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Interacción del antihelmíntico triclabendazol, el analgésico URB937 y sus derivados con transportadores de membrana dependientes de ATP

Memoria presentada por el Licenciado Borja Barrera Cuesta para la obtención del grado de Doctor con Mención Internacional por la Universidad de León

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INFORME DE LOS DIRECTORES DE LA TESIS

(Art. 11.3 del R.D. 56/2005)

El Dr. D. **Julio Gabriel Prieto Fernández** y la Dra. Dña. **Gracia Merino Peláez** como Directores de la Tesis Doctoral titulada "Interacción del antihelmíntico triclabendazol, el analgésico URB937 y sus derivados con transportadores de membrana dependientes de ATP" realizada por D. **Borja Barrera Cuesta** en el programa de doctorado Biomedicina en el departamento de Ciencias Biomédicas, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

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Tesis Doctoral por compendio de Publicaciones

- ✓ **The anthelmintic triclabendazole and its metabolites inhibit the membrane transporter ABCG2/BCRP.**
  Barrera B, Otero JA, Egido E, Prieto JG, Seelig A, Álvarez AI, Merino G.
  Antimicrobial Agents and Chemotherapy (2012)
  56(7): 3535-43
  Impact Factor: 4.57, 31/260 Category: Pharmacology and Pharmacy

- ✓ **Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: Role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter.**
  Barrera B, González-Lobato L, Otero JA, Real R, Prieto JG, Álvarez AI, Merino G.
  The Veterinary Journal (2013)
  doi:pii: S1090-0233(13)00370-5. 10.1016/j.tvjl.2013.07.033.
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- ✓ **The ABC membrane transporter ABCG2 prevents access of FAAH inhibitor URB937 to the central nervous system.**
  Moreno-Sanz G, Barrera B, Guijarro A, d'Elia I, Otero JA, Álvarez AI, Bandiera T, Merino G, Piomelli D.
  Pharmacological Research (2011)
  64(4):359-63
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- ✓ **Identification of structural determinants of peripherally restricted FAAH inhibitors for substrate recognition by blood-brain barrier transporters ABCG2 and P-glycoprotein**
  Barrera B, Moreno-Sanz G, Armirotti A, Scarpelli R, Bandiera T, Prieto JG, Merino G, Piomelli D.
  (Manuscript in preparation)

Parte de los resultados expuestos en la presente memoria han dado lugar a las siguientes C**omunicaciones a Congresos tanto Nacionales como Internacionales:**

- Fernanda Imperiale, Miriam Pérez, Rebeca Real, **Borja Barrera**, Carlos Lanusse, Julio G. Prieto y Gracia Merino. *"Caracterización del triclabendazol sulfóxido como sustrato e inhibidor del transportador BCRP/ABCG2"*. Congreso de la Sociedad Española de Bioquímica y Biología Molecular. Málaga, España 2010.

- **Borja Barrera**, Rebeca Real, Lucía González, Estefanía Egido, Miriam Pérez, Julio G. Prieto, Ana I. Álvarez y Gracia Merino. *"Interaction of the bovine, murine and human ABCG2/BCRP protein by the anthelmintic triclabendazole"*. Congress of the Spanish Society of Pharmacology. León, España 2010.

- **Borja Barrera**, Jon Andoni Otero, Lucía González, Ana I. Álvarez, Julio G. Prieto y Gracia Merino. *"Metabolites of triclabendazole inhibit in vitro drug transport mediated by ABCG2/BCRP"*. Biomedical Transporters 2011. Grindelwald, Suiza 2011.

- Jon Andoni Otero, **Borja Barrera**, Estefanía Egido, Julio G. Prieto, Ana I. Álvarez y Gracia Merino. *"In vivo interaction of the metabolites of the benzimidazole triclabendazole with the ABCG2/BCRP transporter"*. Biomedical Transporters 2011. Grindelwald, Suiza 2011.

- **Borja Barrera**, Jon Andoni Otero, Lucía González, Ana I. Álvarez, Julio G. Prieto y Gracia Merino. *"Interacción in vivo de los principales metabolitos del triclabendazol con dos sustratos conocidos del transportador de membrana ABCG2/BCRP"*. II Congreso Internacional LABIOFAM 2012: Simposio de Productos Naturales en la Terapia contra el Cáncer. La Habana, Cuba 2012.

- **Borja Barrera**, Jon Andoni Otero, Lucía González, Ana I. Álvarez, Julio G. Prieto y Gracia Merino. *"Inhibición in vitro del transportador ABCG2/BCRP bovino y ovino en células MDCKII por los metabolitos del triclabendazol"*. II Congreso Internacional LABIOFAM 2012: Simposio de Productos Naturales en la Terapia contra el Cáncer. La Habana, Cuba 2012.

- **Borja Barrera**. *"Interacción in vitro e in vivo de los metabolitos del antihelmíntico triclabendazol con el transportador ABCG2 de rumiantes"*. II Congreso Internacional LABIOFAM 2012: Simposio de Productos Naturales en la Terapia contra el Cáncer. La Habana, Cuba 2012.

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A mis padres y a mi hermano

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ABREVIATURAS

ABC	*ATP-Binding Cassette* (Casete de unión al ATP)
ADP	Adenosin Difosfato
ATP	Adenosin Trifosfato
ARN	Ácido Ribonucleico
$AUC_{(0\text{-}48h)}$	Área bajo la curva de concentración del fármaco frente al tiempo desde el tiempo cero hasta las 48 h
BBB	*(Blood-Brain Barrier)* Barrera Hematoencefálica
BCRP	*Breast Cancer Resistance Protein* (Proteína asociada al cáncer de mama)
CAR	*Constitutive Androstane Receptor* (Receptor Constitutivo de Androstano)
CB_1	Receptor de Cannabinoides de tipo 1
CB_2	Receptor de Cannabinoides de tipo 2
DMEM	*(Dulbecco's Modified Eagle's Medium)* Medio Eagle modificado
EMEA	*European Medicine Agency* (Agencia Europea del Medicamento)
ESI	*(Electrospray Ionization)* Ionización por electrospray
FAAH	*Fatty-acid amide hydrolase* **(**Amida hidrolasa de ácidos grasos)
FDA	*Food and Drug Administration*
FMO	Flavin Monooxigenasa
HPLC	Cromatografía Líquida de Alta Eficiencia
IC_{50}	Concentración a la que se consigue el 50% de la inhibición máxima
ID_{50}	Mitad de la máxima dosis efectiva
i.p.	Intraperitoneal
i.v.	Intravenoso
MDCKII	*Madin-Darby Canine Kidney Cells* (línea celular derivada de riñón de perro)
MDR	*Multidrug Resistance* (Multirresistencia a fármacos)
MF	Mediana de Fluorescencia

MRP	*Multidrug Resistance Protein* (Proteína asociada a la resistencia de fármacos)
MS	*(Mass Spectrometer)* Espectrómetro de Masas
MW	*Molecular Weight* (Peso Molecular)
MXR	Mitoxantrona
PDA	*(Photodiode Array Detector)* Detector de formación de fotodiodos
PEG400	Polietilenglicol 400
P-gp	Glicoproteína P
PhIP	2-amino-1-metil-6-fenilimidazol [4,5-b] piridina
PSA	*Polar surface area* (Área de superficie polar)
PXR	*Pregnane X receptor* (Receptor de Pregnano X)
SAR	*(Structure-Activity Relationship)* Relación estructura-función
s.c.	Subcutáneo
SNC	Sistema Nervioso Central
SNP	*Single Nucleotide Polymorphism*
SP	*Side Population*
SQD	*Single Quadrupole Detector* (Detector simple Cuadrupolo)
SULT1	Fenol Sulfotransferasas
TCBZ	Triclabendazol
TCBZSO	Triclabendazol sulfóxido
$TCBZSO_2$	Triclabendazol sulfona
$T_{máx}$	Tiempo observado al que se alcanza la concentración máxima
2-AG	2-araquidonoil-*sn*-glicerol

1. RESUMEN

La presente memoria de Tesis Doctoral estudia las interacciones del antihelmíntico triclabendazol (TCBZ) y sus metabolitos y el analgésico en desarrollo URB937 y sus análogos estructurales con el transportador ABCG2. Además, en el caso del URB937 y sus derivados se amplió el estudio a la glicoproteína P.

ABCG2 se expresa en diversos tejidos del organismo presentando una función primordialmente protectora. Además, ABCG2 es el único transportador ABC cuya expresión se induce durante la lactación en la glándula mamaria, estando implicado en el paso de distintos compuestos hacia la leche, y es uno de los transportadores presentes en la barrera hematoencefálica restringiendo la entrada de fármacos hacia el SNC. Su función en ambas localizaciones se delimita a través de la búsqueda de compuestos que interactúen con él como sustratos o inhibidores.

El triclabendazol es un fármaco muy utilizado en terapéutica veterinaria generalmente administrado de forma concomitante junto con otros fármacos. En este caso, estudiamos las interacciones farmacológicas mediadas por ABCG2 de los principales metabolitos del triclabendazol a nivel de glándula mamaria y en relación con la secreción de fármacos a leche. En cuanto al URB937 (analgésico en desarrollo) y sus análogos estructurales, nos hemos centrado en su interacción con ABCG2 y la glicoproteína P, otro transportador ABC, a nivel de la barrera hematoencefálica, donde ambos transportadores funcionan evitando la entrada de compuestos al cerebro. Así, los objetivos resumidos de la presente memoria consistieron por un lado, en el estudio de la capacidad inhibitoria *in vitro* del TCBZ y sus metabolitos (TCBZSO y $TCBZSO_2$) sobre ABCG2 en distintas

especies y el estudio *in vivo* del efecto de la coadministración del TCBZ y sus metabolitos sobre la farmacocinética y secreción en leche de varios sustratos en modelos animales. Por otro lado, determinamos tanto *in vitro* como *in vivo* si el analgésico en desarrollo URB937 es sustrato de ABCG2, así como la interacción *in vitro* de sus análogos estructurales tanto con ABCG2 como con la glicoproteína P.

Para lograr nuestros objetivos hemos llevado a cabo diversos experimentos tanto *in vitro* como *in vivo*. Para los experimentos *in vitro*, hemos utilizado células epiteliales MDCKII transducidas con diversas variantes del transportador ABCG2 (murino, humano, ovino y dos variantes bovinas). Con estas células hemos realizado experimentos de reversión de la acumulación intracelular de mitoxantrona, un sustrato fluorescente de ABCG2 muy utilizado para estos estudios, y experimentos de transporte transepitelial de fármacos en placas Transwell. Así, hemos probado cómo los dos metabolitos del antihelmíntico triclabendazol (TCBZSO y $TCBZSO_2$) eran capaces de inhibir a cada una de las variantes de ABCG2 a distintas concentraciones. Por otra parte, también comprobamos cómo la variante *wild-type* del transportador bovino es inhibida más eficazmente *in vitro* que la variante Y581S. En los experimentos de transporte transepitelial de fármacos utilizamos dos compuestos (nitrofurantoína y danofloxacina), fármacos muy utilizados como sustratos del transportador, y comprobamos cómo los metabolitos TCBZSO y $TCBZSO_2$ eran capaces de revertir el transporte mediado por ABCG2. Por lo tanto, los estudios *in vitro* realizados identificaron al TCBZ y sus metabolitos como inhibidores de las diferentes variantes de ABCG2.

Este tipo de experimentos *in vitro* se utilizaron también para confirmar la interacción del analgésico en desarrollo URB937 y sus análogos estructurales como sustratos de ABCG2 y de glicoproteína P.

En cuanto a los experimentos *in vivo*, hemos utilizado modelos de ratones *wild-type* y *knockout* para el transportador Abcg2 y ovejas lactantes de raza Assaf. Así se ha determinado cómo los metabolitos TCBZSO y $TCBZSO_2$ inhiben *in vivo* al transportador, aumentando los niveles en plasma del antiinflamatorio sulfasalazina y reduciendo los niveles en leche del antibiótico nitrofurantoína en ratones. Estos resultados se han corroborado en el caso de los experimentos con ovejas de producción lechera de raza Assaf, demostrando tras la coadministración con TCBZ, una reducción del 40% en la secreción en leche del endectocida moxidectina, sin verse afectados los perfiles plasmáticos. En el caso de la fluoroquinolona danofloxacina no se observó interacción con el TCBZ.

Los estudios *in vivo* con URB937 realizados en ratones *wild-type* y *knockout* para Abcg2 demostraron que Abcg2 restringe el acceso de URB937 al sistema nervioso central sin afectar a sus niveles en plasma ni a su distribución y actividad en los tejidos periféricos.

Las interacciones farmacológicas con el transportador de membrana ABCG2 descritas en la presente memoria permitirán un uso más seguro de la terapia antihelmíntica en medicina veterinaria y el desarrollo de nuevos analgésicos periféricos sin efectos adversos a nivel de sistema nervioso central.

2. SUMMARY

The present Thesis Dissertation studies the interaction of the anthelmintic triclabendazole (TCBZ) and its metabolites and the analgesic URB937 and its structural analogues with the ABCG2 transporter. Furthermore, in the case of URB937 and its derivatives, the study was extended to P-glycoprotein.

ABCG2 is expressed in several tissues of the organism and has a mainly protection function. Furthermore, ABCG2 is the only ABC transporter whose expression is induced during lactation in the mammary gland; thus it is involved in the transfer of compounds into milk. In addition, it is one of the transporters present in the blood-brain barrier, restricting the access of drugs into the brain. Its function in both localizations is usually studied by searching for compounds that interact as substrates or inhibitors.

Triclabendazole is a drug used in veterinary therapeutics and usually concomitantly administered with other drugs. In this case we studied the pharmacological interactions mediated by ABCG2 of the main triclabendazole metabolites at the level of the mammary gland and related with the secretion of drugs into milk. Regarding URB937 (an underdevelopment analgesic) and its structural analogues, we focused on its interaction with ABCG2 and P-glycoprotein, another ABC transporter at the level of the blood-brain barrier, where both transporters work preventing the entry of compounds into the brain. Thus, the summarized objectives of the present Dissertation consisted on one hand, of the study of the *in vitro* inhibitory potential of TCBZ and its metabolites (TCBZSO and $TCBZSO_2$) over ABCG2 in different species, and the study of the *in vivo* effect of the coadministration of TCBZ and its metabolites on the pharmacokinetics and secretion into milk of different substrates in animal models. On the other hand, we determined *in vitro* and *in vivo* whether the analgesic

URB937 is an ABCG2 substrate. In addition, the *in vitro* interaction of its structural analogues with ABCG2 and P-glycoprotein was studied.

In order to achieve our objectives we performed several *in vitro* and *in vivo* experiments. For *in vitro* experiments, we used MDCKII epithelial cells transduced with several variants of the ABCG2 transporter (murine, human, ovine and two bovine variants). With these cells we performed assays of reversion of the intracellular accumulation of mitoxantrone, a known ABCG2 fluorescence substrate, and experiments of transepithelial transport of drugs with Transwell plates. Then we tested to what extent the two triclabendazole anthelmintic metabolites (TCBZSO and $TCBZSO_2$) were able to inhibit each of the ABCG2 variants at different concentrations. We also tested to what extent the wild-type variant of the ABCG2 bovine transporter is more efficiently inhibited than the Y581S variant. In the transepithelial transport experiments, we used two compounds (nitrofurantoin and danofloxacin), drugs widely used as substrates of the transporter, and we tested in that way how the metabolites TCBZSO and $TCBZSO_2$ were able to inhibit that ABCG2-mediated transport. Therefore, our *in vitro* studies identified TCBZ and its metabolites as inhibitors of the different variants of ABCG2.

Furthermore, these type of *in vitro* experiments were used to confirm the *in vitro* interaction of the analgesic URB937 and its structural analogues as ABCG2 and P-glycoprotein substrates.

Regarding *in vivo* experiments, we used wild-type and knockout mouse models and lactating Assaf sheep. We thus determined how the metabolites TCBZSO and $TCBZSO_2$ *in vivo* inhibit the transporter, increasing the plasma

levels of the anti-inflammatory sulfasalazine and reducing the milk levels of the antibiotic nitrofurantoin in mice.

These results were corroborated with experiments in lactating Assaf sheep, showing, after TCBZ coadministration, a reduction of 40% in secretion into milk of the endectocide moxidectin, with no effect on plasma levels. In the case of the fluoroquinolone danofloxacin no interaction with TCBZ treatment was reported.

In vivo studies with URB937 in wild-type and knockout mice for Abcg2 showed that Abcg2 restricts the access of URB937 to the central nervous system without any effect on its plasma levels and its distribution and activity in peripheral tissues.

The pharmacological interactions with ABC membrane transporters described in this Dissertation will allow a safer use of anthelmintic therapy in veterinary medicine and the development of new peripheral analgesics without adverse effects at the level of the central nervous system.

3. INTRODUCCIÓN

Los transportadores pertenecientes a la familia ABC se caracterizan por presentar un *cassette* de unión al ATP (*ATP-binding cassette,* ABC). Son proteínas transmembrana que funcionan como bombas de expulsión de una gran variedad de compuestos no relacionados estructuralmente. Se considera que estas proteínas de membrana actúan como barreras de protección. Precisamente, su función es la causa de que influyan directamente en la biodisponibilidad de los fármacos. Estos transportadores se expresan en numerosos tejidos que actúan como barreras que limitan el paso de xenobióticos al organismo y la toxicidad tisular, tales como intestino, hígado y riñón. ABCG2 junto con la glicoproteína P (P-gp), ambos miembros de la familia de transportadores ABC, desempeñan un papel importante a nivel de la barrera hematoencefálica, limitando el paso de sustancias tóxicas hacia el sistema nervioso central (SNC) (Poller y cols., 2011; Nakanishi y cols., 2013). En el epitelio mamario se ha demostrado la participación de ABCG2 en la secreción de fármacos hacia la leche (Merino y cols., 2005b; van Herwaarden y Schinkel, 2006; van Herwaarden y cols., 2007), siendo por tanto un determinante fundamental en la aparición de residuos farmacológicos en leche.

El estudio de la influencia de las interacciones farmacológicas mediadas por el transportador ABCG2 en la aparición de residuos en leche es de vital importancia para una práctica clínica más segura. La coadministración de múltiples fármacos se utiliza con frecuencia en clínica veterinaria y puede afectar a la cinética, a la actividad farmacológica e incluso a la excreción de los fármacos a la leche.

Con todo ello, es importante conocer compuestos que sean capaces de inhibir a los transportadores ABC, entre ellos ABCG2. La inhibición de ABCG2 se considera como una estrategia importante en la modulación de la

biodisponibilidad de fármacos, que modificaría la distribución tisular de sus sustratos e incluso su secreción a leche. Por otro lado, se encuentra el tratamiento farmacológico del cáncer, ya que en algunos tumores hay una alta expresión del transportador y su inhibición permitiría la acumulación intracelular de agentes quimioterapéuticos y por lo tanto la mayor destrucción de células malignas.

Uno de los fármacos utilizados en medicina veterinaria para el tratamiento de parasitosis es el triclabendazol. Debido a la ausencia de estudios de su interacción con ABCG2 y a su habitual coadministración con otros fármacos, hemos realizado una caracterización de su capacidad inhibitoria tanto *in vitro* como *in vivo* sobre ABCG2.

Por otra parte, el estudio de las interacciones de los fármacos en fase de desarrollo con transportadores ABC, constituye un pilar fundamental en la industria farmacéutica (Giacomini y cols., 2010). Por lo tanto, conocer si un compuesto es sustrato de transportadores ABC nos permite anticipar su comportamiento farmacocinético y sus interacciones farmacológicas. Esto cobra especial relevancia en el caso de la entrada de compuestos en el SNC, influenciada por transportadores ABC, principalmente ABCG2 y glicoproteína P, que en algunos casos puede ser beneficiosa y deseable (como en el caso del tratamiento de metástasis de cerebro) pero, sin embargo, en otros casos puede querer evitarse debido a los efectos adversos que pueden provocar ciertos compuestos a nivel del SNC (como en el caso de los analgésicos periféricos como el URB937, compuesto objeto de estudio en parte de la presente memoria). Es también interesante la comprensión de las características estructurales de los compuestos que pueden llegar a intervenir en estos procesos. Así, hemos realizado el estudio de la interacción del URB937 y sus análogos estructurales como

sustratos de los dos principales transportadores presentes en la barrera hematoencefálica, la proteína ABCG2 y la glicoproteína P.

4. REVISIÓN BIBLIOGRÁFICA

4.1. TRANSPORTADORES DE MEMBRANA DEPENDIENTES DE ATP.

Los transportadores de membrana dependientes de ATP, también conocidos como transportadores ABC debido a su denominación en inglés (*ATP-Binding Cassette*), son proteínas que actúan como transportadores activos primarios o "bombas exportadoras". Su amplia presencia en prácticamente todos los organismos vivos (Glavinas y cols., 2004), con una estructura muy conservada, sugiere que tienen un papel importante en la función celular; de hecho, están implicados en el transporte de lípidos, sales biliares, colesterol, hormonas, esteroides, metabolitos y otros compuestos considerados como tóxicos (Borst y Elferink, 2002; Eisenblatter y Galla, 2002). Los transportadores ABC exportan, a través de la membrana plasmática, compuestos de diferente naturaleza entre los que se incluyen metabolitos endógenos, fármacos, xenotoxinas y agentes carcinogénicos; de este modo, en general, ejercen una función protectora ya que afectan a la exposición sistémica y la penetración tisular de sus sustratos mediante la excreción de dichos compuestos en hígado, intestino, riñón, etc. Este transporte se produce utilizando la hidrólisis de ATP en contra de un gradiente de concentración. Además, múltiples referencias demuestran la implicación de estos transportadores en el fenómeno de multirresistencia a fármacos antitumorales (Jonker y cols., 2000, 2005) ya que, en tumores, la sobreexpresión de estos transportadores disminuye la entrada de fármacos anticancerígenos en las células, reduciendo la eficacia de estos tratamientos.

En mamíferos, las proteínas ABC están constituidas por doce dominios transmembrana distribuidos en dos mitades homólogas, dos zonas de unión al ATP ubicadas en la parte citoplasmática y una zona altamente glicosilada hacia la parte externa de la membrana (Figura 1). Esta estructura básica puede estar

presente en una única proteína de una cadena polipeptídica (*full-transporters*), o en dos proteínas separadas (*half-transporters*) (Figura 2); en este último caso, los transportadores ABC pasan a ser funcionalmente competentes después de una dimerización específica mediante puentes disulfuro (Robey y cols., 2009).

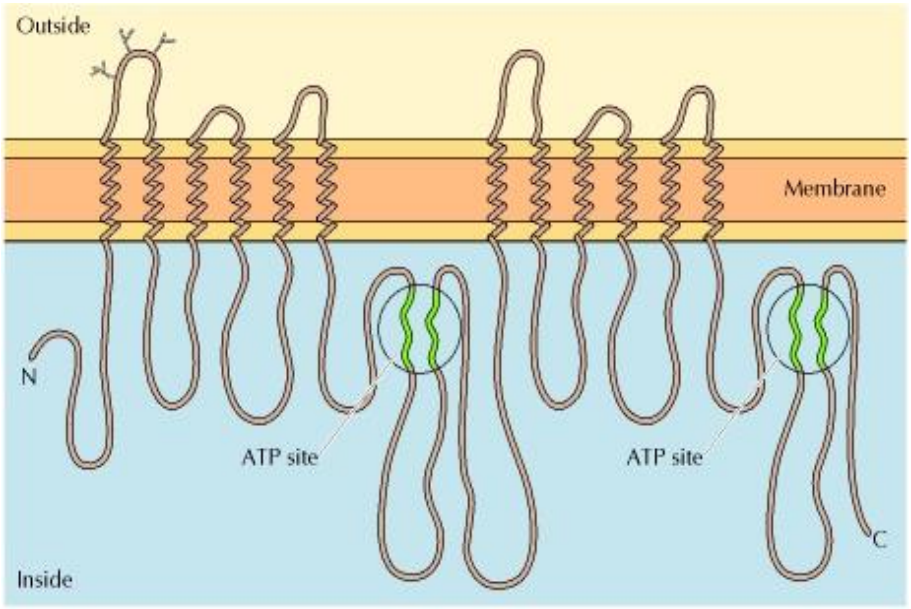


Figura 1. Estructura general de un transportador ABC de mamífero (*full-transporter*).

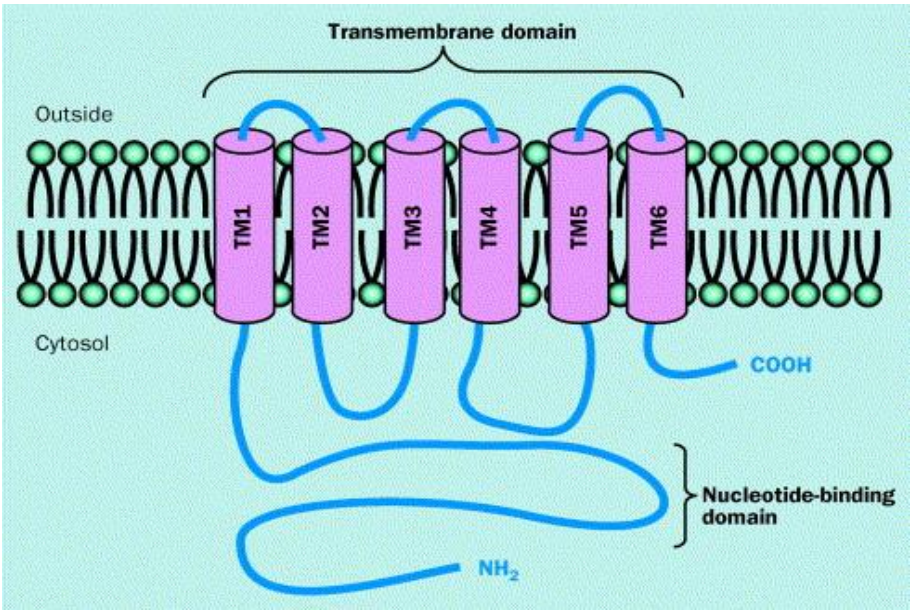


Figura 2. Estructura de un *half-transporter* de la familia ABC (Lage y Dietel, 2000)

Entre todos los transportadores pertenecientes a la familia ABC, hay 13 que se relacionan con el transporte y la resistencia a fármacos (Gillet y cols., 2007). Algunos de estos transportadores se han estudiado en mayor profundidad, como es el caso del transportador ABCB1/MDR1 ó glicoproteína P (P-gp), las proteínas asociadas a la resistencia de fármacos (MRPs 1-6) y el transportador ABCG2/BCRP (*Breast Cancer Resistance Protein*). Todos ellos se localizan en las zonas basolaterales y/o apicales de la membrana plasmática y son capaces de transportar compuestos estructuralmente diferentes, así como metabolitos y derivados conjugados de una amplia variedad de fármacos.

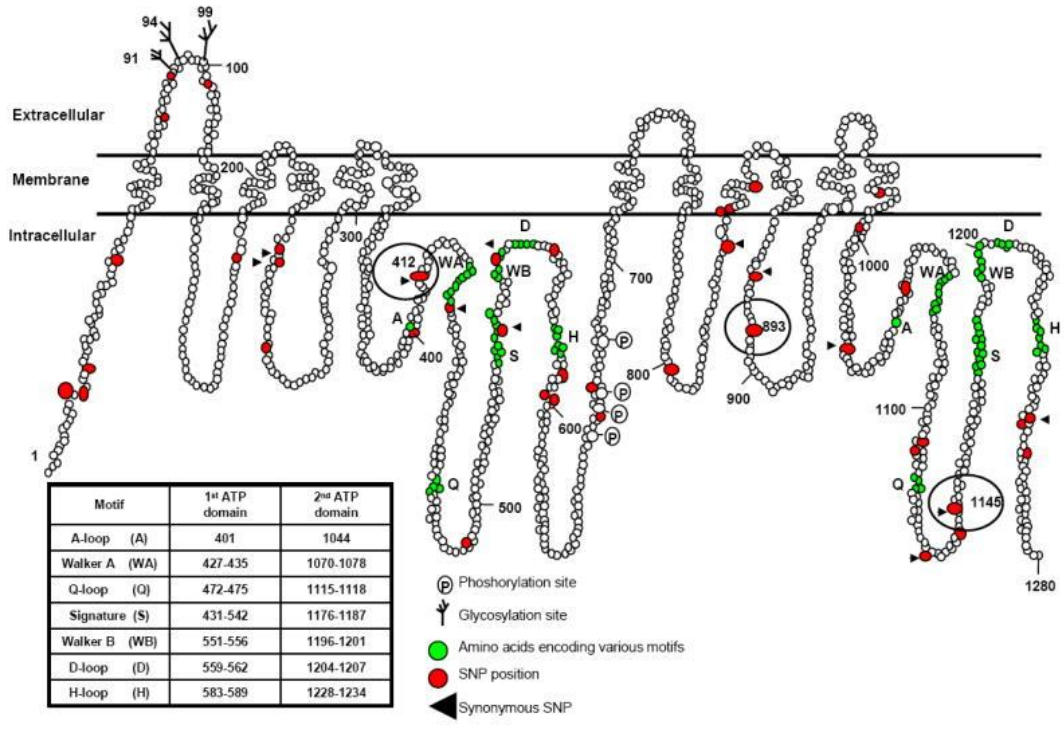
En los últimos años se ha puesto de manifiesto que la interacción entre fármacos y transportadores de membrana dependientes de ATP y otras proteínas transportadoras es uno de los mecanismos clave que sustenta la importancia clínica de las interacciones entre fármacos (Marchetti y cols., 2007).

4.2. TRANSPORTADOR ABCB1/MDR1/GLICOPROTEÍNA P.

El transportador ABCB1 fue el primer miembro de la familia de transportadores ABC que se descubrió y ha sido el que más se ha estudiado a lo largo del tiempo. Su descubrimiento se realizó en 1976, cuando Rudy Juliano y Victor Ling identificaron una glicoproteína de membrana de alto peso molecular que parecía alterar la permeabilidad de la membrana en las quimioterapias y que se sobreexpresaba en células resistentes a la colchicina. A esta proteína de membrana se la denominó glicoproteína P ó P-gp (Juliano y Ling, 1976).

La glicoproteína P (P-gp) está codificada por el gen *ABCB1* (*MDR1* en humanos; *mdr1a* y *mdr1b* en roedores) (Schinkel, 1997) el cual se encuentra localizado en humanos en el cromosoma 7 en la banda p21-21.1 (Fojo y cols.,

1986) y su ADN codificante ocupa unas 4,5 Kb, con 28 exones que varían de tamaño entre 49 y 587 pb (Chen y cols., 1990). Este gen codifica para un polipéptido de 1280 aminoácidos con un peso molecular aparente de 170 kD que posee 12 segmentos transmembrana distribuidos en dos mitades homólogas. Cada una de estas mitades contiene 6 segmentos transmembrana unidos por un gran lazo citoplasmático; además presenta dos sitios de unión para el ATP. Estudios *in vitro* han demostrado que la actividad ATPasa puede ser inducida por la presencia de sustratos del transportador (Kerr y cols., 2001). Este transportador se caracteriza porque el primer lazo extracelular está altamente glicosilado (Figura 3) aunque parece ser que la ausencia de glicosilación en esta zona no afecta a la función de la proteína (Schinkel y cols., 1993). También se han identificado varios sitios de fosforilación, pero estudios con mutantes en cultivos celulares han mostrado que estos sitios no son responsables de la localización ni de la función de la proteína (Gribar y cols., 2000; Paterson y Gottesman, 2007).



Motif	1st ATP domain	2nd ATP domain
A-loop (A)	401	1044
Walker A (WA)	427-435	1070-1078
Q-loop (Q)	472-475	1115-1118
Signature (S)	431-542	1176-1187
Walker B (WB)	551-556	1196-1201
D-loop (D)	559-562	1204-1207
H-loop (H)	583-589	1228-1234

Figura 3. Hipotético modelo bidimensional de P-gp humana. Cada círculo representa un residuo aminoácido (revisado por Fung y Gottesman, 2009).

Hace algunos años, se especulaba con que P-gp podía trabajar como una flipasa, de modo que el sustrato inicialmente interactuaba con la parte interna de la bicapa lipídica y, a continuación P-gp volteaba el compuesto hacia la parte exterior (Higgins, 1992). El modelo más aceptado actualmente es el llamado de la "bomba de vacío hidrofóbica" (Figura 4) en el cual el sustrato interacciona directamente con la zona de unión del fármaco a la proteína y éste se bombea al espacio extracelular asistido por la hidrólisis de ATP a ADP (Ambudkar y cols., 2003). Este modelo fue demostrado en 2009 cuando se consiguió cristalizar la proteína y mediante rayos X se observó una cavidad interna cubicada de aproximadamente 6000 Å y separada unos 30 Å de los dos sitios de unión a nucleótidos (Aller y cols., 2009).

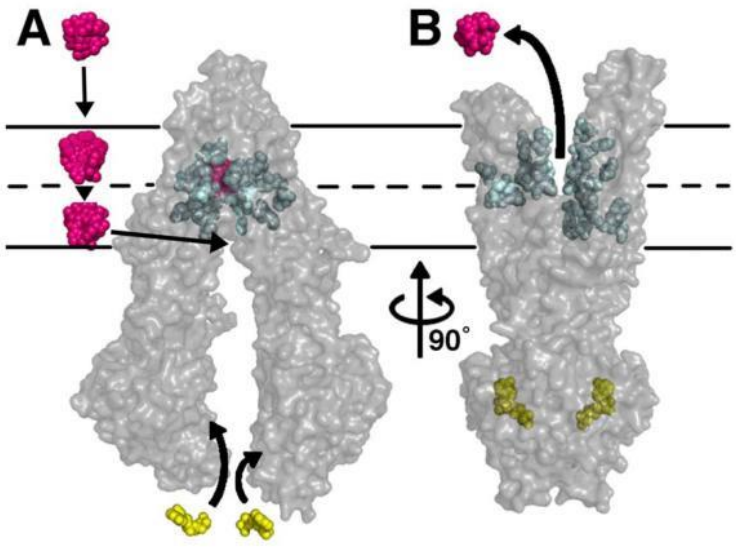


Figura 4. Modelo del transporte de un sustrato por la proteína P-gp. (A) El sustrato (rosa) entra en la bicapa lipídica desde el exterior de la célula y luego pasa a la zona de unión del sustrato situada en la proteína. (B) El ATP (amarillo) se une a los sitios de unión de ATP de la proteína y produce un cambio conformacional de la misma exponiendo al sustrato hacia el exterior y cerrando el paso hacia el interior de la proteína para asegurar el transporte en una sola dirección hacia el exterior (Tamaki y cols., 2011).

El mecanismo de unión del sustrato y el reconocimiento de sustratos e inhibidores por parte de P-gp es complejo. Se ha encontrado que la proteína posee

un espacio grande para la unión del fármaco formado por los dominios transmembrana 5, 6, 11 y 12 que determina la especificidad del sustrato (Kajiji y cols., 1993; Loo y Clarke, 1994; Zhang y cols., 1995). La presencia de esta zona se ve apoyada por los resultados obtenidos en experimentos de marcajes de fotoafinidad con análogos de sustratos de P-gp. Sin embargo, hay mutaciones de aminoácidos en otras partes del transportador que también cambian la especificidad del sustrato, lo que sugiere que el reconocimiento de fármacos es un proceso complejo (Doige y Sharom, 1992; Ambudkar, 1995).

Análisis termodinámicos revelaron que tanto las interacciones del sustrato con P-gp como las del sustrato con los lípidos de la membrana plasmática contribuyen a la afinidad del bombeo. La cantidad bombeada de fármaco depende de la afinidad de la proteína por el sustrato y de los cambios conformacionales de ésta. Los ratios de transporte parecen no estar limitados exclusivamente por la cantidad de ATP hidrolizado y pueden estar parcialmente controlados por la cantidad de fármaco disociado (Clay y Sharom, 2013).

Análisis de las propiedades físico químicas de compuestos, revelaron que un número limitado de los sustratos de P-gp (<10%) poseían un área de superficie polar topológica menor de 60 Å mientras que más del 75% de sus sustratos poseían un área de superficie polar topológica mayor de 60 Å (Desai y cols., 2013).

La proteína se encuentra ampliamente distribuida en muchos tejidos y se localiza en la membrana plasmática, específicamente en la porción apical/luminal de las células epiteliales pertenecientes al borde en cepillo del intestino, en la membrana de los canalículos biliares del hepatocito, la membrana luminal de las células epiteliales del túbulo proximal del riñón y en las células endoteliales de la

barrera hematoencefálica; además, también se ha observado su localización en ovario, testículo, células de la médula espinal y en placenta y endometrio de mujeres embarazadas (Zhu, 1999; Cui y cols., 2009; revisado por Fung y Gottesman, 2009). En particular, en la placenta de humanos, P-gp posee una elevada expresión en la membrana apical de los sincitiotrofoblastos maternos (Mac Farland y cols., 1994; Nakamura y cols., 1997).

La distribución tisular de P-gp y los resultados obtenidos utilizando ratones *knockout* sugieren que ésta juega un papel importante en la excreción de xenobióticos y de sustratos endógenos vía la membrana canalicular de los hepatocitos hacia la bilis y vía la membrana del borde en cepillo de los enterocitos y los túbulos proximales hacia el lumen intestinal y la orina respectivamente (Mayer y cols., 1996; Kim y cols., 1998); además bloquea la transferencia de compuestos xenobióticos hidrofóbicos a través de la placenta (Nakamura y cols., 1997) y previene la entrada de sustratos al SNC, ya que forma parte de la barrera hematoencefálica (Cordon-Cardo y cols., 1989; Schinkel y cols., 1996; Schinkel A.H., 1997), como veremos más adelante. En resumen, la proteína P-gp actúa en defensa del organismo.

Este transportador acepta un amplio espectro de compuestos estructural y funcionalmente no relacionados (Tabla 1).

Tabla 1. Principales sustratos e inhibidores de los transportadores glicoproteína P y ABCG2 (modificada de Ieiri I., 2012).

Transportador	Sustratos	Inhibidores
Glicoproteína-P (Pgp ó ABCB1)	**Antitumorales** (docetaxel, etopósido, paclitaxel, topotecan, vinblastina), **antihipertensivos** (diltiazem, losartan), **antiarrítmicos** (digoxina, verapamil), **antivirales** (indinavir, nelfinavir), **antibióticos** (eritromicina, esparfloxacina), **inmunosupresores** (ciclosporina, tacrolimus), **otros** (cimetidina, fexofenadina, loperamida, fenitoína, morfina, ondansetrón)	Amiodarona, amitriptitila, diltiazem, dipyridamole, fenotiazinas, propafenona, propanolol, quinidina, espironolactona, tamoxifeno
BCRP (ABCG2)	**Antitumorales** (epirrubicina, topotecan, doxorrubicina, daunorrubicina, etopósido, SN-38, gefitinib, imatinib, irinotecan, metotrexato, mitoxantrona), a**ntihipertensivos** (reserpina, prazosin), a**ntiinflamatorios** (sulfasalazina), a**ntibióticos** (nitrofurantoína, ciprofloxacina, ofloxacina, norfloxacina), **antivirales** (zidovudina, lamivudina), **otros** (genisteína, quercetina, pravastatina, rosuvastatina, cimetidina, estrona-3-sulfato, dehidroepiandrosterona sulfato, 17-β-D-estradiol-glucurónido, dinitrofenil-S-glutatión)	Cilclosporina A, tacrolimus, sirolimus, gefitinib, imatinib, naringenina, quercetina, genisteína, 17-β-D-estradiol, estrona, tamoxifeno, reserpina, curcumina, dipiridamol, nicardipeno, nitrendipeno, nimodipeno

4.3. TRANSPORTADOR ABCG2/BCRP.

4.3.1. Estructura de la proteína.

La proteína ABCG2 o BCRP (*Breast Cancer Resistance Protein*) es el segundo miembro de la subfamilia G de las proteínas o transportadores denominados ABC. La subfamilia G de transportadores ABC se caracteriza porque el dominio de unión al ATP se sitúa próximo al extremo N-terminal y no al carboxilo terminal, como ocurre en el resto de subfamilias pertenecientes a este grupo (Krishnamurthy y Schuetz, 2006). El gen *ABCG2* humano se localiza en el cromosoma 4q22, tiene 66 Kb y consta de 16 exones (Figura 5).

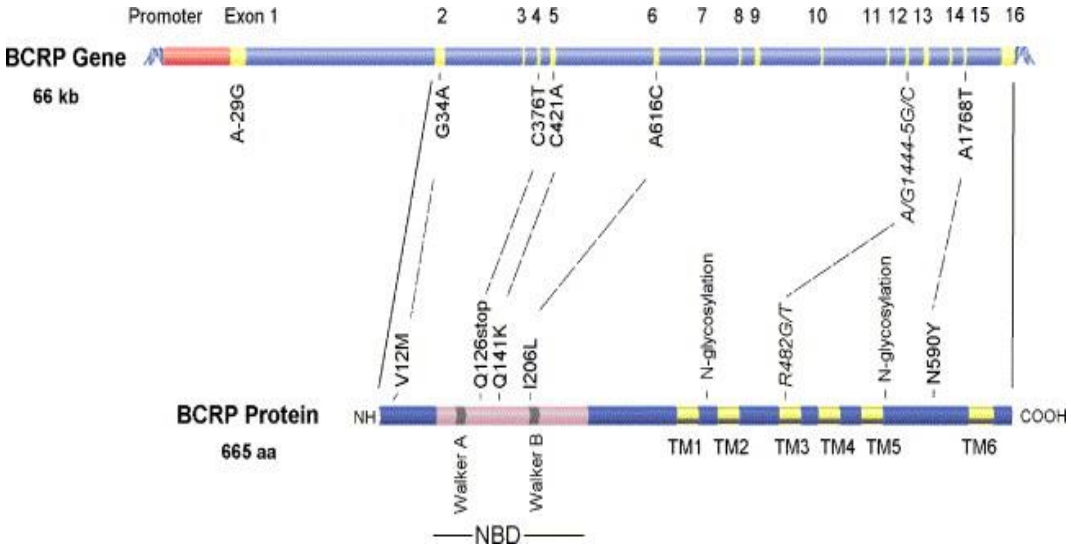


Figura 5. Estructura del gen *ABCG2* humano y de la proteína derivada mostrándose algunos de los polimorfismos descritos en humana (Staud y Pavek, 2005). NBD: *Nucleotide Binding Domain*, región de unión de nucleótidos; TM: *Transmembrane regions*, regiones transmembrana.

El transportador ABCG2 humano consta de 655 aminoácidos y posee 6 hélices transmembrana (residuos 397 a 655) y un sitio de unión a ATP (residuos 1 a 396). De los 3 posibles lugares de glicosilación, sólo una asparagina en posición 596 del lazo extracelular parece estar glicosilada (Figura 6), pero esto no resulta imprescindible para la correcta localización y funcionalidad del transportador (Diop y Hrycyna, 2005). ABCG2 es un *half-transporter* y requiere de una

homodimerización para ser funcional, lo cual fue confirmado mediante ensayos en oocitos de *Xenopus laevis* (Nakanishi y cols., 2003). Estudios bioquímicos posteriores han permitido observar ABCG2 en forma de homotetrámero que actuaría como modulador de la forma ABCG2 homodímera (Xu y cols., 2004). Se ha postulado que la formación de puentes disulfuro, especialmente en la cisteína 603, tiene un importante papel en la generación de dímeros/multímeros (Kage y cols., 2005). Sin embargo, estudios recientes de resonancia con fluorescencia en células han demostrado que la cisteína 603 no es esencial para la formación de dímeros/multímeros; además otros residuos como la cisteína 592 o la cisteína 608, también implicados en la formación de puentes disulfuro, podrían participar en la formación de los oligómeros (Ni y cols., 2010).

