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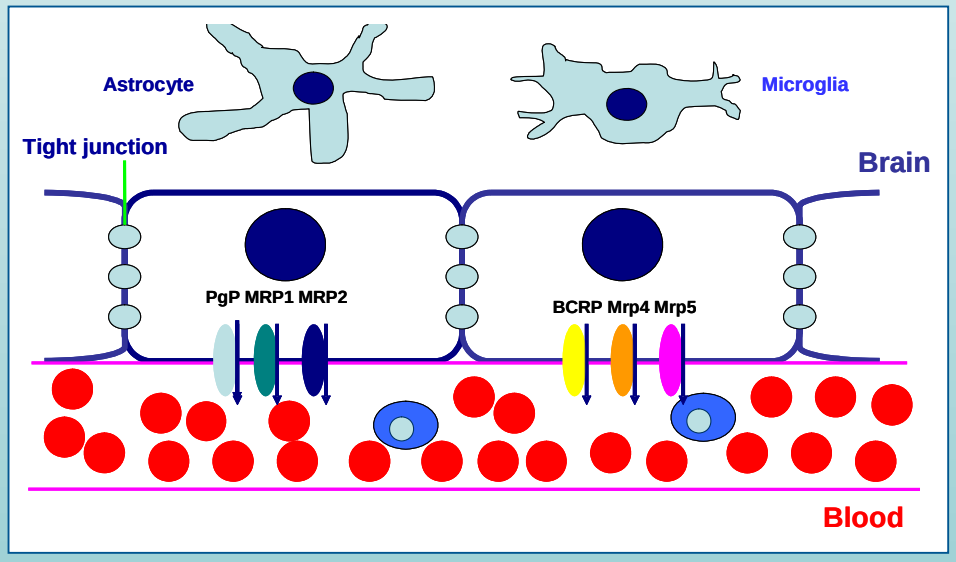

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Drug Discoveries & Therapeutics

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Editorial and Head Office

Wei TANG, MD PhD
Secretary-in-General

TSUIN-IKIZAKA 410
2-17-5 Hongo, Bunkyo-ku
Tokyo 113-0033, Japan
Tel: 03-5840-9697
Fax: 03-5840-9698
E-mail: office@ddtjournal.com
URL: www.ddtjournal.com

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Review

Role of multidrug resistance associated proteins in drug development

Shu-Feng Zhou*

School of Health Sciences, RMIT University, Bundoora, Victoria, Australia.

ABSTRACT: The multidrug resistance associated proteins (MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, MRP7, MRP8 and MRP9) belongs to the ATP-binding cassette superfamily (ABCC family) of transporters expressed differentially in the liver, kidney, intestine and blood-brain barrier. MRPs transport a structurally diverse array of endo- and xenobiotics and their metabolites (in particular conjugates) and are subject to induction and inhibition by a variety of compounds. An increased efflux of natural product anticancer drugs and other anticancer agents by MRPs in cancer cells is associated with tumor resistance. These transporting proteins play a role in the absorption, distribution and elimination of various compounds in the body. There are increased reports on the clinical impact of genetic mutations of genes encoding MRP1-9. Therefore, MRPs have an important role in drug development, and a better understanding of their function and regulating mechanism can help minimize and avoid drug toxicity, unfavourable drug-drug interactions, and to overcome drug resistance.

Keywords: MRP, Drug development, Single nucleotide polymorphism, Toxicity, Pharmacokinetics, Blood-brain barrier, Biliary excretion, Intestinal absorption, Drug transport

1. Introduction

The human body is continuously exposed to a great variety of xenobiotics *via* food, drugs, occupation and environment. Evolution has equipped the body with a plethora of protecting systems to defend itself against the potentially harmful effects of these compounds. One of the important and clinically relevant defense mechanisms include the active extrusion of xenobiotics by commonly shared transport proteins, mainly located in kidney, liver and intestine. The ATP-binding cassette (ABC) superfamily of transporters consist of a large number of functionally diverse transmembrane proteins which have been subdivided into seven families designated A through G (*1-4*). Members of this transport superfamily display high amino acid similarity of the 200 amino acids surrounding the ATP-binding folds. Approximately 1,100 ABC transporters are known at this time. Traffic ATPases and P-glycoproteins (PgPs) are other names used for this family. The family includes bacterial transporters, the cystic fibrosis transmembrane conductance regulator, the *Plasmodium falciparum* drug-resistance gene, and genes apparently involved in peptide transport during antigen presentation.

In humans, members of this family serve a variety of physiological roles in transmembrane transport and cell signalling, many of which are associated with disease phenotypes such as multidrug resistance, cystic fibrosis, Tangier disease, adrenoleukodystrophy and Zellwegers' syndrome (*1,2,4*). The available outline of the human genome contains 48 ABC genes (*5*); 16 of these have a known function and 14 are associated with a defined human disease (*6*). ABC transporters that pump cytotoxic drugs from the cell are also present in micro-organisms, and this is one of the main mechanisms by which pathogenic species can resist antibiotic treatment. The human family of ABC transporters includes at least 48 members with 7 subfamilies (*4*). They facilitate unidirectional translocation of chemically diverse substrates including amino acids, lipids, inorganic ions, peptides, saccharides, metals, drugs, and proteins. Energy derived from the hydrolysis of ATP is used to transport the substrate across the membrane against a concentration gradient (*7*). These transporters are present in almost all tissues and cell types in different amounts. A typical ABC transporter is characterized by the presence of three peptide motifs: *Walker A* and *B* sequences and the so-called ABC-signature sequence ("ALSGGQ") (*1,8*). Most ABC proteins from eukaryotes encode full transporters, consisting of two ATP-binding domains and 12 membrane-spanning regions or half transporters,

**Correspondence to:* Dr. Shu-Feng Zhou, School of Health Sciences, RMIT University, Bundoora, Victoria 3083, Australia;
e-mail: Shufeng.zhou@rmit.edu.au

which are presumed to dimerize (*9*). The MRP family contains at least nine members (MRP1-9, ABCC1-6 and ABCC10-12, respectively) with sizes from 1,325 to 1,545 amino acids. This probably completes the family, as there are no other putative MRP genes among the 52 human ABC transporter genes. ABCC7 (CFTR) is a chloride channel, and channels are not transporters. ABCC8 and 9 (SUR1 and 2), the sulfonylurea receptors, are the ATP-sensing subunits of a complex potassium channel and are not known to transport any substrates. The MRPs, CFTR, and the SURs are considered to evolve from a common ancestor, and these proteins are now grouped together in the C branch of the ABC transporter family. This paper highlights the pharmacological roles of MRPs and their implications in drug development.

2. Topology of MRPs

PgP/MDR1 consists of 1,276 to 1,280 amino acids with a molecular mass of 170 kDa. The commonly accepted model for the topologic structure of PgP has a tandemly duplicated structure, with each half of the molecule contains a nucleotide-binding domain (NBD) and reveals six predicted and highly hydrophobic transmembrane regions (*4*). The *N*- and *C*-termini, as well as the NBDs, are located intracellularly, and the first extracellular loop is *N*-glycosylated. Both NBDs are essential for proper functioning of the protein. Each consists of two core consensus motifs referred to as the *Walker A* and *B* motifs and a *S* signature of ABC transporters (*10*). These motifs generally are found in a wide range of ATPases, and they are involved directly in the binding and hydrolysis of nucleotides. Structures of bacterial ABC transporter proteins suggest that the two NBDs form a common binding site where the energy of ATP is harvested to promote efflux through a pore that is delineated by the transmembrane helice (*11*). The two half molecules are separated by a highly charged "linker region" which is phosphorylated at several sites by protein kinase C. Different topologic orientations of PgP have been reported, and several studies have indicated that conformational changes in the structure of PgP are involved in the mechanism of substrate efflux (*12*).

Like PgP, MRPs belong to ABC transporter superfamily. All MRP members have 2 hydrophobic transmembrane domains (TMD1 and TMD2) and 2 cytoplasmic NBDs (Figure 1) (*13*). The NBDs are responsible for the ATP binding/hydrolysis that drives drug transport, and their structure is conserved independently of the degree of primary-sequence homology (*14*). The TMDs contain the drug-binding sites that are likely located in a flexible internal chamber that is sufficiently large to accommodate different drugs. MRPs can be categorized according to the presence or absence of a third (NH_2-terminal) membrane-spanning domain (TMD_0) in their structure (Figure 1) (*15-17*). This topological feature can be found in MRP1, MRP2, MRP3, MRP6, and MRP7, while it is not possessed by MRP4, MRP5, MRP8, and MRP9 (*18-21*). TMD_0 is not essential for catalytic function or intracellular routing; the function of this domain is unknown (*22*). MRPs with this structural feature have the ability to transport conjugates, while MRPs without it are able to transport cyclic nucleotides. Long MRPs share an L_0 segment (Figure 1) with a highly conserved sequence near its *N* terminus. This sequence is also present near the *N* terminus of the short MRPs. It is essential for function and appears to associate with the membrane.

3. Substrate specificity, resistance profiles and inhibitor selectivity of MRPs

The first member of MRP family, MRP1 (ABCC1), was found in 1992 in lung cancer cell line conferring resistance to doxorubicin which was not related to PgP (*23*). The genes encoding MRP1 and PgP are evolutionarily very distant, and the primary structure of the two proteins is quite dissimilar, sharing only 15 percent amino acid identity (*23*). Most of the sequence similarity between MRP1 and PgP is found within the nucleotide-binding domains that generally are conserved among members of the ABC superfamily (*24*). MRP1 is larger than other full-length ABC proteins, containing approximately 250 additional amino acids in its NH_2-terminal. Thus, in addition to the 12 transmembrane segments characterizing PgP, MRP1 has five transmembrane domains. MRP1 is nearly present in all major tissues and in all peripheral blood cell types (*25,26*). The expression levels of MRP1 are different in various organs and cell lines (*27-30*). Natural product drugs such as vincristine, etoposide and doxorubicin are substrates for MRP1 (*31*). Although MRP1 and PgP have some identical substrates, they show difference in the substrate specificity. PgP can transport drugs in original form, while MRP1 can transport glutathione (GSH), oxidized GSH (GSSG), as well as a number of GSH, glucuronate and sulfate conjugates of drugs (Figure 2) (*31-33*). Additionally, MRP1 has several physiologic substrates, such as 17-β-D-estradiol-glucuronide ($E_2$17βG), the GSH-conjugated cysteinyl leukotriene C_4 (LTC_4), sulfated bile acids, prostaglandin (PG) A GSH conjugates, and unconjugated bilirubin (*34-39*). The high affinity for LTC_4 is a specific feature of MRP1, which may contribute to the distinguished role of MRP1 in immune responses associated with cellular excretion of LTC_4 (*40,41*). In contrast, PgP shows poor resistance to these conjugated organic anions (*32*). Moreover, significant species difference in the substrate specificity of MRP1 has been noted.

Substrates of MRP1 also include neutral and basic cytotoxic compounds without conjugation with GSH or other anionic drugs (*42,43*). However, intracellular GSH is needed when MRP1 transports these chemicals (*44,45*). GSH concentrations increase in some organs of *mrp*

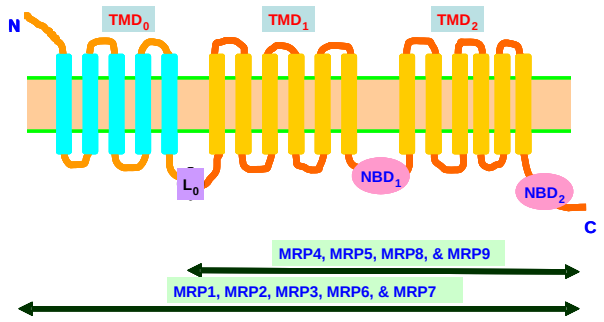

Figure 1. Predicted topological structure of MRP1-9 (ABCC1-6 and 10-12). All MRP members have two hydrophobic transmembrane domains (TMD1 and TMD2) and two cytoplasmic nucleotide binding domains (NBDs) responsible for the ATP binding and hydrolysis that drives drug transport. MRPs can be categorized according to the presence or absence of a third (NH_2-terminal) membrane-spanning domain (TMD_0) in their structure. This topological feature can be found in MRP1, MRP2, MRP3, MRP6, and MRP7, while it is not possessed by MRP4, MRP5, MRP8, and MRP9. TMD_0 is not essential for catalytic function or intracellular routing; the function of this domain is unknown. Long MRPs share an L_0 segment with a highly conserved sequence near its N terminus. This sequence is also present near the N terminus of the short MRPs. It is essential for function and appears to associate with the membrane.

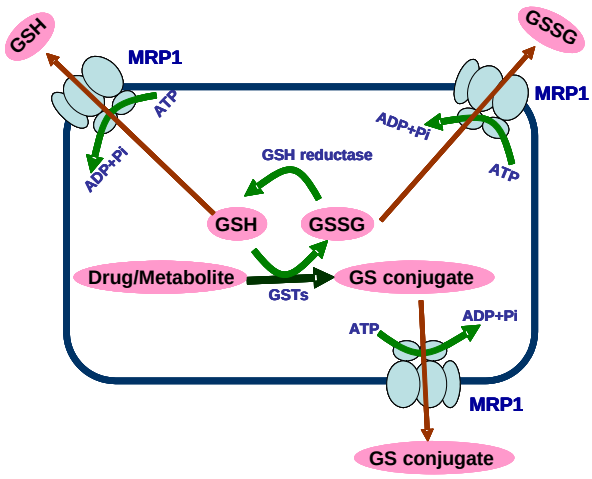

Figure 2. Glutathione (GSH)-dependent transport of drugs and their GSH conjugates by MRP1. P-glycoprotein can transport drugs in original form, while MRP1 can transport GSH, oxidized GSH (GSSG), as well as a number of GSH, glucuronate and sulfate conjugates of drugs. However, MRP1 has low affinity to GSH and GSSH. GSH not only enhances MRP1-mediated transport of hydrophobic xenobiotics, but also certain hydrophilic conjugated endobiotics, which represents a major detoxifying pathway.

knockout mice (*46*), and decrease in cells overexpressing MRP1 (*32,47*). MRP1 may reduce the harm of xenobiotics to cells by co-transporting the xenobiotics and GSH out (*46*). Overexpression of MRP1 is associated with an increased transport activity of compounds conjugated with GSH, glucuronide, or sulfate, which is known as glutathione conjugate pumps (*36,48,49*). GSH not only can enhance MRP1-mediated transport of hydrophobic xenobiotics, but also certain hydrophilic conjugated endobiotics (*30*). However, MRP1 has low affinity to GSH (*50,51*). Drugs including verapamil and apigenin have been demonstrated to increase the affinity of MRP1 to GSH (*52,53*). Vincristine uptake is inhibited by vinblastine but not daunorubicin or doxorubicin. Although GSH or vincristine alone has little effect on the MRP1-mediated transport of LTC_4, the combination of them becomes the potent inhibitor of MRP1-mediated transport of LTC_4 (*50*).

Human MRP1 confers resistance to anthracycline drugs, while Mrp1 from other species do not (*54,55*). Unlike PgP, however, MRP1 appears to cause resistance to some heavy metal ions, including arsenite and antimonials (*56,57*), which is consistent with the extensive homology of *MRP1* with the *Leishmania* arsenite transporter-encoding gene (*ltpgpA*) and the yeast cadmium factor gene (*ycf1*). In addition to alkaloid cytotoxic drugs, MRP1 is resistant to methotrexate (MTX), ZD1694 and GW1843 (*58,59*). The topoisomerase I inhibitors, camptothecin derivative, CPT-11 (irinotecan), and its active metabolite, SN-38 in unconjugated and conjugated forms are also actively effluxed out of cells by MRP1 (*60*). MRP1 confers resistance to doxorubicin, vincristine, etoposide, and mitoxantrone (*61,62*). MRP1 substrates also include conjugates of thiotepa, cyclophosphamide, chlorambucil, and melphalan (*61,63,64*). The resistance capability of MRP1 to melphalan can be increased by co-upregulation of glutathione *S*-transferases or the GSH biosynthetic enzyme, γ-glutamylcysteine synthetase (*63,64*).

MRP1 also confers resistance to arsenic in association with GSH (*56*). The ability of MRP1 to cause arsenite resistance in transfected or selected cellsand the overexpression of MRP1 in cells selected for arsenite (*56*) has raised the question of whether MRP1 might be responsible for the arsenite resistance of patients treated with arsenite for acute promyelocytic leukemia. However, that the *Mrp1*$^{-/-}$ mouse is not hypersensitive to arsenite (*65*), which suggests that MRP1 is not a critical factor in the cellular defense against arsenite. This could be due to the rapid excretion of the complexes of arsenite and methylarsenite with GSH into bile (*66*).

MRP1 transports the protease inhibitors, ritonavir and saquinavir (*67-69*), the antiandrogen drug flutamide and its metabolite hydroxyflutamide (*70*), and the GSH conjugates of ethacrynic acid (a diuretic) (*71*). In addition, the radiopharmaceuticals ^{99m}Tc-Sestamibi, ^{99m}Tc-Tetrofosmin, and the gadolinium chelate B22956/1 are substrates of MRP1 (*72-74*). Such compounds are used in clinical functional imaging studies and recently they may be used for *in vivo* imaging of hepatobiliary transport function.

A number of chemical toxicants and their metabolites are known to be the substrates for MRP1. Aflatoxin B1 and several *S* and *R* GSH conjugate stereoisomers of aflatoxin B1 (*75*), the GSH conjugates of herbicide metolachlor (*76*), and the GSH conjugates of the model toxicants 1-chloro-2,4-dinitrobenzene (*77*) and 4-nitroquinoline 1-oxide (*78*) have been identified as MRP1 substrates. However, a recent study indicated that carcinogen aflatoxin B1 induced a similar number of lung and liver tumors in both *mrp1*-null and wide type mice (*79*). This may be due to the redundancy

of transmembrane export pumps, other pumps may effectively vicariate for MRP1-mediated transport of aflatoxin B1 and its glutathione conjugates. In addition, the 3β-*O*-glucuronide conjugate of the tobacco metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is also a substrate of MRP1 (*53*). Notably, the NNAL-*O*-glucuronide transport by MRP1 requires physiological concentrations of GSH (*53*). NNAL is a lung cancer inducer.

MRP1 and murine Mrp1 are normally located in intracellular vesicles of undefined nature and in the basolateral membrane of epithelial membranes. Hence, MRP1 secretes drugs into the body, rather than moving them out of the body as PgP or MRP2 do. This makes MRP1 a system of cellular defense rather than one of total organism defense like Mdr1 PgP and MRP2, which eliminate drugs from the body. The importance of this cellular function is highlighted by the fact that mice lacking Mrp1 are hypersensitive to etoposide (*65,80*), whereas an increased sensitivity to vincristine is uncovered in the TKO mice (triple knockout mice in which the disrupted *Mrp1* alleles are combined with disruptions of the two drug-transporting PgP (ABCB1) genes, *Mdr1a* and *Mdr1b*) (*80*). In mice, loss of Mrp1 is associated only with increased sensitivity to epipodophyllotoxins (*e.g.* etoposide) and *Vinca* alkaloids (*e.g.* vincristine), the drugs also most affected by the absence of Mrp1 in *Mrp1*$^{-/-}$ embryonic stem cells (*65*). Knockout mice without *mrp1* have a decreased response to inflammatory stimuli, increased levels of GSH, and increased sensitivity to etoposide but are otherwise healthy and fertile (*41,65*).

A variety of inhibitors of MRP1 have been identified, but their specificity as yes to be determined. Some general inhibitors of organic anion transport including probenecid, sulfinpyrazone and indomethacin are able to inhibit MRP1 (*81-83*). The inhibitors of PgP such as verapamil, quercetin, genistein and cyclosporine can also suppress the transport activity of MRP1 (*84-88*). Other PgP and MRP1 dual inhibitors include the dihydropyridine PAK-104P (*89*), the polyhydroxylated sterol acetate agosterol A (*90*), steroid analogs (*91,92*), and imidazothiazole derivatives (*93*). The MRP1 inhibiting bioflavonoids, such as genistein, quercetin, biochanin A, and kaempferol, can also decrease the intracellular GSH levels (*85-88*). The non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine), nucleoside reverse transcriptase inhibitors (abacavir, emtricitabine, and lamivudine), and tenofovir as a nonnucleotide reverse transcriptase inhibitor also inhibited MRP1 *in vitro* (*94*).

There are some inhibitors specific to MRP family members. For example, the LTD_4 receptor antagonist, MK571, is a GSH conjugate inhibiting both MRP1 and MRP2 (*95*). Different to MK571 in structure, the peptide leukotriene receptor antagonist ONO-1078, has also been demonstrated to reduce LTC_4-efflux in lung tumor cells by blocking MRP1 function (*96*). The sulphonylurea, glibenclamide also shows inhibitory activity to both MRP1 and MRP2 (*97*). In addition, several highly specific ad potent MRP1 inhibitors have been identified. These include tricyclic isoxazole derivatives such as LY475776 and LY402913 (*98-100*). It has been reported that some antisense oligonucleotides are also able to inhibit MRP1 activity by reducing MRP1 mRNA levels and the protein synthesis (*101-103*). For instance, some antisense oligonucleotides reduce the expression level of the MRP1 protein by 46% and its mRNA level by 76% (*103*). ISIS 7597, an antisense oligonucleotide, is able to quickly decrease intracellular MRP1 mRNA levels by up to 90% at a low concentration (0.5 μM) (*101*).

MRP2 (ABCC2) is also known as the canalicular multispecific organic anion transporter (cMOAT). The amino acids of MRP2 have 49% identity with MRP1 (*104*). Human *MRP2* maps to chromosome 10q23-24 and consists of 32 exons spanning 65 kb (*105*). The location of MRP2 is unique, as it is present on the apical plasma membranes of polarized cells such as hepatocytes, pneumocytes, kidney proximal tubules, and specialized cells in the intestine and brain (*106,107*), while other MRPs are all located on basolateral membrane of polarized cells. Based on its localization and substrate specificity, it is proposed that the primary physiological function of MRP2 is to export amphiphilic organic anions and xenobiotics into bile and into the lumen of excretory organs (*108*).

Like MRP1, MRP2 transfected cells are resistant to etoposide, vinca alkaloids, anthracyclines, camptothecins, CPT-11 and MTX (*59,109-111*). The substrates of MPR1 and MRP2 have similarity with regard to the transport of GSH and glucuronate, and sulfate conjugates, but there are some important differences. The affinity of MRP2 to GSH conjugates is less than that of MRP1 (*112,113*). For instance, the affinity to MRP2 for both LTC_4 and *N*-ethylmaleimide glutathione is found to be significantly lower than that of MRP1 (*83*), whereas bilirubin mono- and bis-glucuronides have higher affinity for MRP2 (*106,114*). MRP2 is distinct from MRP1 with the ability to confer resistance to cisplatin (*109-111*), probably in the presence of GSH (*48*). Cisplatin resistance in MRP2-overexpressing cells is thus abrogated by MRP2 antisense cDNA. GSH itself appears to be a relatively low affinity substrate for MRP2 (*115*), but the co-transport of GSH with MRP2 substrate is similar to that observed for MRP1 (*113,116*).

MRP2 transports an array of conjugated endogenous metabolites. In addition to LTC_4, GSH, GSSG, and bilirubin conjugates, MRP2 is able to transport LTD_4, LTE_4, and the glucuronide conjugates of estrodiol and triiodo-L-thyronine (*112*). The substrates of MRP2 also include the glucuronide conjugates of grepafloxacin, diclofenac and acetaminophen (*112,117,118*). Moreover, sulfated MRP2 substrates include taurolithocholate sulfate and taurochenodeoxycholate sulfate, but not

estrone 3-sulfate (*119,120*).

MRP2 also transport ampicillin, ceftriaxone, pravastatin, temocaprilat, grepafloxacin and BQ-123 (*119,121*). Olmesartan, a novel angiotensin II blocker, is a substrate of MRP2 (*122*). A previous study reported that the biliary excretion of olmesartan is mediated by Mrp2 based on low biliary excretion in Eisai hyperbilirubinemic rats (EHBR), which are inherited mrp2-deficient rats, compared with Sprague-Dawley rats (*123*). Moreover, the HIV protease inhibitors saquinavir, lopinavir, ritonavir and indinavir are MRP2 substrates (*124,125*). Similar to MRP1, MRP2 can transport ^{99m}Tc-labeled compounds used in functional imaging studies (*126*).

Interestingly, MRP2 shows its ability to transport certain carcinogens and other toxicants as conjugates or as unconjugated organic anions. For example, MRP2 can transport the tobacco carcinogen NNAL, and in contrast to MRP1, GSH is not needed (*53*). MRP2 is also capable of transporting the GSH conjugate of (+)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide, the active metabolite of benzo[a]pyrene (*127*). Other toxicants as substrates of MRP2 include arsenite, cadmium and α-naphthylisothiocyanate with the need of GSH (*128,129*). This suggests a role of MRP2 in chemoprotection in the body.

Many inhibitors of MRP2 have been established, and most of which do not have high selectivity to MRP2. For instance, MK571 can also inhibit MRP1 and MRP3. The organic anions have different inhibitory effects on MRP2. For example, probenecid and furosemide inhibit, whereas under certain conditions, sulfinpyrazone, penicillin G, and indomethacin considerably stimulated MRP2 transport activity (*83*). However, all these compounds inhibit MRP1-ATPase capability. MRP1 may be a more potent transporter of GSH conjugates and free GSH than MRP2, but several anions are preferred substrates for MRP2. This may indicate different modulation selectivity on MRP1 or MRP2 in drug resistant cancer cells (*83*). The MPR2-mediated transport of known substrate $E_2$17βG can be blocked by bile acids and certain amphipathic anions (*130,131*). The antisense cDNA expression is also used to block the drug resistance capability of MRP2 (*132*). The non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine), nucleoside reverse transcriptase inhibitors (abacavir, emtricitabine, and lamivudine), and tenofovir as a nonnucleotide reverse transcriptase inhibitor also inhibited MRP2 *in vitro* (*94*).

Among the MRP family, MRP3 has the highest amino acid sequence resemblance (58%) with MRP1 (*133*). Less is known about this protein than either MRP1 or MRP2. Although most closely related to MRP1 and MRP2, MRP3 has its own particular pattern of tissue localisation and substrate specificity. MRP3 mRNA is mainly detected in small intestine, pancreas, colon, placenta, and adrenal gland, while lower levels are found in liver, brain, kidney and prostate (*134-136*). MRP3 is mainly localized in the basolateral membrane of polarized cells such as cholangiocytes, hepatocytes an enterocytes (*130*).

MRP3 confers resistance to a much narrower spectrum of anticancer drugs compared to MRP1 and MRP2, and the drugs are limited to vincristine, methotrexate, epipodophyllotins (etoposide and teniposide) (*137,138*). MRP3-mediated transport of etoposide is inhibited by some organic anion transport inhibitors, but is not influenced by the reduction of intracellular GSH level. MRP3 is also involved in the transport of $E_2$17βG, LTC_4, dinitrophenyl *S*-glutathione, acetaminophen glucuronide, but not GSH and etoposide glucuronide (*139,140*). Both etoposide and MTX can block the MRP3-mediated transport of $E_2$17βG (*141*). Unlike MRP1 and MRP2, MRP3 has a higher affinity to glucuronate conjugates than to GSH conjugates (*142*). Furthermore, the resistance capacity of MRP3 to etoposide and vincristine is much lower than that of MRP1. However, MRP3 shows poor resistance to some natural product drugs, such as anthracyclines and Taxol (*138*). MRP3 is present in cancer cell lines from many tissues, but initial studies on MRP3 in a panel of drug-resistant cancer cell lines did not turn up any association between MRP3 levels and drug resistance (*143*). However, there was a strong correlation between MRP3 and doxorubicin resistance in lung cancer lines (*144*).

In contrast to MRP1 and MRP2, MRP3 has a greater capacity to transport glucuronate conjugates than GSH conjugates, and it can not increase GSH efflux in transfected cells (*145*). MRP3 also transports monovalent bile salts such as cholate, glycocholate and taurocholate which are not substrates for MRP1 and MRP2 (*138,146*). Conversely, the conjugated cholate 3-*O*-glucronide, taurochenodeoxycholate 3-sulfae and taurolithocholate-3-sulfte are substrates for all three MRP proteins (*139*). Thus, MRP3 may have a role in entero-hepatic circulation of bile salts and it is considered to function as a backup detoxifying pathway for hepatocytes when normal canalicular route is damaged by cholestatic diseases and the function of MRP1 and MRP2 is impaired (*147-149*). The non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine), nucleoside reverse transcriptase inhibitors (emtricitabine, and lamivudine), and tenofovir as a nonnucleotide reverse transcriptase inhibitor also inhibited MRP3 *in vitro* (*94*).

MRP4 (ABCC4) has particular tissue expression profile, drug resistance selectivity, and substrate and inhibitor specificity, in comparison with other MRPs. Although MRP4 mRNA is present in most organs, MRP4 protein is mainly detected in the kidneys (*134*). MRP4 is a lipophilic anion pump capable of transporting some physiological and endogenous compounds. These include cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), GSH (*150*),

and folate (*151-153*). MRP4 is also able to mediate the uptake of PGE_1 and PGE_2, while MRP1, MRP2, MRP3, and MPR5 can not transport PGE_1 and PGE_2 (*154,155*).

MRP4 is able to transport several endogenous organic anions and steroid conjugates, including $E_2$17βG (*35,36,139*), and dehydroepiandrosterone-3-sulfate (DHEAS) which is the major circulating steroid made in the adrenal gland in humans (*156*). The affinity of MRP4 for $E_2$17βG is similar to that of MRP3, while lower than that of MRP1 and MRP2 (*35,36,139*). No transport of DHEAS by MRP2 or MRP3 is found (*156*). MRP4 mediates ATP-dependent co-transport of GSH or *S*-methyl-glutathione together with cholyltaurine, cholylglycine, or cholate (*157*). A recent study has identified conjugated bile acids, especially sulfated derivatives, as substrates of MRP4 (*156*). Bile acids, like the steroid $E_2$17βG, contain a cholesterol backbone structure and may thus represent physiological substrates of MRP4. GSH plays an important role in the function of MRP4, as MRP4 transports many of its substrates in a GSH-dependent manner and depletion of intracellular GSH by the GSH synthesis inhibitor, DL-buthionine-(*S*,*R*)-sulphoximine, blocks the MRP4-mediated export of cAMP and abolishes resistance to nucleoside analogues (*150*). MRP4 participates in the hepatic basolateral excretion of sulfate conjugates (*158*).

A variety of nucleoside (purine and pyrimidine) analogues are found to be substrates for MRP4. These include ganciclovir (*159*), azidiothymidine monophosphate (*160*), 9-(2-phosphonylmethoxyethy l)adenine (PMEA) (*160,161*), bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine, a lipophilic ester prodrug) (*162*), 6-mercaptopurine, and 6-thioguanine (*151*). ATP-dependent uptake of the acyclic nucleotide phosphonates, adefovir and tenofovir but not cidofovir, was observed only in the membrane vesicles expressing MRP4 (*163*). The kidney accumulation of adefovir and tenofovir was significantly greater in Mrp4 knockout mice (130 *versus* 66 and 191 *vs* 87 pmol/g tissue, respectively); thus, the renal luminal efflux clearance was estimated to be 37 and 46%, respectively, of the control (*163*). There was no change in the kinetic parameters of cidofovir in Mrp4 knockout mice. There was no difference in the fraction of mono- and diphosphorylated forms of adefovir in the kidney between wild-type and Mrp4 knockout mice (*163*). These findings indicate that MRP4 is involved in the renal luminal efflux of both adefovir and tenofovir, but it makes only a limited contribution to the urinary excretion of cidofovir. MRP4 is also an efflux pump for urate, the purine end metabolite (*164*) and thioxanthosine monophosphate and thioinosine monophosphate (both thiopurine metabolites) (*165*). Moreover, MRP4 transports the anticancer agents topotecan (*166*), leucovorin (*152*), and MTX (*137,152,161*). Topotecan is a semi-synthetic, water-soluble derivative of camptothecin, a cytotoxic plant alkaloid isolated from the Chinese tree *Camptotheca acuminata* (*167*). It is used as a second-line treatment for patients with ovarian carcinoma. Moreover, MRP4 can mediate the efflux of the glutathione conjugate of monochlorobimane, a bimane that forms fluorescent adduct with thiols (*168*).

A variety of inhibitors for MRP4 have been identified. Like MRP1 and MRP2, MRP4 is also inhibited by the leukotriene antagonist MK571 (*151,153*). The cellular efflux of cGMP by both MRP4 and MRP5 is inhibited by PGA1 and PGE1, the steroid progesterone and the anticancer drug estramustine (a combination of estrogen and mechlorethamine) (*169*). PGA1 inhibited the ATP-dependent efflux of MTX, another MRP4 substrate (*152,170*). PGF1α, PGF2α, PGA1, and thromboxane B2 are high-affinity inhibitors (therefore presumably substrates) of MRP4-mediated transport of PGE1 and PGE2 (*171*). The MRP4-mediated transport of PGE1 and PGE2 is also inhibited by rofecoxib and celecoxib (both COX-2-specific inhibitors), and diclofenac (*171*). Sulfinpyrazone is a potent inhibitor (IC_{50} = 420 μM) of PMEA efflux in MRP4-overexpressing HEK293 cells (*171*). MTX can inhibit the MRP4-mediated transport of $E_2$17βG (*151*). Glucuronide and glutathione conjugates can also inhibit MRP4-mediated transport of MTX (*152,153*). The MRP4-mediated transport of $E_2$17βG is blocked in the presence of estradiol 3,17-disulphate, taurolithocholate 3-sulphate (*156*), or topotecan (*166*). The MRP4-mediated transport of bimane-glutathione is totally inhibited in the presence of carbonylcyanide *m*-chlorophenylhydrasone (an uncoupler of oxidative phosphorylation) and significant inhibition is also observed with known inhibitors of MRP transporters including benzbromarone, verapamil, indomethacin, MTX, and 6-TG (*168*). Such transport is also inhibited by 1-chloro-2,4-dinitrobenzene (CDNB) which is metabolized to the glutathione conjugate after entry into cells.

MRP4 may be regulated at transcriptional, translational and posttranslational level. Its expression is substantially increased in livers of mice with disruption of the farnesyl/bile acid nuclear receptor, which have increased levels of serum and hepatocellular bile acids, and MRP4 can be further upregulated by cholic acid feeding (*172*). The constitutively active nuclear receptor (CAR) is required to coordinately upregulate hepatic expression of MRP4 and an enzyme known to sulfate hydroxy-bile acids and steroids (Sult2a1) (*173*). CAR activators increased MRP4 and Sult2a1 expression in primary human hepatocytes and HepG2, a human liver cell line. Sult2a1 was down-regulated in MRP4-null mice, further indicating an inter-relation between *MRP4* and *Sult2a1* gene expression. Based on the hydrophilic nature of sulfated bile acids and MRP4's capability to transport sulfated steroids, these findings suggest that MRP4 and Sult2a1 participate in an integrated pathway mediating elimination of sulfated steroid and bile-acid metabolites from the liver. In addition, a recent

study in infected human macrophages indicates that azidiothymidine treatment induces MRP4 mRNA (*174*).

Analysis of tissue RNA suggests that *MRP5* is ubiquitously expressed. The highest levels are found in skeletal muscle and brain (*143*). In comparison with MRP1-3, MRP5 (ABCC5) has its particular drug resistance selectivity and shows no resistance to natural anticancer compounds or MTX. MRP5 and MRP4 share only 36% amino acids identity, and their substrate specificity is similar. Both MRP4 and MRP5 are able to mediate the Mg^{++}/ATP-dependent transport of cGMP and cAMP. MRP4 has a higher affinity for cAMP than that of MRP5, while MRP5 has a higher affinity for cGMP that of MRP4 (*151*). Like MRP4, MRP5 is capable to transporting purine derivatives including PMEA and 6-mercaptopurine (*175,176*). However, MRP4 is also able to transport some substrates of MRP1-3, such as $E_2$17βG and MTX (*137*). MRP5 is able to transport *S*-(2,4-dinitrophenyl)glutathione which is inhibited by typical organic anion transport inhibitors, including sulfinpyrazone and benzbromarone (*175*). However, most glutathione and glucuronate conjugates are not substrates of MRP5. Notably, MRP5 shows resistance to heavy metals including cadmium chloride and potassium antimonyl tartrate (*176*). MRP5 can be modulated by general organic anion transport inhibitors, including probenecid, sulfinpyrazone, benzbromarone, and MK571 (*171*). Like MRP4, there are no specific inhibitors of MRP5.

The physiological functions and possible role in drug resistance of MRP4 and 5 remain to be defined. Obviously, the discovery that these pumps can transport cyclic nucleotides, notably cGMP, has raised the question of whether MRP4/5 can affect the signal transduction role of cGMP by removing it from the cell, which would supplement the degradation by phosphodiesterases. There is also evidence for an extracellular signaling role for cGMP in kidney and several other tissues, and MRP4/5 might be involved. No human disease has been associated with alterations in MRP5, and the Mrp5 KO mouse, generated by Wijnholds *et al*. (*175*), has no obvious phenotype. It is possible, however, that the overlapping substrate specificities of MRP5 and MRP4 (and possibly MRP8 and 9) may hide the physiological function of Mrp5, *e.g*., in cyclic nucleotide transport, and that the breeding of mice lacking all these transporters may lead to an understanding of the physiological function of each of them.

Human MRP6 is most closely related to MRP1 and MRP2 with 45% and 43% amino acid identity, respectively. The highest levels of *MRP6* mRNA and protein expression are detected in kidney and liver while low levels are found in most other tissues such as skin and retina (*177-179*). MRP6 is located on the basolateral membranes in hepatocytes and kidney proximal tubules (*180*). Overexpression of MRP6 does lead to weak resistance to chemotherapeutic drugs (*181*). Rat Mrp6 transported the cyclic cyclopentapeptide endothelin-1 receptor antagonist BQ123, although endothelin-1 itself is not a substrate of Mrp6 (*182*). However, rat Mrp6 did not transport glucuronide, sulfate and GSH conjugates, hydrophobic drugs, PGs or aminophospholipids (*182*). More recently, MRP6 was found to transport glutathione conjugates, such as LTC_4, *N*-ethylmaleimide, *S*-glutathione and dinitrophenol glutathione, while $E_2$17βG appears a poor MRP6 substrate (*181,183*). Effective inhibitors of MRP1 and MRP2, including indomethacin, probenecid, and benzbromarone, can block the MRP6-mediated transport (*183*). MRP6 also exhibited low-level resistant activity to a variety of natural product anticancer drugs, such as etoposide, teniposide, doxorubicin, cisplatin, daunorubicin and dactinomycin (*181*). These findings suggest that MRP6 may transport conjugated organic anions and probably confers resistance to anticancer drugs to a less effective extent than MRP1-3.

MRP7 (ABCC10) has the lowest amino acid sequence identity (33-36%) with other MRP family members (*18*). Although MRP7 mRNA can be detected in most tissues, but the expression levels are usually very low (*18*). MRP7 is able to transport $E_2$17βG with a high K_m (58 μM) (*184*). This suggests that MRP7 may be a lipophilic anion transporter. In contrast, MRP7 did not transport other typical MRP substrates, such as cyclic nucleotides, MTX, or bile acids (*184,185*). Interestingly, MRP7 is as closely related to the SUR K_i channel regulators, but the functional implication is yet to be determined.

