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ABSTRACTS

POSTER SECTION 12: BIOTRANSFORMATION AND DRUG TRANSPORTERS

P12.1 | Differential interaction of enterolignans with the ovine ABCG2-mediated transport of danofloxacin

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Introduction and Objective: ABCG2 is an efflux transporter expressed in the apical plasma membrane of the cells in a variety of tissue and performs a protective function. It is involved in the bioavailability and biodistribution of a variety of compounds, such as drugs and diet components. Lignans are dietary polyphenols, which are metabolized by gut microbiota into enterolignans, mainly enterodiol and enterolactone. They are widely distributed in the organism and have beneficial effects on human and animal health. Enterolactone and enterodiol are ABCG2 substrates. However, their potential interaction with other ABCG2 substrates has not been widely studied in veterinary medicine. Danofloxacin is an antimicrobial drug, widely used in veterinary medicine, and it is also an ABCG2 substrate. The aim of this study was to investigate the *in vitro* ovine ABCG2-mediated interaction of enterolignans and the antimicrobial danofloxacin.

Material and Methods: Transepithelial transport assays using MDCKII cells either as parental cells or transduced with ovine ABCG2 were performed. Cells were seeded on Transwell plates (Colstar, Corning, NY) and were grown for 3 days. The day of the experiment medium on both sides of monolayer was replaced with transport medium containing danofloxacin, with or without the inhibitor (50 or 100 μM enterolactone or enterodiol). Aliquots of 100 μl were taken from the opposite compartment at 2 and 4 h. Samples were analyzed by HPLC.

Results and Conclusion: MDCKII cells overexpressing the ovine variant of the ABCG2 transporter showed a relative transport ratio for danofloxacin of 14.50 in the absence of enterolignans, confirming that danofloxacin is an ABCG2 substrate. Enterolactone at 50 and 100 μM inhibited the ovine ABCG2-mediated transport of danofloxacin, reducing the relative transport ratio to 2.48 and 1.4 respectively. However, enterodiol at 50 and 100 μM showed no significant effects on the ovine ABCG2-mediated transport. Therefore, these results show that only the enterolignan enterolactone significantly inhibits the *in vitro* transport of the antimicrobial danofloxacin by ovine ABCG2.

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P12.2 | Role of the enterolignan enterodiol as an *in vitro* inhibitor of the transporter ABCG2/BCRP in ruminants using mitoxantrone accumulation assays

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Introduction: The Breast Cancer Resistance Protein (BCRP/ABCG2) is a member of the ATP-binding cassette (ABC) transporters that extrudes a wide range of substrates from cells in intestine, liver and other organs like mammary gland, thus affecting the bioavailability and milk secretion of many compounds. The development of ABCG2 modulators is critical in order to improve drug pharmacokinetics properties, reduce milk secretion of xenobiotics and toxins and/or increase the effective intracellular concentration of its substrates. Lignans are dietary polyphenols present in ruminant diets with relevant health-beneficial properties, which are metabolized by gut microbiota to enterolignans, enterodiol and enterolactone. Previous studies demonstrated the interaction of enterolactone with ABCG2 (1). Our aim was to study the potential of enterodiol as inhibitor of ovine ABCG2 and two functional variants of bovine transporter, WT-bABCG2 and Y581S-bABCG2, using mitoxantrone accumulation assays.

Materials and Methods: *In vitro* accumulation assays were carried out on MDCKII cells with stable overexpression of the different ruminant variants of the transporter. Mitoxantrone was used as a fluorescent substrate, and Ko143 as a complete inhibitor of ABCG2. Two concentrations of enterodiol (100 μM and 200 μM) were tested.

Accumulation of mitoxantrone was determined by flow cytometry and inhibitory potency was calculated.

Results and Conclusion: The inhibitory potency results in mitoxantrone accumulation assays revealed that enterodiol acts as an inhibitor of ovine ABCG2 at both concentrations (47.0 ± 0.1% at 200 μM and 17.65 ± 0.03% at 100 μM, respectively). However, in bovine ABCG2 expressing cells, the inhibitory potency of enterodiol remained below 10% in both genotypes of ABCG2. In conclusion, we demonstrated inhibitory effects of enterodiol on the sheep ABCG2 transporter, and a lack of effect for the two variants of bovine ABCG2 transporter using the mitoxantrone accumulation assays. The use of enterolignans as modulators of the activity of ABCG2 would allow to control the presence of ABCG2 substrates and thereby undesirable residues in the milk. This would be feasible in the case of sheep, but apparently not in cattle based on our negative data in the case of bovine ABCG2 variants.