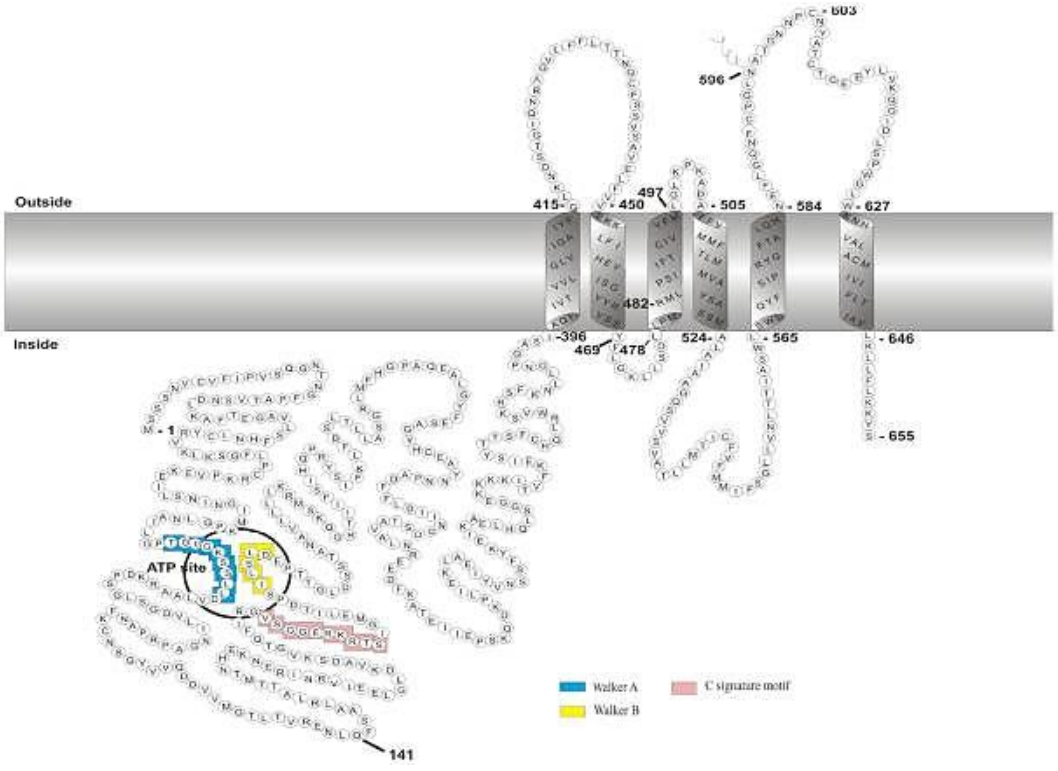


Figura 6. Conformación de ABCG2 en la membrana plasmática. Se observa el sitio de unión a ATP y el dominio transmembrana formado por 6 regiones (Ni y cols., 2010).

4.3.2. Distribución tisular y función fisiológica.

ABCG2 participa en la multirresistencia a fármacos y en su distribución corporal y puede actuar como defensa frente a la acumulación de toxinas en el organismo, algunas de las cuales proceden de la dieta (Eisenblatter y Galla, 2002; Porlgar y cols., 2008).

En mamíferos, esta proteína presenta una amplia localización, se sitúa en la membrana apical de las células epiteliales de intestino, colon, hígado, placenta, médula ósea, cerebro, pulmón, y riñón (revisado por Klaassen y Aleksunes, 2010). Otros tejidos donde se expresa el transportador son el tejido hematopoyético, la barrera hematoencefálica, la barrera placentaria, la barrera hematotesticular y la glándula mamaria (Figura 7). De todos los tejidos donde podemos encontrar ABCG2, la máxima expresión se encuentra en la placenta (Nishimura y Naito, 2005), seguida de intestino delgado.

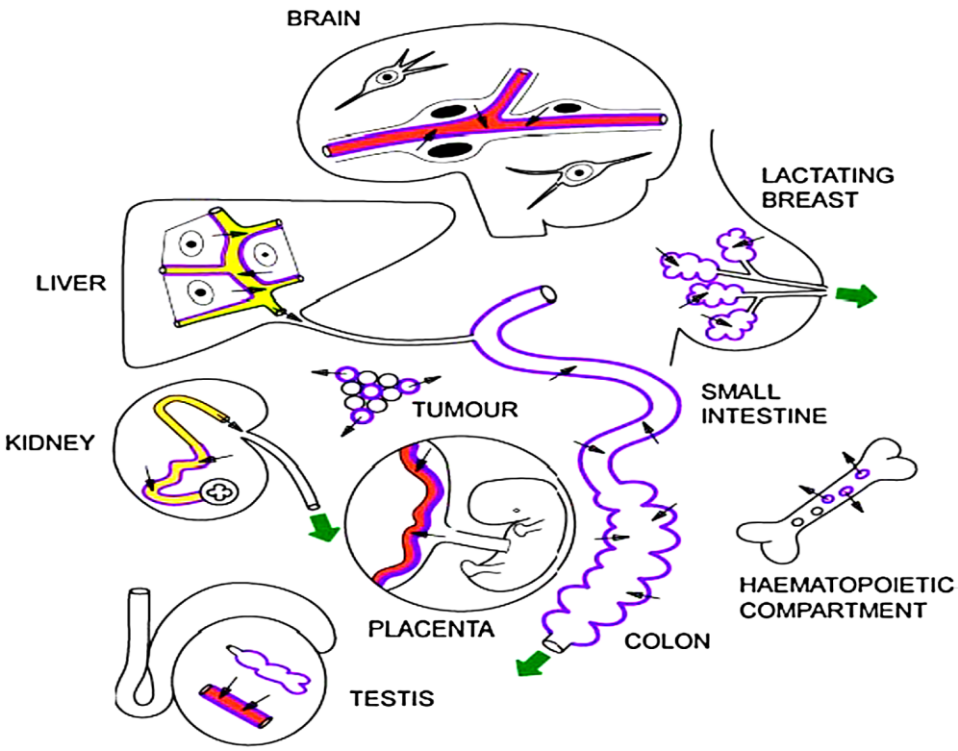


Figura 7. Órganos en los que se localiza el transportador ABCG2. Las flechas pequeñas indican la dirección del transporte mediado por ABCG2 en cada órgano. Las flechas grandes indican la excreción neta en el organismo de los sustratos de ABCG2. La expresión en células endoteliales, capilares y venas en los distintos tejidos no aparece indicada (Vlaming y cols., 2009).

En el **epitelio mamario** se ha demostrado su participación en la secreción de fármacos hacia la leche (Merino y cols., 2005b; van Herwaarden y Schinkel, 2006; van Herwaarden y cols., 2007) y debido a su importancia para el desarrollo de la presente memoria dicha función será tratada en el próximo apartado.

En **placenta**, el transportador está localizado en la membrana plasmática de los sincitiotrofoblastos, limitando la exposición del feto a toxinas, fármacos y xenobióticos y ejerciendo, por tanto, un papel protector al expulsar los fármacos y toxinas hacia el torrente sanguíneo de la madre. En estudios *ex vivo*, ABCG2 humana y de rata han demostrado disminuir significativamente el transporte materno-fetal de PhIP (Myllynen y cols., 2008), glibenclamida (Pollex y cols., 2008) y cimetidina (Staud y Pavek, 2005). En ratones gestantes Mdr1a/b$^{-/-}$, la coadministración del inhibidor de Abcg2 GF120918 causó un aumento significativo en la concentración fetal de topotecan (Jonker y cols., 2000). Además, se examinó el papel de Abcg2 en la exposición fetal de nitrofurantoína en ratones gestantes, sugiriendo claramente que Abcg2 limita significativamente la distribución de nitrofurantoína al feto (Zhang y cols., 2007). También se observó que los niveles fetales de glibenclamida en los ratones Abcg2$^{-/-}$ en gestación fue dos veces mayor que en el caso de ratones *wild-type* (Zhou y cols., 2008). Estos estudios respaldan el papel de ABCG2/Abcg2 en la limitación de la exposición fetal a xenobióticos.

En el **intestino delgado**, tanto en ratón como en humanos, ABCG2 se localiza en el borde en cepillo de los enterocitos, limitando la absorción intestinal de determinados compuestos ingeridos (Maliepaard y cols., 2001; Jonker y cols., 2002). Se ha demostrado también un aumento de la absorción intestinal de antibióticos (Merino y cols., 2006), flavonoides como la quercetina (Sesink y

cols., 2005), antiinflamatorios como la sulfasalazina (Zaher y cols., 2006) y de compuestos carcinógenos como la aflatoxina B1 y la toxina 2-amino-1-metil-6 phenylimidazo [4,5-b] piridina (PhIP) (van Herwaarden y cols., 2006) en ratones Abcg2$^{-/-}$ en comparación con ratones *wild-type*, proporcionando una sólida evidencia para justificar el papel de ABCG2 en la absorción de fármacos y otros compuestos tras su administración por vía oral.

En **hígado**, ABCG2 se expresa en la membrana canalicular de los hepatocitos (Fetsch y cols., 2006), en los conductos y conductillos biliares, los vasos sanguíneos del endotelio hepático (Vander Borght y cols., 2006) y en la membrana luminal de las células epiteliales de la vesícula biliar (Aust y cols., 2004). ABCG2 interviene en el transporte de diversos compuestos hacia el canalículo biliar y por tanto, en su excreción a bilis (Merino y cols., 2005b; Zamek-Gliszczynski y cols., 2006; Ando y cols., 2007).

En **riñón,** la proteína se localiza en el túbulo cortical (Fetsch y cols., 2006) y en el borde en cepillo de las células del túbulo proximal (Huls y cols., 2008), participando en la excreción de fármacos y compuestos endógenos como el urato a este nivel (Mizuno y cols., 2004 y 2007; Woodward y cols., 2009).

ABCG2, junto con P-gp, desempeña un papel importante a nivel de la **barrera hematoencefálica,** limitando el paso de sustancias tóxicas hacia el SNC, función que será tratada en profundidad más adelante en la presente memoria.

La expresión de ABCG2 en las células madre multipotentes (*stem*) tiene también una especial relevancia. Los trabajos de Zhou y cols. (2002) en médula ósea de ratón y, posteriormente de Scharenberg y cols. (2002) en humanos permitieron identificar a ABCG2 como un marcador de **células *stem*** (multipotentes) a través de la expulsión del Hoechst 33342, un colorante

fluorescente, sustrato del transportador. ABCG2 se expresa en una subpoblación de las células *stem*, denominada SP (*side population cells*), presente en la mayoría de los tejidos corporales (Fatima y cols., 2012) incluyendo tejido muscular y óseo. Esta subpoblación SP presenta características de células *stem* ya que pueden autorrenovarse y diferenciarse al responder a las vías de señalización propias de las células *stem*; además son resistentes a agentes quimioterapéuticos, lo cual puede ser debido a la expresión de este transportador. Las células SP procedentes de ratones Abcg2$^{-/-}$ son más sensibles a la mitoxantrona, un agente quimioterapéutico sustrato de Abcg2 (Zhou y cols., 2002). La expresión de ABCG2 parece que se correlaciona con el mantenimiento de la población en situación de pluripotencialidad, participando en la autorrenovación, así como en la regeneración de tejido dañado (Huls y cols., 2008).

4.3.3. Sustratos e inhibidores del transportador.

El transportador BCRP/ABCG2 posee la capacidad de transportar un amplio espectro de compuestos, solapándose en algunos casos con sustratos de otros transportadores como P-gp (Tang y cols., 2012). ABCG2 transporta fundamentalmente moléculas hidrofóbicas cargadas positiva o negativamente. Cabe destacar que la mayoría de las células que sobreexpresan ABCG2 son resistentes a agentes quimioterapéuticos como la mitoxantrona, topotecan, irinotecan, indolcarbazoles, antifolatos e inhibidores de tirosina quinasas como imatinib, gefitinib y erlotinib (Polgar y cols., 2008). Otros sustratos del transportador (Tabla 1) son algunos antibióticos (nitrofurantoína, fluoroquinolonas) (Merino y cols., 2005b; Álvarez y cols., 2008), la vitamina B2 y la biotina (van Herwaarden y cols., 2007), el ácido úrico (Woodward y cols., 2009) y los ácidos biliares (Blazquez y cols., 2012) entre otros (revisado por Ieiri

I., 2012). Incluso algunos antiinflamatorios como por ejemplo la sulfasalazina han sido descritos como sustratos tipo *in vivo* de Abcg2 (Zhang y cols., 2006). ABCG2 también interacciona con las porfirinas y análogos de porfirinas (Krishnamurthy y cols., 2004) como la feoforbida A, metabolito clorofílico empleado como fotosensibilizador en el tratamiento de tumores (Robey y cols., 2004).

Además de la búsqueda de sustratos del transportador, también es importante conocer compuestos que sean capaces de inhibirlo. La inhibición de ABCG2 se considera como una estrategia para el tratamiento farmacológico del cáncer, ya que en algunos tumores hay una alta expresión del transportador y su inhibición permitiría la acumulación intracelular de agentes quimioterapéuticos y por lo tanto la mayor destrucción de células malignas. También es importante la modulación de la biodisponibilidad de sus sustratos, que modifica su distribución tisular e incluso su secreción a leche, como veremos más adelante. Por ejemplo, el empleo de curcumina, un producto natural no tóxico, como inhibidor de Abcg2 intestinal produce un aumento en la biodisponibilidad del sustrato sulfasalazina (Shukla y cols., 2009). Efectos secundarios adversos asociados con el retraso en la eliminación de metotrexato han sido atribuidos a la coadministración de protectores gástricos (por ejemplo, omeprazol y pantoprazol) (Reid y cols., 1993; Troger y cols., 2002; Santucci y cols., 2010), y la inhibición de la secreción renal vía ABCG2 fue propuesta como el mecanismo de interacción (Breedveld y cols., 2004). Además, el diclofenaco puede inhibir el transporte de metotrexato mediado por ABCG2 *in vitro* (Lagas y cols., 2009), lo que puede explicar al menos parcialmente, la disminución del aclaramiento plasmático en pacientes en los que el metotrexato es coadministrado con diclofenaco (Thyss y cols., 1986).

En cuanto a los inhibidores de ABCG2, el primero descrito fue la fumitremorgina C (Rabindran y cols., 2000), una toxina aislada de *Aspergillus fumigatus*. Su análogo Ko143, es un inhibidor de ABCG2 ampliamente utilizado en ensayos de laboratorio (Allen y cols., 2002) debido a su menor toxicidad. Además se conoce un amplio número de sustancias inhibidoras (Tabla 1) entre los que se encuentran compuestos esteroideos como corticosterona y digoxina, antivirales como el ritonavir y saquinavir, inmunosupresores como el tacrolimus y sirulimus, inhibidores de tirosina quinasa como imatinib y nilotinib, flavonoides como naringenina, daidzeína y genisteína y otros compuestos como tamoxifeno, reserpina, curcumina, etc (revisado por Ieiri I., 2012 y Schnepf y Zolk., 2013). En el caso de los antirretrovirales, se han descrito tanto como sustratos (zidovudine y abacavir) como inhibidores (lopinavir, nelfinavir, delavirdine, efavirenz, atazanavir, amprenavir y abacavir) del transportador (Wang y cols., 2004; Pan y cols., 2007; Weiss y cols., 2007).

La estructura de los compuestos va a ser determinante a la hora de interaccionar con ABCG2. Se ha sugerido que la presencia de un grupo amino unido a un anillo heterocíclico, así como la fusión de anillos heterocíclicos, es determinante en las interacciones de compuestos con el transportador (Giacomini y cols., 2010). Hay estudios que demuestran que el coeficiente de reparto octanol-agua a un pH de 7,4 y la polarizabilidad molecular son componentes importantes para las interacciones de fármacos inhibidores con ABCG2 (Nicolle y cols., 2009).

A pesar de que el número de sustratos e inhibidores de ABCG2 descritos es elevado, todavía no está clara la relación entre la estructura de la proteína y las características específicas de sus sustratos e inhibidores.

4.3.4. Importancia de los polimorfismos del transportador.

En cuanto a los polimorfismos de *ABCG2* en humanos se han realizado diversos estudios en varios grupos étnicos y se han encontrado más de 50 SNPs (*Single Nucleotide Polimorfisms*) (Figura 8) (Iida y cols., 2002; Zamber y cols., 2003; Kobayashi y cols., 2005). Al igual que para otros transportadores, la frecuencia de los SNPs muestra diferencias inter-étnicas. Notablemente, dos mutaciones no sinónimas, 34G>A (12Val>Met en el exón 2) y 421C>A (141Gly>Lys en el exón 5) presentan una mayor frecuencia en la población asiática en comparación con la caucásica y la africano americana. Curiosamente, tres SNPs no sinónimos, 34G>A, 376C>T, (126Gln>codón de stop en el exón 4), y 421C>A ocurren simultáneamente (Revisado por Ieiri I., 2012).

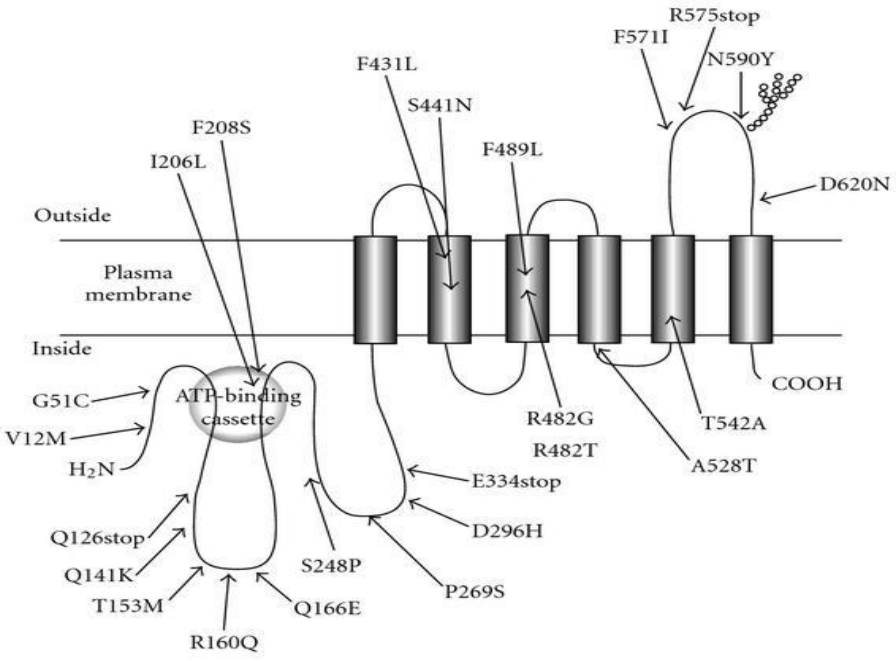


Figura 8. Representación esquemática del transportador ABCG2 humano y localización de varios de sus polimorfismos no sinónimos descritos (revisado por Ishikawa y cols., 2010).

Los estudios farmacogenéticos están enfocados principalmente en el SNP común de ABCG2 421C>A, que tiene profundos efectos potenciales en la farmacocinética de sustratos de ABCG2 en la mayoría de la población. De hecho,

el SNP 421C>A reduce los niveles de expresión de ABCG2 y altera la especificidad de sus sustratos, y se ha asociado con una mayor acumulación en plasma de los sustratos de ABCG2 administrados de forma oral, tales como el topotecan, diflomotecan, gefitinib y sunitinib (revisado por Giacomini y cols., 2013). Recientemente, se ha demostrado una importante asociación entre este polimorfismo y el desarrollo de gota debido a niveles elevados de ácido úrico (Woodward y cols., 2009).

En cuanto a los polimorfismos en otras especies, como los rumiantes, Cohen-Zinder y cols. (2005) describieron un polimorfismo en vacuno que afecta a ABCG2, el polimorfismo no sinónimo Y581S, que se localizó en el exón 14 del gen bovino. Estos autores relacionaron este SNP con un mayor contenido en grasa y proteínas en leche y una menor producción en vacas de raza Holstein.

En cuanto al efecto del SNP Y581S bovino sobre el transporte de fármacos, estudios iniciales de nuestro grupo de investigación, demostraron diferencias significativas en la acumulación de mitoxantrona entre ambas variantes tras su clonación y expresión transitoria (Merino y cols., 2009). Posteriormente, la generación de células MDCKII con expresión estable de ambas variantes, permitió demostrar que la variante Y581S bovina muestra un mayor transporte *in vitro* de algunas fluoroquinolonas como la danofloxacina, la enrofloxacina, la difloxacina (Real y cols., 2011), la marbofloxacina, la orbifloxacina, la sarafloxacina y la vitamina riboflavina (Gonzalez-Lobato, 2012, Tesis Doctoral). Además, también se han observado diferencias en la inhibición de dichas variantes bovinas de ABCG2 por parte de la lactona macrocíclica ivermectina mostrando una mayor potencia inhibitoria en la proteína *wild-type* a las concentraciones probadas (Real y cols., 2011). Recientemente, se ha

demostrado también *in vivo* en nuestro laboratorio una mayor secreción a leche de la fluoroquinolona danofloxacina por parte de los animales portadores de la variante Y581S (Otero y cols., 2013).

4.4. PAPEL DE ABCG2 EN EL TRANSPORTE DE COMPUESTOS A LECHE.

Es importante conocer los mecanismos por los que fármacos y metabolitos pasan al tejido mamario, siendo muchos de ellos secretados activamente en la leche, ya que el tratamiento con fármacos en los animales de producción lechera puede ocasionar la aparición de residuos en leche. Los peligros para la salud a causa de estos residuos pueden ser tóxicos, microbiológicos e inmunopatológicos, al provocar alergias, hipersensibilidad o ser mutagénicos, teratogénicos y/o carcinogénicos (revisado por McManaman y Neville, 2003). Además, existen cada vez más estudios (Martin y cols., 2000; Williams y Philips, 2000; Mukherjee y cols., 2006) que evidencian un origen epigenético en el incremento del cáncer de mama como resultado de alteraciones medioambientales y presencia de tóxicos en los alimentos. Aparte de esto, es importante destacar que estos residuos causan graves daños económicos en la industria láctea, ya que la elaboración de productos derivados de la leche, como queso y yogurt, necesitan del desarrollo de la flora ácido-láctica, la cual es inhibida por pequeñas concentraciones de antibióticos que puedan estar presentes en esa leche.

En el caso de los animales, los niveles de residuos de fármacos en leche están regulados, de forma explícita, a través de las agencias de control del medicamento. Así, la normativa legal dictaminada por la EMEA (Agencia Europea del Medicamento) exige la espera, por parte de los productores, de un

tiempo mínimo que garantice la eliminación del fármaco hasta conseguir niveles inferiores a los límites máximos de residuos. Sin embargo, este tiempo de espera representa un tiempo muerto para los ganaderos, ya que se asocia con pérdidas económicas y, por tanto, no siempre se cumplen (Imperiale y cols., 2004).

La naturaleza del transporte activo implicado en estos procesos permaneció sin dilucidar hasta que los estudios de Jonker y cols. (2005) probaron, por primera vez, el papel funcional de ABCG2 en la glándula mamaria. Estudios inmunohistoquímicos y por *western blot* realizados en diferentes estados de desarrollo de la glándula mamaria, revelaron que Abcg2 no se expresa en ratones hembra de 8 ó 14 semanas de edad, pero durante la gestación y, especialmente en la lactación, aumenta su expresión considerablemente, disminuyendo rápidamente durante la involución de la glándula (Figura 9).

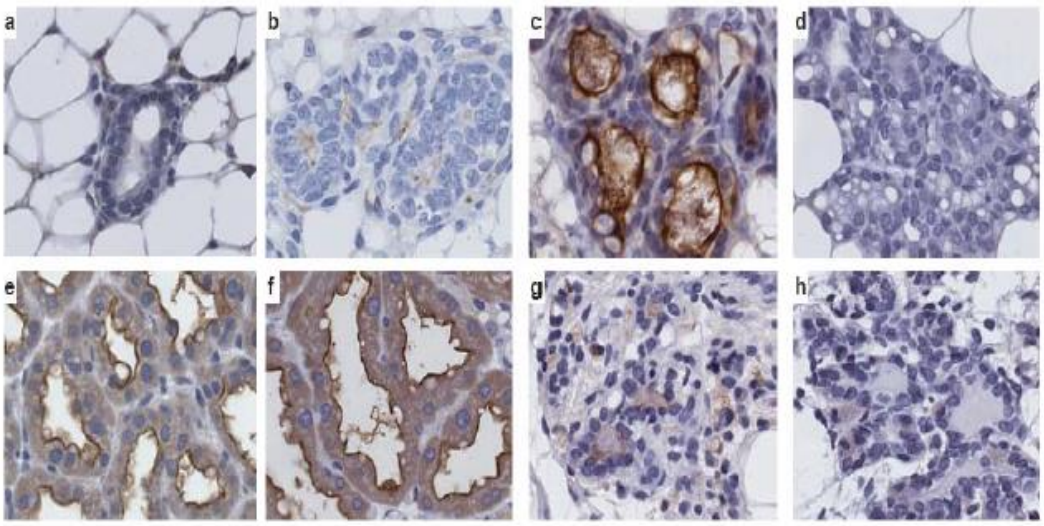


Figura 9. Inmunolocalización del transportador Abcg2 en muestras de glándula mamaria de ratón. (a) hembra virgen de 14 semanas; (b) 5,5 días tras la cópula; (c) 15,5 días tras la cópula; (d) control negativo ($Abcg2^{-/-}$); (e) 1 semana de lactación; (f) 2 semanas de lactación; (g) 1 semana de involución; (h) 4 semanas de involución. La presencia del transportador Abcg2 se pone de manifiesto empleando estreptavidina-biotina inmunoperoxidasa (Jonker y cols., 2005).

Estos estudios además demostraron que Abcg2 se localiza principalmente en la membrana apical de las células epiteliales alveolares, no detectándose en los

conductos principales. Este patrón de expresión de ABCG2 fue confirmado en vacas, ovejas, cabras y humanos (Jonker y cols., 2005; Pulido y cols., 2006; Wu y cols., 2008).

En contraposición con el papel protector propuesto en la placenta y otros órganos, indicado anteriormente, varios estudios demostraron que ABCG2 en la glándula mamaria actúa concentrando compuestos en la leche materna. Jonker y cols. evaluaron en 2005 la excreción hacia la leche de la toxina PhIP (2-amino-1-metil-6-fenilimidazol [4,5-b] piridina), el antitumoral topotecan y el protector gástrico cimetidina, después de la administración intravenosa de estos tres sustratos del transportador a ratones *wild-type* y ratones *knockout* ($Abcg2^{-/-}$) en lactación. Los estudios pusieron de manifiesto que, en ratones *wild-type* estos compuestos eran secretados activamente hacia la leche y que esta secreción disminuía drásticamente en los ratones deficientes en Abcg2; además, la secreción de topotecan hacia la leche era inhibida eficazmente mediante la administración oral del inhibidor de ABCG2 GF120918. Posteriormente, también utilizando ratones *knockout*, se demostró la implicación de Abcg2 en la excreción a leche de carcinógenos de la dieta (van Herwaarden y cols., 2003), los antibióticos nitrofurantoína, ciprofloxacina y danofloxacina (Merino y cols., 2005b; Merino y cols., 2006; Real y cols., 2011) y el antiparasitario moxidectina (Pérez y cols., 2009a). Además, algunas vitaminas como la riboflavina (vitamina B2), la biotina y la vitamina K también se ha demostrado que son transportadas hacia la leche mediante ABCG2 (van Herwaarden y cols., 2007; Vlaming y cols., 2009).

Si se tiene en cuenta que otros transportadores ABC localizados en la membrana apical como ABCB1 y MRP2 no están presentes en la glándula mamaria en lactación, los resultados de todos estos estudios identifican a ABCG2

como responsable de la transferencia de fármacos, carcinógenos y compuestos endógenos y de la dieta hacia la leche.

Es obvio que la concentración de compuestos de naturaleza tóxica en la leche materna que sirve para la alimentación de las crías no es una función precisamente protectora. Por eso, vamos a considerar algunas hipótesis sobre la función de ABCG2 en la glándula mamaria que pueden ayudarnos a explicar esta paradoja (Vlaming y cols., 2009):

A. **La expresión de ABCG2 en la glándula mamaria es una coincidencia:** esta hipótesis asume que la expresión de ABCG2 en la glándula mamaria no es biológicamente positiva; sin embargo, los posibles efectos negativos que pueda tener la secreción de toxinas hacia la leche, son mitigados por el hecho de que la madre puede prevenir la acumulación sistémica de estos compuestos mediante la expresión de ABCG2 en otros órganos (hígado, intestino, riñón). Por tanto, sólo unos pocos compuestos nocivos pasarán a la leche. Por otro lado, como ya hemos indicado anteriormente, la riboflavina y otras vitaminas son sustratos de ABCG2, lo cual parece una coincidencia debido a la amplia variedad de sustratos del transportador, pero sabemos que el transportador se induce durante el embarazo y la lactación en al menos 5 especies de mamíferos separadas filogenéticamente (ratón, vaca, oveja, cabra y humanos) (Jonker y cols., 2005; Pulido y cols., 2006; Wu y cols., 2008) y sin embargo, se ha demostrado en ratones que esta inducción no se produce en otros órganos (hígado, intestino, riñón) durante estos estadíos (Merino y cols., 2005b); así que debe de haber algún factor de inducción

específico en la glándula mamaria. Todo esto sugiere que es necesaria la expresión de ABCG2 en la glándula mamaria.

B. **La expresión de ABCG2 en glándula mamaria es útil como mecanismo de aclaramiento de xenobióticos para la madre:** según esta hipótesis, las madres en lactación son más vulnerables y necesitan rutas extras de eliminación de compuestos tóxicos, papel que puede desarrollar ABCG2 en la glándula mamaria. En contrapartida los recién nacidos recibirán estos compuestos, pero se ha demostrado la expresión de ABCG2 en la submucosa intestinal del feto (Kalabis y cols., 2007), lo cual debería ser suficiente para restringir la entrada de sustratos de ABCG2 presentes en la leche a la cría. De todas formas, si la madre está expuesta a xenobióticos, es más efectivo que desarrolle otras rutas de aclaramiento (hígado, intestino, riñón).

C. **La exposición a xenobióticos de los recién nacidos lactantes les prepara para el cambio a la comida sólida:** esta hipótesis asume que la transferencia moderada de xenobióticos a través de la leche ayuda a los recién nacidos a desarrollar buenos sistemas de detoxificación (van Herwaarden y cols., 2007). Esta hipótesis parece correcta excepto si tenemos en cuenta que todavía no hay muchos sustratos conocidos de ABCG2 que sean también buenos activadores de PXR y CAR, que parecen ser los mediadores más obvios para la preventiva inducción de los sistemas de detoxificación en los recién nacidos (Urquhart y cols., 2007).

D. **La transferencia de xenobióticos a la leche puede reducir el desarrollo de alergias a estas moléculas más adelante en la vida del**

recién nacido (Verhasselt y cols., 2008)**:** esta inducción de la tolerancia depende de la presencia de TGF-β, que normalmente está presente en la leche materna. La presencia de esta proteína sugiere que la inducción de la tolerancia oral es una función de la leche. Existe una limitación a esta hipótesis y es que se debería aplicar a moléculas relativamente pequeñas (que sean transportadas por ABCG2) y no a proteínas. Las proteínas y los conjugados de proteínas son más usualmente alergenos que las moléculas pequeñas, aunque también hay moléculas pequeñas que pueden ser muy alergénicas (por ejemplo la penicilina) (Greenberger., 2006) y verdaderas alergias a otros fármacos también han sido descritas (cefalosporinas, sulfas, anticonvulsionantes, agentes neuromusculo bloqueantes, novocaína,etc.) (Greenberger., 2006; Bohan y cols., 2007). Esta hipótesis puede ser válida cuando una molécula pequeña que sea alergénica sea identificada como sustrato de ABCG2.

E. **La expresión de ABCG2 en la glándula mamaria es necesaria para la transferencia de nutrientes a la leche:** esta hipótesis asume que todavía no se han aplicado las condiciones apropiadas a los ratones Abcg2 $^{-/-}$ lactantes para revelar una fuerte necesidad de ABCG2 en el suministro de vitaminas y otros nutrientes a la leche. Las pruebas de esta hipótesis pueden variar en la búsqueda de las condiciones apropiadas en el laboratorio o quizás identificando nutrientes adicionales que puedan ser transportados por ABCG2 a la leche. Sin embargo, esta hipótesis no aclara la cuestión de porqué los mamíferos no han desarrollado sistemas especializados de transporte dedicados al

bombeo de nutrientes a la leche en vez de utilizar un transportador multiespecífico como es ABCG2.

Por supuesto se pueden formular otras hipótesis que sean más tentativas y unas no tienen por qué excluir a las otras.

La implicación de ABCG2 en la secreción de fármacos a la leche también ha sido estudiada en animales domésticos por nuestro grupo de investigación junto con la posibilidad de modulación para disminuir los residuos farmacológicos en leche. Pulido y cols. (2006) probaron que la coadministración de enrofloxacina con inhibidores de ABCG2 en ovejas de producción lechera disminuía el paso del fármaco a la leche sin verse afectados los niveles plasmáticos del mismo. También estudios de Pérez y cols. (2009b y 2013) demostraron que la excreción de los antibióticos nitrofurantoína y danofloxacina a leche en ovejas podía ser modulada mediante dietas que presentaban diferentes niveles de flavonoides, inhibidores de ABCG2. Además, se estudió la interacción de la lactona macrocíclica ivermectina, inhibidor de ABCG2, en ovejas tras la coadministración de danofloxacina (Real y cols., 2011). La ivermectina no produjo cambios en la concentración de danofloxacina en plasma, pero sí una disminución del 40% en leche.

4.5. PAPEL DE ABCG2 Y GLICOPROTEÍNA P EN LA BARRERA HEMATOENCEFÁLICA.

La barrera hematoencefálica es un mecanismo de protección que controla la homeostasis cerebral y proporciona al SNC una protección frente a cualquier sustancia extraña. La barrera hematoencefálica previene la entrada al cerebro del 98% de las moléculas pequeñas y el 100% de las grandes. Se trata de una barrera

protectora importantísima pero que a su vez impide la entrada de fármacos para el tratamiento de enfermedades del SNC tales como metástasis en cerebro o epilepsia.

Esta barrera, se localiza a nivel de los capilares, entre la sangre y el tejido cerebral, y se caracteriza por la presencia de uniones estrechas intercelulares y la expresión de muchos sistemas de transporte polarizados. De entre estos transportadores, el papel de P-gp en la barrera hematoencefálica está bien establecido, y fue demostrado directamente por primera vez utilizando ratones Abcb1a$^{-/-}$, los cuales presentaron una mayor acumulación de ivermectina y abamectina en el cerebro y, en consecuencia, neurotoxicidad (Schinkel y cols., 1994; Lankas y cols., 1997).

Posteriormente, se observó que además de P-gp, el transportador ABCG2 se expresaba también en los capilares del cerebro de humanos y de muchas otras especies (Maliepaard y cols., 2001; Cooray y cols., 2002; Eisenblätter y cols., 2003; Aronica y cols., 2005; Sisodiya y cols., 2006; Daood y cols., 2008; Kamiie y cols., 2008). En ratones normales, la expresión de la proteína Abcg2 fue tres veces menor que la de P-gp (Kamiie y cols., 2008). Sin embargo, en capilares de cerebro porcino, se demostró que la expresión de ABCG2 era mayor que la de P-gp y ABCC1 basándose en el análisis de ARN mensajero (Eisenblätter y cols., 2003). De forma similar, capilares de cerebro extraídos de secciones de tejido de cerebros humanos sanos mediante microdisección, revelaron mayor expresión de ARN mensajero de ABCG2 en comparación con el de P-gp y ABCC1 (Zhang y cols., 2003). Recientemente, usando cromatografía líquida acoplada a espectrometría de masas, Uchida y cols. (2011) obtuvieron un perfil de expresión proteico cuantitativo de los transportadores de membrana en capilares de cerebro

humano y observaron, que de entre los transportadores, ABCG2 fue la proteína más abundante y su nivel de expresión fue 1,33 veces más alto que el de P-gp. Se debe decir, que dadas las obvias diferencias entre especies en la expresión relativa de ABCG2, debería recordarse que la contribución relativa de ABCG2 en la barrera hematoencefálica probablemente esté subestimada cuando se extrapolan datos de estudios con ratones a humanos (revisado por Schnepf y Zolk, 2013).

Independientemente de esta limitación, los estudios con ratones *knockout* nos han proporcionado una profunda visión de la actividad protectora de ABCG2 y P-gp en la barrera hematoencefálica. Por ejemplo, comparado con los ratones control, la penetración en cerebro del antitumoral imatinib administrado por vía intravenosa fue de 2,5 veces y 3,6 veces mayor en ratones Abcg2$^{-/-}$ y en ratones Abcb1a/1b$^{-/-}$ respectivamente, lo cual demostró que tanto Abcg2 como P-gp limitan la penetración de imatinib en cerebro (Breedveld y cols., 2005). Uno de los mayores efectos observados de ABCG2 se encuentra recogido en el trabajo de Enokizono y cols. (2007), donde describen un aumento de 9,2 veces en la entrada del flavonoide genisteína en el cerebro de ratones Abcg2$^{-/-}$ en comparación con ratones *wild-type*. Una gran variedad de compuestos, tales como el imatinib y el topotecan, son sustratos compartidos tanto por P-gp como por ABCG2; la generación del modelo de ratón *knockout* combinado abcb1a/1b/abcg2 permitió el estudio de la interacción entre ambos transportadores en la barrera hematoencefálica (Poller y cols., 2011; Nakanishi y cols., 2013). Comparado con los ratones *wild-type,* los ratones *knockout* para un solo transportador, no mostraron incremento o este fue muy leve en la acumulación en cerebro de topotecan, imatinib y sunitinib, mientras que en los ratones *knockout* para ambos transportadores, la acumulación en el cerebro de estos compuestos aumentó de

manera desproporcionada (de Vries y cols., 2007; Oostendorp y cols., 2009; Tang y cols., 2012). Estos datos sugieren que en los ratones *knockout* para uno de los transportadores, el transportador que sí se expresa puede compensar la pérdida de función del que no se expresa. En los ratones doblemente *knockout* para ambos transportadores, sin embargo, la capacidad de compensación parece no producirse, debido a que no hay ningún otro transportador adicional capaz de hacerlo. Se ha especulado que la compensación funcional en la barrera hematoencefálica entre Abcg2 y Abcb1 puede deberse a una sobreexpresión compensatoria del transportador. En efecto, se observó un nivel de ARN mensajero codificante para Abcg2 en los capilares del cerebro de ratones Abcb1a$^{-/-}$ tres veces mayor que en ratones *wild-type* (Cisternino y cols., 2004). Sin embargo, estos cambios en los niveles de ARN mensajero no se traducían en cambios en la expresión de la proteína, como se comprobó mediante análisis por *western blot* y más recientemente por proteómica cuantitativa basada en cromatografía líquida acoplada a espectrometría de masas, el cual es un método muy sensible (de Vries y cols., 2007; Agarwal y cols., 2012). Por lo tanto, la cooperación observada entre Abcg2 y Abcb1 en el modelo de ratón no se debe a un mecanismo compensatorio de sobreexpresión.

En estudios posteriores se demostró la relevancia de Abcb1 y/o Abcg2 en la penetración a cerebro de una gran variedad de compuestos, incluyendo varios inhibidores de tirosin quinasas como el lapatinib, dasatinib, sorafenib, gefitinib, erlotinib, dabrafenib y CYT387 (Marchetti y cols., 2008; Polli y cols., 2008; Lagas y cols., 2009; Agarwal y cols., 2010; Lagas y cols., 2010; Kodaira y cols., 2010; Mittapalli y cols., 2012; Tang y cols., 2012; Durmus y cols., 2013), otros antitumorales como el prazosin o la mitoxantrona (Cisternino y cols., 2004),

antivirales como el abacavir (Giri y cols., 2008) y fármacos antiepilépticos (Cerveny y cols., 2006). Aunque ambos transportadores parecen contribuir de forma similar en la excreción de algunos fármacos en la barrera hematoencefálica, incluyendo topotecan e imatinib, la penetración en cerebro de dasatinib, sunitinib y abacavir parece estar limitada principalmente por Abcb1. En comparación, la acumulación en cerebro de prazosin, mitoxantrona y sorafenib fue restringida principalmente por Abcg2 (Cisternino y cols., 2004).

Todos estos datos en conjunto sugieren la cooperación de ABCG2 y P-gp en la barrera hematoencefálica. Ésta conclusión se basa en los siguientes descubrimientos (Poller y cols., 2011):

1) La localización principal de ABCG2 en la superficie luminal de los capilares del endotelio se asemeja a la de P-gp.
2) ABCG2 y P-gp tienen una significante superposición en los perfiles de especificidad de sus sustratos.
3) ABCG2 puede compensar la pérdida de ABCB1 en la barrera hematoencefálica.

La respuesta al mecanismo de cooperación existente entre P-gp y ABCG2 puede ser descubierta examinando las afinidades relativas de cada transportador por sus sustratos y los niveles relativos de cada transportador en la barrera hematoencefálica. Experimentos en líneas celulares con sobreexpresión estable de P-gp y ABCG2 sugieren que la saturación del transporte mediado por P-gp y ABCG2, que depende de la especificidad por el fármaco y de su concentración, es otra variable que influye en la contribución relativa de cada transportador a la excreción de fármacos que sean sustratos de ambos (Poller y cols., 2011).

Por último, destacar que también estos transportadores están implicados en la patogenia y tratamiento de numerosas enfermedades relacionadas con el SNC como por ejemplo la epilepsia, el cáncer cerebral y el alzheimer (Hartz y Bauer, 2011). Una de las claves de esta última enfermedad es la acumulación de un péptido β-amiloide en el cerebro, posiblemente debido a una disminución en la eliminación de dicho péptido en la que participarían varios transportadores ABC (Wolf y cols., 2012). Además, la epilepsia refractaria, que se observa en uno de cada cuatro pacientes epilépticos, estuvo directamente relacionada con la sobreexpresión de P-gp y otros transportadores ABC como ABCG2 en cerebros epileptogénicos (Lazarowski y cols., 2007).

4.6. EL TRICLABENDAZOL Y SUS METABOLITOS.

El antihelmíntico triclabendazol (TCBZ) (5-cloro-6-(2,3-diclorofenoxi)-2-metil-tiobencimidazol), se ha convertido en el principal fármaco de elección en humana y en veterinaria para el tratamiento de la fasciolosis desde su comercialización a principios de los años 80.

La fasciolosis es una zoonosis muy importante que afecta al ganado de las zonas templadas y que es causada por el parásito *Fasciola hepatica*. Esta enfermedad ha experimentado un fuerte aumento en los últimos años en países en vías de desarrollo, lo cual ha sido atribuido al cambio climático. Aunque es una enfermedad principalmente de los animales herbívoros, los humanos nos convertimos en huéspedes accidentales mediante la ingestión de plantas crudas. La forma humana de la enfermedad se ha convertido en un problema de salud pública en muchas partes del mundo (Mas-coma y cols., 2005), con los problemas que ello conlleva de anemia y desnutrición durante la infancia. Presenta un amplio

espectro de cuadros clínicos que van desde fiebre, eosinofilia y síntomas gastrointestinales vagos en la fase aguda, a colangitis, colecistitis, obstrucción biliar extrahepática e infestación o infección asintomática por eosinofilia en la fase crónica. Sin embargo, a menudo puede ser pasada por alto, especialmente en la fase aguda, debido a síntomas leves. Recientemente se han introducido ensayos serológicos para facilitar el diagnóstico y como consecuencia ha habido un aumento en el número de casos notificados (Arslan y cols., 2012).