MRP8 (ABCC11) has 40% amino acids identity with MRP5, and has been characterized as an amphipathic anion transporter. MRP8 is mainly present in normal breast and testis, while little is present in liver, brain, and placenta (*19*). With the ability to efflux cAMP and cGMP, MRP8 confers resistance to purine and pyrimidine nucleotide derivatives, including anticancer fluoropyrimidines, and several antiviral agents. Similar to the case for other MRPs that possess only two membrane spanning domains (MRP4 and MRP5), MRP8 is a cyclic nucleotide efflux pump that is able to confer resistance to nucleoside-based agents, such as PMEA and 5-FU (*186*). In contrast, little resistance is found for some natural product anticancer drugs (*187*). Recently, MRP8 is found to transport a variety of physiological and synthetic lipophilic anions, including the LTC_4, steroid sulfates such as dehydroepiandrosterone (DHEAS) and estrone 3-sulfate, $E_2$17βG, leukotriene C_4 and dinitrophenyl-*S*-glutathione, the monoanionic bile acids glycocholate and taurocholate, and MTX (*188-191*).

Both *MRP8* and *MRP9* genes are identified using a functional genomic approach and bioinformatics tools. Both MRP8 and MRP9 (ABCC12) have the highest degree of similarity with MRP5. One major difference between MRP8 and MRP9 is that MRP9 has only one ATP-binding domain but two transmembrane domains

each with four membrane-spanning regions. The *MRP9* gene is unusual because it encodes two transcripts of different sizes (*192*). The larger 4.5-kb RNA is found in breast cancer, normal breast, and testis and encodes an MRP-like protein that lacks transmembrane domains 3, 4, 11, and 12 and the second nucleotide-binding domain. The smaller 1.3-kb RNA is detected in brain, skeletal muscle, and ovary and seems to encode the second nucleotide-binding domain. There is a lack of information on the substrate specificity of MRP9. It is speculated that MRP9 may have a different function from other family members. Because both MRP8 and MRP9 are membrane proteins with very restricted expression in essential tissues (*21*), they may represent potential molecular targets for targeted therapy with antibodies, antibody conjugates, and immunotoxins.

Various MRPs show considerable differences in their tissue distribution, substrate specificities, and proposed physiological and pharmacological functions. The tissue distribution, substrates and inhibitors of MRPs are listed in Table 1. MRPs are capable of transporting a structurally diverse array of endo- and xenobiotics including many therapeutic drugs and their metabolites across cell membranes. They play an important role in the absorption, disposition and elimination of many therapeutic agents in the body.

4. Induction of MRPs

Regulation of ABC transporter gene expression involves participation of numerous nuclear receptors (*193-195*). Nuclear receptors constitute a family of transcription factors that act as heterodimers, which bind to promoter elements and induce gene expression. Transporter genes are regulated at several levels, including membrane retrieval and reinsertion, translation, and transcription. Nuclear receptors relevant for the expression of ABC transporters are liver X receptor (LXR), farnesoid receptor (FXR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) (*4*). The induction of *CYP3A4* and *CYP2B6* genes by numerous xenobiotics is well known to be mediated through activation of PXR (*196*). PXR is activated by a diverse number of compounds, including rifampicin, phenobarbital, and mifepristone in humans. PXR mediates the expression of rodent Oatp1a4 (*194*), Oatp2 (*197,198*), human MDR1 (*199*), mouse MRP1 (*200*), Mrp2 (*200*) and Mrp3 (*201*). Furthermore, CAR activation induces Mrp2-7 mRNA in mouse liver (*202*) and is involved in the regulation of Mrp4 and sulfotransferase 2A1 (*173*). The PPARα agonist clofibrate induces gene and protein expression of Mrp3 and Mrp4 efflux transporters in a PPARα-dependent manner while having little effect on mRNA expression of Ntcp, Oatp1a1, Oatp1a4, and Oatp1b2 uptake transporters in mouse liver (*203*).

In primary cultures of human hepatocytes, MRP1 was increased by rifampin (*204*). In mouse liver, carbon tetrachloride induced Mrp1 (*205*). MRP1 is up-regulated when exposed to rifampin (*206,207*) or mitoxantrone in tumor cells (*208*). The expression of MRP1 in human colorectal cancer cell lines was induced by sulindac (*209*).

The promoter regions of the human *MRP2* and the rat *Mrp2* gene contain a number of putative consensus binding sites for AP1, SP1, HNF1, and HNF3β (*210*). The -431 to -258 region also contains important elements that control expression in HepG2 cells, particularly the CCAAT-enhancer binding protein β. AhR ligands (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polychlorinated biphenyl 126, and β-naphthoflavone), the CAR activator 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, and nuclear factor-E2-related factor 2 (Nrf2) activators (butylated hydroxyanisole, oltipraz, and ethoxyquin) increased Mrp2 expression in mouse liver, suggesting that AhR, CAR, and Nrf2 may be important for modulating Mrp2 expression by chemicals (*202*). Induction of rat Mrp2 has been observed with numerous chemicals, such as pregnenolone-16α-carbonitrile, spironolactone, and dexamethasone (all PXR ligands), phenobarbital (CAR ligand), and oltipraz (Nrf2 activator) (*211,212*). Similar induction of Mrp2 with indole-3-carbinol and β-naphthoflavone, both AhR ligands, has also been observed in rat liver (*213*). Ligands for FXR, PXR, and CAR all induced Mrp2 mRNA in primary cultures of rat hepatocytes and characterized a putative ER-8 at -401 to -376 of the rat Mrp2 promoter that bound the corresponding FXR/RXR, PXR/RXR, and CAR/RXR heterodimers (*214*). Treatment with the chemical carcinogen 2-acetylaminofluorene, cisplatin, and the protein-synthesis inhibitor cycloheximide increased expression of Mrp2 in rat liver (*215*). *trans*-Stilbene oxide also induced rat Mrp2 expression *via* CAR-independent manner (*216*).

The inducibility of *Mrp2* gene expression in primate liver was investigated in rhesus monkeys treated with tamoxifen or rifampin (*217*). Both tamoxifen and rifampin strongly induced Mrp2 mRNA in two male and two female rhesus; tamoxifen induced Mrp2 protein in both male and female rhesus, whereas rifampin showed some inducing effect in a female but was inactive in a male monkey. Carotenoids and retinol also induced MRP2 through PXR activation (*218*). Human MRP2 is similarly up-regulated by the PXR activators rifampicin and tamoxifen, which differ from known rodent ligands for PXR (*219*). Similarly, MRP2 is induced by phenobarbital (*220*) and by *tert*-butyl hydroquinone in HepG2 cells (*200*), which suggests that CAR and Nrf2, respectively, may regulate expression of the human *MRP2* gene. These results suggest that the gene for *Mrp2* may be similarly up-regulated by PXR agonists in human and rat, but mouse Mrp2 may not be as sensitive to PXR ligands. In clinical studies, expression of MRP2 mRNA and protein was decreased in patients with obstructive cholestasis who were poorly drained by percutaneous

Table 1. Tissue distribution, substrates and inhibitors of MRPs

Name	Symbol	Tissue location	Expression levels	Major drug substrates	Physiologic substrates	Inhibitors
MRP1	ABCC1	All major tissues	Differ in various organs and cell lines	Doxorubicin, vincristine, etoposide, MTX, camptothecin, CPT-11, SN-38, cyclophosphamide, conjugates	Glutathione, LTC4, $E_2$17βG, sulfated bile acids, bilirubin, PGA GSH conjugate, GSH, GSSG	Probenecid, sulfinpyrazone, indomethacin, verapamil, quercetin, genistein, cyclosporine, PAK-104P, steroid analogs, MK571, ONO-1078, sulphonylurea, glibenclamide
MRP2	ABCC2, cMOAT	Liver, kidney, intestine, brain		Conjugates, cisplatin, etoposide, vinca alkaloids, anthracyclines, Camptothecins, MTX, lopinavir, olmesartan	LTC4, GSH, GSSG, bilirubin conjugates, LTD4, LTE4	MK571, furosemide
MRP3	ABCC3	Small intestine, pancreas, colon, placenta, adrenal gland	Low level in liver, brain, kidney and prostate	Etoposide, teniposide, dinitrophenyl *S*-glutathione, acetaminophen glucuronide, vincristine, MTX	LTC4, $E_2$17βG, cholate, glycocholate, taurocholate	Etoposide, MTX
MRP4	ABCC4	Kidneys	Low levels in other tissues	MTX, 6-thioguanine, PMEA, 6-mercaptopurine, topotecan	cGMP, cAMP, DHEAS, $E_2$17βG, PGE_1, PGE_2	MK571, celecoxib, rofecoxib, diclofenac
MRP5	ABCC5	Most tissues	Low levels	6-Mercaptopurine, 6-thioguanine, PMEA, heavy metals, *S*-(2,4-dinitrophenyl)glutathione	cGMP, cAMP	Probenecid, sulfinpyrazone, benzbromarone, MK571
MRP6	ABCC6	Liver, kidney	Low levels in other tissues	LTC4, *N*-ethylmaleimide *S*-glutathione, dinitrophenol glutathione, etoposide, doxorubicin, cisplatin, daunorubicin	?	Indomethacin, probenecid, benzbromarone
MRP7	ABCC10	Most tissues	Very low levels	?	$E_2$17βG	?
MRP8	ABCC11	Normal breast, testis	Low levels in liver, brain, and placenta	5-FU, ddC, PMEA, MTX, bile acids	cGMP, cAMP, LTC4, DHEAS,	?
MRP9	ABCC12	Breast cancer, normal breast, testis, brain, skeletal muscle, ovary	Low levels	?	?	?

transhepatic biliary drainage (*221*). In another clinical study, rifampin treatment of normal human subjects increased MRP2 mRNA and protein in the duodenum (*222*). Additionally, induction of chronic renal failure in rats increased Mrp2 mRNA and protein levels in both the kidney and the liver (*223*). This may represent a compensatory mechanism during renal failure, although the human response has not yet been documented.

The expression of MRP3 in rat and human liver is low under normal conditions but is induced during cholestasis and in the absence of MRP2 or bile salt export pump (BSEP) (*172*,*224*). Bile acids, in particular lithocholic acid, have been demonstrated to activate PXR likely as a mechanism to control their production and metabolism to prevent their accumulation to toxic levels (*225*). In rats, mice, and humans, Mrp3 has been shown to be regulated by phenobarbital, diallyl sulfide, and polychlorinated biphenyl 99 (*226*), compounds that induce Cyp2B1/2 and are known or hypothesized CAR activators. *trans*-Stilbene oxide also induced rat Mrp3 expression *via* CAR-independent manner (*216*). Similar to Mrp2, Mrp3 is highly up-regulated by oltipraz (*202*), suggesting that Nrf2 might be an important transcription factor that regulates Mrp3 (*226*). In humans, induction by β-naphthoflavone and rifampicin suggests that MRP3 might be regulated *via* AhR or PXR, respectively (*220*). Using a large collection of human liver tissues, it was found that omeprazole was an inducer of MRP3 expression, probably through a AhR-dependent pathway (*227*). This effect could be reproduced with HepG2 hepatoma cells, which showed a concentration-dependent induction of MRP3 expression by omeprazole. Overall, Mrp3 seems to be regulated similarly in rats, mice, and humans, with potential transcriptional regulation by AhR, PXR, CAR, PPARα, and Nrf2.

The CAR activator 1,4-bis[2-(3,5-dichloro-pyridyloxy)]benzene and Nrf2 activators (butylated hydroxyanisole, oltipraz, and ethoxyquin) induced Mrp4 in mouse liver (*202*), indicating potential roles for CAR and Nrf2 in the regulation of mouse Mrp4. In rats, Mrp4 is induced in liver by the Nrf2 activators oltipraz, and ethoxyquin (*228*). *trans*-Stilbene oxide also induced rat Mrp4 expression *via* CAR-independent manner (*216*). Little data exists on induction of MRP4 in humans. However, studies in CAR-null mice have definitively shown that induction of Mrp4 by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene and phenobarbital is *via* CAR (*173*). Taken together, the most likely means of induction of Mrp4 is by transcriptional activation by CAR and Nrf2.

Few chemicals have been observed to modulate expression of MRP6 in rats or humans. However, AhR, CAR, and Nrf2 activators induced expression of Mrp6 in mouse liver (*202*). A recent study found that the expression of this gene in cells of hepatic origin is significantly upregulated by retinoids, acting as agonists of the retinoid X receptor (RXR) rather than the retinoid A receptor (RAR) (*229*).

One of the patterns of Mrp expression of note is that AhR and Nrf2 activators often induce the same transporter (*i.e.*, Mrp2, 3, 5, and 6). Several genes known to be regulated by Nrf2 are also regulated in a similar manner compared with these Mrps. Rat UDP-glucuronosyltransferase 1A6 is induced by oltipraz, a classical Nrf2 activator, and oltipraz induction of UDP-glucuronosyltransferase 1A6 is dependent on the binding of AhR to the xenobiotic response element (*230*). Furthermore, one of the known target genes of Nrf2 activation, Nqo1, can be induced by the classical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and that induction was Nrf2-dependent (*231*). Although the mechanism of this cross-activation is not well defined, MRPs may share a similar pattern of inducibility to the phase I and II enzymes known to be regulated by these two receptors. Thus, it is unclear whether the induction of MRPs by some of the microsomal enzyme inducers is mediated through direct mechanisms (transcription factor binding to its cognate response element) or indirect mechanisms that involve some sort of "cross talk" (activation of multiple receptors by a chemical and/or transcriptional up-regulation of another gene or transcription factor that acts on the gene of interest.

5. MRPs and intestinal absorption of drugs

Many orally administered drugs must overcome several barriers before reaching their target site (*232*). The first major obstacle to cross is the intestinal epithelium. MRP2 and MRP4, together with PgP/MDR1 (ABCB1) and BCRP/MXR (ABCG2), have been shown to localize at the apical/lumenal membrane of enterocytes, and thus are thought to form a barrier to intestinal absorption of substrate drugs (Figure 3) (*232*). Their expression level varies between different segments of the intestine. In general, BCRP/MXR (ABCG2), MRP2 (ABCC2) and PgP/MDR1 (ABCB1) are expressed at high level in the small intestine (*232*), considered by many in the field as the rate limiting barrier to oral drug absorption.

Regarding their role in limiting intestinal absorption, MDR1 is the most thoroughly characterized and well accepted. Although the expression levels of both the MRP2 and MXR are higher in the small intestine than the expression of MDR1, there are much fewer data available on their role in drug absorption (*232*). MRP2 has been shown to limit absorption of a phenylimidazo[4,5-b]pyridine (PhIP) derivative, a food-derived carcinogen, and MXR has been shown to limit absorption of topotecan.

6. MRPs and biliary excretion of drugs

Hepatic transporters are involved in the regulation of bile formation and disposition of xenobiotics. The hepatocyte has a polarized plasma membrane with basolateral

and apical domains, enabling vectorial movement of endogenous and exogenous compounds from blood into bile. Drugs that reach the blood are then passed to the liver, where they are metabolized and subject to biliary excretion, often by MRPs and other important ABC transporters (Figure 4) (*4,232,233*). Canalicular secretion of bile components represents the rate-limiting step in bile formation. Bile acids, glutathione conjugates, and xenobiotics are removed from hepatocytes and concentrated into the bile by canalicular efflux transporters in an ATP-dependent manner.

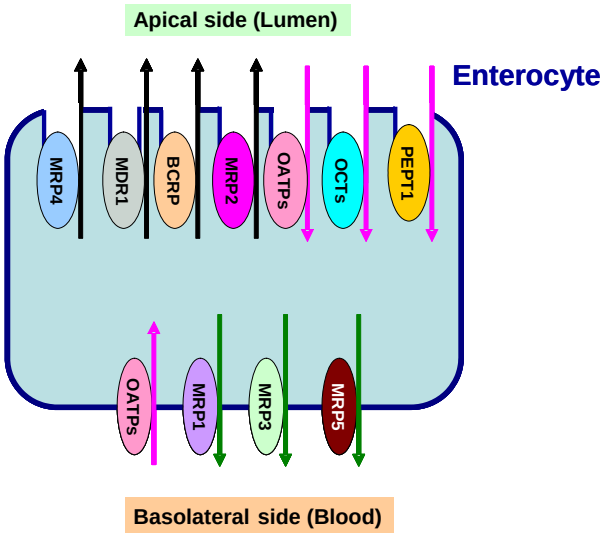

Figure 3. MRP2 and MRP4, together with PgP/MDR1 (ABCB1) and BCRP/MXR (ABCG2), are localize at the apical/lumenal membrane of enterocytes, and thus are thought to form a physical barrier to intestinal absorption of a number of substrate drugs. OATPs, OCTs and PEPT1 are also located at this side. Their expression level varies between different segments of the intestine. In general, BCRP/MXR (ABCG2), MRP2 (ABCC2) and PgP/MDR1 (ABCB1) are expressed at high level in the small intestine, considered by many in the field as the rate limiting barrier to oral drug absorption. MRP1, 3, and 5, and OATPs are expressed at the basolateral membrane of enterocytes.

Four MRP transporters (MRP2, 3, 4, and 6) are expressed to an appreciable extent in liver. In liver, MRP2 is the only MRP localized to the canalicular membrane and participates in excretion of chemicals into bile. Alternatively, MRP3 and MRP4 are localized to the basolateral membrane and efflux chemicals from hepatocytes into blood. MRP6 is thought to be localized to the basolateral membrane as well, but a high-affinity substrate for this transporter has not been identified. The MRPs play an important role in the hepatic elimination of metabolites, and modulation of MRP expression in liver can alter drug disposition.

Both organic cations and anions are taken up into the hepatocyte by groups of transport proteins (OCTs and OATPs respectively) with overlapping specificity. None of the known OATPs import unconjugated bilirubin. Organic anions (including bilirubin and glutathione) are transported across the hepatocyte into bile, usually after being modified by covalent conjugation in the microsomes. These conjugates are secreted into bile by MRP2. After uptake, some compounds may reflux back into the plasma, either by passive diffusion, by MRPs and export by the newly discovered, dimeric organic solute tranporter (OSTα,β) (*234*); these are expressed at the basolateral membrane of the hepatocyte and show considerable overlap of substrate specificity. MRP1 exports both unconjugated and conjugated bilirubins, whereas MRP3 and 4, and OSTα,β best

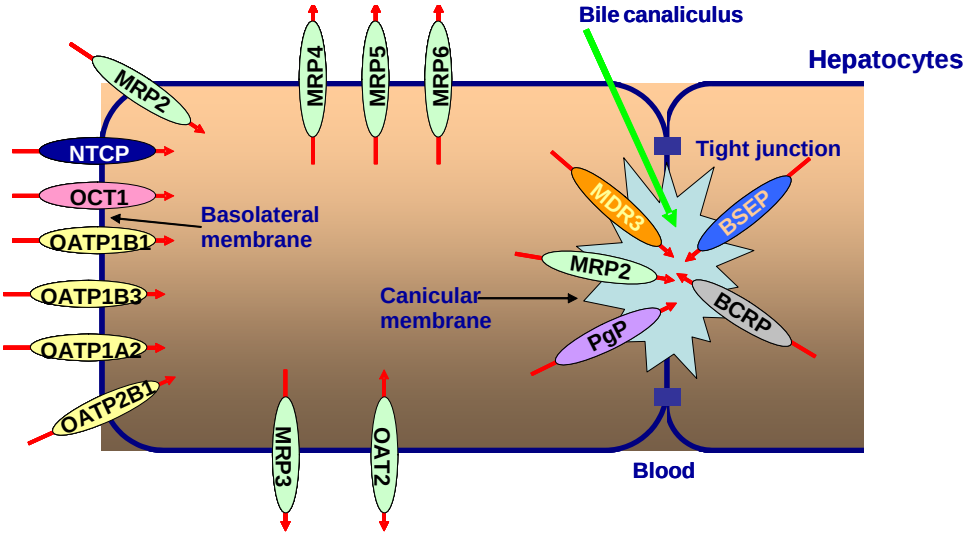

Figure 4. Localization of MRP transporters in hepatocytes. MRP2, localized on the basolateral (sinusoidal) membrane of hepatocytes, plays a critical role in the hepatic excretion of drugs and their metabolites (mainly conjugates). MRP3-6 facilitate the efflux of non-membrane-permeable molecules out of the hepatic cells. Human NTCP (Na^+-taurocholate co-transporting polypeptide) is a Na^+-dependent taurocholate uptake transporter located on the basolateral (sinusoidal) membrane of hepatocytes. NTCP mediates the Na^+-coupled uptake of bile salts from the space of Disse. The conjugated bile salts are then secreted into bile by the canalicular bile salt export pump (BSEP). Phosphatidylcholine (lecithin) is transported to the outer leaflet of the canalicular membrane by the phospholipid flippase, MDR3, from where it is stripped into bile by secreted bile salts. Uptake of organic cations is mediated by a family of organic cation transporters (OCTs). Uptake or organic anions is mediated by families of organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs). Human OATPs, located on the basolateral membrane of hepatocytes, are responsible for the uptake of bile salts, organic anions, hormones, cholates along with their metabolites and conjugates. After conjugation, the organic anions, as well as glutathione, are then secreted into bile by MRP2. A wide variety of amphipathic compounds (including many drugs and organic cations) are exported from the hepatocytes into bile by apical MDR1.

export conjugated bile salts (*234*). All of them have low expression in the normal liver, but are upregulated in cholestasis (*233*,*235*).

Some MRPs (*e.g*. MRP2) play a critical role in the hepatic excretion of drugs and their metabolites (*233*). Decreased MRP function can thus impair hepatic capacity to excrete drugs and their metabolites. For example, altered MRP2 function can change the clearance of many clinically important drugs, including cancer chemotherapeutics (irinotecan, methotrexate, and vinblastine), antibiotics (ampicillin, ceftriaxone, and rifampin), antihyperlipidemics, and angiotensin-converting enzyme inhibitors, as well as many toxins and their conjugates (*236*).

MRP3-6 facilitate the efflux of non-membrane-permeable molecules out of the hepatic cells. Human NTCP (Na^+-taurocholate co-transporting polypeptide) is a Na^+-dependent taurocholate uptake transporter located on the basolateral (sinusoidal) membrane of hepatocytes. The conjugated bile salts are then secreted into bile by the canalicular bile salt export pump (BSEP/ABCB11) (*233*). Phosphatidylcholine (lecithin) is transported to the outer leaflet of the canalicular membrane by the phospholipid flippase (*237*), MDR3, from where it is stripped into bile by secreted bile salts (*238*). Uptake of organic cations is mediated by a family of organic cation transporters (OCTs). Uptake or organic anions is mediated by families of organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs) (*233*,*235*). Human OATPs, located on the basolateral membrane of hepatocytes, are responsible for the uptake of bile salts, organic anions, hormones, cholates along with their metabolites and conjugates (*235*). After conjugation, the organic anions, as well as glutathione, are then secreted into bile by MRP2. A wide variety of amphipathic compounds (including many drugs and organic cations) are exported from the hepatocytes into bile by apical MDR1 (*239*). With regard to the transporters involved in biliary excretion, it is known that PgP (MDR1/ABCB1), MRP2 (ABCC2), the bile salt export protein (BSEP/ABCB11), and BCRP/ABCG2 are predominantly expressed on canalicular membrane (*232*).

7. MRPs and renal drug excretion

PgP/MDR1 (ABCB1), MRP2 (ABCC2), MRP4 (ABCC4) primarily localize to the apical (luminal) membrane of renal epithelial cells, while MRP1 (ABCC1) and MRP6 have been shown to be expressed on the basolateral membrane (Figure 5) (*104*,*240-243*). Substrates of MRP2 and MRP4 have been shown to have altered renal clearance in animals lacking transporter function (*241*). These transporters export compounds from the cytoplasm of renal tubular cells to the urine, therefore, substrates of these transporters are expected to have higher renal elimination than it is expected by glomerular filtration. Tenofovir, an anti-HIV agent, is actively excreted from the proximal tubule cells by MRP2 and MRP4 (*244*). Further studies are needed to understand the detailed role of these transporters in pharamacokinetics.

Additionally, members of the OATP, OCT (OCT1-3) and OAT (OAT1, 3, 4) transporter families have been identified in the basolateral membrane of proximal tubule cells (*241*,*245-247*). OAT3 has shown to be responsible for the renal elimination of pravastatin (*248*). Substrates of OCTs have been shown to have greatly reduced renal clearance and increased plasma concentration in mice lacking OCT1 and OCT2 (*249*). On the other hand, the two peptide transporters PEPT1 and PEPT2 are present on the luminal membrane of proximal tubule cells and were shown to be responsible for the tubular re-absorption of peptide-like drugs such as β-lactam antibiotics across the brush-border membranes (*250*). The reabsorbtion process results in lower renal clearance than it is expected by glomerular filtration. Furthermore, the uptake process might result in increased concentration of drugs in the cytoplasm of proximal tubular cells, leading to toxic effects in the kidney. The nephorotoxic effect of the antibiotic cephaloridine was linked to OAT3 function (*251*,*252*), while OCT2 was identified as the major determinant of the nephrotoxicity of the anti-cancer drug cisplatin (*253*).

MRP2 inhibition by tenofovir may contribute to the known interaction between tenofovir and didanosine. Coadministration of these two antiretroviral drugs leads to an increase of the area under the didanosine concentration-time curve (AUC) by 44 to 60% (*254*). This may occur through tenofovir-induced inhibition of the active uptake of didanosine into the proximal tubule cells by the human organic anion transporter 1 (*255*) or by inhibition of purine nucleoside phosphorylase, an enzyme involved in the degradation of didanosine (*244*,*256*). However, assuming that the MRP2 inhibitor didanosine is also an MRP2 substrate, the increase in didanosine AUC could also be achieved by inhibition of MRP2-mediated efflux in the tubular brush-border membrane or in other tissues. Inhibition of several MRP could also have contributed to the life-threatening toxicity (*e.g*. neutropenia) of the MRP substrate vinblastine in a patient with HIV-associated multicentric Castleman's disease who was maintained on lamivudine, abacavir, and nevirapine (*257*). Another patient with HIV-associated Hodgkin's disease also experienced life-threatening neutropenia when treated with ABVD (doxorubicine, bleomycine, vinblastine, dacarbazine) chemotherapy and lopinavir-ritonavir based antiretroviral therapy (*258*). Vinblastine and lopinavir-ritonavir interaction was managed with lopinavir-ritonavir interruption around chemotherapy administration, with complete remission and immunovirological success after six cycles.

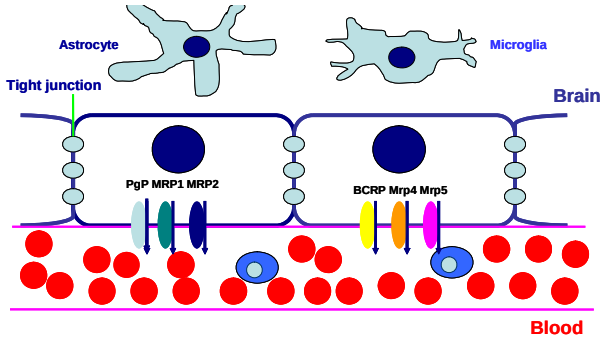

Figure 5. MRP1, MRP2, MRP4, and MRP5 are clearly localized to the luminal (apical) side of brain capillary endothelial cells of the blood-brain barriers. It is well established that the PgP/MDR1 (ABCB1) and BCRP protein localized in the apical/luminal membrane of the brain capillary endothelial cells are a major barrier of brain penetration of drugs. These transporters are also expressed in astrocytes and microglias.

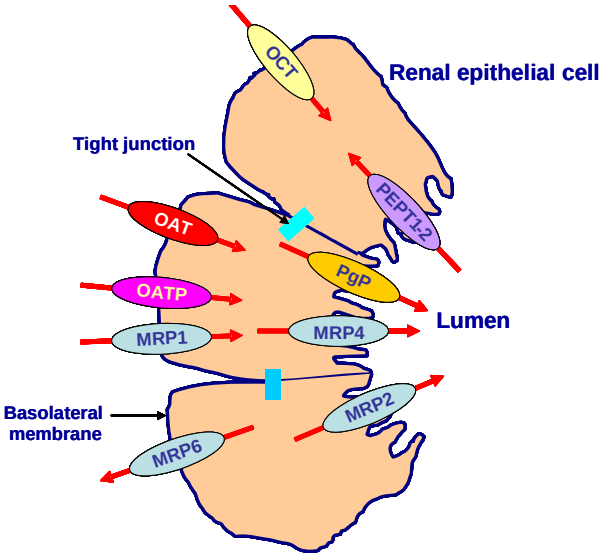

Figure 6. MRP2 (ABCC2) and MRP4 (ABCC4) are primarily localized to the apical (luminal) membrane of renal epithelial cells, while MRP1 (ABCC1) and MRP6 are expressed on the basolateral membrane of proximal tubule cells. PgP/MDR1 (ABCB1) is also located to the apical membrane of renal epithelial cells. Moreover, OATP, OCT (OCT1-3) and OAT (OAT1, 3, and 4) transporters have been identified in the basolateral membrane of proximal tubule cells.

8. MRPs and the blood-brain barrier (BBB)

The BBB is formed by the tight junctions that connect the brain endothelial cells, thus restricting the entry of compounds from the circulating blood to the brain *via* paracellular and transcellular routes (*259-264*). The BBB acts as an anatomical and transporter barrier notably due to the presence of tight junctions and a multitude of ABC transporters such as PgP, BCRP, and MRP1, 2, 4, and 5 (Figure 6) (*4,260,261,264-266*). As such, the BBB contributes to brain homeostasis by protecting the brain from potentially harmful endogenous and exogenous substances (*267*). It is well established that the PgP/MDR1 (ABCB1) and BCRP/MXR(ABCG2) localized in the apical/luminal membrane of the brain capillary endothelial cells are a major barrier of brain penetration of drugs.

Functional studies have assigned a role for human MRP2 (ABCC2) in the blood-brain barrier. MRP1 (ABCC1) is also implicated in protecting the brain tissue against xenobiotics (*e.g.* somatostatin analogs). MRP1 is localized in the basolateral membrane of the choroid epithelial cells and prevents the penetration of drugs and toxicants into the cephalo-spinal fluid. Similarity between the localization of MRP2, MXR (BCRP) and MDR1 in the brain microvessel endothelial cells and in the enteral epithelial cells suggests that these transporters function together to serve as physiological barriers against xenobiotics at the intestinal brush-border membrane and at the blood-brain barrier. MRP4 and MRP5 have been located in the brain capillary endothelial cells forming the blood-brain barrier. MRP1, MRP4, and MRP5 are clearly localized to the luminal side of brain capillary endothelial cells.

Despite advances in brain research, central nervous system (CNS) disorders remain very difficult to treat because the majority of drugs do not cross the BBB. The BBB blocks delivery of more than 98% of CNS acting drugs (*262,268*). Successful brain penetration is a prerequisite for the design of chemical lead substances for CNS acting drugs. To restrict CNS adverse effects, brain penetration properties are also important for the development of non-CNS acting drugs. Therefore, for both drug classes their BBB penetration is useful to be tested in advance. PgP and other ABC transporters can limit the penetration of drugs into the brain and thus modulate effectiveness and central nervous system toxicity of numerous drugs (*262,269,270*). The drug delivery challenge posed by the BBB is compelling, particularly as the population ages and the incidence of neurodegenerative diseases such as stroke, Alzheimer's disease, and Parkinson's disease increase in prevalence. Despite advances in brain research, central nervous system disorders remain very difficult to treat because the majority of drugs do not cross the BBB. The BBB limits the ability of many drugs to penetrate brain tissue by restricting paracellular and transcellular transport (*262*). To circumvent the limited access of drugs into the brain, different approaches have been investigated, including drug delivery systems such as liposomes, nanoparticles, peptide-vector strategy, MDR1 modulators, modulators of endothelial tight junctions, or osmotic pressure modification (*271*).

MRP alterations may also affect the distribution of their substrates, thus altering therapeutics or toxicology. For example, MRP4-deficient mice had enhanced accumulation of topotecan in brain tissue and cerebrospinal fluid (*166*). On the other hand, modulation of MRPs in blood-brain barrier may facilitate the management of diseases of the central nervous system by enhancing penetration of drugs into the brain. Such MRP-based barrier may be circumvented by targeted site-specific drug delivery systems, such

as immunoliposome and nanoparticulate systems (*272*). Moreover, development of novel approaches for bypassing the impact of these drug transporters and for the design of effective drugs that are not substrates and the development of selective and potent inhibitors for the MRP transporters becomes a high imperative for the pharmaceutical industry (*269*).

MRPs enhanced the ability of tumor cells to efflux chemotherapy drugs out of cells to reduce the cellular drug concentration leading to resistance to anticancer drugs. Increased expression of these drug transporters in tumor cells is associated with resistance to a number of important chemotherapeutic agents. With the accumulation of information on drug resistance profile and physiological function of MRP family, the relationship between drug selectivity and specific transporter level will be more and more significant and helpful in clinical cancer treatment and development of novel anticancer agents.

MRPs can be regulated at the level of transcription, translation and post-translation. Like PgP, MRPs are also subject to induction and inhibition by a number of compounds. Not surprisingly, the induction and inhibition of MRPs by various agents are of pharmacokinetic and pharmacodynamic importance. The identification of induces and inhibitors for each MRP may also allow the prediction of potential drug-drug interactions.

9. MRPs and drug toxicity

MRPs can efflux the GSH conjugated xenobiotics and endobiotics from the intracellular compartment into extracellular medium. This can protect cells from the toxic effects of xenobiotics and endobiotics. Therefore, screening the substrates and inhibitors of MRPs could point out the physiological function for each member of MRPs. Also, this could give information on toxicity and efficacy of individual drug. Modulation of MRPs activity seems to be significant to find new mechanism of drug-drug interaction and optimize drug bioavailability.

In addition to playing an important role in drug excretion through the bile, MRPs serve as protective shields by preventing uptake or facilitating clearance of toxic substances in the liver. Anti-toxic effects of MRP1-3 have been studied in more details. MRP2 (ABCC2) is involved in hepato-biliary excretion of GSH conjugates of inorganic arsenic and its chemical derivatives. In addition, some food-derived carcinogens and pre-carcinogens and their glucuronide conjugates are also transported by MRP2 (ABCC2), MRP1 (ABCC1) and MRP3 (ABCC3) may also contribute to the toxicological defense function by eliminating a number of toxic agents and their conjugates from epithelial tissues. It has been observed, that MRP3 (ABCC3) expression is strongly upregulated in the liver of the MRP2 (ABCC2) deficient patients and animals implying that basolateral MRP1 and MRP3-mediated efflux of toxicants may become of pivotal importance when administering MRP2-interacting drugs. ABC pumps play important function in the homeostasis of their own endogenous substrates. At pharmacological blockade of the transport, endogenous substrates may cause toxicity and adverse effects. MRP2 (ABCC2), which transports sulfated bile salts as well as bilirubin conjugates, and MDR3 (ABCB4), the phosphatidyl choline flippase, in particular carry important functions, therefore full or partial blockade of these proteins may evoke toxicity and adverse effects.

10. Pharmacogenetics of MRPs

In vitro site-directed mutagenesis studies indicate that mutants of MRPs may exhibit an altered substrate specificity, plasma membrane trafficking, ATP binding and transport activity (*12*,*273*-*275*). The replacement of Glu^{1089} with a neutral or positive charged amino acid reduced or completely eliminated the anthracycline resistance of MRP1 without influencing transport of LTC_4 and $E_2$17βG (*12*). Substitution of the aromatic residue (Trp^{653} in NBD1 and Tyr^{1302} in NBD2) with a polar cysteine residue, such as W653C or Y1302C, decreased the affinity for ATP, resulting in greatly increased K_d values for ATP binding or K_m values for ATP in ATP-dependent LTC_4 transport (*273*). In addition, the mutation N597A near transmembrane helix increased and decreased resistance to vincristine and VP-16, respectively, while S605A decreased resistance to vincristine, VP-16 and doxorubicin and S604A selectively increased $E_2$17βG transport (*274*).

A number of mutations in MRP1 have been found in different ethnic populations (Table 2), but these are not associated with any known genetic diseases. Nevertheless, some of these *MRP1* mutations may be associated with altered drug disposition. Substitution of Arg^{433} with Ser predicted to be close to TM8 of MRP1 caused by the low frequency G1299T polymorphism in exon 10 leads to a substrate selective change in organic anion transport activity and drug resistance using MRP1-expressing HeLa cells (*276*) or human leukemia CEM-7A cells (*277*). The 128C *MRP1* polymorphism in exon 2 resulting in Cys43Ser substitution disrupted plasma membrane trafficking and reduced resistance to doxorubicin, vincristine and arsenite in HeLa cells expressing this MRP1 mutant while the transport of conjugated organic anion remained comparable to wild type MRP1 (*278*,*279*). Further studies are needed to explore the pharmacological role of *MRP1* polymorphism in humans.