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Reference: 1. Miguel V *et al*. (2014) *Drug Metab Dispos*; 42: 943–946.

P12.3 | *In vitro* transepithelial transport of the fluoroquinolone flumequine by the ruminant ABCG2 transporter

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Introduction/Objective: Flumequine is a fluoroquinolone drug with antimicrobial activity against gram-negative bacteria, widely used in veterinary medicine, especially in farm animals. ABCG2 is an efflux membrane transporter responsible for the distribution and excretion of different drugs in several tissues, including secretion into milk. Previous studies showed the *in vitro* and *in vivo* interaction between this transporter and other fluoroquinolones. Our purpose was to study the *in vitro* interaction between flumequine and the ruminant variants of the ABCG2 transporters, i.e. ovine and both functional bovine variants (wild-type and Y581S).

Material and Methods: Transepithelial transport assays using parental and MDCKII cells, over-expressing ovine and two variants of bovine ABCG2 proteins, were used. Cells were seeded on microporous polycarbonate membrane filters (Transwell, Costar, Corning, NY) and were grown for 3 days. The day of the experiment, medium in both sides of the monolayer was replaced with OptiMEM medium (Life Technologies) containing 10 μM flumequine, with or without of the specific ABCG2 inhibitor Ko143 (1 μM). Aliquots of 100 μl were taken from the opposite compartment at 2 and 4 hours and stored at -20°C for HPLC analysis.

Results and Conclusion: MDCKII cells over-expressing ovine, wild-type bovine and Y581S bovine ABCG2 variants showed a relative transport ratio for flumequine of 4.59 ± 0.42, 1.88 ± 0.25 and 3.59 ± 0.08 respectively. Differences were statistically significant compared with parental MDCKII and between both bovine ABCG2 variants, with higher relative transport ratio in Y581S variant. When the selective ABCG2 inhibitor was used, ABCG2-mediated transport was completely inhibited. These results show that ruminant ABCG2 plays an important role in the flumequine transport and species differences and genetic variability in expression in bovines could be responsible for undesirable residues in meat and milk after flumequine treatment in farm animals.

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P12.4 | A preliminary investigation on the effects of quercetin on aflatoxin B1 (AFB1) toxicity and transport in an *in vitro* model of functional Caco-2 intestinal cells

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Introduction/Objective: The Caco-2 cell line is a well characterized human intestinal *in vitro* model exhibiting metabolic and transport activities. Due to its ability to spontaneously differentiate into a polarized monolayer resembling typical functional enterocytes, it has been widely employed to study the intestinal absorption of several xenobiotics, including mycotoxins. AFB1 is a food and feed contaminant of particular concern because of the generation of highly toxic metabolites (i.e. AFB1-exo-8,9-epoxide and AFM1). Flavonoids have been shown to modulate biotransformation enzymes and/or efflux pumps involved in AFB1 metabolism and transport, respectively. Our aim was to investigate the effects of Quercetin (QUER) on both AFB1 toxicity and transport in Caco-2 cells.

Materials and Methods: Cell viability assays (i.e. CTG or Neutral Red Uptake or TEER) were performed on either un-differentiated (UD) and differentiated (D) cells pre-incubated with 10 or 30 μM of QUER for 16 h, and then treated with different concentrations of AFB1 (1–30 μM for UD, and 10 and 30 μM for DC cells) in the presence of the antioxidant at the same concentrations. To evaluate the AFB1 transport and its possible modulation by QUER, experiments were performed on D cells grown in transwell inserts. Cells were

exposed to different concentrations of AFB1 (3 or 10 μM) in the presence of QUER after pre-incubation with 10 or 30 μM of the antioxidant. Media from apical and basolateral chambers were collected for chemical analysis (LC/MS-MS) at 1, 4 and 24 h. Data were analyzed by ANOVA followed by Dunnett's post hoc test.