Con el fin de mejorar el abordaje terapéutico de esta parasitosis, se necesita un mejor conocimiento del mecanismo de acción del TCBZ, de los factores que afectan a su biodisponibilidad y de sus posibles interacciones farmacológicas. El TCBZ es un bencimidazol halogenado antihelmíntico que posee elevada actividad frente a estados inmaduros y adultos de *Fasciola hepatica*, ya que bloquea a la tubulina y afecta a los mecanismos de transporte intracelular, interfiriendo en la síntesis de proteínas del parásito (Boray y cols., 1983), habiendo demostrado una elevada eficacia fasciolicida en ovinos, caprinos y bovinos (Wolff y cols., 1983; Misra y cols., 1987). Existen estudios recientes en humanos con una infección combinada de *Schistosoma mansoni* y *Fasciola hepatica* en los que se han probado distintas dosis de TCBZ, obteniéndose a las 8 semanas tras el tratamiento un porcentaje de curación del 96% para la fasciolosis y del 32,7% para la eschistosomiasis, no presentando el hígado ninguna alteración de su función tras las 8 semanas de tratamiento (Osman y cols., 2011). Otros estudios realizados en una zona del altiplano boliviano donde la prevalencia de la infección por *Fasciola hepatica* es alta, han demostrado que el tratamiento con TCBZ es fiable, seguro y eficaz para la salud pública (Villegas y cols., 2012). Esta acción se asocia principalmente con el metabolito activo triclabendazol sulfóxido

(TCBZSO), ya que el fármaco original TCBZ es rápidamente metabolizado en el hígado tanto por la enzima flavin-monooxigenasa (FMO) como por el citocromo P450 (Mottier y cols., 2004; Virkel y cols., 2006) hacia TCBZSO y posteriormente a triclabendazol sulfona ($TCBZSO_2$) (Figura 10). El TCBZ puede ser oxidado a TCBZSO por la microflora intestinal antes de su absorción, o por la pared intestinal durante su absorción (Mestorino y cols., 2008). Es evidente que los mecanismos que afectan a la farmacocinética del TCBZ son complejos, pero sirven (junto con una fuerte unión a las proteínas del plasma) para mantener concentraciones activas del fasciolicida en el hospedador durante largos periodos de tiempo, lo cual hace que la eficacia del fármaco sea mayor.

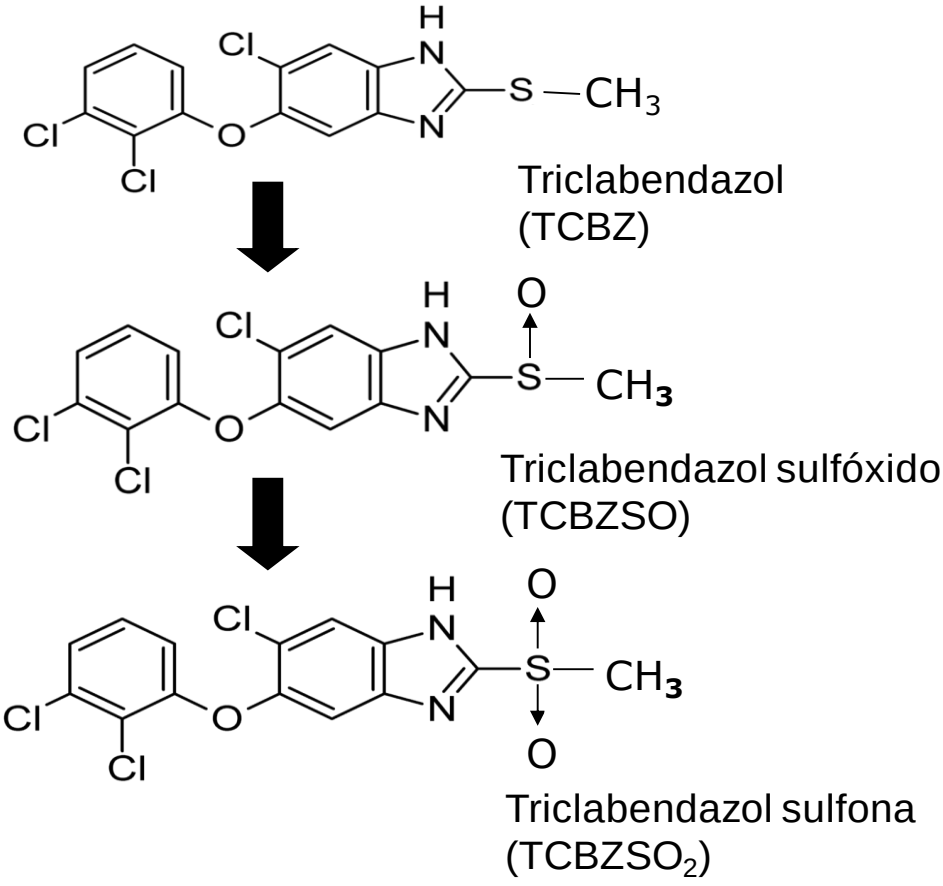


Figura 10. Estructura química del triclabendazol y sus metabolitos

Se han realizado diversos estudios *in vitro* para comparar la acción de TCBZ, TCBZSO y $TCBZSO_2$ frente a *Fasciola hepatica*. En uno de estos estudios se utilizó el microscopio electrónico de barrido para observar los cambios que se producían en la superficie del parásito y el microscopio electrónico de transmisión para ver los cambios internos y así determinar los daños producidos en el tegumento del parásito por cada uno de los tres compuestos (Halferty y cols., 2009). El nivel de daño en la superficie del parásito varía de una región a otra, pero en general, el daño que produce el TCBZ en la superficie es mayor. Sin embargo, los resultados obtenidos en los cambios internos producidos después del tratamiento con cada uno de los tres compuestos, demostraron que el que menor efecto tenía en este sentido era el TCBZ, seguido del TCBZSO y el que más cambios internos producía era la $TCBZSO_2$. Combinando los resultados obtenidos en cuanto a los cambios que producen estos compuestos en la superficie del parásito y en su interior, se llega a la conclusión de que el más activo es la $TCBZSO_2$, seguida del TCBZSO y por último el TCBZ (Halferty y cols., 2009). Por lo tanto, la $TCBZSO_2$ también contribuye a la acción del fármaco *in vivo*, y no es por tanto, el metabolito inactivo que se pensaba hace unos años.

Respecto a la interacción del TCBZ y sus metabolitos con los transportadores de membrana dependientes de ATP, se ha demostrado que el TCBZ y su metabolito TCBZSO inhiben el transporte mediado por P-gp en células en cultivo transfectadas con este transportador (Dupuy y cols., 2010). Además, también se ha descrito, que se puede modular la farmacocinética de este antiparasitario mediante su coadministración con inhibidores de P-gp (Lifschitz y cols., 2009; Virkel y cols., 2009). Como ejemplo, se ha observado en ovejas que la coadministración de TCBZ junto con el antiparasitario ivermectina afecta al

perfil de la concentración en plasma de TCBZ, pero también se vio afectada la eliminación de la propia ivermectina y su biodisponibilidad plasmática fue 3 veces mayor que al administrarla de forma individual (Lifschitz y cols., 2009). Estos estudios muestran una nueva estrategia para aumentar la eficacia del TCBZ mediante la modulación de su farmacocinética utilizando inhibidores de los transportadores ABC y también su papel como inhibidor de los mismos.

Todos los trabajos publicados hasta el momento, se habían centrado en la interacción del TCBZ y sus metabolitos con P-gp, y poco se sabía de lo que ocurre con otros transportadores ABC como ABCG2, lo que formará parte de los objetivos de la presente memoria.

4.7. URB937.

El URB937 (Figura 11), es un compuesto en fase de desarrollo con importantes propiedades analgésicas en tejidos periféricos. Se sabe que la percepción del dolor puede ser controlada eficazmente por neurotransmisores que operan en el SNC. Esta modulación ha sido bien caracterizada en el asta dorsal de la médula espinal, donde los impulsos transmitidos por fibras nociceptivas (sensibles al dolor) son procesados antes de ser transmitidos al cerebro. Además de estos mecanismos centrales, puede tener lugar un control intrínseco de la transmisión del dolor en los terminales de las fibras nerviosas aferentes fuera del SNC. Un destacado ejemplo de la regulación periférica lo proporcionan los opioides endógenos, que son liberados de células inmunes activadas durante la inflamación e inhiben el inicio del dolor mediante la interacción con receptores de opioides localizados en los extremos de los nervios sensoriales (Stein y cols., 2003). Los mediadores celulares del sistema endocannabinoide podrían cumplir

así una función análoga a la de los opioides, ya que la activación farmacológica de los receptores cannabinoides periféricos de tipo 1 (CB_1) y de tipo 2 (CB_2) inhibe los comportamientos relacionados con el dolor (Calignano y cols., 1998; Jaggar y cols., 1998; Nackley y cols., 2003; Stein y Zollner, 2009), mientras que la disrupción genética de la expresión del receptor CB_1 en las neuronas nociceptivas primarias aumenta tales comportamientos (Dziadulewicz y cols., 2007). Por otra parte, existen pruebas de que las condiciones clínicas asociadas con el dolor neuropático o la inflamación van acompañadas de incrementos en los niveles del endocannabinoide araquidonoiletanolamida (anandamida) en las zonas periféricas (por ejemplo, en el síndrome de dolor regional complejo o la artritis) (Agarwal y cols., 2007; Anand y cols., 2009).

La explotación terapéutica del sistema endocannabinoide con agonistas exógenos, está limitada por los efectos secundarios no deseados causados por la activación indiscriminada de receptores CB_1, particularmente en cerebro (Mechoulam y Parker, 2013). Una estrategia alternativa dirigida directamente al receptor CB_1 es la de aumentar la actividad de señalización de los ligandos cannabinoides endógenos (anandamida) (Di Marzo y cols., 1994) y 2-araquidonoil-*sn*-glicerol (2-AG) (Stella y cols., 1997), bloqueando su degradación intracelular. La anandamida se libera en demanda de neuronas estimuladas (Di Marzo y cols., 1994). Inhibidores de la enzima responsable de su escisión hidrolítica, la amida hidrolasa de ácidos grasos (FAAH), han demostrado un incremento de los niveles de anandamida y la activación de receptores CB_1 centrales y periféricos (Piomelli y cols., 2006; Bisogno y Maccarrone, 2013).

Fuera del SNC, los receptores CB_1 se encuentran en órganos como el hígado, riñón e intestino, así como en terminales sensoriales periféricos y

neuronas de los ganglios de la raíz dorsal (Kunos y cols., 2009). La evidencia sugiere que el beneficio terapéutico en el que no intervenga el SNC puede lograrse en condiciones tales como el dolor y el síndrome metabólico mediante la focalización en estos receptores periféricos (Agarwal y cols., 2007; Osei-Hyiaman y cols., 2008). Por lo tanto, el desarrollo de agentes farmacológicos que no crucen la barrera hematoencefálica, proporciona un posible enfoque para identificar terapias basadas en endocannabinoides que sean efectivas y seguras (Moreno-Sanz y cols., 2013).

La activación selectiva de receptores CB_1 periféricos por la anandamida endógena producida se logró por primera vez con el compuesto URB937 (Figura 11), el cual bloquea la actividad FAAH solamente fuera del SNC a través de un mecanismo irreversible (Clapper y cols., 2010). URB937 inhibe respuestas a dolor visceral e inflamatorio en roedores mediante la reducción de entradas nociceptivas a la médula espinal (Clapper y cols., 2010).

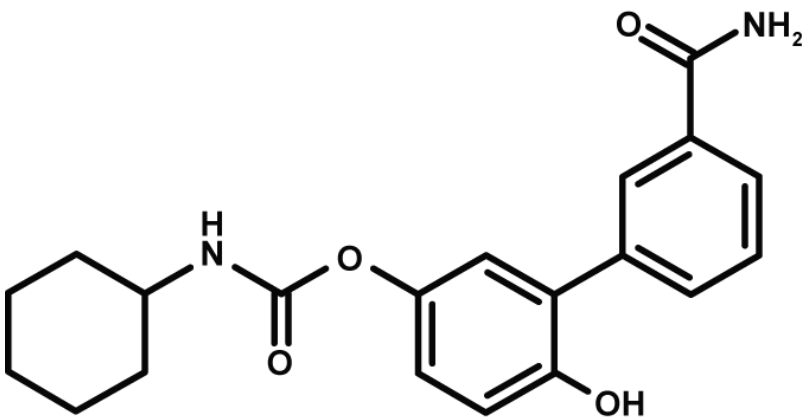


Figura 11. Estructura química del compuesto URB937

Después de su administración sistémica en ratones, URB937 inhibe la actividad FAAH en hígado, con una mitad de la máxima dosis efectiva (ID_{50}) de

0,1 mg/Kg, pero falla al alterar la actividad FAAH en prosencéfalo, hipotálamo y médula espinal. De hecho, URB937 es 400 veces más potente inhibiendo la actividad FAAH en el hígado que en el cerebro del ratón (Clapper y cols., 2010). Según estudios de Clapper y cols. (2010), la distribución de URB937 tras administración sistémica en ratones a una dosis de 1 mg/Kg fue rápida en sangre e hígado, pero no se detectó este compuesto en cerebro. Así, la inhibición *in vivo* de FAAH por parte de URB937 está acompañada de un incremento de los niveles de anandamida exclusivamente en tejidos periféricos, evitándose así los efectos psicotrópicos a nivel de SNC. Sus efectos son mayores que los observados para inhibidores de FAAH globalmente activos, tales como el URB597 o el PF-04457845 (Sasso y cols., 2012), destacando la potencial significancia terapéutica del bloqueo periférico de FAAH (Moreno-Sanz y cols., 2012).

Debido a su lipofilicidad, URB937 debería difundir pasivamente hacia el SNC a menos que esta difusión sea contrarrestada de forma activa por algún mecanismo (Clapper y cols., 2010). Existen evidencias farmacológicas que sugieren que la expulsión de URB937 del cerebro de ratones podría estar mediada por el transportador de membrana ABCG2 (Clapper y cols., 2010). Estos autores administraron varios inhibidores del transporte de fármacos a través de la barrera hematoencefálica, junto con una dosis sistémica alta de URB937 que no lograse entrar en cerebro en condiciones normales (25 mg/Kg intraperitoneal). El tratamiento con Ko143 (10 mg/Kg intraperitoneal), un potente y conocido inhibidor de ABCG2, hizo decrecer notablemente la actividad de FAAH en cerebro. Por el contrario, la administración de otros inhibidores del transporte de fármacos incluidos el verapamilo, el probenecid y la rifampicina (Clapper y cols.,

2010) fueron inefectivos. El demostrar si efectivamente URB937 es un compuesto sustrato de ABCG2 es uno de los objetivos de la presente memoria.

Sin embargo, como ya hemos comentado anteriormente, no podemos ignorar la importancia de la glicoproteína P en el paso de compuestos a través de la barrera hematoencefálica. Por otra parte, estudios recientes enfocados hacia el estudio de las características estructurales que evitan la entrada de URB937 en cerebro mediante la administración de análogos estructurales a ratones han demostrado la importancia en el bloqueo de la entrada a cerebro de estos compuestos de diversos sustituyentes en los anillos fenólicos proximal y distal de la molécula de URB937 (Moreno-Sanz y cols., 2013). El estudio *in vitro* de la interacción de URB937 y diferentes análogos estructurales con los principales transportadores ABC presentes en la barrera hematoencefálica, ABCG2 y glicoproteína P, ayudaría a encontrar los determinantes estructurales cruciales para la interacción compuesto-transportador explicando el restringido acceso al SNC del URB937, lo cual forma parte de los objetivos de la presente memoria.

5. OBJETIVOS

Estos antecedentes han puesto de manifiesto la función del transportador ABCG2, señalando de forma específica las implicaciones terapéuticas derivadas de la interacción con fármacos en la glándula mamaria y en la barrera hematoencefálica, por ello, la presente memoria tiene como objetivo general determinar las interacciones del antihelmíntico TCBZ y sus metabolitos (TCBZSO y $TCBZSO_2$) así como las del analgésico en desarrollo URB937 y sus análogos estructurales con el transportador de membrana ABCG2, ampliándose el estudio en el caso del URB937 y sus derivados a la glicoproteína P. Este objetivo general se desarrollará en los siguientes objetivos específicos:

- Estudiar la capacidad inhibitoria *in vitro* del TCBZ y sus metabolitos (TCBZSO y TCBZSO2) sobre el transportador de membrana ABCG2 en distintas especies animales utilizando para ello células epiteliales que sobreexpresan las distintas variantes del transportador.

- Estudiar el efecto *in vivo* de la coadministración del TCBZ y sus metabolitos sobre la farmacocinética y excreción en leche de varios sustratos del transportador utilizando para ello modelos animales como los ratones y las ovejas.

- Determinar tanto *in vitro* como *in vivo* si el compuesto URB937 es sustrato de ABCG2 utilizando para ello células epiteliales que sobreexpresan el transportador y modelos animales de ratones *wild-type* y *knockout* para Abcg2.

- Caracterizar *in vitro* los análogos estructurales de URB937 como sustratos de los principales transportadores ABC presentes en la barrera hematoencefálica, Abcg2 y glicoproteína P, para realizar un estudio de la relación estructura-función de la molécula del URB937, utilizando para ello células epiteliales que sobreexpresan dichos transportadores.

6. ARTÍCULO I

The anthelmintic triclabendazole and its metabolites inhibit the membrane transporter ABCG2/BCRP.

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The Anthelmintic Triclabendazole and Its Metabolites Inhibit the Membrane Transporter ABCG2/BCRP

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ABCG2/BCRP is an ATP-binding cassette transporter that extrudes compounds from cells in the intestine, liver, kidney, and other organs, such as the mammary gland, affecting pharmacokinetics and milk secretion of antibiotics, anticancer drugs, and other compounds and mediating drug-drug interactions. In addition, ABCG2 expression in cancer cells may directly cause resis-tance by active efflux of anticancer drugs. The development of ABCG2 modulators is critical in order to improve drug pharmaco-kinetic properties, reduce milk secretion of xenotoxins, and/or increase the effective intracellular concentrations of substrates. Our purpose was to determine whether the anthelmintic triclabendazole (TCBZ) and its main plasma metabolites triclabenda-zole sulfoxide (TCBZSO) and triclabendazole sulfone ($TCBZSO_2$) inhibit ABCG2 activity. ATPase assays using human ABCG2-enriched membranes demonstrated a clear ABCG2 inhibition exerted by these compounds. Mitoxantrone accumulation assays using murine Abcg2- and human ABCG2-transduced MDCK-II cells confirmed that TCBZSO and $TCBZSO_2$ are ABCG2 inhibi-tors, reaching inhibitory potencies between 40 and 55% for a concentration range from 5 to 25 μM. Transepithelial transport assays of ABCG2 substrates in the presence of both TCBZ metabolites at 15 μM showed very efficient inhibition of the Abcg2/ABCG2-mediated transport of the antibacterial agents nitrofurantoin and danofloxacin. TCBZSO administration also inhibited nitrofurantoin Abcg2-mediated secretion into milk by more than 2-fold and increased plasma levels of the sulfonamide sul-fasalazine by more than 1.5-fold in mice. These results support the potential role of TCBZSO and $TCBZSO_2$ as ABCG2 inhibitors to participate in drug interactions and modulate ABCG2-mediated pharmacokinetic processes.

ABCG2/BCRP is a described member of the ABC transporter family, a group of proteins that transport certain chemicals out of cells (29). These ABC drug efflux transporters extrude a wide range of xenotoxins from cells in intestine, liver, and other organs and thus affect the bioavailability of many compounds and participate in drug-drug interactions. In ad-dition, ABCG2 mediates secretion into the milk of its sub-strates (both therapeutic and toxic), such as antibiotics, anti-tumoral agents, carcinogens, or vitamins (31, 32). Recently, the International Transporter Consortium has included ABCG2 in the group of transporters that are clinically relevant (8). More-over, the overexpression of ABC transporters has been associ-ated with multidrug resistance (MDR), a major impediment to successful cancer chemotherapy. Increasing interest has been given to the development of inhibitors to overcome MDR and to increase oral bioavailability and tissue penetration or to de-crease milk secretion of its substrates (21, 28).

Some benzimidazole drugs, such as the anthelmintics albenda-zole sulfoxide and oxfendazole and the antacid pantoprazole, have been reported to interact with ABCG2 (3, 19). In the case of pan-toprazole, its use as an ABCG2 inhibitor to improve plasma phar-macokinetics and brain penetration of ABCG2 substrates has been reported (2, 3). Triclabendazole (TCBZ) is a flukicidal haloge-nated benzimidazole thiol derivative used for treating liver fluke infections in livestock and is the drug of choice against human Fascioliasis (6). The TCBZ parent drug is not detected in plasma after its oral administration because it is rapidly metabolized into its metabolites triclabendazole sulfoxide (TCBZSO) and tricla-bendazole sulfone ($TCBZSO_2$) (10) (Fig. 1). TCBZ and TCBZSO have been shown to interact with other ABC transporters *in vitro* (4); however, the interaction of TCBZ and its metabolites with ABCG2 has not yet been investigated.

In this paper, we studied whether TCBZ and its metabolites (TCBZSO and $TCBZSO_2$) *in vitro* inhibit the ABCG2 transporter in ATPase assays using ABCG2-enriched membranes and in mitoxantrone (MXR) accumulation and transepithelial transport assays using ABCG2-transduced cell lines. *In vivo* inhibition of the transporter was assessed by studying the Abcg2-mediated effect of TCBZSO coadministration on the secretion into milk of the anti-bacterial agent nitrofurantoin and on plasma levels of the sulfon-amide sulfasalazine using $Abcg2^{-/-}$ and wild-type mice. Experi-ments with murine Abcg2-transduced cells and mice are included in this study, as mice are extensively used as experimental models to study the transporter function *in vivo*.

MATERIALS AND METHODS

Reagents and drugs. Mitoxantrone, sulfasalazine, and nitrofurantoin were purchased from Sigma-Aldrich (St. Louis, MO), danofloxacin was purchased from Fluka Chemie (Buchs, Switzerland), TCBZ was purchased from Sequoia Research Products (Pangbourne, United Kingdom), TCBZSO and $TCBZSO_2$ were purchased from LGC Standards (Barcelona, Spain), isoflurane (Isovet) was purchased from Schering-Plough (Madrid, Spain), oxytocin (Oxiton) was purchased from Ovejero (León, Spain), and Ko143 was purchased from Tocris (Bristol, United Kingdom). All the other chemicals were analytical grade and available from commercial sources.

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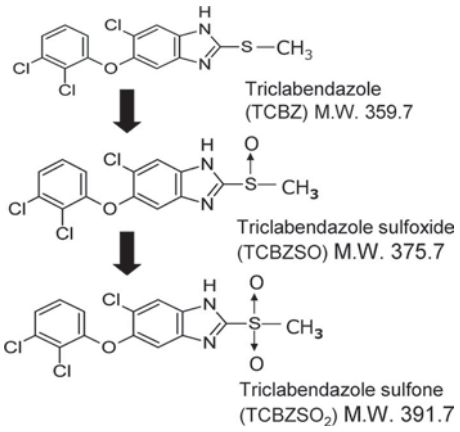

FIG 1 Chemical structures of triclabendazole (TCBZ) and its metabolites triclabendazole sulfoxide (TCBZSO) and triclabendazole sulfone ($TCBZSO_2$). The molecular weight (M.W.) for each compound is shown.

Animals. Animals were housed and handled according to procedures approved by the Research Committee of Animal Use of the University of León (Spain) and carried out according to the Principles of Laboratory Animal Care and the European guidelines described in the EC Directive 86/609. The animals used were male or lactating female Abcg2$^{-/-}$ and wild-type mice, all of >99% FVB genetic background and between 9 and 13 weeks of age. Animals were kindly provided by A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands), were kept in a temperature-controlled environment with a 12-h-light/12-h-dark cycle, and received a standard diet (Panlab; Barcelona, Spain) and water *ad libitum*.

Cell cultures. MDCK-II cells and their human ABCG2- and murine Abcg2-transduced subclones were kindly provided by A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Culture conditions were as previously described (12, 23).

Transport studies. Transepithelial transport assays using Transwell plates were carried out as previously described (19) with minor modifica-tions. Transepithelial resistance was measured in each well using a Milli-cell ERS ohmmeter (Millipore, Bedford, MA); wells registering a resis-tance of 150 Ω or greater, after correcting for the resistance obtained in blank control wells, were used in the transport experiments. The measure-ment was repeated at the end of the experiment to check the tightness of the monolayer. Experiments were performed using Opti-MEM medium, a reduced serum medium that is a modification of Eagle's minimum es-sential medium, buffered with HEPES and sodium bicarbonate. Active transport across MDCK-II monolayers was expressed by the relative transport ratio, defined as the apically directed transport percentage di-vided by the basolaterally directed translocation percentage, after 4 h (30).

ATPase assay. ABCG2-associated ATP hydrolysis was determined by quantifying the release of inorganic phosphate (P_i) with a colorimetric assay with small modifications (1). Experiments were carried out in 96-well microtiter plates (F96 MicroWell plates, nontreated; Nalge Nunc, Rochester, NY). Plasma membrane vesicle preparations from isolated mammalian cells containing human ABCG2 (BCRP-M-ATPase) were obtained from Solvo Biotechnology (Budapest, Hungary) (9). Vesicles were diluted in reaction volumes of 60 μl containing a protein concentration of 0.075 mg/ml in ice-cold phosphate release assay buffer (25 mM Tris-HCl including 50 mM KCl, 3 mM ATP, 2.5 mM MgSO4, 3 mM dithiothreitol [DTT], 0.5 mM EGTA, 2 mM ouabain, and 3 mM sodium azide) adjusted to pH 7 at 37°C (1). Incubation of compounds and membranes was started by transferring the plate from ice to a water bath kept at 37°C for 1 h and was terminated by rapidly cooling the plate on ice. The phosphate release assays were performed in parallel in the presence of vanadate to inhibit ABCG2 ATPase activity, and the vanadate values were subtracted from the measurements. At least two independent measurements in plasma membrane vesicles were performed. Each independent experiment consisted of one 96-well plate with two measurements.

Accumulation assays. *In vitro* accumulation assays were carried out as previously described (23). Mitoxantrone (MXR; 10 μM) was used as a fluorescent substrate. Relative cellular accumulation of MXR of at least 5,000 cells was determined by flow cytometry using a CyAn cytometer (Beckman Coulter, Fullerton, CA). The fluorescence of the accumulated substrate in tested populations was quantified from histogram plots using the median of fluorescence (MF). Flow cytometry data were processed and analyzed using SUMMIT version 4.3 software (Innovation Drive, Fort Collins, CO). Inhibitory potencies of compounds were calculated as previously described (23) in MDCK-II-ABCG2 or MDCK-II-Abcg2 cells according to the following equation: inhibitory potency = (MF with tested compound − MF without inhibitor)/(MF with Ko143 − MF without inhibitor) × 100%.

Plasma levels of sulfasalazine. Sulfasalazine (20 mg/kg of body weight) was intragastrically administered to wild-type and Abcg2$^{-/-}$ male mice by oral gavage feeding in 4-h-fasted mice as a solution of 6% ethanol, 42% PEG400, and 52% water. Oral administration consisted of 300 μl of solution per 30 g of body weight. TCBZSO (50 mg/kg) or the vehicle (6% ethanol, 42% PEG400, and 52% water) was orally administered 15 min before oral administration of sulfasalazine (20 mg/kg). Blood was collected after 30 min of administration of sulfasalazine by cardiac puncture after anesthesia with isoflurane. At the end of the experiment, the mice were killed by cervical dislocation. Heparinized blood samples were centrifuged immediately at 1,500 × *g* for 10 min, and collected plasma was stored at −20°C until high-performance liquid chromatography (HPLC) analysis. Between 4 and 7 animals were used for each experimental group.

Milk secretion experiments. Pups of approximately 10 days old were separated from their mother approximately 4 h before the start of the experiment. Nitrofurantoin (5 mg/kg) was administered in the tail vein to wild-type and Abcg2$^{-/-}$ lactating female mice as a solution of 6% ethanol, 42% PEG400, and 52% water. The intravenous (i.v.) administration consisted of 150 μl of solution per 30 g of body weight. TCBZSO (50 mg/kg or 100 mg/kg) or the vehicle (6% ethanol, 42% PEG400, and 52% water) was administered intraperitoneally (i.p.) (500 μl of solution per 30 g of body weight) 5 min before intravenous administration of nitrofurantoin. Oxytocin (200 μl of a 1-IU/ml solution) was administered subcutaneously to lactating dams in order to stimulate milk secretion 20 min after the administration of nitrofurantoin. Blood and milk were collected 30 min after substrate administration under anesthesia with isoflurane. Blood was collected by orbital bleeding, and heparinized blood samples were centrifuged immediately at 1,500 × *g* for 10 min. Milk was collected from the mammary glands by gentle pinching. At the end of the experiment, mice were subsequently killed by cervical dislocation. Collected plasma and milk samples were stored at −20°C until HPLC analysis. Between 4 and 7 animals were used for each experimental group.

HPLC analysis. The chromatographic system consisted of a Waters 2695 separation module and a Waters 2998 UV photodiode array detector.

The conditions for HPLC analysis of danofloxacin were modified according to previously published methods (17, 18). Samples from the transport assays were not processed, and 50 μl of the culture medium was injected directly into the HPLC system. Separation of the samples was performed on a reverse-phase column (Phenomenex Synergi 4-μm Hydro-RP 80A). The mobile phase consisted of 25 mM orthophosphoric acid (pH 3.0)-acetonitrile (75:25), the flow rate of the mobile phase was set to 1.5 ml/min, and UV absorbance was measured at 278 nm. The temperature of the samples was 4°C. Standard samples were prepared in the appropriate drug-free matrix, yielding a concentration range from 0.02 μg/ml to 5 μg/ml.

The conditions for HPLC analysis of nitrofurantoin were modified according to a previously published method (20). Samples from the trans-

port assays were not processed, and 50 μl of the culture medium was injected directly into the HPLC system. For the mouse samples of nitrofurantoin, to each 50-μl aliquot of plasma or milk, 5 μl of furazolidone (12.5 μg/ml) was incorporated as an internal standard and 50 μl of cold methanol was added. Samples were shaken and kept at −20°C for 15 min, the organic and water phases were separated by centrifugation at 16,000 × *g* for 5 min, and 50 μl of the supernatant was injected into the HPLC system. Separation of the samples was performed on a reverse-phase column (Phenomenex Synergi 4-μm Hydro-RP 80A). The mobile phase consisted of 25 mM potassium phosphate buffer (pH 3)-acetonitrile (75:25), the flow rate of the mobile phase was set to 1.2 ml/min, and UV absorbance was measured at 366 nm. The temperature of the samples was 4°C, and the temperature of the column was 30°C. Standard samples in the appropriate drug-free matrix were prepared, yielding concentration ranges from 0.039 μg/ml to 5 μg/ml for transport samples, from 0.125 μg/ml to 4 μg/ml for plasma mouse samples, and from 0.0312 μg/ml to 4 μg/ml for milk mouse samples.

The conditions for HPLC analysis of sulfasalazine were modified according to previously published methods (13). For the mouse samples of sulfasalazine, to each 100-μl aliquot of plasma, 10 μl of probenecid (37.5 μg/ml in methanol) was incorporated as an internal standard and 300 μl of methanol was added. Samples were shaken and kept at −20°C for 15 min, and the organic and water phases were separated by centrifugation at 1,500 × *g* for 2 min. The supernatant was collected in a new Eppendorf tube and evaporated to dryness under a nitrogen stream. The samples were resuspended in 100 μl of methanol and injected into the HPLC system. Separation of the samples was performed on a reverse-phase column (Chemcobond 5-ODS-H, 5-μm particle size, 4.6 by 250 mm). The mobile phase consisted of 12 mM phosphate buffer containing 0.06% tetrabutylammonium hydrogen sulfate (pH 7.4)-methanol (50:50), the flow rate of the mobile phase was set to 1 ml/min, and UV absorbance was measured at 260 nm. The temperature of the samples was 4°C, and the temperature of the column was 40°C. Standard samples in the appropriate drug-free matrix were prepared, yielding a concentration range from 0.04 μg/ml to 40 μg/ml. Integration was performed using Empower software (Waters).

Statistical analysis. The two-sided unpaired Student *t* test was used throughout to assess the statistical significance of differences between the two sets of data. Results are presented as the means and standard deviations (SDs). Differences were considered to be statistically significant when P was <0.05.

RESULTS

Effect of TCBZ and its metabolites TCBZSO and TCBZSO$_2$ on ABCG2 ATPase activity. To characterize the interaction of TCBZ and its metabolites (TCBZSO and TCBZSO$_2$) with ABCG2, drug-stimulated ATPase activity in inside-out plasma membrane vesicles from isolated mammalian cells containing human ABCG2 was measured by monitoring the phosphate release rate at pH 7 and 37°C. Figure 2 shows the rate of ABCG2 ATPase activity as a function of compound concentration (log scale). Drug-stimulated ABCG2 ATPase activity is expressed as a percentage of the basal activity (taken as 100%). ABCG2 titration curves of the three compounds showed typical bell-shaped curves previously observed for P-glycoprotein (1), with activation at lower drug concentrations and clear inhibition at higher drug concentrations, indicating an important interaction with the transporter. Maximum activity increases in the order of TCBZ to TCBZSO to TCBZSO$_2$, and the concentration of half-maximum inhibition increases in the same order. The higher the half-maximum inhibition, the lower the inhibitory power of the compound. Note that, in all cases, the inhibition in ABCG2 ATPase activity is achieved at rather low concentrations. As has been seen for ATPase activity, all three compounds are probably effectively transported by ABCG2, with the best activation curve being for TCBZSO$_2$.

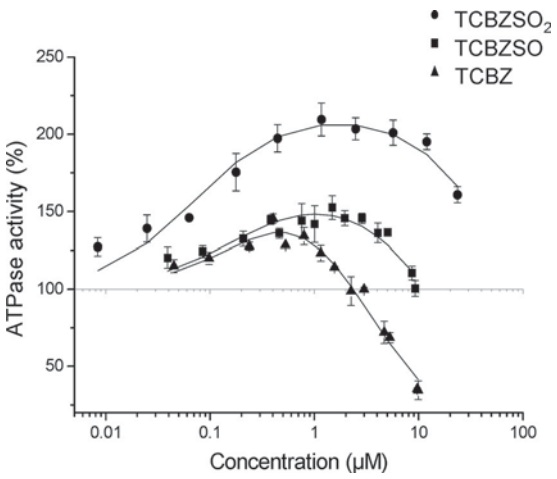

FIG 2 ATPase activity in inside-out plasma membrane vesicles as a function of the compound concentration for ABCG2. The titration curves shown represent the averages of two to four measurements; standard deviations are given. Curves are fits to the modified Michaelis-Menten equation proposed by Litman et al. (16).

Mitoxantrone accumulation assays. To further study the Abcg2/ABCG2 inhibitory effect of the major plasma metabolites TCBZSO and TCBZSO$_2$, the ability of these compounds to reverse the reduced mitoxantrone accumulation in murine Abcg2- and human ABCG2-expressing cell lines was tested in flow cytometry experiments. Abcg2/ABCG2 inhibition with the model inhibitor Ko143 increased the accumulation of mitoxantrone in Abcg2- and ABCG2-transduced cells and thus increased the median of fluorescence (MF) to levels similar to those in the parental cells.

Our results showed that the addition of TCBZSO or TCBZSO$_2$ at different concentrations (0.01 to 25 μM; higher concentrations were cytotoxic) (Fig. 3) increased, in a concentration-dependent manner, the accumulation of mitoxantrone (10 μM) in Abcg2/ABCG2-transduced cells. The strongest inhibitory potency for TCBZSO was reached at 25 μM for murine Abcg2-transduced cells (40%) and at 10 μM in the human ABCG2-transduced cells (55%). In the case of TCBZSO$_2$, the strongest inhibitory potency was reached at 25 μM for Abcg2-transduced cells and at 5 μM for ABCG2-transduced cells, with values of 55% in both cases. All these data indicate that TCBZSO and TCBZSO$_2$ are inhibitors of Abcg2/ABCG2.

***In vitro* transport of nitrofurantoin and danofloxacin in the presence of TCBZSO and TCBZSO$_2$.** To complete the characterization of the inhibitory behavior of the TCBZ metabolites on Abcg2/ABCG2 using other assays and Abcg2/ABCG2 substrates, we tested the effect of these compounds (15 μM TCBZSO and 15μM TCBZSO$_2$) on the Abcg2/ABCG2-mediated *in vitro* transport of two known Abcg2/ABCG2 substrates, the antibacterial agents nitrofurantoin (10 μM) and danofloxacin (10μM). As has already been reported (20, 26), we observed for nitrofurantoin (Fig. 4) and danofloxacin (Fig. 5) that in the MDCK-II parental cell line, apically and basolaterally directed translocations were similar (Fig. 4A and 5A), but that in the Abcg2/ABCG2-transduced MDCK-II cell lines, apically directed translocation was highly in-creased and basolaterally directed translocation dramatically decreased (Fig. 4D and G and 5D and G), since these drugs are excellent Abcg2/ABCG2 substrates.

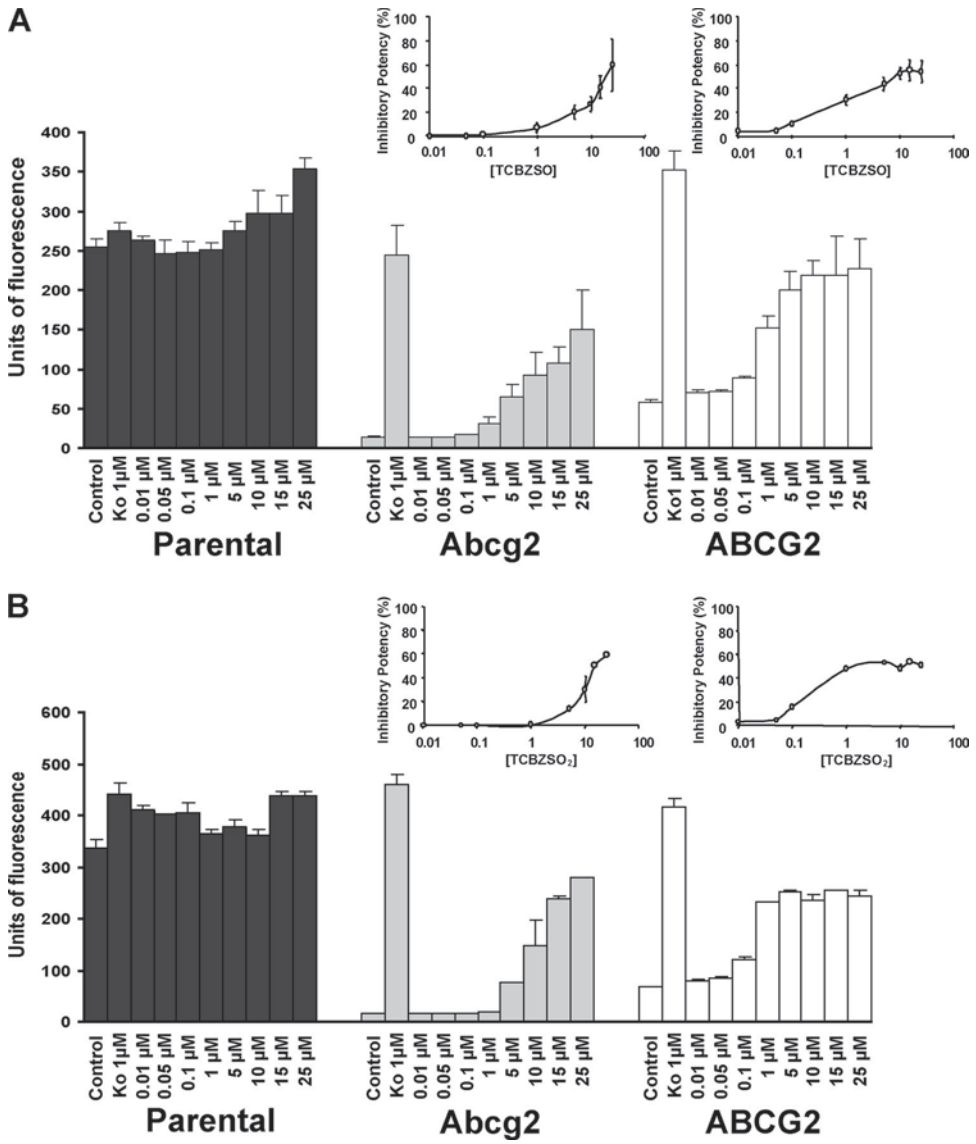

FIG 3 Effect of TCBZSO (A) and $TCBZSO_2$ (B) on accumulation of mitoxantrone (10 μM) at different concentrations in parent MDCK-II cells and in their murine Abcg2- and human ABCG2-transduced derivatives. Cells were preincubated with or without Ko143 (1 μM). Results (units of fluorescence) are expressed as the means of at least three experiments; error bars indicate SDs. In addition, inhibitory potencies of the different concentrations of the tested compounds for Abcg2 and ABCG2 are represented at the top of each graph. Inhibitory potency was related to the effect of the reference inhibitor Ko143 (set at 100% inhibition of Abcg2/ABCG2).

When we added TCBZSO (15 μM) and $TCBZSO_2$ (15 μM) as inhibitors, apically directed trans-location decreased and, subsequently, basolaterally directed trans-location increased compared to the control situation without an inhibitor in Abcg2/ABCG2-transduced cells (Fig. 4E, F, H, and I and 5E, F, H, and I). Murine Abcg2-mediated transport was mod-erately inhibited, and in the case of the human ABCG2, transport was almost completely inhibited in both cases, with relative trans-port ratios similar to those of the parental cells.

These results therefore showed that TCBZSO (15 μM) and $TCBZSO_2$ (15 μM) very efficiently inhibit the Abcg2/ABCG2-mediated transport of antibacterial substrates such as nitrofurantoin and danofloxacin.