Spontaneous mutant strains of hyperbilirubinemic rat, the Groningen yellow/transport deficient Wistar rat and the Eisai hyperbilirubinemic Sprague-Dawley rat are deficient in biliary excretion of bilirubin glucuronides and glucuronide and glutathione conjugates of xenobiotics due to mutations of *Mrp2*

Table 2. Important single nucleotide polymorphisms (SNPs) of *MRP* genes

MRP genes	Chromosomal location	Amino acid variation	Nucleotide variation	Location	Reference
MRP1	16p13.11 - p13.12	Cys43Ser	G128C	Exon2	*287*
		Thr73Ile	C218T	Exon2	
		Arg433Ser	G1299T	Exon10	*276*
		Gly671Val	G2012T	Exon16	*307*
		Arg723Gln	G2168A	Exon17	*287*
		Arg1058Gln	G3173A	Exon23	*287*
MRP2	10q23 - 24		C-24T	Promoter	*118*, *287*
		Val417Ile	G1249A	Exon10	*118*, *286*, *287*
		Gly676Arg	G2026C	Exon16	*285*
		Try709Arg	T2125C	Exon17	*284*
		Arg768Trp	C2302T	Exon18	*118*, *286*, *287*
		Ser789Phe	C2366T	Exon18	
		I1173F	A3517T	Exon25	*288*
		R1150H	G3449A	Exon25	
		Ile1324Ile	C3972T	Exon28	*118*, *287*
		Ala1450Thr	G4348A	Exon31	*118*, *286*, *287*
MRP3	17q21.3	Lys13Asn	G39GC	Exon1	*290*
		His68Tyr	C202T	Exon2	
		Ser346Phe	C1037T	Exon9	
		Gln513Lys	C1537A	Exon12	
		Arg1297His	G3890A	Exon27	
		Gly1423Arg	G4267A	Exon29	
MRP4	13q32.1	Unknown	Unknown	Unknown	
MRP5	3q27	Unknown	Unknown	Unknown	
MRP6	16p13.1	L63L	G189G > C	Exon2	*299*
		W64R	190T > C	Exon2	
		T364R	1091C > G	Exon9	*308*, *309*
		Q378X	1132C > T	Exon9	
		R518X	1552 C > T	Exon12	*296*, *310*
		R518Q	1553G > A	Exon12	
		R1141X	3421C > T	Exon24	*295*, *296*
		R1138Q	3413G > A	Exon24	
		T1130M	3389C > T	Exon24	
		R1114C	3340C > T	Exon24	
		M1127T	3380C > T	Exon24	
		R1275X	3823C > T	Exon27	*295*
		P1346S	4036C > T	Exon28	*295*
		E1400K	4198G > A	Exon29	*296*
MRP7	6p12 - 21	Unknown	Unknown	Unknown	
MRP8	16q12.1	Unknown	Unknown	Unknown	
MRP9	16q12.1	Unknown	Unknown	Unknown	

gene (*280-282*). Such mutations in the *Mrp2* gene cause premature termination codons. Cloning of *mrp2* has made possible an understanding of its structure-function relationships, localization and regulation of expression, and characterization of the defect in patients with the Dubin-Johnson Syndrome (DJS). Mutations of MRP2 are responsible for DJS, which is characterized with impairment of hepatobiliary elimination of organic anions such as conjugated hyperbilirubinaemia, increased urinary coproporphyrin I fraction (> 80%), and deposition of melanin-like pigment in the liver (*105*,*282*,*283*). Patients with DJS may also have a decreased biliary clearance of bromosulfophthalein and some degree of jaundice (*105*). The absence of functional MRP2 is the molecular basis of transport defect of DJS (*283*). Many single nucleotide polymorphisms in DJS patients have been reported (Table 2) (*118*,*284-288*). These include C-24T (promoter), G1249A (exon 10), G2026C (exon 16), T2125C (exon 17), C2302T (exon 18), C2366 (exon 18), A3517T (exon 25), G3449 (exon 25), C3972T (exon 28) and G4348A (Exon 31) (Table 2). Many of these mutations are localized to NBD1 or NBD2. For instance, G4348A may affect MRP2 function because it is located in the *Walker C* motif within the carboxyl terminal NBD region of MRP2 (*118*). S789F and A1450T which are less frequently than V417I substitution may be more relevant to the *in vivo* function of MRP2 than V417I (*286*). Homozygous mutations lead to classic Dubin-Johnson syndrome, whereas heterozygous mutants have moderately elevated urinary coproporphyrin 1 fraction (~40%) with normal total and direct bilirubin (*105*). Unlike other mutations, R1150H mutants of the MRP2 protein mature and are properly localized, but transport activity is impaired (*288*). In addition, a significant allelic association between the 1249G > A SNP in *MRP2* gene and tenofovir-induced tenofovir-induced proximal

tubulopathy (*289*). Future studies are needed to identify any polymorphisms and their impact on MRP2 function.

Lang *et al*. (*290*) have reported the *MRP3* gene polymorphisms in 103 Caucasians. A total of 51 mutations were identified and 15 SNPs were located in the coding exons of MRP3, six of which are nonsynonymous mutations. The SNPs G39GC (allele frequency = 0.5%, in exon 1), C202T (1.6%, exon 2), C1037T (0.5%, exon 9), C1537A (0.5%, exon 12), G3890A (5.2%, exon 27) and G4267A (0.6%, exon 29) led to Lys13Asn, His68Tyr, Ser346Phe, Gln513Lys, Arg1297His and Gly1423Arg amino acid substitutions, respectively (Table 2). A splice site mutation (G1339-1T) was found at the intron 10-exon 11 boundary. There was a significant correlation of C-211T with *MRP3* mRNA expression, with individuals homozygous and heterozygous for the C-211T promoter polymorphism having significantly lower *MRP3* transcript levels compared to wild-type individuals.

Pseudoxanthoma elasticum (PXE) is an autosomally inherited disorder characterized by accumulation of mineralized and fragmented elastic fibers in the skin, Bruch's membrane in the retina, and vessel walls with abnormalities of collagen and matrix constituents in the soft connective tissues (*291-293*). The ophthalmic and dermatologic expression of PXE and its vascular complications are heterogeneous, with considerable variation in phenotype, progression, and mode of inheritance. Clinical manifestations mainly include coalesced papules and laxity in the flexural areas of skin, retinal angioid streaks and recurrent hemorrhage and vessel alterations similar to those in atherosclerosis (*294*). Lower expression of MRP6 was found in tissues affected by PXE, including skin, retina, and vessel walls. PXE is considered to be caused by mutations in *MRP6*. Small peptides transported by MRP6 in humans may be essential for extracellular matrix deposition or turnover of connective tissue at specific sites in the body.

Mutant alleles of *MRP6* occurred in homozygous, compound heterozygous and heterozygous forms. The great majority of mutations were located from exon 24 to 30, with exon 24 being the most affected (*295-298*). Among the others, exons 2, 9, and 12 were particularly involved (*295,299*). Almost all mutations were located in the intracellular site of MRP6.

A physiological function has only been established for MRP8, for which a single nucleotide polymorphism determines wet *vs* dry earwax type (*189*). However, the constituent of earwax that is susceptible to transport by MRP8 has not been identified. The functional characteristics and its genetic mutations of MRP9 are currently unknown.

Since MRPs are able to transport a wide range of drugs with various structures, the analysis of polymorphisms of these drug transporters may provide a potent tool for improving the risk assessment, prevention, early diagnosis and treatment of diseases. Naturally occurring mutations in MRP/ABCC-related drug transporters have been reported, some of which are non-synonymous single nucleotide polymorphisms (*275*). The consequences of the resulting amino acid changes can sometimes be predicted from *in vitro* site-directed mutagenesis studies or from knowledge of mutations of analogous (conserved) residues in ABCC proteins that cause DJS, PXE (ABCC6), cystic fibrosis (CFTR/ABCC7) or persistent hyperinsulinemic hypoglycemia of infancy (SUR1/ABCC8) (*275*). Polymorphisms of MRPs could be recognized as an important source of interindividual variability of pharmacokinetics and pharmacodynamics of many drugs. Also, this could help to establish a more powerful patient orientated drug therapy against severe adverse effects and for better therapeutic outcome. Eventually, this may provide a powerful tool for drug development, particularly for those with a narrow therapeutic window, such as anticancer drugs.

11. MRPs as potential therapeutic targets in multidrug resistance

PgP-mediated or classic multidrug resistance (MDR), which was identified in the 1970s, is a well-characterized experimental phenomenon. Classic MDR is characterized by: a) cross-resistance between a series of chemically unrelated drugs, b) decreased drug accumulation in cancer cells, c) increased expression of PgP, and d) reversal of the phenotype by a variety of different compounds (*300*). The drugs most often involved in PgP-mediated MDR are of fungal or plant origin, including the anthracyclines (*e.g*. primarily daunorubicin and doxorubicin) and vinca alkaloids. Apart from drugs within these groups, a number of other, nonrelated compounds are able to induce PgP-mediated MDR (*e.g*., epipodophyllotoxins, actinomycin D, colchicine, the taxanes, and the anthracenedione derivatives (*300*). All these drugs are hydrophobic, and most are weak bases.

MRP members play an important role in cancer chemotherapy. The differences in substrate selectivity, organ distribution, and membrane localization of these pumps play major function in related cancer drug transports. The knowledge about the mechanism of drug resistance may be useful in predicting the human response of chemotherapy. The overlapping substrates range of MRPs may have significant contributions for the clinical use of modulators aimed to block the resistant activity of pumps and increase the intracellular drug levels.

Most compounds that efficiently block PgP have only low affinity for MRP1, MRP2 and other MRPs. Despite that there are only a few effective and specific MRP inhibitors available, drug targeting of these transporters may play a role in cancer chemotherapy and in the pharmacokinetics of substrate drugs (*301*). The perfect reversing agent is efficient, lacks unrelated pharmacological effects, shows no pharmacokinetic interactions with other drugs, tackles specific

mechanisms of resistance with high potency and is readily administered to patients. Selective down-regulation of resistance genes in cancer cells by antisense or interfering RNA is an emerging approach in therapeutics. Because there is sufficient evidence to implicate several MRPs as negative prognostic markers during cancer chemotherapy, the pharmacological reversal of MRP1 function becomes a possible approach for overcoming tumor resistance. Disulfiram, a drug approved for use in treating alcoholism, reverses either MDR1- or MRP1-mediated efflux of fluorescent drug substrates *via* inhibiting ATP hydrolysis and the binding of [α-^{32}P]8-azidoATP to P-glycoprotein and MRP1 (*302*).

Design of novel anticancer agents that evade transporter-mediated efflux is a potential approach to avoid multidrug resistance. Epothilones are novel microtubule-targeting agents with a paclitaxel-like mechanism of action that are not recognized by PgP, providing proof of the concept that new classes of anticancer agents that do not interact with the multidrug transporters can be developed to improve response to therapy. As most anticancer agents subject to efflux are currently irreplaceable in chemotherapy regimens, an attractive solution would be to chemically modify their susceptibility to being transported while retaining antineoplastic activity. Although such modifications frequently decrease the bioavailability or efficacy of drugs, some novel agents have been developed using this approach (*303*). The intracellular concentration of drugs can also be elevated by increasing the rate of influx by improving the formulation. Encapsulation of doxorubicin in polyethylene glycol-coated liposomes might be safer and occasionally more effective than conventional doxorubicin (*304*). Overexpression of ABC transporters, particularly PgP, BCRP and MRPs, has consistently been implicated as a cause for MDR both *in vitro* and *in vivo*.

New and effective strategies are needed to engage, evade or exploit these transporters to improve cancer therapy.

12. Conclusions and future directions

MRPs which belong to the ABC transporter family are able to transport a remarkable array of diverse endo- and xenobiotics and their metabolites. MRP1, MRP2 and MRP3 are lipophilic anion transporters with similar substrate ranges and confer resistance to some natural compounds and methotrexate. MRP4, MRP5, and MRP8 are cyclic nucleotide transporters. Each member of MRP family has its own specified substrates. Notably, the 190 kDa MRP and PgP only have 15% the same amino acid and differ greatly in many aspects. Substrates for PgP are mainly neutral or mildly positive lipophilic compounds, while MRP is able to pump conjugated organic anions and neutral organic compounds.

Differences in substrate range, subcellular localization, expression profiles and kinetic parameters of transport dictate distinct physiological functions for MRPs (*4*). For example, MRP1 is distinguished from MRP2 and MRP3 by its higher affinity for LTC_4, a feature that is reflected in the specific role that MRP1 plays in mediating immune responses involving cellular export of this cystinyl leukotriene (*41*). By contrast with MRP1, MRP2 is primarily expressed at canalicular (apical) surfaces of hepatocytes where it functions in the extrusion of endogenous organic anions such as bilirubin glucuronide and certain anticancer agents and in the provision of the biliary fluid constituent glutathione. In addition to the transport of glutathione and glucuronate conjugates, MRP3 has the additional capability of mediating the transport of monoanionic bile acids. The latter feature, in combination with its induction at basolateral surfaces of hepatocytes and cholangiocytes under cholestatic conditions, support the notion that it functions as a compensatory backup mechanism to eliminate from these cells potentially toxic compounds that are ordinarily excreted into the bile. With regard to drug-resistance capabilities, MRP1, MRP2, and MRP3 are able to confer cellular resistance to natural product agents to varying extents, and all three pumps are potent methotrexate resistance factors (*9*). Recent investigations of MRP4 and MRP5 indicate that they have the facility for mediating the transport of cyclic nucleotides, a property that has implicated the two pumps in the regulation of intracellular levels of these second messengers as well as in the cellular extrusion of cAMP involved in intercellular signalling (*4*). In accord with their capacity to transport cyclic nucleotides, MRP4 and MRP5 have the facility for conferring resistance to certain antiviral and anticancer nucleotide analogs but do not seem to be capable of effluxing natural product agents (*9*). MRP6, whose hereditary deficiency results in PXE, a disease that affects elastic tissues in the skin, eyes, and cardiovascular system, has recently been determined to be competent in the transport of glutathione conjugates and the cyclic pentapeptide BQ123 (*182*). MRP7 was able to catalyze the MgATP-energized transport of the glucuronide $E_2$17βG. By comparison with $E_2$17βG, only modest transport was observed for LTC_4, and transport of a range of other compounds that are established substrates of other MRP family members was not detected to any extent (*184*). Further studies are needed to elucidate the clinical, pharmacological and toxicological relevance of all these MRPs.

Interindividual differences of drug response are an important cause of treatment failures and adverse drug reactions. The identification of polymorphisms explaining distinct phenotypes of drug metabolizing enzymes contributed in part to the understanding of individual variations of drug plasma levels. However, bioavailability also depends on a major extent from the expression and activity of drug transport across cellular membranes. In particular, the ABC family such as PgP/ABCB1, MRPs and BCRP/ABCG2 have been identified as major determinants of chemoresistance in

tumor cells. They are expressed in the apical membranes of many barrier tissues such as the intestine, liver, blood-brain barrier, kidney, placenta, testis and in lymphocytes, thus contributing to plasma, liquor, but also intracellular drug disposition (*305*). Since expression and function exhibit a broad variability, it was hypothesized that hereditary variances in the genes of ABC transporters could explain at least in part interindividual differences of pharmacokinetics and clinical outcome of a variety of drugs (*275*,*305*). The pharmacogenetic studies on MRPs including the single nucleotide polymorphism may provide powerful tools for drug development. Studies on the functions of MRPs may give more information on drug toxicity and drug-drug interaction. Continual updating of databases of sequence variants and haplotype analysis, together with *in vitro* biochemical validation assays and pharmacological studies in knockout animals, should make it possible to determine how genetic variation in the MRP-related transporters contributes to the range of responses to drugs and chemicals observed in different human populations. However, the mechanisms of MRPs activity and the substrates of some members of MRP family are unclear. In the future, we need to do more molecular, proteomic and genetic studies on MRPs to identify the regulation mechanism for individual MRPs.

The ability of transport proteins including MDR1, BCRP and MRPs to reduce oral bioavailability and alter tissue distribution has obvious implications for drug design (*306*). Indeed, the identification of transporters that influence the disposition and safety of drugs has become a new challenge for drug discovery programmes. It is essential to know, first, whether drugs can freely cross pharmacological barriers or whether their passage is restricted by ABC transporters; and, second, whether drugs can influence the passage of other compounds through the inhibition of ABC transporters. Consequently, the evaluation of transport susceptibility of drug candidates has become an important step in the development of novel therapeutics, and the pharmaceutical industry has adopted routine evaluation of PgP susceptibility in the drug discovery process. In the early stages of drug development, it is important to identify drugs as substrates, inducers, inhibitors, or modulators for MRPs, as this may help to avoid drug toxicity, drug resistance and drug-drug interactions and to optimize cancer chemotherapy. The identification always involves the application of proper models and probes, such as *in vitro* (*e.g*. purified MRP protein or MRP-overexpressing cells) and *in vivo* models.

References

1. Locher KP. Structure and mechanism of ABC transporters. Curr Opin Struct Biol 2004; 14:426-443.
2. Tian Q, Zhang J, Chan E, Duan W, Zhou SF. Multidrug resistance proteins (MRPs) and implication in drug development. Drug Dev Res 2005; 51:1-18.
3. Piddock LJ. Multidrug - resistance efflux pumps - not just for resistance. Nat Rev Microbiol 2006; 4:629-636.
4. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002; 71:537-592.
5. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 2001; 11:1156-1166.
6. Müller M. 49 Human ATP-Binding Cassette Transporters. *http://www.nutrigene.4t.com/humanabc.htm*. (access date: December 5, 2008).
7. Jones PM, George AM. The ABC transporter structure and mechanism: perspectives on recent research. Cell Mol Life Sci 2004; 61:682-699.
8. Klein I, Sarkadi B, Váradi A. An inventory of the human ABC proteins. Biochim Biophys Acta 1999; 1461:237-262.
9. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 2000; 92:1295-1302.
10. Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. Embo J 1982; 1:945-951.
11. Chang G, Roth CB. Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science 2001; 293:1793-1800.
12. Zhang DW, Cole SP, Deeley RG. Identification of a nonconserved amino acid residue in multidrug resistance protein 1 important for determining substrate specificity: evidence for functional interaction between transmembrane helices 14 and 17. J Biol Chem 2001; 276:34966-34974.
13. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992; 8:67-113.
14. Altenberg GA. Structure of multidrug-resistance proteins of the ATP-binding cassette (ABC) superfamily. Curr Med Chem Anticancer Agents 2004; 4:53-62.
15. Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. Oncogene 2003; 22:7537-7552.
16. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 2001; 11:1156-1166.
17. Belinsky MG, Bain LJ, Balsara BB, Testa JR, Kruh GD. Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. J Natl Cancer Inst 1998; 90:1735-1741.
18. Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. Cancer Lett 2001; 162:181-191.
19. Bera TK, Lee S, Salvatore G, Lee B, Pastan I. MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Mol Med 2001; 7:509-516.
20. Tammur J, Prades C, Arnould I, Rzhetsky A, Hutchinson A, Adachi M, Schuetz JD, Swoboda KJ, Ptácek LJ, Rosier M, Dean M, Allikmets R. Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. Gene 2001; 273:89-96.
21. Yabuuchi H, Shimizu H, Takayanagi S, Ishikawa T. Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. Biochem

Biophys Res Commun 2001; 288:933-939.
22. Bakos E, Evers R, Szakács G, Tusnády GE, Welker E, Szabó K, de Haas M, van Deemter L, Borst P, Váradi A, Sarkadi B. Functional multidrug resistance protein (MRP1) lacking the *N*-terminal transmembrane domain. J Biol Chem 1998; 273: 32167-32175.
23. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1992; 258:1650-1654.
24. Cole SP, Deeley RG. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. Bioessays 1998; 20:931-940.
25. Zaman GJ, Versantvoort CH, Smit JJ, Eijdems EW, de Haas M, Smith AJ, Broxterman HJ, Mulder NH, de Vries EG, Baas F, Borst P. Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. Cancer Res 1993; 53:1747-1750.
26. Burger H, Nooter K, Zaman GJ, Sonneveld P, van Wingerden KE, Oostrum RG, Stoter G. Expression of the multidrug resistance-associated protein (MRP) in acute and chronic leukemias. Leukemia 1994; 8:990-997.
27. Stride BD, Valdimarsson G, Gerlach JH, Wilson GM, Cole SP, Deeley RG. Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter. Mol Pharmacol 1996; 49:962-971.
28. Flens MJ, Zaman GJ, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJ, Scheper RJ. Tissue distribution of the multidrug resistance protein. Am J Pathol 1996; 148:1237-1247.
29. Wijnholds J, Scheffer GL, van der Valk M, van der Valk P, Beijnen JH, Scheper RJ, Borst P. Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. J Exp Med 1998; 188:797-808.
30. Qian YM, Song WC, Cui H, Cole SP, Deeley RG. Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1. J Biol Chem 2001; 276:6404-6411.
31. Leslie EM, Deeley RG, Cole SP. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology 2001; 167:3-23.
32. Lautier D, Canitrot Y, Deeley RG, Cole SP. Multidrug resistance mediated by the multidrug resistance protein (*MRP*) gene. Biochem Pharmacol 1996; 52:967-977.
33. Hammond CL, Marchan R, Krance SM, Ballatori N. Glutathione export during apoptosis requires functional multidrug resistance-associated proteins. J Biol Chem 2007; 282:14337-14347.
34. Leier I, Jedlitschky G, Buchholz U, Center M, Cole SP, Deeley RG, Keppler D. ATP-dependent glutathione disulphide transport mediated by the *MRP* gene-encoded conjugate export pump. Biochem J 1996; 314:433-437.
35. Loe DW, Almquist KC, Cole SP, Deeley RG. ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids. J Biol Chem 1996; 271:9683-9689.
36. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the *MRP* gene-encoded conjugate export pump. Cancer Res 1996; 56:988-994.
37. Rigato I, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C. The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. Biochem J 2004; 383:335-341.
38. Evers R, Cnubben NH, Wijnholds J, van Deemter L, van Bladeren PJ, Borst P. Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. FEBS Lett 1997; 419:112-116.
39. Akimaru K, Kuo MT, Furuta K, Suzuki M, Noyori R, Ishikawa T. Induction of MRP/GS-X pump and cellular resistance to anticancer prostaglandins. Cytotechnology 1996; 19:221-227.
40. Robbiani DF, Finch RA, Jäger D, Muller WA, Sartorelli AC, Randolph GJ. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. Cell 2000; 103:757-768.
41. Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P. Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat Med 1997; 3:1275-1279.
42. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 1994; 54:5902-5910.
43. Zaman GJ, Flens MJ, van Leusden MR, de Haas M, Mülder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci U S A 1994; 91:8822-8826.
44. Versantvoort CH, Broxterman HJ, Bagrij T, Scheper RJ, Twentyman PR. Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. Br J Cancer 1995; 72:82-89.
45. Zaman GJ, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RP, Baas F, Borst P. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. Proc Natl Acad Sci U S A 1995; 92:7690-7694.
46. Rappa G, Lorico A, Flavell RA, Sartorelli AC. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. Cancer Res 1997; 57:5232-5237.
47. Cole SP, Downes HF, Mirski SE, Clements DJ. Alterations in glutathione and glutathione-related enzymes in a multidrug-resistant small cell lung cancer cell line. Mol Pharmacol 1990; 37:192-197.
48. Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, Keppler D. The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. J Biol Chem 1994; 269:27807-27810.
49. Müller M, Meijer C, Zaman GJ, Borst P, Scheper RJ, Mulder NH, de Vries EG, Jansen PL. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATPdependent glutathione *S*-conjugate transport. Proc Natl Acad Sci U S A 1994; 91:13033-13037.
50. Loe DW, Deeley RG, Cole SP. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res 1998; 58:5130-5136.
51. Salerno M, Garnier-Suillerot A. Kinetics of glutathione and daunorubicin efflux from multidrug resistance protein overexpressing small-cell lung cancer cells. Eur J

Pharmacol 2001; 421:1-9.
52. Leslie EM, Deeley RG, Cole SP. Bioflavonoid stimulation of glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). Drug Metab Dispos 2003; 31:11-15.
53. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP. Transport of the beta-*O*-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog. J Biol Chem 2001; 276:27846-27854.
54. Yang Z, Horn M, Wang J, Shen DD, Ho RJ. Development and characterization of a recombinant Madin-Darby canine kidney cell line that expresses rat multidrug resistance-associated protein 1 (rMRP1). AAPS PharmSci 2004; 6:E8.
55. Nunoya K, Grant CE, Zhang D, Cole SP, Deeley RG. Molecular cloning and pharmacological characterization of rat multidrug resistance protein 1 (mrp1). Drug Metab Dispos 2003; 31:1016-1026.
56. Leslie EM, Haimeur A, Waalkes MP. Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. J Biol Chem 2004; 279:32700-32708.
57. Ito K, Olsen SL, Qiu W, Deeley RG, Cole SP. Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport. J Biol Chem 2001; 276:15616-15624.
58. Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Res 2001; 61:7225-7232.
59. Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res 1999; 59:2532-2535.
60. Chu XY, Suzuki H, Ueda K, Kato Y, Akiyama S, Sugiyama Y. Active efflux of CPT-11 and its metabolites in human KB-derived cell lines. J Pharmacol Exp Ther 1999; 288:735-741.
61. Morrow CS, Smitherman PK, Diah SK, Schneider E, Townsend AJ. Coordinated action of glutathione *S*-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. J Biol Chem 1998; 273:20114-20120.
62. Morrow CS, Peklak-Scott C, Bishwokarma B, Kute TE, Smitherman PK, Townsend AJ. Multidrug resistance protein 1 (MRP1, ABCC1) mediates resistance to mitoxantrone *via* glutathione-dependent drug efflux. Mol Pharmacol 2006; 69:1499-1505.
63. Paumi CM, Ledford BG, Smitherman PK, Townsend AJ, Morrow CS. Role of multidrug resistance protein 1 (MRP1) and glutathione *S*-transferase A1-1 in alkylating agent resistance. Kinetics of glutathione conjugate formation and efflux govern differential cellular sensitivity to chlorambucil *versus* melphalan toxicity. J Biol Chem 2001; 276:7952-7956.
64. Barnouin K, Leier I, Jedlitschky G, Pourtier-Manzanedo A, König J, Lehmann WD, Keppler D. Multidrug resistance protein-mediated transport of chlorambucil and melphalan conjugated to glutathione. Br J Cancer 1998; 77:201-209.
65. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. Disruption of the murine *MRP* (*multidrug resistance protein*) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. Cancer Res 1997; 57:5238-5242.
66. Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RP. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. Biochem J 1999; 338:393-401.
67. Meaden ER, Hoggard PG, Newton P, Tjia JF, Aldam D, Cornforth D, Lloyd J, Williams I, Back DJ, Khoo SH. P-glycoprotein and MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals. J Antimicrob Chemother 2002; 50:583-588.
68. Williams GC, Liu A, Knipp G, Sinko PJ. Direct evidence that saquinavir is transported by multidrug resistance-associated protein (MRP1) and canalicular multispecific organic anion transporter (MRP2). Antimicrob Agents Chemother 2002; 46:3456-3462.
69. Dallas S, Ronaldson PT, Bendayan M, Bendayan R. Multidrug resistance protein 1-mediated transport of saquinavir by microglia. Neuroreport 2004; 15:1183-1186.
70. Grzywacz MJ, Yang JM, Hait WN. Effect of the multidrug resistance protein on the transport of the antiandrogen flutamide. Cancer Res 2003; 63:2492-2498.
71. Zaman GJ, Cnubben NH, van Bladeren PJ, Evers R, Borst P. Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP. FEBS Lett 1996; 391:126-130.
72. Hendrikse NH, Kuipers F, Meijer C, Havinga R, Bijleveld CM, van der Graaf WT, Vaalburg W, de Vries EG. *In vivo* imaging of hepatobiliary transport function mediated by multidrug resistance associated protein and P-glycoprotein. Cancer Chemother Pharmacol 2004; 54:131-138.
73. Chen WS, Luker KE, Dahlheimer JL, Pica CM, Luker GD, Piwnica-Worms D. Effects of MDR1 and MDR3 P-glycoproteins, MRP1, and BCRP/MXR/ABCP on the transport of (99m)Tc-tetrofosmin. Biochem Pharmacol 2000; 60:413-426.
74. Lorusso V, Pascolo L, Fernetti C, Visigalli M, Anelli P, Tiribelli C. *In vitro* and *in vivo* hepatic transport of the magnetic resonance imaging contrast agent B22956/1: role of MRP proteins. Biochem Biophys Res Commun 2002; 293:100-105.
75. Loe DW, Stewart RK, Massey TE, Deeley RG, Cole SP. ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (*MRP*) gene. Mol Pharmacol 1997; 51:1034-1041.
76. Geisler M, Girin M, Brandt S, Vincenzetti V, Plaza S, Paris N, Kobae Y, Maeshima M, Billion K, Kolukisaoglu UH, Schulz B, Martinoia E. Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. Mol Biol Cell 2004; 15:3393-3405.
77. Diah SK, Smitherman PK, Townsend AJ, Morrow CS. Detoxification of 1-chloro-2,4-dinitrobenzene in MCF7 breast cancer cells expressing glutathione *S*-transferase P1-1 and/or multidrug resistance protein 1. Toxicol Appl Pharmacol 1999; 157:85-93.
78. Morrow CS, Diah S, Smitherman PK, Schneider E, Townsend AJ. Multidrug resistance protein and glutathione *S*-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. Carcinogenesis 1998;

19:109-115.
79. Lorico A, Nesland J, Emilsen E, Fodstad O, Rappa G. Role of the multidrug resistance protein 1 gene in the carcinogenicity of aflatoxin B1: investigations using mrp1-null mice. Toxicology 2002; 171:201-205.
80. Johnson DR, Finch RA, Lin ZP, Zeiss CJ, Sartorelli AC. The pharmacological phenotype of combined multidrug-resistance mdr1a/1b- and mrp1-deficient mice. Cancer Res 2001; 61:1469-1476.
81. Gollapudi S, Kim CH, Tran BN, Sangha S, Gupta S. Probenecid reverses multidrug resistance in multidrug resistance-associated protein-overexpressing HL60/AR and H69/AR cells but not in P-glycoprotein-overexpressing HL60/Tax and P388/ADR cells. Cancer Chemother Pharmacol 1997; 40:150-158.
82. Draper MP, Martell RL, Levy SB. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. Br J Cancer 1997; 75:810-815.
83. Bakos E, Evers R, Sinkó E, Váradi A, Borst P, Sarkadi B. Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. Mol Pharmacol 2000; 57:760-768.
84. Barrand MA, Rhodes T, Center MS, Twentyman PR. Chemosensitisation and drug accumulation effects of cyclosporin A, PSC-833 and verapamil in human MDR large cell lung cancer cells expressing a 190k membrane protein distinct from P-glycoprotein. Eur J Cancer 1993; 29A:408-415.
85. Hooijberg JH, Broxterman HJ, Heijn M, Fles DL, Lankelma J, Pinedo HM. Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. FEBS Lett 1997; 413:344-348.
86. Hooijberg JH, Broxterman HJ, Scheffer GL, Vrasdonk C, Heijn M, de Jong MC, Scheper RJ, Lankelma J, Pinedo HM. Potent interaction of flavopiridol with MRP1. Br J Cancer 1999; 81:269-276.
87. Versantvoort CH, Broxterman HJ, Lankelma J, Feller N, Pinedo HM. Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. Biochem Pharmacol 1994; 48:1129-1136.
88. Nguyen H, Zhang S, Morris ME. Effect of flavonoids on MRP1-mediated transport in Panc-1 cells. J Pharm Sci 2003; 92:250-257.
89. Marbeuf-Gueye C, Salerno M, Quidu P, Garnier-Suillerot A. Inhibition of the P-glycoprotein- and multidrug resistance protein-mediated efflux of anthracyclines and calceinacetoxymethyl ester by PAK-104P. Eur J Pharmacol 2000; 391:207-216.
90. Aoki S, Chen ZS, Higasiyama K, Setiawan A, Akiyama S, Kobayashi M. Reversing effect of agosterol A, a spongean sterol acetate, on multidrug resistance in human carcinoma cells. Jpn J Cancer Res 2001; 92:886-895.
91. Bandi N, Kompella UB. Budesonide reduces multidrug resistance-associated protein 1 expression in an airway epithelial cell line (Calu-1). Eur J Pharmacol 2002; 437:9-17.
92. Payen L, Delugin L, Courtois A, Trinquart Y, Guillouzo A, Fardel O. Reversal of MRP-mediated multidrug resistance in human lung cancer cells by the antiprogestatin drug RU486. Biochem Biophys Res Commun 1999; 258:513-518.
93. Naito S, Koike K, Ono M, Machida T, Tasaka S, Kiue A, Koga H, Kumazawa J. Development of novel reversal agents, imidazothiazole derivatives, targeting MDR1- and MRP-mediated multidrug resistance. Oncol Res 1998; 10:123-132.
94. Weiss J, Theile D, Ketabi-Kiyanvash N, Lindenmaier H, Haefeli WE. Inhibition of MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 by nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors. Drug Metab Dispos 2007; 35:340-344.
95. Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995; 208:345-352.
96. Nakano R, Oka M, Nakamura T, Fukuda M, Kawabata S, Terashi K, Tsukamoto K, Noguchi Y, Soda H, Kohno S. A leukotriene receptor antagonist, ONO-1078, modulates drug sensitivity and leukotriene C_4 efflux in lung cancer cells expressing multidrug resistance protein. Biochem Biophys Res Commun 1998; 251:307-312.
97. Payen L, Delugin L, Courtois A, Trinquart Y, Guillouzo A, Fardel O. The sulphonylurea glibenclamide inhibits multidrug resistance protein (MRP1) activity in human lung cancer cells. Br J Pharmacol 2001; 132:778-784.
98. Mao Q, Qiu W, Weigl KE, Lander PA, Tabas LB, Shepard RL, Dantzig AH, Deeley RG, Cole SP. GSH-dependent photolabeling of multidrug resistance protein MRP1 (ABCC1) by [^{125}I]LY475776. Evidence of a major binding site in the COOH-proximal membrane spanning domain. J Biol Chem 2002; 277:28690-28699.
99. Norman BH, Dantzig AH, Kroin JS, Law KL, Tabas LB, Shepard RL, Palkowitz AD, Hauser KL, Winter MA, Sluka JP, Starling JJ. Reversal of resistance in multidrug resistance protein (MRP1)-overexpressing cells by LY329146. Bioorg Med Chem Lett 1999; 9:3381-3386.
100. Qian YM, Grant CE, Westlake CJ, Zhang DW, Lander PA, Shepard RL, Dantzig AH, Cole SP, Deeley RG. Photolabeling of human and murine multidrug resistance protein 1 with the high affinity inhibitor [^{125}I]LY475776 and azidophenacyl-[^{35}S]glutathione. J Biol Chem 2002; 277:35225-35231.
101. Stewart AJ, Canitrot Y, Baracchini E, Dean NM, Deeley RG, Cole SP. Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. Biochem Pharmacol 1996; 51:461-469.
102. Peaston AE, Gardaneh M, Franco AV, Hocker JE, Murphy KM, Farnsworth ML, Catchpoole DR, Haber M, Norris MD, Lock RB, Marshall GM. *MRP1* gene expression level regulates the death and differentiation response of neuroblastoma cells. Br J Cancer 2001; 85:1564-1571.
103. Niewiarowski W, Gendaszewska E, Rebowski G, Wójcik M, Mikołajczyk B, Goss W, Soszyński M, Bartosz G. Multidrug resistance-associated protein--reduction of expression in human leukaemia cells by antisense phosphorothioate oligonucleotides. Acta Biochim Pol 2000; 47:1183-1188.
104. Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH, Borst P. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J Clin Invest 1998; 101:1310-1319.
105. Toh S, Wada M, Uchiumi T, Inokuchi A, Makino Y, Horie Y, Adachi Y, Sakisaka S, Kuwano M. Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin-Johnson syndrome. Am J Human Genet 1999; 64:739-746.