Results and Conclusions: As expected, AFB1 decreases the viability of both UD and D Caco-2 cells in a time and dose-dependent manner. QUER did not seem to convey any protective effect against AFB1-induced cytotoxicity neither in UD nor in D cells. Chemical analyses showed a time and concentration-dependent increase ($P \leq 0.001$) of AFB1 in the basolateral compartment accompanied by a decrease in the apical one ($P \leq 0.001$), demonstrating the transport of AFB1 through the cell monolayer. The detection of AFM1 in the apical compartment indicates that Caco-2 cells are able to metabolize AFB1 into AFM1.

P12.5 | Metabolism and efflux of enrofloxacin in primary bovine mammary epithelial cells: a pilot study

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Introduction: Knowledge on disposition and metabolism of drugs in bovine mammary epithelial cells (bMEC) may support the optimization of treatment protocols. The study aimed to investigate the biotransformation and transport of enrofloxacin (ENR) in primary bMECs. ENR was chosen as a model drug as it is metabolized to ciprofloxacin (CPR) by cytochrome P450 (CYP) enzymes and actively effluxed by Brest Cancer Research Protein (BCRP).

Material and Methods: Primary bMECs were isolated from healthy udders of lactating cows ($n = 5$) and cultured on DMEM (supplemented with insulin, transferrin and sodium selenite) with a seeding density of 1.5×10^5 cells. bMECs cultures were incubated after the sixth passage with ENR (10 μM, 3.96 mg ml^{-1}), dexamethasone (25 μM DEX) and quercetin (25 μM QCT) alone or in combinations. The experiments were done in duplicate with incubation periods of 0.5, 1, 1.5, 2, 2.5 and 3 h, respectively. ENR and CPR concentrations in medium and in the cells were determined by HPLC-FL analysis. Quantitative RT-PCR analysis of mRNA expression of bovine ABCG2, CYP1A1, CYP1A2, CYP3A4-like, α-casein, lactoferrin and cyclophilin B (reference gene) was performed by SybrGreen protocols.

Results: Primary bMECs formed typical cobblestone shape colonies and expressed α-casein and lactoferrin mRNA, genes highly specific for MECs from lactating animals. CYP3A4-like mRNA showed the highest level of expression ($P < 0.05$). QCT and DEX inhibited the conversion of ENR to CPR to some extent. Maximum CPR levels in the medium of ENR treated cells were 0.375 ± 0.01 μg ml^{-1} at 1.5 h. Thereafter, CPR levels gradually decreased. The medium of ENR-QCT or ENR-DEX treated bMECs contained similar lower CPR levels ranging between 0.19 ± 0.07–0.36 ± 0.06 μg ml^{-1}, with a maximum at 1.5–2 h.

Conclusion: The results showed that ENR penetrates into primary bMECs. Data suggest that ENR is converted to CPR in bMECs. CYP3A4, CYP1A1 and CYP1A2 are involved in the metabolism of ENR to CPR in these cells. Significant changes of ENR conversion to CPR was not observed by DEX treatment. Metabolism of the parent compound to CPR was statistically insignificantly inhibited by QCT.

P12.6 | Effect of flavonoids on cytochrome P450 activity in porcine intestinal epithelial cells - *in vitro* interaction study

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Introduction/Objective: World production of pork has increased rapidly in recent decades. The use of low-dose dietary antibiotics has been banned by the EU for growth promotion of livestock. Subsequently, there is a growing interest to replace the use of antimicrobial growth promoters by different alternatives. Among these alternatives are flavonoids, which seem to improve gut health by improving feed intake and epithelial integrity and by reducing inflammatory processes in the gut. Previous investigations, indicated interactions between some flavonoids and CYP450 enzymes, which play a critical role in the xenobiotic metabolism. As a consequence, simultaneous consumption of flavonoids and drugs can alter the pharmacokinetics of the latter. In case of oral administration, CYP450 enzymes of small intestine play an important role in the pre-systemic metabolism, therefore our studies focus on this organ.