Effect of coadministration of TCBZSO on plasma levels of sulfasalazine. To assess whether the *in vitro* Abcg2/ABCG2 inhib-itory role of the major plasma metabolites TCBZSO and $TCBZSO_2$ was also relevant *in vivo*, we studied the effect of the coadministration of TCBZSO on plasma levels of the sulfonamide sulfasalazine, a model ABCG2 substrate (35). Danofloxacin was not used for these pharmacokinetic experiments because Abcg2 does not affect plasma levels of danofloxacin in mice (26), and therefore, this antibacterial cannot be considered as an *in vivo* model substrate to study Abcg2-mediated effects on plasma levels. TCBZSO (50 mg/kg) or the vehicle was orally administered to wild-type and $Abcg2^{-/-}$ male mice 15 min prior to oral adminis-tration of sulfasalazine (20 mg/kg), and plasma samples were col-lected 30 min after sulfasalazine administration. The plasma con-

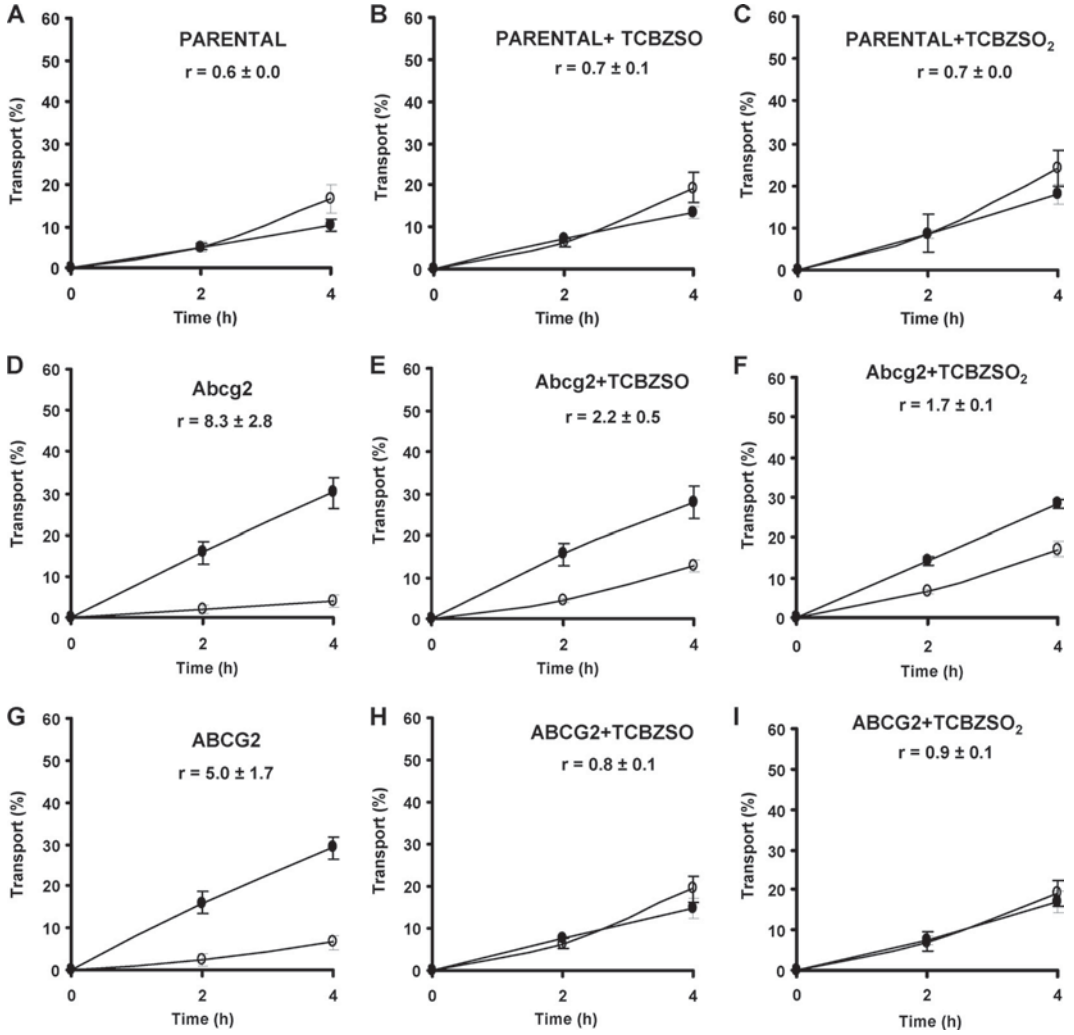

FIG 4 Transepithelial transport of nitrofurantoin (10 μM) in parent MDCK-II (A) and in their murine Abcg2- and human ABCG2-transduced derivatives (D and G) in the absence or presence of TCBZSO (15 μM) or $TCBZSO_2$ (15 μM). The experiment was started with the addition of nitrofurantoin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. TCBZSO (B, E, and H) and $TCBZSO_2$ (C, F, and I) were present as indicated. Results are means, and error bars (sometimes smaller than the symbols) indicate SDs ($n = 3$). ●, translocation from the basolateral to the apical compartment; ○, translocation from the apical to the basolateral compartment. *r* represents the relative transport ratio (i.e., the apically directed translocation divided by the basolaterally directed translocation) at 4 h.

centration of sulfasalazine was more than 1.5-fold higher in wild-type mice coadministered with TCBZSO than in control wild-type mice (0.63± 0.11 μg/ml versus 0.40± 0.13 μg/ml, respectively; $P < 0.05$) (Fig. 6A). No significant differences in plasma concen-trations of sulfasalazine were observed with TCBZSO treatment in the $Abcg2^{-/-}$ mice (4.91± 1.67 and 5.83± 1.70 μg/ml for control and TCBZSO-treated animals, respectively), indicating that the TCBZSO effect is Abcg2 specific. Plasma concentrations of sul-fasalazine in $Abcg2^{-/-}$ mice were approximately 10-fold higher than those in the wild-type animals (4.91± 1.67 μg/ml versus 0.40± 0.13 μg/ml, respectively) according to the results obtained by Zaher et al. (35), confirming that this compound is a very good *in vivo* substrate of Abcg2. We thus demonstrated that the coad-ministration of TCBZSO affects oral plasma levels of sulfasalazine through inhibition of Abcg2 at the dosage used.

Effect of TCBZSO coadministration on plasma and milk levels of nitrofurantoin. To further demonstrate an *in vivo* Abcg2/ABCG2 inhibitory role of TCBZ metabolites in other relevant drug-drug interactions and biological processes, the effect of the coadministration of TCBZSO on the secretion into milk of the antibacterial nitrofurantoin, an *in vivo* Abcg2/ABCG2 model substrate, was studied. Nitrofurantoin transfer into milk has been previously used as an experimental setting to test the *in vivo* effect of ABCG2 inhibitors (21, 33).

TCBZSO (50 and 100 mg/kg) was administered i.p. to lactating $Abcg2^{-/-}$ and wild-type females 5 min prior to an intravenous administration of nitrofurantoin (5 mg/kg). Thirty minutes after nitrofurantoin administration, milk and blood were collected. No significant differences were observed in plasma concentrations in wild-type mice after coadministration of TCBZSO at both doses (Fig. 6A). Plasma concentrations of nitrofurantoin in $Abcg2^{-/-}$ mice were approximately 3-fold higher than those in wild-type animals (1.70± 0.71 versus 0.59± 0.25 μg/ml, respectively; $P <$ 0.05), confirming that this compound is a very good *in vivo* sub-

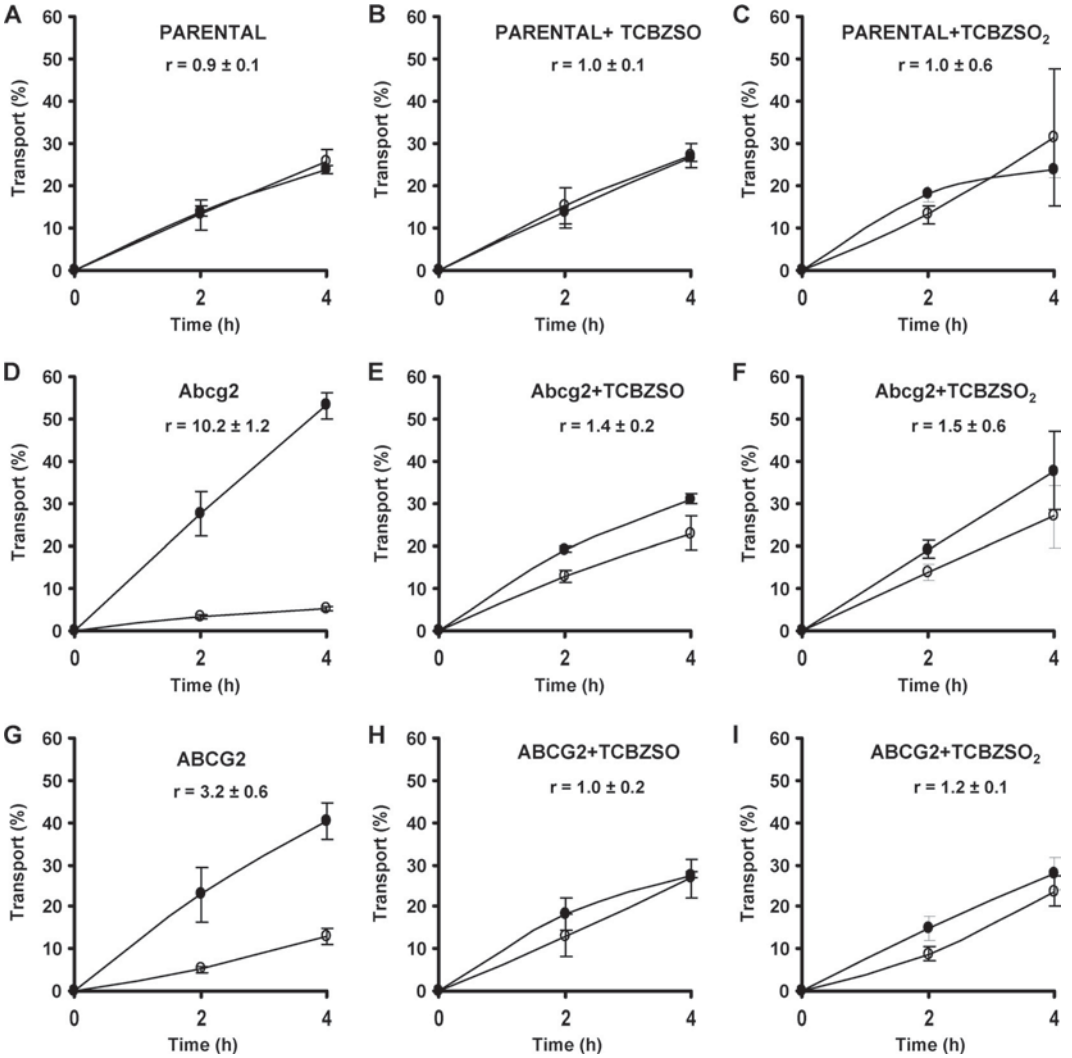

FIG 5 Transepithelial transport of danofloxacin (10 μM) in parent MDCK-II (A) and in their murine Abcg2- and human ABCG2-transduced derivatives (D and G) in the absence or presence of TCBZSO (15 μM) or $TCBZSO_2$ (15 μM). The experiment was started with the addition of danofloxacin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. TCBZSO (B, E, and H) and $TCBZSO_2$ (C, F, and I) were present as indicated. Results are means, and error bars (sometimes smaller than the symbols) indicate SDs ($n = 3$). ●, translocation from the basolateral to the apical compartment; ○, translocation from the apical to the basolateral compartment. *r* represents the relative transport ratio (i.e., the apically directed translocation divided by the basolaterally directed translocation) at 4 h.

strate of Abcg2. The milk concentration of nitrofurantoin (Fig. 6B) was more than 2-fold lower in wild-type mice treated with TCBZSO (50 mg/kg) (0.74± 0.44 μg/ml) and more than 4-fold lower in wild-type mice treated with TCBZSO (100 mg/kg)(0.38± 0.18 μg/ml) than in control wild-type mice (1.61± 0.53 μg/ml) ($P < 0.05$). No differences were observed after TCBZSO treatment in $Abcg2^{-/-}$ mice, indicating that the TCBZSO effect is Abcg2 specific. Consequently, TCBZSO inhibits Abcg2-mediated secretion of nitrofurantoin into milk since the milk-to-plasma ratio of this compound (Fig. 6C) was 3-fold lower in wild-type mice treated with TCBZSO (50 mg/kg) (0.93± 0.25) and almost 4-fold lower in wild-type mice treated with TCBZSO (100 mg/kg)(0.75± 0.49) than in control wild-type mice (2.79± 1.42) ($P < 0.05$).

Our results show that coadministration of TCBZSO inhibits Abcg2/ABCG2-mediated secretion of nitrofurantoin into milk at the dosage used.

DISCUSSION

The concomitant administration of multiple drugs is often used in pharmacotherapy and may affect their kinetics and pharmacolog-ical activity. There is increasing evidence to suggest that interfer-ence between drugs and ATP-binding cassette (ABC) proteins is a key mechanism underpinning clinically important drug interac-tions (17). It is therefore of interest to study the potential effect of the major active plasma metabolites of the widely used fas-ciolicide TCBZ (TCBZSO and $TCBZSO_2$) in drug interactions with Abcg2/ABCG2 substrates affecting pharmacokinetics and milk secretion. In this study, we have shown that TCBZSO and $TCBZSO_2$ efficiently inhibit *in vitro* and *in vivo* ABCG2 trans-porter activity by using different *in vitro* and *in vivo* assays with different substrates.

In ATPase assays (Fig. 2), ABCG2 inhibition was observed for all three compounds studied, TCBZ, TCBZSO, and $TCBZSO_2$, a t

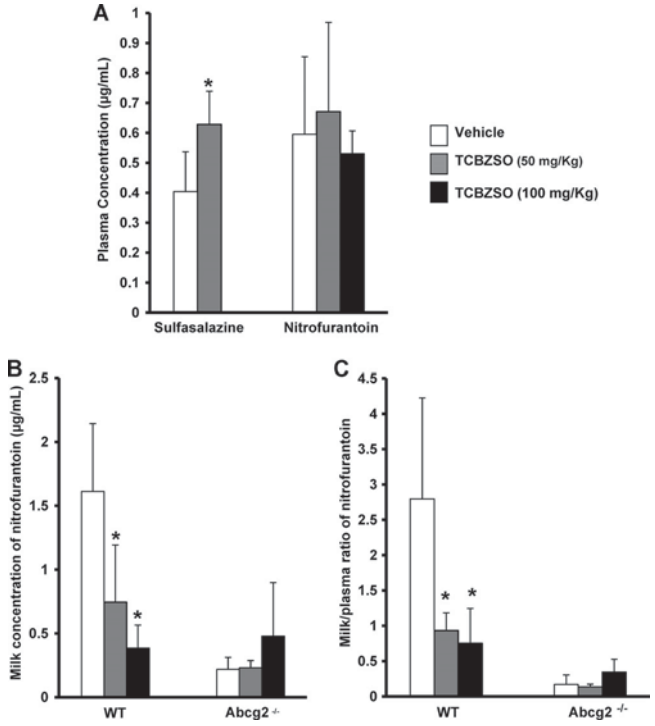

FIG 6 *In vivo* effect of TCBZSO coadministration. (A) Plasma concentrations of sulfasalazine and nitrofurantoin in wild-type mice. TCBZSO (50 mg/kg) or the vehicle was administered orally to males 15 min prior to oral administration of sulfasalazine (20 mg/kg). TCBZSO (50 or 100 mg/kg) or the vehicle was administered i.p. to lactating females 5 min prior to i.v. administration of nitrofurantoin (5 mg/kg). (B and C) Milk concentrations (B) and milk/plasma ratios (C) of nitrofurantoin in wild-type and Abcg2$^{-/-}$ lactating females. TCBZSO (50 or 100 mg/kg) or the vehicle was administered i.p. to mice 5 min prior to i.v. administration of nitrofurantoin (5 mg/kg). Plasma and milk were collected after 30 min of drug administration and analyzed by HPLC. Results are means, and error bars indicate SDs (n = 4 to 7). *, $P < 0.05$ (significant differences between control and TCBZSO treatments in wild-type mice).

concentrations higher than 1 μM, with the strongest inhibition observed in the case of TCBZ, the most hydrophobic compound. Subsequent inhibition studies were performed with the major plasma metabolites TCBZSO and $TCBZSO_2$, since due to its high metabolism, the TCBZ parent drug is not detected in plasma. In mitoxantrone accumulation assays with a concentration range from 5 to 25 μM, both compounds showed inhibitory potencies between 40 and 55% for murine Abcg2/human ABCG2. Some drugs considered to be good ABCG2 inhibitors showed 50% in-hibitory concentrations (IC_{50}s) in the same range for the same cell line (34): for lopinavir, 7.66 μM; for nelfinavir, 13.50 μM; for saquinavir, 27.40 μM; and for delavirdine, 18.60 μM. For other benzimidazole drugs considered to interact with ABCG2, such as pantoprazole and omeprazole, the IC_{50}s were 13 μM and 36 μM, respectively (3). Our concentration values with an inhibitory potency close to 50% are in the same range as the plasma concentrations of the active metabolite TCBZSO that were reported in humans (25 μM≈9.4 μg/ml) (5) and in veterinary species (30 μM≈ 11.3 μg/ml) (7) after treatment at the therapeutic dose.

The Abcg2/ABCG2 inhibitory potential of the TCBZ metabolites was also confirmed for other known Abcg2/ABCG2 substrates, such as the antibacterial agents nitrofurantoin and danofloxacin, in transepithelial transport experiments at a concentration of 15 μM, showing a moderate inhibition for murine Abcg2 and a complete inhibition for human ABCG2 (Fig. 4 and 5). The 15 μM concentration was chosen based on the stronger inhibition observed in the mitoxantrone accumulation assays for human ABCG2. Inhibition of the *in vitro* transepithelial transport of both compounds at concentrations of TCBZ metabolites below 15 μM could not be excluded. In-hibition in transepithelial transport experiments can be ex-pected as long as the concentration of the drug is higher than the concentration at maximum activity in an ATPase assay (for all three compounds, in the ABCG2 ATPase activity profiles, the maximum activity was reported at around 1 μM) (27). The similar inhibitory power of TCBZSO and $TCBZSO_2$ that was observed in transport assays is due to the similar concentration of half-maximum inhibition in ATPase assays (Fig. 2). Al-though the interaction of these compounds with other ABC transporters, such as P-glycoprotein, has been previously re-ported (4), a lack of effect of these compounds on vectorial transport in parental cells (Fig. 4A, B, and C and 5A, B, and C) indicates that this interaction is probably ABCG2 specific in our experimental setting. All these data indicate that both TCBZ metabolites are good *in vitro* inhibitors of Abcg2/ABCG2.

Furthermore, we demonstrated the relevance of the ABCG2 inhibition properties of these compounds in mice using two different ABCG2 substrates in two different pharmacokinetic processes. Plasma levels of sulfasalazine and milk levels of nitrofuran-toin (Fig. 6) were significantly affected by the coadministration of TCBZSO only in wild-type animals, with no effect on Abcg2$^{-/-}$ mice, indicating the Abcg2-specific effect. This effect is most likely due not only to the inhibition exerted by TCBZSO itself but also to that by its metabolite $TCBZSO_2$. TCBZSO coadministration did not affect nitrofurantoin plasma levels. Some authors have re-ported local effects mediated by Abcg2 (fetal distribution and milk secretion) but no differences in plasma systemic profiles between wild-type and knockout mice for some substrates (24, 30, 36). Unlike the nitrofurantoin experiment, there seems to have been an Abcg2-mediated effect of TCBZSO coadministration on plasma levels of sulfasalazine, since the difference in plasma concentrations of this compound after oral administration be-tween untreated Abcg2$^{-/-}$ and wild-type mice was approxi-mately 10-fold, whereas in the case of nitrofurantoin (i.v. ad-ministration), it was only 3-fold, thus indicating a higher effect of Abcg2 on the systemic disposition of sulfasalazine after oral administration. In addition, the different routes of TCBZSO administration (oral for the sulfasalazine experiment and intraperitoneal for the nitrofurantoin experiment) and/or the gender or physiological status of the animals may influence the TCBZSO inhibitory effect.

This *in vivo* interaction between drugs resulting in higher plasma levels or lower secretion of the substrate into milk can be applied not only to the substrates tested but also to other ABCG2 substrates. This finding is highly relevant considering that concurrent administration of different drugs is a usual clinical practice. In addition, TCBZ is marketed in combination with other anthelmintics to improve efficacy, to broaden the spectrum of activity, and to limit resistance emergence (4). Some of these drug combinations include drugs, such as ivermectin (15) or oxfendazole

(19), that are known to interact with ABC transporters. It will therefore be of interest to further study the possible *in vivo* effect of these TCBZ metabolites in the potential drug interactions with other known Abcg2/ABCG2 substrates in therapeutic target species (humans and livestock).

ABCG2 inhibitors can be used in combination therapy with substrates of the transporter in order to modulate their pharmacokinetics, brain penetration, milk secretion, and, thus, their efficacy. Several studies have managed to increase the bioavailability and milk secretion of antibacterial agents, such as nitrofurantoin, or antitumorals, such as topotecan, or to improve brain penetration of the antitumoral imatinib with the use of ABCG2 and P-glycoprotein inhibitors, such as elacridar, the benzimidazole pantoprazole, or isoflavones (2, 11, 14, 21, 25). However, it has to be noted that the use of TCBZ for this purpose may be controversial in animals whose products are destined for human consumption or in areas of parasite endemicity due to the potential development of resistance.

In addition, inhibitors of ABCG2 may be useful in other appli-cation fields, e.g., for reversal resistance in chemotherapy (22). Further studies are needed to show the application of these compounds in this field.

In summary, in this study, we have shown clear *in vitro* and *in vivo* interactions between the major plasma metabolites of TCBZ and ABCG2. These compounds are excellent ABCG2 inhibitors, and their relevance may be important for ABCG2-mediated drug-drug interactions affecting drug bioavailability.

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REFERENCES

1. **Aanismaa P, Seelig A.** 2007. P-glycoprotein kinetics measured in plasma membrane vesicles and living cells. Biochemistry **46**:3394–3404.
2. **Breedveld P, et al.** 2005. The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res. **65**:2577–2582.
3. **Breedveld P, et al.** 2004. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res. **64**: 5804–5811.
4. **Dupuy J, Alvinerie M, Menez C, Lespine A.** 2010. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. Chem. Biol. Interact. **186**:280–286.
5. **El-Tantawy WH, Salem HF, Mohammed Safwat NA.** 2007. Effect of Fascioliasis on the pharmacokinetic parameters of triclabendazole in human subjects. Pharm. World Sci. **29**:190–198.
6. **Fairweather I.** 2009. Triclabendazole progress report, 2005-2009: an advancement of learning? J. Helminthol. **83**:139–150.
7. **Fairweather I, Boray JC.** 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet. J. **158**:81–112.
8. **Giacomini KM, et al.** 2010. Membrane transporters in drug development. Nat. Rev. Drug Discov. **9**:215–236.
9. **Glavinas H, et al.** 2007. ABCG2 (breast cancer resistance protein/mitoxantrone resistance-associated protein) ATPase assay: a useful tool to detect drug-transporter interactions. Drug Metab. Dispos. **35**:1533–1542.
10. **Hennessy DR, Lacey E, Steel JW, Prichard RK.** 1987. The kinetics of triclabendazole disposition in sheep. J. Vet. Pharmacol. Ther. **10**:64–72.
11. **Jonker JW, et al.** 2005. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat. Med. **11**:127–129.
12. **Jonker JW, et al.** 2000. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J. Natl. Cancer Inst. **92**:1651–1656.
13. **Kita T, et al.** 2001. N-Acetyltransferase 2 genotype correlates with sulfasalazine pharmacokinetics after multiple dosing in healthy Japanese subjects. Biol. Pharm. Bull. **24**:1176–1180.
14. **Kruijtzer CM, et al.** 2002. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J. Clin. Oncol. **20**:2943–2950.
15. **Lifschitz A, Virkel G, Ballent M, Sallovitz J, Lanusse C.** 2009. Combined use of ivermectin and triclabendazole in sheep: in vitro and in vivo characterisation of their pharmacological interaction. Vet. J. **182**:261–268.
16. **Litman T, Zeuthen T, Skovsgaard T, Stein WD.** 1997. Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity. Biochim. Biophys. Acta **1361**:169–176.
17. **Marchetti S, Mazzanti R, Beijnen JH, Schellens JH.** 2007. Concise review: clinical relevance of drug-drug and herb-drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). Oncologist **12**:927–941.
18. **Merino G, et al.** 2006. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. Drug Metab. Dispos. **34**:690–695.
19. **Merino G, et al.** 2005. Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). Drug Metab. Dispos. **33**:614–618.
20. **Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH.** 2005. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Mol. Pharmacol. **67**:1758–1764.
21. **Merino G, et al.** 2010. In vivo inhibition of BCRP/ABCG2 mediated transport of nitrofurantoin by the isoflavones genistein and daidzein: a comparative study in Bcrp1 (−/−) mice. Pharm. Res. **27**:2098–2105.
22. **Noguchi K, Katayama K, Mitsuhashi J, Sugimoto Y.** 2009. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. Adv. Drug Deliv. Rev. **61**:26–33.
23. **Pavek P, et al.** 2005. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. J. Pharmacol. Exp. Ther. **312**:144–152.
24. **Perez M, et al.** 2009. In vitro and in vivo interaction of moxidectin with BCRP/ABCG2. Chem. Biol. Interact. **180**:106–112.
25. **Perez M, et al.** 2009. Milk secretion of nitrofurantoin, as a specific BCRP/ABCG2 substrate, in assaf sheep: modulation by isoflavones. J. Vet. Pharmacol. Ther. **32**:498–502.
26. **Real R, et al.** 2011. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: interaction with ivermectin. J. Vet. Pharmacol. Ther. **34**:313–321.
27. **Seelig A.** 2007. The role of size and charge for blood-brain barrier permeation of drugs and fatty acids. J. Mol. Neurosci. **33**:32–41.
28. **Shukla S, Ohnuma S, Ambudkar SV.** 2011. Improving cancer chemotherapy with modulators of ABC drug transporters. Curr. Drug Targets **12**:621–630.
29. **Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM.** 2006. Targeting multidrug resistance in cancer. Nat. Rev. Drug Discov. **5**:219–234.
30. **Tang SC, et al.** 2012. Brain accumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration. Int. J. Cancer **130**:223–233.
31. **van Herwaarden AE, Schinkel AH.** 2006. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. Trends Pharmacol. Sci. **27**:10–16.
32. **van Herwaarden AE, et al.** 2006. Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. Carcinogenesis **27**:123–130.
33. **Wang L, Leggas M, Goswami M, Empey PE, McNamara PJ.** 2008.

N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) as a chemical ATP-binding cassette transporter family G member 2 (Abcg2) knockout model to study nitrofurantoin transfer into milk. Drug Metab. Dispos. **36**:2591–2596.

34. **Weiss J, et al.** 2007. Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. J. Antimicrob. Chemother. **59**:238–245.
35. **Zaher H, et al.** 2006. Breast cancer resistance protein (Bcrp/abcg2) is a major determinant of sulfasalazine absorption and elimination in the mouse. Mol. Pharm. **3**:55–61.
36. **Zhou L, et al.** 2008. The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an Obstetric-Fetal Pharmacology Research Unit Network and University of Washington Specialized Center of Research Study. Mol. Pharmacol. **73**:949–959.

7. ARTÍCULO II

Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: Role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter.

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Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: Role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter

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ABSTRACT

ATP-binding cassette transporter G2/breast cancer resistance protein (ABCG2/BCRP) mediates drug–drug interactions that affect the secretion of drugs into milk. The aims of this study were: (1) to determine whether the major plasma metabolites of the flukicide triclabendazole (TCBZ), triclabendazole sulfoxide (TCBZSO) and triclabendazole sulfone ($TCBZSO_2$), inhibit ovine and bovine ABCG2 and its Y581S variant in vitro, and (2) to examine whether coadministration of TCBZ with the ABCG2 substrates danofloxacin (a fluoroquinolone) and moxidectin (a milbemycin) affects the secretion of these drugs into the milk of sheep.

TCBZSO and $TCBZSO_2$ inhibited ruminant ABCG2 in vitro by reversing the reduced mitoxantrone accumulation and reducing basal to apical transport of nitrofurantoin in cells transduced with bovine variants (S581 and Y581) and the ovine variant of ABCG2. Coadministration of TCBZ with moxidectin or danofloxacin to sheep resulted in significantly reduced levels of moxidectin, but not danofloxacin, in the milk of TCBZ-treated sheep compared to sheep administered moxidectin or danofloxacin alone. The milk area under concentration time curve (AUC 0–48 h) was 2.99 ± 1.41 μg h/mL in the group treated with TCBZ and moxidectin, and 7.75 ± 3.58 μg h/mL in the group treated with moxidectin alone. The AUC (0–48 h) milk/plasma ratio was 37% lower in the group treated with TCBZ and moxidectin (7.34 ± 1.51) than in the group treated with moxidectin alone (11.68 ± 3.61). TCBZ metabolites appear to inhibit ruminant ABCG2 and affect the secretion of ABCG2 substrates into milk of sheep.

Introduction

ATP-binding cassette (ABC) transporters are major determinants of the efflux of a wide variety of drugs, protecting against the toxicity of xenobiotics and influencing pharmacokinetics and pharmacodynamics. ABC efflux proteins in parasites are important in anthelmintic resistance (Lespine et al., 2012). Milbemycins (such as moxidectin), benzimidazoles (such as albendazole sulfoxide and oxfendazole) and fluoroquinolones (such as enrofloxacin and danofloxacin) are substrates of the ABC transporter ABCG2 (Perez et al., 2009a; Real et al., 2011a; Mealey, 2012). Induced expression of ABCG2 during lactation is related to its role in active drug secretion into milk (Jonker et al., 2005).

Multiple drugs are often administered concomitantly in veterinary therapy, which may affect their kinetics and pharmacological activity. Drug–drug interactions mediated by ABC transporters include increased plasma availability and potentiation of the macrocyclic lactones ivermectin and moxidectin after coadministration of the anthelmintic triclabendazole (TCBZ) or inhibitors of the ABC transporter P-glycoprotein (Lifschitz et al., 2009; McKellar and Gokbulut, 2012). In Assaf sheep, secretion of danofloxacin into milk is reduced on coadministration with the macrocyclic lactone ivermectin (Real et al., 2011a) or by supplementation with a soy-enriched diet (Perez et al., 2013).

Genetic variations in ABCG2 should also be considered, since the bovine ABCG2 Y581S single nucleotide polymorphism (SNP) is associated with increased secretion of danofloxacin into milk (Otero et al., 2013), differential inhibition of the macrocyclic lactone ivermectin in vitro and increased transepithelial transport of antibiotics in vitro (Real et al., 2011b).

Triclabendazole (TCBZ) is a halogenated benzimidazole thiol derivative used for the treatment of liver fluke (*Fasciola* spp.) infestation (Fairweather, 2009). The parent drug TCBZ is not detected in

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plasma or milk after its oral administration in sheep, because it is rapidly metabolised into triclabendazole sulfoxide (TCBZSO) and triclabendazole sulfone ($TCBZSO_2$) (Hennessy et al., 1987; Imperi-ale et al., 2011). Triclabendazole metabolites are good inhibitors of the murine and human variants of ABCG2 (Barrera et al., 2012). However, their relevance for drug–drug interactions in ruminants remains to be established.

The aims of the present study were (1) to determine whether the major plasma TCBZ metabolites (TCBZSO and $TCBZSO_2$) in vitro inhibit the two bovine and the ovine ABCG2 variants, and (2) to assess the relevance of TCBZ metabolites in in vivo drug–drug interactions by studying the effect of TCBZ coadministration on the disposition of the ABCG2 substrates moxidectin (Perez et al., 2009a) and danofloxacin (Real et al., 2011a) in the plasma and milk of sheep.

Materials and methods

Cell cultures

Madin-Darby canine kidney epithelial cell (MDCKII) parental cells were provided by Dr A.H. Schinkel, Netherlands Cancer Institute, Amsterdam, The Netherlands. MDCKII cells were stably transduced with both bovine variants (S581 and Y581) and the ovine variant of ABCG2 (Real et al., 2011b). Culture conditions were as described previously (Jonker et al., 2000; Pavek et al., 2005).

Accumulation assays

In vitro accumulation assays were carried out as described previously (Pavek et al., 2005). Mitoxantrone (MXR, 10 μM; Sigma–Aldrich) was used as a fluorescent substrate and the compounds tested were used as inhibitors. Relative cellular accumulation of MXR was determined by flow cytometry using a CYAN cytometer (Beckman Coulter) from histogram plots using the median of fluorescence (MF). ABCG2 inhibition increases the accumulation of MXR in ABCG2-transduced cells and thus increases MF. Inhibitory potencies were calculated as described previously (Pavek et al., 2005): Inhibitory potency = (MF with tested compound − MF without inhibitor)/(MF with Ko143 − MF without inhibitor) × 100%. Ko143 (1 μM; Tocris) has been described previously as a potent and specific ABCG2 inhibitor (Allen et al., 2002).

Transport studies

Transport assays using Transwell plates were carried out as described previously (Merino et al., 2010; Real et al., 2011b), with minor modifications. Cells were seeded on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar) at a density of 1.0×10^6 cells per well and grown for 3 days. Transepithelial resistance, as measured in each well using a Millicell ERS ohmmeter (Millipore), was used to check the integrity of the monolayer. The appearance of nitrofurantoin (Sigma–Aldrich) in the acceptor compartment was presented as the fraction of total nitrofurantoin added to the donor compartment at the beginning of the experiment.

Pharmacokinetic studies

The experiments were performed on the Experimental Farm of the University of Leon, Spain, and were approved by the Research Committee for Animal Use of the University of Leon (approval number 13-2011, date of approval 7 November 2011).

Twenty-four lactating Assaf sheep (3–4 months in lactation), aged 2–3 years and weighing 70–75 kg, were used in this study. The animals were parasite-free and drinking water was available ad libitum. The experimental design was per-formed with animals divided into four groups: (1) the first group (n = 6) received a single dose of 1.25 mg/kg danofloxacin (Advocin 2.5%, Pfizer) IM; (2) the second group (n = 6) was injected with 1.25 mg/kg danofloxacin (Advocin 2.5%) IM coad-ministered with 1 mg/kg TCBZ (Sequoia Research Products) IV; (3) the third group (n = 6) received a single dose of 0.2 mg/kg moxidectin (Cydectin 1%, Fort Dodge) SC;(4) and the fourth group (n = 5) was injected with 0.2 mg/kg moxidectin (Cydectin 1%) SC coadministered with 2 mg/kg TCBZ (Fasinex 10%) PO. The dosages and routes of administration of TCBZ were chosen in an attempt to achieve similar plasma profiles of the TCBZ metabolites and the coadministered drugs and to avoid concentrations in milk >0.20 μg/mL (close to the maximum limits of TCBZ residues in target tissues) (EMEA, 2006).

Blood samples were collected from the jugular vein and milk samples were collected after complete milking of the gland before each treatment, at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h after danofloxacin administration (groups 1 and 2), and at 1, 4, 8, 12, 24, 36, 48, 72, 96, 120, 192 and 360 h after moxidectin administration (groups 3 and 4). Plasma was separated by centrifugation at 1200 *g* for 15 min and plasma and milk samples were stored at −20 °C until analysis.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was used to determine concentrations of danofloxacin in plasma and milk in experimental groups 1 and 2, and concentrations of moxidectin in plasma and milk in experimental groups 3 and 4. Levels of the major plasma and milk TCBZ metabolites were analysed in the TCBZ-treated groups (groups 2 and 4) by HPLC.

Conditions for HPLC of nitrofurantoin were modified in accordance with Merino et al. (2005). The mobile phase consisted of 25 mM potassium phosphate buffer (pH 3):acetonitrile (75:25), the flow rate of the mobile phase was set to 1.2 mL/min and ultraviolet (UV) absorbance was measured at 366 nm. Standard samples for calibration curves were prepared at concentrations of 0.04–5 μg/mL, with coefficients of correlation >0.99. The limit of quantification (LOQ) was 40 ng/mL.

Conditions for HPLC analysis of danofloxacin were modified in accordance with Perez et al. (2013). Marbofloxacin (Sigma–Aldrich) was used as an internal standard and samples were extracted with chloroform. The mobile phase consisted of 25 mM orthophosphoric acid (pH 3.0):acetonitrile (75:25) at a flow rate of 1.5 mL/min. Fluorescence was detected at 338 nm (excitation) and 425 nm (emission). Standard samples of danofloxacin (Sigma–Aldrich) for calibration curves were prepared at concentrations of 0.01–0.64 μg/mL for plasma and 0.16–10 μg/mL for milk, with coefficients of correlation >0.99. The extraction recovery levels for concentrations in the standard curve were 85% for plasma and 86% for milk. LOQs were 8.2 ng/mL for plasma and 100 ng/mL for milk.

Conditions for HPLC analysis of moxidectin were modified in accordance with Prieto et al. (2003). Ivermectin (Sigma–Aldrich) was used as an internal standard and samples were extracted with methanol and derivatised. The mobile phase consisted of acetonitrile:methanol:water with 0.2% acetic acid (45%:50%:5%, V/V/V) at a flow rate of 1.8 mL/min. Fluorescence was detected at 365 nm (excitation) and 475 nm (emission). Standard samples of moxidectin (Sigma–Aldrich) for calibration curves were prepared at concentrations of 0.001–0.06 μg/mL for plasma and 0.004–0.36 μg/mL for milk, with coefficients of correlation >0.99. The extraction recovery levels for concentrations in the standard curve were 82% for plasma and 85% for milk. LOQs were 1 ng/mL for plasma and 3 ng/mL for milk.

Conditions for HPLC analysis of TCBZ metabolites were modified in accordance with Imperiale et al. (2011). Oxibendazole (Sigma–Aldrich) was used as an internal standard and samples were extracted with acetonitrile. The mobile phase consisted of 25 mM ammonium acetate (pH: 6.6):acetonitrile (48:52) at a flow rate of 1.2 mL/min and UV absorbance was measured at 300 nm. Standard samples of TCBZSO (LGC Standards) and $TCBSO_2$ (LGC Standards) for calibration curves were prepared at concentrations of 0.08–10 μg/mL for plasma and 0.04–0.625 μg/mL for milk, with coefficients of correlation >0.99. The extraction recovery levels for concentrations in the standard curve for TCBZSO were 72% for plasma and 71% for milk, and for $TCBZSO_2$ were 77% for plasma and 76% for milk. LOQs for TCBZSO were 52.8 ng/mL for plasma and 24.1 ng/mL for milk. LOQs for $TCBZSO_2$ were 9.6 ng/mL for plas-ma and 21.6 ng/mL for milk.

Statistical analysis

Results are reported as the mean ± standard deviation (SD). Statistical analysis for significant differences was performed using the two-tailed Student's t test. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Inhibitory potency of the two TCBZ metabolites in mitoxantrone accumulation assays

To demonstrate the potential inhibitory effect of major plasma metabolites of TCBZ (TCBZSO and $TCBZSO_2$) on ruminant ABCG2, the ability of these compounds to reverse the reduced mitoxantrone accumulation in cells transduced with both bovine variants (S581 and Y581) and the ovine variant of ABCG2 was tested in flow cytometry experiments. The presence of either TCBZ metabolite inhibited all the ruminant ABCG2 variants, increasing the accumulation of mitoxantrone in ABCG2-transduced cells in a concentration-dependent manner. The highest inhibitory potency (60–70%) appeared at 25 μM for the bovine Y581 variant (Figs. 1A and B).

In vitro transport of nitrofurantoin in the presence of both TCBZ metabolites

To further characterise the inhibitory properties of the TCBZ metabolites on ruminant ABCG2, transepithelial transport assays were carried out with MDCKII cells transduced with the two bovine and the ovine variants of ABCG2 using the antibacterial nitrofurantoin (10 μM) as a known ABCG2 model substrate and TCBZSO (15 μM) and $TCBZSO_2$ (15 μM) as inhibitors (Figs. 2 and 3; Table 1). As has already been reported for bovine ABCG2 (Real et al., 2011b), the basal to apical transport increased greatly, while the apical to basal transport decreased in the cells expressing the ruminant transporters (Figs. 2D, G and 3D), with basal–apical:apical–basal ratios >2 compared with the parent cells (Table 1). This ruminant ABCG2-mediated transport was inhibited when TCBZSO (15 μM) (Figs. 2E, H and 3E) and $TCBZSO_2$ (15 μM) (Figs. 2F, I and 3F) were added, since the basal to apical transport decreased, while apical to basal transport increased, in comparison with the control (no inhibitor) in cells transduced with bovine and ovine ABCG2 (Figs. 2 and 3; Table 1). These results demonstrated that TCBZSO and $TCBZSO_2$ are in vitro inhibitors of the ovine and the two bovine variants of ABCG2.

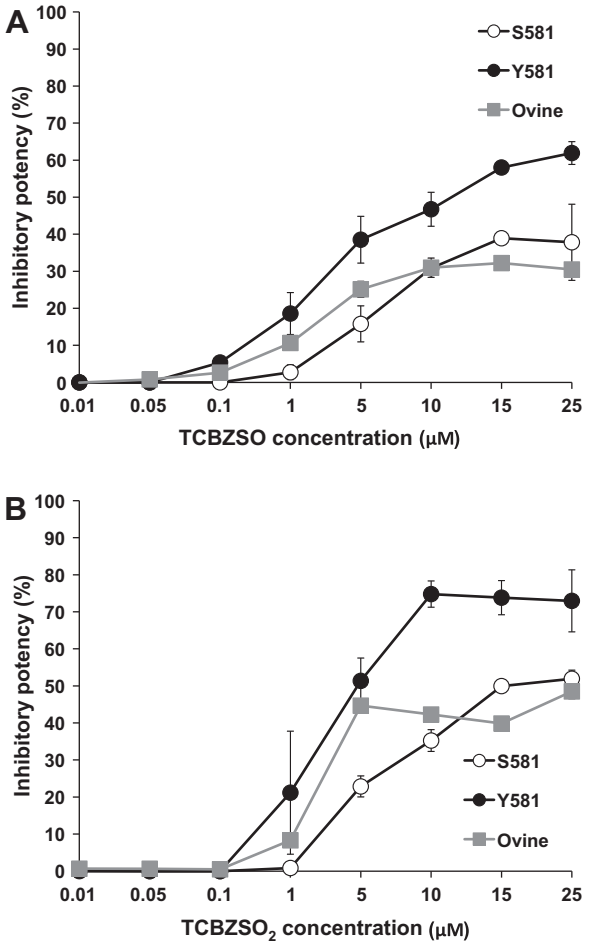

Fig. 1. Inhibitory potencies of the different concentrations of the compounds tested, TCBZSO (A) and $TCBZSO_2$ (B), for ovine and the two bovine variants of ABCG2 in mitoxantrone accumulation assays. Bars indicate standard deviations ($n = 3$). Inhibitory potency was related to the effect of reference inhibitor Ko143 (1 μM) (set at 100% inhibition of ABCG2).

Effect of TCBZ coadministration on pharmacokinetics and milk secretion of the antibacterial danofloxacin and the endectocide moxidectin

To demonstrate further the in vivo ABCG2 inhibitory role of TCBZ metabolites in clinically relevant drug–drug interactions, the effect of the coadministration of the parent drug TCBZ on the pharmacokinetics and secretion into milk of two well-known ABCG2 substrates widely used in veterinary medicine was studied in sheep. These were the fluoroquinolone danofloxacin and the macrocyclic lactone moxidectin. In animals treated with danofloxacin, no significant differences were noted between the control group and the TCBZ-treated group in plasma and milk pharmacokinetics (Figs. 4A and C; Table 2). In the TCBZ-treated group, concentrations of TCBZSO and $TCBZSO_2$ in milk were 20× lower than danofloxacin concentrations (Figs. 4B and D).

In sheep treated with moxidectin, plasma levels did not differ significantly between TCBZ-treated animals and the control group (Fig. 5A; Table 3). However, there were significant differences in milk levels between the control group (moxidectin only) and the TCBZ-treated group (moxidectin plus TCBZ) at 12, 24 and 36 h (Fig. 5C). The milk maximum concentration (C_{max}), area under concentration (AUC 0–48 h) values and the AUC (0–48 h) milk:plasma ratio decreased significantly in TCBZ-treated animals (Table 3). Furthermore, the dose secreted in milk at 48 h was 2.3 times lower in the TCBZ-treated group. Pharmacokinetic profiles of TCBZ metabolites in plasma and milk in the TCBZ-treated group were similar to those for moxidectin (Fig. 5B and D). These results indicate that coadministration of TCBZ decreases the secretion of moxidectin into milk.

Discussion

Interaction between drugs and ABC transporters, including ABCG2, is a mechanism of relevance for clinically important drug–drug interactions (Marchetti et al., 2007). In this study, effi-cient in vitro inhibition of both variants of bovine ABCG2 and the ovine ABCG2 by the major plasma metabolites of the anthelmintic TCBZ (TCBZSO and $TCBZSO_2$) was demonstrated. Furthermore, the extent of in vivo ABCG2-mediated drug–drug interactions involv-ing TCBZ metabolites and veterinary ABCG2 substrates was assessed in sheep.

In vitro results showed efficient inhibition of ovine ABCG2 and both bovine variants of ABCG2 by these metabolites. A differential inhibition pattern previously observed between the two variants for ivermectin (Real et al., 2011b) was confirmed in this study by the higher inhibitory potencies observed for the bovine Y581 variant compared to S581 (Fig. 1). Both TCBZ metabolites showed inhibitory potencies of 30–80% with concentrations from 10 to 25 μM. This last concentration is very close to the plasma concentration of the active metabolite TCBZSO reported for sheep (30 μM) after therapeutic treatment with TCBZ (Fairweather and Boray, 1999). Transport experiments for the ABCG2 substrate nitrofurantoin in the presence of the two TCBZ metabolites confirmed the inhibitory properties of these compounds at a concentration of 15 μM, although it is not possible to rule out inhibition at lower concentrations.