106. Keppler D, Leier I, Jedlitschky G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. Biol Chem 1997; 378:787-791.
107. Masuda M, I'izuka Y, Yamazaki M, Nishigaki R, Kato Y, Ni'inuma K, Suzuki H, Sugiyama Y. Methotrexate is excreted into the bile by canalicular multispecific organic anion transporter in rats. Cancer Res 1997; 57:3506-3510.
108. Keppler D, Kartenbeck J. The canalicular conjugate export pump encoded by the cmrp/cmoat gene. Prog Liver Dis 1996; 14:55-67.
109. Cui Y, König J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999; 55:929-937.
110. Kawabe T, Chen ZS, Wada M, Uchiumi T, Ono M, Akiyama S, Kuwano M. Enhanced transport of anticancer agents and leukotriene C_4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). FEBS Lett 1999; 456:327-331.
111. Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res 1997; 57:5475-5479.
112. Xiong H, Turner KC, Ward ES, Jansen PL, Brouwer KL. Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR(-) rats. J Pharmacol Exp Ther 2000; 295:512-518.
113. Evers R, de Haas M, Sparidans R, Beijnen J, Wielinga PR, Lankelma J, Borst P. Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. Br J Cancer 2000; 83:375-383.
114. Konno T, Ebihara T, Hisaeda K, Uchiumi T, Nakamura T, Shirakusa T, Kuwano M, Wada M. Identification of domains participating in the substrate specificity and subcellular localization of the multidrug resistance proteins MRP1 and MRP2. J Biol Chem 2003; 278:22908-22917.
115. Rebbeor JF, Connolly GC, Henson JH, Boyer JL, Ballatori N. ATP-dependent GSH and glutathione *S*-conjugate transport in skate liver: role of an Mrp functional homologue. Am J Physiol Gastrointest Liver Physiol 2000; 279:G417-425.
116. Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RP. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. Biochem J 1999; 338:393-401.
117. Xiong H, Suzuki H, Sugiyama Y, Meier PJ, Pollack GM, Brouwer KL. Mechanisms of impaired biliary excretion of acetaminophen glucuronide after acute phenobarbital treatment or phenobarbital pretreatment. Drug Metab Dispos 2002; 30:962-969.
118. Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. Adv Drug Deliv Rev 2002; 54:1311-1331.
119. Sasaki M, Suzuki H, Ito K, Abe T, Sugiyama Y. Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and Multidrug resistance-associated protein 2 (MRP2/ABCC2). J Biol Chem 2002; 277:6497-6503.
120. Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, Sugiyama Y. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 2001; 1511:7-16.
121. Kobayashi N, Tani T, Hisaka A, Hara K, Yasumori T. Hepatobiliary transport of a nonpeptidic endothelin antagonist, (+)-(5*S*,6*R*,7*R*)-2-butyl-7-[2((2*S*)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl) cyclopentenol[1,2-*b*]pyridine-6-carboxylic acid: uptake by isolated rat hepatocytes and canalicular membrane vesicles. Pharm Res 2003; 20:89-95.
122. Nakagomi-Hagihara R, Nakai D, Kawai K, Yoshigae Y, Tokui T, Abe T, Ikeda T. OATP1B1, OATP1B3, and mrp2 are involved in hepatobiliary transport of olmesartan, a novel angiotensin II blocker. Drug Metab Dispos 2006; 34:862-869.
123. Takayanagi M, Sano N, Takikawa H. Biliary excretion of olmesartan, an anigotensin II receptor antagonist, in the rat. J Gastroenterol Hepatol 2005; 20:784-788.
124. Huisman MT, Smit JW, Crommentuyn KM, Zelcer N, Wiltshire HR, Beijnen JH, Schinkel AH. Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. Aids 2002; 16:2295-2301.
125. Agarwal S, Pal D, Mitra AK. Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. Int J Pharm 2007; 339:139-147.
126. Hendrikse NH, Franssen EJ, van der Graaf WT, Vaalburg W, de Vries EG. Visualization of multidrug resistance *in vivo*. Eur J Nucl Med 1999; 26:283-293.
127. Srivastava SK, Watkins SC, Schuetz E, Singh SV. Role of glutathione conjugate efflux in cellular protection against benzo[a]pyrene-7,8-diol-9,10-epoxide-induced DNA damage. Mol Carcinog 2002; 33:156-162.
128. Terlouw SA, Graeff C, Smeets PH, Fricker G, Russel FG, Masereeuw R, Miller DS. Short- and long-term influences of heavy metals on anionic drug efflux from renal proximal tubule. J Pharmacol Exp Ther 2002; 301:578-585.
129. Dietrich CG, Ottenhoff R, de Waart DR, Oude Elferink RP. Role of MRP2 and GSH in intrahepatic cycling of toxins. Toxicology 2001; 167:73-81.
130. Bodo A, Bakos E, Szeri F, Varadi A, Sarkadi B. Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. J Biol Chem 2003; 278:23529-23537.
131. Zelcer N, Huisman MT, Reid G, Wielinga P, Breedveld P, Kuil A, Knipscheer P, Schellens JH, Schinkel AH, Borst P. Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). J Biol Chem 2003; 278:23538-23544.
132. Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res 1997; 57:5475-5479.
133. Kiuchi Y, Suzuki H, Hirohashi T, Tyson CA, Sugiyama Y. cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3). FEBS Lett 1998; 433:149-152.
134. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues

of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 1997; 57:3537-3547.
135. Zimmermann C, Gutmann H, Hruz P, Gutzwiller JP, Beglinger C, Drewe J. Mapping of MDR1 and MRP1-5 mRNA expression along the human intestinal tract. Drug Metab Dispos 2005; 33:219-224.
136. Nies AT, Jedlitschky G, König J, Herold-Mende C, Steiner HH, Schmitt HP, Keppler D. Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. Neuroscience 2004; 129:349-360.
137. Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A 1999; 96:6914-6919.
138. Zeng H, Bain LJ, Belinsky MG, Kruh GD. Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. Cancer Res 1999; 59:5964-5967.
139. Zeng H, Liu G, Rea PA, Kruh GD. Transport of amphipathic anions by human multidrug resistance protein 3. Cancer Res 2000; 60:4779-4784.
140. Chu XY, Huskey SE, Braun MP, Sarkadi B, Evans DC, Evers R. Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2, and MRP3. J Pharmacol Exp Ther 2004; 309:156-164.
141. Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P. Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). J Biol Chem 2001; 276:46400-46407.
142. Hirohashi T, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J Biol Chem 1999; 274:15181-15185.
143. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 1997; 57:3537-3547.
144. Young LC, Campling BG, Voskoglou-Nomikos T, Cole SP, Deeley RG, Gerlach JH. Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin Cancer Res 1999; 5:673-680.
145. Ishikawa T. The ATP-dependent glutathione *S*-conjugate export pump. Trends Biochem Sci 1993; 17:463-468.
146. Hirohashi T, Suzuki H, Takikawa H, Sugiyama Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem 2000; 275:2905-2910.
147. Kullak-Ublick GA, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. Gastroenterology 2004; 126:322-342.
148. Ros JE, Libbrecht L, Geuken M, Jansen PL, Roskams TA. High expression of MDR1, MRP1, and MRP3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease. J Pathol 2003; 200:553-560.
149. Zollner G, Fickert P, Silbert D, Fuchsbichler A, Marschall HU, Zatloukal K, Denk H, Trauner M. Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. J Hepatol 2003; 38:717-727.
150. Lai L, Tan TM. Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. Biochem J 2002; 361:497-503.
151. Chen ZS, Lee K, Kruh GD. Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. J Biol Chem 2001; 276:33747-33754.
152. Chen ZS, Lee K, Walther S, Raftogianis RB, Kuwano M, Zeng H, Kruh GD. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res 2002; 62:3144-3150.
153. van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. The *MRP4/ABCC4* gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol 2002; 13:595-603.
154. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 2003; 100:9244-9249.
155. Rius M, Thon WF, Keppler D, Nies AT. Prostanoid transport by multidrug resistance protein 4 (MRP4/ABCC4) localized in tissues of the human urogenital tract. J Urol 2005; 174:2409-2414.
156. Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, Borst P. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). Biochem J 2003; 371:361-367.
157. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. Hepatology 2003; 38:374-384.
158. Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD, Brouwer KL. Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3$^{-/-}$ and Abcc4$^{-/-}$ mice. J Pharmacol Exp Ther 2006; 319:1485-1491.
159. Adachi M, Sampath J, Lan LB, Sun D, Hargrove P, Flatley R, Tatum A, Edwards MZ, Wezeman M, Matherly L, Drake R, Schuetz J. Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. J Biol Chem 2002; 277:38998-39004.
160. Sampath J, Adachi M, Hatse S, Naesens L, Balzarini J, Flatley RM, Matherly LH, Schuetz JD. Role of MRP4 and MRP5 in biology and chemotherapy. AAPS PharmSci 2002; 4:E14.
161. Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. J Natl Cancer Inst 2000; 92:1934-1940.
162. Dallas S, Schlichter L, Bendayan R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-pho sphonylmethoxyethyl)adenine by microglia. J Pharmacol Exp Ther 2004; 309:1221-1229.
163. Imaoka T, Kusuhara H, Adachi M, Schuetz JD, Takeuchi K, Sugiyama Y. Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. Mol Pharmacol 2007; 71:619-627.
164. Van Aubel RA, Smeets PH, van den Heuvel JJ, Russel FG. Human organic anion transporter MRP4 (ABCC4) is

an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites. Am J Physiol Renal Physiol 2005; 288:F327-333.
165. Wielinga PR, Reid G, Challa EE, van der Heijden I, van Deemter L, de Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, Borst P. Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. Mol Pharmacol 2002; 62:1321-1331.
166. Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, Mercer KE, Zhuang Y, Panetta JC, Johnston B, Scheper RJ, Stewart CF, Schuetz JD. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. Mol Cell Biol 2004; 24:7612-7621.
167. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA. Plant antitumor agents: 1, the isolation and structure of camptothecin, anovel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc 1966; 88:3888-3890.
168. Bai J, Lai L, Yeo HC, Goh BC, Tan TM. Multidrug resistance protein 4 (MRP4/ABCC4) mediates efflux of bimane-glutathione. Int J Biochem Cell Biol 2004; 36:247-257.
169. Wielinga PR, van der Heijden I, Reid G, Beijnen JH, Wijnholds J, Borst P. Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. J Biol Chem 2003; 278:17664-17671.
170. Assaraf YG, Sierra EE, Babani S, Goldman ID. Inhibitory effects of prostaglandin A1 on membrane transport of folates mediated by both the reduced folate carrier and ATP-driven exporters. Biochem Pharmacol 1999; 58:1321-1327.
171. Reid G, Wielinga P, Zelcer N, De Haas M, Van Deemter L, Wijnholds J, Balzarini J, Borst P. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003; 63:1094-1103.
172. Schuetz EG, Strom S, Yasuda K, *et al*. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. J Biol Chem 2001; 276:39411-39418.
173. Assem M, Schuetz EG, Leggas M, Sun D, Yasuda K, Reid G, Zelcer N, Adachi M, Strom S, Evans RM, Moore DD, Borst P, Schuetz JD. Interactions between hepatic Mrp4 and Sult2a as revealed by the constitutive androstane receptor and *Mrp4* knockout mice. J Biol Chem 2004; 279:22250-22257.
174. Jorajuria S, Dereuddre-Bosquet N, Becher F, Martin S, Porcheray F, Garrigues A, Mabondzo A, Benech H, Grassi J, Orlowski S, Dormont D, Clayette P. ATP binding cassette multidrug transporters limit the anti-HIV activity of zidovudine and indinavir in infected human macrophages. Antivir Ther 2004; 9:519-528.
175. Wijnholds J, Mol CA, van Deemter L, de Haas M, Scheffer GL, Baas F, Beijnen JH, Scheper RJ, Hatse S, De Clercq E, Balzarini J, Borst P. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc Natl Acad Sci U S A 2000; 97:7476-7481.
176. McAleer MA, Breen MA, White NL, Matthews N. pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. J Biol Chem 1999; 274:23541-23548.
177. Kool M, van der Linden M, de Haas M, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene *MRP1*, in tissues and cancer cells. Cancer Res 1999; 59:175-182.
178. Scheffer GL, Hu X, Pijnenborg AC, Wijnholds J, Bergen AA, Scheper RJ. MRP6 (ABCC6) detection in normal human tissues and tumors. Lab Invest 2002; 82:515-518.
179. Boraldi F, Quaglino D, Croce MA, Garcia Fernandez MI, Tiozzo R, Gheduzzi D, Bacchelli B, Pasquali Ronchetti I. Multidrug resistance protein-6 (MRP6) in human dermal fibroblasts. Comparison between cells from normal subjects and from Pseudoxanthoma elasticum patients. Matrix Biol 2003; 22:491-500.
180. Sinkó E, Iliás A, Ujhelly O, Homolya L, Scheffer GL, Bergen AA, Sarkadi B, Váradi A. Subcellular localization and *N*-glycosylation of human ABCC6, expressed in MDCKII cells. Biochem Biophys Res Commun 2003; 308:263-269.
181. Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). Cancer Res 2002; 62:6172-6177.
182. Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 2000; 57:634-641.
183. Iliás A, Urbán Z, Seidl TL, Le Saux O, Sinkó E, Boyd CD, Sarkadi B, Váradi A. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). J Biol Chem 2002; 277:16860-16867.
184. Chen ZS, Hopper-Borge E, Belinsky MG, Shchaveleva I, Kotova E, Kruh GD. Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). Mol Pharmacol 2003; 63:351-358.
185. Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG, Kruh GD. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. Cancer Res 2004; 64:4927-4930.
186. Oguri T, Bessho Y, Achiwa H, Ozasa H, Maeno K, Maeda H, Sato S, Ueda R. MRP8/ABCC11 directly confers resistance to 5-fluorouracil. Mol Cancer Ther 2007; 6:122-127.
187. Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG, Kruh GD. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl) adenine. J Biol Chem 2003; 278:29509-29514.
188. Chen LM, Wu XP, Ruan JW, Liang YJ, Ding Y, Shi Z, Wang XW, Gu LQ, Fu LW. Screening novel, potent multidrug-resistant modulators from imidazole derivatives. Oncol Res 2004; 14:355-362.
189. Kruh GD, Guo Y, Hopper-Borge E, Belinsky MG, Chen ZS. ABCC10, ABCC11, and ABCC12. Pflugers Arch 2007; 453:675-684.
190. Bortfeld M, Rius M, König J, Herold-Mende C, Nies AT, Keppler D. Human multidrug resistance protein 8 (MRP8/ABCC11), an apical efflux pump for steroid sulfates, is an axonal protein of the CNS and peripheral nervous system. Neuroscience 2006; 137:1247-1257.
191. Chen ZS, Guo Y, Belinsky MG, Kotova E, Kruh GD. Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C_4 by human multidrug resistance protein 8 (ABCC11). Mol Pharmacol 2005; 67:545-557.
192. Bera TK, Iavarone C, Kumar V, Lee S, Lee B, Pastan

I. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proc Natl Acad Sci U S A 2002; 99:6997-7002.
193. Urquhart BL, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. J Clin Pharmacol 2007; 47:566-578.
194. Staudinger JL, Madan A, Carol KM, Parkinson A. Regulation of drug transporter gene expression by nuclear receptors. Drug Metab Dispos 2003; 31:523-527.
195. Klaassen CD, Slitt AL. Regulation of hepatic transporters by xenobiotic receptors. Curr Drug Metab 2005; 6:309-328.
196. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Bäckman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci U S A 1998; 95:12208-12213.
197. Kliewer SA, Willson TM. Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. J Lipid Res 2000; 43:359-364.
198. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A 2001; 98:3369-3374.
199. Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001; 276:14581-14587.
200. Kauffmann HM, Pfannschmidt S, Zöller H, Benz A, Vorderstemann B, Webster JI, Schrenk D. Influence of redox-active compounds and PXR-activators on human *MRP1* and *MRP2* gene expression. Toxicology 2002; 171:137-146.
201. Teng S, Jekerle V, Piquette-Miller M. Induction of ABCC3 (MRP3) by pregnane X receptor activators. Drug Metab Dispos 2003; 31:1296-1299.
202. Maher JM, Cheng X, Slitt AL, Dieter MZ, Klaassen CD. Induction of the multidrug resistance-associated protein family of transporters by chemical activators of receptor-mediated pathways in mouse liver. Drug Metab Dispos 2005; 33:956-962.
203. Moffit JS, Aleksunes LM, Maher JM, Scheffer GL, Klaassen CD, Manautou JE. Induction of hepatic transporters multidrug resistance-associated proteins (Mrp) 3 and 4 by clofibrate is regulated by peroxisome proliferator-activated receptor alpha. J Pharmacol Exp Ther 2006; 317:537-545.
204. Nishimura M, Koeda A, Suzuki E, Kawano Y, Nakayama M, Satoh T, Narimatsu S, Naito S. Regulation of mRNA expression of MDR1, MRP1, MRP2 and MRP3 by prototypical microsomal enzyme inducers in primary cultures of human and rat hepatocytes. Drug Metab Pharmacokinet 2006; 21:297-307.
205. Aleksunes LM, Scheffer GL, Jakowski AB, Pruimboom-Brees IM, Manautou JE. Coordinated expression of multidrug resistance-associated proteins (Mrps) in mouse liver during toxicant-induced injury. Toxicol Sci 2006; 89:370-379.
206. Magnarin M, Morelli M, Rosati A, Bartoli F, Candussio L, Giraldi T, Decorti G. Induction of proteins involved in multidrug resistance (P-glycoprotein, MRP1, MRP2, LRP) and of CYP 3A4 by rifampicin in LLC-PK1 cells. Eur J Pharmacol 2004; 483:19-28.
207. Pfrunder A, Gutmann H, Beglinger C, Drewe J. Gene expression of CYP3A4, ABC-transporters (MDR1 and MRP1-MRP5) and hPXR in three different human colon carcinoma cell lines. J Pharm Pharmacol 2003; 55:59-66.
208. Nieth C, Lage H. Induction of the ABC-transporters Mdr1/P-gp (Abcb1), mrpl (Abcc1), and bcrp (Abcg2) during establishment of multidrug resistance following exposure to mitoxantrone. J Chemother 2005; 17:215-223.
209. Tatebe S, Sinicrope FA, Kuo MT. Induction of multidrug resistance proteins MRP1 and MRP3 and gamma-glutamylcysteine synthetase gene expression by nonsteroidal anti-inflammatory drugs in human colon cancer cells. Biochem Biophys Res Commun 2002; 290:1427-1433.
210. Kauffmann HM, Schrenk D. Sequence analysis and functional characterization of the 5'-flanking region of the rat multidrug resistance protein 2 (*mrp2*) gene. Biochem Biophys Res Commun 1998; 245:325-331.
211. Johnson DR, Klaassen CD. Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. Toxicol Sci 2002; 67:182-189.
212. Courtois A, Payen L, Le Ferrec E, Scheffer GL, Trinquart Y, Guillouzo A, Fardel O. Differential regulation of multidrug resistance-associated protein 2 (MRP2) and cytochrornes P4502B1/2 and 3A1/2 in phenobarbital-treated hepatocytes. Biochem Pharmacol 2002; 63:333-341.
213. Cherrington NJ, Hartley DP, Li N, Johnson DR, Klaassen CD. Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2 and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. J Pharmacol Exp Ther 2002; 300:97-104.
214. Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 2002; 277:2908-2915.
215. Kauffmann HM, Keppler D, Kartenbeck J, Schrenk D. Induction of *cMrp/cMOAT* gene expression by cisplatin, 2-acetylaminofluorene, or cycloheximide in rat hepatocytes. Hepatology 1997; 26:980-985.
216. Slitt AL, Cherrington NJ, Fisher CD, Negishi M, Klaassen CD. Induction of genes for metabolism and transport by trans-stilbene oxide in livers of Sprague-Dawley and Wistar-Kyoto rats. Drug Metab Dispos 2006; 34:1190-1197.
217. Kauffmann HM, Keppler D, Gant TW, Schrenk D. Induction of hepatic *MRP2* (*cMRP/cMOAT*) gene expression in nonhuman primates treated with rifampicin or tamoxifen. Arch Toxicol 1998; 72:763-768.
218. Rühl R, Sczech R, Landes N, Pfluger P, Kluth D, Schweigert FJ. Carotenoids and their metabolites are naturally occurring activators of gene expression *via* the pregnane X receptor. Eur J Nutr 2004; 43:336-343.
219. Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT. The pregnane x receptor: a promiscuous xenobiotic receptor that has diverged during evolution. Mol Endocrinol 2000; 14:27-39.
220. Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B, Kauffmann HM. Up-regulation of transporters of the MRP family by drugs and toxins. Toxicol Lett 2001; 120:51-57.
221. Shoda J, Kano M, Oda K, Kamiya J, Nimura Y, Suzuki

H, Sugiyama Y, Miyazaki H, Todoroki T, Stengelin S, Kramer W, Matsuzaki Y, Tanaka N. The expression levels of plasma membrane transporters in the cholestatic liver of patients undergoing biliary drainage and their association with the impairment of biliary secretory function. Am J Gastroenterol 2001; 96:3368-3378.
222. Fromm MF, Kauffmann HM, Fritz P, Burk O, Kroemer HK, Warzok RW, Eichelbaum M, Siegmund W, Schrenk D. The effect of rifampin treatment on intestinal expression of human MRP transporters. Am J Pathol 2000; 157:1575-1580.
223. Laouari D, Yang R, Veau C, Blanke I, Friedlander G. Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure. Am J Physiol Renal Physiol 2001; 280:F636-645.
224. König J, Rost D, Cui Y, Keppler D. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology 1999; 29:1156-1163.
225. Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. Proc Natl Acad Sci U S A 2001; 98:3375-3380.
226. Cherrington NJ, Slitt AL, Maher JM, Zhang XX, Zhang J, Huang W, Wan YJ, Moore DD, Klaassen CD. Induction of multidrug resistance protein 3 (mrp3) *in vivo* is independent of constitutive androstane receptor. Drug Metab Dispos 2003; 31:1315-1319.
227. Hitzl M, Klein K, Zanger UM, Fritz P, Nüssler AK, Neuhaus P, Fromm MF. Influence of omeprazole on multidrug resistance protein 3 expression in human liver. J Pharmacol Exp Ther 2003; 304:524-530.
228. Chen C, Klaassen CD. Rat multidrug resistance protein 4 (Mrp4, Abcc4): molecular cloning, organ distribution, postnatal renal expression, and chemical inducibility. Biochem Biophys Res Commun 2004; 317:46-53.
229. Ratajewski M, Bartosz G, Pulaski L. Expression of the human *ABCC6* gene is induced by retinoids through the retinoid X receptor. Biochem Biophys Res Commun 2006; 350:1082-1087.
230. Auyeung DJ, Kessler FK, Ritter JK. Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. Mol Pharmacol 2003; 63:119-127.
231. Ma Q, Kinneer K, Bi Y, Chan JY, Kan YW. Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. Biochem J 2004; 377:205-213.
232. Chan LM, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur J Pharm Sci 2004; 21:25-51.
233. Alrefai WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res 2007; 24:1803-1823.
234. Ballatori N, Christian WV, Lee JY, Dawson PA, Soroka CJ, Boyer JL, Madejczyk MS, Li N. OSTalpha-OSTbeta: a major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia. Hepatology 2005; 42:1270-1279.
235. Geier A, Wagner M, Dietrich CG, Trauner M. Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. Biochim Biophys Acta 2007; 1773:283-308.
236. Gerk PM, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. J Pharmacol Exp Ther 2002; 302:407-415.
237. Zachowski A, Henry JP, Devaux PF. Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. Nature 1989; 340:75-76.
238. Smith AJ, Timmermans-Hereijgers JL, Roelofsen B, Wirtz KW, van Blitterswijk WJ, Smit JJ, Schinkel AH, Borst P. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett 1994; 354:263-266.
239. Smit JW, Weert B, Schinkel AH, Meijer DK. Heterologous expression of various P-glycoproteins in polarized epithelial cells induces directional transport of small (type 1) and bulky (type 2) cationic drugs. J Pharmacol Exp Ther 1998; 286:321-327.
240. Matsuzaki Y, Nakano A, Jiang QJ, Pulkkinen L, Uitto J. Tissue-specific expression of the *ABCC6* gene. J Invest Dermatol 2005; 125:900-905.
241. van de Water FM, Masereeuw R, Russel FG. Function and regulation of multidrug resistance proteins (MRPs) in the renal elimination of organic anions. Drug Metab Rev 2005; 37:443-471.
242. Kim WJ, Kakehi Y, Kinoshita H, Arao S, Fukumoto M, Yoshida O. Expression patterns of multidrug-resistance (MDR1), multidrug resistance-associated protein (MRP), glutathione-*S*-transferase-pi (GST-pi) and DNA topoisomerase II (Topo II) genes in renal cell carcinomas and normal kidney. J Urol 1996; 156:506-511.
243. Inui KI, Masuda S, Saito H. Cellular and molecular aspects of drug transport in the kidney. Kidney Int 2000; 58:944-958.
244. Ray AS, Cihlar T, Robinson KL, Tong L, Vela JE, Fuller MD, Wieman LM, Eisenberg EJ, Rhodes GR. Mechanism of active renal tubular efflux of tenofovir. Antimicrob Agents Chemother 2006; 50:3297-3304.
245. Sekine T, Miyazaki H, Endou H. Molecular physiology of renal organic anion transporters. Am J Physiol Renal Physiol 2006; 290:F251-261.
246. Launay-Vacher V, Izzedine H, Karie S, Hulot JS, Baumelou A, Deray G. Renal tubular drug transporters. Nephron Physiol 2006; 103:97-106.
247. Terada T, Inui K. Peptide transporters: structure, function, regulation and application for drug delivery. Curr Drug Metab 2004; 5:85-94.
248. Nishizato Y, Ieiri I, Suzuki H, Kimura M, Kawabata K, Hirota T, Takane H, Irie S, Kusuhara H, Urasaki Y, Urae A, Higuchi S, Otsubo K, Sugiyama Y. Polymorphisms of *OATP-C* (*SLC21A6*) and *OAT3* (*SLC22A8*) genes: consequences for pravastatin pharmacokinetics. Clin Pharmacol Ther 2003; 73:554-565.
249. Jonker JW, Wagenaar E, Van Eijl S, Schinkel AH. Deficiency in the organic cation transporters 1 and 2 (Oct1/Oct2 [Slc22a1/Slc22a2]) in mice abolishes renal secretion of organic cations. Mol Cell Biol 2003; 23:7902-7908.
250. Ganapathy ME, Brandsch M, Prasad PD, Ganapathy V, Leibach FH. Differential recognition of beta -lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. J Biol Chem 1995; 270:25672-25677.
251. Jung KY, Takeda M, Shimoda M, Narikawa S, Tojo A, Kim DK, Chairoungdua A, Choi BK, Kusuhara H,

Sugiyama Y, Sekine T, Endou H. Involvement of rat organic anion transporter 3 (rOAT3) in cephaloridine-induced nephrotoxicity: in comparison with rOAT1. Life Sci 2002; 70:1861-1874.
252. Deguchi T, Kusuhara H, Takadate A, Endou H, Otagiri M, Sugiyama Y. Characterization of uremic toxin transport by organic anion transporters in the kidney. Kidney Int 2004; 65:162-174.
253. Yonezawa A, Masuda S, Nishihara K, Yano I, Katsura T, Inui K. Association between tubular toxicity of cisplatin and expression of organic cation transporter rOCT2 (Slc22a2) in the rat. Biochem Pharmacol 2005; 70:1823-1831.
254. Kearney BP, Sayre JR, Flaherty JF, Chen SS, Kaul S, Cheng AK. Drug-drug and drug-food interactions between tenofovir disoproxil fumarate and didanosine. J Clin Pharmacol 2005; 45:1360-1367.
255. Kearney BP, Flaherty JF, Shah J. Tenofovir disoproxil fumarate: clinical pharmacology and pharmacokinetics. Clin Pharmacokinet 2004; 43:595-612.
256. Ray AS, Olson L, Fridland A. Role of purine nucleoside phosphorylase in interactions between 2',3'-dideoxyinosine and allopurinol, ganciclovir, or tenofovir. Antimicrob Agents Chemother 2004; 48:1089-1095.
257. Kotb R, Vincent I, Dulioust A, Peretti D, Taburet AM, Delfraissy JF, Goujard C. Life-threatening interaction between antiretroviral therapy and vinblastine in HIV-associated multicentric Castleman's disease. Eur J Haematol 2006; 76:269-271.
258. Makinson A, Martelli N, Peyrière H, Turriere C, Le Moing V, Reynes J. Profound neutropenia resulting from interaction between antiretroviral therapy and vinblastine in a patient with HIV-associated Hodgkin's disease. Eur J Haematol 2007; 78:358-360.
259. Banks WA. Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. J Neurovirol 1999; 5:538-555.
260. Löscher W, Potschka H. Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx 2005; 2:86-98.
261. Couture L, Nash JA, Turgeon J. The ATP-binding cassette transporters and their implication in drug disposition: a special look at the heart. Pharmacol Rev 2006; 58:244-258.
262. Girardin F. Membrane transporter proteins: a challenge for CNS drug development. Dialogues Clin Neurosci 2006; 8:311-321.
263. de Boer AG, Gaillard PJ. Drug targeting to the brain. Annu Rev Pharmacol Toxicol 2007; 47:323-355.
264. Dallas S, Miller DS, Bendayan R. Multidrug resistance-associated proteins: expression and function in the central nervous system. Pharmacol Rev 2006; 58:140-161.
265. de Boer AG, van der Sandt IC, Gaillard PJ. The role of drug transporters at the blood-brain barrier. Annu Rev Pharmacol Toxicol 2003; 43:629-656.
266. Hawkins BT, Davis TP. The blood-brain barrier/ neurovascular unit in health and disease. Pharmacol Rev 2005; 57:173-185.
267. Terasaki T, Ohtsuki S. Brain-to-blood transporters for endogenous substrates and xenobiotics at the blood-brain barrier: an overview of biology and methodology. NeuroRx 2005; 2:63-72.
268. Taylor EM. The impact of efflux transporters in the brain on the development of drugs for CNS disorders. Clin Pharmacokinet 2002; 41:81-92.
269. Begley DJ. ABC transporters and the blood-brain barrier. Curr Pharm Des 2004; 10:1295-1312.
270. Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006; 86:849-899.
271. Siegal T, Zylber-Katz E. Strategies for increasing drug delivery to the brain: focus on brain lymphoma. Clin Pharmacokinet 2002; 41:171-186.
272. Fricker G, Miller DS. Modulation of drug transporters at the blood-brain barrier. Pharmacology 2004; 70:169-176.
273. Zhao Q, Chang XB. Mutation of the aromatic amino acid interacting with adenine moiety of ATP to a polar residue alters the properties of multidrug resistance protein 1. J Biol Chem 2004; 279:48505-48512.
274. Zhang DW, Nunoya K, Vasa M, Gu HM, Theis A, Cole SP, Deeley RG. Transmembrane helix 11 of multidrug resistance protein 1 (MRP1/ABCC1): identification of polar amino acids important for substrate specificity and binding of ATP at nucleotide binding domain 1. Biochemistry 2004; 43:9413-9425.
275. Conseil G, Deeley RG, Cole SP. Polymorphisms of MRP1 (ABCC1) and related ATP-dependent drug transporters. Pharmacogenet Genomics 2005; 15:523-533.
276. Conrad S, Kauffmann HM, Ito K, Leslie EM, Deeley RG, Schrenk D, Cole SP. A naturally occurring mutation in MRP1 results in a selective decrease in organic anion transport and in increased doxorubicin resistance. Pharmacogenetics 2002; 12:321-330.
277. Assaraf YG, Rothem L, Hooijberg JH, Stark M, Ifergan I, Kathmann I, Dijkmans BA, Peters GJ, Jansen G. Loss of multidrug resistance protein 1 expression and folate efflux activity results in a highly concentrative folate transport in human leukemia cells. J Biol Chem 2003; 278:6680-6686.
278. Leslie EM, Létourneau IJ, Deeley RG, Cole SP. Functional and structural consequences of cysteine substitutions in the NH_2 proximal region of the human multidrug resistance protein 1 (MRP1/ABCC1). Biochemistry 2003; 42:5214-5224.
279. Ito K, Oleschuk CJ, Westlake C, Vasa MZ, Deeley RG, Cole SP. Mutation of Trp^{1254} in the multispecific organic anion transporter, multidrug resistance protein 2 (MRP2) (ABCC2), alters substrate specificity and results in loss of methotrexate transport activity. J Biol Chem 2001; 276:38108-38114.
280. Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMRP, reveals a novel conjungate export pump deficient in hyperbilirubinemic rats. J Biol Chem 1996; 271:15091-15098.
281. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, Sugiyama Y. Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. Am J Physiol 1997; 272(1 Pt 1):G16-22.
282. Paulusma CC, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RP. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 1996; 271:1126-1128.
283. Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the *MRP* gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. Hepatology 1996; 23:1061-1066.
284. Machida I, Inagaki Y, Suzuki S, Hayashi H, Wakusawa S.

Mutation analysis of the multidrug resistance protein 2 (*MRP2*) gene in a Japanese patient with Dubin-Johnson syndrome. Hepatol Res 2004; 30:86-90.
285. Wakusawa S, Machida I, Suzuki S, Hayashi H, Yano M, Yoshioka K. Identification of a novel 2026G→C mutation of the *MRP2* gene in a Japanese patient with Dubin-Johnson syndrome. J Hum Genet 2003; 48:425-429.
286. Hirouchi M, Suzuki H, Itoda M, Ozawa S, Sawada J, Ieiri I, Ohtsubo K, Sugiyama Y. Characterization of the cellular localization, expression level, and function of SNP variants of MRP2/ABCC2. Pharm Res 2004; 21:742-748.
287. Ito S, Ieiri I, Tanabe M, Suzuki A, Higuchi S, Otsubo K. Polymorphism of the ABC transporter genes, *MDR1*, *MRP1* and *MRP2/cMOAT*, in healthy Japanese subjects. Pharmacogenetics 2001; 11:175-184.
288. Mor-Cohen R, Zivelin A, Rosenberg N, Shani M, Muallem S, Seligsohn U. Identification and functional analysis of two novel mutations in the multidrug resistance protein 2 gene in Israeli patients with Dubin-Johnson syndrome. J Biol Chem 2001; 276:36923-36930.
289. Izzedine H, Hulot JS, Villard E, Goyenvalle C, Dominguez S, Ghosn J, Valantin MA, Lechat P, Deray AG. Association between *ABCC2* gene haplotypes and tenofovir-induced proximal tubulopathy. J Infect Dis 2006; 194:1481-1491.
290. Lang T, Hitzl M, Burk O, Mornhinweg E, Keil A, Kerb R, Klein K, Zanger UM, Eichelbaum M, Fromm MF. Genetic polymorphisms in the multidrug resistance-associated protein 3 (*ABCC3, MRP3*) gene and relationship to its mRNA and protein expression in human liver. Pharmacogenetics 2004; 14:155-164.
291. Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, de Paepe A, Boyd CD. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 2000; 25:223-227.
292. Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. Pseudoxanthoma elasticum: mutations in the *MRP6* gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci U S A 2000; 97:6001-6006.
293. Uitto J. Pseudoxanthoma elasticum-a connective tissue disease or a metabolic disorder at the genome/environment interface? J Invest Dermatol 2004; 122: ix-x.
294. Hu X, Plomp AS, van Soest S, Wijnholds J, de Jong PT, Bergen AA. Pseudoxanthoma elasticum: a clinical, histopathological, and molecular update. Surv Ophthalmol 2003; 48:424-438.
295. Gheduzzi D, Guidetti R, Anzivino C, Tarugi P, Di Leo E, Quaglino D, Ronchetti IP. ABCC6 mutations in Italian families affected by pseudoxanthoma elasticum (PXE). Hum Mutat 2004; 24:438-439.
296. Chassaing N, Martin L, Mazereeuw J, Barrié L, Nizard S, Bonafé JL, Calvas P, Hovnanian A. Novel ABCC6 mutations in pseudoxanthoma elasticum. J Invest Dermatol 2004; 122:608-613.
297. Hu X, Peek R, Plomp A, ten Brink J, Scheffer G, van Soest S, Leys A, de Jong PT, Bergen AA. Analysis of the frequent R1141X mutation in the *ABCC6* gene in pseudoxanthoma elasticum. Invest Ophthalmol Vis Sci 2003; 44:1824-1829.
298. Hendig D, Schulz V, Eichgrün J, Szliska C, Götting C, Kleesiek K. New *ABCC6* gene mutations in German pseudoxanthoma elasticum patients. J Mol Med 2005; 83:140-147.
299. Germain DP, Remones V, Perdu J, Jeunemaitre X. Identification of two polymorphisms (c189G>C; c190T>C) in exon 2 of the human *MRP6* gene (ABCC6) by screening of Pseudoxanthoma elasticum patients: possible sequence correction? Hum Mutat 2000; 16:449.
300. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002; 2:48-58.
301. Teodori E, Dei S, Martelli C, Scapecchi S, Gualtieri F. The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). Curr Drug Targets 2006; 7:893-909.
302. Sauna ZE, Peng XH, Nandigama K, Tekle S, Ambudkar SV. The molecular basis of the action of disulfiram as a modulator of the multidrug resistance-linked ATP binding cassette transporters MDR1 (ABCB1) and MRP1 (ABCC1). Mol Pharmacol 2004; 65:675-684.
303. Perego P, De Cesare M, De Isabella P, Carenini N, Beggiolin G, Pezzoni G, Palumbo M, Tartaglia L, Pratesi G, Pisano C, Carminati P, Scheffer GL, Zunino F. A novel 7-modified camptothecin analog overcomes breast cancer resistance protein-associated resistance in a mitoxantrone-selected colon carcinoma cell line. Cancer Res 2001; 61:6034-6037.
304. Vail DM, Amantea MA, Colbern GT, Martin FJ, Hilger RA, Working PK. Pegylated liposomal doxorubicin: proof of principle using preclinical animal models and pharmacokinetic studies. Semin Oncol 2004; 31:16-35.
305. Cascorbi I. Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. Pharmacol Ther 2006; 112:457-473.
306. Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006; 5:219-234.
307. Conrad S, Kauffmann HM, Ito K, Deeley RG, Cole SP, Schrenk D. Identification of human multidrug resistance protein 1 (MRP1) mutations and characterization of a G671V substitution. J Hum Genet 2001; 46:656-663.
308. Cai L, Lumsden A, Guenther UP, Neldner SA, Zäch S, Knoblauch H, Ramesar R, Hohl D, Callen DF, Neldner KH, Lindpaintner K, Richards RI, Struk B. A novel Q378X mutation exists in the transmembrane transporter protein ABCC6 and its pseudogene: implications for mutation analysis in pseudoxanthoma elasticum. J Mol Med 2001; 79:536-546.
309. Pulkkinen L, Nakano A, Ringpfeil F, Uitto J. Identification of ABCC6 pseudogenes on human chromosome 16p: implications for mutation detection in pseudoxanthoma elasticum. Hum Genet 2001; 109:356-365.
310. Meloni I, Rubegni P, De Aloe G, Bruttini M, Pianigiani E, Cusano R, Seri M, Mondillo S, Federico A, Bardelli AM, Andreassi L, Fimiani M, Renieri A. Pseudoxanthoma elasticum: Point mutations in the *ABCC6* gene and a large deletion including also ABCC1 and MYH11. Hum Mutat 2001; 18:85.

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Review

Pharmacogenomics-based clinical studies using a novel, fully automated genotyping system

Setsuo Hasegawa*, Sayaka Kimura, Ai Kobayashi, Naoko Imanishi

Research and Development, Sekino Clinical Pharmacology Clinic, Tokyo, Japan.

ABSTRACT: Clinical investigations into single nucleotide polymorphisms (SNPs) in drug metabolism have already been set out for clinical trials in subject groups classified as extensive metabolizers or poor metabolizers. In particular, the frequency of CYP2C19 in poor metabolizers within the Japanese population is relatively high, and genetic variations result in differences in kinetics and pharmacological action, *e.g.* clinical response to proton pump inhibitors which are mainly metabolized by 2C19 in the liver. We introduced a novel, fully automated genotyping system and used it in the genotyping of CYP2C19. The completed system is based on the analysis of a melting curve of probe DNA which is bound to the target SNP site using a fluorescence quenching probe. The system enables automated and multiple SNP-genotyping from sample preparation. This fully automated system of analysis can be adapted to clinical studies, *e.g.* classification of genes related to pharmaco-kinetics and target receptors by genetic variations.

Keywords: Pharmacogenomics, Automated genotyping system, Single nucleotide polymorphism, CYP2C19

1. Introduction

With the advent of a post-genome era, clinical studies associated with drug development are likely to change drastically. In particular, noticeable improvements are expected in phase-1 clinical studies where healthy adults are enrolled as trial subjects. With the introduction of pharmacogenomics, evidence-based medications which are based on genome information will become available and result in improved safety and efficacy (*1*). Sekino Clinical Pharmacology Clinic (SCPC) has developed into one of the leading facilities for Phase I clinical trials in Japan. In response to drug development in the post-genomic era, this Clinic is taking a proactive approach to the introduction of pharmacogenomics into clinical trials.

Since determination of the human genome sequence, two main streams of personalized medicine have appeared in the post-genomic era: one is genomic-based drug discovery and the other is individual single nucleotide polymorphism (SNP) typing. SNP is the most common form of DNA sequence variation occurring when a single nucleotide in the genome differs between members of the species, ethnicity, and individuals. Genotyping of SNPs is of great value to biomedical research and in developing personalized medicine because it can affect how humans respond to pathogens, chemicals, and drugs in particular (*2-5*).

Conventionally, gene-typing was performed using several large pieces of equipment that must be operated manually. In addition to relatively large space, it also required technical experts to properly operate the complicated system. Outsourcing of gene-typing is not a viable option because of ethical concerns relating to safety management of DNA and security of genomic information. Recently, this Clinic has implemented a novel, fully automated, easy-to-use, compact genotyping system in Pharmacogenomics (PGx)-based clinical studies, and this system could contribute to in-house analysis of SNPs by non-technical experts.

2. Background of PGx-related clinical trials and the current state of PGx-trials in Japan

Clinical trials are usually classified into three phases. Phase I trials are primarily concerned with assessing a drug's safety. This initial phase of testing in humans is done in a small number of healthy volunteer subjects. The study is mainly designed to determine what happens to the drug in the human body; in other words, it is a pharmacokinetics study. Phase II and III trials for patients continue to test the safety of the drug and begin to evaluate how well the new drug works, which are known as pharmacodynamics studies. In basic terms, pharmacokinetics is the study of what a body does to

**Correspondence to:* Dr. Setsuo Hasegawa, Sekino Clinical Pharmacology Clinic, 3-28-3 Ikebukuro, Toshima-ku, Tokyo 171-0014, Japan;
e-mail: hasegawa_setsuo@seri.co.jp

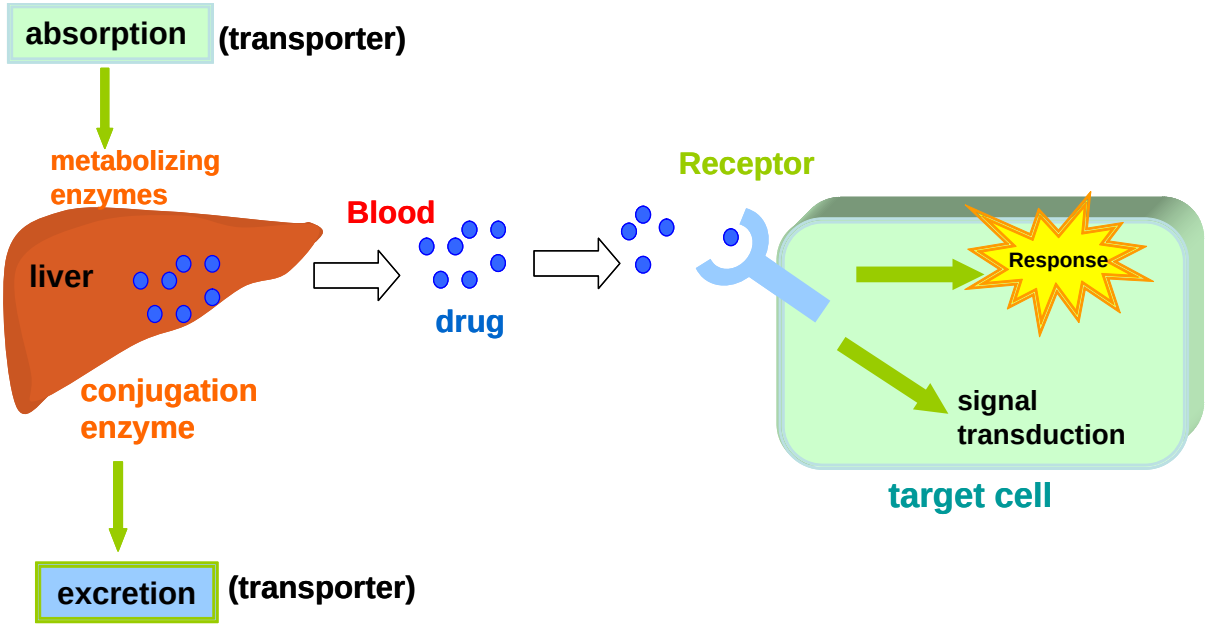
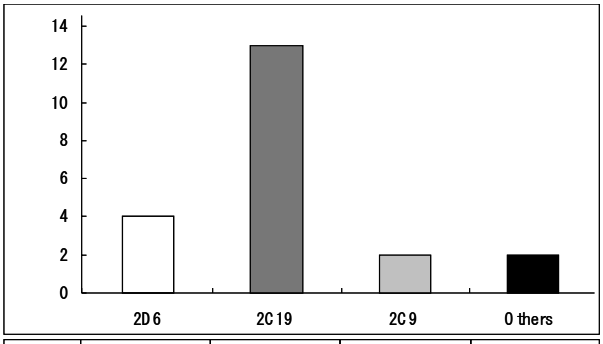


Figure 1. Inter-individual variation in drug response. Pharmacogenomics is the study of the role of inheritance in inter-individual variation in drug response. The field of pharmacogenomics began with a focus on drug metabolism, but it has been extended to cover the full spectrum of drug disposition, including transporters that influence drug absorption, distribution, and excretion. Furthermore, genetic variation in drug targets (*e.g.*, receptors) can have a profound effect on drug efficacy.

a drug, as opposed to pharmacodynamics, which is the study of what a drug does to a body. Pharmacogenomics influences both, so it can be used during all phases of clinical trials to assess safety and efficacy.