Materials and Methods: Porcine intestinal epithelial cells (IPEC-J2) were treated by flavonoids (apigenin, 4′,5,7-trimethyl-apigenin, quercetin, 3-methylquercetin and 3′7-dimethylquercetin) in 25 and 50 μM concentration as well as with known CYP inhibitors (ketoconazole 25 μM, α-naphthoflavone 50 μM) per inducer (phenobarbital 1 mM) compounds. The CYP1A1, CYP1A2 and CYP3A4 activities were measured by chemiluminescent assay. Simultaneous treatment of enterocytes with flavonoids and antipyrine was also performed in order to test possible drug interactions.

Results and Conclusions: Both apigenin derivatives acted as significant CYP3A4 inhibitors. 4′,5,7-trimethyl-apigenin combined with phenobarbital resulted also in a significant CYP3A4 inhibition. Both quercetin and 3-methyl-quercetin inhibited CYP3A4. When cells were treated with phenobarbital, CYP3A4 enzyme activity was also inhibited by quercetin and 3-o-methyl-quercetin. Antipyrine decreased the CYP450 activity, and this effect was enhanced, when antipyrine and apigenin or quercetin were administered simultaneously. These results suggest that flavonoids can

inhibit intestinal CYP450 activity, in particular porcine CYP3A4 equivalent. As the CYP3A family plays a significant role in the metabolism of clinically important drugs, further investigations should focus on the intestinal metabolism of flavonoids in other species, and afterwards, *in vivo* studies should be performed in this topic.

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P12.7 | Determining mefloquine's *in vitro* phase II conjugative hepatic metabolism using feline microsomes

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Introduction: Feline infectious peritonitis (FIP) is a systemic, fatal, viral-induced immune-mediated disease of cats caused by virulent biotypes of feline coronaviruses (FCoV), known as the feline infectious peritonitis virus (FIPV). Currently, there is no effective treatment for FIP. Traditional therapies rely on the use of immunosuppressive agents to dampen clinical signs temporarily but none address the underlying problem of viral replication. Recent *in vitro* studies seeking an efficacious FCoV treatment identified mefloquine, a human anti-malarial drug, as a potential FIP anti-viral agent; in fact, it substantially reduced the viral load of FIPV in infected Crandell Rees feline kidney cells without cytotoxic effects at low concentrations (1). However, in cats, some therapeutic agents are known to have delayed phase II hepatic metabolism, resulting in delayed elimination and consequent toxicity. As there are no pharmacokinetic studies on the use of mefloquine in the cat, the overall aim of this study was to develop an *in vitro* model of feline conjugative metabolism, using hepatic microsomes to predict whether mefloquine is likely to accumulate in the live cat. Microsomes extracted from the common brush-tailed possum (*Trichosurus vulpecula*) were used as a comparison species.

Materials and Methods: Hepatic microsomes (0.5 mg ml^{-1}) were incubated over 6 hours with 10 μM of mefloquine in 0.1 M phosphate buffer (pH 7.4) containing a UDPGA regenerating system and alamethicin. For the quantification of mefloquine and the screening of its potential conjugated metabolites, an HPLC-UV assay was developed. Using HPLC, the rate of hepatic metabolism (*in vitro* Cl_{int}) was calculated by quantifying the depletion of mefloquine during the microsomal incubation.

Results and Conclusions: There was no evidence of mefloquine depletion in either the cat or common brush-tailed possum when evaluated over 6 hours. Similarly, no phase II metabolite formation was observed. Although further studies are required, this initial investigation into the phase II hepatic metabolism of mefloquine suggests that the drug may not undertake this route of elimination in the cat or common brush-tailed possum. Consequently, once the true nature of its conjugative metabolism is verified, mefloquine's potential as an anti-viral treatment for FIP can be explored more extensively in healthy and FIP affected cats.

Reference: 1. McDonagh P *et al.* (2014) *Vet Microbiol*; 174: 438–447.

P12.8 | Cytochrome P450 gene expression of porcine jejunal cells exposed to feed additives

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Introduction: The intestinal epithelium serves as the first determining barrier to the drugs administered *per os*. Cytochrome P450 (CYP) enzymes play a key role in the initial step of xenobiotic metabolism; therefore, intestinal CYP enzyme activities could be an important influencing factor of the oral utilization of xenobiotic substances. Fulvic acid is one of the most active fractions of humates which have recently been considered as feed supplements. Fulvic acid contains many reactive functional groups, including carboxyls, hydroxyls, carbonyls, phenols, quinones, and semiquinones. Furthermore, drinking water acidifiers (DWA) have also been used as feed additives. The aim of this study was to examine the effect of a DWA (volatile fatty acids, amino acids, phosphoric acid, zinc and copper salt complexes mixture) and fulvic acid upon CYP mRNA levels of porcine intestinal epithelial cells.