Inhibition of ruminant ABCG2 by the two TCBZ metabolites was confirmed in an in vivo setting when TCBZ was coadministered to sheep jointly with the endectocide moxidectin, an ABCG2 substrate. Levels of moxidectin in milk were significantly decreased by TCBZ, with no significant effect in on plasma levels (Figs. 5A and C). Although the interaction of these compounds with other ABC transporters, such as P-glycoprotein, has been reported previ-ously (Dupuy et al., 2010), the effect of the TCBZ metabolites on

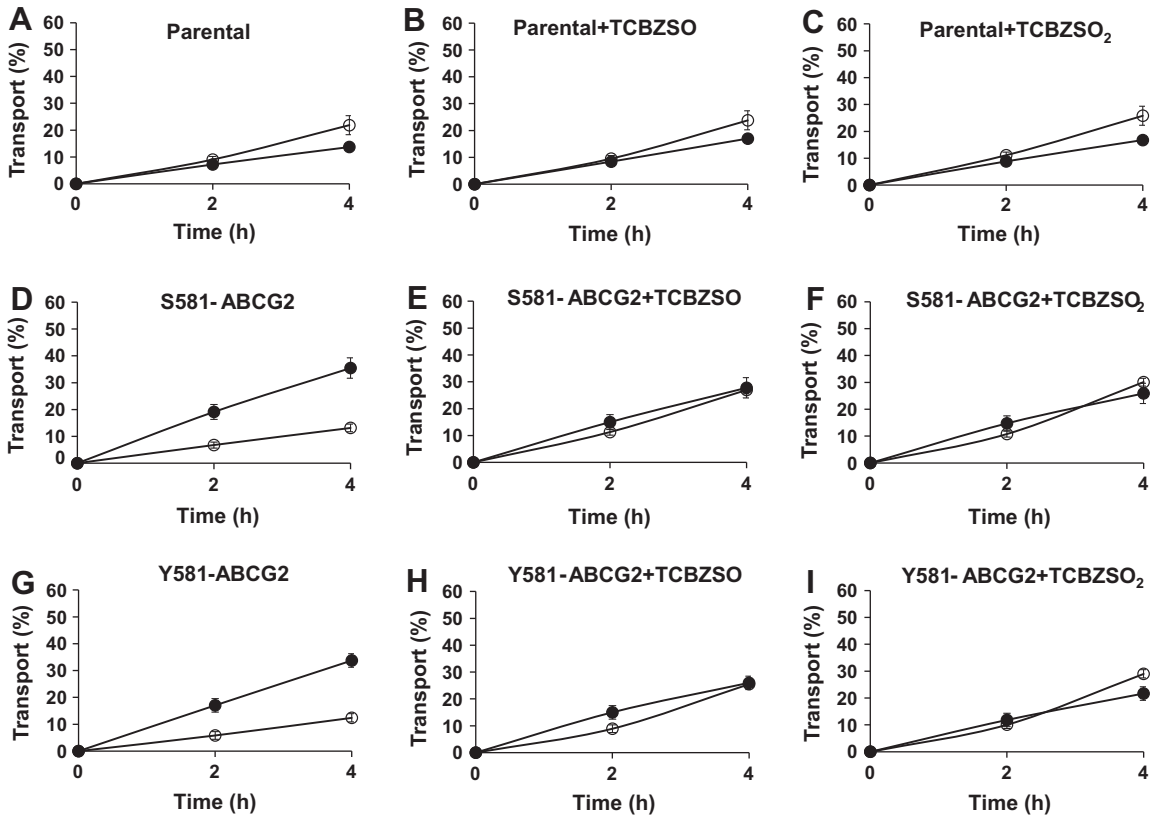

Fig. 2. Transepithelial transport of nitrofurantoin (10 μM) in MDCKII parental (non-transduced) (A), MDCKII-S581-ABCG2 (D) and MDCKII-Y581-ABCG2 (G) monolayers in the absence or presence of TCBZSO (15 μM) or $TCBZSO_2$ (15 μM). S581 and Y581 are the two variants of bovine ABCG2. The experiment was started with the addition of nitrofurantoin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. TCBZSO (B, E and H) and $TCBZSO_2$ (C, F and I) were present as indicated. Results are means; bars (sometimes smaller than the symbols) indicate standard deviations ($n = 3$). ● Translocation from the basolateral to the apical compartment. ○ Translocation from the apical to the basolateral compartment.

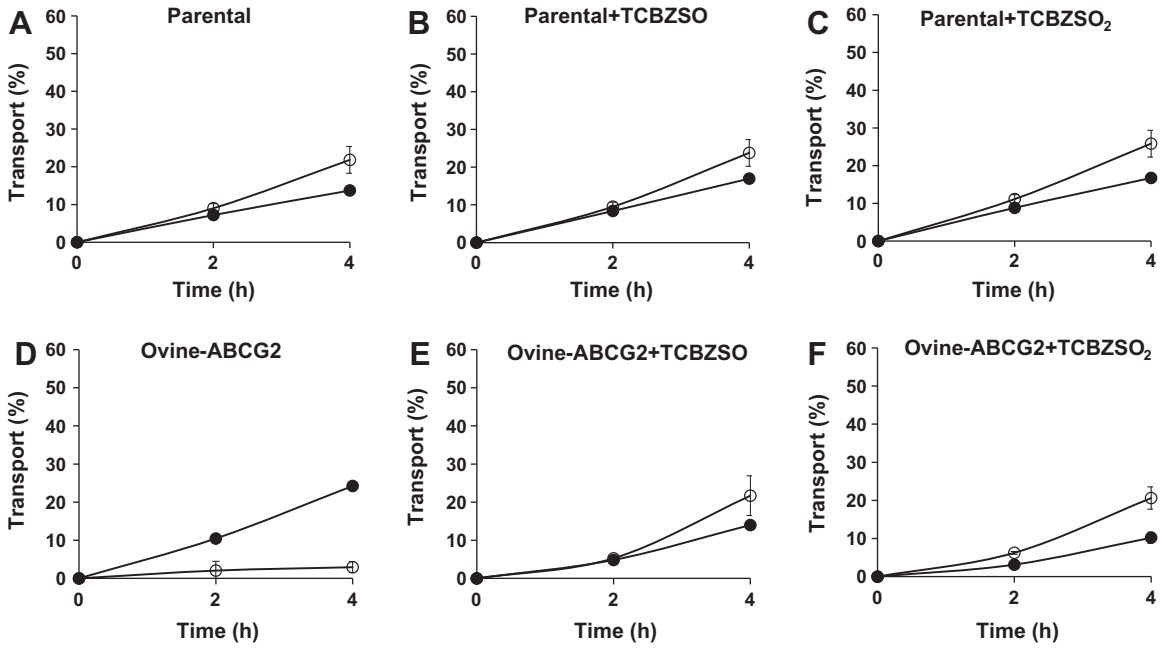

Fig. 3. Transepithelial transport of nitrofurantoin (10 μM) in MDCKII parental (non-transduced) (A) and MDCKII-Ovine-ABCG2 (D) monolayers in the absence or presence of TCBZSO (15 μM) or $TCBZSO_2$ (15 μM). The experiment was started with the addition of nitrofurantoin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. TCBZSO (B and E) and $TCBZSO_2$ (C and F) were present as indicated. Results are means; bars (sometimes smaller than the symbols) indicate standard deviations ($n = 3$). ● Translocation from the basolateral to the apical compartment. ○ Translocation from the apical to the basolateral compartment.

Table 1
Nitrofurantoin transport permeated towards apical (BL–AP transport) or basal (AP–BL transport) compartments in cell cultures in the absence and presence of inhibitors.

Drug treatment	Subclone	Sampling time (h)	Transport BL–AP (%)	Transport AP–BL (%)	Ratio BL–AP:AP–BL
Nitrofurantoin (10 μM)	MDCKII	2	7.22 ± 0.50	9.03 ± 2.03	
		4	13.72 ± 1.10	21.84 ± 0.65	0.63 ± 0.06
	Y581	2	17.05 ± 0.69	5.83 ± 1.99	
		4	33.75 ± 1.95	12.40 ± 1.00	2.73 ± 0.10
	S581	2	19.11 ± 0.97	6.78 ± 1.62	
		4	35.45 ± 0.47	13.16 ± 0.88	2.70 ± 0.20
	Ovine	2	10.45 ± 0.43	2.05 ± 2.44	
		4	24.23 ± 0.25	2.95 ± 1.42	9.26 ± 4.37
Nitrofurantoin (10 μM) + TBZSO (15 μM)	MDCKII	2	8.39 ± 0.05	9.52 ± 2.46	
		4	16.96 ± 1.16	23.78 ± 0.43	0.71 ± 0.04
	Y581	2	14.99 ± 4.99	8.91 ± 2.24	
		4	25.98 ± 3.90	25.50 ± 2.04	1.03 ± 0.23*
	S581	2	14.99 ± 2.16	11.28 ± 2.33	
		4	27.76 ± 3.05	26.95 ± 0.90	1.03 ± 0.12*
	Ovine	2	4.82 ± 0.79	5.27 ± 0.17	
		4	13.99 ± 0.92	21.69 ± 5.20	0.67 ± 0.19*
Nitrofurantoin (10 μM) + $TBZSO_2$ (15 μM)	MDCKII	2	8.80 ± 0.52	11.12 ± 1.23	
		4	16.73 ± 0.76	25.82 ± 2.10	0.65 ± 0.05
	Y581	2	11.82 ± 0.64	10.07 ± 1.05	
		4	21.65 ± 1.28	28.99 ± 1.52	0.74 ± 0.01*
	S581	2	14.74 ± 2.13	10.78 ± 1.79	
		4	25.90 ± 2.88	30.09 ± 1.88	0.86 ± 0.12*
	Ovine	2	3.15 ± 0.48	6.25 ± 0.35	
		4	10.23 ± 1.46	20.62 ± 2.91	0.51 ± 0.12*

* Significantly decreased compared to the counterparts of nitrofurantoin group ($P < 0.05$). Results are expressed as mean ± standard deviation ($n = 3$).

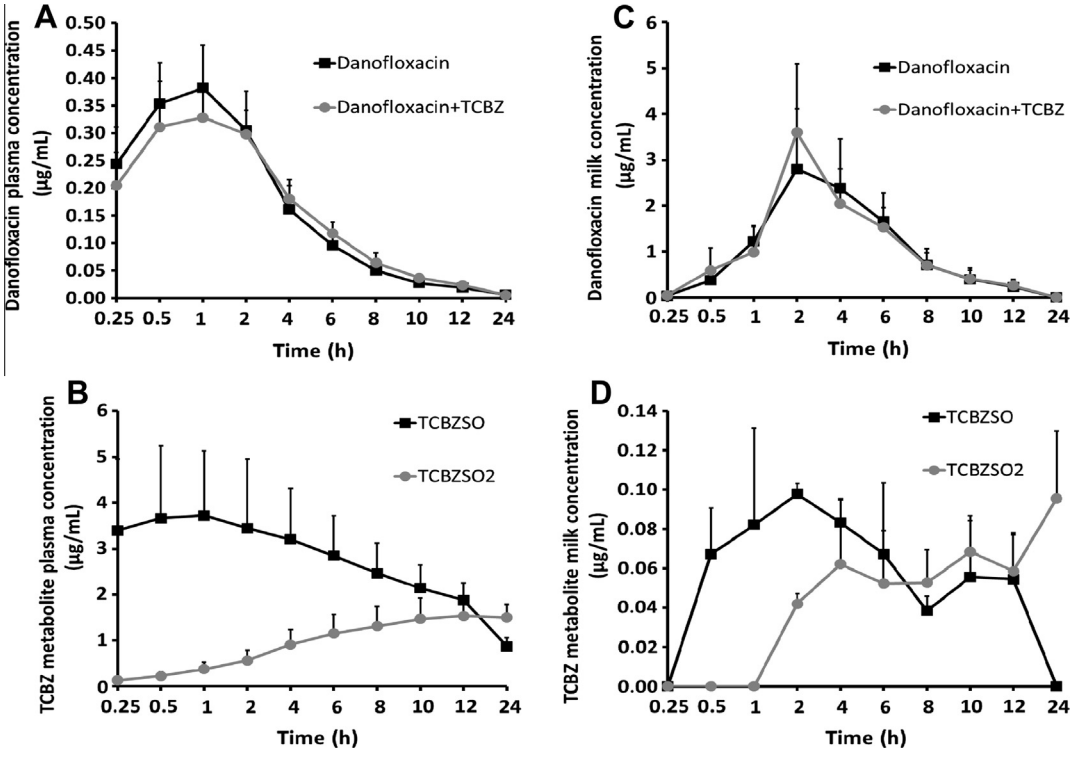

Fig. 4. Concentrations in plasma (A) and milk (C) vs. time curves for danofloxacin obtained from animals treated with a single dose of 1.25 mg/kg (IM) of danofloxacin and animals injected with danofloxacin 1.25 mg/kg (IM) and TCBZ 1 mg/kg (IV). Concentrations in plasma (B) and milk (D) vs. time curves for the main TCBZ metabolites (TCBZSO and $TCBZSO_2$) obtained from the animals injected with danofloxacin 1.25 mg/kg (IM) and TCBZ 1 mg/kg (IV). Each point represents a mean; bars indicate the standard deviation ($n = 6$).

moxidectin secretion into ovine milk must be attributed to an ABCG2-mediated interaction because other ABC transporters are not expressed to a substantial degree in lactating mammary glands (van Herwaarden and Schinkel, 2006). This inhibition effect may be considered to be almost total, because the reduction of 37% in the AUC (0–48 h) milk/plasma ratio for the TCBZ-treated group (Table 3) is similar to the decrease of 35–42% previously reported for milk secretion of moxidectin in $Abcg2^{-/-}$ mice compared to wild-type mice (Perez et al., 2009a).

Furthermore, since TCBZ is not detected either in plasma or in milk, this ABCG2-mediated effect must be produced by the two major TCBZ plasma metabolites, which showed a similar

Table 2
Pharmacokinetic parameters obtained in plasma and milk after IM administration of danofloxacin (1.25 mg/kg) in the two experimental groups.

	Danofloxacin	Danofloxacin + TCBZ
Plasma		
AUC (0–24 h) (μg h/mL)	1.77 ± 0.41	1.85 ± 0.28
C_{max} (μg/mL)	0.38 ± 0.08	0.34 ± 0.05
T_{max} (h)	1.00 ± 0.00	1.08 ± 0.73
MRT (h)	4.78 ± 0.79	5.00 ± 0.21
Milk		
AUC (0–24 h) (μg h/mL)	17.24 ± 7.27	17.13 ± 6.12
C_{max} (μg/mL)	2.92 ± 1.27	3.38 ± 1.44
T_{max} (h)	2.34 ± 0.82	2.67 ± 1.03
MRT (h)	5.03 ± 0.22	5.05 ± 0.22
AUC (0–24 h) (milk/plasma)	9.63 ± 2.84	9.11 ± 2.12
Dose secreted (%) in milk (24 h)[a]	1.27 ± 0.38	0.91 ± 0.15

AUC, area under the curve: C_{max}, maximum concentration; T_{max}, time to maximum concentration; MRT, mean residence time.
Results are expressed as mean ± standard deviation (n = 6).
[a] Individual milk yield approximately 3 L.

pharmacokinetic profile (similar T_{max}) in plasma and milk, and similar levels in milk to moxidectin (Fig. 5). This was not the case for danofloxacin, since $TCBZSO_2$ showed different plasma and milk profiles from danofloxacin and both metabolites were present at much lower concentrations than danofloxacin in milk (Fig. 4), with no evidence of interaction. Several reports showed a lack of ABCG2-mediated effect in plasma levels of danofloxacin, whereas milk levels of this antibiotic are widely affected by ABCG2 activity (Real et al., 2011a; Otero et al., 2013). However, in the present study, the low concentrations of TCBZ metabolites in milk pre-vented ABCG2-mediated interaction with danofloxacin.

The in vivo drug–drug interaction involving TCBZ and moxidectin might be also applied to other veterinary ABCG2 substrates. Lactating dairy cattle could be treated with TCBZ only during drying-off periods to avoid drug residues in milk for consumption. However, in endemic areas, parasite control programmes are implemented as regular anthelmintic treatments, even during the lactating period (Imperiale et al., 2011). In these situations, concomitant administration of multiple drugs would be likely to lead to modifications in secretion of drugs and other ABCG2 substrates, such as some vitamins, into milk (van Herwaarden et al., 2007), with an important effect on milk quality.

The use of combinations of anthelmintics, with different mechanisms of action, is a frequent practice in veterinary medicine due to the potential reduction of the frequency of treatment and to delay the development of resistance (Barnes et al., 1995). TCBZ is marketed in combination with other anthelmintics, such as ivermectin, a macrocyclic lactone that interacts with ABCG2 (Jani et al., 2011; Real et al., 2011a), and interactions between both drugs have been reported. Ivermectin elimination was delayed and its plasma availability was three-fold higher when coadministered with TCBZ (Lifschitz et al., 2009), although this drug–drug interaction was attributed to the inhibition of P-glycoprotein and/or metabolic enzymes.

Furthermore, TCBZ might be used in combination with other ABCG2 substrates, to adjust their secretion into milk. This modulation strategy has already been attempted for a number of drugs by coadministration of P-glycoprotein inhibitors (Fairweather, 2009) and ABCG2 inhibitors (Perez et al., 2009b; Real et al., 2011a). However, it should be noted that the use of TCBZ for this purpose may be controversial in animals whose products are destined for human consumption, or in areas of parasite endemicity, owing to the potential development of resistance (Barrera et al., 2012).

Much of the information related to ABCG2-mediated interactions derived from human and rodent studies appears to be applicable to other mammalian species (Mealey, 2012), since previous

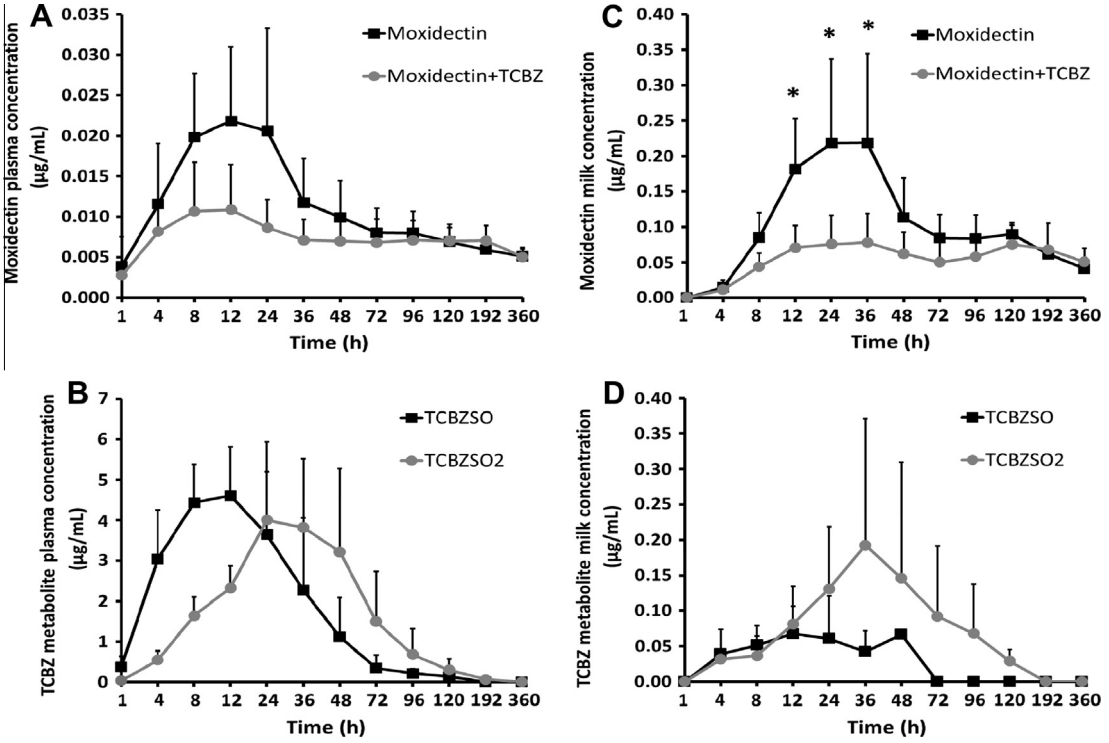

Fig. 5. Concentrations in plasma (A) and milk (C) vs. time curves for moxidectin obtained from animals treated with a single dose of 0.2 mg/kg (SC) of moxidectin and animals injected with moxidectin 0.2 mg/kg (SC) and TCBZ 2 mg/kg (PO). Concentrations in plasma (B) and milk (D) vs. time curves for the main TCBZ metabolites (TCBZSO and $TCBZSO_2$) obtained from the animals injected with moxidectin 0.2 mg/kg (SC) and TCBZ 2 mg/kg (PO). Each point represents a mean; bars indicate the standard deviation (n = 5–6).

Table 3
Pharmacokinetic parameters obtained in plasma and milk after SC administration of moxidectin (0.2 mg/kg) in the two experimental groups.

	Moxidectin	Moxidectin + TCBZ
Plasma		
AUC (0–48 h) (μg h/mL)	0.65 ± 0.38	0.40 ± 0.16
C_{max} (μg/mL)	0.02 ± 0.01	0.01 ± 0.00
T_{max} (h)	12.00 ± 7.48	9.60 ± 2.19
MRT (h)	273.30 ± 76.00	340.58 ± 83.42
Milk		
AUC (0–48 h) (μg h/mL)	7.75 ± 3.58	2.99 ± 1.41*
C_{max} (μg/mL)	0.24 ± 0.11	0.10 ± 0.04*
T_{max} (h)	27.00 ± 11.49	24.00 ± 12.00
MRT (h)	283.12 ± 84.64	355.44 ± 130.74
AUC (0–48 h) (milk/plasma)	11.68 ± 3.61	7.34 ± 1.51*
Dose secreted (%) in milk (48 h) [a]	3.84 ± 1.76	1.65 ± 0.96*
Dose secreted (%) in milk (360 h) [b]	6.38 ± 2.28	3.94 ± 1.94

AUC, area under the curve: C_{max}, maximum concentration; T_{max}, time to maximum concentration; MRT, mean residence time.
Results are expressed as mean ± standard deviation (n = 5–6).
* Significantly different from control group, $P < 0.05$.
[a] Individual milk yield approximately 6 L.
[b] Individual milk yield approximately 15 L.

work has demonstrated that TCBZSO and $TCBZSO_2$ are murine and human ABCG2 inhibitors (Barrera et al., 2012). In addition, the in vivo experiments reported here using sheep may be also applicable to other ruminant species, such as cattle, where a study of the differences dependent upon the Y581S genotype might be of interest in the light of the differences in the inhibition profile for the two bovine variants observed in the present study (Fig. 1). Our re-sults suggest that the effects of TCBZ metabolites on the disposi-tion of ABCG2 substrates, including drug secretion into milk, may vary among individuals due to the presence of the bovine Y581S SNP, thus potentially affecting drug efficacy and risk of drug resi-dues in milk.

Conclusions

TCBZ metabolites are good in vitro inhibitors of ovine and bovine variants of ABCG2. Coadministration of TCBZ to sheep results in a reduction in the secretion into milk of the endectocide moxidectin, with no significant effect on plasma levels, and shows an ABCG2 drug interaction attributed to the two major TCBZ metabolites. The relevance of our results may be important for drug interactions reducing secretion into milk of other ABCG2 substrates after TCBZ coadministration, thus affecting drug disposition and efficacy. These new findings will contribute to understanding of the factors that influence the transfer of drugs into milk.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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References

Allen, J.D., van Loevezijn, A., Lakhai, J.M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J.H., Koomen, G.J., Schinkel, A.H., 2002. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Molecular Cancer Therapeutics 1, 417–425.

Barnes, E.H., Dobson, R.J., Barger, I.A., 1995. Worm control and anthelmintic resistance: Adventures with a model. Parasitology Today 11, 56–63.

Barrera, B., Otero, J.A., Egido, E., Prieto, J.G., Seelig, A., Alvarez, A.I., Merino, G., 2012. The anthelmintic triclabendazole and its metabolites inhibit the membrane transporter ABCG2/BCRP. Antimicrobial Agents and Chemotherapy 56, 3535–3543.

Dupuy, J., Alvinerie, M., Menez, C., Lespine, A., 2010. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. Chemico-Biological Interactions 186, 280–286.

EMEA, 2006. Committee for Veterinary Medicinal Products. Triclabendazole (Modification of Maximum Residue Limits). Summary Report 4. EMEA/CVMP/320386/2005-Final, June 2006. http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500015673.pdf (accessed 10 June 2013).

Fairweather, I., 2009. Triclabendazole progress report, 2005–2009: An advancement of learning? Journal of Helminthology 83, 139–150.

Fairweather, I., Boray, J.C., 1999. Fasciolicides: Efficacy, actions, resistance and its management. The Veterinary Journal 158, 81–112.

Hennessy, D.R., Lacey, E., Steel, J.W., Prichard, R.K., 1987. The kinetics of triclabendazole disposition in sheep. Journal of Veterinary Pharmacology and Therapeutics 10, 64–72.

Imperiale, F., Ortiz, P., Cabrera, M., Farias, C., Sallovitz, J.M., Iezzi, S., Perez, J., Alvarez, L., Lanusse, C., 2011. Residual concentrations of the flukicidal compound triclabendazole in dairy cows' milk and cheese. Food additives and Contaminants Part A: Chemistry, Analysis, Control, Exposure and Risk Assessment 28, 438–445.

Jani, M., Makai, I., Kis, E., Szabo, P., Nagy, T., Krajcsi, P., Lespine, A., 2011. Ivermectin interacts with human ABCG2. Journal of Pharmaceutical Sciences 100, 94–97.

Jonker, J.W., Smit, J.W., Brinkhuis, R.F., Maliepaard, M., Beijnen, J.H., Schellens, J.H., Schinkel, A.H., 2000. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. Journal of the National Cancer Institute 92, 1651–1656.

Jonker, J.W., Merino, G., Musters, S., van Herwaarden, A.E., Bolscher, E., Wagenaar, E., Mesman, E., Dale, T.C., Schinkel, A.H., 2005. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nature Medicine 11, 127–129.

Lespine, A., Ménez, C., Bourguinat, C., Prichard, R.K., 2012. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: Prospects for reversing transport-dependent anthelmintic resistance. International Journal for Parasitology: Drugs and Drug Resistance 2, 58–75.

Lifschitz, A., Virkel, G., Ballent, M., Sallovitz, J., Lanusse, C., 2009. Combined use of ivermectin and triclabendazole in sheep: In vitro and in vivo characterisation of their pharmacological interaction. The Veterinary Journal 182, 261–268.

Marchetti, S., Mazzanti, R., Beijnen, J.H., Schellens, J.H., 2007. Clinical relevance of drug and herb drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). Oncologist 12, 927–941.

McKellar, Q.A., Gokbulut, C., 2012. Pharmacokinetic features of the antiparasitic macrocyclic lactones. Current Pharmaceutical Biotechnology 13, 888–911. Mealey, K.L., 2012. ABCG2 transporter: Therapeutic and physiologic implications in veterinary species. Journal of Veterinary Pharmacology and Therapeutics 35, 105–112.

Merino, G., Jonker, J.W., Wagenaar, E., van Herwaarden, A.E., Schinkel, A.H., 2005. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Molecular Pharmacology 67, 1758–1764.

Merino, G., Perez, M., Real, R., Egido, E., Prieto, J.G., Alvarez, A.I., 2010. In vivo inhibition of BCRP/ABCG2 mediated transport of nitrofurantoin by the isoflavones genistein and daidzein: A comparative study in $Bcrp1^{-/-}$ mice. Pharmaceutical Research 27, 2098–2105.

Otero, J.A., Real, R., de la Fuente, A., Prieto, J.G., Marques, M., Alvarez, A.I., Merino, G., 2013. The bovine ATP-binding cassette transporter ABCG2 Y581S single nucleotide polymorphism increases milk secretion of the fluoroquinolone danofloxacin. Drug Metabolism and Disposition 41, 546–549.

Pavek, P., Merino, G., Wagenaar, E., Bolscher, E., Novotna, M., Jonker, J.W., Schinkel, A.H., 2005. Human breast cancer resistance protein: Interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. Journal of Pharmacology and Experimental Therapeutics 312, 144–152.

Perez, M., Blazquez, A.G., Real, R., Mendoza, G., Prieto, J.G., Merino, G., Alvarez, A.I., 2009a. In vitro and in vivo interaction of moxidectin with BCRP/ABCG2. Chemico-Biological Interactions 180, 106–112.

Perez, M., Real, R., Mendoza, G., Merino, G., Prieto, J.G., Alvarez, A.I., 2009b. Milk secretion of nitrofurantoin, as a specific BCRP/ABCG2 substrate, in Assaf sheep: Modulation by isoflavones. Journal of Veterinary Pharmacology and Therapeutics 32, 498–502.

Perez, M., Otero, J.A., Barrera, B., Prieto, J.G., Merino, G., Alvarez, A.I., 2013. Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein: Effect on

plasma and milk levels of danofloxacin in sheep. The Veterinary Journal 196, 203–208.

Prieto, J.G., Merino, G., Pulido, M.M., Estevez, E., Molina, A.J., Vila, L., Alvarez, A.I., 2003. Improved LC method to determine ivermectin in plasma. Journal of Pharmaceutical and Biomedical Analysis 31, 639–645.

Real, R., Egido, E., Perez, M., Gonzalez-Lobato, L., Barrera, B., Prieto, J.G., Alvarez, A.I., Merino, G., 2011a. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: Interaction with ivermectin. Journal of Veterinary Pharmacology and Therapeutics 34, 313–321.

Real, R., Gonzalez-Lobato, L., Baro, M.F., Valbuena, S., de la Fuente, A., Prieto, J.G., Alvarez, A.I., Marques, M.M., Merino, G., 2011b. Analysis of the effect of the bovine adenosine triphosphate-binding cassette transporter G2 single nucleotide polymorphism Y581S on transcellular transport of veterinary drugs using new cell culture models. Journal of Animal Science 89, 4325–4338.

van Herwaarden, A.E., Schinkel, A.H., 2006. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. Trends in Pharmacological Sciences 27, 10–16.

van Herwaarden, A.E., Wagenaar, E., Merino, G., Jonker, J.W., Rosing, H., Beijnen, J.H., Schinkel, A.H., 2007. Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. Molecular and Cellular Biology 27, 1247–1253.

8. ARTÍCULO III

The ABC membrane transporter ABCG2 prevents access of FAAH inhibitor URB937 to the central nervous system.

Moreno-Sanz G, Barrera B, Guijarro A, d'Elia I, Otero JA, Alvarez AI, Bandiera T, Merino G, Piomelli D

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The ABC membrane transporter ABCG2 prevents access of FAAH inhibitor URB937 to the central nervous system

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ABSTRACT

The *O*-arylcarbamate URB937 is a potent inhibitor of fatty-acid amide hydrolase (FAAH), an intracellular serine hydrolase responsible for the deactivation of the endocannabinoid anandamide. URB937 is unique among FAAH inhibitors in that is actively extruded from the central nervous system (CNS), and therefore increases anandamide levels exclusively in peripheral tissues. Despite its limited distribution, URB937 exhibits marked analgesic properties in rodent models of pain. Pharmacological evidence suggests that the extrusion of URB937 from the CNS may be mediated by the ABC membrane transporter ABCG2 (also called Breast cancer resistance protein, BCRP). In the present study, we show that URB937 is a substrate for both mouse and human orthologues of ABCG2. The relative transport ratios for URB937 in Madin-Darby canine kidney (MDCKII) cells monolayers over-expressing either mouse Abcg2 or human ABCG2 were significantly higher compared to parental monolayers (13.6 and 13.1 *vs.* 1.5, respectively). Accumulation of the compound in the luminal/apical side was prevented by co-administration of the selective ABCG2 inhibitor, Ko-143. *In vivo* studies in mice showed that URB937 (25 mg kg^{-1}) readily entered the brain and spinal cord of Abcg2-deficient mice following intraperitoneal administration, whereas the same dose of drug remained restricted to peripheral tissues in wild-type mice. By identifying ABCG2 as a transport mechanism responsible for the extrusion of URB937 from the CNS, the present results should facilitate the rational design of novel peripherally restricted FAAH inhibitors.

1. Introduction

The intracellular hydrolysis of the endocannabinoid anandamide is catalyzed by the enzyme fatty-acid amide hydrolase (FAAH) [1], a membrane-bound serine hydrolase that also catalyzes the cleavage of other non-cannabinoid fatty-acid amides such as oleoylethanolamide and palmitoylethanolamide [1,2]. The *O*-arylcarbamate URB937 (cyclohexylcarbamic acid 3′-carbamoyl-6-hydroxybiphenyl-3-yl ester; also referred to as ARN354) is a potent and selective FAAH inhibitor. Following systemic administration in mice, URB937 inhibits liver FAAH activity with a half-maximal effective dose (ID_{50}) of 0.1 mg kg^{-1}, but fails to alter FAAH activity in forebrain, hypothalamus and spinal cord. Furthermore, *in vivo* inhibition of FAAH by URB937 is accompanied by an elevation of anandamide levels, which occurs exclusively in peripheral tissues. Despite its restricted access to the central nervous system (CNS), URB937 exerts profound antinociceptive effects in rodent models, which are prevented by blockade of CB_1 cannabinoid receptors [3]. Pharmacological evidence suggests that the extrusion of URB937 from the mouse brain may be mediated by ABCG2 (Breast cancer resistance protein, BCRP), a member of the ATP-binding cassette (ABC) superfamily of efflux transporters. ABCG2 was first identified in 1998 in the multidrug resistant human breast cancer cell line MCF-7/AdrVp [4]. ABCG2 can transport a large number of structurally unrelated compounds and is increasingly recognized for its role in drug disposition and tis-sue protection [5,6]. ABCG2 is highly expressed in organs that are important for the absorption (small intestine), elimination (liver and kidney), and distribution (blood–brain and placental barriers) of drugs and other xenobiotics [7]. Despite its substantial medical significance, the transport mechanism of ABCG2 remains poorly understood.

Abbreviations: FAAH, fatty-acid amide hydrolase; CNS, central nervous system; BCRP/ABCG2, breast cancer resistance protein; Pgp/ABCB1, P-glycoprotein; MDCKII, Madin-Darby canine kidney; ABC, ATP-binding cassette; SULT1, phenol sulfotransferases; i.p., intraperitoneal; s.c., subcutaneous.

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In the present study, we used both *in vitro* and *in vivo* approaches to examine whether ABCG2 mediates the transport of URB937 and its extrusion from the CNS. We measured the transport rate of URB937 through polarized monolayers of Madin-Darby canine kid-ney (MDCK II) cells that over-express either mouse Abcg2 or human ABCG2. The effect of Ko143, a selective ABCG2 inhibitor [8], was also assessed. Additionally, we used Abcg2-deficient (Abcg2$^{-/-}$) mice to further explore the role of ABCG2 in the distribution of URB937.

2. Materials and methods

2.1. Animals

Adult male Swiss-Webster mice (25–30 g) and adult male Abcg2$^{-/-}$ mice and wild-type littermates (9–13 weeks, >99% FVB genetic background) were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard chow and water *ad libitum.* Abcg2$^{-/-}$ mice were kindly provided by Dr. A.H. Schinkel, Netherlands Cancer Institute (Amsterdam, The Netherlands). All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and the "Principles of Laboratory Animal Care" and the European guidelines described in the EC Directive 86/609. Procedures were also approved by the Institutional Animal Care and Use Committee of the University of California, Irvine, and the Research Committee of Animal Use of the University of León (Spain).

2.2. Chemicals

Ko143 was purchased from Tocris (Bristol, UK), isoflurane (Isovet®) from Schering-Plough (Madrid, Spain), anandamide-[ethanolamine-^{3}H] (10,000 cpm, specific activity 60 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO, USA). URB937 was synthesized as described [3]. All other chemicals were of analytical grade and available from commercial sources.

2.3. Cell cultures

MDCKII cells and their human ABCG2- and murine Abcg2-transduced subclones were a kind gift of Dr. A.H. Schinkel. Culture conditions were as previously described [9,10]. The cells were cultured in Dulbecco-modified Eagles's medium (DMEM) supplemented with Glutamax (Life Technologies, Inc., Carlsbad, CA, USA), penicillin (50 units/mL), streptomycin (50 μg/mL), and 10% (v/v) fetal calf serum (MP Biomedicals, Solon, OH, USA). Cells were cultured at 37 °C in the presence of 5% CO_2. Cells were trypsinized every 3–4 days for subculturing.

2.4. Transport studies

Transepithelial transport assays were carried out using Transwell plates as previously described [11], with minor modifications. Cells were seeded on microporous polycarbonate membrane fil-ters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY, USA) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and the medium was replaced every day. Transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Bedford, MA, USA); wells reg-istering a resistance of 150 Ω or greater, after correcting for the resistance obtained in blank control wells, were used in trans-port experiments. The measurements were repeated at the end of the experiment to check the tightness of the monolayer. Two hours before the start of the experiment, medium on both sides of the monolayer was replaced with 2 mL of Optimem medium (Life Technologies, Inc., Carlsbad, CA, USA), without serum, either with or without Ko143 (1 μM). The experiment was started ($t = 0$) by replacing the medium in either the apical or basolateral compartment with fresh Optimem medium, either with or without Ko143 (1 μM), and containing 5 μM URB937. Aliquots of 100 μL were taken from the drug opposite compartment at $t = 2$ and 4 h, and stored at −20 °C until analysis. The appearance of the compound in the acceptor compartment is presented as fraction of total compound added to the donor compartment at the beginning of the experiment. Active transport across MDCKII monolayers was expressed by the relative transport ratio, defined as the percentage apically directed transport divided by the percentage basolaterally directed translocation, after 4 h [12].

2.5. Tissue processing

Animals were killed by decapitation under slight anesthesia with isoflurane and brain, spinal cord, liver and kidney tissue were snap frozen in liquid nitrogen. Blood was collected through a left cardioventricular puncture and centrifuged at 2000 × *g* for 30 min to obtain plasma. Samples were weighed and homogenized in ice-cold Tris–HCl buffer (50 mM, 5–9 vol., pH 7.5) containing 0.32 M sucrose. Homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants were collected (250 μL) and protein concentration determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). Remaining supernatant and pellet where further extracted with methanol/chloroform for URB937 analysis.

2.6. FAAH activity

FAAH activity was measured at 37 °C for 30 min in 0.5 mL of Tris–HCl buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (BSA) (0.05%, w/v), tissue homogenates (50 μg protein from brain, spinal cord and kidney and 100 μg from liver), 10 μM anandamide, and anandamide-[ethanolamine-^{3}H](10,000 cpm, specific activity 60 Ci/mmol; American Radiolabeled Chemicals, MO, USA). The reactions were stopped with chloroform/methanol (1:1, 1 mL) and radioactivity was measured in the aqueous layers by liquid scintillation counting.

2.7. Lipid extraction and URB937 quantification by liquid chromatography/mass spectrometry (LC/MS)

Tissue and plasma levels of URB937 were determined as previously described [3] with minor modifications. In brief, aqueous tissue homogenates and plasma samples were extracted with methanol/chloroform (1:2) containing cyclohexyl biphenyl-3-ylacetamide as internal standard. Organic phases were evaporated under nitrogen and reconstituted in 60 μL of methanol. Sam-ples were analyzed using an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an electrospray ionization interface. URB937 and *N*-cyclohexyl biphenyl-3-ylacetamide (mass-to-charge ratio, m/z = 377 and 294, respectively) were eluted on an XDB Eclipse C18 column (50 mm× 4.6 mm inner diameter, 1.8 μm, Zorbax, Agi-lent Technologies, Inc., Palo Alto, CA, USA) using a linear gradient of 60% to 100% of A in B over 3 min at a flow rate of 1.0 mL/min. Mobile phase A consisted of methanol containing 0.25% acetic acid and 5mM ammonium acetate; mobile phase B consisted of water containing 0.25% acetic acid and 5mM ammonium acetate.

2.8. Experimental design for in vivo studies

URB937 was dissolved in saline/PEG400/Tween-80 (18:1:1) to a concentration of 2.5 mg mL^{-1} and injected subcutaneously between the shoulder blades at the volume of 10 mL kg^{-1} for a final dosing of 25 mg kg^{-1}.

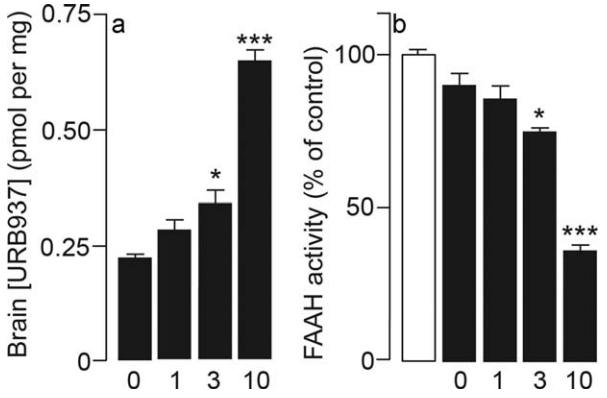

Fig. 1. Effects of the selective ABCG2 inhibitor Ko143 on URB937 access to brain in mice. Intraperitoneal administration of Ko143 (0, 1, 3 and 10 mg kg^{-1}) (a) increases the levels of URB937 (administered at 25 mg kg^{-1}, s.c.) in mouse brain tissue; and (b) allows URB937 to inhibit brain FAAH activity. *$P < 0.05$; ***$P < 0.001$ *vs.* vehicle-treated animals; ANOVA with Dunnett's *post hoc* test; $n = 4$. Data are expressed as mean ± SEM.

2.8.1. Effects of ABCG2 blockade on URB937 penetration in the brain

Sixteen Swiss-Webster male mice were randomly divided into 4 groups, each receiving a single dose of Ko143 (0, 1, 3 and 10 mg kg^{-1}, i.p.;10 mL kg^{-1}) dissolved in saline/PEG400/Tween-80 (18:1:1) with DMSO (30%), 20 min prior to URB937 administration. Animals were killed 1 h later and brains were collected for URB937 levels and FAAH activity determination.

2.8.2. Distribution of URB937 in Abcg2-deficient mice (Abcg2$^{-/-}$)

Wild-type ($n = 5$) and Abcg2$^{-/-}$ mice ($n = 4$) received a single dose of URB937 (25 mg kg^{-1}, subcutaneous, s.c.) and were killed after 1 h for tissue collection.

2.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) or standard deviation (S.D.) and the significance of differences was determined using one-way analysis of variance (ANOVA) followed by Dunett test as *post hoc*, and Student's *t*-test. Differences were considered significant if $P < 0.05$. Statistical analyses were conducted using GraphPad Prism Version 4.0 (San Diego, CA, USA).

3. Results

3.1. Effects of ABCG2 blockade on URB937 penetration in the brain

Systemic administration of the selective ABCG2 inhibitor, Ko143 (0, 1, 3 and 10 mg kg^{-1}, i.p.), 20 min prior to injection of URB937 (25 mg kg^{-1}, s.c.), dose-dependently increased access of URB937 to the brain (Fig. 1a). The elevation in brain URB937 levels was accompanied by a dose-dependent decrease in brain FAAH activity (Fig. 1b), which was assessed *ex vivo* 1 h after URB937 adminis-tration. These results confirm and extend previous observations suggesting that pharmacological blockade of ABCG2 activity allows URB937 to enter the CNS [3].

3.2. Transcellular transport of URB937 in vitro

To assess whether URB937 is a substrate for Abcg2/ABCG2, we investigated the polarized transport of this compound using wild-type MDCKII cells as well as MDCKII sub-clones stably over-expressing either mouse Abcg2 or human ABCG2. Relative transport ratios for URB937 were significantly increased in Abcg2 and ABCG2-transduced MDCKII cells, compared to parental cells (Fig. 2a–c). Inclusion on the incubations of the ABCG2 inhibitor Ko143 (1 μM), completely inhibited Abcg2/ABCG2-mediated trans-port (Fig. 2d–f). These findings indicate that URB937 is an *in vitro* substrate for both the mouse and human orthologues of ABCG2.