Pharmacogenomics is the study of the role of inheritance in inter-individual variation in drug response. The field of pharmacogenomics began with a focus on drug metabolism, but it has been extended to cover the full spectrum of drug disposition, including transporters that influence drug absorption, distribution, and excretion. Furthermore, genetic variation in drug targets (*e.g.* receptors) can have a profound effect on drug efficacy (Figure 1). However, the current state of clinical trials, and especially in the case of genotype-based subject stratification, is such that metabolizing enzyme-related studies are proceeding. Cytochrome P450 is a family of the body's more powerful detoxification enzymes. Of these, the CYP2D6, CYP2C19, and CYP2C9 genotypes are termed Known Valid Biomarkers, which are genetically associated with changes in drug effects and accepted by the scientific community as predicting clinical outcome.

Figure 2 shows a cumulative number of biomarkers investigated in the trials at this institute. CYP2C19-related studies are predominant in Japan. Research on CYP2C19 poor metabolizers in different ethnic populations has clearly revealed that the frequency of poor metabolizers is relatively high, approximately 20%, in the Japanese population. These genetic variations result in differences in kinetics and pharmacological action, *e.g.* clinical responses to proton pump inhibitors that are mainly metabolized by 2C19 in the liver (*6*).



	2D6	2C19	2C9	Other
Total	4	13	2	2

Figure 2. Biomarkers in PGx-related clinical studies. This bar graph shows a cumulative number of biomarkers investigated in trials at Sekino Clinical Pharmacology Clinic. CYP2C19-related studies are predominant in Japan.

3. Implementation of a novel, fully automated genotyping system

3.1. *Research and development phase*

A Research and Development laboratory has been established for in-house analysis of gene polymorphism. A novel automatic SNP-typing system has been developed in collaboration with ARKRAY, Inc, Kyoto, Japan. ARKRAY handles a wide range of analytical equipment for use in environments as diverse as major hospitals, diagnostic centers, and point-of-care testing. ARKRAY provides the latest equipment for major hospitals and diagnostic centers, easy-to-use, compact testing systems for clinics, and testing equipment for

convenient measurement at home or elsewhere for home care. The genotyping system that was developed is not a finished product but an experimental model. The most attractive features of this system are its compact design and automatic analysis including pre-treatment.

CYP2C19 is involved in the metabolism of several important groups of drugs including many proton pump inhibitors and antiepileptics. The CYP2C19 gene is located on chromosome 10q24. Twenty-one SNPs are found on CYP2C19. Within the Japanese population, however, variants except for *2 and *3 are absent. Thus, only these 2 non-functional alleles had to be genotyped. The *2 (m1) alleles (subtypes A and B) have a defining mutation of a G681 to A substitution that results in a splicing defect. Subtypes are not differentiated. The *3 (m2) allele has a defining mutation of a G636 to A substitution that results in a Trp212 to stop codon change.

In the current system, three-color real-time optical detection is possible. Thus, multiplex SNP genotyping can be performed at one time within 90 min. These results provided are consistent with the results obtained by allele-specific primer PCR as is conventionally used (*7*).

3.2. *Overview of the newly finished product*

Following improvements and upgrades, the system will be completed in the near future. It has a more compact design: 41 cm (width) by 45 cm (depth) by 41 cm (height) (Figure 3).

The system allows fully integrated automatic gene-typing from sample pretreatment to gene amplification and signal detection. The equipment incorporates computer-free analysis so that measurement results can also be analyzed with a single system. The working time has been reduced by using a newly developed technique for sample pretreatment that requires no DNA extraction. Following placement of the reagent pack and the sample, gene-typing results are available in 80 min.

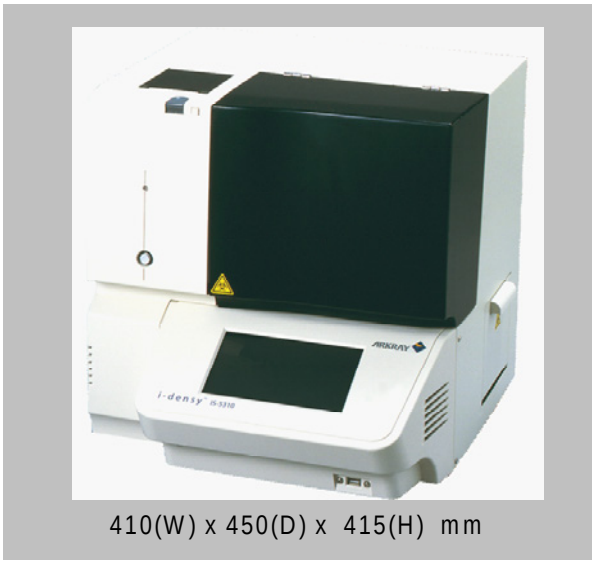


Figure 3. Fully integrated and automatic gene-typing system. Following improvements and upgrades, the system will be completed in the near future. It has a more compact design: 41 cm (width) by 45 cm (depth) by 41 cm (height).

3.3. *Features and specifications*

Key features
- Pretreatment, amplification, and detection are all automated.
- Automated testing procedures setup using bar-codes on the disposable reagent.
- Closed system for reagents.
- Rapid processing from preparation to detection. <Within 80 min>
- 4 independently programmable reaction sites.
- 3-color optical detection for each site.

Specifications
- EMC compliant
- Computer linkage *via* USB/Ethernet

3.4. *The principle of signal detection*

PCR is performed at the site with the SNP. The reagent contains Guanine Quenching Probes that have either of the complementary sequences of the target SNP. As the temperature is decreased following PCR, the probe and amplified product are hybridized regardless of whether a mismatch is present or not. Then, using a gradual temperature increase, the loosely bound mismatch sequences and probes detach and fluorescence is emitted. When the temperature is increased further, perfect match sequences and probes will detach and fluorescence strength will increase. In this way, an SNP can be detected by the difference in temperature and fluorescence. This method is known as 'Melting Point Analysis' or 'Tm Analysis' (Figure 4).

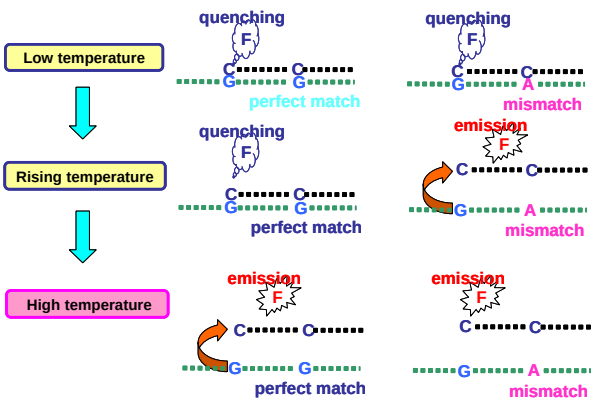


Figure 4. The principle of signal detection. SNPs can be detected by the difference in temperature and fluorescence. This method is known as "Melting Point Analysis" or "Tm Analysis".

Figure 5 shows an example of interpreted measurement results in the case of CYP2C19. With one pack of CYP2C19 reagent, the SNP in both *2 and *3 can be analyzed simultaneously. This example is of 'wild' in *2 investigation and 'hetero' or heterogeneous in *3 investigation, so the genotype was determined to be *1/*3.

3.5. *Operation of the equipment*

Prior to measurement, the necessary number of tips, reaction tubes and reagent packs are put in place. The equipment contains 4 independently programmable reaction sites (Figure 6). Reagent packs, reaction tubes, and tips are all included in one package. The

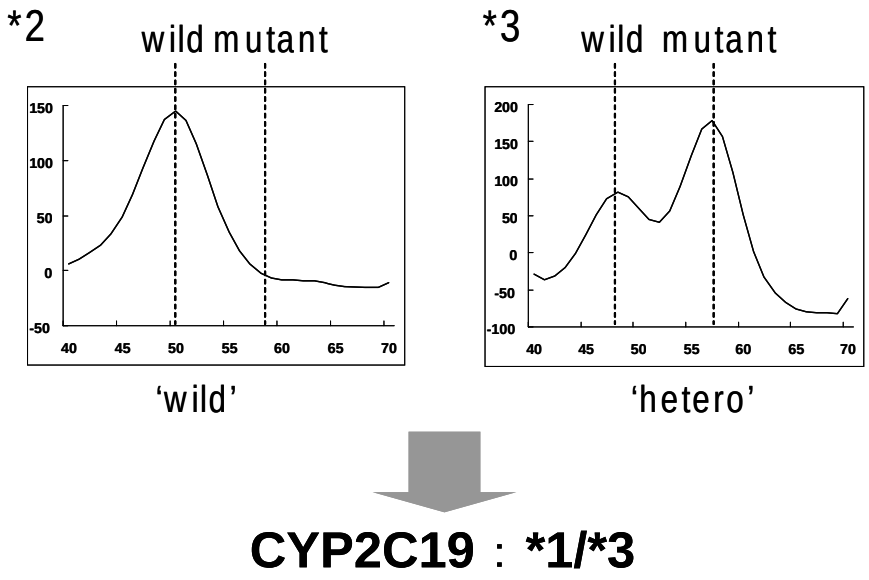


Figure 5. Interpreted measurement results in the case of CYP2C19. With one pack of CYP2C19 reagent, the SNP in both *2 and *3 can be analyzed simultaneously. This example is of 'wild' in *2 investigation and 'hetero' in *3 investigation, so the genotype was determined to be *1/*3.

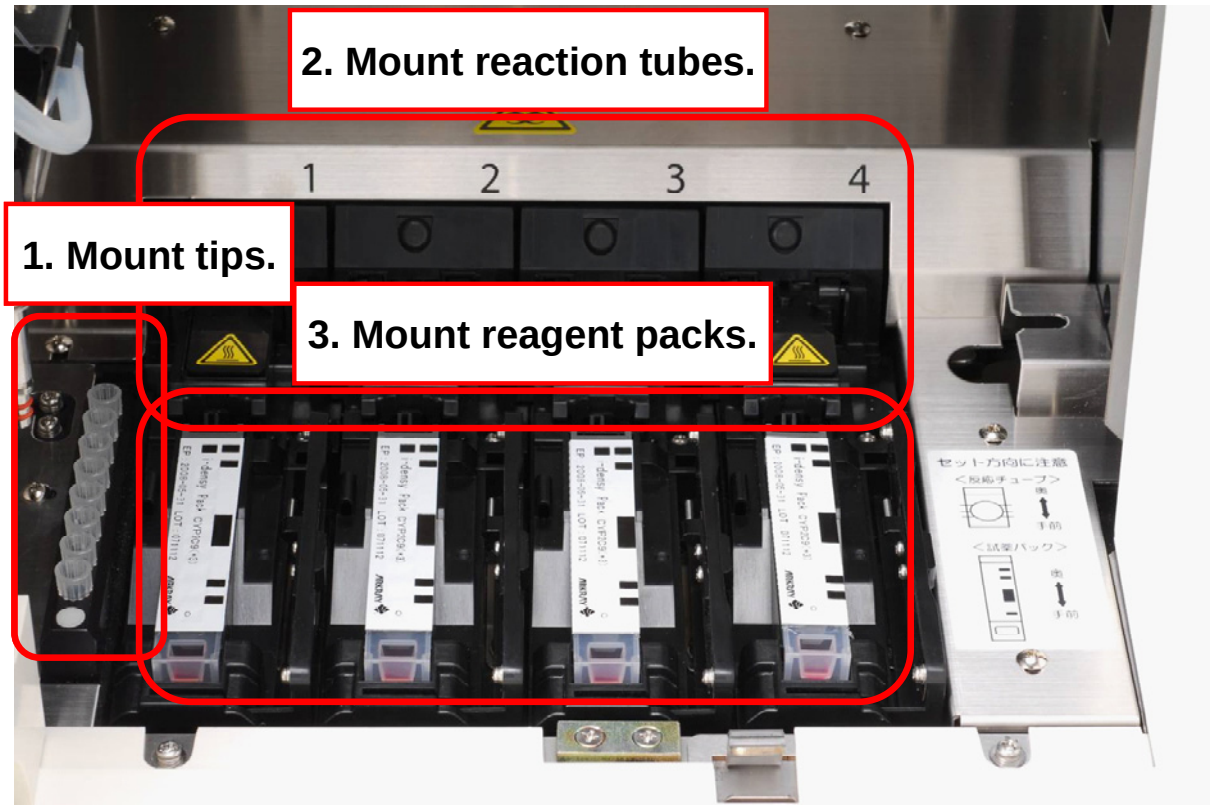


Figure 6. Operation of the equipment. Prior to measurement, the necessary number of tips, reaction tubes, and reagent packs are put in place. The equipment contains 4 independently programmable reaction sites.

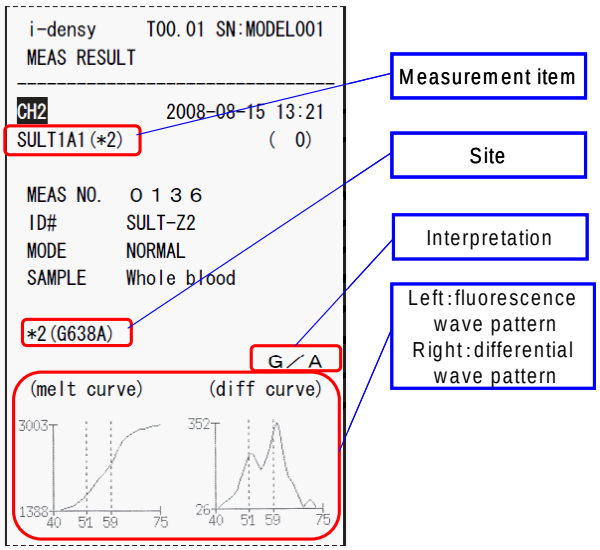


Figure 7. End of measurement. After measurement is complete, measurement results can be printed out. The equipment can store a maximum of 500 results per user. Previous results can also be printed.

reagent pack contains a solution for dilution and reagent for pretreatment, amplification and detection. Contamination can be avoided because each cell is designed to be separated so that the solution cannot flow into adjacent cells. The sample is applied to the sample block. In the current system, whole blood as well as oral swabs can be used as SNP-typing materials. When a blood sample is used, the sample block is filled with just 50 μL of whole blood.

Measurement is started by simply pressing the start button. The system is equipped with automatic recognition using barcodes printed on the reagent packs and an easy-to-use color LCD with touch screen. These features allow rapid and simple gene-typing. After measurement is complete, measurement results can be printed out. The equipment can store a maximum of 500 results per user. Previous results can also be printed (Figure 7).

3.6. *Forward-looking approach for clinical use*

Figure 8 shows the prospective development of SNP-analysis. According to the order shown here, valid genomic biomarkers will expand from PK-related genes, *via* genes related to target receptors, to genes that cause disease in the future.

For example, disposition of warfarin provides an interesting model for a suitable clinical application of the system. Warfarin has received a great deal of attention in recent years as a target of personalized medicine (*8*). Warfarin is metabolized into inactive metabolites by CYP2C9, and warfarin inhibits the Vitamin K-dependent carboxylation of coagulation factor *via* vitamin K epoxide reductase complex 1 (VKORC1) (*9,10*). An extensive amount of clinical data suggests that the risk of bleeding is particularly

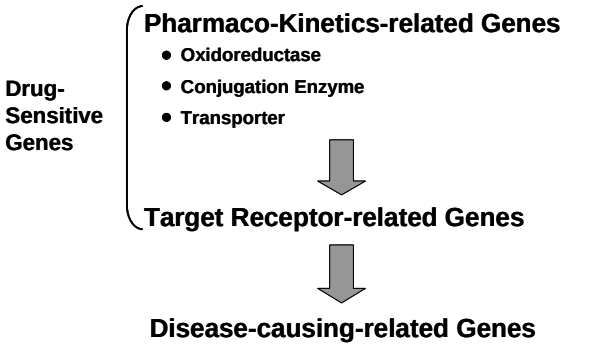


Figure 8. Prospective development of SNP-analysis. This diagram shows the prospective development of SNP-analysis. According to the order shown here, valid genomic biomarkers will expand from PK-related genes, *via* genes related to target receptors, to genes related to genes that cause disease in the future.

high in patients with gene variants in CYP2C9 and/or VKORC1 (*11,12*). In addition to the analytical program for CVP2C9, one has also been established for VKORC1. Preliminary investigations revealed data in general agreement with reference data. The system allows 2C9 and VKORC1-genotyping to be performed simultaneously, which can provide a clinically significant improvement to current practices. New software content related to gene polymorphism is currently being developing to meet the needs of clinical practice.

4. Conclusion

The SNP genotyping system thus developed is very unique in contrast to previous methods or systems for SNP genotyping. Rapid, simple and contamination-free genotyping system could contribute not just to PGx-related clinical trials but also to order-made therapy in the near future.

Acknowledgements

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References

1. Huang SM, Goodsaid F, Rahman A, Frueh F, Lesko LJ. Application of pharmacogenomics in clinical pharmacology. Toxicol Mech Methods 2006; 16:89-99.
2. Evans WE, Relling MV. Pharmacogenomics: Translating functional genomics into rational therapeutics. Science 1999; 286:487-491.
3. Evans WE, McLeod HL. Pharmacogenomics - Drug disposition, drug targets, and side effects. N Engl J Med 2003; 348:529-537.
4. Marsh S, McLeod HL. Pharmacogenomics: from bedside

to clinical practice. Hum Mol Genet 2006; 15:89-93.
5. Weinshilboum R. Inheritance and drug response. N Engl J Med 2003; 348:529-537.
6. Furuta T, Ohashi, Kamata T, Takashima M, Kosuge K, Kawasaki T, Hanai H, Kubota T, Ishizaki T, Kaneko E. Effect of genetic differences in omeprazole metabolism on cure rates for Helicobacter pylori infection and peptic ulcer. Ann Intern Med 1998; 129:1027-1030.
7. Matsumoto N, Kakihara F, Kimura S, Kurebayashi Y, Hirai M, Yohda, Hasegawa S. Single nucleotide polymorphism genotyping of CYP2C19 using a new automated system. Anal Biochem 2007; 370:121-123.
8. Lesko LJ. The critical path of warfarin dosing: Finding an optimal dosing strategy using pharmacogenetics. Clin Pharmacol Ther 2008; 84:301-303.
9. Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Muller CR, Strom TM, Oldenburg J. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. Nature 2004; 427:537-541.
10. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. Nature 2004; 427:541-544.
11. D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V, Grandone E, Margaglione M. A polymorphism in the *VKORC1* gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. Blood 2005; 105:645-649.
12. Mushiroda T, Ohnishi Y, Saito S, Takahashi A, Kikuchi Y, Saito S, Shimomura H, Wanibuchi Y, Suzuki T, Kamatani N, Nakamura Y. Association of VKORC1 and CYP2C9 polymorphisms with warfarin dose requirements in Japanese patients. J Hum Genet 2006; 51:249-253.

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Brief Report

Apoptosis-inducing effect of cinobufacini, *Bufo bufo gargarizans* Cantor skin extract, on human hepatoma cell line BEL-7402

Fanghua Qi[1], Anyuan Li[1,*], Hong Lv[1], Lin Zhao[1], Jijun Li[1], Bo Gao[2], Wei Tang[3,*]

[1] *Department of Traditional Chinese Medicine, Shandong Provincial Hospital, Shandong University, Ji'nan, Shandong, China;*
[2] *Anhui Jinchan Biochemical Co., Ltd., Huaibei, Anhui, China;*
[3] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan.*

ABSTRACT: Cinobufacini, a water-soluble preparation of Chinese medicine, is extracted from the skin of *Bufo bufo gargarizans* Cantor. The present study sought to investigate the effects of Cinobufacini on apoptosis of the hepatocellular carcinoma cell line BEL-7402. Cell viability was measured by methyl thiazolyl tetrazolium assay. Cell morphology was observed by Hoechst 33258 staining. Western blotting analysis was used to detect Bax and Bcl-2 expression. Results indicated that Cinobufacini inhibited the proliferation of BEL-7402 cells in a dose and time-dependent manner. Marked morphological changes indicative of apoptosis were observed after treatment with different concentrations of Cinobufacini. Western blot analysis showed that Bcl-2 expression was down-regulated while Bax expression was up-regulated. Thus, Cinobufacini may have a significant apoptosis-inducing effect on BEL-7402 cells, and this could prove useful for further anti-cancer research.

Keywords: Cinobufacini, *Bufo bufo gargarizans* Cantor, Proliferation, Apoptosis, Human hepatoma cell line BEL-7402

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide and its incidence has been increasing over the past few decades in areas such as Europe, the US, and eastern Asia (*1*). Although there are many advanced diagnosis and treatment methods such as surgery, radiation, and chemotherapy, HCC remains a formidable challenge for clinical therapy (*2-5*). Recently, traditional Chinese medicines have attracted attention as candidates for new cancer therapeutics with a low level of toxicity.

The skin of the Chinese toad, *Bufo bufo gargarizans* Cantor, has been used as an effective traditional Chinese medicine for thousands years in China (*6*). Cinobufacini, as it is typically designated in Chinese references, is a water-soluble preparation made from toad skin (*7*). Cinobufacini has been reported to possess a variety of biological effects, such as anti-tumor and anti-virus effects, and enhance physical immunity according to clinical data (*8,9*). Although Cinobufacini has proven to be effective against a variety of malignancies, and especially gastrointestinal tumors, its anti-tumor mechanisms have yet to be identified for the most part. No detailed data on the role and mechanisms of Cinobufacini in HCC cells have been available thus far. Thus, the current study investigated the effects of Cinobufacini on apoptosis in the human hepatoma cell line BEL-7402 and its mechanisms of action.

2. Materials and Methods

2.1. *Reagents*

Cinobufacini, which was prepared by an extraction of 20 g of toad's skin with boiling water followed by a concentration to 1 mL, was obtained from Anhui Jinchan Biochemical Co., Ltd., Anhui, China. High-glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen, Carlsbad, CA, USA. Fetal calf serum (FCS) was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China. Hoechst 33258 was purchased from Sigma-Aldrich, St Louis, MO, USA. The anti-Bax, anti-BCL-2 and β-actin antibodies were purchased from

**Correspondence to:* Dr. Anyuan Li, Department of Traditional Chinese Medicine, Shandong Provincial Hospital, Shandong University, No 324, Jingwuweiqi Road, Ji'nan 250021, Shandong, China;
e-mail: sdslyy999@163.com;
Dr. Wei Tang, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Hongo 7-3-1, Tokyo 113-8655, Japan;
e-mail: TANG-SUR@h.u-tokyo.ac.jp

Santa Cruz Biotechnology, Santa Cruz, CA, USA and the second antibodies were purchased from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China. BCA-100 Protein Quantitative Analysis Kits were obtained from Shenergy BioScience & Technology Company.

2.2. *Cells and culture conditions*

The Bel-7402 cell line, which was established from a specimen obtained from a 53-year-old male with HCC (*10*), was obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Science, Shanghai, China. The cells were incubated in DMEM medium supplemented with 10% FCS, 100 U/mL of penicillin and 100 mg/mL of streptomycin in a humidified atmosphere with 5% CO_2 in air at 37°C. Cells in the logarithmic growth phase were collected for the following experiments.

2.3. *MTT assay*

Cells were plated at a density of 6×10^4 cells/mL in 96-well plates. Twenty-four hours later, the cultures were incubated with different concentrations of Cinobufacini (0, 0.005, 0.01, 0.05, 0.1, and 0.5 mg/mL) prepared by diluting the Cinobufacini stock solution with serum-free medium. At times of 24 h, 48 h and 72 h after addition of Cinobufacini solutions, 20 μL of methyl thiazolyl tetrazolium (MTT: 5 mg/mL) were added to each well and plates were then incubated for 4 h. Water-insoluble formazan was dissolved by adding 100 μL dimethyl sulfoxide (DMSO) to each well. Finally, optical densities were monitored at 490 nm with 570 nm as a reference wavelength using an ELISA plate reader. The inhibitory rate (IR) and IC_{50} (concentration of drug that inhibits cell growth by 50%) were then calculated.

2.4. *Hoechst 33258 staining*

Hoechst 33258 staining was used to observe the apoptotic morphology of cells. Briefly, 3×10^5 cells/mL cells were seeded in six-well plates and incubated for 24 h. Then the cells were treated with Cinobufacini at 0, 0.01, 0.05, and 0.1 mg/mL, respectively, for 48 h. Finally, cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 15 min and stained with Hoechst 33258 at room temperature for 10 min. After cells were washed with PBS, morphological changes including a reduction in volume and nuclear chromatin condensation were observed under a fluorescence microscope and photographed at a magnification of 200×.

2.5. *Western blotting analysis*

Cells were seeded in culture dishes (35 mm) at a density of 6×10^4 cells/mL and incubated for 24 h. Then, select cells were treated with Cinobufacini at a concentration of 0.1 mg/mL for 24 h and 48 h. At times of 24 h and 48 h, cells were washed with ice-cold PBS twice and lysed with lysis buffer for 30 min at 4°C, and then debris was removed by centrifugation for 10 min at 20,000 × g at 4°C. The protein concentrations of supernatant were determined with a BCA-100 Protein Quantitative Analysis Kit. Equal amounts of protein (30 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were first incubated in blocking solution (5% skim milk) for 1 h at room temperature and then incubated overnight at 4°C with the first antibodies: anti-Bax (1:750-dilution) or anti-BCL-2 (1:750-dilution). After they were washed with TBST (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% tween-20, pH 8.0) three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000-dilution) for 1 h and then again washed with TBST three times. Finally, protein bands were visualized with an enhanced chemiluminescence (ECL) detection system. As an internal control, β-actin was detected with anti-β-actin antibodies. The ratio of Bax/BCL-2 was analyzed using an Alphalmager (IS-2200). (NatureGene Corp., USA).

2.6. *Statistical analysis*

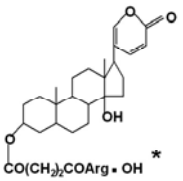
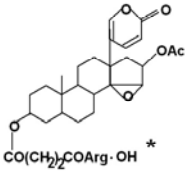
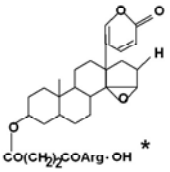
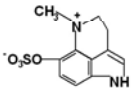
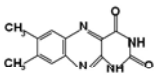
All experiments were performed in triplicate and the results were expressed as mean ± S.D. Statistical analysis was performed by ANOVA using SPSS.11.5 software.

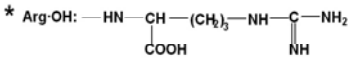
3. Results and Discussion

Despite recent advances in exploring the molecular biology of HCC cells and searching for new chemotherapeutic agents for the treatment of this malignant disease, there are still few efficient therapeutic measures for patients in the advanced stages (*11-13*). Therefore, a significant step would be to find new drugs and effective therapies for the clinical treatment of HCC. Recent reports indicate that traditional Chinese medicines may have a curative potential.

Cinobufacini is a water-soluble preparation of Chinese medicine. Reportedly, there are five mainly compounds that have been isolated from Cinobufacini. Their chemical components and compositions are shown in Table 1 (*14*). Recent studies have shown that Cinobufacini's active ingredients, bufalin and cinobufagin, have a significant effect on inducing apoptosis in a number of cancer cells such as human leukaemia cell lines HL-60 and U937, human gastric cancer cell line BGC-823, human prostate cancer

Table 1. Major chemical constituents of Cinobufacini

Constituents	Structure
Bufalin 3-succinoyl-arginine ester	
Cinobufagin 3-succinoyl-arginine ester	
Resibufogenin 3-succinoyl-arginine ester	
Bufothionine	
Lumichrome	



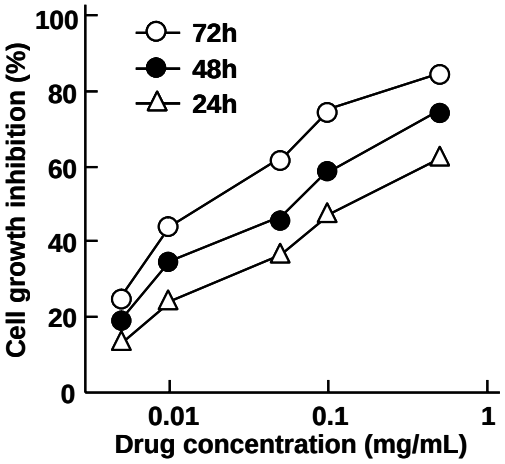


Figure 1. Growth-inhibiting effects of Cinobufacini on Bel-7402 cells. Cells were treated with different concentrations of Cinobufacini as indicated.

cell lines, and human endometrial and ovarian cancer cell lines (*15-17*). Therefore, further study of the active ingredients in Cinobufacini and their effect on HCC is also of great significance. Clinical data on Cinobufacini indicates that it acts to protect liver function and improves the quality of life for patients with little toxicity and few side effects except when treating tumors (*18-20*). However, Cinobufacini has also been reported to inhibit growth in a time-dependent manner in the human normal liver L-02 cell line (*21*). Therefore, another significant step would be to investigate the effects and mechanisms of action of Cinobufacini in normal human liver cells with anti-inflammatory action, anti-viral action, and so on.

The current study found that Cinobufacini extracted from the skin of *Bufo bufo gargarizans* Cantor inhibited proliferation and induced apoptosis in the human hepatoma cell line BEL-7402. MTT assays were performed to investigate the effect of Cinobufacini on the proliferation of BEL-7402 cells. As shown in Figure 1, after treatment with different concentrations (0.01, 0.05, and 0.1 mg/mL) of Cinobufacini for 48 h, the growth inhibition rates of cells were 34.8%, 45.5% and 58.5%, respectively, while the growth inhibition rates of cells treated with 0.1 mg/mL of Cinobufacini for 24 h, 48 h, and 72 h were 47.0%, 58.5% and 74.9%, respectively. In addition, the IC_{50} of BEL-7402 cells at times of 24 h, 48 h, and 72 h were 0.15, 0.06, and 0.02 mg/mL, respectively. These findings indicate that Cinobufacini has a significant growth-inhibiting effect on cells in a dose and time-dependent manner. After treatment with different concentrations (0.01, 0.05, and 0.1 mg/mL) of Cinobufacini for 48 h, marked morphological changes of cell apoptosis including chromatin aggregation, nuclear and cytoplasmic condensation, and partition of cytoplasm and nucleus into membrane-bound vesicles (apoptotic bodies) were observed with Hoechst 33258 staining (Figure 2).

To clarify the mechanisms of apoptosis caused by Cinobufacini, the protein expression of BCL-2 and Bax was examined in BEL-7402 cells after treatment with 0.1 mg/mL Cinobufacini for 24 h and 48 h. As shown in Figure 3A, Western blotting analysis showed that anti-apoptotic protein Bcl-2 expression was down-regulated while pro-apoptotic protein Bax expression was up-regulated in a time-dependent manner. Moreover, the ratio of Bax/BCL-2 significantly increased at all measured times compared to the control (Figure 3B). Apoptosis is now recognized as a key step in the evolution of tumors (*22*). Inducers of apoptosis have recently been used in cancer therapy, and activation of apoptosis pathways is a significant mechanism by which cytotoxic drugs kill tumor cells (*23*). The ratio of Bax to BCL-2, rather than BCL-2 alone, is crucial to the survival of drug-induced apoptosis (*24*). The current results indicated that an increased ratio of Bax to BCL-2 may be a significant mechanism by which Cinobufacini induces apoptosis of BEL-7402 cells.

In conclusion, the present study indicated that

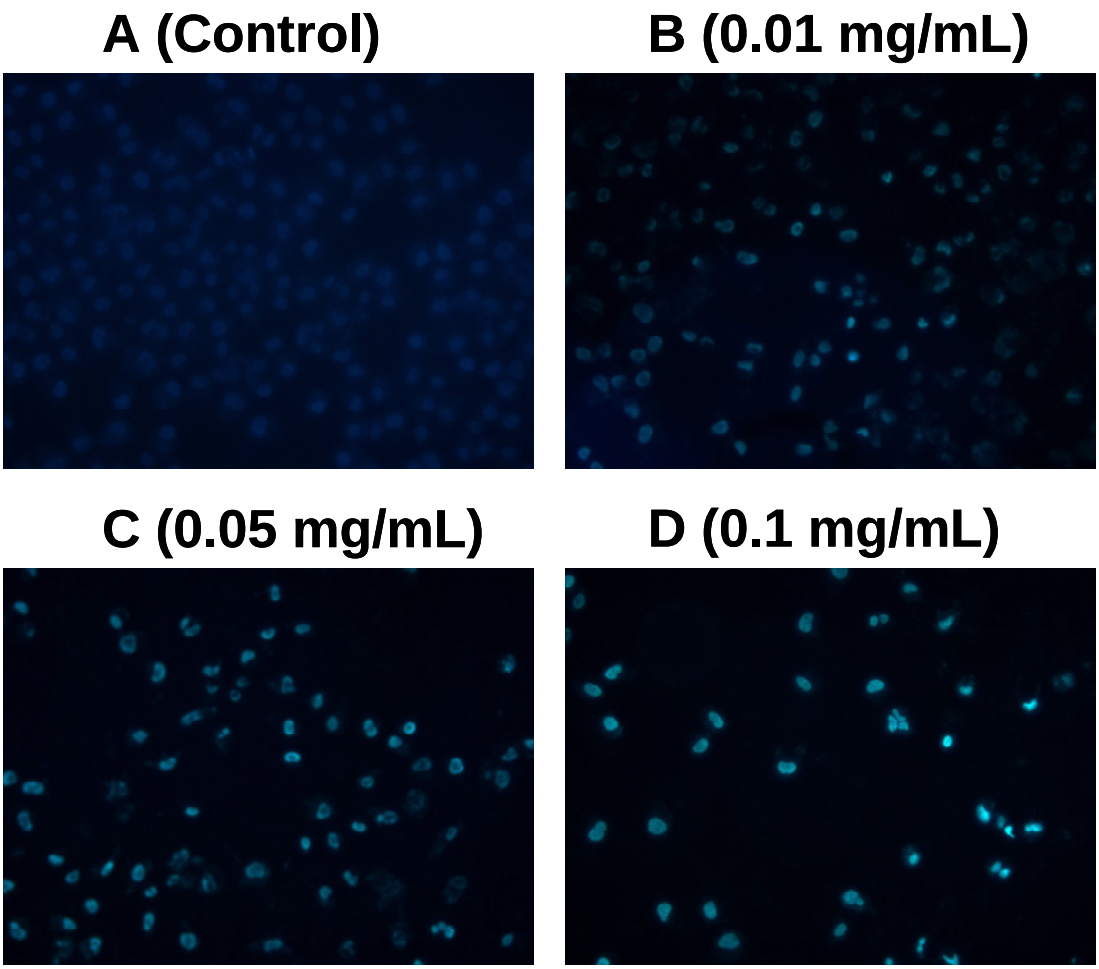


Figure 2. Apoptosis observed with Hoechst 33258 staining. Cells were treated with 0 (A), 0.01 (B), 0.05 (C), and 0.1 mg/mL (D), respectively, for 48 h. Original magnification, 200×.

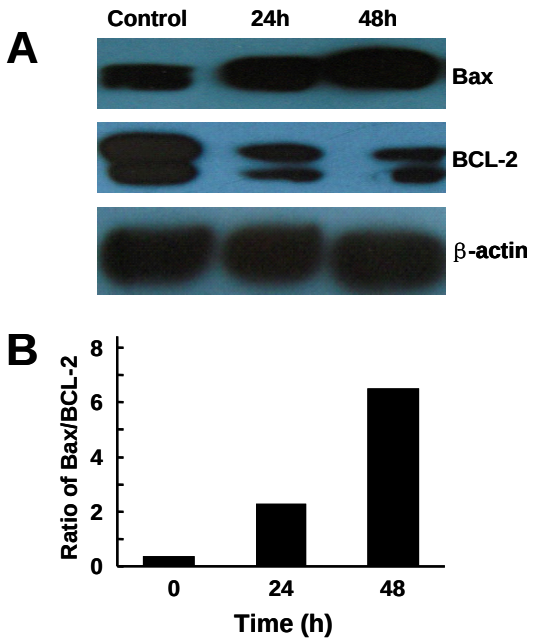


Figure 3. Western blotting analysis of apoptosis-related factors. (A) Effects of Cinobufacini treatment on protein levels of Bax, BCL-2, and β-actin. Cells were treated with 0.1 mg/mL for 24 h and 48 h. (B) The ratio of Bax/BCL-2.

Cinobufacini significantly inhibited the proliferation and induced the apoptosis of BEL-7402 cells. Moreover, apoptosis induced by Cinobufacini may be regulated by the expression of Bax and BCL-2. However, further study is needed to clarify the mechanisms by which Cinobufacini and its active ingredients induce apoptosis.

Acknowledgments

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References

1. Marrero JA. Hepatocellular carcinoma. Curr Opin Gastroenterol 2006; 22:248-253.
2. Tian Z, Si J, Chang Q, Zhou L, Chen S, Xiao P, Wu E. Antitumor activity and mechanisms of action of total glycosides from aerial part of Cimicifuga dahurica targeted against hepatoma. BMC Cancer 2007; 7:237-246.
3. Okita K. Clinical aspects of hepatocellular carcinoma in Japan. Intern Med 2006; 45:229-233.

