pm 0.08
	MDCKII ovine ABCG2	2	43.31 \pm 4.96	2.43 \pm 1.40	
		4	62.87 \pm 4.72	2.77 \pm 0.75	24.85 \pm 4.62 ^a
Meloxicam + Eprinomectin (5 μ M)	MDCKII	2	29.34 \pm 0.49	21.83 \pm 2.58	
		4	38.32 \pm 2.02	35.36 \pm 1.49	1.08 \pm 0.01
	MDCKII ovine ABCG2	2	50.70 \pm 1.44	3.14 \pm 0.36	
		4	66.01 \pm 4.74	4.27 \pm 0.57	15.69 \pm 3.21 ^b
Meloxicam + Eprinomectin (10 μ M)	MDCKII	2	25.11 \pm 3.96	17.72 \pm 0.96	
		4	36.70 \pm 1.55	33.00 \pm 1.36	1.11 \pm 0.04
	MDCKII ovine ABCG2	2	43.61 \pm 0.98	6.94 \pm 2.99	
		4	62.40 \pm 3.93	13.81 \pm 6.61	5.33 \pm 2.35 ^b

416 Results are means \pm SDs.

417 ^a $p \leq 0.05$, significant differences from parental MDCKII cells

418 ^b $p \leq 0.05$, significant differences from MDCKII ovine ABCG2 cells without eprinomectin.

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1183 **420 Table 3.**
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1186 421 Mean (\pm SD) pharmacokinetic parameters in plasma of sheep after subcutaneous
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1188 422 administration of danofloxacin at a dosage of 1.25 mg/kg in sheep coadministered with
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1190 423 eprinomectin (0.5 mg/kg s.c.)
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		Danofloxacin	Danofloxacin + Eprinomectin
Plasma	AUC (0-48 h)	1.40 \pm 0.04	1.80 \pm 0.23*
	Cmax (μ g/mL)	0.16 \pm 0.05	0.2 \pm 0.06
	Tmax (h)	2.67 \pm 0.93	2.27 \pm 1.26
	MRT (h)	9.66 \pm 3.22	9.15 \pm 2.39
Milk	AUC (0-48 h)	16.2 \pm 2.96	15.41 \pm 1.79
	Cmax (μ g/mL)	2.00 \pm 0.81	1.88 \pm 0.37
	Tmax (h)	2.66 \pm 2.08	4.5 \pm 1.71
	MRT (h)	6.38 \pm 0.32	6.7 \pm 0.49
Milk/plasma	AUC	11.9 \pm 4.49	10.19 \pm 2.51

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1242 **427 Table 4.**
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1245 428 Mean (\pm SD) pharmacokinetic parameters in plasma of sheep after subcutaneous
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1247 429 administration of meloxicam at a dosage of 0.5 mg/kg in sheep coadministered with
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1249 430 eprinomectin (1.0 mg/kg s.c.)
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		Meloxicam	Meloxicam + Eprinomectin
Plasma	AUC (0-72 h)	23.7 \pm 3.94	33.1 \pm 6.77*
	C _{max} (μ g/mL)	1.53 \pm 0.29	1.83 \pm 0.24*
	T _{max} (h)	4.33 \pm 0.82	6.00 \pm 0.00*
	MRT (h)	16.9 \pm 0.85	17.6 \pm 3.89
Milk	AUC (0-72 h)	4.33 \pm 0.92	5.13 \pm 1.87
	C _{max} (μ g/mL)	0.48 \pm 0.23	0.37 \pm 0.08
	T _{max} (h)	4.33 \pm 0.82	4.50 \pm 1.97
	MRT (h)	13.8 \pm 4.05	18.08 \pm 7.68
Milk/plasma	AUC	0.19 \pm 0.03	0.17 \pm 0.02