3.3. Distribution of URB937 in Abcg2$^{-/-}$ mice

To further examine the role of Abcg2 in the peripheral segregation of URB937, we administered the compound (25 mg kg^{-1}, s.c.) to Abcg2$^{-/-}$ mice and their wild-type littermates. There were no differences between wild-type and mutant mice in either circulating levels of URB937 (Table 1) or tissue-to-blood ratio (liver and kidney, Fig. 3a). However, levels of URB937 in brain and spinal cord,

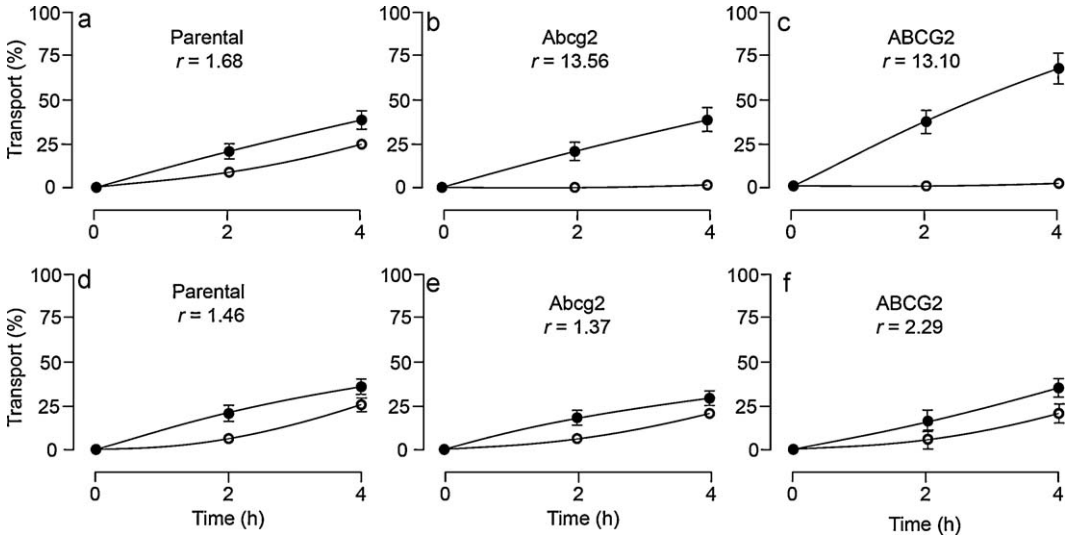

Fig. 2. Transepithelial transport of URB937. Transport of URB937 (5 μM) in MDCKII parent (a), MDCKII-Abcg2 (b), and MDCKII-ABCG2 (c) monolayers. The experiment was started with the addition of URB937 to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC/MS and plotted. ABCG2 inhibitor Ko143 (1 μM) (d–f) was present as indicated. Results are means; error bars (sometimes smaller than the symbols) indicate S.D.($n = 3$). (●) Translocation from the basolateral to the apical compartment; (○) translocation from the apical to the basolateral compartment. *r* represents the relative transport ratio (apically direct transport divided by the basolaterally directed translocation) at $t = 4$ h.

Table 1
Levels of URB937 in plasma, peripheral tissues and CNS of wild-type and Abcg2$^{-/-}$ mice measured 1 h after systemic administration (25 mg kg^{-1}, s.c).

Genotype/tissue	Plasma (pmol/μL)	Liver (pmol/mg)	Kidney (pmol/mg)	Brain (pmol/mg)	Spinal cord (pmol/mg)
Wild-type	8.34 ± 1.7	7.51 ± 3.1	33.88 ± 3.3	0.46 ± 0.12	0.29 ± 0.04
Abcg2$^{-/-}$	6.95 ± 0.9	7.75 ± 2.3	34.97 ± 0.35	1.15 ± 0.04*	0.80 ± 0.10*

Student *t*-test; *n* = 4–5. Data are expressed as mean ± SEM.
* $P < 0.05$ *vs.* wild-type littermates.

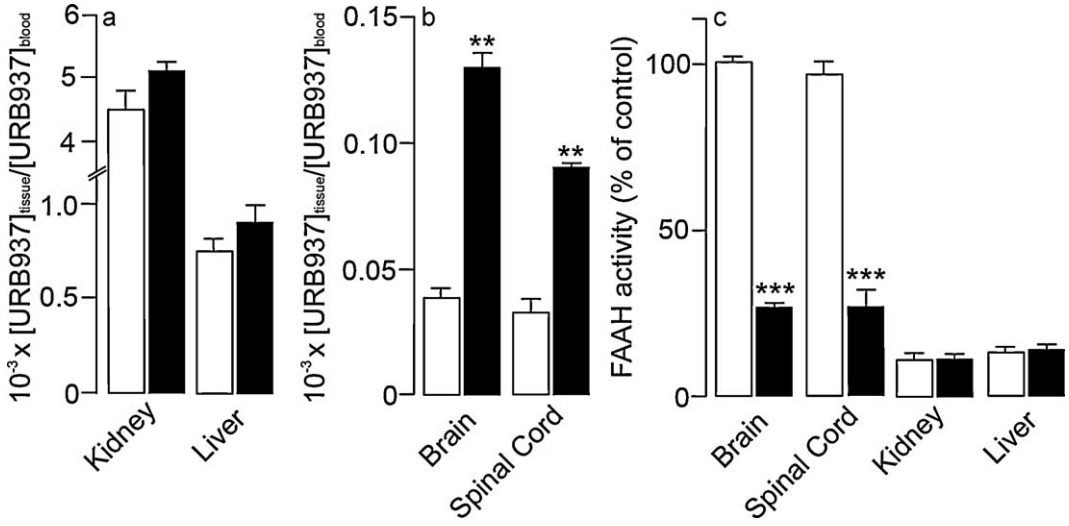

Fig. 3. Effects of URB937 in Abcg2$^{-/-}$ mice. Panels a and b show the ratio between tissue and plasma levels of URB937 1 h after administration of URB937 (25 mg kg^{-1}, s.c.) in wild-type (open bars) and Abcg2$^{-/-}$ mice (filled bars). Ablation of Abcg2 had no effect on the distribution of URB937 in peripheral tissues (a), but significantly increased CNS permeability of the compound (b). Furthermore, URB937 (25 mg kg^{-1}, s.c.) abolished FAAH activity after 1 h in peripheral tissues of both Abcg2$^{-/-}$ and wild-type mice (c). Brain FAAH activity is not affected in wild-type mice, but is strongly reduced in Abcg2$^{-/-}$ mutants (c). $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* wild-type littermates; Student's *t*-test; *n* = 4–5. Data are expressed as mean ± SEM.

two organs protected by the blood–brain barrier, were substantially higher in mice lacking Abcg2 (Fig. 3b). Consistent with those find-ings, Abcg2$^{-/-}$ mice displayed a marked reduction (80%) in FAAH activity in brain and spinal cord compared to wild-type animals, which showed almost no inhibition of FAAH activity in CNS (Fig. 3c). On the other hand, FAAH activity was nearly abolished in liver and kidney tissue in wild-type and Abcg2$^{-/-}$ mice (Fig. 3c). These find-ings provide conclusive evidence that Abcg2 restricts the access of URB937 to the mouse CNS *in vivo*.

4. Discussion

The *O*-arylcarbamate URB937 is a potent and selective inhibitor of intracellular FAAH activity. In rodents, systemic administration of URB937 inhibits FAAH and elevates anandamide levels in periph-eral tissues, but not in forebrain, hypothalamus or spinal cord. Indeed, URB937 is 400 times more potent at inhibiting FAAH activ-ity in mouse liver than in mouse brain [3]. Despite its peripheral distribution, URB937 exerts substantial analgesic effects in mice and rats, which are dependent on CB_1 receptor activation. Previous pharmacological experiments have suggested that the extrusion of URB937 from brain might be mediated by the ABC-transporter ABCG2 [3]. In the present study, we tested this hypothesis uti-lizing both *in vitro* and *in vivo* approaches. First, we used the polarized canine kidney cell line MDCKII and its subclones trans-duced with murine Abcg2 and human ABCG2 cDNAs to determine whether URB937 interacts with ABCG2 *in vitro*. Cells were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of URB937 across the monolayers was assessed. In general, Abcg2/ABCG2 substrates show a higher rela-tive transport ratio in the Abcg2/ABCG2 transduced cells compared to the parental cells due to both heightened transport to the apical side and diminished transport to the basolateral side. Our results show that the relative transport ratio for URB937 was increased nearly 10 folds by over-expression of either mouse or human ABCG2. This effect was prevented by the addition of the selective ABCG2 inhibitor, Ko143. We interpret these findings to indicate that URB937 is an *in vitro* substrate for ABCG2.

To further investigate the role of ABCG2 in the extrusion of URB937, we examined the distribution of this compound in Abcg2$^{-/-}$ mice and their wild-type littermates. Irrespective of genotype, URB937 abrogated FAAH activity in peripheral tissues. By contrast, the drug strongly inhibited FAAH activity in the CNS of Abcg2$^{-/-}$ mice, but failed to do so in wild-type mice. CNS levels and tissue/blood ratio of URB937 were also significantly increased in Abcg2$^{-/-}$ mice. Together with our *in vitro* data, these results indicate that ABCG2 restricts the access of URB937 to the CNS, with-out affecting plasma levels or peripheral tissue distribution of this compound.

This interpretation is consistent with several studies reporting local effects mediated by Abcg2, including brain penetration, but no differences in systemic profiles between wild-type and Abcg2$^{-/-}$ mice [13–16]. Although URB937 is an *in vitro* substrate for ABCG2, additional factors could affect the systemic disposition of this com-pound due to profound differences in tightness and drug uptake systems between systemic membranes and the blood–brain bar-rier endothelial luminal membrane [16]. Hence, the relevance of ABCG2 for brain penetration of any given substrate will depend upon other constituents of the blood–brain barrier [17]. Among these, the ABC transporter P-glycoprotein (Pgp/ABCB1) is known to overlap with ABCG2 in substrate specificity [16]. By using Abcg2$^{-/-}$ mice in which Pgp is still present, it could be difficult to unequiv-ocally demonstrate a functional role for ABCG2 at the blood–brain barrier [18]. However, in our study, we could demonstrate the role

of ABCG2 in the brain extrusion of URB937 together with a difference in FAAH activity depending on murine genotype. The role of P-glycoprotein in this process is currently under investigation.

Preliminary structure–activity relationship explorations suggest that the *para*-hydroxyl substituent in the proximal ring of URB937 is a key determinant of the constrained brain penetra-tion of this compound [3]. The importance of the phenol moiety raises the question as to whether phenol sulfotransferases (SULT1) might be involved in the extrusion of URB937 from the CNS. ABC-transporters commonly co-localize with sulfotransferases [19,20]. The ability of ABCG2 to transport sulfate-conjugated phenol compounds has been previously described [5,21]. Moreover, ABCG2 has been recently reported to be an important physiological mediator of phenolic conjugation [22]. In the same study, Zhu et al. demon-strated that both sulfotransferase activity and ABCG2-mediated efflux of sulfate conjugates are saturable processes. Our previous findings, showing that administration of a non-selective SULT1 inhibitor increases the entry of URB937 into the brain [3], suggest that this compound might be a substrate for SULT1 activity. Fur-ther experimentation is needed, however, to conclusively test this hypothesis.

Significant synthetic efforts have been directed at targeting ABC-transporters, to develop compounds that are either able to cir-cumvent transport-mediated resistance (e.g. in chemotherapy) or show improved bioavailability and therapeutic index through spe-cific transporter recognition [20,23,24]. The present results indicate that ABCG2 is responsible for the extrusion of URB937 from the CNS. This finding provides the structural information needed to discover novel peripherally restricted FAAH inhibitors devoid of central side effects.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

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References

[1] Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 1996;384:83–7.
[2] Ahn K, McKinney MK, Cravatt BF. Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev 2008;108:1687–707.
[3] Clapper JR, Moreno-Sanz G, Russo R, Guijarro A, Vacondio F, Duranti A, et al. Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. Nat Neurosci 2010;13:1265–70.
[4] Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998;95:15665–70.
[5] Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J 2005;7:E118–33.
[6] Polgar O, Robey RW, Bates SE. ABCG2: structure, function and role in drug response. Expert Opin Drug Metab Toxicol 2008;4:1–15.
[7] Van Herwaarden AE, Schinkel AH. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. Trends Pharmacol Sci 2006;27:10–6.
[8] Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 2002;1(6):417–25.
[9] Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH, et al. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst 2000;92:1651–6.
[10] Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, et al. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. J Pharmacol Exp Ther 2005;312:144–52.
[11] Huisman MT, Smit JW, Wiltshire HR, Hoetelmans RM, Beijnen JH, Schinkel AH. P-glycoprotein limits oral availability, brain, and fetal penetration of saquinavir even with high doses of ritonavir. Mol Pharmacol 2001;59:806–13.
[12] Huisman MT, Chhatta AA, van Tellingen O, Beijnen JH, Schinkel AH. MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. Int J Cancer 2005;116:824–9.
[13] Zhang Y, Wang H, Unadkat JD, Mao Q. Breast cancer resistance protein 1 limits fetal distribution of nitrofurantoin in the pregnant mouse. Drug Metab Dispos 2007;35:2154–8.
[14] Zhou L, Naraharisetti SB, Wang H, Unadkat JD, Hebert MF, Mao Q. The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an Obstetric-Fetal Pharmacology Research Unit Network and University of Washington Specialized Center of Research Study. Mol Pharmacol 2008;73:949–59.
[15] Real R, Egido E, Perez M, Gonzalez-Lobato L, Barrera B, Prieto JG, et al. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: interaction with ivermectin. J Vet Pharmacol Ther
2010.[16] Tang SC, Lagas JS, Lankheet NA, Poller B, Hillebrand MJ, Rosing H, et al.
Brainaccumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration. Int J Cancer 2011.
[17] Robey RW, To KK, Polgar O, Dohse M, Fetsch P, Dean M, et al. ABCG2: a perspective. Adv Drug Deliv Rev 2009;61:3–13.
[18] Vlaming ML, Lagas JS, Schinkel AH. Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice. Adv Drug Deliv Rev 2009;61:14–25.
[19] Enokizono J, Kusuhara H, Sugiyama Y. Regional expression and activity of breast cancer resistance protein (Bcrp/Abcg2) in mouse intestine: overlapping distribution with sulfotransferases. Drug Metab Dispos 2007;35:922–8.
[20] Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, et al. Membrane transporters in drug development. Nat Rev Drug Discov 2010;9:215–36.
[21] Imai Y, Asada S, Tsukahara S, Ishikawa E, Tsuruo T, Sugimoto Y. Breast cancer resistance protein exports sulfated estrogens but not free estrogens. Mol Pharmacol 2003;64:610–8.
[22] Zhu W, Xu H, Wang SW, Hu M. Breast cancer resistance protein (BCRP) and sulfotransferases contribute significantly to the disposition of genistein in mouse intestine. AAPS J 2010;12:525–36.
[23] Nakagawa H, Saito H, Ikegami Y, Aida-Hyugaji S, Sawada S, Ishikawa T. Molecular modeling of new camptothecin analogues to circumvent ABCG2-mediated drug resistance in cancer. Cancer Lett 2006;234:81–9.
[24] Pick A, Muller H, Mayer R, Haenisch B, Pajeva IK, Weigt M, et al. Structure–activity relationships of flavonoids as inhibitors of breast cancer resistance protein (BCRP). Bioorg Med Chem 2011;19(6):2090–102.

9. ARTÍCULO IV

Identification of structural determinants of peripherally restricted FAAH inhibitors for substrate recognition by blood-brain barrier transporters ABCG2 and P-glycoprotein

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(Manuscript in preparation)

Identification of structural determinants of peripherally restricted FAAH inhibitors for substrate recognition by blood-brain barrier transporters ABCG2 and P-glycoprotein

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Abstract

The blood-brain barrier (BBB) is a unique system composed by the endothelial cells that form the cerebral capillaries, which represents the main entry route for molecules into the central nervous system (CNS). Breast Cancer Resistance Protein (BCRP/ABCG2) and P-glycoprotein (P-gp) are the main transporters of the ATP-binding cassette (ABC) family in the BBB that regulate the efflux of potentially harmful xenobiotics, preventing their access to the brain. Previous studies have identified ABCG2 as responsible for the peripheral distribution of the selective fatty-acid amidohydrolase (FAAH) inhibitor URB937 in rodents. However, the involvement of P-glycoprotein in this process is unknown. To fill this gap and to extend the structure-activity relationship (SAR) on the substrate specificity of Abcg2, in the present study we employed monolayers of MDCKII cells over-expressing either Abcg2 or P-gp to test a selected cohort of peripheral and non-peripheral URB937 analogues as substrates for these efflux transporters. Our results indicate that most of these compounds are very good substrates for both transporters *in vitro*. Only compounds lacking some key structural features showed no changes in the vectorial transport in the Abcg2- and P-gp-transduced MDCKII cell lines compared to parental cells, remarking the importance of those substituents for the substrate recognition by these ABC transporters. Our results confirm previous *in vivo* data suggesting a crucial role for the hydrogen bond donors of the carboxamide moiety in the distal phenyl ring of O-byphenyl carbamate-based FAAH inhibitors on restricting their access to the CNS and their interaction with Abcg2 and P-gp. The present data also give valuable insight of the intricate bioarchitecture underpinning BBB efflux systems.

Introduction

The blood-brain barrier (BBB) is a unique system composed by the endothelial cells that form the cerebral capillaries, together with other perivascular elements (associated astrocytic end-feet processes, perivascular neurons and pericytes), which represents the main entry route for molecules into the central nervous system (CNS). Brain endothelial cells are distinctive in that, while forming complex tight junctions that effectively seal the paracellular pathway, contain numerous apical and basolateral membrane transporters that regulate the transcellular traffic of essential molecules between brain and blood, as well as the efflux of potentially harmful substances and waste products (Cecchelli et al., 2007). The efflux system, localized in the apical membrane of the endothelial cells, and mainly composed by the ATP-binding cassette (ABC) family of transporters, has raised great interest as a strategic target to control the delivery of drugs to the CNS, particularly in the case of P-glycoprotein (P-gp, ABCB1) and Breast Cancer Resistance Protein (BCRP, ABCG2) (Marquez & van Bambeke, 2011). These two transporters are highly expressed in normal human tissues such as small intestine, liver, kidney, brain endothelium, and placenta, where they play an important role in the absorption, elimination and systemic distribution of drugs and xenobiotics, (König et al., 2013) as well as in tumors, contributing critically to multidrug resistance (MDR) in the treatment of cancer (Sharom, 2008). Significant drug discovery efforts have been aimed at developing inhibitors for these transporters in order to overcome such MDR (Pick et al., 2008; Falasca & Linton., 2012) but less is known about their substrate specificity. While recent advances in the structural characterization of P-gp have helped to close the gaps of its substrate polyspecificity (Gutmann et al., 2010), the lack of high-resolution crystal structures for ABCG2 renders the pharmacophoric characterization of its substrates problematic. Current approaches to elucidate the structure of the substrate-binding domain of ABCG2 combine docking experiments with homology models (Rosenberg et al., 2010) and single–residue mutagenesis studies (Ni et al., 2010; Cai et al., 2010; Ni et al., 2011).

ABCG2 transports a wide range of molecules including chemotherapeutic (mitoxantrone, methotrexate) and non-chemotherapy agents (nitrofurantoin, cimetidine, fluoroquinolones), as well as non-therapeutic compounds such as dietary flavonoids, porphyrins and estrone 3-sulfate (Alvarez et al., 2008; Ni et al., 2010). Genetic and pharmacological studies have identified Abcg2 (ABCG2 in humans) as responsible for the peripheral distribution of the selective fatty-acid amidohydrolase (FAAH) inhibitor URB937 in rodents (Moreno-Sanz et al., 2011). URB937 exerts potent analgesic effects in mice and rats, which are dependent upon activation of type-1 cannabinoid (CB1) receptors exclusively in peripheral tissues, avoiding CNS-related side effects. Previous ligand-based studies have reported the presence of hydroxyl or amine groups on the outer ring of camptothecin analogs (Yoshikawa et al., 2004), imidazoacridinones (Bram et al., 2009), mitoxantrone (Rosenberg et al., 2010) and urolithins (Gonzalez-Sarrias et al., 2013) to be essential for the substrate recognition and efflux via ABCG2, possibly facilitating hydrogen bond formation. This is in agreement with our previous results showing that analogues of the globally active FAAH inhibitor cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester, URB597, bearing a hydroxyl (URB937) or amine group in the -para position of the proximal phenyl ring (R1) had a peripherally restricted systemic distribution (Clapper et al., 2010). Conversely, removing the carbamoyl moiety from the distal phenyl ring (R) yields cyclohexylcarbamic acid 6-hydroxybiphenyl-3-yl ester (URB694), a compound that readily enters the brain (Clapper et al., 2009). These observations suggest that the R and R1/R2 regions (Figure 1) are involved in limiting the access of *O*-biphenylcarbamate-based FAAH inhibitors to the CNS.

To deepen into the pharmacophoric properties that prevent URB937 from crossing the BBB in mice, we synthesized a series of structural analogues aimed to extend the structure-activity relationship (SAR) of its peripheral segregation *in vivo*, and assessed the implication of Abcg2 in such process by blocking the transporter with its selective inhibitor Ko143 (Moreno-Sanz et al., 2013). However, distribution across tissue barriers can barely be unequivocally attributed to a single transporter, given the considerable overlap in substrate specificity between ABCG2 and other ABC transporters (P-gp in particular) that can lead to synergistic effects (Marquez & van Bambeke, 2011; Tang et al., 2011). Furthermore, other factors such as the polar surface area (PSA) of the compound (Shityakov et al., 2013) or the local lipid composition of the membrane (Pal et al., 2007) could also affect the interaction of the drug with the BBB.

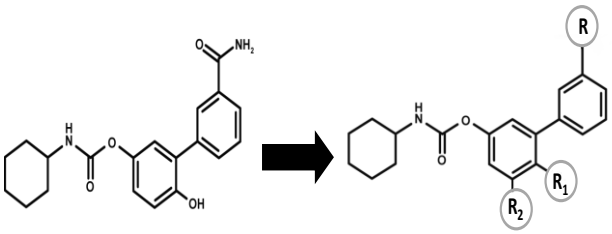


Fig.1 Chemical structure of the compound URB937. Circle zones are the regions that are substituted in the different tested analogues.

To overcome this limitation, in the present study we employed monolayers of MDCKII cells over-expressing either Abcg2 or P-gp (ABCB1) to test a selected cohort of peripheral and non-peripheral URB937 analogues for their affinity towards these efflux transporters. Our results extend the SAR of cyclohexylcarbamic acid biphenyl esters as substrates for Abcg2 and identify them, for the first time, as substrates for P-gp, pointing out critical structural determinants that are crucial for drug-protein interaction. Also, discrepancies with the available *in vivo* data on these compounds are further discussed in an attempt to better characterize the synergistic actions of these two transporters on restricting the access of *O*-byphenyl carbamate-based FAAH inhibitors to the CNS.

Material and methods

Chemicals

Ko143 was purchased from Tocris (Bristol, United Kingdom). URB937 and its derivatives were synthesized as described (Moreno-Sanz et al., 2013). All the other chemicals were analytical grade and available from commercial sources.

Cell cultures

MDCKII cells and their murine Abcg2- and human ABCB1- transduced subclones were a kind gift of Dr. A.H. Schinkel. Culture conditions were as previously described (Jonker et al., 2000; Pavek et al., 2005). The cells were cultured in Dulbecco-modified Eagles's medium (DMEM) supplemented with Glutamax (Life Technologies, Inc., Carlsbad, CA, USA), penicillin (50 units/mL), streptomycin (50µg/mL), and 10% (v/v) fetal calf serum (MP Biomedicals, Solon, OH, USA). Cells were cultured at 37 ◦C in the presence of 5% CO_2. Cells were trypsinized every 3–4 days for subculturing.

Transport studies

Transepithelial transport assays using Transwell plates were carried out as previously described (Merino et al., 2005) with minor modifications. Cells were seeded on microporous polycarbonate membrane filters (3.0 µm pore size, 24mm diameter; Transwell 3414; Costar, Corning, NY, USA) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and the medium was replaced every day. Before starting the experiment transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Bedford, MA); wells registering a resistance of 150 Ω or greater, after correcting for the resistance obtained in blank control wells, were used in the transport experiments. The measurement was repeated at the end of the experiment to check the tightness of the monolayer. Experiments were performed using Optimem medium, a reduced serum medium that is a modification of Eagle's minimum essential medium, buffered with HEPES and sodium bicarbonate. Two hours before the start of the experiment, medium on both sides of the monolayer was replaced with 2mL of Optimem medium (Life Technologies, Inc., Carlsbad, CA, USA), without serum, either with or without Ko143 (1µM) in the case of Abcg2 experiments. The experiment was started (t=0) by replacing the medium in either the apical or basolateral compartment with fresh Optimem medium, either with or without Ko143 (1µM) in the case of Abcg2 experiments, and containing 5 µM of each of the tested compounds. Aliquots of 100 µL were taken from the drug opposite compartment at t=2 and 4h, and stored at −20 ºC until analysis. The appearance of the compound in the acceptor compartment is presented as fraction of total compound added to the donor compartment at the beginning of the experiment. Active transport across MDCKII monolayers was expressed by the relative transport ratio (r), defined as the percentage apically directed transport divided by the percentage basolaterally directed translocation, after 4 h (Huisman et al., 2005).

HPLC/MS analysis

To determine URB937 and its metabolites HPLC/MS analyses were run on a Waters ACQUITY UPLC/MS system consisting of a Single Quadropole Detector (SQD) Mass Spectrometer (MS) equipped with an Electrospray Ionization (ESI) interface and a Photodiode Array (PDA) Detector. PDA range was 210-400 nm. ESI in positive and negative mode was applied. Mobile phases: (A) 10mM NH4OAc in H2O, pH 5; (B) 10mM NH4OAc in CH3CN/H2O (95:5) pH 5. Analyses were performed either with method A, B or C. *Method A*: gradient 5 to 95% B over 3 min; flow rate 0.5 mL/min; temperature 40 °C. Pre column: Vanguard BEH C18 (1.7µm 2.1x5mm). Column: BEH C18 (1.7µm 2.1x50mm). *Method B*: gradient 0 to 50% B over 3 min; flow rate 0.5 mL/min; temperature 40 °C. Pre column: VanGuard HSS T3 C18 (1.7µm 2.1x5 mm). Column HSS T3 (1.8µm 2.1 x 50mm). *Method C*: gradient: 50 to 100% B over 3 min, flow rate 0.5 mL/min; temperature 40 °C. Pre column: Vanguard BEH C18 (1.7µm 2.1x5mm). Column: BEH C18 (1.7µm 2.1x50mm). HPLC system included a 2747 Sample Manager, 2545 Binary Gradient Module, System Fluidic Organizer and 515 HPLC Pump. PDA range was 210-400 nm.

Results

Screening of the Transport of URB937 and Some Representative Analogues on Parental, Abcg2- and ABCB1-transduced MDCKII Cell Models

We used polarized monolayers of canine kidney cell line MDCKII and its subclones, transduced with either murine Abcg2 or human ABCB1 cDNAs, to test the role of these proteins in the transport of *O*-biphenyl carbamate-based FAAH inhibitors *in vitro* (Figure 2).

In the MDCKII parental cell line, while most of the molecules showed similar apically and basolaterally directed translocations (Figure 2A), compounds URB937, ARN2454, ARN0716 and ARN1289 reported a somewhat higher apically-directed transport compared to the basolaterally-directed in the parental cells, with relative transport ratios close to 2 (Tables 1 and 2), probably due to the presence of basal levels of endogenous transporters in the MDCKII cells such as P-gp. However it is worth noticing that among this set of molecules, these four compounds in particular share two common features: i) have been previously described to be peripherally restricted in mice and ii) its access to the CNS to be mediated by Abcg2 (Moreno-Sanz et al., 2013).

In the murine Abcg2-transduced MDCKII cell line (Tables 1 and 2), most compounds displayed an increased translocation from the basolateral to the apical compartment and a diminished translocation from the apical to the basolateral compartment compared to parental cells (Figure 2B), with relative transport ratios

higher than 13 in the Abcg2-transduced cells (Table 1 and Table 2), indicating that these compounds are very good substrates of Abcg2. Only the compounds ARN1290 and ARN8874 showed no changes in the vectorial transport in the Abcg2-transduced MDCKII cell line compared to parental cells, with relative transport ratios lower than 2 (Figure 2B, Tables 1 and 2), indicating that these molecules are not Abcg2 substrates. In the cases in which we observed a vectorial transport mediated by Abcg2, we were able to revert it by the addition of the specific Abcg2 inhibitor Ko143, thus confirming the role of Abcg2 in the directed translocation of these compounds (data not shown).

In the human ABCB1-transduced MDCKII cell line, compounds ARN1290 and ARN8874 also showed no changes in the vectorial transport compared to the parental cell line (Figure 2C, Tables 1 and 2) similarly to what found on the Abcg2-transduced cells, which suggests that they are not transported either by P-gp. The rest of the compounds displayed a significant increase in the translocation from the basolateral to the apical compartment and a significantly decreased translocation from the apical to the basolateral compartment compared to the parental cells (Figure 2C, Tables 1 and 2), with relative transport ratios higher than 6. We interpret these results to indicate that these molecules are also excellent P-gp substrates.

Altogether, these results indicate that compounds ARN1290 and ARN8874 are not *in vitro* substrates of either of the transporters tested, the murine Abcg2 and the human ABCB1. Taking into account the statement of the International transporter consortium which considers a compound as a substrate of a transporter if the relative transport ratio is higher than 2 (Giacomini et al., 2010), we can confirm that the rest of the tested compounds are good *in vitro* substrates for each of the tested transporters (Table 1 and Table 2).

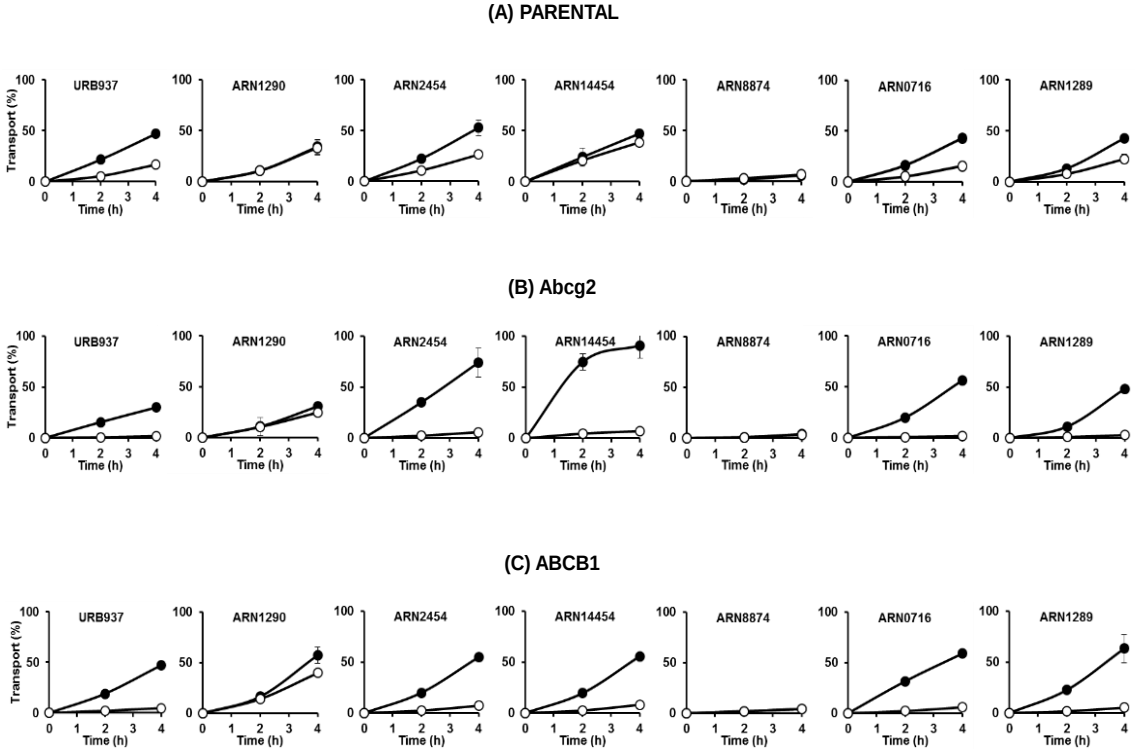
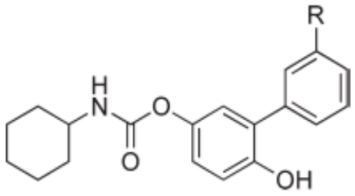


Figure 2. Transepithelial transport of tested compounds (5 µM) in (A) parental MDCKII cells, (B) their murine Abcg2 and their human ABCB1-transduced derivatives:(○) translocation from the basolateral to the apical compartment; (●) translocation from the apical to the basolateral compartment. The experiment was started with the addition of each compound to one compartment (basolateral or apical). After 2 and 4 h, the percentage of each compound appearing in the opposite compartment was measured by LC-MS and plotted. Data are expressed as mean values ± SD (n = 3 for parental and Abcg2 and n = 2 for ABCB1 experiments).

Table 1. Characteristics of the URB937 analogues with a substituent in the distal phenyl ring.



Compound	R	Pheripheral	PSA (Å²)[a]	Parental (r)[b]	Abcg2 (r)[b]	P-gp (r)[b]
URB937	$CONH_2$	YES	83	2.00 ± 1.08	17.44 ± 3.45	9.91 ± 0.31
ARN1290	$COCH_3$	NO	62	1.13 ± 0.03	1.25 ± 0.13	1.44 ± 0.13
ARN2454	$CONHCH_3$	YES	73	2.11 ± 0.00	16.18 ± 10.89	7.66 ± 0.57
ARN14454	$CON(CH_3)_2$	YES	65	1.31 ± 0.00	13.12 ± 2.04	6.84 ± 0.24

[a] PSA data obtained from Moreno-Sanz et al. (2013).
[b] (r), relative transport ratio, defined as the percentage apically directed transport divided by the percentage basolaterally directed translocation, after 4 h, in the corresponding cell line (parental, Abcg2- and ABCB1- transduced cells)

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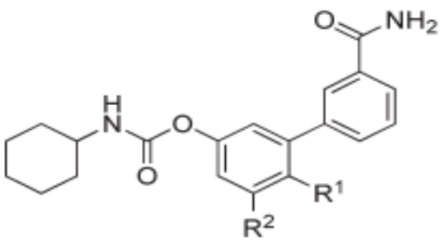

Compound	R_1	R_2	Pheripheral	PSA (Å²)[a]	Parental (r)[b]	Abcg2 (r)[b]	P-gp (r)[b]
URB937	OH	H	YES	83	2.00 ± 1.08	17.44 ± 3.45	9.91 ± 0.31
ARN8874	OSO_3NH_4	H	YES	117	0.91 ± 0.06	1.30 ± 0.43	0.96 ± 0.25
ARN0716	CH_2OH	H	YES	83	2.76 ± 0.06	31.43 ± 3.39	9.93 ± 0.06
ARN1289	H	OH	YES	84	2.27 ± 0.00	17.58 ± 2.75	12.00 ± 1.19

[a] PSA data obtained from Moreno-Sanz et al. (2013).
[b] (r), relative transport ratio, defined as the percentage apically directed transport divided by the percentage basolaterally directed translocation, after 4 h, in the corresponding cell line (parental, Abcg2- and ABCB1- transduced cells)

Discussion

The serendipitous discovery of the FAAH inhibitor URB937 having a restricted access to the CNS unveiled a relevant role for peripherally produced anandamide at controlling pain initiation and transmission of nociceptive inputs from peripheral nerves into the spinal cord (Clapper et al., 2010). The identification of the ABC transporter Abcg2 as responsible for the extrusion of URB937 from the BBB (Moreno-Sanz et al., 2011), opened the possibility for a novel strategy to develop safer therapeutic agents devoid of unwanted, CNS-mediated side effects. Recent synthetic efforts aimed at elucidating the structural determinants responsible for the peripheral segregation of URB937 rendered a small group of novel peripheral FAAH inhibitors (Moreno-Sanz et al., 2013). To further investigate the mediation of Abcg2 in this process, we used MDCKII cells over-expressing the murine isoform of Abcg2 to test a selected cohort of peripheral and non-peripheral URB937 analogues for their interaction with this efflux transporter. Since not only Abcg2 but also P-gp is functionally present in the BBB, we also tested this set of molecules in MDCKII cells over expressing the human isoform of ABCB1. Our results indicate that most of these

FAAH inhibitors are substrates for both transporters and only compounds ARN1290 and ARN8874 have no interaction with any of them. It is worth noticing the high relative transport ratios reported for these molecules in the transduced cell lines (Tables 1 and 2), higher than 6 and achieving more than 30 for ARN0716 in Abcg2 cells, which indicates that they are excellent substrates for both transporters, since compounds generally used as positive control, such as topotecan, display relative transport ratios between 5.7 and 6.9 in the same cell lines (Poller et al., 2011).

Our results are in accordance with what found by Moreno-Sanz et al. (2013) in mice, showing that the methylketone derivative ARN1290 displayed good brain penetration *in vivo*, suggesting no interaction with efflux ABC transporters at the BBB. The present data confirm the key role of the amino within the carbamoyl group in the distal ring of URB937 (absent in ARN1290) for the peripheral distribution of this compound. In addition, our data show that the primary (URB937) and secondary amides (ARN2454) with restricted access to the brain in mice, (Moreno-Sanz et al., 2013) are substrates for Abcg2 and P-gp, indicating the important role of both transporters in the transport across the BBB for these compounds. However, in our hands, the tertiary amide (ARN14454) behaves like a good substrate for Abcg2 and P-gp *in vitro*, but its restricted access into the brain was not reverted by the Abcg2 inhibitor Ko143 (Moreno-Sanz et al. 2013). Probably, P-gp is the predominant *in vivo* transporter in the BBB for this compound. A plausible interpretation for this finding is that the carboxamide hydrogen bond donors may be important for the substrate specificity of URB937 and ARN2454 for the Abcg2 transporter, as it has already been shown for other functional groups (Pick et al., 2008). As a result, the affinity for Abcg2 of the tertiary amide (ARN14454) may decrease and P-gp could play a predominant role *in vivo*, when both transporters are active in the BBB. This more relevant role of P-gp in BBB, which can completely compensate for the loss of Abcg2 in the case of some dual substrates, has been previously reported (Poller et al., 2011).

Sulfated derivative ARN8874 was originally synthetized to test the mediation of a detoxifying phenolsulfotransferase in the extrusion mechanism. Our results indicate that ARN8874 does not interact with either of the transporters *in vitro*. This compound failed to enter the brain when administered to mice and pre-treatment with Ko143 did not affect its peripheral segregation (Moreno-Sanz et al. 2013), which appears not to be mediated by Abcg2. The present *in vitro* results also confirm that this compound is not a substrate for either Abcg2 or P-gp. In this case, it is likely that the presence of absolute charges on the surface (with a high PSA, Table 2) of the compound hinders its diffusion through cellular membranes, as suggested by the low percentage of transport in MDCKII cells, precluding its entry into the brain epithelial cells and its interaction with ABC membrane transporters. Our results also confirm that Abcg2 and P-gp substrate specificity allows some flexibility in the location of the hydroxyl group in the proximal ring of URB937, given that both the m-hydroxyl (ARN1289) and the p-hydroxyethyl (ARN0716) derivatives are Abcg2 and P-gp *in vitro* substrates. This is in agreement with previous *in vivo* data showing that these compounds have a restricted access to the mice brain (Moreno-Sanz et al. 2013).

In conclusion, our *in vitro* results largely support previous findings about the restricted entry of the analogues with carboxamide hydrogen bond donors in the distal phenyl ring due to the fact that they are substrates of Abcg2 and P-gp, with the exception of ARN8874. The substitutions in the proximal phenyl ring seem not to be so critical for the recognition by these transporters. In the case of the *O*-biphenyl-3-yl carbamates tested, there is a complete overlap in transport specificity for Abcg2 and P-gp *in vitro*.

Although further experiments are required to fully characterize the mechanisms that preclude these compounds from entering the brain, our results may be relevant to the design of new pharmacological strategies to avoid CNS-mediated side effects of analgesic drugs.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

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References

Alvarez, A.I., Perez, M., Prieto, J.G., Molina, A.J., Real, R., Merino, G., 2008. Fluoroquinolone efflux mediated by ABC transporters. *Journal of pharmaceutical sciences* **97**, 3483-3493.

Bram, E.E., Adar, Y., Mesika, N., Sabisz, M., Skladanowski, A., Assaraf, Y.G., 2009. Structural determinants of imidazoacridinones facilitating antitumor activity are crucial for substrate recognition by ABCG2. *Molecular pharmacology* **75**, 1149-1159.

Cai, X., Bikadi, Z., Ni, Z., Lee, E.W., Wang, H., Rosenberg, M.F., Mao, Q., 2010. Role of basic residues within or near the predicted transmembrane helix 2 of the human breast cancer resistance protein in drug transport. *The Journal of pharmacology and experimental therapeutics* **333**, 670-681.

Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, M.P., Fenart, L., 2007. Modelling of the blood-brain barrier in drug discovery and development. *Nature reviews.Drug discovery* **6**, 650-661.

Clapper, J.R., Vacondio, F., King, A.R., Duranti, A., Tontini, A., Silva, C., Sanchini, S., Tarzia, G., Mor, M., Piomelli, D., 2009. A second generation of carbamate-based fatty acid amide hydrolase inhibitors with improved activity *in vivo*. *ChemMedChem* **4**, 1505-1513.

Clapper, J.R., Moreno-Sanz, G., Russo, R., Guijarro, A., Vacondio, F., Duranti, A., Tontini, A., Sanchini, S., Sciolino, N.R., Spradley, J.M., 2010. Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature neuroscience* **13**, 1265-1270.

Falasca, M., Linton, K.J., 2012. Investigational ABC transporter inhibitors. *Expert opinion on investigational drugs* **21**, 657-666.

Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V., 2010. Membrane transporters in drug development. *Nature reviews.Drug discovery* **9**, 215-236.

Gonzalez-Sarrias, A., Miguel, V., Merino, G., Lucas, R., Morales, J.C., Tomas-Barberan, F., Alvarez, A.I., Espin, J.C., 2013. The gut microbiota ellagic acid-derived metabolite urolithin A and its sulfate conjugate are substrates for the drug efflux transporter breast cancer resistance protein (ABCG2/BCRP). *Journal of Agricultural and Food Chemistry* **61**, 4352-4359.

Gutmann, D.A., Ward, A., Urbatsch, I.L., Chang, G., van Veen, H.W., 2010. Understanding polyspecificity of multidrug ABC transporters: closing in on the gaps in ABCB1. *Trends in biochemical sciences* **35**, 36-42.

Huisman, M.T., Chhatta, A.A., van Tellingen, O., Beijnen, J.H., Schinkel, A.H., 2005. MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *International journal of cancer.Journal international du cancer* **116**, 824-829.

Jonker, J.W., Smit, J.W., Brinkhuis, R.F., Maliepaard, M., Beijnen, J.H., Schellens, J.H., Schinkel, A.H., 2000. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *Journal of the National Cancer Institute* **92**, 1651-1656.

Marquez, B., Van Bambeke, F., 2011. ABC multidrug transporters: target for modulation of drug pharmacokinetics and drug-drug interactions. *Current Drug Targets* **12**, 600-620.

Merino, G., Jonker, J.W., Wagenaar, E., Pulido, M.M., Molina, A.J., Alvarez, A.I., Schinkel, A.H., 2005. Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). *Drug metabolism and disposition: the biological fate of chemicals* **33**, 614-618.

Moreno-Sanz, G., Barrera, B., Guijarro, A., d'Elia, I., Otero, J.A., Alvarez, A.I., Bandiera, T., Merino, G., Piomelli, D., 2011. The ABC membrane transporter ABCG2 prevents access of FAAH inhibitor URB937 to the central nervous system. *Pharmacological research : the official journal of the Italian Pharmacological Society* **64**, 359-363.