4. Liu H, Qin CY, Han GQ, Xu HW, Meng M, Yang Z. Mechanism of apoptotic effects induced selectively by ursodeoxycholic acid on human hepatoma cell lines. World J Gastroenterol 2007; 13:1652-1658.
5. Zhu AX. Hepatocellular carcinoma: are we making progress? Cancer Invest 2003; 21:418-428.
6. Yu HY. The application summary of arenobufagin from ancient periods to nowadays. Journal of Liaoning Uinversity of TCM 2008; 10:52-54. (In Chinese)
7. Liang ZH, Zhang WB, Wang B. The significance of Cinobutacini in the prevention and treatment of tumor. Modern Journal of Integrated Traditional Chinese and Western Medicine 2002; 11:215-217. (In Chinese)
8. Meng ZQ, Shen YH, Yang PY, Robert NM, Bei WY, Zhang Y, Ge YQ, Lorenzo C, Razelle KR, Liu LM. Phase I study of huachansu in hepatocellular carcinoma, non-small cell lung cancer, and pancreatic cancer: a preliminary report. China Oncology 2007; 17:6-9. (In Chinese)
9. Zuo XD, Cui YA, Qin SK, Wang JH. Clinical research progress on the antitumor effects of Cinobutacini. Chin Clin Oncol 2003; 8:232-235. (In Chinese)
10. Liu H, Qin CK, Han GQ, XU HW, Ren WH, Qin CY. Synthetic chenodeoxycholic acid derivative, HS-1200, induces apoptosis of human hepatoma cells *via* a mitochondrial pathway. Cancer Lett 2008; 270:242-249.
11. Zhang JF, Liu PQ, Chen GH, Lu MQ, Cai CJ, Yang Y, Li H. Ponicidin inhibits cell growth on hepatocellular carcinoma cells by induction of apoptosis. Dig Liver Dis 2007; 39:160-166.
12. Thomas MB, Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. J Clin Oncol 2005; 23:8093-8108.
13. Kudo M. Early detection and curative treatment of early-stage hepatocellular carcinoma. Clin Gastroenterol Hepatol 2005; 3:S144-148.
14. Yang LH, Jing XQ, Zhang W. Studies on the Chemical Constituents from the skin of *Bufo bufo gargarizans* Cantor. Journal of Shenyang Pharmaceutical University 2000; 17:292-295. (In Chinese)
15. Takai N, Ueda T, Nishida M, Nasu K, Narahara H. Bufalin induces growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cells. Int J Mol Med 2008; 21:637-643.
16. Ye JY, Huang WJ, Kan SF, Wang PS. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. Prostate 2003; 54:112-124.
17. Yang HY, Zhu NX, Hong YW, Yu RX. Experimental research on cinobufagin induces the apoptosis of human leukaemia cell line HL-60. Fujian Journal of TCM 2002; 33:43-44. (In Chinese)
18. Yang YG, Li J, Ma YH, Liu JS. Clinical observation of Cinobutacini combining with chemotherapeutic drugs treating advanced hepatoma. Journal of Practical Traditional Chinese Medicine 2006; 22:2-3. (In Chinese)
19. Wang X, Yuan FQ. Observation of clinical effect in treating primary hepatocellular carcinoma with Cinobutacini. Journal of Practical Traditional Chinese Internal Medicine 2005; 19:379-380. (In Chinese)
20. Liu YX, Kuang TH, Jiang SJ. Clinical observation of Cinobutacini injection on improving the life quality of patients with advanced hepatoma. Chinese Journal of Traditional Medical Science and Technology 2005; 12:54-55. (In Chinese)
21. Shen XB, Li B, Yu CQ. Effects of five traditional Chinese medicine injections on the proliferation of human liver L-02 cell line. Journal of Anhui TCM College 2004; 23:38-39. (In Chinese)
22. Fracchia M, Galatola G, Sarotto V, Perona M, Pera A, Risio M. Serum bile acids, programmed cell death and cell proliferation in the mucosa of patients with colorectal adenomas. Dig Liver Dis 2005; 37:509-514.
23. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, and Sakamoto H, Haga N. Molecular targeting therapy of cancer: drug resistance, apoptosis, and survival signal. Cancer Sci 2003; 94:15-21.
24. Kirkin V, Joos S, Zornig M. The role Bcl-2 family members in tumorigenesis. Biochim Biophys Acta 2004; 1644:229-249.

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Original Article

Part 2. Long term *in vivo*/*in vitro* evaluation of the Cholecystokinin antagonists: *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenylurea MPP and carboxamide MPM

Eric Lattmann[1,*], Yodchai Boonprakob[2], Jintana Sattayasai[3]

[1] *The School of Pharmacy, Aston University, Aston Triangle, Birmingham B4 7ET, UK;*
[2] *School of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand;*
[3] *Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Tailand.*

ABSTRACT: The mixed CCK antagonist *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-indole-carboxamide MPP with a binding affinity of 25 nM/20 nM and the CCK_1 selective 3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenyl-urea MPM (IC_{50} = 25 nM) represent the best two compounds of an amide and a urea pyrazoline series, which were previously evaluated in mice (Part 1) for their CNS activity.

The long term *in vivo* and *in vitro* evaluation is described in this part. Stress was induced for a 4 week period daily. A dose of 0.5 mg/kg of MPP and MPM showed a significant antidepressant effect in the foced swim test in rats, which was in enhanced within a 4 week test period. The mixed CCK antagonist MPM only occurred anxiolytic properties in the elevated X-maze in rats at a 0.5 mg/kg dose. For the stress induced rats, the MPP and MPM treatment reversed the effects of stress on the dendritic atrophy in hippocampal CA3 pyramidal neurons. A reduction of organ weight was reversed for the adrenal gland, when the animals were treated with the CCK antagonists MPP and MPM over a period of 4 weeks.

Keywords: CCK-antagonists, *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-indole-carboxamides, 3-Oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenyl-urea, Forced swim test, Elevated plus-maze, Hippocampal CA3 pyramidal neurons

1. Introduction

Cholecystokinin (CCK) is a peptide neuromodulator and/or neurotransmitter. It was originally discovered from the gastrointestinal system, and is extensively and abundantly distributed within the central nervous system (CNS). CCK was initially isolated as 33 amino acid peptide from the porcine duodenum (*1*). Species specific molecular variants of the CCK have also been identified (CCK-58, CCK-39, CCK-22, sulfated CCK-8, unsulfated CCK-8, CCK-7, CCK-5 and CCK-4) in pig, monkey, rat, cat, dog, chicken and man (*2*).

Receptors for CCK were divided into two subtypes, CCK_A (CCK_1) and CCK_B (CCK_2), which reflected their initial localization in the gastrointestinal tract and the brain, respectively (*3*). However, the presence of CCK_A receptors was demonstrated in various regions of the brain, such as the dorsomedial hypothalamic and habenular nuclei. In addition, CCK_B receptors were identified in the gastrointestinal tract. The CCK_A and CCK_B receptors, both belonging to the class of G protein-coupled receptors, were characterized by seven transmembrane domains (*4*).

The biological roles of peripheral CCK_A receptors were well characterized. They included contraction of the gall bladder, stimulation of pancreatic enzymes secretion, and the potentiation of insulin secretion (*5*).

The peripheral CCK_B receptors were primarily responsible for the stimulation of gastric acid secretion. The central CCK_B receptors were involved in the control of nociception (*6*), the development of anxiety (*7*), panic attacks and satiety (*8*).

Since the CCK-discovery in the CNS, anatomical, physiological and pharmacological studies of cholecystokinin continued steadily. During the last decade, more than 1,000 scientific papers were published on CCK. Interestingly, CCK was not only widely expressed in virtually all CNS regions, it was the most abundant neuropeptide system in the brain of several mammals, especially in the human brain (*9*). In the brain, CCK (*10*) was co-localized with many classical neurotransmitters, such as dopamine (*11*),

**Correspondence to:* Dr. Eric Lattmann, The School of Pharmacy, Aston University, Aston Triangle, Birmingham B4 7ET, UK;
e-mail: e.lattmann@aston.ac.uk

GABA and glutamate (*12*). The co-localization of CCK and GABA in some areas of CNS, especially the cortex and the hippocampus proposed possible roles of CCK in many psychiatric disorders (*13*), including anxiety, depression, attention deficit disorder and in the negative symptoms and cognitive deficits of schizophrenia. Considerable interest was devoted to the pharmacology of CCK_B receptors, since administration of selective agonists produced panic-like attacks in human (*14*). Moreover, CCK_B antagonists had been shown to inhibit panic attacks induced in humans by systemic administration of CCK-4 (*15*). These results led to the conclusion that CCK_B receptors were involved in the regulation of anxiety.

One potential role, which was proposed for CCK, was to act as a modulator of pain (*16*). Indeed, studies have shown, that CCK antagonists potentiated opioid analgesia (*17*) and might also have intrinsic analgesic activity (*18*). A study (*19*) showed that CCK antagonists blocked the development of morphine tolerance (Part 1, *20*).

Specific and highly potent CCK antagonists for both receptor subtypes were developed and suggested to have much pharmacological and therapeutic potential. The discovery of asperlicin (*21*) was the initial point for this new discovery programme. CCK_A antagonists, such as the amino acid derivatives lorglumide and loxiglumide (*22*), the benzodiazepines devazepide (*23*) and FK-480 (*24*) have been developed. Moreover, the pharmacological properties of the potent selective CCK_A antagonists, TP-680 (*25*) and T-0632 (*26*), have been reported. Some CCK_B receptor antagonists such as L-365,260 reached clinical trials and had clinical utility as anxiolytics (*27*), antipsychotics (*28*) or analgesics (*29*). Although various CCK antagonists were produced and studied continuously, toxicity, lack of efficiency and poor pharmaceutical properties of the substances made new compounds still be needed. We have reported the antinociceptive, anxiolytic and antidepressant effects of our *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenylureas and carboxamides in Part 1.

It is focused in this part of the publication on the long term evaluation of two pyrazoline based antagonists, a CCK_A selective amide and a mixed phenyl ureido-antagonist.

2. Materials and Methods

2.1. *Animals*

Experiments were conducted in male IRC mice obtained from the Animal House, Faculty of Medicine, Khon Kaen University. Each experimental group consisted of 6 animals and the treatment procedures were approved by the ethical committee, Faculty of Medicine, Khon Kaen University.

Mice were intraperitoneal injected with test compounds dissolved in 5% DMSO and not more than 0.2 mL/animal. After 30 min animals were tested, as described in the following sections.

2.2. *Antidepression test*

The forced swim test: The forced swim test was carried out in a glass cylinder filled with water and the water temperature was approximately 25-28℃. Rats were gently placed into the water and the immobility time was recorded by an observer during the period of 5 min. Immobility was defined as absence of all movement and rats remained floating passively in the water with its head just above the water surface.

2.3. *Anxiolytic activity test*

The elevated plus-maze: The elevated plus-maze consisted of two open arms without any walls, two enclosed arms, an end wall and the central arena interconnecting all of the arms. The maze was elevated from the floor. At the beginning of the experiment the rat was placed in the central arena facing one of the enclosed arms. During a 5 min interval, the time rats spent in the open arms of the plus-maze was recorded. The rat was considered to be in the open part when it had clearly crossed the line between the central arena and the open arm with its 4 legs.

2.4. *Effect of the CCK antagonists **MPM** and **MPP** in the stress model*

Male Sprague-Dawley rats, weighting 250-300 g at the beginning of the experiment, were housed in groups of three. They were kept in a 12 h light/dark cycle and given food and tap water ad libitum. Rats were divided into 2 conditions, stress (s) and non-stress (ns) and 6 rats/group were used for each test. Stress groups of rats were subjected to chronic restraint stress over a period of 28 days. On each day, rats were individually restraint in wire mesh cages for 6 h (10 am - 4 pm). Prior to the restraint sessions, the rats received either 5% DMSO or the synthetic CCK antagonists at a dose of 0.5 mg/kg BW orally at 9:00 am. On day 1, 7, 14, 21 and 28, the animals were evaluated in the elevated plus maze and the forced swim tests for studying behavioural changes under stress and non- stress conditions.

At the end of the treatment period, rats were deeply anesthetized with thiopental sodium 60 mg/kg intraperitoneally. The adequacy of anesthesia was monitored by checking for the absence of corneal reflexes and the flexor withdrawal response. Anesthetic rats were transcardially perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After the fixative perfusion, the brain was removed rapidly and cut into 2 sides, which

were subsequently used for the Golgi-Cox method and immunohistochemistry.

2.5. *Golgi-Cox method*

Preservative perfused slices were cut into 4-5 mm thick slices with a sharp razor blade and impregnated in the Golgi-Cox solution for 20-30 days in the dark. The impregnated blocks of tissue were embedded in paraffin before sectioning. The coronal sections, 100 µm thick were cut on a microtome. The sections were put on a clean drop of water on glass slides. Subsequently the sections were spread at 40°C on a hot plate. They were dried at 40°C in the oven for 1 h, rinsed in xylene, covered with mounting media, which was slipped. In order to be selected for analysis, golgi impregnated neurons had to possess the following characteristics:

(i) Location within the CA3 region of the dorsal hippocampus
(ii) Dark and consistent impregnation throughout the extent of all of the dendrites
(iii) Relative isolation from neighboring impregnated cells, which could interfere with the analysis

From each animal, 8-10 pyramidal cells from CA3 were selected. Each selected neuron was traced at 10× magnification, using a light microscope with a camera lucida drawing tube attached. From these drawings, the number of dendritic branch (bifurcation) points tree was determined for each selected neuron within a 100 µm thick section of each dendritic.

2.6. *Immunohistochemistry method*

The left side of the brain was postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. Tissues were rinsed with phosphate buffer and infiltrated with a 30% sucrose solution in order to provide cryprotection. The specimens were frozen rapidly with deep freeze at -25°C in a cryostat. After freezing, coronal section of 35 µm thick specimens were cut on a cryostat and stored in phosphate buffer. The specimens were stained with monoclonal antibody against choline acetyltransferase (ChAT) enzyme, a marker for cholinergic neurons and the density of immunoreactive neurons was determined in hippocampal areas.

2.7. *Weights of certain organs affected by stress*

After the brain was removed, adrenal glands and the spleen were dissected out. The surrounding fat and extrageneous tissues were removed and the organs were pat dried and weighed using a weighing-machine. The results were expressed as mg/100 g BW.

2.8. *Statistical analysis*

All data were expressed as mean ± SD. Significant difference between control and treatment was determined by using unpaired Student *t*-test. The differences among various groups were compared by ANOVA. Turkey test for pair wise comparison was performed to determine any significant difference at p-value < 0.05.

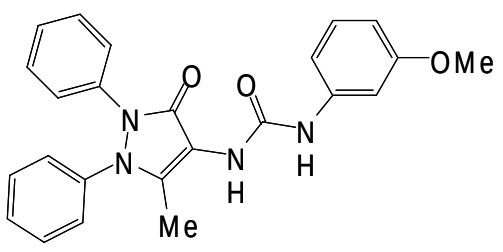
3. Results

The potent CCK_1 selective antagonist **MPP** and the mixed CCK antagonist **MPM** were selected and tested for the long term effects on stress responses in rats. The chemical structures of **MPP** and **MPM** are outlined in Figure 1.

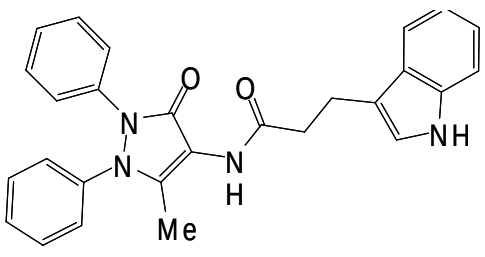
3.1. *Behavioural effects*

In the forced swim test, immobility times (in s) of the control non-stress rats, which received 5% DMSO were 149.15 ± 6.34, 155.99 ± 14.10, 155.09 ± 0.57, 155.31 ± 7.47 and 152.93 ± 6.78, respectively, when tested on day 1, 7, 14, 21 and 28. No significant difference could be observed among the tests carried out at various times in the control non-stress group.

Non-stress rats receiving either **MPM** or **MPP** had a significant decrease in immobility time, when observed on day 7, 14, 21 and 28, but not day 1, compared to the control non-stress group on the same day.



MPM



MPP

Figure 1. Selected structures of *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-1*H*-indole-carboxamides and ureido-pyrazolines.

Restrained rats (stress group) had a significant increase in immobility time when observed on day 7 and further until the end of treatment, when compared to the control non-stress group on the same day of the test. From day 7 until the end of experiment, stress rats receiving either **MPM** or **MPP** showed a significant reduction of immobility time, when compared to stress rats that received 5% DMSO. In addition, the immobility time of stress rats receiving either **MPM** or **MPP** was found also significantly lower than the non-stress control rats, especially at day 28.

In the elevated plus maze test (Figure 3), no change in time in the open arms and number of entry could be observed in the non-stress rats that received 5% DMSO (as control) until the end of the experiment. On day 21 and 28, non-stress rats, which received 0.5 mg/kg BW of **MPM**, had a significant increase in time spent in the open arms and the number of entry, when compared to the control non-stress group of the same day.

From day 7 until the end of the experiment, stress rats showed a significant reduction of time in the open arms and number of entry, when compared to the control non-stress group tested at the same day. It was observed that **MPM** treatment reduced the anxiogenic effect of stress significantly when tested on day 14, 21 and 28.

The results showed that restraining stress could produce depression and anxiety in rats, which could be observed as early as 7 days of restraint. Oral treatment with the mixed CCK antagonist **MPM** and the CCK_A selective antagonist **MPP** reduced depression and **MPP** reduced the anxiogenic effect of stress in rats in our experiments.

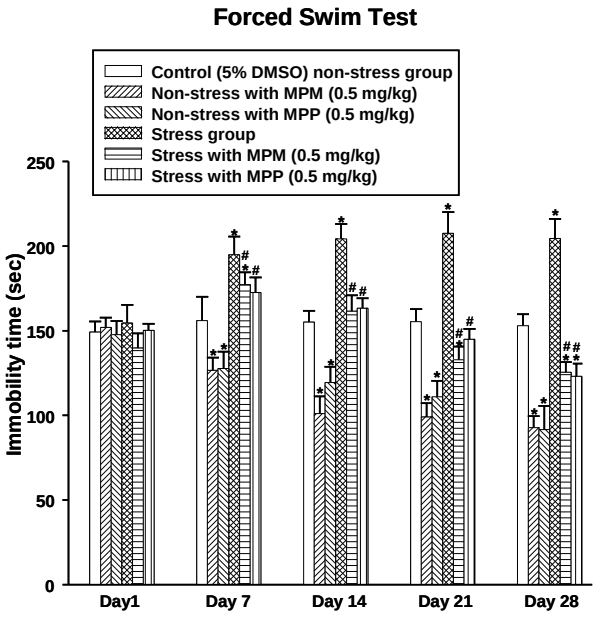


Figure 2. Effects of **MPM** and **MPP** on immobility time of non-stress and stress rats tested in the forced swim test. *P*-value < 0.05; * compared to the control non-stress (5% DMSO); # compared to the stress group on the same day of the test.

3.2. *Effects of **MPP**/**MPM** and stress on hippocampal CA3 pyramidal neurons*

Figure 4 showed the hippocampal CA3 pyramidal neurons impregnated with Golgi-Cox solution for 20-30 days from various treatment groups. In the control non-stress group, both basal and apical dendritic trees were highly branched (Figure 4A). Pyramidal neurons from rats, which were restrained for 28 days, showed atrophic changes of dendrites especially in the apical branches (Figure 4B). **MPM** (Figure 4C) and **MPP** (Figure 4D) treatment reversed the effect of stress on dendritic atrophy and the neurons appeared normal.

Each selected neuron from the sections was drawn on paper with a 10 µm sector from the centre (neuronal cell body) using a camera lucida drawing tube, attached to the microscope under 10× objective magnification and the drawings were shown in Figure 5.

The total number of branch points and the length of the dendrites, as estimated by the radius of the field, were

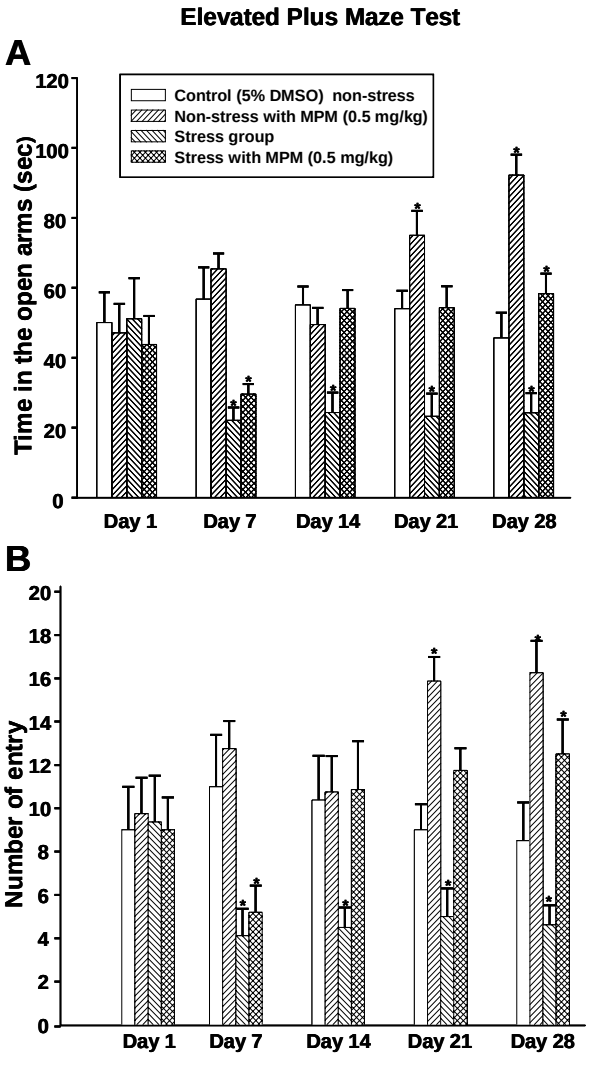


Figure 3. Effects of **MPM** on time spent in the open arms (A) and the number of entries (B) of non-stress and stress rats tested in the elevated plus maze test. *P*-value < 0.05; compared to the control non-stress (5% DMSO).

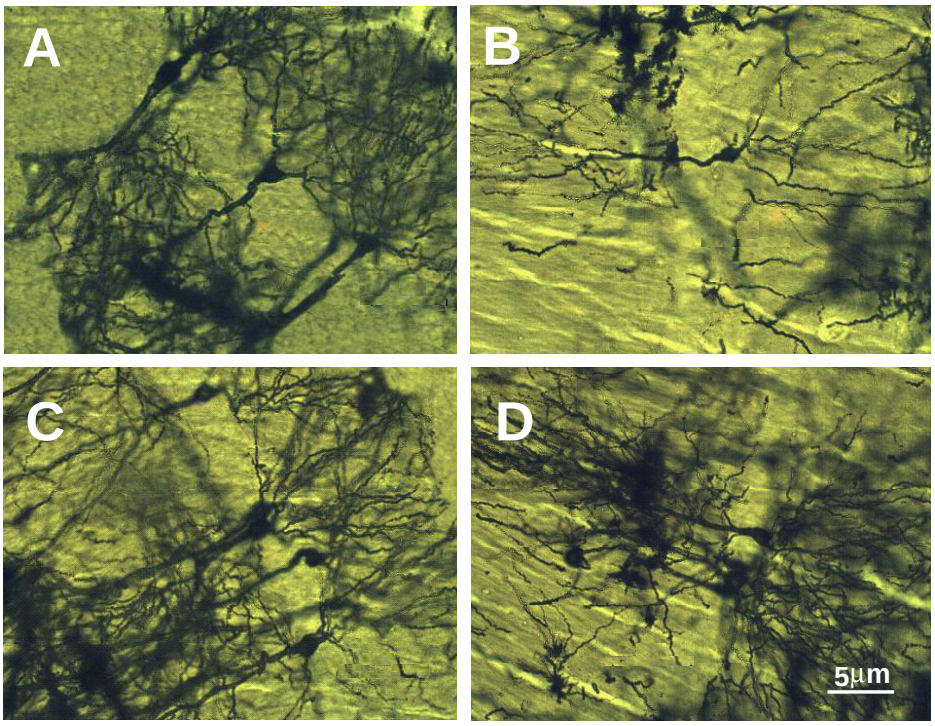


Figure 4. The Golgi-impregnated CA3 pyramidal neurons from rat's hippocampus at 20× magnification. A: the control, non-stress group; B: stress group; C: stress with **MPM** group; D: stress with **MPP** group.

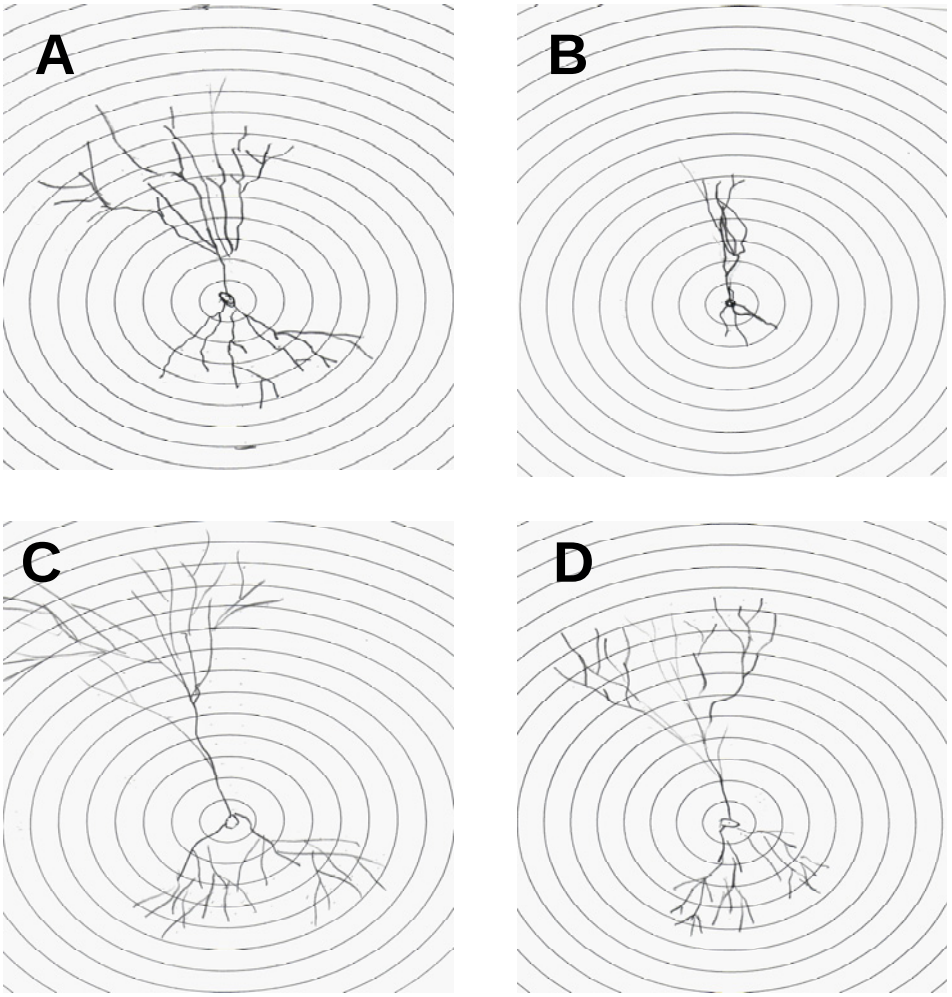


Figure 5. Camera lucida drawings of Golgi-impregnated CA3 pyramidal neurons from rat's hippocampus. Each sector of the drawing was equal to 10 µm. A: Control, non-stress group; B: Stress group; C: Stress with **MPM** group; D: Stress with **MPP** group.

determined from the drawings (10 neurons were traced from each group). In the stress group, both the number of branch points (Figure 5) and the radius (Figure 6) of the dendritic field of the apical tree, but not basal tree, were found significantly reduced compared to the control non-stress group. Stress rats, which received either **MPM** or **MPP**, showed no difference from the control group in both observed parameters. This suggested that both antagonists, **MPM** and **MPP** were able to antagonize the induced dendritic atrophy caused by stress.

3.3. *Effects of **MPM**/**MPP** and stress on organ weight*

Normally, the adrenal glands and the spleen are two of many organs affected by stress conditions. The weights of the adrenal glands and the spleen, expressed as mg/100 g BW, were outlined in Figure 8 for various groups of rats. Non-stress rats receiving 5% DMSO, served as control and the wet weights of the adrenal glands and the spleen were recorded as 20.00 ± 2.97 and 324.25 ± 18.49 mg/100 g BW, respectively. No effect of either **MPM** or **MPP** treatment on the weights of the two organs was observed in non-stress rats. Restraining the rats for 28 days increased the wet weights of the adrenal glands significantly, without having any effect on the weight of the spleen. Treatment with either **MPM** or **MPP**, at a dose of 0.5 mg/kg BW/day, antagonized in stress rats the effects of stress on the wet weights of the adrenal glands, which was found comparable to the control group after this 28 day treatment period.

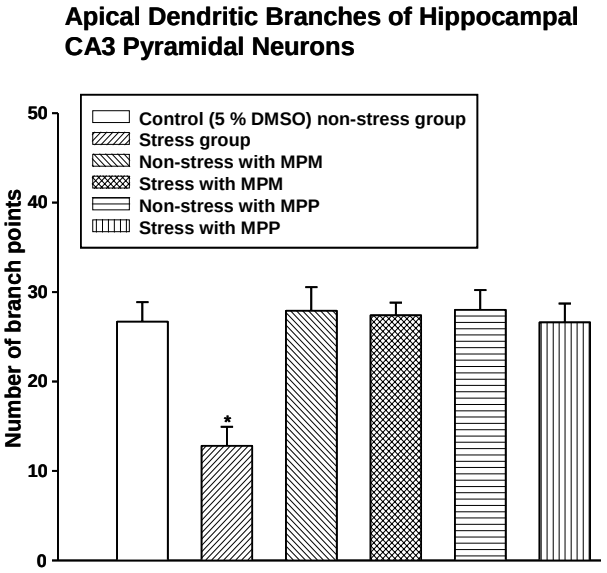


Figure 6. Effects of **MPM** and **MPP** on the number of apical dendritic branch points observed in hippocampal CA3 pyramidal neurons for non-stress and stress rats. * *P*-value < 0.05 when compared to the control non-stress group.

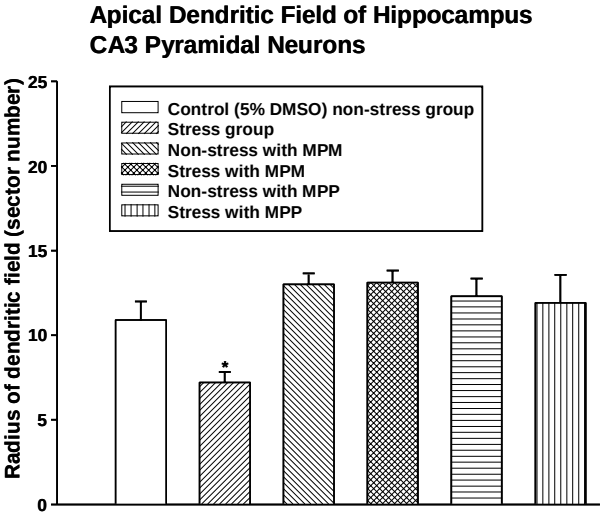


Figure 7. Effects of **MPM** and **MPP** on the radius of apical dendritic fields observed in hippocampal CA3 pyramidal neurons in non-stress and stress rats. * *P*-value < 0.05 when compared to the control non-stress group.

4. Discussion

In the present 4 week-study in rats, the antidepressant-like and anxiolytic-like long term effects of a mixed (**MPP**) and CCK_1 selective antagonist (**MPM**) were further evaluated, using effective and reliable animal models, such as the Porsolt swim test and the elevated X-maze.

The antagonistic effects against stress on rat's behaviours and the hippocampal neurons were clearly determined for the previously found active dose of 0.5 mg/kg of both CCK antagonist. Animal models of anxiety and depression, based on emotional reactivity, have been designed and proven to be bidirectional sensitive to stressful manipulations (*30*) and after the determination of effective doses in part 1 it was now investigated, what long term effects were observed when used at an effective dose. By simple, rapid and inexpensive ways of evaluating an animal's conditions, the forced swim test was used for testing antidepressant-like effects, whereas the elevated plus maze was used

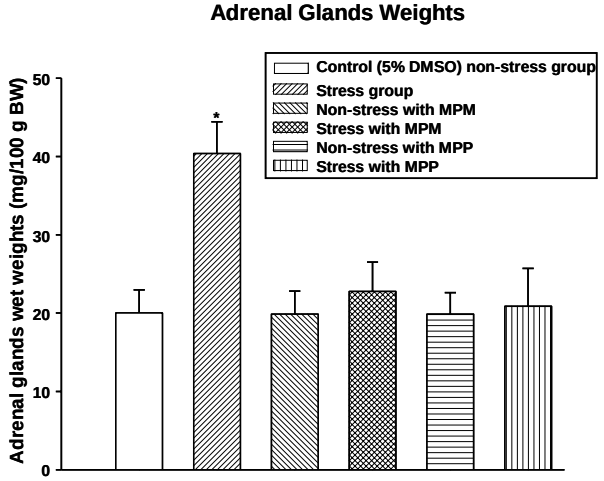


Figure 8. Effects of **MPM** and **MPP** on the wet weights of adrenal glands of non-stress and stress rats. Data were presented as mean ± SD. * *P*-value < 0.05 when compared to the control non-stress group.

for anxiolytic-like effects showing better effect when used long term. The aim was to investigate, if tolerance or further improvement, was observed towards the positive biological effects. Among the experimental models used for testing the antidepressant-like effect of the compounds, the forced swim test (also known as the Porsolt swim test) is one of commonly used and best model. The test is easily to perform and there is no need to use any expensive instruments. In our study, the forced swim test was found sensitive and reliable in detecting the antidepressant-like of the CCK_1 selective amide **MPP** and the mixed antagonist **MPM**.

The elevated plus maze and the light and dark box tests are also classified as a test, suitable for studying the acute stress effects. The elevated plus maze test, a well-validated animal model has become the most widely used model for the study of drug effects on anxiety (*31*) and only **MPM** showed anxiolytic effects.

The tail flick and the hot plate tests are widely used in pain assessment in animals and considered to be supraspinally integrated responses to heat (*32*) and the antinociceptive effects were discussed in part one of this series of publications (*20*).

Despite many findings, however, conflicting results concerning the types of CCK receptors involved in those mood disorders have been reported. The stimulation of CCK_1 or CCK_2 receptors was implicated in the physical and psychological responses of CCK to stress. Furthermore, several selective CCK_2 agonists produced anxiogenic-like effects, while CCK_2 antagonists induced anxiolytic-like effect in several models of anxiety (*33*). However, there was evidence indicated, that CCK_1 receptors were involved in the mediation of anxiolytic-like effects in the light and dark box model of exploration in mice (*34*). In the same model CCK_2 antagonists also showed an anxiolytic-like effect (*35*). Thus, both CCK_1 and CCK_2 receptors could have roles in the modulation of anxiety-related behaviour in animal models (*36*) as seen for **MPM**. The anxiolytic-like effect of only the mixed CCK antagonists is rather complex, as discussed by Hendrie *et al*., 1993. It has been reported, that CCK through CCK_1 receptor could potentiate the effect of amines, while CCK_2 receptor could inhibit the amine release (*37*). It might be the case, that the optimal ratio of the binding affinity among CCK_1 and CCK_2 receptors reflects best the results on mood disorders, as seen here with **MPM**.

As mood disorders are the abnormal behaviours, mostly found as response to stress conditions, it is interesting to see the effects of CCK antagonists in antagonizing the effects of stress. In the present study, 28 days of chronic restraint stress produced significant hippocampal dendritic atrophy, especially in the CA3 area, as previously shown (*38*). Atrophic changes (*39*) were clearly seen in apical, but not basal dendrites. Changes in basal dendrites were reported with prolonged stress (*40*). The effects of stress on hippocampal neurons were suggested to mediate through many mechanisms including glucocorticoid (*41*), glutamate (*42*), serotonin (*43*) and GABA (*44*). Glutamate, as an excitotoxin, might be a very important pathway in the hippocampal damage by stress, by acting through NMDA receptors. Serotonin released by stress may interacted pre-or post-synaptically with glutamate release and also potentiate NMDA receptor binding *via* $5\text{-}HT_2$ receptors.

Restraint stress also showed effects on the adrenal glands, but not the spleen (*45*). The enlargement of adrenal glands, observed after restraint stress, might indicate an increase in glucocorticoid synthesis / release in response to stress. However, it is still not known, whether the enlargement was due to hypertrophy or cellular hyperplasia and if the findings were sub-region specific or not.

The spleen size was not changed by stress in this study. Although a lower number of spleen cells were present, which correlate with a decreased number of lymphocytes in the circulation (*46*), the changes in cell numbers may not be detectable by measuring the wet weight of the organ.

MPM and **MPP**, prevented the effects of stress on mood changes, hippocampal dendrites and adrenal gland weight. The anti-stress effects of CCK antagonists could possibly act at many sites. The interaction of CCK-8S with glutamate was studied in the hippocampal CA3 and suggested, that excitatory amino acids may be enhanced by CCK-8S (*47*). Moreover, CCK was also able to regulate the limbic hypothalamo-pituitary-adrenal (LHPA) axis, acting on both, its central and peripheral parts.

CCK stimulated aldosterone secretion *via* CCK_1 and CCK_2 receptors in zona glumerulosa cells in the adrenal cortex and therefore, enhanced glucocorticoid secretion from zona fasciculata-reticularis cells *via* an indirect mechanism, involving a CCK_2 receptor mediated stimulation of ACTH release (*48*). Accordingly, CCK antagonists might antagonize stress effects through both types of receptors at hippocampus, pituitary and adrenal glands and break the LHPA axis in response to stress. As suggested earlier, the effects of CCK antagonists against stress may need the proper ratio of the effect against CCK_1 and CCK_2 receptors, since that receptor could inhibit and stimulate corticosteroid secretion, respectively (*49*).

5. Conclusions

Significant antidepressant-like effects were clearly observed and improved over time in rodents, treated with **MPM** or **MPP** in the forced swim-tests.

Anxiolytic-like effects were determined in rodents treated with **MPM**. The effects could be seen best in the elevated plus maze and no tolerance was observed.

MPM and **MPP** at a dose of 0.5 mg/kg BW in rats, antagonized all the effects of chronic restraint stress *in vivo* and *in vitro*. The CCK antagonists antagonised mood disorders (depression/anxiety) in rats *in vivo* and antagonised the stress induced hippocampal dendritic atrophy and an increased in adrenal glands weight *in vitro* over a 4 week period. These non-chiral, readily available agents, such as **MPM**, will play an exciting new role as novel substances in clinical trials for mood disorders and/or, in combination with morphine in various types of pain (part 1 and part 2).