Materials and Methods: The non-transformed porcine intestinal epithelial cells (IPEC-J2 cell line), originally isolated from jejunal epithelia of a neonatal unsuckled piglet, were grown on six-well transwell polyester membrane inserts, that serves as a model of intestinal epithelium. Cell cultures were treated at day 21 with DWA (0.1 and 1 μl ml^{-1}) and fulvic acid (25 and 250 μg ml^{-1}) for 1 h. Cells were washed with culture medium and incubated for additional 1 h before total RNA isolation. CYP1A1, CYP1A2 and CYP3A29 mRNA levels were measured using quantitative real-time PCR (qPCR). Hypoxanthine phosphoribosyl transferase (HPRT) and Cyclophilin-A (CycA) were used as reference genes.

Results and Conclusions: The relative expression of CYP1A1 was upregulated by 25 and 250 μg ml^{-1} fulvic acid and 1 μl ml^{-1} DWA (11.06 ± 4.04, 35.52 ± 7.16 and 10.55 ± 0.91, respectively). CYP1A2 mRNA levels tripled and doubled in response to the high doses of DWA and fulvic acid treatments. None of the treatments changed CYP3A29 gene expression. In conclusion, the applied supplements increased the intestinal gene expression of certain CYP genes, especially at higher doses; consequently, it may lead to enhanced levels of CYP protein, potentiating possible drug-drug interactions, in particular with those substances which are metabolized via the CYP1A subfamily.

P12.9 | Examination of the interspecies differences in microsomal drug metabolism in chickens and rabbits

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Introduction/Objective: Existing interspecies differences in drug metabolism can lead to significant alterations of the efficacy and safety of the medicinal substances. *In vitro* methods may be useful for a preliminary detection of the species dependent characteristics of drug decomposition. In our experiment we compared the effect of three model drugs (monensin, tylosin and tiamulin) on hepatic microsomal cytochrome P450 (CYP) enzyme activities of chicken and rabbit by luminometry.

Materials and Methods: Rabbit and chicken microsome suspensions were prepared from the livers of previously non-medicated young adult animals (n = 4 for each species) with the help of two-step differential ultracentrifugation method of tissue homogenates. The model substrates (monensin, tiamulin and tylosin) were incubated in different concentrations in a range from 0.001 μM to 5 mM with the microsomal fractions of each species and with NADPH regenerating system, respectively. The alteration of the CYP activities were detected by selective CYP luminescent assays (P450-Glo™, Promega, Madison, WI, USA) and the formed luminescent signal was measured by Victor X2 luminometer (PerkinElmer, Waltham, MA, USA).

Results and Conclusions: The activity of chicken CYP1A was inhibited by 0.001 μM monensin while the rabbit CYP1A activity was decreased by 1 μM monensin. Furthermore, the activity of chicken CYP2C was declined by 0.001 μM monensin, but in rabbit microsomes monensin caused the inhibition of this enzyme only in concentration of 0.1 μM. CYP3A6 activity of rabbit was remarkably inhibited by 1 μM monensin. Tiamulin decreased the rabbit CYP3A6, CYP2C and CYP1A activities at 0.1 μM, 1.25 mM and 5 mM, respectively. In chickens, tiamulin diminished the activity of CYP2C and CYP1A at 0.5 μM and 0.5 mM, respectively. Tylosin only decreased the rabbit CYP3A6 and CYP2C activities in 0.625 mM and 2 mM concentrations. From these results, we concluded that there is a significant difference between rabbits and chickens in the metabolic processing of xenobiotics. For example, CYP2C and CYP3A were the most clearly inhibited enzyme subfamilies in rabbits, while the CYP1A and CYP2C were in chickens. In microsomes of both species monensin caused the strongest CYP-inhibition, followed by tiamulin. Tylosin was a weak inhibitor of rabbit CYP enzymes, and it did not have a clinically relevant inhibitory effect on the chicken CYP enzymes.