Moreno-Sanz, G., Duranti, A., Melzig, L., Fiorelli, C., Ruda, G.F., Colombano, G., Mestichelli, P., Sanchini, S., Tontini, A., Mor, M., 2013. Synthesis and Structure-Activity Relationship Studies of O-Biphenyl-3-yl Carbamates as Peripherally Restricted Fatty Acid Amide Hydrolase Inhibitors. *Journal of medicinal chemistry* .

Ni, Z., Bikadi, Z., Cai, X., Rosenberg, M.F., Mao, Q., 2010. Transmembrane helices 1 and 6 of the human breast cancer resistance protein (BCRP/ABCG2): identification of polar residues important for drug transport. *American journal of physiology.Cell physiology* **299**, C1100-9.

Ni, Z., Bikadi, Z., Shuster, D.L., Zhao, C., Rosenberg, M.F., Mao, Q., 2011. Identification of proline residues in or near the transmembrane helices of the human breast cancer resistance protein (BCRP/ABCG2) that are important for transport activity and substrate specificity. *Biochemistry* **50**, 8057-8066.

Pal, A., Mehn, D., Molnar, E., Gedey, S., Meszaros, P., Nagy, T., Glavinas, H., Janaky, T., von Richter, O., Bathori, G., 2007. Cholesterol potentiates ABCG2 activity in a heterologous expression system: improved *in vitro* model to study function of human ABCG2. *The Journal of pharmacology and experimental therapeutics* **321**, 1085-1094.

Pavek, P., Merino, G., Wagenaar, E., Bolscher, E., Novotna, M., Jonker, J.W., Schinkel, A.H., 2005. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *The Journal of pharmacology and experimental therapeutics* **312**, 144-152.

Pick, A., Muller, H., Wiese, M., 2008. Structure-activity relationships of new inhibitors of breast cancer resistance protein (ABCG2). *Bioorganic & medicinal chemistry* **16**, 8224-8236.

Poller, B., Iusuf, D., Sparidans, R.W., Wagenaar, E., Beijnen, J.H., Schinkel, A.H., 2011. Differential impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on axitinib brain accumulation and oral plasma pharmacokinetics. *Drug metabolism and disposition: the biological fate of chemicals* **39**, 729-735.

Rosenberg, M.F., Bikadi, Z., Chan, J., Liu, X., Ni, Z., Cai, X., Ford, R.C., Mao, Q., 2010. The human breast cancer resistance protein (BCRP/ABCG2) shows conformational changes with mitoxantrone. *Structure* **18**, 482-493.

Sharom, F.J., 2008. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* **9**, 105-127.

Shityakov, S., Neuhaus, W., Dandekar, T., Forster, C., 2013. Analysing molecular polar surface descriptors to predict blood-brain barrier permeation. *International journal of computational biology and drug design* **6**, 146-156.

Tang, S.C., Lagas, J.S., Lankheet, N.A., Poller, B., Hillebrand, M.J., Rosing, H., Beijnen, J.H., Schinkel, A.H., 2012. Brain accumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration. *International journal of cancer.Journal international du cancer* **130**, 223-233.

Weinmann, A., Koch, S., Niederle, I.M., Schulze-Bergkamen, H., Konig, J., Hoppe-Lotichius, M., Hansen, T., Pitton, M.B., Duber, C., Otto, G., 2013. Trends in Epidemiology, Treatment, and Survival of Hepatocellular Carcinoma Patients Between 1998 and 2009: An Analysis of 1066 Cases of a German HCC Registry. *Journal of clinical gastroenterology.*

Yoshikawa, M., Ikegami, Y., Hayasaka, S., Ishii, K., Ito, A., Sano, K., Suzuki, T., Togawa, T., Yoshida, H., Soda, H., 2004. Novel camptothecin analogues that circumvent ABCG2-associated drug resistance in human tumor cells. *International journal of cancer.Journal international du cancer* **110**, 921-927.

10. DISCUSIÓN

El transportador ABCG2 ejerce una función de protección del organismo frente a xenobióticos y tóxicos de la dieta, limitando su absorción intestinal, facilitando su eliminación biliar y urinaria y restringiendo su entrada a diversos órganos a través de barreras fisiológicas tales como la barrera hematoencefálica. Esta función protectora queda en entredicho en el caso de la implicación de este transportador en la secreción de compuestos a la leche, desconociéndose hasta el momento su papel fisiológico en este órgano, pero destacando su importancia en el riesgo de aparición de residuos de fármacos en leche. Nuestro estudio se ha centrado precisamente en el estudio del tipo de interacción con este transportador (como sustratos o inhibidores) del antihelmíntico triclabendazol y sus metabolitos y el analgésico en desarrollo URB937 y sus análogos estructurales, a estos dos últimos niveles orgánicos (barrera hematoencefálica y glándula mamaria). Además, en el caso del URB937 y sus derivados se amplió el estudio *in vitro* a la glicoproteína P, por la importancia de este transportador en el funcionamiento de la barrera hematoencefálica.

La identificación de un compuesto en desarrollo como sustrato de los principales transportadores presentes en la barrera hematoencefálica cobra especial relevancia si su seguridad está relacionada con su entrada o expulsión del SNC, como en el caso del URB937, analgésico periférico que muestra ausencia de actividad en SNC, evitando así efectos adversos a este nivel. De gran relevancia para el diseño de nuevas terapias analgésicas es también, en este caso, el estudio mediante análogos estructurales de las características de este compuesto implicadas en la interacción con los principales transportadores ABC presentes en la barrera hematoencefálica. También tiene especial importancia la identificación, como inhibidores del transportador, de fármacos que se utilizan habitualmente en

la terapéutica veterinaria, como en el caso del triclabendazol, dando así respuesta al posible mecanismo de interacción entre fármacos en el caso de terapias combinadas, que afectan a la secreción de sustratos de ABCG2 en leche.

10.1. INTERACCIÓN DEL TCBZ Y SUS METABOLITOS CON EL TRANSPORTADOR ABCG2/BCRP.

La administración concomitante de múltiples fármacos es una práctica utilizada de forma general en farmacoterapia, y puede afectar a la actividad farmacológica y a la cinética de los compuestos que se administran. La interacción entre fármacos y transportadores ABC, incluyendo ABCG2 es un mecanismo de relevante importancia clínica cuando hablamos de las interacciones entre fármacos (Marchetti y cols., 2007).

El TCBZ es un fasciolicida que se usa de una forma general en medicina humana y veterinaria, siendo frecuente su coadministración con otros fármacos, por lo que es interesante estudiar sus interacciones con compuestos que sean sustratos del transportador ABCG2 y ver cómo puede afectar a su farmacocinética y a su secreción en leche. En nuestro estudio nos centramos en el efecto potencial de los metabolitos mayoritarios en plasma (TCBZSO y $TCBZSO_2$) del TCBZ, ya que debido a su elevado metabolismo, el fármaco parental TCBZ no es detectado en plasma.

En esta memoria se incluyen dos trabajos en los que, mediante experimentos *in vitro* con células MDCKII transfectadas con diversas variantes del transportador ABCG2 de diferentes especies y experimentos *in vivo* con

modelos animales como los ratones y con animales de interés ganadero como ovejas de raza Assaf, comprobamos cómo los metabolitos del TCBZ (TCBZSO y $TCBZSO_2$) inhiben este transportador y afectan a las farmacocinéticas y/o secreción en leche de otros sustratos conocidos de ABCG2.

En cuanto a los estudios *in vitro,* utilizamos células MDCKII que sobreexpresan el transportador Abcg2 murino y el transportador ABCG2 humano (artículo I) y las dos variantes bovinas (*wild-type*, Y581S) y la ovina (artículo II) en experimentos de transporte transepitelial y ensayos de acumulación intracelular de mitoxantrona.

Los resultados obtenidos mostraron que estos compuestos son capaces de inhibir eficientemente todas las variantes probadas del transportador ABCG2. En los ensayos de acumulación intracelular de mitoxantrona, con un rango de concentraciones desde 5 a 25 μM, ambos compuestos (TCBZSO y $TCBZSO_2$) mostraron potencias inhibitorias de entre 30 y 70% dependiendo de la variante probada. La mayor potencia inhibitoria (60-70%) aparece a 25 μM para la variante *wild-type* (Y581) bovina (Figura 1 del artículo II). A esta concentración, Abcg2 murina y ABCG2 humana mostraron inhibiciones de alrededor del 60% (Figura 3 del artículo I) y ABCG2 ovina mostró una inhibición de entre el 30% (TCBZSO) y el 50% ($TCBZSO_2$) (Figura 1 del artículo II). Además se ha observado diferente inhibición por parte de estos compuestos de ambas variantes de la ABCG2 bovina, con valores inferiores en la variante bovina Y581S (S581) (35-50% de inhibición a 25 μM, Figura 1 del artículo II), lo cual confirma la inhibición diferencial de ambas variantes observada previamente por nuestro grupo de investigación para otros inhibidores de ABCG2 (Real y cols., 2011). Las concentraciones efectivas que obtuvimos, están en el mismo rango que las IC_{50} de

otros fármacos considerados buenos inhibidores del transportador ABCG2 humano en la misma línea celular (Weiss y cols., 2007): para lopinavir, 7,66 μM; para nelfinavir, 13,50 μM; para saquinavir, 27,40 μM; y para delavirdina, 18,60 μM. Para otros fármacos benzimidazólicos que interaccionan con ABCG2, tales como pantoprazol y omeprazol, las IC_{50} fueron de 13 μM y 36 μM, respectivamente (Breedveld y cols., 2004). La concentración de 25 μM que en nuestro caso puede llegar a producir una inhibición del 70% en ABCG2 bovina es similar a las concentraciones plasmáticas del metabolito activo TCBZSO que se han descrito para oveja (30 μM) (Fairweather y Boray, 1999) o humana (25 □M) (El-Tantawy y cols., 2007) tras el tratamiento terapéutico con TCBZ.

El potencial de inhibición de los metabolitos del TCBZ fue también confirmado para otros sustratos conocidos de ABCG2, como los antibióticos nitrofurantoína (todas las variantes) y danofloxacina (sólo variantes de ratón y de humana) en experimentos de transporte transepitelial a una concentración de 15 μM, mostrando una moderada inhibición de la variante Abcg2 murina (Figuras 4 y 5 del artículo I) y ABCG2 bovina (TCBZSO) (Figura 2 del artículo II) y una inhibición completa para las variantes ABCG2 humana, bovina ($TCBZSO_2$) y ovina (Figuras 4 y 5 del artículo I y Figuras 2 y 3 del artículo II). La concentración de 15 μM se eligió teniendo en cuenta la elevada inhibición obtenida a esta concentración en los ensayos de acumulación intracelular de mitoxantrona en casos como el de ABCG2 humana (55-60% de inhibición) (Figura 3 del artículo I) o el de la variante bovina *wild-type* (Y581) (55-70% de inhibición) (Figura 1 del artículo II), pero no podemos excluir inhibiciones del transporte transepitelial *in vitro* por parte de ambos metabolitos a concentraciones inferiores a 15 μM. Aunque se han descrito interacciones de estos compuestos con

otros transportadores como la P-gp (Dupuy y cols., 2010), la ausencia de efecto de estos compuestos en el transporte vectorial en las células parentales (Figuras 4 y 5 del artículo I y Figura 1 del artículo II) nos indica que esta interacción es específica de ABCG2 para nuestras condiciones experimentales. Todos estos datos en conjunto indican que ambos metabolitos del TCBZ son buenos inhibidores *in vitro* del transportador ABCG2.

Una vez realizados los experimentos *in vitro* y teniendo en cuenta los prometedores resultados obtenidos, decidimos comprobar lo que ocurría en la situación real *in vivo*, comenzando con el modelo animal de ratón. Utilizamos ratones *wild-type* y ratones *knockout* para el transportador Abcg2 y estudiamos cómo modifican los metabolitos de estudio la farmacocinética del sustrato tipo de Abcg2 sulfasalazina cuando se coadministran con él. Los resultados obtenidos mostraron que los niveles en plasma de sulfasalazina aumentaron significativamente con la coadministración de TCBZSO sólo en los ratones *wild-type*, no observándose efecto en los ratones Abcg2 $^{-/-}$, lo cual nos indica que el efecto es específico de Abcg2 (Figura 6 del artículo I). Este efecto no se debe solamente a la inhibición ejercida por el TCBZSO, ya que éste se metaboliza a $TCBZSO_2$ que también ejerce efecto inhibitorio.

Además estudiamos el efecto de los metabolitos del TCBZ sobre la secreción en leche de sustratos de ABCG2, primero en el modelo de ratón y después utilizando ovejas de raza Assaf. Así, en los ratones *wild-type* la secreción en leche de nitrofurantoína disminuyó significativamente tras la coadministración de TCBZSO (Figura 6 del artículo I). En el caso de las ovejas, ocurrió lo mismo con los niveles de moxidectina en leche, que fueron significativamente inferiores en el grupo tratado con TCBZ (Figura 5 del artículo II). En ambos casos, no se

vieron afectados los niveles plasmáticos. Como ya hemos dicho anteriormente, aunque la interacción de estos compuestos con otros transportadores ABC ya ha sido descrita anteriormente (Dupuy y cols., 2010), el efecto de los metabolitos del TCBZ en la secreción en leche de nitrofurantoína y moxidectina se debe atribuir a la interacción con ABCG2, ya que otros transportadores ABC no se expresan de manera sustancial en glándula mamaria (van Herwaarden y Schinkel, 2006). En el caso de las ovejas, este efecto inhibitorio puede ser considerado como casi total, porque la reducción de un 37% en la AUC (0-48h) del ratio leche/plasma de la moxidectina para el grupo de ovejas tratadas con TCBZ (Tabla 3 del artículo II) es similar a la reducción de un 35-42% publicada previamente para la secreción en leche de moxidectina en ratones Abcg2$^{-/-}$ comparados con ratones *wild-type* (Pérez y cols., 2009a). Además, debido a que el fármaco parental TCBZ no se detecta en plasma ni en leche, podemos decir que este efecto mediado por ABCG2 es producido por los dos metabolitos mayoritarios en plasma (TCBZSO y $TCBZSO_2$), que en el caso del experimento con moxidectina en ovejas mostraron un perfil farmacocinético similar (similar $T_{máx}$) en plasma y en leche y niveles similares en leche a los de la moxidectina (Figura 5 del artículo II). No fue así para el caso del experimento con la danofloxacina, donde la $TCBZSO_2$ mostró perfiles diferentes a los de la danofloxacina en plasma y en leche y la concentración de ambos metabolitos en leche fue mucho menor que la de danofloxacina (Figura 4 del artículo II), sin mostrar por tanto interacción.

Diversos estudios han mostrado una falta de efecto de ABCG2 en los niveles en plasma de danofloxacina, mientras que los niveles en leche de este antibiótico sí que se vieron ampliamente afectados por la actividad de este transportador (Real y cols., 2011; Otero y cols., 2013). Sin embargo, en la

presente memoria, la baja concentración de los metabolitos del TCBZ en leche hace que no exista interacción de los mismos con el transportador ABCG2 y por lo tanto no se vea afectada la concentración de danofloxacina secretada a la leche.

Ni en ratones ni en ovejas se encontraron diferencias significativas en los niveles en plasma de nitrofurantoína y moxidectina tras el tratamiento con TCBZSO o TBZ respectivamente, a pesar de observarse cambios en los niveles en leche. Algunos autores han descrito efectos locales mediados por Abcg2 (distribución fetal y secreción a leche) sin encontrar diferencias en los perfiles de las concentraciones en plasma entre ratones *wild-type* y *knockout* para algunos sustratos (Pérez y cols., 2009a; Tang y cols., 2012; Zhou y cols., 2008) e incluso en ovejas tras coadministración de inhibidores como la ivermectina (Real et al., 2011). En el caso de nuestros ratones, a diferencia del experimento con nitrofurantoína, la coadministración con TCBZSO parece haber tenido un efecto mediado por Abcg2 en los niveles plasmáticos de sulfasalazina (Figura 6 del artículo I), probablemente debido a un mayor efecto de Abcg2 en la disposición sistémica de sulfasalazina tras la administración oral, ya que la diferencia en las concentraciones en plasma de este compuesto después de una administración oral entre los ratones Abcg2$^{-/-}$ no tratados y los ratones *wild-type* fue aproximadamente de 10 veces (4,91±1,67 µg/mL vs. 0,40±0,13 µg/mL), mientras que en el caso de la nitrofurantoína (administración intravenosa), fue solamente de 3 veces (2,32±0,75 µg/mL vs. 0,60±0,10 µg/mL). Además, las diferentes rutas de administración del TCBZSO (oral en el caso del experimento con la sulfasalazina e intraperitoneal en el caso del experimento con la nitrofurantoína) y/o el género o el estado fisiológico de los animales puede influir en el efecto inhibidor del TCBZSO.

Esta interacción *in vivo* entre fármacos en la que está implicado el TCBZ, puede ser aplicada no sólo para el caso de la moxidectina en ovejas, sino también para otros sustratos de ABCG2 de uso veterinario. Este descubrimiento es muy relevante si consideramos que la administración concurrente de diferentes fármacos es una práctica clínica usual. Además, el TCBZ se comercializa combinado con otros antihelmínticos para mejorar su eficacia, ampliar su espectro de actividad y limitar la resistencia emergente (Dupuy y cols., 2010). Algunas de estas combinaciones de fármacos incluyen fármacos como la ivermectina (Lifschitz y cols., 2009) o el oxfendazol (Merino y cols., 2005a), de los que se conoce su interacción con los transportadores ABC. Por ejemplo, cuando se coadministró ivermectina junto con TCBZ, la eliminación de ésta se retrasó y su biodisponibilidad plasmática fue 3 veces mayor (Lifschitz y cols., 2009), aunque en este caso esta interacción entre fármacos fue atribuida a la inhibición de P-gp y/o enzimas metabólicas.

El ganado de producción lechera puede ser tratado con TCBZ solamente durante los periodos de secado para evitar residuos en la leche de consumo. Sin embargo, en áreas endémicas, los programas de control de parásitos incluyen tratamientos regulares con antihelmínticos incluso durante los periodos de lactación (Imperiale y cols., 2011). En estas situaciones, la administración concomitante de múltiples fármacos podría dar lugar a modificaciones en la secreción de otros compuestos y otros sustratos de ABCG2 como por ejemplo algunas vitaminas a la leche (van Herwaarden y cols., 2007), lo que conlleva un importante efecto en la calidad de la misma.

Por otra parte, el TCBZ podría ser utilizado en combinación con otros sustratos de ABCG2, con el fin de inhibir el transportador y modificar la

distribución farmacológica en el organismo. Esta estrategia de modulación ya ha sido probada en animales con algunos antihelmínticos coadministrados con inhibidores de P-gp (Fairweather, 2009) o con inhibidores de ABCG2 (Pérez y cols., 2009b; Real y cols., 2011). Diversos estudios han logrado incrementar la biodisponibilidad y la secreción a leche de antibióticos como la nitrofurantoína o la danofloxacina, o de antitumorales como el topotecan, o aumentar la entrada en cerebro del antitumoral imatinib mediante el uso de inhibidores de ABCG2 y P-gp, como el elacridar, el pantoprazol, o las isoflavonas (Kruijtzer y cols., 2002; Breedveld y cols., 2005; Jonker y cols., 2005; Pérez y cols., 2009b, 2013; Merino y cols., 2010). Sin embargo, debe señalarse que el uso de TCBZ para este propósito puede ser controvertido en animales cuyos productos sean destinados para el consumo humano o en áreas donde el parásito es endémico, debido al potencial desarrollo de resistencias por la exposición de los consumidores a la leche contaminada.

Además, los inhibidores de ABCG2 pueden ser útiles en otras aplicaciones como por ejemplo la reversión de la resistencia a la quimioterapia (Noguchi y cols., 2009) aunque son necesarios más estudios que demuestren la aplicación de estos compuestos en este campo. Recientemente, el Consorcio Internacional de Transportadores (formado por científicos industriales, reguladores (FDA) y científicos académicos con experiencia en metabolismo de fármacos, transporte y farmacocinéticas) ha incluido a ABCG2 en el grupo de transportadores clínicamente relevantes (Giacomini y cols., 2010), y ha destacado la falta de inhibidores conocidos de transportadores ABC, indicando que esto supone un área de conocimiento que requiere de más estudios de investigación.

La mayoría de la información relacionada con las interacciones mediadas por ABCG2 proveniente de estudios con humanos y roedores podría ser aplicable también a otras especies de mamíferos (Mealey, 2012). Así, los experimentos *in vivo* descritos aquí usando ratones y ovejas, pueden ser también aplicables a otras especies de rumiantes como por ejemplo el ganado vacuno, donde el estudio de las diferencias existentes dependiendo del genotipo Y581S puede ser de gran importancia, teniendo en cuenta las diferencias observadas *in vitro* en el segundo de los presentes trabajos para el perfil de inhibición de las dos variantes bovinas. Sería por tanto interesante, estudiar los posibles efectos *in vivo* de estos metabolitos del TCBZ en cuanto a las potenciales interacciones con fármacos sustratos conocidos de ABCG2 en otras especies terapéuticas de interés, como por ejemplo ganado vacuno o humanos.

10.2. INTERACCIÓN DEL URB937 Y SUS ANÁLOGOS ESTRUCTURALES CON LOS TRANSPORTADORES ABCG2 Y GLICOPROTEÍNA P.

URB937 es un potente inhibidor de FAAH, enzima que degrada el endocannabinoide anandamida. URB937 no penetra en el SNC, y por lo tanto interrumpe la desactivación de la anandamida solamente en los tejidos periféricos. En roedores, la administración sistémica de URB937 inhibe la actividad FAAH y eleva los niveles de anandamida en tejidos periféricos pero no en prosencéfalo, hipotálamo o médula espinal (Clapper y cols., 2010). A pesar de su restringido rango de acción, URB937 provoca marcados efectos antinociceptivos en modelos de roedores con dolores agudos y persistentes que fueron impedidos por el bloqueo del receptor cannabinoide CB_1 (Clapper y cols., 2010). Estos

descubrimientos sugieren que la inhibición de la actividad periférica de FAAH pone de manifiesto un mecanismo analgésico endógeno, mediado por anandamida, que regula la transmisión de las señales emergentes de dolor a la médula espinal y al cerebro (Clapper y cols., 2010).

Experimentos farmacológicos previos han sugerido que la expulsión de URB937 del cerebro puede estar mediada por el transportador de membrana ABCG2 (Clapper y cols., 2010). En la presente memoria, comprobamos dicha hipótesis mediante estudios tanto *in vitro* como *in vivo* (Artículo III).

Los resultados obtenidos *in vitro* mostraron que el transporte relativo de URB937 mediado por ABCG2 se incrementó cerca de 10 veces en las células que sobreexpresaban tanto el transportador murino como el humano (Figura 2 del Artículo III). Este efecto fue revertido cuando se añadió el inhibidor específico de ABCG2, Ko143. Estos resultados nos indican que URB937 es un sustrato *in vitro* de ABCG2.

Para descubrir más sobre el papel de ABCG2 en la extrusión de URB937, examinamos la distribución de este compuesto y su actividad FAAH en ratones Abcg2$^{-/-}$ y en ratones *wild-type*. Independientemente del genotipo, URB937 inhibía la actividad FAAH en los tejidos periféricos (Figura 3 del artículo III). Por el contrario, el fármaco inhibía fuertemente la actividad FAAH en el SNC de los ratones Abcg2$^{-/-}$ pero no se producía esta inhibición en los ratones *wild-type*. Los niveles en SNC de URB937 también se incrementaron significativamente en los ratones Abcg2$^{-/-}$. En conjunto, los resultados obtenidos *in vivo* indican que ABCG2 restringe el acceso de URB937 al SNC sin afectar a sus niveles en plasma ni a su distribución y actividad en los tejidos periféricos.

Además, se ha demostrado que esta restricción en cuanto al acceso al SNC de URB937 mediado por ABCG2 es también extrapolable a otras especies como la rata (Moreno-Sanz y cols., 2012) y muy probablemente a humanos.

Recientemente, se ha confirmado el papel limitador de Abcg2 en cuanto al paso de URB937 a través de barreras fisiológicas, ya que Abcg2 no sólo limita el acceso de URB937 a nivel de SNC, sino también a la unidad fetoplacentaria protegiendo por lo tanto al feto (Moreno-Sanz y cols., 2012). Al igual que Abcg2, FAAH se expresa también en los sincitiotrofoblastos placentarios, donde sus niveles están estrictamente controlados durante la gestación (Helliwell y cols., 2004), probablemente debido al papel clave que desempeña la anandamida en la implantación del blastocisto (Wang y cols., 2003). El hecho de que la expresión de FAAH se mantenga elevada durante el desarrollo placentario sugiere que esta enzima puede proteger al feto de la anandamida materna circulante hasta que la barrera placentaria sea funcional (Habayeb y cols., 2008) y por tanto Abcg2 pueda realizar su función protectora.

Hemos demostrado que ABCG2 afecta a la entrada de URB937 a nivel de SNC sin afectar a sus niveles en plasma o tejidos periféricos. Esta interpretación es consistente de nuevo con diversos estudios que demuestran efectos locales mediados por Abcg2, incluyendo penetración en cerebro, pero no diferencias en los perfiles sistémicos entre ratones *wild-type* y Abcg2$^{-/-}$ (Real y cols., 2011; Tang y cols., 2012, Nakanishi y cols., 2013). Aunque URB937 es un sustrato *in vitro* de ABCG2, existen otros factores adicionales que podrían afectar a la disposición sistémica de este compuesto debido a diferencias en la permeabilidad y en los sistemas de captación de fármacos entre las membranas sistémicas y la membrana luminal endotelial de la barrera hematoencefálica (Tang y cols., 2012). Por lo

tanto, la relevancia de ABCG2 en la penetración en cerebro de cualquiera de sus sustratos, dependerá también de otros constituyentes de la barrera hematoencefálica (Robey y cols., 2009). Entre estos, el transportador ABC conocido como P-gp se sabe que se solapa con ABCG2 en la especificidad de sus sustratos (Tang y cols., 2012) y nuestros estudios *in vitro* demuestran que URB937 también es sustrato *in vitro* de P-gp (Figura 2 del artículo IV) y es muy probable que este transportador también contribuya *in vivo* al acceso restringido al SNC de este compuesto. Usando ratones Abcg2$^{-/-}$ en los que P-gp está todavía presente, puede ser difícil demostrar inequívocamente un papel funcional de ABCG2 en la barrera hematoencefálica debido a la capacidad de compensación entre transportadores ABC (Vlaming y cols., 2009). Sin embargo, en nuestro estudio pudimos demostrar el papel de ABCG2 en la extrusión del cerebro de URB937 junto con una diferencia en la actividad FAAH dependiendo del genotipo del ratón.

Debido a la aparición de efectos adversos, entre ellos psicotrópicos, a nivel del SNC, de muchos derivados opiodes, es de vital importancia el diseño de nuevas terapias analgésicas para lo que es de gran utilidad el conocimiento de las características estructurales que permiten al URB937 evitar su entrada a SNC. Para ello determinamos si diferentes análogos estructurales de URB937 son o no sustratos *in vitro* tanto de Abcg2 como de P-gp (artículo IV).

Los resultados muestran que la mayoría de estos compuestos interaccionan con ambos transportadores (Abcg2 murino y ABCB1 humano), incluido el compuesto periférico URB937, y solamente los compuestos ARN1290 y ARN8874 no tienen ninguna interacción con ellos (Figura 2 y Tablas 1 y 2 del artículo IV). Especial atención requieren los elevados ratios relativos de transporte

observados para los sustratos en las líneas celulares transducidas (Tablas 1 y 2 del artículo IV), mayores de 6 y alcanzando valores mayores de 30 para el compuesto ARN0716 en las células transducidas con el transportador Abcg2, indicando que estos compuestos son excelentes sustratos de ambos transportadores, ya que sustratos modelo usados como controles positivos, tales como el topotecan muestran ratios relativos de transporte entre 5,7 y 6,9 en el mismo tipo celular (Poller y cols., 2011).

Nuestros resultados están en concordancia con los resultados *in vivo* obtenidos por Moreno-Sanz y cols. (2013) que mostraron que el derivado metilketona ARN1290 manifestó buena penetración en cerebro *in vivo*, sugiriendo que no interacciona con los transportadores ABC presentes en la barrera hematoencefálica. Nuestros resultados confirman el papel fundamental que juega el amino en el grupo carbamoil en la region R del anillo distal del URB937 (ARN0354) (ausente en ARN1290) en la distribución periférica de este compuesto. Además, nuestros datos confirman que las amidas primaria (ARN0354) y secundaria (ARN2454) con acceso restringido a cerebro en ratones (Moreno-Sanz y cols., 2013) son sustratos de Abcg2 y de P-gp, indicando el importante papel que tienen ambos transportadores en el transporte a través de la barrera hematoencefálica de estos compuestos. Sin embargo, en nuestro trabajo, la amida terciaria (ARN14454) es un buen sustrato *in vitro* de Abcg2 y de P-gp, pero su acceso restringido al cerebro no fue revertido por el inhibidor de Abcg2 Ko143 (Moreno-Sanz y cols., 2013). Probablemente, P-gp es el transportador *in vivo* predominante en la barrera hematoencefálica para este compuesto. Una posible interpretación de este hecho es que los donadores de enlaces de hidrógeno de la carboxamida deben de ser importantes para la afinidad de ARN0354 y ARN2454

por el transportador Abcg2, como ya ha sido visto para otros grupos funcionales (Pick y cols., 2008). Por lo tanto, para la amida terciaria (ARN14454), la afinidad de Abcg2 debe decrecer y P-gp debe jugar un papel predominante *in vivo* cuando ambos transportadores están presentes en la barrera hematoencefálica. Este papel más relevante de P-gp en la barrera hematoencefálica, que puede compensar completamente la pérdida de Abcg2 en el caso de algunos sustratos de ambos transportadores, ya ha sido descrito previamente (Poller y cols., 2011).

El derivado sulfatado ARN8874 fue sintetizado inicialmente para comprobar la implicación de una fenolsulfotransferasa detoxificante en el mecanismo de extrusión del URB937. La importancia del resto fenólico plantea la cuestión de si las fenol sulfotransferasas (SULT1) pueden estar involucradas en la extrusión del URB937 del SNC. Los transportadores ABC están comúnmente localizados en los mismos lugares que las sulfotransferasas (Enokizono y cols., 2007; Giacomini y cols., 2010). La capacidad de ABCG2 para transportar fenol conjugados ya ha sido descrita anteriormente (Imai y cols., 2003; Mao y Unadkat, 2005). Además, ABCG2 ha sido descrito recientemente como un mediador fisiológico importante de la conjugación fenólica (Zhu y cols., 2010). En este mismo estudio, estos autores demostraron que tanto la actividad sulfotransferasa como el bombeo de conjugados sulfato por parte de ABCG2 eran procesos saturables. Existen estudios previos que demuestran que la administración de un inhibidor no selectivo de SULT1 incrementa la entrada de URB937 en cerebro (Clapper y cols., 2010), sugiriendo que este compuesto puede ser un sustrato de SULT1. Por otra parte, se sabe que la conjugación con sulfatos incrementa la afinidad de ABCG2 por sus sustratos (Suzuki y cols., 2003). Sin embargo, la transferencia del grupo sulfato se espera que ocurra en el citosol de las células

epiteliales, donde se localizan las sulfotransferasas, una vez que el compuesto ha difundido a través de la membrana apical. Nuestros resultados muestran que ARN8874 no interacciona *in vitro* con ninguno de los dos transportadores (Figura 2 del artículo IV). Este compuesto no penetra en cerebro cuando es administrado a ratones y el pre-tratamiento con Ko143 no afecta a su distribución periférica (Moreno-Sanz y cols., 2013), por lo que parece que su transporte no está mediado por Abcg2. En este trabajo (artículo IV), también confirmamos que este compuesto no es sustrato *in vitro* ni de Abcg2 ni de P-gp. En este caso, es probable que la presencia de cargas absolutas en superficie (con un alto PSA, Tabla 2 del artículo IV) del compuesto obstaculiza su difusión a través de las membranas celulares (Moreno-Sanz y cols., 2013), tal y como sugiere el bajo porcentaje de transporte en las células MDCKII (Figura 2 del artículo IV), evitando su entrada en cerebro y su interacción con los transportadores de membrana dependientes de ATP. Nuestros resultados también confirman que existe algo de flexibilidad en cuanto al reconocimiento de los sustratos de Abcg2 y P-gp según la localización del grupo hidroxilo en el anillo fenólico proximal del URB937, ya que ambos derivados, el m-hidroxil (ARN1289) y el p-hidroxietil (ARN0716) son sustratos *in vitro* tanto de Abcg2 como de P-gp, lo que concuerda con los resultados previos *in vivo* que muestran su acceso restringido a cerebro (Moreno-Sanz y cols., 2013).

Se han realizado importantes esfuerzos focalizados en la síntesis de compuestos que sean capaces de evitar la resistencia terapéutica debida al transporte de los mismos por transportadores ABC (por ejemplo en quimioterapia) o que muestren mejor biodisponibilidad o mejor índice terapéutico a través del reconocimiento específico de los transportadores (Nakagawa y cols., 2006;

Giacomini y cols., 2010; Pick y cols., 2011). Los resultados mostrados en el presente estudio indican que el transportador ABCG2, y probablemente P-gp son responsables de la expulsión de URB937 del SNC. También se confirman resultados previos *in vivo* en los que se indica la importancia del grupo carboxamida del anillo fenólico distal de la molécula de URB937 en la restricción de acceso de estos compuestos al SNC debido a su relevancia para la interacción con los transportadores ABC objeto de estudio.

Este descubrimiento proporciona la información estructural necesaria para encontrar nuevos inhibidores de la actividad FAAH que sean restringidos periféricamente evitando efectos secundarios a nivel del SNC y que pueden ser utilizados de forma segura durante la gestación (Moreno-Sanz y cols., 2012).

11. CONCLUSIONES

De los resultados obtenidos en los trabajos presentados podemos obtener las siguientes conclusiones:

CONCLUSIÓN PRIMERA: Los metabolitos del antihelmíntico TCBZ, el TCBZSO y la $TCBZSO_{2,}$ son inhibidores eficientes *in vitro* del transportador ABCG2 humano, murino, ovino y de ambas variantes del transportador bovino, en modelos de células MDCKII que sobreexpresan las diferentes variantes del transportador.

CONCLUSION SEGUNDA: La variante *wild-type* del transportador bovino es inhibida más eficazmente *in vitro* que la variante Y581S por los metabolitos TCBZSO y $TCBZSO_2$ para todas las concentraciones de compuestos probadas, en experimentos de acumulación intracelular de mitoxantrona.

CONCLUSIÓN TERCERA: Los estudios farmacocinéticos realizados comparando las concentraciones en plasma y en leche entre ratones *wild-type* y ratones deficientes en el transportador Abcg2 mostraron que los metabolitos del TCBZ son inhibidores *in vivo* del transportador Abcg2 murino, aumentando significativamente los niveles plasmáticos del antiinflamatorio sulfasalazina y reduciendo la secreción a leche del antibiótico nitrofurantoína en ratones *wild-type* tras su coadministración, no observándose estos efectos en los ratones deficientes en el transportador.

CONCLUSIÓN CUARTA: Los estudios farmacocinéticos realizados en ovejas de producción lechera de raza Assaf mostraron que la coadministración de TCBZ

reduce en casi un 40% la secreción a leche del antiparasitario moxidectina, debido al efecto inhibitorio de los metabolitos TCBZSO y $TCBZSO_2$ sobre el transportador ABCG2 ovino.

CONCLUSIÓN QUINTA: El compuesto inhibidor de la actividad FAAH, URB937, es sustrato *in vitro* del transportador ABCG2 murino y humano y de la glicoproteína P humana en experimentos de transporte transepitelial con células MDCKII transducidas con estos transportadores, obteniéndose ratios de transporte direccional basal-apical/apical-basal a las 4 horas superiores a 13 en el caso del transportador ABCG2 humano.

CONCLUSIÓN SEXTA: El transportador ABCG2 es responsable de que el compuesto URB937 no sea capaz de atravesar la barrera hematoencefálica y entrar al SNC, restringiéndose su presencia solamente a tejidos periféricos en los ratones *wild-type,* observándose tras su administración un aumento significativo de sus niveles en cerebro y una inhibición de la actividad FAAH a nivel de SNC en ratones deficientes en el transportador. No podemos descartar que la glicoproteína P también tenga un papel importante en este proceso.

CONCLUSIÓN SÉPTIMA: Nuestros estudios de relación estructura-función con análogos estructurales del URB937 confirmaron la importancia del grupo carboxamida del anillo fenólico distal de la molécula de URB937 en la restricción de acceso de estos compuestos al SNC debido a su relevancia en la interacción con los transportadores ABC. También se confirmó cierta flexibilidad en la localización del grupo hidroxilo en el anillo fenólico proximal del URB937 ya que

tanto el análogo estructural m-hidroxil (ARN1289) como el p-hidroxietil (ARN0716) fueron sustratos tanto de Abcg2 como de la glicoproteína P.

12. CONCLUSIONS

From the results shown in this Dissertation we obtain the following conclusions:

FIRST CONCLUSION: The TCBZ metabolites TCBZSO and $TCBZSO_2$ are efficient *in vitro* inhibitors of human, murine, ovine and both bovine variants of the ABCG2 transporter, in MDCKII cells models that overexpressed the different variants of the transporter.

SECOND CONCLUSION: The wild-type bovine variant of the ABCG2 transporter is more efficiently inhibited *in vitro* than the variant Y581S by the metabolites TCBZSO and $TCBZSO_2$ for all the concentrations tested in mitoxantrone intracellular accumulation experiments.

THIRD CONCLUSION: The pharmacokinetic studies performed comparing plasma and milk concentrations between wild-type and knockout mice for Abcg2 showed that TCBZ metabolites are *in vivo* inhibitors of the murine Abcg2 transporter, since they increase significantly the plasma levels of the anti-inflammatory agent sulfasalazine and reduce the secretion into milk of the antibiotic nitrofurantoin in wild-type mice after its coadministration with no effects in knockout mice for the Abcg2 transporter.

FOURTH CONCLUSION: The pharmacokinetic studies made with lactating Assaf sheep showed that the coadministration of TCBZ reduces by almost 40% the secretion into milk of the endectocide moxidectin, due to the inhibitory effect of the metabolites TCBZSO and $TCBZSO_2$ on the ovine ABCG2 transporter.

FIFTH CONCLUSION: The inhibiting compound of FAAH activity URB937 is an *in vitro* substrate of the murine and human ABCG2 transporter in transepithelial transport experiments with MDCKII cells that overexpressed these variants of the transporter, with basal apical/apical basal directional transport ratios at 4 hours higher than 13 in the case of the human transporter.

SIXTH CONCLUSION: The ABCG2 transporter is responsible that the compound URB937 isn't able to cross the blood-brain barrier and entry to the CNS, being its presence restricted only in peripheral tissues in wild-type mice, showing after its administration a significantly increase in its levels and an inhibition of the FAAH activity at the level of the central nervous system in knockout mice for the transporter. We can't discard that P-glycoprotein also has a relevant role on this process.

SEVENTH CONCLUSION: Our structure-function relationship studies with structural analogues of URB937 confirmed the relevance of the carboxamide group in the distal phenyl ring of the molecular structure of URB937 for the restricted access of these compounds to the central nervous system due to its interaction with ABC transporters. Some flexibility in the location of the hydroxyl group in the proximal phenyl ring of URB937 was confirmed, since both the m-hydroxyl (ARN1289) and the p-hydroxyethyl (ARN0716) derivatives were Abcg2 and P-glycoprotein *in vitro* substrates.

13. BIBLIOGRAFÍA

Agarwal, N., Pacher, P., Tegeder, I., Amaya, F., Constantin, C.E., Brenner, G.J., Rubino, T., Michalski, C.W., Marsicano, G., Monory, K. *and others*, 2007. Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nature neuroscience* **10**, 870-879.

Agarwal, S., Sane, R., Gallardo, J.L., Ohlfest, J.R., Elmquist, W.F., 2010. Distribution of gefitinib to the brain is limited by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)-mediated active efflux. *The Journal of pharmacology and experimental therapeutics* **334**, 147-155.

Agarwal, S., Uchida, Y., Mittapalli, R.K., Sane, R., Terasaki, T., Elmquist, W.F., 2012. Quantitative proteomics of transporter expression in brain capillary endothelial cells isolated from P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp), and P-gp/Bcrp knockout mice. *Drug metabolism and disposition: the biological fate of chemicals* **40**, 1164-1169.

Allen, J.D., van Loevezijn, A., Lakhai, J.M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J.H., Koomen, G.J., Schinkel, A.H., 2002. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Molecular cancer therapeutics* **1**, 417-425.

Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L. *and others*, 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science (New York, N.Y.)* **323**, 1718-1722.

Alvarez, A.I., Perez, M., Prieto, J.G., Molina, A.J., Real, R., Merino, G., 2008. Fluoroquinolone efflux mediated by ABC transporters. *Journal of pharmaceutical sciences* **97**, 3483-3493.

Ambudkar, S.V., 1995. Purification and reconstitution of functional human P-glycoprotein. *Journal of Bioenergetics and Biomembranes* **27**, 23-29.

Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E., Gottesman, M.M., 2003. P-glycoprotein: from genomics to mechanism. *Oncogene* **22**, 7468-7485.

Anand, P., Whiteside, G., Fowler, C.J., Hohmann, A.G., 2009. Targeting CB2 receptors and the endocannabinoid system for the treatment of pain. *Brain Research Reviews* **60**, 255-266.

Ando, T., Kusuhara, H., Merino, G., Alvarez, A.I., Schinkel, A.H., Sugiyama, Y., 2007. Involvement of breast cancer resistance protein (ABCG2) in the biliary excretion mechanism of fluoroquinolones. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 1873-1879.

Aronica, E., Gorter, J.A., Redeker, S., van Vliet, E.A., Ramkema, M., Scheffer, G.L., Scheper, R.J., van der Valk, P., Leenstra, S., Baayen, J.C. *and others*, 2005. Localization of breast cancer resistance protein (BCRP) in

microvessel endothelium of human control and epileptic brain. *Epilepsia* **46**, 849-857.

Arslan, F., Batirel, A., Samasti, M., Tabak, F., Mert, A., Ozer, S., 2012. Fascioliasis: 3 cases with three different clinical presentations. *The Turkish journal of gastroenterology : the official journal of Turkish Society of Gastroenterology* **23**, 267-271.

Aust, S., Obrist, P., Jaeger, W., Klimpfinger, M., Tucek, G., Wrba, F., Penner, E., Thalhammer, T., 2004. Subcellular localization of the ABCG2 transporter in normal and malignant human gallbladder epithelium. *Laboratory investigation; a journal of technical methods and pathology* **84**, 1024-1036.

Bisogno, T., Maccarrone, M., 2013. Latest advances in the discovery of fatty acid amide hydrolase inhibitors. *Expert opinion on drug discovery* **8**, 509-522.

Blazquez, A.G., Briz, O., Romero, M.R., Rosales, R., Monte, M.J., Vaquero, J., Macias, R.I., Cassio, D., Marin, J.J., 2012. Characterization of the role of ABCG2 as a bile acid transporter in liver and placenta. *Molecular pharmacology* **81**, 273-283.

Bohan, K.H., Mansuri, T.F., Wilson, N.M., 2007. Anticonvulsant hypersensitivity syndrome: implications for pharmaceutical care. *Pharmacotherapy* **27**, 1425-1439.

Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. *The Veterinary record* **113**, 315-317.

Borst, P., Elferink, R.O., 2002. Mammalian ABC transporters in health and disease. *Annual Review of Biochemistry* **71**, 537-592.

Breedveld, P., Zelcer, N., Pluim, D., Sonmezer, O., Tibben, M.M., Beijnen, J.H., Schinkel, A.H., van Tellingen, O., Borst, P., Schellens, J.H., 2004. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug-drug interactions. *Cancer research* **64**, 5804-5811.