Acknowledgement

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References

1. Mutt V, Jorpes JE. Structure of porcine cholecystokinin-pancreozymin. 1. Cleavage with thrombin and with trypsin. Eur J Biochem 1968; 6:156-162.
2. Reeve JR. Relative bioactivities of cholecystokinins-8 and -33 on rat pancreaticacini. Peptides 1986; 7:723-727.
3. Innis RB, Snyder SH. Distinct cholecystokinin receptors in brain and pancreas. Proc Natl Acad Sci U S A 1980; 77:6917-6921.
4. Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ. Model systems for the study of seven-transmembrane-segment receptor. Annu Rev Biochem 1991; 60:653-680.
5. Gardner JD, Jensen RT. Derivatives of CCK-(26-32) as cholecystokinin receptor antagonists in guinea pig pancreatic acini. Am J Physiol 1984; 246:292-295.
6. Baber NS, Dourish CT, Hill DR. The role of CCK caerulein, and CCK antagonists in nociception. Pain 1989; 39:307-328.
7. Singh L, Lewis AS, Field MJ, Hughes J, Woodruff GN. Evidence for an involvement of the brain cholecystokinin B receptor in anxiety. Proc Natl Acad Sci U S A 1991; 88:1130-1133.
8. Kulkosky PJ, Sanchez MR, Foderaro MA, Chiu N. Cholecystokinin and satiation with alcohol. Alcohol 1989; 6:395-402.
9. Larsson LI. Innervation of the pancreas by substance P, enkephalin, vasoactive intestinal polypeptide and gastrin/CCK immunoractive nerves. J Histochem Cytochem 1979; 27:1283-1284.
10. Rehfeld JF. Gastrin and cholecystokinin in human cerebrospinal fluid. Immunochemical determination of concentrations and molecular heterogeneity. Brain Res 1978; 155:19-26.
11. Crawley JN. Subtype-selective cholecystokinin receptor antagonists block cholecystokinin modulation of dopamine-mediated behaviors in the rat mesolimbic pathway. J Neurosci 1992; 12:3380-3391.
12. Abelson JL, Nesse RM, Vinik AI. Pentagastrin infusions in patients with panic disorder. II. Neuroendocrinology. Biol Psychiatry 1994; 36:84-96.
13. Hendry SHC, Jones EG, DeFilipe J, Schmechel D, Brandon C, Emson PC. Neuropeptide containing neurons on the cerebral cortex are also GABAergic. Proc Natl Acad Sci U S A 1984; 81:6526-6530.
14. De Montigny C. Cholecystokinin tetrapeptide induces panic-like attacks in healthy volunteers. Preliminary findings. Arch Gen Psychiatry 1989; 46:511-517.
15. Bradwejn, Koszycki D, Shriqui C. Enhanced sensitivity to cholecystokinin tetrapeptide in panic disorder. Clinical and behavioral findings. Arch Gen Psychiatry 1991; 48:603-610.
16. Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. Evidences for the neuropeptide cholecystokinin as an antagonists of opiate analgesia. Science 1983; 219:310-312.
17. Dourish CT, Clark ML, Iverson SD. Analgesia induced by restraint stress in attenuated by CCK and enhanced by the CCK antagonists MK-329, L-365,031. Soc Neurosci 1988; 14:290.
18. O'Neill MF, Dourish CT, Iverson SD. Hypolocomotion induced by peripheral or central injection of CCK in the mouse is blocked by the CCK_A receptor antagonist devazepide but not by the CCK_B receptor antagonist L-365,260. Eur J Pharmacol 1990; 193:203-208.
19. Dourish CT, O'Neill MF, Coughlan J, Kitchener SJ, Hawley D, Iverson SD. The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. Eur J Pharmacol 1990; 176:35-44.
20. Lattmann E,Sattayasai J, Boonprakob Y, Singh H, Lattmann P, Dunn S. Cholecystokinin antagonists (part 1): Antinociceptive, anxiolytic and antidepressant effects of *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenylureas and carboxamides. Drug Discov Ther 2008; 2:156-167.
21. Chang RS, Lotti VJ. A potent nonpeptide cholecystokinin antagonist selective for peripheral tissues isolated from Asperigillus alliaceus. Science 1985; 230:177-180.
22. Makovec F, Bani M, Chisté R, Revel L, Rovati LC, Rovati LA. Differentiation of central and peripheral cholecystokinin receptors by new glutaramic acid derivatives with cholecystokinin-antagonistic activity. Arzneimittelforschung 1986; 36:98-102.
23. Chang RS, Lotti VJ. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. Proc Natl Acad Sci U S A 1986; 83:4923-4926.
24. Ito H, Sogabe H, Nakarai T, Sato Y, Tomoi M, Kadowaki M, Matsuo M, Tokoro K, Yoshida K. Pharmacological profile of FK-480, a novel cholecystokinin type-A receptor antagonist: comparison to loxiglumide. J Pharmacol Exp Ther 1994; 268:571-575.
25. Akiyama T, Tachibana I, Hirohata Y, Shirohara H, Yamamoto M, Otsuki M. Pharmacological profile of TP-680, a new cholecystokininA receptor antagonist. Br J Pharmacol 1996; 117:1158-1164.
26. Taniguchi H, Yazaki N, Yomota E, Shikano T, Endo T, Nagasaki M. Pharmacologicalprofile of T-0632, a novel potent and selective CCK_A receptor antagonist, *in vivo*. Eur J Pharmacol 1996; 312:227-233.
27. Josselyn SA, Frankland PW, Petrisano S, Bush DE, Yeomans JS, Vaccarino FJ. The CCK_B antagonist, L-365,260, attenuates fear-potentiated startle. Peptides 1995; 16:1313-1315.
28. Feifel D, Reza T, Robeck S. Antipsychotic potential of CCK-based treatments: an assessment using the prepulse inhibition model of psychosis. Neuropsychopharmacology 1999; 20:141-149.
29. McCleane GJ. A phase 1 study of the cholecystokinin

(CCK) B antagonist L-365,260 in human subjects taking morphine for intractable non-cancer pain. Neurosci Lett 2002; 332:210-212.
30. Espejo EF. Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behav Brain Res 1997; 87:233-238.
31. Hogg S. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 1996; 54:21-30.
32. Dubner R, Ren K. Endogenous mechanisms of sensory modulation. Pain 1999; 6:S45-53.
33. Wang H, Wong PT, Spiess J, Zhu YZ. Cholecystokinin-2 (CCK_2) receptor-mediated anxiety-like behaviors in rats. Neurosci Biobehav Rev 2005; 29:1361-1373.
34. Hendrie CA, Neill JC, Dourish CT. The effect of CCK_A and CCK_B antagonists on activity in the black/white exploration model of anxiety in mice. Physiol Behav 1993; 54:689-693.
35. Bickerdike MJ, Marden CA, Dourish CT, Fletcher A. The influence of 5-hydroxytryptamine re-uptake blockade on CCK receptor antagonist effects in the rat elevated zero-maze. Eur J Pharmacol 1994; 271:403-411.
36. Rotzinger S, Vaccarino FJ. Cholecystokinin receptor subtypes: role in the modulation of anxiety-related and reward-related behaviours in animal models. J Psychiatry Neurosci 2003; 28:171-181.
37. Crawley JN. Cholecystokinin-dopamine interactions. Trends Pharmacol Sci 1991; 12:232-236.
38. Cook SC, Wellman CL. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. J Neurobiol 2004; 60:236-248.
39. Radley JJ, Morrison JH. Repeated stress and structural plasticity in the brain. Ageing Res Rev 2005; 4:271-287.
40. Shankaranarayana Rao BS, Govindaiah, Laxmi TR, Meti BL, Raju TR. Subicular lesions cause dendritic atrophy in CA1 and CA3 pyramidal neurons of the rat hippocampus. Neuroscience 2001; 102:319-327.
41. Magarinos AM, McEwen BS. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors. Neuroscience 1995; 69:83-88.
42. Magarinos AM, McEwen BS, Flugge G, Fuchs E. Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shews. J Neurosci 1996; 16:3534-3540.
43. Watanabe Y, Gould E, Cameron HA, Daniels DC, McEwen BS. Stress and antidepressant effects on hippocampus. Eur J Pharmacol 1992; 222:157-162.
44. Magarinos AM, Deslandes A, McEwen BS. Effects of antidepressant and benzodiazepine treatments on dendritic structure of CA3 pyramidal neurons after chronic stress. Eur J Pharmacol 1999; 371:113-122.
45. Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman J. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. Am J Physiol Endocrinol Metab 2006; 291:E965-973.
46. Welsh CJ, Bustamante L, Nayak M, Welsh TH, Dean DD, Meagher MW. The effects of restraint stress on the neuropathogenesis of Theiler's virus infection II: NK cell function and cytokine levels in acute disease. Brain Behav Immun 2004; 18:166-174.
47. Gabriel S, Grutzmann R, Lemke M, Gabriel HJ, Henklein P, Davidowa H. Interaction of cholecystokinin and glutamate agonists within the dLGN, the dentate gyrus, and the hippocampus. Brain Res 1996; 39:381-389.
48. Nussdorfer GG, Spinazzi R, Mazzocchi G. Cholecystokinin and adrenal-cortex secretion. Vitam Horm 2005; 71:433-453.
49. Malendowicz LK, Spinazzi R, Majchrzak M, Nowak M, Nussforder GG, Ziolkowska A, Macchi C, Trejter M. Effects of prolonged cholecystokinin administration on rat pituitary-adrenocortical axis: role of the CCK receptor subtypes 1 and 2. Int J Mol Med 2003; 12:903-909.

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Original Article

Pioglitazone attenuates tactile allodynia and microglial activation in mice with peripheral nerve injury

Shigeki Iwai[1], Takehiko Maeda[1,*], Norikazu Kiguchi[1], Yuka Kobayashi[1], Yohji Fukazawa[1], Masanobu Ozaki[2], Shiroh Kishioka[1]

[1] *Department of Pharmacology, Wakayama Medical University, Wakayama, Japan;*
[2] *Department of Toxicology, Niigata University of Pharmacy and Applied Life Science, Niigata, Japan.*

ABSTRACT: To test the possibility of a peroxisome proliferator activated receptor (PPAR)γ agonist to treat neuropathic pain, we examined the effects of pioglitazone, a PPARγ agonist, on tactile allodynia and expression of activated microglia in the dorsal horn of spinal cord using neuropathic pain model. The unilateral sciatic nerve was partially ligated (PSL) in male ICR mice. Pioglitazone (1-25 mg/kg p.o.) was administrated to mice once daily for five days immediately after PSL. We stimulated the footpad of the hind paw of mice using a von Frey filament to estimate tactile allodynia on day 5 of PSL. The activated microglia in the lumbar spinal cord was observed by immunohistochemistry with anti-Iba1 antibody, a marker for activated microglia. The number of Iba1-immunoreactive cells was counted in the dorsal horn spinal cord. On day 5, significant allodynia was developed in PSL mice. Pioglitazone significantly attenuated the tactile allodynia in a dose of 1-25 mg/kg. However, these doses of pioglitazone did not affect nociceptive responses in sham mice. Moreover, on day 6, the number of activated microglia was significantly increased in the ipsilateral dorsal horn of mice. The increase in the number of activated microglia induced by PSL was significantly suppressed by pioglitazone (1-25 mg/kg p.o.). Pioglitazone did not affect the number of activated microglia in sham mice. These results suggest that PPARγ activation inhibits the development of tactile allodynia and the expression of activated microglia in the dorsal horn of spinal cord in mice with PSL-induced peripheral nerve injury.

Keywords: Ligation, Neuropathic pain, Sciatic nerve, Spinal cord, Thiazolidinedione

**Correspondence to:* Dr. Takehiko Maeda, Department of Pharmacology, Wakayama Medical University, Wakayama 641-0012, Japan;
e-mail: tmaeda@wakayama-med.ac.jp

1. Introduction

Neuropathic pain is characterized by pain in the absence of a stimulus and/or by reduced nociceptive thresholds so that normally innocuous stimuli produce pain. This is a burdensome and potentially debilitating pain state. Numerous studies using animal models have proposed candidates for therapeutic targets to reduce neuropathic pain. The therapeutic strategies for neuropathic pain aim to reduce the excitability of neurons in the peripheral nervous system and/or the CNS by modulating the activity of ion channels or by mimicking and enhancing endogenous inhibitory mechanism. However, currently, there are no effective pharmacotherapies for neuropathic pain (*1*).

Microglial cells have a key role in the response to direct injuries of the central nervous system elicited by trauma or ischemia, in autoimmune diseases, and in neurodegenerative disorders (*2*). Recent evidence indicates that activated microglia are key cellular intermediates in the pathogenesis of nerve injury-induced pain hypersensitivity. Microglial activation leads to increased synthesis of the protease (*3*) and the cytokines (*4*). Direct modulation of dorsal horn neuron activity by these cytokines may be involved in the development of neuropathic pain. Therefore, targeting glia could provide opportunities for disease modification by aborting neurological alterations that support the development of persistent pain.

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor belonging to a nuclear hormone receptor superfamily, containing three isoforms (α, β/δ and γ). PPARγ plays a critical physiological role as a primary lipid sensor and regulator of lipid metabolism. Thus, its ligands are clinically used for treatment of some diseases, including type 2 diabetes (*5*). However, PPARγ has additional effects on cellular physiology. Activation of PPAR has been shown to suppress inflammation in peripheral macrophages and in models of human autoimmune disease (*6*). Recently, it has been found

that PPARγ ligands have promising therapeutic use in neurological diseases involving neuroinflammation, such as Alzheimer's disease and multiple sclerosis (*7*). There are two reports indicating that PPARγ ligands can reduce neuropathic pain in animal models (*8,9*). Nonetheless, further information on activation of microglia and neuropathic pain induced by peripheral nerve injury is not available. In the present study, we examined the correlation of effect of PPARγ agonist pioglitazone on tactile allodynia and on microglia activation in the dorsal horn of spinal cord elicited by partial sciatic nerve ligation (PSL).

2. Materials and Methods

2.1. *Subjects and surgery*

Male ICR mice (5-week-old: Japan SLC, Hamamatsu) were anesthetized with pentobarbital (80 mg/kg, i.p., Dainippon Pharmaceuticals Co., Osaka, Japan). The sciatic nerve (SCN) was exposed just below the hip bone, and half of the sciatic nerve was tightly ligated with silk suture thread (PSL), according to the modified method of Seltzer *et al.* (*10*). The procedures used in these studies were approved by the Animal Research Committee of Wakayama Medical University in accordance with Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and The Guidelines for Animal Experiments in Wakayama Medical University (approval number 271).

2.2. *Behavioral test*

We observed the withdrawal responses of hind paw of which the plantar surface was applied with calibrated von Frey filaments (0.4 g; Stolting, Wood Dale, IL, USA) on day 5 following PSL. Tactile allodynia was calculated as the ratio of the number of hind paw withdrawals of 5 stimulations.

2.3. *Immunohistochemistry*

Six days following PSL, the mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 20 mL of PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar spinal cord were dissected out and cut transversely (20-μm-thick) with a cryostat. The sections were incubated with a rabbit antibody against mouse Iba1 (Wako, Osaka, Japan). Then, the sections were incubated with secondary antibody solution (Alexa Fluor 488-conjugated antibody to the rabbit IgG, Molecular Probes, Eugene, OR, USA). Fluorescent images for a mouse were captured with a fluorescence microscope. Four to five images (400 μm × 400 μm) were taken in an area including the dorsal horn. All Iba1-positive cells were counted per the area, and the number from all the sections was averaged for each mouse.

2.4. *Drug administration*

Pioglitazone (1-25 mg/kg p.o.) or its vehicle (0.5% carboxymethyl cellulose, CMC) was given once daily from immediately after PSL to day 4 of PSL. Pioglitazone hydrochloride was kindly donated by Takeda Pharmaceutical Company (Osaka, Japan).

2.5. *Statistical test*

Statistical significance was determined by ANOVA followed by Tukey multiple comparisons' test, and set at $p < 0.05$.

3. Results

3.1. *Effect of pioglitazone on PSL-induced tactile allodynia*

We tested the effects of pioglitazone on tactile allodynia elicited by peripheral nerve injury on day 5 following PSL. Pioglitazone was administered once daily from immediately after PSL to day 4 following PSL. PSL significantly increased the ratio of withdrawal response of hindpaw to innocuous mechanical stimulation, compared to in sham group. The PSL-induced tactile allodynia were significantly attenuated by pioglitazone (1-25 mg/kg), which did not affect the ratio of nociceptive responses in sham group (Figure 1).

3.2. *Effect of pioglitazone on expression of PSL-activated microglia*

Immunohistochemistry using anti-Iba1 antibody revealed the expression of activated microglia with

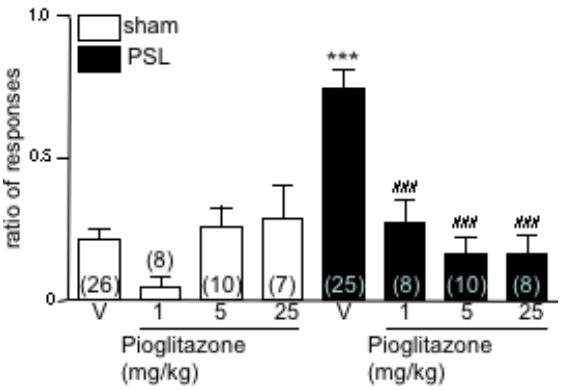

Figure 1. Effect of pioglitazone on tactile allodynia in mice subjected to PSL. Pioglitazone (1-25 mg/kg, p.o.) was administered once daily immediately after PSL to day 4 following PSL. Behavioral test was performed on day 5 of PSL. V denotes vehicle. The number in parentheses indicates the number of experiments. *** $p < 0.001$ *vs.* V/sham. ### $p < 0.001$ *vs.* V/PSL.

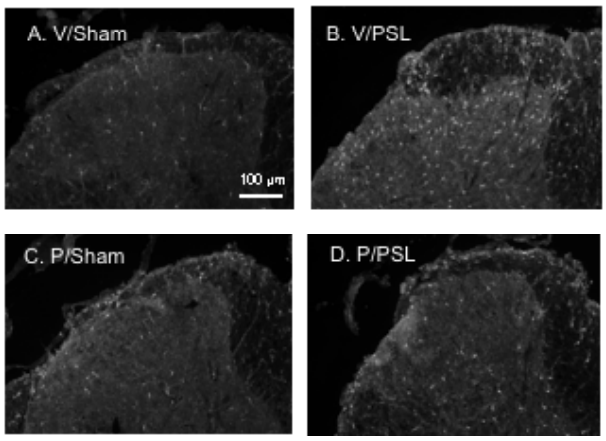

Figure 2. Pioglitazone attenuates PSL-induced up-regulation of activated microglia in the dorsal horn of spinal cord. Mice were perfused with 4% paraformaldehyde on day 6 of PSL. The sections were prepared from the dissected lumbar spinal cord and stained with anti-Iba1 antibody, a specific marker for activated microglia. Micrographs were representative of sham and PSL treated with vehicle (V) or 25 mg/kg pioglitazone (P). Dose regimen is shown at the legend in Figure 1.

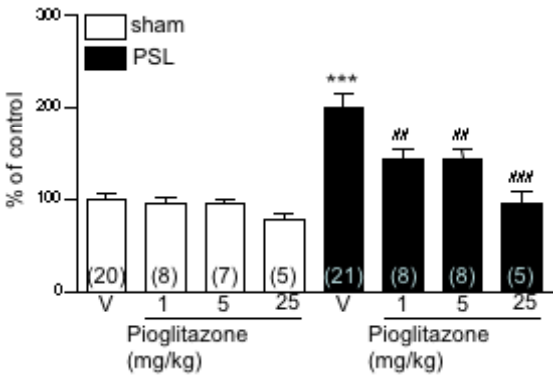

Figure 3. Effect of pioglitazone on up-regulation of activated microglia in mice subjected to PSL. The number of cells with immunoreactivity for Iba1 was counted in the dorsal horn of spinal cord. Dose regimen and immunostaining procedures are shown at the legend in Figures 1 and 2, respectively. The number of immunoreacitve cells were normalized relative to sham group treated with vehicle (control), and expressed in percentage (% of control). V denotes vehicle. The number in parentheses indicates the number of experiments. $^{***}p < 0.001$ *vs*. V/sham. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs*. V/PSL.

amoeboid morphology in the dorsal horn of spinal cord (Figure 2A). The number of cells with immunoreactivity to anti-Iba1 antibody was significantly greater in PSL group treated with vehicle than in sham group with vehicle (Figure 2B). PSL-induced increase in the number of activated microglia was significantly attenuated by administration of pioglitazone at 1-25 mg/kg (Figures 2D and 3).

4. Discussion

We examined the effect of PPARγ agonist, pioglitazone, on development of tactile allodynia and expression of activated microglia in mice subjected to peripheral nerve injury. Administration of pioglitazone for five days immediately after PSL attenuated tactile allodynia, associated with inhibition of PSL-induced expression of activated microglia in the dorsal horn of spinal cord. These results suggest that relief of tactile allodynia *via* PPARγ stimulation may be mediated by the inhibition of central sensitization through reduced activation of microglia in the spinal cord.

Pioglitazone reportedly attenuated thermal hyperalgesia and microglial activation in spinal cord injury model of rats (*9*). This model is clinically useful for study of serious motor dysfunction after spinal cord injury. The motor dysfunction, however, makes it difficult to evaluate withdrawal responses of an injured side of paw to nociceptive stimuli. In our study, mice subjected to PSL showed motor paralysis immediately after PSL, but recovered within a few days (*10*). Additionally, other finding that pioglitazone had improved motor paralysis in the spinal cord injury model (*9*) might make it even more complicated to interpret the influence of pioglitazone on thermal hyperalgesia. The PSL model, with less severe motor paralysis, is likely to be more useful studies of neuropathic pain.

The present study agrees with the well-established paradigm that peripheral nerve injury up-regulates activated microglia in the dorsal horn of spinal cord. The activated spinal microglia is required for the expression of neuropathic pain after nerve injury (*11*). Microglial activation leads to increased production of the proinflammatory cytokines, which subsequently act directly on the terminals of primary afferent neurons and on the dorsal horn neurons (*1*). The proinflammatory cytokines have another important autocrine feedback signal to microglial cells themselves, which results in fueling of the microglial inflammatory response (*12*). Proinflammatory cytokines contribute to increased spontaneous nociceptor activity and stimulus sensitivity, called central sensitization underlying neuropathic pain (*13-15*). Study on neuroinflammatory has shown that PPARγ agonist with anti-inflammatory activity suppresses production of proinflammatory mediators in the brain (*2*). We also found that pioglitazone blocked PSL-induced upregulation of proinflammatory cytokines in the dorsal horn of spinal cord, such as IL-6 and TNF-alpha, which are believed to be essential for neuropathic pain (data not shown). These facts propose a hypothesis that pioglitazone prevents development of tactile allodynia through inhibition of PSL-induced upregulation of the proinflammatory cytokines in the dorsal horn of spinal cord. On the other hand, PPARγ is expressed in the dorsal horn of spinal cord (*8,16*). These reports suggest that spinal PPARγ plays a possible role for inhibition of microglial activation. Further evidence supports the action of orally given pioglitazone on CNS: 18% of pioglitazone crosses the blood-brain barrier in rats when administered p.o. (*17*).

In conclusion, PPARγ synthetic ligands such as pioglitazone appear to be a promising drug to treat neuropathic pain involving through interfering

with microglial activation. A deep knowledge of the molecular mechanisms evoked by pioglitazone either dependent or independent of the receptor activation and of PPARγ expression in activated microglia is mandatory for the clinical use of pioglitazone with regimen for increased efficacy and safety.

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References

1. Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 2007; 10:1361-1368.
2. Bernardo A, Minghetti L. Regulation of Glial Cell Functions by PPAR-gamma Natural and Synthetic Agonists. PPAR Res 2008; 2008:864140.
3. Clark AK, Yip PK, Grist J, Gentry C, Staniland AA, Marchand F, Dehvari M, Wotherspoon G, Winter J, Ullah J, Bevan S, Malcangio M. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc Natl Acad Sci U S A 2007; 104:10655-10660.
4. Winkelstein BA, Rutkowski MD, Sweitzer SM, Pahl JL, DeLeo JA. Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. J Comp Neurol 2001; 439:127-139.
5. Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. Nat Med 2004; 10:355-361.
6. Straus DS, Glass CK. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. Trend Immunol 2007; 28:551-558.
7. Heneka MT, Landreth GE, Hull M. Drug insight: effects mediated by peroxisome proliferator-activated receptor-gamma in CNS disorders. Nat Clin Pract 2007; 3:496-504.
8. Churi SB, Abdel-Aleem OS, Tumber KK, Scuderi-Porter H, Taylor BK. Intrathecal rosiglitazone acts at peroxisome proliferator-activated receptor-gamma to rapidly inhibit neuropathic pain in rats. J Pain 2008; 9:639-649.
9. Park SW, Yi JH, Miranpuri G, Satriotomo I, Bowen K, Resnick DK, Vemuganti R. Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. J Pharmacol Exp Ther 2007; 320:1002-1012.
10. Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990; 43:205-218.
11. Tsuda M, Inoue K, Salter MW. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. Trend Neurosci 2005; 28:101-107.
12. Pocock JM, Liddle AC. Microglial signalling cascades in neurodegenerative disease. Prog Brain Rese 2001; 132:555-565.
13. Cunha TM, Verri WA, Jr., Silva JS, Poole S, Cunha FQ, Ferreira SH. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proc Natl Acad Sci U S A 2005; 102:1755-1760.
14. Schafers M, Lee DH, Brors D, Yaksh TL, Sorkin LS. Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation. J Neurosci 2003; 23:3028-3038.
15. Wolf G, Gabay E, Tal M, Yirmiya R, Shavit Y. Genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. Pain 2006; 120:315-324.
16. Moreno S, Farioli-Vecchioli S, Ceru MP. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 2004; 123:131-145.
17. Maeshiba Y, Kiyota Y, Yamashita K, Yoshimura Y, Motohashi M, Tanayama S. Disposition of the new antidiabetic agent pioglitazone in rats, dogs, and monkeys. Arzneimittel-Forschung 1997; 47:29-35.

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Original Article

Phenolphthalein treatment in pregnant women and congenital abnormalities in their offspring: A population-based case-control study

Ferenc Bánhidy[1], Nándor Ács[1], Erzsébet H. Puhó[2], Andrew E. Czeizel[2,*]

[1] *Second Department of Obstetrics and Gynecology, Semmelweis University, School of Medicine, Budapest, Hungary;*
[2] *Foundation for the Community Control of Hereditary Diseases, Budapest, Hungary.*

ABSTRACT: Phenolphthalein is frequently used laxative drug since 1930s, but the possible teratogenic effect of phenolphthalein was not checked in case-control eptedmiological study. In addition US Food and Drug Administration (FDA) declared the mutagenic and carcinogenic effect of phenolphthalein in 1999, thus we decided to evaluate the birth outcomes particularly congenital abnormalities (CAs) of newborn infants born to women treated with phenolphthalein during pregnancy. Cases with CA and their matched controls without CA born to mothers with phenolphthalein use during pregnancy were compared in the population-based large data set of the Hungarian Case-Control Surveillance System of Congenital Abnormalities. Of 22,843 cases with CA, 191 (0.83%) while of 38,151 controls, 247 (0.64%) were born to mothers with phenolphthalein treatment (adjusted OR with 95% CI: 1.3, 1.0-1.5). The mean gestational week at delivery was somewhat longer in both the case (0.3 week) and control (0.2 week) groups while the mean birth weight was somewhat larger in cases (46 g) and controls (12 g) born to mothers with phenolphthalein treatment during the study pregnancy compared with mothers without phenolphthalein treatment. These differences were in agreement with the lower rate of preterm births and low birth weight in controls born to mothers with phenolphthalein treatment during pregnancy. The detailed analysis of different CA groups showed an association between maternal phenolphthalein treatment during pregnancy and a higher risk for Hirschsprung's disease (p = 0.01) based on 4 cases in the so-called other isolated CA-group. In conclusion phenolphthalein treatment in pregnant women associates with a higher risk for Hirschsprung's disease in their children, but this finding is only a signal which needs confirmation or rejection in other studies.

Keywords: Phenolphthalein, Congenital abnormalities, Hirschsprung's disease, Birth outcomes, Population-based case-control study

**Correspondence to:* Dr. Andrew E. Czeizel, 1026 Budapest, Törökvész lejtõ 32. Hungary;
e-mail: czeizel@interware.hu

1. Introduction

Among maternal diseases during pregnancy, constipation is one of the most frequent pathological conditions which affects 11-38% of pregnant women (*1,2*). However, some clinical reports mentioned the complaints of constipation in over half of pregnant women (*3*). The recommended first line therapy of constipation includes diet with increased intake of bran and wheat fibre, in addition of fluid intakes, regular defecation and increased exercise. The second line of therapy comprises of osmotic laxatives such as magnesium hydrochloride and lactulose. The third line of therapy is based on stimulant medications, mainly senna (*4-6*), however, phenolphthalein was also used for the treatment of constipation in Hungary during the study period frequently by pregnant women as well.

The phenolphthalein is diphenylmethane ($C_{20}H_{14}O_4$) derivative laxative that act as a relatively nontoxic stimulant on the colon and take at least 6 hours to produce a fecal evacuation (*7*). Phenolphthalein was discovered as a laxative in 1902 by Zoltan Vámossy (1868-1953) in Hungary (*8,9*) and marketed in 1937 as laxative tablet without prescription. However, FDA declared the mutagenic and carcinogenic effect of phenolphthalein in 1999, and though EMEA did not accept this statement, the use of phenolphthalein was recommended only after prescription (*10*).

We found only one study regarding the human teratogenic effect of phenolphthalein that did not indicate

any increase in the rate of structural birth defects, *i.e*. congenital abnormalities (CAs) (*11*). The objective of our study was to compare the occurrence of pregnant women by phenolphthalein treatment during pregnancy who had later informative offspring ("cases") with different CAs and their matched control newborns without CA in the population-based large data set of the Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA) (*12*).

2. Materials and Methods

2.1. *Protocols*

The protocol of the HCCSCA included five steps.

The first step was the selection of cases with CA from the data set of the Hungarian Congenital Abnormality Registry (HCAR), 1980-1996 (*13*) for the HCCSCA. Notification of CAs is mandatory for physicians from the birth until the end of first postnatal year to the HCAR in Hungary. Most cases with CA are reported by obstetricians and pediatricians. In Hungary practically all deliveries take place in inpatient obstetric clinics and the birth attendants are obstetricians. In addition all infants affected with CA are treated in the neonatal units of inpatient obstetric clinics, or in various general and special (surgical, cardiologic, orthopedic, *etc*.) inpatient and outpatient pediatric clinics. Autopsy was mandatory for all infant deaths and common in stillborn fetuses during the study period. Pathologists sent a copy of the autopsy report to the HCAR if defects were identified in stillbirths and infant deaths. Since 1984 fetal defects diagnosed in prenatal diagnostic centres with or without elective termination of pregnancy have also been included into the HCAR. Isolated minor anomalies (*e.g.*, umbilical hernia, small hemangioma, hydrocele) were recorded in the HCAR but not evaluated at the calculation of different CA rates. The total (birth + fetal) prevalence of cases with CA diagnosed from the second trimester of pregnancy through the age of one year was 35 per 1,000 *informative offspring* (live-born infants, stillborn fetuses and electively terminated malformed fetuses) in the HCAR, 1980-1996, and about 90% of major CAs were recorded in the HCAR during the 17 years of the study period (*14*).

The major objective of the HCCSCA is a postmarketing surveillance of drug teratogenicity (*12*). Thus there were three exclusion criteria of cases with CAs from the HCAR for the data set of the HCCSCA. (i) Cases reported after three months of birth or pregnancy termination were excluded. The longer time between birth or pregnancy termination and data collection decreases the accuracy of information about pregnancy history. However, 77% of cases were reported during the first three-month time window, and the rest of most cases were affected with mild CA. (ii) Three mild CAs (such as congenital dysplasia of hip, congenital inguinal hernia, and large hemangioma), and (iii) CA-syndromes caused by major gene mutations or chromosomal aberrations with preconceptional origin were also excluded.

The second step was to ascertain appropriate *controls* from the National Birth Registry of the Central Statistical Office for the HCCSCA. Controls were defined as newborn infants without CA. In general two controls were matched to every case according to sex, birth week in the year when the case was born, and district of parents' residence.

The third step was to obtain the necessary *maternal and exposure* data from three sources:

(1) *Prospective medically recorded data*: an explanatory letter was mailed to mothers immediately after the selection of cases and controls to inform them on the purpose of the HCCSCA, the benefit of this public health activity for them and in general for the prevention of CAs. Mothers were asked to send us the *prenatal care logbook* and other *medical records* particularly discharge summaries concerning their diseases during the study pregnancy and their child's CA for three weeks. Prenatal care was mandatory for pregnant women in Hungary (if somebody did not visit prenatal care clinic, she did not receive a maternity grant and leave), thus nearly 100% of pregnant women visited prenatal care clinics, an average 7 times in their pregnancies. The first visit was between the 6th and 12th gestational week. The role of licensed obstetricians is to record all pregnancy complications, maternal diseases and related drug prescriptions in the prenatal care logbook.

(2) *Retrospective self-reported maternal information*: a structured *questionnaire* with a list of medicinal products (drugs and pregnancy supplements) and diseases, plus a printed informed consent form were also mailed to the mothers. The questionnaire requested information on pregnancy complications and maternal diseases, on medicinal products taken during pregnancy according to gestational months, and on family history of CAs. To standardize the answers, mothers were asked to read the enclosed lists of medicinal products and diseases as a memory aid before they filled in the questionnaire. We also asked mothers to give a signature for informed consent form which permitted us to record their name and address in the HCCSCA.

The mean ± S.D. time elapsed between the birth or pregnancy termination and the return of the "information package" (questionnaire, logbook, discharge summary, and informed consent form) in our prepaid envelope was 3.5 ± 1.2 and 5.2 ± 2.9 months in the case and control groups, respectively.

(3) *Supplementary data collection*: regional nurses were asked to visit all non-respondent case mothers, in addition 200 non-respondent control mothers. Regional nurses helped mothers to fill in the same questionnaire used in the HCCSCA; obtained data regarding smoking and drinking habit through cross interview of mothers and their close relatives; they evaluated the available

medical records and asked mothers to sign informed consent form. Regional nurses did not visit all non-respondent control mothers because the committee on ethics considered this follow-up to be disturbing to the parents of all healthy children (*15*).

The flow of cases from the HCAR and controls from the Central Statistical Office to the HCCSCA and the achievement of final data set were published previously (*16*). Overall, the necessary information was available on 96.3% of cases (84.4% from reply to the mailing, 11.9% from the nurse visit) and 83.0% of the controls (82.6% from reply, 0.4% from visit). Prenatal care logbooks were available in 88.4% of cases and in 93.8% of controls who were evaluated. Informed consent form was signed by 98% of mothers, names and addresses were deleted in the rest of subjects.

The fourth step was the *evaluation of phenolphthalein treatment* according to 12 different aspects.

1) The source of information. Three groups of phenolphthalein treatments were differentiated: (a) data only from the prenatal care logbooks and/or other medical record; (b) data from the questionnaire, and (c) concordant data from both medical records and the questionnaire.

2) The type of treatment. Two groups were differentiated: (a) phenolphthalein alone and (b) phenolphthalein plus other drugs.

3) The route of administration. In Hungary phenolphthalein was used in three medicinal products: (i) Phenolphthaleinum® (Alkaloida) tablets contain 500 mg, (ii) Bilagit® (Chinoin) tablets contain phenolphthalein 20 mg, methylhomatropine 1 mg, papaverine 20 mg, methenamine 80 mg, sodium choleinicum 60 mg and menthol 7.5 mg and (iii) Artin® (Biogal) tablets contain phenolphthalein 32 mg, aloin 16 mg, ipecacuanhae radix 4 mg, strychnin siccum extr. 4 mg, and belladonae siccum extr. 2.4 mg for oral treatment. However, Artin® was used only by 3 control and 2 case mothers, thus these pregnant women were excluded from the study due to the small numbers of subjects, in addition we wanted to evaluate a homogeneous sample as much as possible.

4) The dose of phenolphthalein treatment. The recommended oral treatment is ½-1 tablet of phenolphthalein in the evening, *i.e*. 250-500 mg per day or 2-3 times 1-2 tablets of Bilagit® tablets per day.

5) The duration of treatment.

6) Maternal diseases as underlying medical conditions particularly constipation as confounders.

7) Pregnancy complications.

8) Other drug uses as confounders.

9) Pregnancy supplements. The use of pregnancy supplements may indicate the level of pregnancy care, and indirectly may show the socio-economic status and the motivation of mothers to prepare and/or to achieve a healthy baby. In addition it is necessary to consider folic acid and folic acid-containing multivitamins in the evaluation of preventable CAs (*17-19*).

10) The *gestational age* was calculated from the first day of the last menstrual period. Three time intervals were considered: (i) First month of gestation because it is before the organogenesis. The first two weeks are before conception while the third and fourth weeks comprise the pre- and implantation period of zygotes and blastocysts including omnipotent stem cells. Thus CAs cannot be induced by environmental agents in the first month of gestation and it explains the "all-or-nothing effect" rule, *i.e*. total loss or normal further development. (ii) The second and third months of gestation. This is the sensitive, the so-called critical period for most major CAs. (iii) The fourth through ninth months of gestation, *i.e*. pregnancy after the organforming period.

11) Medically recorded birth weight and gestational age in the discharge summary of mothers after delivery. In addition the rate of low birth weight (less than 2,500 gram) and preterm birth (less than 37th gestational week) was also calculated and evaluated.

12) Other confounding factors, such as maternal age, birth order, marital and employment status. Employment status of mothers showed a strong correlation with their education and a moderate correlation with their income (*20*), thus this variable was considered as indicator of socioeconomic status.

2.2. *Statistical analyses*

Statistical analyses were performed using the software package SAS version 8.02 (SAS Institute Inc., Cary, NC, USA). First, the occurrence of phenolphthalein treatment during the study pregnancy was compared between the study groups and crude odds ratios (OR) with 95% confidence interval (CI) was calculated. Second, frequency tables were made for the main maternal variables in order to describe the study groups of mothers with phenolphthalein treatment and of mothers without phenolphthalein treatment as reference. Third, the prevalence of pregnancy complications, acute and chronic maternal diseases, other drug treatments and pregnancy supplements used during the study pregnancy were compared between case and control mothers with phenolphthalein treatment, and crude OR with 95% CI were calculated. Fourth, the prevalence of phenolphthalein treatment was evaluated according to gestational period in 16 different CA groups (including at least 2 cases born to mothers with phenolphthalein treatment during pregnancy) in the second and/or third gestational months and this prevalence was compared with the frequency of phenolphthalein treatment in their all matched controls, and adjusted OR with 95% CI were evaluated in a conditional logistic regression model. The latter OR were adjusted for maternal age (< 20 yr *vs*. 20-29 yr *vs*. 30 yr or more), birth order (first delivery *vs*. one or more previous deliveries), maternal employment status (professional-managerial-skilled worker *vs*. semiskilled worker-unskilled worker-housewife *vs*.

others) use of folic acid and fever related acute maternal diseases (as a dichotomous variable).