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1301 **435 Figures legends**
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1303 **436** Fig 1. Effect of eprinomectin on accumulation of mitoxantrone (10 μ M) at 1.25, 2.5, 5,
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1305 **437** 7.5 or 10 μ M in parent MDCKII cells and in their ovine ABCG2 transduced cells.
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1307 **438** Results (units of fluorescence, median); error bars indicate SD. (n=3-6). Inhibitory
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1309 **439** potency (%) of eprinomectin in ovine ABCG2-transduced cells is also represented.
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1311 **440**
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1314 **441** Fig 2. Concentrations in plasma and milk (embedded) vs. time curves for danofloxacin
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1316 **442** obtained from lactating Assaf sheep treated with a single dose of Advocin® at 1.25
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1318 **443** mg/kg (sc) and coadministered with Eprecis® at 0.5 mg/kg (sc). Each point represents a
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1320 **444** mean; bars indicate standard deviation (n=5-6). (*) $p \leq 0.05$.
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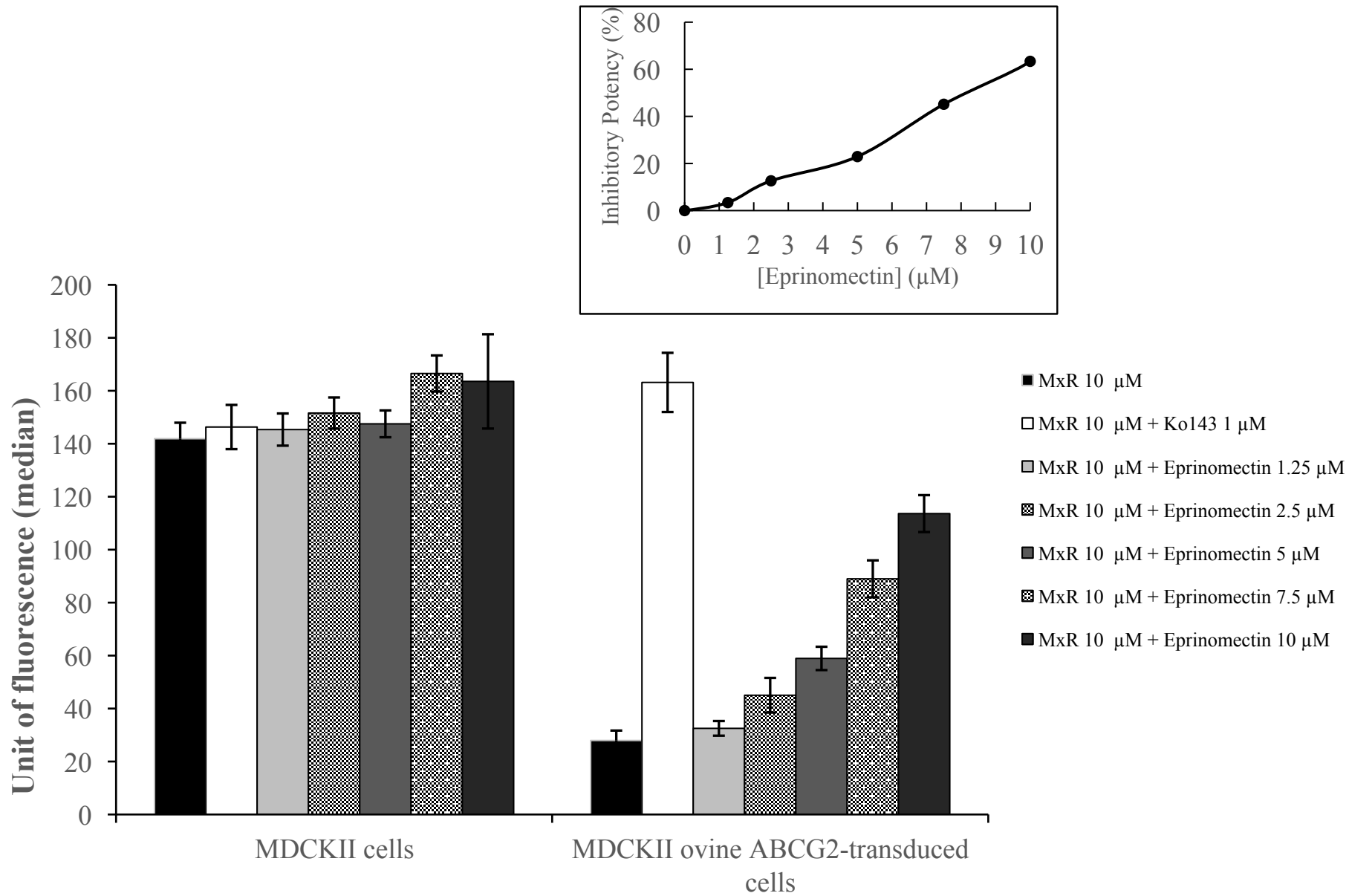
1322 **445**
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1324 **446** Fig 3. Concentrations in plasma and milk (embedded) vs. time curves for meloxicam