P12.10 | Cytotoxicity and biotransformation of enrofloxacin in HepG2 cell culture

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Introduction: Enrofloxacin is the most used fluoroquinolone to treat infections in animals. At the same time, fluoroquinolones are among those antimicrobial chemotherapeutics, which are frequently detected in the environment at relatively high concentrations ranging from ng l^{-1} to μg l^{-1}. Moreover, their accumulation, toxicity and the resistance of pathogenic microorganisms are of major concern. The present study was performed to determine the cytotoxic effects of enrofloxacin and its biotransformation in an *in vitro* study using human HepG2 cells.

Materials and Methods: The cytotoxic potential of enrofloxacin and its biotransformation was investigated using human hepatoma cells (HepG2). Four biochemical endpoints were assessed: mitochondrial (MTT assay) and lysosomal (NRU assay) activity, total cell protein content (TPC assay), and membrane integrity (LDH leakage assay) after 72 h incubation with the drug at concentrations ranging from 0.78 to 100 μg ml^{-1}. Additionally, the metabolites of the fluoroquinolone in the cell culture medium were determined using LC-MS/MS.

Results: The cytotoxicity of enrofloxacin was concentration dependent. The results suggest that the drug has a low cytotoxic potential. The cytotoxic concentrations values of the fluoroquinolone (< 100 μg ml^{-1}) in NRU, TPC and LDH assay for hepatoma (HepG2) cells after 72 h incubation were calculated. The most affected endpoint was damage in cellular membrane integrity in the studied cell cultures after the treatment with enrofloxacin. The LC-MS/MS analysis of medium from HepG2 cell cultures exposed to the drug revealed that the main metabolite of enrofloxacin was ciprofloxacin. Additionally, the concentration of ciprofloxacin increased with the concentration of enrofloxacin. The amount of the metabolite formed was closely related to the drug cytotoxicity in HepG2 cells.

Conclusions: The exposure of HepG2 cells to enrofloxacin led to concentration-dependent cytotoxicity, which correlated with the rate of biotransformation of enrofloxacin to ciprofloxacin.

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P12.11 | Interaction of enrofloxacin and deoxynivalenol and effects on their biotransformation in HepG2 cells

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Introduction: Exposure to chemical contaminants is an important concern worldwide. Most of the toxicological data apply to the effects of chemical contaminants when they are present alone; however, humans are usually exposed to multiple toxic compounds, which might impact health *via* food or the environment. Enrofloxacin (ENRO) is the most persistent fluoroquinolone in the environment, due to input from veterinary use. This aspect may have important consequences, such as undesirable antibiotic residues and increase in bacterial resistance, which can directly influence consumer's health. Deoxynivalenol (DON) is the most prevalent mycotoxin naturally present in grains and other food commodities. Humans and animals are mainly exposed to DON through cereal-derived food and feed, respectively. Subsequently, humans can be simultaneously exposed to ENRO and DON through food consumption.

Materials and Methods: To improve the understanding of the toxicity of ENRO and DON alone or in combination, various combinations of both substances were tested in human HepG2 cell. Cytotoxicity was assessed through: MTT, NRU, TPC and LDH assays and types of interactions determined by the isobologram-combination index method after 72 h of exposure. Additionally, the metabolites of the fluoroquinolone and the mycotoxin in the medium from the culture were determined using LC-MS/MS.

Results: HepG2 cells were exposed to combination of ENRO and DON at different ratios (ENRO/DON of 1/1; 1/0.01). The cytotoxic concentrations values of these combination in four assays were calculated. The most affected endpoint was inhibition of lysosomal activity (NRU assay) in HepG2 cells after simultaneous exposure to ENRO and DON. Regardless of the ratio, the type of interaction observed in HepG2 cells ranged from antagonism to nearly additive with increasing cytotoxicity was dependent from concentrations and used assay. The LC-MS/MS analysis of medium from HepG2 cell cultures exposed to this mixture revealed that the main metabolite of ENRO was ciprofloxacin, whereas no metabolites of DON were detected, as expected.

Conclusions: This study provides an example for exposure scenarios that occur in daily life. Interactions between different contaminants and residual drug concentrations should be included in forthcoming risk assessment approaches.

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