Breedveld, P., Pluim, D., Cipriani, G., Wielinga, P., van Tellingen, O., Schinkel, A.H., Schellens, J.H., 2005. The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer research* **65**, 2577-2582.

Calignano, A., La Rana, G., Giuffrida, A., Piomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. *Nature* **394**, 277-281.

Cerveny, L., Pavek, P., Malakova, J., Staud, F., Fendrich, Z., 2006. Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. *Epilepsia* **47**, 461-468.

Chen, C.J., Clark, D., Ueda, K., Pastan, I., Gottesman, M.M., Roninson, I.B., 1990. Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. *The Journal of biological chemistry* **265**, 506-514.

Cisternino, S., Mercier, C., Bourasset, F., Roux, F., Scherrmann, J.M., 2004. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer research* **64**, 3296-3301.

Clapper, J.R., Moreno-Sanz, G., Russo, R., Guijarro, A., Vacondio, F., Duranti, A., Tontini, A., Sanchini, S., Sciolino, N.R., Spradley, J.M. *and others*, 2010. Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature neuroscience* **13**, 1265-1270.

Clay, A.T., Sharom, F.J., 2013. Lipid bilayer properties control membrane partitioning, binding, and transport of p-glycoprotein substrates. *Biochemistry* **52**, 343-354.

Cohen-Zinder, M., Seroussi, E., Larkin, D.M., Loor, J.J., Everts-van der Wind, A., Lee, J.H., Drackley, J.K., Band, M.R., Hernandez, A.G., Shani, M. *and others*, 2005. Identification of a missense mutation in the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yield and composition in Holstein cattle. *Genome research* **15**, 936-944.

Cooray, H.C., Blackmore, C.G., Maskell, L., Barrand, M.A., 2002. Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13**, 2059-2063.

Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R., Bertino, J.R., 1989. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 695-698.

Cui, Y.J., Cheng, X., Weaver, Y.M., Klaassen, C.D., 2009. Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 203-210.

Daood, M., Tsai, C., Ahdab-Barmada, M., Watchko, J.F., 2008. ABC transporter (P-gp/ABCB1, MRP1/ABCC1, BCRP/ABCG2) expression in the developing human CNS. *Neuropediatrics* **39**, 211-218.

de Vries, N.A., Zhao, J., Kroon, E., Buckle, T., Beijnen, J.H., van Tellingen, O., 2007. P-glycoprotein and breast cancer resistance protein: two dominant

transporters working together in limiting the brain penetration of topotecan. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 6440-6449.

Desai, P.V., Sawada, G.A., Watson, I.A., Raub, T.J., 2013. Integration of in silico and *in vitro* tools for scaffold optimization during drug discovery: predicting P-glycoprotein efflux. *Molecular pharmaceutics* **10**, 1249-1261.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Piomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**, 686-691.

Diop, N.K., Hrycyna, C.A., 2005. N-Linked glycosylation of the human ABC transporter ABCG2 on asparagine 596 is not essential for expression, transport activity, or trafficking to the plasma membrane. *Biochemistry* **44**, 5420-5429.

Doige, C.A., Sharom, F.J., 1992. Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells. *Biochimica et biophysica acta* **1109**, 161-171.

Dupuy, J., Alvinerie, M., Menez, C., Lespine, A., 2010. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. *Chemico-biological interactions* **186**, 280-286.

Durmus, S., Xu, N., Sparidans, R.W., Wagenaar, E., Beijnen, J.H., Schinkel, A.H., 2013. P-glycoprotein (MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) restrict brain accumulation of the JAK1/2 inhibitor, CYT387. *Pharmacological research : the official journal of the Italian Pharmacological Society* **76C**, 9-16.

Dziadulewicz, E.K., Bevan, S.J., Brain, C.T., Coote, P.R., Culshaw, A.J., Davis, A.J., Edwards, L.J., Fisher, A.J., Fox, A.J., Gentry, C. *and others*, 2007. Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone: a potent, orally bioavailable human CB1/CB2 dual agonist with antihyperalgesic properties and restricted central nervous system penetration. *Journal of medicinal chemistry* **50**, 3851-3856.

Eisenblatter, T., Galla, H.J., 2002. A new multidrug resistance protein at the blood-brain barrier. *Biochemical and biophysical research communications* **293**, 1273-1278.

Eisenblatter, T., Huwel, S., Galla, H.J., 2003. Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the blood-brain barrier. *Brain research* **971**, 221-231.

El-Tantawy, W.H., Salem, H.F., Mohammed Safwat, N.A., 2007. Effect of Fascioliasis on the pharmacokinetic parameters of triclabendazole in human subjects. *Pharmacy world & science : PWS* **29**, 190-198.

Enokizono, J., Kusuhara, H., Sugiyama, Y., 2007. Regional expression and activity of breast cancer resistance protein (Bcrp/Abcg2) in mouse intestine: overlapping distribution with sulfotransferases. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 922-928.

Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. *Veterinary journal (London, England : 1997)* **158**, 81-112.

Fairweather, I., 2009. Triclabendazole progress report, 2005-2009: an advancement of learning? *Journal of helminthology* **83**, 139-150.

Fatima, S., Zhou, S., Sorrentino, B.P., 2012. Abcg2 expression marks tissue-specific stem cells in multiple organs in a mouse progeny tracking model. *Stem cells (Dayton, Ohio)* **30**, 210-221.

Fetsch, P.A., Abati, A., Litman, T., Morisaki, K., Honjo, Y., Mittal, K., Bates, S.E., 2006. Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues. *Cancer letters* **235**, 84-92.

Fojo, A., Lebo, R., Shimizu, N., Chin, J.E., Roninson, I.B., Merlino, G.T., Gottesman, M.M., Pastan, I., 1986. Localization of multidrug resistance-associated DNA sequences to human chromosome 7. *Somatic cell and molecular genetics* **12**, 415-420.

Fung, K.L., Gottesman, M.M., 2009. A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function. *Biochimica et biophysica acta* **1794**, 860-871.

Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V. *and others*, 2010. International transporter consortium. Membrane transporters in drug development. *Nature reviews.Drug discovery* **9**, 215-236.

Giacomini, K.M., Balimane, P.V., Cho, S.K., Eadon, M., Edeki, T., Hillgren, K.M., Huang, S.M., Sugiyama, Y., Weitz, D., Wen, Y. *and others*, 2013. International transporter consortium commentary on clinically important transporter polymorphisms. *Clinical pharmacology and therapeutics* **94**, 23-26.

Gillet, J.P., Efferth, T., Remacle, J., 2007. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochimica et biophysica acta* **1775**, 237-262.

Giri, N., Shaik, N., Pan, G., Terasaki, T., Mukai, C., Kitagaki, S., Miyakoshi, N., Elmquist, W.F., 2008. Investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) on pharmacokinetics and central nervous system penetration of abacavir and zidovudine in the mouse. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 1476-1484.

Glavinas, H., Krajcsi, P., Cserepes, J., Sarkadi, B., 2004. The role of ABC transporters in drug resistance, metabolism and toxicity. *Current drug delivery* **1**, 27-42.

González-Lobato, L. Diferencias específicas y polimórficas en la actividad y modulación a través de inhibidores del transportador ABCG2/BCRP. *Tesis Doctoral,* **2012**. Universidad de León

Greenberger, P.A., 2006. 8. Drug allergy. *The Journal of allergy and clinical immunology* **117**, S464-70.

Gribar, J.J., Ramachandra, M., Hrycyna, C.A., Dey, S., Ambudkar, S.V., 2000. Functional characterization of glycosylation-deficient human P-glycoprotein using a vaccinia virus expression system. *The Journal of membrane biology* **173**, 203-214.

Habayeb, O.M., Taylor, A.H., Bell, S.C., Taylor, D.J., Konje, J.C., 2008. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. *Endocrinology* **149**, 5052-5060.

Halferty, L., Brennan, G.P., Trudgett, A., Hoey, L., Fairweather, I., 2009. Relative activity of triclabendazole metabolites against the liver fluke, Fasciola hepatica. *Veterinary parasitology* **159**, 126-138.

Hartz, A.M., Bauer, B., 2011. ABC transporters in the CNS - an inventory. *Current Pharmaceutical Biotechnology* **12**, 656-673.

Helliwell, R.J., Chamley, L.W., Blake-Palmer, K., Mitchell, M.D., Wu, J., Kearn, C.S., Glass, M., 2004. Characterization of the endocannabinoid system in early human pregnancy. *The Journal of clinical endocrinology and metabolism* **89**, 5168-5174.

Higgins, C.F., 1992. ABC transporters: from microorganisms to man. *Annual Review of Cell Biology* **8**, 67-113.

Huls, M., Brown, C.D., Windass, A.S., Sayer, R., van den Heuvel, J.J., Heemskerk, S., Russel, F.G., Masereeuw, R., 2008. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney international* **73**, 220-225.

Ieiri, I., 2012. Functional significance of genetic polymorphisms in P-glycoprotein (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2). *Drug metabolism and pharmacokinetics* **27**, 85-105.

Iida, A., Saito, S., Sekine, A., Mishima, C., Kitamura, Y., Kondo, K., Harigae, S., Osawa, S., Nakamura, Y., 2002. Catalog of 605 single-nucleotide polymorphisms (SNPs) among 13 genes encoding human ATP-binding cassette transporters: ABCA4, ABCA7, ABCA8, ABCD1, ABCD3, ABCD4, ABCE1, ABCF1, ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8. *Journal of human genetics* **47**, 285-310.

Imai, Y., Asada, S., Tsukahara, S., Ishikawa, E., Tsuruo, T., Sugimoto, Y., 2003. Breast cancer resistance protein exports sulfated estrogens but not free estrogens. *Molecular pharmacology* **64**, 610-618.

Imperiale, F.A., Busetti, M.R., Suarez, V.H., Lanusse, C.E., 2004. Milk excretion of ivermectin and moxidectin in dairy sheep: assessment of drug residues during cheese elaboration and ripening period. *Journal of Agricultural and Food Chemistry* **52**, 6205-6211.

Imperiale, F., Ortiz, P., Cabrera, M., Farias, C., Sallovitz, J.M., Iezzi, S., Perez, J., Alvarez, L., Lanusse, C., 2011. Residual concentrations of the flukicidal compound triclabendazole in dairy cows' milk and cheese. *Food additives & contaminants.Part A, Chemistry, analysis, control, exposure & risk assessment* **28**, 438-445.

Ishikawa, T., Nakagawa, H., Hagiya, Y., Nonoguchi, N., Miyatake, S., Kuroiwa, T., 2010. Key Role of Human ABC Transporter ABCG2 in Photodynamic Therapy and Photodynamic Diagnosis. *Advances in pharmacological sciences* **2010**, 587306.

Jaggar, S.I., Sellaturay, S., Rice, A.S., 1998. The endogenous cannabinoid anandamide, but not the CB2 ligand palmitoylethanolamide, prevents the viscero-visceral hyper-reflexia associated with inflammation of the rat urinary bladder. *Neuroscience letters* **253**, 123-126.

Jonker, J.W., Smit, J.W., Brinkhuis, R.F., Maliepaard, M., Beijnen, J.H., Schellens, J.H., Schinkel, A.H., 2000. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *Journal of the National Cancer Institute* **92**, 1651-1656.

Jonker, J.W., Buitelaar, M., Wagenaar, E., Van Der Valk, M.A., Scheffer, G.L., Scheper, R.J., Plosch, T., Kuipers, F., Elferink, R.P., Rosing, H. *and others*, 2002. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15649-15654.

Jonker, J.W., Merino, G., Musters, S., van Herwaarden, A.E., Bolscher, E., Wagenaar, E., Mesman, E., Dale, T.C., Schinkel, A.H., 2005. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nature medicine* **11**, 127-129.

Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et biophysica acta* **455**, 152-162.

Kage, K., Fujita, T., Sugimoto, Y., 2005. Role of Cys-603 in dimer/oligomer formation of the breast cancer resistance protein BCRP/ABCG2. *Cancer science* **96**, 866-872.

Kajiji, S., Talbot, F., Grizzuti, K., Van Dyke-Phillips, V., Agresti, M., Safa, A.R., Gros, P., 1993. Functional analysis of P-glycoprotein mutants identifies predicted transmembrane domain 11 as a putative drug binding site. *Biochemistry* **32**, 4185-4194.

Kalabis, G.M., Petropoulos, S., Gibb, W., Matthews, S.G., 2007. Breast cancer resistance protein (Bcrp1/Abcg2) in mouse placenta and yolk sac: ontogeny and its regulation by progesterone. *Placenta* **28**, 1073-1081.

Kamiie, J., Ohtsuki, S., Iwase, R., Ohmine, K., Katsukura, Y., Yanai, K., Sekine, Y., Uchida, Y., Ito, S., Terasaki, T., 2008. Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharmaceutical research* **25**, 1469-1483.

Kerr, K.M., Sauna, Z.E., Ambudkar, S.V., 2001. Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in Human P-glycoprotein. Evidence for ADP release as the rate-limiting step in the catalytic cycle and its modulation by substrates. *The Journal of biological chemistry* **276**, 8657-8664.

Kim, R.B., Fromm, M.F., Wandel, C., Leake, B., Wood, A.J., Roden, D.M., Wilkinson, G.R., 1998. The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *The Journal of clinical investigation* **101**, 289-294.

Klaassen, C.D., Aleksunes, L.M., 2010. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacological reviews* **62**, 1-96.

Kobayashi, D., Ieiri, I., Hirota, T., Takane, H., Maegawa, S., Kigawa, J., Suzuki, H., Nanba, E., Oshimura, M., Terakawa, N. *and others*, 2005. Functional assessment of ABCG2 (BCRP) gene polymorphisms to protein expression in human placenta. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 94-101.

Kodaira, H., Kusuhara, H., Ushiki, J., Fuse, E., Sugiyama, Y., 2010. Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *The Journal of pharmacology and experimental therapeutics* **333**, 788-796.

Krishnamurthy, P., Ross, D.D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K.E., Sarkadi, B., Sorrentino, B.P., Schuetz, J.D., 2004. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *The Journal of biological chemistry* **279**, 24218-24225.

Krishnamurthy, P., Schuetz, J.D., 2006. Role of ABCG2/BCRP in biology and medicine. *Annual Review of Pharmacology and Toxicology* **46**, 381-410.

Kruijtzer, C.M., Beijnen, J.H., Rosing, H., ten Bokkel Huinink, W.W., Schot, M., Jewell, R.C., Paul, E.M., Schellens, J.H., 2002. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **20**, 2943-2950.

Kunos, G., Osei-Hyiaman, D., Batkai, S., Sharkey, K.A., Makriyannis, A., 2009. Should peripheral CB(1) cannabinoid receptors be selectively targeted for therapeutic gain? *Trends in pharmacological sciences* **30**, 1-7.

Lagas, J.S., van der Kruijssen, C.M., van de Wetering, K., Beijnen, J.H., Schinkel, A.H., 2009. Transport of diclofenac by breast cancer resistance protein (ABCG2) and stimulation of multidrug resistance protein 2 (ABCC2)-mediated drug transport by diclofenac and benzbromarone. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 129-136.

Lagas, J.S., van Waterschoot, R.A., Sparidans, R.W., Wagenaar, E., Beijnen, J.H., Schinkel, A.H., 2010. Breast cancer resistance protein and P-glycoprotein limit sorafenib brain accumulation. *Molecular cancer therapeutics* **9**, 319-326.

Lage, H., Dietel, M., 2000. Effect of the breast-cancer resistance protein on atypical multidrug resistance. *The lancet oncology* **1**, 169-175.

Lankas, G.R., Cartwright, M.E., Umbenhauer, D., 1997. P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and applied pharmacology* **143**, 357-365.

Lazarowski, A., Czornyj, L., Lubienieki, F., Girardi, E., Vazquez, S., D'Giano, C., 2007. ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. *Epilepsia* **48** Suppl **5**, 140-149.

Lifschitz, A., Virkel, G., Ballent, M., Sallovitz, J., Lanusse, C., 2009. Combined use of ivermectin and triclabendazole in sheep: *in vitro* and *in vivo* characterisation of their pharmacological interaction. *Veterinary journal (London, England : 1997)* **182**, 261-268.

Loo, T.W., Clarke, D.M., 1994. Mutations to amino acids located in predicted transmembrane segment 6 (TM6) modulate the activity and substrate specificity of human P-glycoprotein. *Biochemistry* **33**, 14049-14057.

MacFarland, A., Abramovich, D.R., Ewen, S.W., Pearson, C.K., 1994. Stage-specific distribution of P-glycoprotein in first-trimester and full-term human placenta. *The Histochemical journal* **26**, 417-423.

Maliepaard, M., Scheffer, G.L., Faneyte, I.F., van Gastelen, M.A., Pijnenborg, A.C., Schinkel, A.H., van De Vijver, M.J., Scheper, R.J., Schellens, J.H., 2001. Subcellular localization and distribution of the breast

cancer resistance protein transporter in normal human tissues. *Cancer research* **61**, 3458-3464.

Mao, Q., Unadkat, J.D., 2005. Role of the breast cancer resistance protein (ABCG2) in drug transport. *The AAPS journal* **7**, E118-33.

Marchetti, S., Mazzanti, R., Beijnen, J.H., Schellens, J.H., 2007. Concise review: Clinical relevance of drug drug and herb drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). *The oncologist* **12**, 927-941.

Marchetti, S., de Vries, N.A., Buckle, T., Bolijn, M.J., van Eijndhoven, M.A., Beijnen, J.H., Mazzanti, R., van Tellingen, O., Schellens, J.H., 2008. Effect of the ATP-binding cassette drug transporters ABCB1, ABCG2, and ABCC2 on erlotinib hydrochloride (Tarceva) disposition in *in vitro* and *in vivo* pharmacokinetic studies employing Bcrp1-/-/Mdr1a/1b-/- (triple-knockout) and wild-type mice. *Molecular cancer therapeutics* **7**, 2280-2287.

Martin, C., Berridge, G., Higgins, C.F., Mistry, P., Charlton, P., Callaghan, R., 2000. Communication between multiple drug binding sites on P-glycoprotein. *Molecular pharmacology* **58**, 624-632.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. *International journal for parasitology* **35**, 1255-1278.

Mayer, U., Wagenaar, E., Beijnen, J.H., Smit, J.W., Meijer, D.K., van Asperen, J., Borst, P., Schinkel, A.H., 1996. Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British journal of pharmacology* **119**, 1038-1044.

McManaman, J.L., Neville, M.C., 2003. Mammary physiology and milk secretion. *Advanced Drug Delivery Reviews* **55**, 629-641.

Mealey, K.L., 2012. ABCG2 transporter: therapeutic and physiologic implications in veterinary species. *Journal of veterinary pharmacology and therapeutics* **35**, 105-112.

Mechoulam, R., Parker, L.A., 2013. The endocannabinoid system and the brain. *Annual Review of Psychology* **64**, 21-47.

Merino, G., Jonker, J.W., Wagenaar, E., Pulido, M.M., Molina, A.J., Alvarez, A.I., Schinkel, A.H., 2005a. Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). *Drug metabolism and disposition: the biological fate of chemicals* **33**, 614-618.

Merino, G., Jonker, J.W., Wagenaar, E., van Herwaarden, A.E., Schinkel, A.H., 2005b. The breast cancer resistance protein (BCRP/ABCG2) affects

pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. *Molecular pharmacology* **67**, 1758-1764.

Merino, G., Alvarez, A.I., Pulido, M.M., Molina, A.J., Schinkel, A.H., Prieto, J.G., 2006. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 690-695.

Merino, G., Real, R., Baro, M.F., Gonzalez-Lobato, L., Prieto, J.G., Alvarez, A.I., Marques, M.M., 2009. Natural allelic variants of bovine ATP-binding cassette transporter ABCG2: increased activity of the Ser581 variant and development of tools for the discovery of new ABCG2 inhibitors. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 5-9.

Merino, G., Perez, M., Real, R., Egido, E., Prieto, J.G., Alvarez, A.I., 2010. *In vivo* inhibition of BCRP/ABCG2 mediated transport of nitrofurantoin by the isoflavones genistein and daidzein: a comparative study in Bcrp1 (-/-) mice. *Pharmaceutical research* **27**, 2098-2105.

Mestorino, N., Formentini, E.A., Lucas, M.F., Fernandez, C., Modamio, P., Hernandez, E.M., Errecalde, J.O., 2008. Pharmacokinetic disposition of triclabendazole in cattle and sheep; discrimination of the order and the rate of the absorption process of its active metabolite triclabendazole sulfoxide. *Veterinary research communications* **32**, 21-33.

Misra SC, Swain G, Panda MR, Panda DN & Das Mohapatra NB, 1987. Efficacy of Fasinex (Ciba-Geigy) against fasciolasis in cattle, buffaloes and goats. *Indian Vet J* **64,** 701-704.

Mittapalli, R.K., Vaidhyanathan, S., Sane, R., Elmquist, W.F., 2012. Impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on the brain distribution of a novel BRAF inhibitor: vemurafenib (PLX4032). *The Journal of pharmacology and experimental therapeutics* **342**, 33-40.

Mizuno, N., Suzuki, M., Kusuhara, H., Suzuki, H., Takeuchi, K., Niwa, T., Jonker, J.W., Sugiyama, Y., 2004. Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 898-901.

Mizuno, N., Takahashi, T., Kusuhara, H., Schuetz, J.D., Niwa, T., Sugiyama, Y., 2007. Evaluation of the role of breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance-associated protein 4 (MRP4/ABCC4) in the urinary excretion of sulfate and glucuronide metabolites of edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one). *Drug metabolism and disposition: the biological fate of chemicals* **35**, 2045-2052.

Moreno-Sanz, G., Sasso, O., Guijarro, A., Oluyemi, O., Bertorelli, R., Reggiani, A., Piomelli, D., 2012. Pharmacological characterization of the peripheral FAAH inhibitor URB937 in female rodents: interaction with the Abcg2 transporter in the blood-placenta barrier. *British journal of pharmacology* **167**, 1620-1628.

Moreno-Sanz, G., Duranti, A., Melzig, L., Fiorelli, C., Ruda, G.F., Colombano, G., Mestichelli, P., Sanchini, S., Tontini, A., Mor, M. ***and others,*** 2013. Synthesis and Structure-Activity Relationship Studies of O-Biphenyl-3-yl Carbamates as Peripherally Restricted Fatty Acid Amide Hydrolase Inhibitors. *Journal of medicinal chemistry*.

Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J., Lanusse, C., 2004. Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into Fasciola hepatica. *Xenobiotica; the fate of foreign compounds in biological systems* **34**, 1043-1057.

Mukherjee, S., Koner, B.C., Ray, S., Ray, A., 2006. Environmental contaminants in pathogenesis of breast cancer. *Indian journal of experimental biology* **44**, 597-617.

Myllynen, P., Kummu, M., Kangas, T., Ilves, M., Immonen, E., Rysa, J., Pirila, R., Lastumaki, A., Vahakangas, K.H., 2008. ABCG2/BCRP decreases the transfer of a food-born chemical carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in perfused term human placenta. *Toxicology and applied pharmacology* **232**, 210-217.

Nackley, A.G., Suplita, R.L.,2nd, Hohmann, A.G., 2003. A peripheral cannabinoid mechanism suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. *Neuroscience* **117**, 659-670.

Nakagawa, H., Saito, H., Ikegami, Y., Aida-Hyugaji, S., Sawada, S., Ishikawa, T., 2006. Molecular modeling of new camptothecin analogues to circumvent ABCG2-mediated drug resistance in cancer. *Cancer letters* **234**, 81-89.

Nakamura, Y., Ikeda, S., Furukawa, T., Sumizawa, T., Tani, A., Akiyama, S., Nagata, Y., 1997. Function of P-glycoprotein expressed in placenta and mole. *Biochemical and biophysical research communications* **235**, 849-853.

Nakanishi, T., Doyle, L.A., Hassel, B., Wei, Y., Bauer, K.S., Wu, S., Pumplin, D.W., Fang, H.B., Ross, D.D., 2003. Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of Xenopus laevis. *Molecular pharmacology* **64**, 1452-1462.

Nakanishi, H., Yonezawa, A., Matsubara, K., Yano, I., 2013. Impact of P-glycoprotein and breast cancer resistance protein on the brain distribution of antiepileptic drugs in knockout mouse models. *European journal of pharmacology* **710**, 20-28.

Ni, Z., Bikadi, Z., Rosenberg, M.F., Mao, Q., 2010. Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Current Drug Metabolism* **11**, 603-617.

Nicolle, E., Boumendjel, A., Macalou, S., Genoux, E., Ahmed-Belkacem, A., Carrupt, P.A., Di Pietro, A., 2009. QSAR analysis and molecular modeling of ABCG2-specific inhibitors. *Advanced Drug Delivery Reviews* **61**, 34-46.

Nishimura, M., Naito, S., 2005. Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. *Drug metabolism and pharmacokinetics* **20**, 452-477.

Noguchi, K., Katayama, K., Mitsuhashi, J., Sugimoto, Y., 2009. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. *Advanced Drug Delivery Reviews* **61**, 26-33.

Oostendorp, R.L., Buckle, T., Beijnen, J.H., van Tellingen, O., Schellens, J.H., 2009. The effect of P-gp (Mdr1a/1b), BCRP (Bcrp1) and P-gp/BCRP inhibitors on the *in vivo* absorption, distribution, metabolism and excretion of imatinib. *Investigational new drugs* **27**, 31-40.

Osei-Hyiaman, D., Liu, J., Zhou, L., Godlewski, G., Harvey-White, J., Jeong, W.I., Batkai, S., Marsicano, G., Lutz, B., Buettner, C. *and others*, 2008. Hepatic CB1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. *The Journal of clinical investigation* **118**, 3160-3169.

Osman, M.M., Shehab, A.Y., Zaki, A., Farag, H.F., 2011. Evaluation of two doses of triclabendazole in treatment of patients with combined schistosomiasis and fascioliasis. *Eastern Mediterranean health journal = La revue de sante de la Mediterranee orientale = al-Majallah al-sihhiyah li-sharq al-mutawassit* **17**, 266-270.

Otero, J.A., Real, R., de la Fuente, A., Prieto, J.G., Marques, M., Alvarez, A.I., Merino, G., 2013. The bovine ATP-binding cassette transporter ABCG2 Tyr581Ser single-nucleotide polymorphism increases milk secretion of the fluoroquinolone danofloxacin. *Drug metabolism and disposition: the biological fate of chemicals* **41**, 546-549.

Pan, G., Giri, N., Elmquist, W.F., 2007. Abcg2/Bcrp1 mediates the polarized transport of antiretroviral nucleosides abacavir and zidovudine. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 1165-1173.

Paterson, J.K., Gottesman, M.M., 2007. P-Glycoprotein is not present in mitochondrial membranes. *Experimental cell research* **313**, 3100-3105.

Perez, M., Blazquez, A.G., Real, R., Mendoza, G., Prieto, J.G., Merino, G., Alvarez, A.I., 2009a. *In vitro* and *in vivo* interaction of moxidectin with BCRP/ABCG2. *Chemico-biological interactions* **180**, 106-112.

Perez, M., Real, R., Mendoza, G., Merino, G., Prieto, J.G., Alvarez, A.I., 2009b. Milk secretion of nitrofurantoin, as a specific BCRP/ABCG2 substrate, in assaf sheep: modulation by isoflavones. *Journal of veterinary pharmacology and therapeutics* **32**, 498-502.

Perez, M., Otero, J.A., Barrera, B., Prieto, J.G., Merino, G., Alvarez, A.I., 2013. Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein: Effect on plasma and milk levels of danofloxacin in sheep. *Veterinary journal (London, England : 1997)* **196**, 203-208.

Pick, A., Muller, H., Wiese, M., 2008. Structure-activity relationships of new inhibitors of breast cancer resistance protein (ABCG2). *Bioorganic & medicinal chemistry* **16**, 8224-8236.

Pick, A., Muller, H., Mayer, R., Haenisch, B., Pajeva, I.K., Weigt, M., Bonisch, H., Muller, C.E., Wiese, M., 2011. Structure-activity relationships of flavonoids as inhibitors of breast cancer resistance protein (BCRP). *Bioorganic & medicinal chemistry* **19**, 2090-2102.

Piomelli, D., Tarzia, G., Duranti, A., Tontini, A., Mor, M., Compton, T.R., Dasse, O., Monaghan, E.P., Parrott, J.A., Putman, D., 2006. Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS drug reviews* **12**, 21-38.

Polgar, O., Robey, R.W., Bates, S.E., 2008. ABCG2: structure, function and role in drug response. *Expert opinion on drug metabolism & toxicology* **4**, 1-15.

Poller, B., Iusuf, D., Sparidans, R.W., Wagenaar, E., Beijnen, J.H., Schinkel, A.H., 2011. Differential impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on axitinib brain accumulation and oral plasma pharmacokinetics. *Drug metabolism and disposition: the biological fate of chemicals* **39**, 729-735.

Pollex, E., Lubetsky, A., Koren, G., 2008. The role of placental breast cancer resistance protein in the efflux of glyburide across the human placenta. *Placenta* **29**, 743-747.

Polli, J.W., Humphreys, J.E., Harmon, K.A., Castellino, S., O'Mara, M.J., Olson, K.L., John-Williams, L.S., Koch, K.M., Serabjit-Singh, C.J., 2008. The role of efflux and uptake transporters in [N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino }methyl)-2-furyl]-4-quinazolinamine (GW572016, lapatinib) disposition and drug interactions. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 695-701.

Pulido, M.M., Molina, A.J., Merino, G., Mendoza, G., Prieto, J.G., Alvarez, A.I., 2006. Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ABCG2): influence of flavonoids and role in milk secretion in sheep. *Journal of veterinary pharmacology and therapeutics* **29**, 279-287.

Rabindran, S.K., Ross, D.D., Doyle, L.A., Yang, W., Greenberger, L.M., 2000. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer research* **60**, 47-50.

Real, R., Egido, E., Perez, M., Gonzalez-Lobato, L., Barrera, B., Prieto, J.G., Alvarez, A.I., Merino, G., 2011. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: interaction with ivermectin. *Journal of veterinary pharmacology and therapeutics* **34**, 313-321.

Reid, T., Yuen, A., Catolico, M., Carlson, R.W., 1993. Impact of omeprazole on the plasma clearance of methotrexate. *Cancer chemotherapy and pharmacology* **33**, 82-84.

Robey, R.W., Steadman, K., Polgar, O., Morisaki, K., Blayney, M., Mistry, P., Bates, S.E., 2004. Pheophorbide a is a specific probe for ABCG2 function and inhibition. *Cancer research* **64**, 1242-1246.

Robey, R.W., To, K.K., Polgar, O., Dohse, M., Fetsch, P., Dean, M., Bates, S.E., 2009. ABCG2: a perspective. *Advanced Drug Delivery Reviews* **61**, 3-13.

Santucci, R., Leveque, D., Kemmel, V., Lutz, P., Gerout, A.C., N'guyen, A., Lescoute, A., Schneider, F., Bergerat, J.P., Herbrecht, R., 2010. Severe intoxication with methotrexate possibly associated with concomitant use of proton pump inhibitors. *Anticancer Research* **30**, 963-965.

Sasso, O., Bertorelli, R., Bandiera, T., Scarpelli, R., Colombano, G., Armirotti, A., Moreno-Sanz, G., Reggiani, A., Piomelli, D., 2012. Peripheral FAAH inhibition causes profound antinociception and protects against indomethacin-induced gastric lesions. *Pharmacological research : the official journal of the Italian Pharmacological Society* **65**, 553-563.

Scharenberg, C.W., Harkey, M.A., Torok-Storb, B., 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* **99**, 507-512.

Schinkel, A.H., Kemp, S., Dolle, M., Rudenko, G., Wagenaar, E., 1993. N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein. *The Journal of biological chemistry* **268**, 7474-7481.

Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J.H., Wagenaar, E., van Deemter, L., Mol, C.A., van der Valk, M.A., Robanus-Maandag, E.C., te Riele, H.P., 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.

Schinkel, A.H., Wagenaar, E., Mol, C.A., van Deemter, L., 1996. P-glycoprotein in the blood-brain barrier of mice influences the brain

penetration and pharmacological activity of many drugs. *The Journal of clinical investigation* **97**, 2517-2524.

Schinkel, A.H., 1997. The physiological function of drug-transporting P-glycoproteins. *Seminars in cancer biology* **8**, 161-170.

Schnepf, R., Zolk, O., 2013. Effect of the ATP-binding cassette transporter ABCG2 on pharmacokinetics: experimental findings and clinical implications. *Expert opinion on drug metabolism & toxicology* **9**, 287-306.

Sesink, A.L., Arts, I.C., de Boer, V.C., Breedveld, P., Schellens, J.H., Hollman, P.C., Russel, F.G., 2005. Breast cancer resistance protein (Bcrp1/Abcg2) limits net intestinal uptake of quercetin in rats by facilitating apical efflux of glucuronides. *Molecular pharmacology* **67**, 1999-2006.

Shukla, S., Zaher, H., Hartz, A., Bauer, B., Ware, J.A., Ambudkar, S.V., 2009. Curcumin inhibits the activity of ABCG2/BCRP1, a multidrug resistance-linked ABC drug transporter in mice. *Pharmaceutical research* **26**, 480-487.

Sisodiya, S.M., Martinian, L., Scheffer, G.L., van der Valk, P., Scheper, R.J., Harding, B.N., Thom, M., 2006. Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathology and applied neurobiology* **32**, 51-63.

Staud, F., Pavek, P., 2005. Breast cancer resistance protein (BCRP/ABCG2). *The international journal of biochemistry & cell biology* **37**, 720-725.

Stein, C., Schafer, M., Machelska, H., 2003. Attacking pain at its source: new perspectives on opioids. *Nature medicine* **9**, 1003-1008.

Stein, C., Zollner, C., 2009. Opioids and sensory nerves. *Handbook of Experimental Pharmacology* **194**, 495-518.

Stella, N., Schweitzer, P., Piomelli, D., 1997. A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388**, 773-778.

Suzuki, M., Suzuki, H., Sugimoto, Y., Sugiyama, Y., 2003. ABCG2 transports sulfated conjugates of steroids and xenobiotics. *The Journal of biological chemistry* **278**, 22644-22649.

Tamaki, A., Ierano, C., Szakacs, G., Robey, R.W., Bates, S.E., 2011. The controversial role of ABC transporters in clinical oncology. *Essays in biochemistry* **50**, 209-232.

Tang, S.C., Lagas, J.S., Lankheet, N.A., Poller, B., Hillebrand, M.J., Rosing, H., Beijnen, J.H., Schinkel, A.H., 2012. Brain accumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib

coadministration. *International journal of cancer.Journal international du cancer* **130**, 223-233.

Thyss, A., Milano, G., Kubar, J., Namer, M., Schneider, M., 1986. Clinical and pharmacokinetic evidence of a life-threatening interaction between methotrexate and ketoprofen. *Lancet* **1**, 256-258.

Troger, U., Stotzel, B., Martens-Lobenhoffer, J., Gollnick, H., Meyer, F.P., 2002. Drug points: Severe myalgia from an interaction between treatments with pantoprazole and methotrexate. *BMJ (Clinical research ed.)* **324**, 1497.

Uchida, Y., Ohtsuki, S., Katsukura, Y., Ikeda, C., Suzuki, T., Kamiie, J., Terasaki, T., 2011. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *Journal of neurochemistry* **117**, 333-345.

Urquhart, B.L., Tirona, R.G., Kim, R.B., 2007. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *Journal of clinical pharmacology* **47**, 566-578.

van Herwaarden, A.E., Jonker, J.W., Wagenaar, E., Brinkhuis, R.F., Schellens, J.H., Beijnen, J.H., Schinkel, A.H., 2003. The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Cancer research* **63**, 6447-6452.

van Herwaarden, A.E., Schinkel, A.H., 2006. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends in pharmacological sciences* **27**, 10-16.

van Herwaarden, A.E., Wagenaar, E., Karnekamp, B., Merino, G., Jonker, J.W., Schinkel, A.H., 2006. Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. *Carcinogenesis* **27**, 123-130.

van Herwaarden, A.E., Wagenaar, E., Merino, G., Jonker, J.W., Rosing, H., Beijnen, J.H., Schinkel, A.H., 2007. Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. *Molecular and cellular biology* **27**, 1247-1253.

Vander Borght, S., Libbrecht, L., Katoonizadeh, A., van Pelt, J., Cassiman, D., Nevens, F., Van Lommel, A., Petersen, B.E., Fevery, J., Jansen, P.L. and others, 2006. Breast cancer resistance protein (BCRP/ABCG2) is expressed by progenitor cells/reactive ductules and hepatocytes and its expression pattern is influenced by disease etiology and species type: possible functional consequences. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **54**, 1051-1059.

Verhasselt, V., Milcent, V., Cazareth, J., Kanda, A., Fleury, S., Dombrowicz, D., Glaichenhaus, N., Julia, V., 2008. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nature medicine* **14**, 170-175.

Villegas, F., Angles, R., Barrientos, R., Barrios, G., Valero, M.A., Hamed, K., Grueninger, H., Ault, S.K., Montresor, A., Engels, D. *and others*, 2012. Administration of triclabendazole is safe and effective in controlling fascioliasis in an endemic community of the Bolivian Altiplano. *PLoS neglected tropical diseases* **6**, e1720.

Virkel, G., Lifschitz, A., Sallovitz, J., Pis, A., Lanusse, C., 2006. Assessment of the main metabolism pathways for the flukicidal compound triclabendazole in sheep. *Journal of veterinary pharmacology and therapeutics* **29**, 213-223.

Virkel, G., Lifschitz, A., Sallovitz, J., Ballent, M., Scarcella, S., Lanusse, C., 2009. Inhibition of cytochrome P450 activity enhances the systemic availability of triclabendazole metabolites in sheep. *Journal of veterinary pharmacology and therapeutics* **32**, 79-86.

Vlaming, M.L., Lagas, J.S., Schinkel, A.H., 2009. Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice. *Advanced Drug Delivery Reviews* **61**, 14-25.

Wang, H., Matsumoto, H., Guo, Y., Paria, B.C., Roberts, R.L., Dey, S.K., 2003. Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14914-14919.

Wang, X., Nitanda, T., Shi, M., Okamoto, M., Furukawa, T., Sugimoto, Y., Akiyama, S., Baba, M., 2004. Induction of cellular resistance to nucleoside reverse transcriptase inhibitors by the wild-type breast cancer resistance protein. *Biochemical pharmacology* **68**, 1363-1370.

Weiss, J., Rose, J., Storch, C.H., Ketabi-Kiyanvash, N., Sauer, A., Haefeli, W.E., Efferth, T., 2007. Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. *The Journal of antimicrobial chemotherapy* **59**, 238-245.

Williams, J.A., Phillips, D.H., 2000. Mammary expression of xenobiotic metabolizing enzymes and their potential role in breast cancer. *Cancer research* **60**, 4667-4677.

Wolf, A., Bauer, B., Hartz, A.M., 2012. ABC Transporters and the Alzheimer's Disease Enigma. *Frontiers in psychiatry* **3**, 54.

Wolff, K., Eckert, J., Schneiter, G., Lutz, H., 1983. Efficacy of triclabendazole against Fasciola hepatica in sheep and goats. *Veterinary parasitology* **13**, 145-150.

Woodward, O.M., Kottgen, A., Coresh, J., Boerwinkle, E., Guggino, W.B., Kottgen, M., 2009. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 10338-10342.

Wu, H.J., Luo, J., Wu, N., Matand, K., Zhang, L.J., Han, X.F., Yang, B.J., 2008. Cloning, sequence and functional analysis of goat ATP-binding cassette transporter G2 (ABCG2). *Molecular biotechnology* **39**, 21-27.

Xu, J., Liu, Y., Yang, Y., Bates, S., Zhang, J.T., 2004. Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *The Journal of biological chemistry* **279**, 19781-19789.

Zaher, H., Khan, A.A., Palandra, J., Brayman, T.G., Yu, L., Ware, J.A., 2006. Breast cancer resistance protein (Bcrp/abcg2) is a major determinant of sulfasalazine absorption and elimination in the mouse. *Molecular pharmaceutics* **3**, 55-61.

Zamber, C.P., Lamba, J.K., Yasuda, K., Farnum, J., Thummel, K., Schuetz, J.D., Schuetz, E.G., 2003. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics* **13**, 19-28.

Zamek-Gliszczynski, M.J., Hoffmaster, K.A., Humphreys, J.E., Tian, X., Nezasa, K., Brouwer, K.L., 2006. Differential involvement of Mrp2 (Abcc2) and Bcrp (Abcg2) in biliary excretion of 4-methylumbelliferyl glucuronide and sulfate in the rat. *The Journal of pharmacology and experimental therapeutics* **319**, 459-467.

Zhang, X., Collins, K.I., Greenberger, L.M., 1995. Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. *The Journal of biological chemistry* **270**, 5441-5448.

Zhang, W., Mojsilovic-Petrovic, J., Andrade, M.F., Zhang, H., Ball, M., Stanimirovic, D.B., 2003. The expression and functional characterization of ABCG2 in brain endothelial cells and vessels. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 2085-2087.

Zhang, Y., Zhou, G., Wang, H., Zhang, X., Wei, F., Cai, Y., Yin, D., 2006. Transcriptional upregulation of breast cancer resistance protein by 17beta-estradiol in ERalpha-positive MCF-7 breast cancer cells. *Oncology* **71**, 446-455.

Zhang, Y., Wang, H., Unadkat, J.D., Mao, Q., 2007. Breast cancer resistance protein 1 limits fetal distribution of nitrofurantoin in the pregnant mouse. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 2154-2158.

Zhou, S., Morris, J.J., Barnes, Y., Lan, L., Schuetz, J.D., Sorrentino, B.P., 2002. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12339-12344.

Zhou, L., Naraharisetti, S.B., Wang, H., Unadkat, J.D., Hebert, M.F., Mao, Q., 2008. The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an Obstetric-Fetal Pharmacology Research Unit Network and University of Washington Specialized Center of Research Study. *Molecular pharmacology* **73**, 949-959.

Zhu, B.T., 1999. A novel hypothesis for the mechanism of action of P-glycoprotein as a multidrug transporter. *Molecular carcinogenesis* **25**, 1-13.

Zhu, W., Xu, H., Wang, S.W., Hu, M., 2010. Breast cancer resistance protein (BCRP) and sulfotransferases contribute significantly to the disposition of genistein in mouse intestine. *The AAPS journal* **12**, 525-536.

14. ANEXOS

14.1. CERTIFICADO DE REALIZAZIÓN DE UNA ESTANCIA BREVE.

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THE RUSSELL H. MORGAN DEPARTMENT OF
RADIOLOGY AND RADIOLOGICAL SCIENCE

1. Becario/ *Applicant:*
Nombre y apellidos/ *Name: Borja Barrera Cuesta*
D.N.I./ *National identity Card: 71448681D*
Centro de aplicación de la beca/ *Home Institución: Universidad de León*
2. Centro en el que se ha realizado la estancia/ *Host institution:*
Nombre/ *Name: Johns Hopkins School of Medicine*
Dirección/ *Adress: 1550 Orleans Street, 492 CRBII, ZIP 21231 Baltimore, MD (USA)*
Localidad/ *Country: Baltimore, Maryland (USA)*
3. Investigador responsable en el centro de la estancia/ *Responsable person in the Host*
Institución/ *Institution: Johns Hopkins School of Medicine*
Nombre/ *Name: Martin G. Pomper*
Cargo/ *Post: Medico y Doctor/ Medical Doctor, PhD*
CERTIFICO: que el becario arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde 06 / 09 / 2011 hasta 21 / 12/ 2011 ***THIS IS TO CERTIFY:*** *that the above mentioned person has performed a stay in this Institution in the following dates: From: 09 / 06 / 2011 To: 12 / 21 / 2011* Lugar y fecha: Baltimore, MD (USA) 21/12/2011 *City and date:* Baltimore, MD (USA) 12/21/2011

Yours sincerely,

Martin G. Pomper, M.D., Ph.D.