3. Results

The case group consisted of 22,843 malformed newborns or fetuses ("informative offspring") with CA, of whom 191 (0.83%) had mothers with oral phenolphthalein treatment (43 pregnant women were treated by Phenolphthalein® and 148 pregnant women by Bilagit® tablets). The total number of births in Hungary was 2,146,574 during the study period between 1980 and 1996. Thus the 38,151 controls without CA represented 1.8% of all Hungarian births, and among those controls, 247 (0.64%) were born to mothers treated orally with phenolphthalein tablets (crude OR with 95% CI: 1.3, 0.7-1.6). Of these 247 pregnant women, 49 were treated by Phenolphthalein® and 198 by Bilagit® tablets.

Of 191 case and 247 control mothers, 7 (3.7%) and 38 (15.4%) had medically recorded oral phenolphthalein treatments in the prenatal logbooks and/or discharge summaries ($\chi^2_1 = 16.0$; $p < 0.0001$). Most pregnant women took one Phenolphthalein® tablet (*i.e.* 500 mg) or 3 times 1-2 Bilagit® tablets (60-120 mg) per day. Of 191 case and 247 controls mothers, only 2 and 1 used only Phenolphthalein® or Bilagit® tablets during the study pregnancy, respectively, thus pregnant women with phenolphthalein plus other drug treatments were evaluated together.

The onset and duration of phenolphthalein treatments in case and control mothers are shown in Table 1. About one-third of pregnant women used phenolphthalein in the first gestational month, however, of these 63 cases and 87 control mothers, only 22 and 35 continued this treatment in the second gestational month, respectively. The mean duration of phenolphthalein treatment was 2.2 and 2.5 months in the case and control mothers ($t = 1.2$; $p = 0.23$), respectively, but it depended on the onset of treatment. The earlier onset associated with the longer duration of treatment. The distribution of gestational months according to the onset of phenolphthalein treatment did not show significant difference between case and control mothers ($\chi^2_8 = 4.1$; $p = 0.85$).

Table 2 summarises the birth data of cases and controls born to mothers with oral phenolphthalein treatment during the study pregnancy. There was no difference in the sex ratio between treated and untreated case and control subgroups. The obvious general male excess is explained by the higher rate of CAs in male genital organs such as hypospadias and undescended testis and controls were matched to the sex of cases.

Here mainly the birth outcomes of controls are commented because CAs may have a more drastic effect for birth outcomes than phenolphthalein itself. The mean gestational week at delivery was somewhat longer in both the case (0.3 week) and control (0.2 week) groups

Table 1. Onset and duration of phenolphthalein treatment according to gestational month and mean duration of treatment in case and control mothers

Gestational month	Case mothers				Control mothers			
	No.	%	Mean	S.D.	No.	%	Mean	S.D.
I.	63	33.0	3.3	3.4	87	35.2	3.8	3.7
II.	17	8.9	1.8	1.1	21	8.5	2.4	2.4
III.	19	9.9	2.2	1.8	17	6.9	2.4	2.3
IV.	11	5.8	3.3	2.3	16	6.5	2.1	1.9
V.	23	12.0	1.4	0.9	32	13.0	1.9	1.6
VI.	16	8.4	1.4	0.7	29	11.7	1.7	1.3
VII.	22	11.5	1.3	0.6	26	10.5	1.5	0.8
VIII.	12	6.3	1.3	0.5	13	5.3	1.4	0.5
IX.	8	4.2	0.0	0.0	6	2.4	0.0	0.0
Total	191	100.0	2.2	2.4	247	100.0	2.5	2.7

Table 2. Birth outcomes of cases and controls born to mothers with or without phenolphthalein treatment (PT) during pregnancy

Variables	Cases				Controls				Comparison of cases and controls born to mothers with PT
	with PT ($N = 191$)		without PT ($N = 22,652$)		with P T ($N = 247$)		without PT ($N = 37,904$)		
Categorical	No.	%	No.	%	No.	%	No.	%	OR (95% CI)
Sex ratio (boy)	124	64.9	14,773	65.2	174	70.4	24,625	65.0	0.7 (0.5 - 1.2)
Stillbirths	5	2.6	392	1.7	0	0.0	0	0.0	-
Elective terminations	2	1.1	102	0.5	0	0.0	0	0.0	-
Livebirths	184	96.3	22,158	97.8	247	100.0	37,904	100.0	-
Twins	5	2.7	416	1.9	2	0.8	408	1.1	3.3 (0.6 - 17.2)
Preterm births	33	17.9	3,732	16.8	21	8.5	3,475	9.2	2.2 (1.3 - 4.0)
Low birthweight newborns	38	20.7	4,591	20.7	11	4.5	2,156	5.7	5.3 (2.6 - 10.7)
Quantitative	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Student *t* test
Gestational age at delivery (wk)*	38.9	3.1	38.6	3.2	39.6	2.1	39.4	2.1	$t = 2.8$, $p = 0.006$
Birth weight (g)*	3,023	766	2,977	704	3,288	508	3,276	511	$t = 4.3$, $p < 0.0001$

* calculated for livebirths

while the mean birth weight was somewhat larger in cases (46 g) and controls (12 g) born to mothers with phenolphthalein treatment during the study pregnancy compared with mothers without phenolphthalein treatment. These differences were in agreement with the lower rate of preterm births and low birth weight in controls born to mothers with phenolphthalein treatment during pregnancy.

Table 3 shows the basic characteristics of mothers with phenolphthalein treatment and without phenolphthalein treatment as reference. The mean maternal age was somewhat higher in pregnant women with phenolphthalein treatment due to the larger proportion of women over 30 years of age. However, the mean birth order was somewhat lower in case mother with phenolphthalein treatment due to the much lower proportion of primiparae. On the other hand the mean birth order was higher in treated control than untreated control mothers. There was no significant difference in the proportion of marital status of mothers among the study groups. Maternal employment status as an indicator of socioeconomic status showed also some differences because treated mothers were more frequent among professional and managerial than among untreated mothers. However, there was no significant difference in the distribution of employment status between case and control mothers with phenolphthalein treatment.

Among pregnancy supplements, the use of folic acid and multivitamins was higher in control mothers with phenolphthalein treatment than in the untreated reference group while their use was less frequent in treated case mothers. Thus, there was a significant difference in the occurrence of folic acid supplementation between treated case and control mothers.

Non-respondent 2,822 mothers who delivered malformed babies were visited at home and the proportion of phenolphthalein treatment occurred in 26 (0.9%) pregnant women. Of these 26 pregnant women, 5 (19.2%) smoked, while of 2,796 pregnant women without phenolphthalein treatment, 551 (19.7%) were smoker. In the control groups, only 200 non-respondent pregnant women were visited at home. The rate of smokers was 19% while pregnant women with phenolphthalein treatment did not occur among them. The proportion of regular/hard drinkers during the study pregnancy was 1.2% and 1.0% in the non-respondent case and control mothers.

We evaluated those pregnancy complications which were recorded in the prenatal care logbook; the exception was nausea and vomiting in pregnancy because this variable was analyzed on the basis of maternal information as well. Nearly all pregnancy complications showed a higher incidence in both case and control pregnant women with phenolphthalein treatment than in case and control mothers without the use of phenolphthalein (Table 4). The exception was pre-eclampsia that showed a lower occurrence in both case and control pregnant women with phenolphthalein treatment. The higher occurrence of anaemia was connected with the frequent haemorrhoids of treated pregnant women due to their chronic constipation. However, there was no significant difference in the incidence of pregnancy complications between case and control mother with phenolphthalein treatment.

The prevalence of all acute specified maternal disease groups was higher in pregnant women with

Table 3. Main variables of case and control pregnant women with phenolphthalein treatment (PT) and without phenolphthalein treatment as reference

Variables	Case mothers				Control mothers				Comparison of cases and controls born to mothers with PT
	without PT (N = 22,652)		with PT (N = 191)		without PT (N = 37,904)		with PT (N = 247)		
Quantitative	No.	%	No.	%	No.	%	No.	%	
Maternal age (yr)									
- 19	11,326	50.0	84	44.0	18,611	49.1	96	38.9	
20 - 29	7,407	32.7	58	30.2	13,304	35.1	97	39.3	$\chi^2_2 = 3.8$, $p = 0.15$
30 -	3,919	17.3	49	25.8	5,989	15.8	54	21.8	
Mean, S.D.	25.5 ± 5.3		26.6 ± 5.2		25.5 ± 4.9		26.5 ± 4.8		$t = 0.2$, $p = 0.84$
Birth order									
1	10,624	46.9	79	41.4	18,111	47.8	98	39.7	$\chi^2_1 = 0.1$, $p = 0.72$
2 or more	12,028	53.1	112	58.6	19,793	52.2	149	60.3	
Mean, S.D.	1.9 ± 1.1		1.8 ± 1.0		1.7 ± 0.9		1.9 ± 0.9		$t = 1.1$, $p = 0.27$
Categorical	No.	%	No.	%	No.	%	No.	%	
Unmarried	1,259	5.6	10	5.2	1,464	3.9	7	2.8	
Employment status									
Professional	1,883	8.3	18	9.4	4,317	11.4	36	14.6	
Managerial	4,905	21.7	63	33.0	10,038	26.5	96	38.9	
Skilled worker	6,270	27.7	59	30.9	11,631	30.7	59	23.9	
Semiskilled worker	3,844	17.0	25	13.1	5,751	15.2	32	13.0	$\chi^2_6 = 7.0$, $p = 0.32$
Unskilled worker	1,495	6.6	8	4.2	1,850	4.9	9	3.6	
Housewife	2,121	9.4	7	3.7	2,032	5.4	6	2.4	
Others	2,134	9.4	11	5.8	2,285	6.0	9	3.6	
Pregnancy supplements									OR (95% CI)
Folic acid	11,188	49.4	91	47.6	20,632	54.4	143	57.9	0.7 (0.5 - 0.9)
Multivitamins	1,321	5.8	9	4.7	2,490	6.6	19	7.7	0.6 (0.3 - 1.3)

phenolphthalein treatment compared with pregnant women without phenolphthalein treatment both in the case and in the control group (Table 5). However, only the rate of influenza-common cold was significantly higher in treated mothers particularly among case mothers.

Among chronic maternal disorders (Table 5), constipation was reported by nearly all pregnant women with phenolphthalein treatment, therefore these data are not shown in Table 5. There was a higher prevalence of haemorrhoids in treated case and control mothers than in untreated mothers.

Table 6 summarizes the frequently used other drugs (at least 4 pregnant women either in case or control mothers with phenolphthalein treatment). There was a much higher frequency of drugs used for the treatment of pregnancy complications, *i.e*. threatened abortion (promethazine) and preterm delivery (pholedrin), in addition nausea and vomiting in pregnancy (vitamin B6). Some others drugs such as acetylsalicylic acid, clotrimazole, dipyrone, penamecillin were used for the treatment of acute maternal diseases. Only the higher use of Reparon® and Demalgon® suppositories was used for the treatment of haemorrhoids, but the treatment of spasmodic drotaverine, analgesic Quarelin® and digesting Dipankrin® tablet might also be associated with complications of constipation. Three drugs (acetylsalicylic acid, Demalgon® and dipyrone) were used somewhat more frequently by case mothers than by control mothers with phenolphthalein treatment.

The main objective of the study was to evaluate cases with different CA groups and their *all matched controls* (Table 7). Our study protocol includes 25 CA-groups, but only 16 had at least 2 cases born to mother with phenolphthalein treatment. There was a higher rate of phenolphthalein treatment during the entire pregnancy in the mothers of cases with total CAs (OR with 95% CI: 1.3, 1.0-1.5) but among different CA-groups only cases with neural-tube defects were born to mother with significantly higher rate of phenolphthalein treatment. However, we focused our analysis into the second and/or third gestational months because most major CAs have the critical period in this time window. (Pregnant women who used phenolphthalein in the first gestational month and continued in the second gestational month were included.) There was no CA-group with higher

Table 4. Occurrence of pregnancy complications in case and control mothers with phenolphthalein treatment (PT) and without PT as reference

Pregnancy complications	Cases mothers				Controls mothers				Comparison of cases and controls born to mothers with PT
	without PT (*N* = 22,692)		with PT (*N* = 191)		without PT (*N* = 37,904)		with PT (*N* = 247)		
	No.	%	No.	%	No.	%	No.	%	OR (95% CI)
Nausea and vomiting									
All	10,772	47.6	98	51.3	19,826	52.3	142	57.5	0.8 (0.5 - 1.1)
Medically recorded (severe)	1,729	7.6	17	8.9	3,831	10.1	38	15.4	0.5 (0.3 - 0.9)
Threatened abortion	3,463	15.3	38	19.9	6,459	17.0	53	21.5	0.9 (0.6 - 1.4)
Pre-eclampsia*	1,928	8.4	11	5.7	3,486	9.1	22	8.8	0.6 (0.3 - 1.3)
Placental disorders**	290	1.3	4	2.1	587	1.5	5	2.0	1.0 (0.3 - 3.9)
Polyhydramnios	206	0.9	6	3.1	188	0.5	3	1.2	2.6 (0.7 - 10.7)
Threatened preterm delivery	2,820	12.5	20	15.7	5,945	15.7	41	16.6	0.6 (0.3 - 1.0)
Gestational diabetes	139	0.6	2	1.0	269	0.7	1	0.4	2.6 (0.2 - 28.9)
Anaemia	3,198	14.1	42	22.0	6,302	16.6	54	21.9	1.0 (0.6 - 1.6)

* hypertonia, edema, albuminuria; ** placenta previa, premature separation of placenta, antepartum hemorrhage.

Table 5. Prevalence of acute and chronic maternal diseases in case and control mothers with phenolphthalein treatment (PT) and without PT as reference

Maternal diseases	Cases mothers				Controls mothers				Comparison of cases and controls born to mothers with PT
	without PT (*N* = 22,652)		with PT (*N* = 191)		without PT (*N* = 37,904)		with PT (*N* = 247)		
	No.	%	No.	%	No.	%	No.	%	OR (95% CI)
Acute									
Influenza - common cold	4,893	21.6	74	38.7	7,001	18.5	60	24.3	2.0 (1.3 - 3.0)
Respiratory system	2,095	9.2	23	12.0	3,418	9.0	37	15.0	0.8 (0.4 - 1.3)
Digestive system	712	3.1	30	15.7	903	2.4	30	12.1	1.3 (0.8 - 2.3)
Urinary tract	1,574	6.9	15	7.9	2,292	6.0	16	6.5	1.2 (0.6 - 2.5)
Genital organs	1,665	7.4	15	7.9	2,878	7.6	20	8.1	1.0 (0.5 - 1.9)
Others	384	1.7	5	2.6	507	1.3	8	3.2	0.8 (0.3 - 2.5)
Chronic									
Diabetes mellitus	55	0.2	1	0.5	51	0.1	1	0.4	1.3 (0.1 - 20.8)
Epilepsy	76	0.3	0	0.0	76	0.2	1	0.4	-
Headache	551	2.4	14	7.3	701	1.8	12	4.9	1.5 (0.7 - 3.4)
Varicose veins in lower extremities	305	1.3	7	3.7	910	2.4	11	4.5	0.8 (0.3 - 2.1)
Thrombophlebitis	327	1.4	5	2.6	565	1.5	1	0.4	6.6 (0.8 - 57.1)
Haemorrhoids	548	2.4	21	11.0	1,244	3.3	24	9.7	1.1 (0.6 - 2.1)

rate of phenolphthalein treatment in the second and/or third gestational months according to adjusted OR. Cases with CA of eyes had different buphthalmos and congenital cataract. However, it is necessary to mention that the critical period of some CAs such as hypospadias, undescended testis, clubfoot is after the third gestational month. Our further analysis calculated with their specific critical periods without any positive associations.

Finally we evaluated 19 cases with other isolated CAs in detail (Table 8). Of these 19 cases, 6 belonged to one of CA groups in the protocol of the HCCSCA but had only one case therefore they were omitted from Table 7. Among further 13 cases, 4 were affected with Hirschsprung's disease (Table 9) and 3 with torticollis.

Table 6. Occurrence of other frequent drug treatments in case and control mothers with phenolphthalein treatment (PT) and without PT as reference

Drugs	Cases mothers				Controls mothers				Comparison between case and control mothers with PT
	without PT (*N* = 22,652)		with PT (*N* = 191)		without PT (*N* = 37,904)		with PT (*N* = 247)		
	No.	%	No.	%	No.	%	No.	%	OR (95% CI)
Acetylsalicylic acid	979	4.3	22	11.5	1,380	3.6	15	6.1	2.0 (1.0 - 4.0)
Allylestrenol	3,449	15.2	32	16,8	5,320	14.0	37	15.0	1.1 (0.7 - 1.9)
Aminophenazone+carbromal (Demalgon®)	371	1.6	12	6.3	336	0.9	5	2.0	3.2 (1.1 - 9.4)
Aminophylline	1,362	6.0	12	6.3	2,267	6.0	17	6.9	0.9 (0.4 - 1.9)
Ampicillin	1,607	7.1	17	8.9	2,573	6.8	19	7.7	1.2 (0.6 - 2.3)
Bacterium coli + phenol (Reparon®)	169	0.7	9	4.7	400	1.1	16	6.5	0.7 (0.3 - 1.6)
Chlordiazepoxide	190	0.8	11	5.8	261	0.7	6	2.4	2.5 (0.9 - 6.8)
Clotrimazole	1,619	7.1	22	11.5	3,051	8.0	26	10.5	1.1 (0.6 - 2.0)
Diazepam	2,723	12.0	23	12.0	4,098	10.8	32	13.0	0.9 (0.5 - 1.6)
Dimenhydrinate	898	4.0	16	8.4	1,711	4.5	15	6.1	1.4 (0.7 - 2.9)
Dipyrone	1,336	5.9	46	24.1	1,872	4.9	39	15.8	1.7 (1.1 - 2.7)
Drotaverine	2,005	8.9	48	25.1	3,428	9.0	53	21.5	1.2 (0.8 - 1.9)
Hydroxyethylrutoside	563	2.5	4	2.1	1,129	3.0	14	5.7	0.4 (0.1 - 1.1)
Irons	14,624	64.6	120	62.8	26,589	70.1	185	74.9	0.6 (0.4 - 0.8)
Noraminophenazone + caffeine + droteverine (Quarelin®)	195	0.9	10	5.2	270	0.7	15	6.1	0.9 (0.4 - 1.9)
Pancreatin+duodenum siccum (Dipankrin®)	44	0.2	9	4.7	88	0.2	15	6.1	0.8 (0.3 - 1.8)
Penamecillin	1,570	6.9	26	13.6	2,223	5.9	23	9.3	1.5 (0.8 - 2.8)
Pholedrin	758	3.3	10	5.2	1,490	3.9	19	7.7	0.7 (0.3 - 1.5)
Potassium + magnesium (Panangin®)	765	3.4	7	3.7	1,392	3.7	13	5.3	0.7 (0.3 - 1.7)
Promethazine	3,608	15.9	40	20.9	5,974	5.8	51	20.6	1.0 (0.6 - 1.6)
Senna	443	2.0	27	14.1	818	2.2	37	15.0	0.9 (0.5 - 1.6)
Terbutalin	2,325	10.3	25	13.1	3,966	10.5	28	11.3	1.2 (0.7 - 2.1)
Vitamin B6	1,988	8.8	25	13.1	4,045	10.7	41	16.6	0.8 (0.4 - 1.3)

Table 7. Results of conditional logistic regression analysis of cases and their all matched controls without CA born to mothers with phenolphthalein treatment during the entire pregnancy and in the second and/or third gestational month

Study groups	Grand total	Entire pregnancy				II-III months			
	No.	No.	%	OR*	95% CI	No.	%	OR*	95% CI
Controls	38,151	247	0.6	Referent		73	0.2	Referent	
Isolated CAs									
Neural-tube defects	1,202	15	1.3	2.7	1.2 - 6.4	4	0.3	1.3	0.3 - 5.2
Hydrocephaly, congenital	314	3	1.0	2.3	0.4 - 11.7	2	0.6	3.5	0.3 - 39.1
Eye CAs	99	2	2.0	-	-	2	2.0	-	-
Cleft lip ± palate	1,374	15	1.1	1.3	0.6 - 2.8	5	0.4	1.3	0.3 - 4.7
Cleft palate only	582	2	0.3	0.8	0.1 - 5.1	0	0.0	-	-
Cardiovascular CAs	4,479	33	0.7	1.1	0.7 - 1.7	13	0.3	1.8	0.8 - 4.0
Obstructive CAs of urinary tract	271	2	0.7	0.7	0.1 - 3.9	0	0.0	-	-
Hypospadias	3,038	22	0.7	1.3	0.7 - 2.2	8	0.3	1.3	0.5 - 3.2
Undescended testis	2,051	10	0.5	0.9	0.4 - 1.9	3	0.2	3.8	0.4 - 38.4
Poly/syndactyly	1,744	20	1.1	1.8	0.9 - 3.4	4	0.2	1.0	0.3 - 3.7
Limb deficiencies	548	7	1.3	2.0	0.6 - 6.0	4	0.7	3.6	0.6 - 19.9
Clubfoot	2,424	25	1.0	1.3	0.8 - 2.2	2	0.1	0.3	0.1 - 1.2
Diaphragmatic CAs	243	2	0.8	0.6	0.1 - 3.1	1	0.4	0.6	0.0 - 6.6
Exomphalos/gastroschisis	238	3	1.3	1.1	0.2 - 5.2	1	0.4	0.7	0.1 - 8.7
Other isolated CAs	2,887	19	0.7	1.0	0.5 - 1.8	6	0.2	0.9	0.3 - 2.7
Multiple CAs	1,349	11	0.8	1.3	0.5 - 3.0	3	0.2	1.3	0.2 - 6.7
Total CAs	22,843	191	0.8	1.3	1.0 - 1.5	58	0.3	1.2	0.9 - 1.8

* adjusted for maternal age, birth order, maternal employment status, use of folic acid and influenza - common cold during pregnancy.

Table 8. Distribution of cases with "other isolated CA"

CA groups/cases	Grand total	Entire pregnancy		Comments
		No.	%	
CA-groups evaluated				
CAs of ear: microtia	354	1	0.3	Bilagit (VIII-IX)
Esophageal atresia/stenosis	217	1	0.5	Bilagit (V)
Pyloric stenosis, congenital	241	1	0.4	Phenolphthalein (IV-IX)
Anal atresia	220	1	0.5	Bilagit (I-II)
CAs of genital organs: intermediate sex	211	1	0.5	Bilagit (III-VIII)
CAs of skeletal system: pectus excavatum	155	1	0.7	Bilagit (I-II)
Subtotal	1,398	6	0.4	
Cases with isolated CA				
Branchial cyst	21	1	4.8	Bilagit (I-II)
Hirschsprung's disease	35	4	11.4	See Table 9
Other CAs of digestive system: Megaloduodenum with transposition of intestine	64	1	1.6	Bilagit (IX)
CA of gallbladder, bile ducts, liver: Congenital cystic liver, Atresia of bile duct	26	2	7.7	Bilagit (I-II), Bilagit (IX)
Torticollis	301	3	1.0	Bilagit (I-IX), Bilagit (VII-VIII), Bilagit (VI)
CA of urachus	6	1	16.7	Phenolphthalein (VIII)
Teratoma	58	1	1.7	Bilagit (I-III)
Others	978	0	0.0	
Subtotal	1,489	13	0.9	
Total	2,887	19	0.7	

The critical period of torticollis caused by the intrauterine deformation of sternocleidomastoid muscle is during the last months of pregnancy, and all cases had mothers with phenolphthalein treatment between the 6th and 9th months. All other CAs occurred only once, thus they are not analysed due to the old rule: one case – no case. However it is worth mentioning, that of these 19 cases, 10 are connected with the digestive system.

The data of four cases with Hirschsprung's disease with a male predominance are shown in detail in Table 9, and there is a 5th case with megaloduodenum (Table 8). The data set of the HCCSCA includes 35 cases with Hirschsprung's disease and 4 (11.4%) had mothers with phenolphthalein treatment while the mothers of 54 matched controls were not treated with phenolphthalein ($\chi^2_1 = 6.4$; $p = 0.01$).

We attempted to evaluate only medically recorded phenolphthalein treatments during the critical period of the previously discussed specified CAs, but the number of cases was too low for any estimation.

4. Discussion

The objective of our study was to evaluate the possible association between oral phenolphthalein treatment during the critical period of different CA groups and the risk for different CAs. Our data did not show an association of phenolphthalein treatment in second and/or third gestational month of pregnancy with any CA group. However, the detailed analysis of the group of the so-called other isolated CAs showed that 4 cases with Hirschsprung's disease and 3 cases with torticollis had mother with phenolphthalein treatment. In addition we analyzed birth outcomes of controls born to mothers with phenolphthalein treatment and we did not find any clinically important association.

At the evaluation of these findings we have to consider the indication of phenolphthalein and Bilagit® treatment, *i.e.* constipation. (However, Bilagit® is used for the treatment of gallbladder's diseases as well.) Severe constipation which needed drug treatment is more frequent in elder primiparae with a higher socioeconomic status and higher proportion of folic acid supplementation (at least in control mothers). However, these pregnant women had a controversial pattern. On the one hand nearly all pregnancy complications and maternal diseases occurred more frequently in pregnant women with phenolphthalein treatment. On the other hand the birth/pregnancy outcomes of these pregnant women did not show the adverse affect of the above risk factors. The explanation for this discrepancy may be the more health conscious lifestyle of pregnant women with severe constipation and their more attentive care from medical doctors. In addition it is worth mentioning that pre-eclampsia occurred less frequently in pregnant women with constipation and phenolphthalein treatment.

There were 3 cases with torticollis, but the prevalence at birth of this deformation type CA is 0.88 per l,000 in Hungary (*14,21*), thus this observed rate did not result in a significant deviation from the expected rate.

The expected incidence of cases with Hirschsprung's disease (HSCR) is between 1 in 8,000 (*22*) and 1 in 25,000 (*23*) births. The data set of the HCCSCA is not appropriate for the estimation of HSCR's incidence but it is worth noting that the rate of cases with HSCR born to mothers with phenolphthalein treatment was higher in our data set than the rate of congenital hydrocephaly (0.7/1,000), cleft palate (0.5/1,000), and diaphragmatic CAs (0.2/1,000) (*14*) with much higher prevalence at birth (their Hungarian rates are shown in brackets). In addition the comparison of cases with HSCR and their matched controls indicated a very significant association with maternal phenolphthalein (mainly Bilagit®) treatment. However, we have to consider that

Table 9. Data of cases with Hirschsprung's disease

No.	Sex[a]	Cases		Mother		Father		Sibs		Pregnancy cmplications	Maternal diseases	Drug treatments	Pregnancy supplements
		Gestational age (wk)	Birth weight (g)	Age (yr)	ES[b]	Age (yr)	ES[b]	No.	CA				
4407	M	37	3,000	26	SSW	28	M	1	None	TPD (V-VII)[c]	Tonsillitis (I) Cholelithiasis (I) Constipation (V) Common cold (V)	Acetylsalicylic acid (I) Bilagit® (I)[e] Senna (V) Allylestrenol (V-VII) Diazepam (V-VII) Terbutaline (V-VII)	Iron (V) Multivitamin (V)
2216	M	37	3,750	23	M	23	P	0	–	–	Common cold (I) Constipation - haemorrhoid (I) Cholelithiasis (VIII)	Almagel® = aluminium hydroxide + magnesium hydroxide (VIII) Reparon®(ung) = Bacterium coli + phenol Bilagit® (VIII)[e]	Folic acid (VIII) Iron (VIII) Multivitamin (II)
1415	M	36	1,900	23	M	29	P	1	None	Mild NVP (I)[d] anaemia (V)	Migraine (III) Cholecystitis (III)	Kefalgin® = ergotamine + aminophenazone + caffeine + belladonna extr. (III) Bilagit® (III)[e]	Iron (V) Vitamin D (V-VIII)
1455	F	36	1,900	39	SW	31	SW	0	–	Mild NVP (II-III)[d]	Constipation (I-IX)	Ampicillin (III) Penamecillin (III) Dipyrone (III) Drotaverine (III) Acetylsalicylic acid (III) Phenolphthalein (I-IX) Senna (I-IX)	Vitamin B6 (II-III) Caldea® = retinol + ergocalciferol + calcium hydrogenphosphate + calcium lactate (IV)

[a] M = male, F = female; [b] ES = Employment status; P = professional; M = managerial; SW = skilled worker; SSW = semiskilled worker; [c] TPD = threatened preterm delivery; [d] NVP = nausea and vomiting in pregnancy; [e] Bilagit® = papaverine + methylhomatropine + phenolphthalein + sodium choleinicum + methenamine + menthol.

multiple comparisons result in a statistically significant association in every 20th estimation because of chance.

HSCR or congenital aganglionic megacolon or colon aganglionosis was described by Harald Hirschsprung in 1888 (*24*) and is caused by the congenital absence of the intramural myenteric parasympathetic nerve ganglia and sympathetic nerve plexus in a segment of colon that extends proximally from the anus for a varying distance. Aganglionosis is limited to the recto-sigmoid colon in 70% of cases (short-segment HSCR), but total colonic aganglionosis and small intestinal aganglionosis were found in 1-10% of cases (long-segment HSCR) in different studies. The aganglionic colon is unable to transmit the coordinated peristaltic waves from the proximal colon producing variable degrees of intestinal obstruction. Hyperperistaltic activity results in increasing hypertrophy and dilatation of the normal colon.

HSCR is common in males (3-5:1). Familial occurrence is obvious because the recurrence risk for sibs is 4% though the occurrence of HSCR cases is 0.02% in the population (*25*). The etiology of this CA can be explained by gene-environmental interactions. The polygenic background of HSCR is supported by the observation that (i) the recurrence risk increases with the number of affected first degree relatives, and (ii) greater when involvement (long-segment) is more severe, in addition (iii) the familial risk is higher in the relatives of females (7.2% *vs*. 2.6% in males) (*26*). The locus of major genes of HSCR was localized in chromosome 10q11.2 (*27*). Recently the mutations of genes operating either alone or in combination in the origin of HSCR have been revealed. These mutations include dominant mutations in the RET gene (*28*) and a recessive mutation in the endothelium receptor type B gene (EDNR-B) (*29*). However, the triggering environmental factors are less known, previously hyperthermia in early gestation was described as a triggering factor in the origin of HSCR (*30*) but later this finding was not confirmed (*31*). Our study showed a higher risk for HSCR thus further studies will be needed to differentiate the possible teratogenic/ triggering or mutagenic effect of phenolphthalein in the origin of this disease.

The strengths of HCCSCA can be explained by the population-based large data set including 438 pregnant women with phenolphthalein treatment in the ethnically homogeneous Hungarian (Caucasian) people. Additional strengths include the matching of cases to controls without CA, available data for potential confounders, and finally that the diagnosis of medically reported CAs was checked in the HCAR (*13*) and later modified, if necessary, on the basis of recent medical examination within the HCCSCA (*12*). Our study design regarding birth outcomes were based on medically recorded gestational age at delivery and birth weight.

However, this data set also has limitations. (i) Most pregnant women were treated by Bilagit® containing methylhomatropine, papaverine, methenamine, sodium choleinicum, and menthol beyond phenolphthalein and of 4 cases with HSCR, 3 had mothers with Bilagit® treatment. The other components of Bilagit® had no teratogenic effect (*32*), nevertheless a drug interaction cannot be excluded. (ii) The response rate was 83% in controls and 84% in cases, but there was an active follow up for all non-respondent case mothers, but for only 200 non-respondent control mothers. However, it is worth noting that there was no significant difference in the prevalence of other frequent maternal diseases and drug treatments between the subgroups of respondents and non-respondents (*15*), thus, the effect of selection bias seems to be limited in the study. (iii) The mean time between the birth/pregnancy termination and the return of the information package was 1.7 months longer in the group of control mothers ($t = 4.4$; $p < 0.001$). However, this degree of time difference does not cause recall bias in long term treatment such as phenolphthalein (*15*). (iv) Most women with phenolphthalein treatment were treated with other drugs as well, but in general their proportion was similar in case and control mothers. (v) Only a very small proportion of case and control mothers had prospectively and medically recorded phenolphthalein treatment during the study pregnancy because these drugs were not prescribed in the prenatal care clinic. Thus, we have to consider recall bias, because the birth of an infant with CA is a serious traumatic event for most mothers who therefore try to find a causal explanation such as diseases or drug uses during pregnancy for CA of their babies. This does not occur after the birth of a healthy newborn infant. Thus recall bias might inflate an increased risk for CAs. Our previous analysis showed that a case-control surveillance of this type may cause spurious association between drugs and CAs with biased OR up to a factor of 1.9 (*33*). However, at the planning of our study design we wanted to limit recall bias. Thus we evaluated different CAs separately because if we find a significant association of phenolphthalein treatment with only one or a few CA, it is an argument against the recall bias because it is general for all CAs. In addition we focused our analysis for the critical period of CAs because we expect an underreporting of phenolphthalein treatment in both the critical and non-critical periods of CAs in the control group. Unfortunately of our 834 malformed controls (Down syndrome), only 11 had mothers with phenolphthalein treatment, thus we were not able for the comparative analysis of cases and malformed controls.

The mechanism of action of the laxative phenolphthalein is similar to that of the anthraquinone purgatives such as senna. Small amounts of the laxatives are absorbed into the systematic circulation. As far as we know results of investigation regarding the cross of phenolphthalein through the placenta have not been published, however, its molecular weight is low enough (approximately 318) for placental transfer (*34*). No investigations reporting the use of phenolphthalein in

experimental animals have been located (*32*). Previously only Heinonen *et al.* (*11*) studied the teratogenic effect of phenolphthalein, and they did not find an increase in the expected rate of CAs among offspring of 236 women who took this laxative during the first four lunar months compared to the expected rate. They reported similar findings in 806 women who took phenolphthalein anytime during pregnancy.

Phenolphthalein is an old-fashioned drug but its mutagenic/carcinogenic effect is debated, therefore the data of our study may contribute the final conclusion.

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References

1. Williamson C. Gastrointestinal disease. Best Practice and Research Clinical Obstet Gynaecol 2001; 15:937-952.
2. Welsh A. Hyperemesis, gastrointestinal and liver disorders in pregnancy. Curr Obstet Gynaecol 2005; 15:123-131.
3. Fagan EA. Disorders of the liver, biliary system and pancreas. Disorders of the gastrointestinal tract. In: de Swiet EF (ed.): Medical Disorders in Obstetric Practice. 4th, Blackwell, Oxford, 2002.
4. Mahadevan U, Kane S. American gastroenterological association institute technical review on the use of gastrointestinal medications in pregnancy. Gastroenterology 2006; 131:283-311.
5. Thukral C, Wolf JL. Therapy insight: drugs for gastrointestinal disorders in pregnant woman. Nat Clin Pract Gastroenterol Hepatol 2006; 3:256-266.
6. Jewell DJ, Young G. Interventions for treating constipation in pregnancy. Cochrane Database Syst Rev 1998; 3:001142.
7. Ganinella TS, Bass P. Laxatives: An update on mechanism of action. Life Sci 1978; 23:1001-1010.
8. Vámossy Z. Egy új hashajtószerrről a purgoról. (A new laxative, Purgo.) Orvosi Hetilap 1902; 43:147-148.
9. Szalkay Z. From Kalmopyrin to Cavinton - the history of pharmaceutical factory in Hungary. *http://www.scitech.mtesz.hu/25gyogyszeripar/index.html*
10. Dobson SZ. History of drug treatment. Dictum, Budapest, 2001.
11. Heinoinen OP, Slone D, Shapiro S. Birth Defects and Drugs in Pregnancy. Publishing Sciences Groups Inc., Littleton, Mass. 1977.
12. Czeizel AE, Rockenbauer M, Siffel C, Varga E. Description and mission evaluation of the Hungarian Case Control Surveillance of Congenital Abnormalities, 1980-1996. Teratology 2001; 63:176-185.
13. Czeizel AE. The first 25 years of the Hungari: an Congenital Abnormality Registry. Teratology 1997; 55:299-305.
14. Czeizel AE, Intôdy Z, Modell B. What proportion of congenital abnormalities can be prevented? BMJ 1993; 306:499-503.
15. Czeizel AE, Petik D, Vargha P. Validation studies of drug exposures in pregnant women. Pharmacoepidemiol Drug Saf 2003; 12:409-416.
16. Ács N, Bánhidy F, Puho E, Czeizel AE. Maternal influenza during pregnancy and risk of congenital abnormalities. Birth Defects Res (Part A) 2005; 73:989-996.
17. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by perconceptional vitamin supplementation. N Engl J Med 1992; 327:1832-1835.
18. Czeizel AE. Reduction of urinary tract and cardiovascular defects by periconceptional multivitamin supplementation. Am J Med Genet 1996; 62:179-183.
19. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural-tube defects. Am J Med Genet 2004; 125:12-21.
20. Puho E, Métneki J, Czeizel AE. Maternal employment status and isolated orofacial clefts in Hungary. Cent Eur J Publ Health 2005; 13:144-148.
21. Pazonyi I, Bod M, Czeizel AE. Postural association. Acta Paediatr Acad Sci Hung 1983; 23:431-445.
22. Bergsma D. Birth Defects Compendium. 2nd ed. Alan R Liss, New York 1979; pp. 240-241.
23. Swenson O. Hirschprung's disease (aganglionic megacolon). N Eng J Med 1959; 260:972-975.
24. Hirschprung H. Stuhltragheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons, Jahrb Kinderheilk 1888; 27:1-7.
25. Badner JA, Sieber WK, Garver KL, Chakravarti A. A genetic study of Hirschsprung diseases. Am J Hum Genet 1990; 46:568-580.
26. Passarge E. The genetics of Hirschsprung's disease: evidence for heterogeneous etiology and a study of sixty-three families. N Engl J Med 1967; 276:138-143.
27. Martucciello G, Bicocchi MP, Dodero P, Lerone M, Cirillo MS, Puliti A, Gimelli G, Romeo G, Jasonni V. Total colonic aganglionosis associated with interstitial deletion of the long arm of chromosome 10. Pediat Surg Int 1992; 7:308-310.
28. Edery P, Pelet A, Mulligan LM, Abel L, Attié T, Dow E, Bonneau D, David A, Flintoff W, Jan D. Long segment and short segment familial Hirschsprung's disease: variable clinical expression at the RET locus. J Med Genet 1994; 31:602-606.
29. Puffenberger EG, Hosoda K, Washington SS. A missense mutation of the endothel-B receptor gene in multigenic Hirschsprung's disease. Cell 1994; 79:1257-1266.
30. Lipson A. Hischsprung disease in the offspring of mothers exposed to hyperthermia during pregnancy. Am J Med Genet 1988; 29:117-124.
31. Larsson LT, Okmian L, Kristoffersson U. No correlation between hyperthermia during pregnancy and Hirschsprung disease in the offspring. Am J Med Genet 1989; 32:260-261.
32. Sheppard TH, Lemire RJ. Catalog of Teratogenic Agents. 11th edition. The Jonhs Hopkins Univ Press, Baltimore-London, 2004.
33. Rockenbauer M, Olsen J, Czeizel AE, Pedersen L, Sorensen HT, EuroMAP Group. Recall bias in a case-control study on the use of medicine during pregnancy. Epidemiology 2001; 12:401-406.
34. Odenthal KP, Ziegler D. *In vitro* effects of antraquinones on rat intestine and uterus. Pharmacology 1988; 36 Supp l:57-65.

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