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1326 **447** obtained from lactating Assaf sheep treated with a single dose of Metacam® at 0.5
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1328 **448** mg/kg (sc) and co-administered with Eprecis® at 1 mg/kg (sc). Each point represents a
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1330 **449** mean; bars indicate standard deviation (n=5-6). (*) $p \leq 0.05$.
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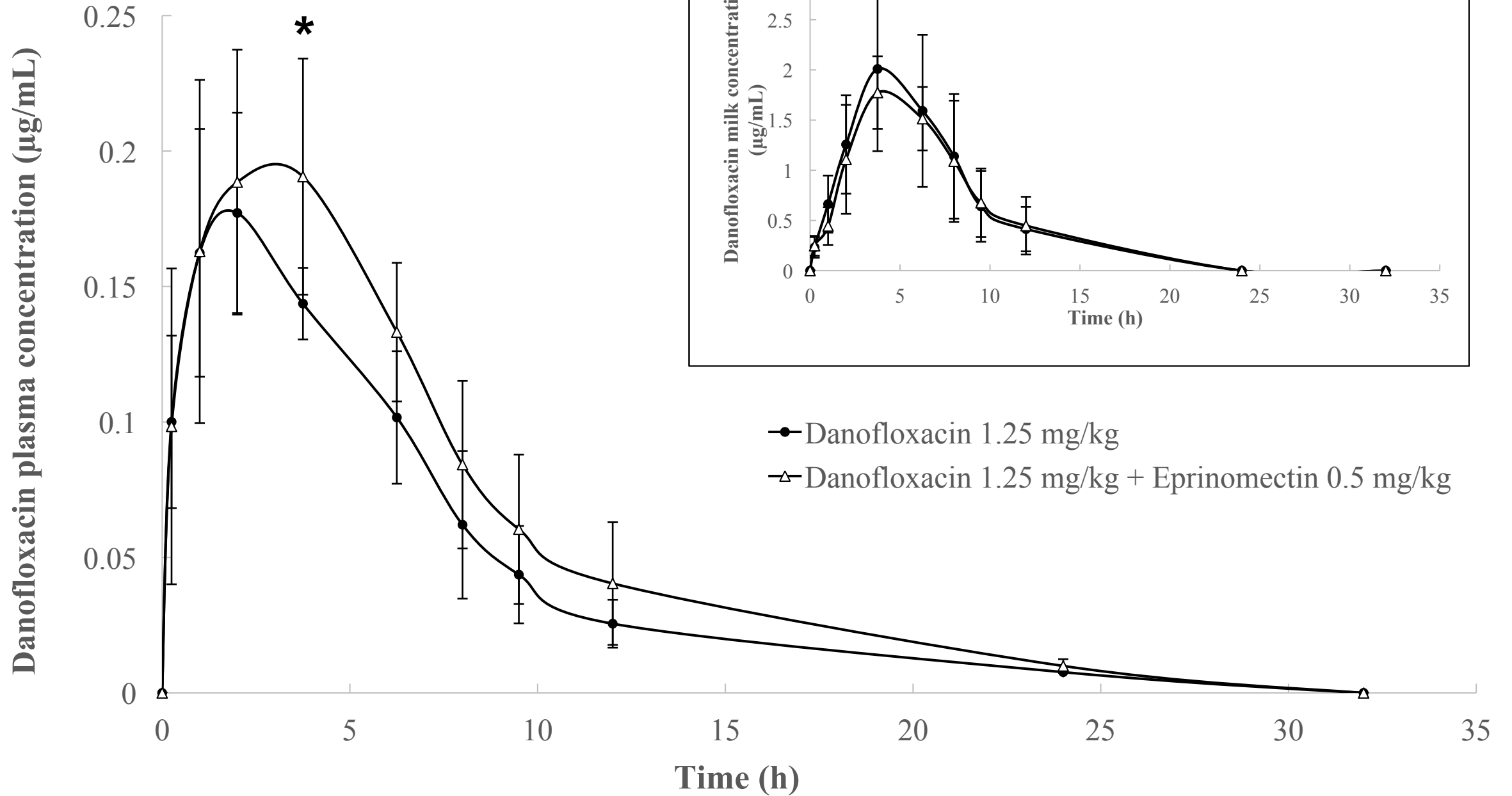
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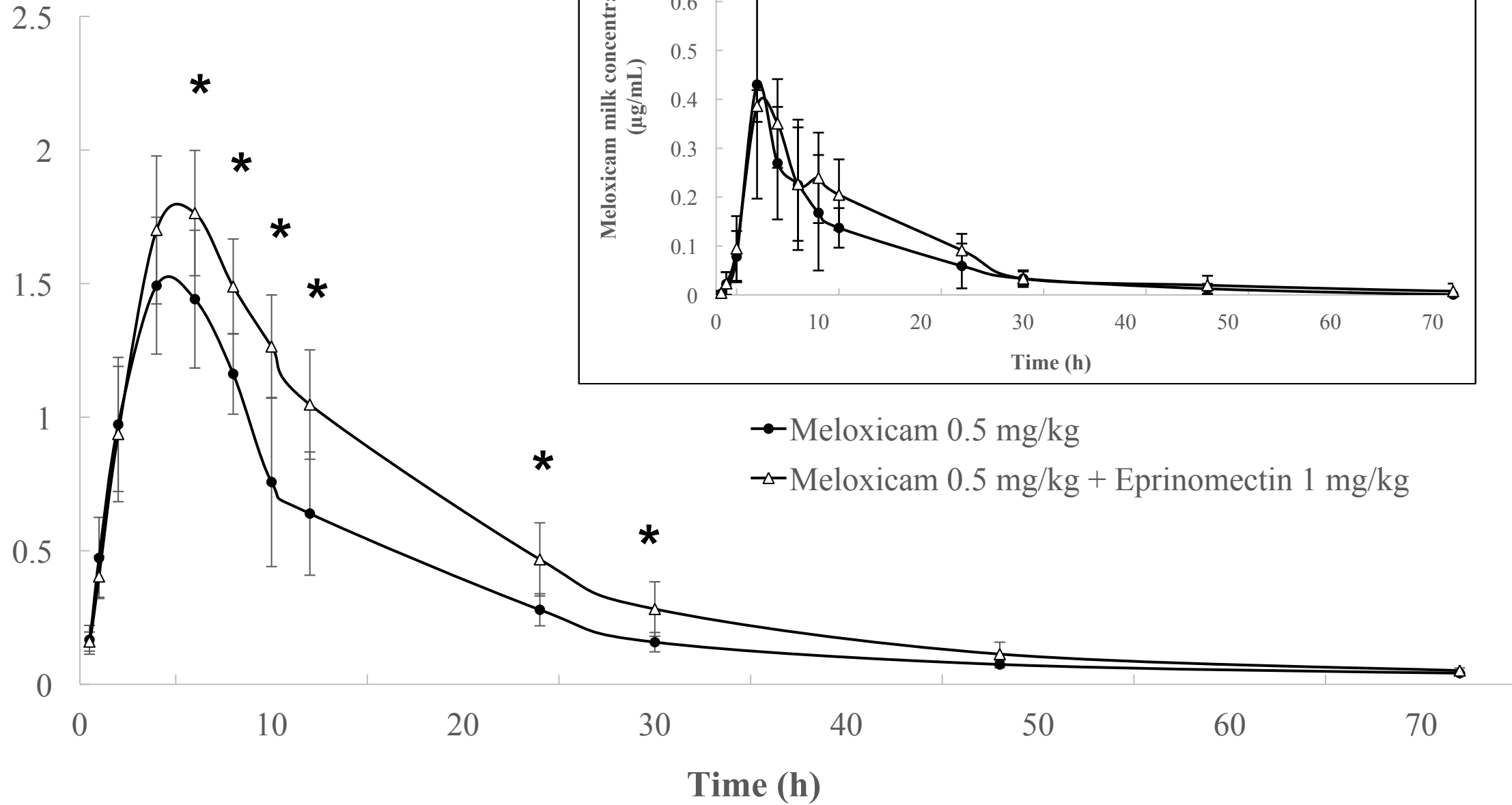
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Meloxicam plasma concentration ($\mu\text{g/mL}$)



● Meloxicam 0.5 mg/kg

△ Meloxicam 0.5 mg/kg + Eprinomectin 1 mg/kg