The Novel Antibacterial Compound Walrycin A Induces Human PXR Transcriptional Activity

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The human pregnane X receptor (PXR) is a ligand-regulated transcription factor belonging to the nuclear receptor superfamily. PXR is activated by a large, structurally diverse, set of endogenous and xenobiotic compounds and coordinates the expression of genes central to metabolism and excretion of potentially harmful chemicals and therapeutic drugs in humans. Walrycin A is a novel antibacterial compound targeting the WalK/WalR two-component signal transduction system of Gram (+) bacteria. Here, we report that, in hepatoma cells, walrycin A potently activates a gene set known to be regulated by the xenobiotic sensor PXR. Walrycin A was as efficient as the reference PXR agonist rifampicin to activate PXR in a transactivation assay at noncytotoxic concentrations. Using a limited proteolysis assay, we show that walrycin A induces conformational changes at a concentration which correlates with walrycin A ability to enhance the expression of prototypic target genes, suggesting that walrycin A interacts with PXR. The activation of the canonical human PXR target gene CYP3A4 by walrycin A is dose and PXR dependent. Finally, in silico docking experiments suggest that the walrycin A oxidation product Russig’s blue is the actual ligand for PXR. Taken together, these results identify walrycin A as a novel human PXR activator.

Key Words: walrycin A; pregnane X receptor; nuclear receptor; CYP3A4; ligand-binding domain; xenobiotic.

Xenobiotics, such as drugs and environmental chemicals, exert a profound influence on human health. Xenobiotics can alter homeostasis and induce deleterious metabolic perturbations. In order to promote the metabolic inactivation and excretion of these compounds, multiple signaling pathways are activated to trigger hepatic biotransformation, biliary excretion, and renal elimination. Part of these clearance mechanisms is coordinately controlled by nuclear receptors such as pregnane X receptor (PXR/NR1I2) and constitutive androstane receptor (CAR/NR1I3). Being important transcription factors controlling xenobiotic detoxification, CAR and PXR display a strong expression in the primarily exposed organs, the liver and the intestine (Lamba et al., 2004; Savkur et al., 2003).

As for many nuclear receptors, CAR and PXR possess a conserved DNA-binding domain (DBD) and a variable C terminal ligand-binding domain (LBD). Within the LBD, the ligand-binding pocket of CAR and PXR accommodates a wide range of structurally unrelated endogenous and exogenous ligands (di Masi et al., 2009). For instance, human PXR and human CAR are both activated by endogenous ligands such as bile acids and steroid hormones (Guo et al., 2003; Timsit and Negishi, 2007; Xie et al., 2003), xenobiotics such as drugs (e.g., rifampicin, dexamethasone, and phenobarbital), endocrine disrupters (bisphenol A and phthalates), and natural plant compounds (hyperforine and zearalenone) (Ayed-Boussema et al., 2011; DeKeyser et al., 2011; Lehmann et al., 1998; Moore et al., 2000; Sueyoshi et al., 1999).

Through their DBD, CAR, and PXR bind to various response elements (direct repeats DR3, DR4, and DR5 as well as everted repeats ER6 and ER8), thereby controlling the expression of a large set of target genes involved in energy metabolism and hormone homeostasis, inflammation, cell differentiation, bile acids, and bilirubin detoxification (Moreau et al., 2008; Pascussi and Vilarem, 2008; Wada et al., 2009). Moreover, this versatile DNA-binding property allows cross talks between CAR and PXR and also with other nuclear receptors such as FXR, LXR, VDR, PPAR, ER, GR, COUP-TFI, and II (Breuker et al., 2010; di Masi et al., 2009; Faucette et al., 2006; Ihunnah et al., 2011; Istrate et al., 2010).
PXR and CAR have been initially described as xenobiotic sensors modulating the expression of several hepatic target genes driven by a so-called xenobiotics response element and involved in detoxification pathways, including drug-metabolizing enzymes and transporters (Omiecinski et al., 2011; Wada et al., 2009). For instance, human cytochrome P450 2B6 (CYP2B6) and 3A4 (CYP3A4) expression is under the control of CAR and PXR, respectively (Kliwer et al., 2002; Lehmann et al., 1998; Maglich et al., 2003; Mo et al., 2009; Sueyoshi et al., 1999). The CYP3A subfamily member CYP3A4A is a key player in detoxification pathways, because about 50% of therapeutically used drugs are metabolized by this enzyme (Istrate et al., 2010; Kliwer et al., 2002).

Moreover, the PXR/CYP3A4 pathway is involved in 60% of known drug-drug interactions (Evans, 2005). Rifampicin, an antibiotic used to treat tuberculosis as well as nosocomial pneumonia caused by methicillin-resistant Staphylococcus aureus (MRSA), is a human PXR agonist inducing CYP3A4 expression. CYP3A4 metabolizes more than 100 drugs including oral contraceptives, anti-HIV protease inhibitors (Baciewicz et al., 2008; Ivanovic et al., 2008; Ma et al., 2008), and antibiotics (Jung et al., 2010). Thus, activation of the PXR signaling pathway leads to a diminished therapeutic efficacy of many drugs and also potentially produces toxic metabolites. There is therefore a need to determine the effects of each novel therapeutic compound on PXR activity.

Recently, a novel antibacterial compound called walrycin A (4-methoxy-1-naphthol) has been identified through a high-throughput screening approach and shown to target the WalK/WalR two-component signal transduction system of Gram (+) bacteria such as S. aureus and Bacillus subtilis (Gotoh et al., 2010). Given that walrycin A belongs to a potential novel class of antibacterial compounds, effects on human xenobiotics metabolism and hepatotoxicity remain to be studied. Here, we report that walrycin A modulates human PXR activity and impacts on hepatic cell viability.

**MATERIALS AND METHODS**

**Materials**

Rifampicin, 6-methoxy-1-naphthol (6MNoL), and 4-methoxy-1-naphthol (walrycin A) were purchased from Sigma-Aldrich (St Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). The housekeeping gene ribosomal protein large P0 (RPLP0, NM_001002.3) forward (CATGCTCAACATCTCCCCCTCC) and reverse (ATAGCAGCCCAATGCT CCTCATGTTGCC) primers, CYP3A4 (NM_017460.5) forward (CATTCCTCATCCCA TTCTTAGGAT) and reverse (CCACTCTGGCTTTTGGTAGCT) primers, and PXR isoforms 1 (NM_003889.3) and 2 (NM_022002.2) forward (ACCTTTGACACTACCTTCTC) and reverse (CGCAGCCACTGCTAAGCA) primers were purchased from Sigma-Aldrich (St Quentin-Fallavier, France).

**Cell Culture and Treatment**

The immortalized human hepatocyte (IHH) cell line was established by F. Kuipers (University Medical Center, Groningen, Netherlands) from primary human hepatocytes. Cells were routinely maintained as previously described (Schippers et al., 1997). Twenty-four hours before treatment, cells were seeded (3 × 10^5 cells per well) in 12-well plates in seeding medium (phenol red–free Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 2 g/l glucose, 2 mM glutamine (Invitrogen Life Technologies, Carlsbad, CA), 7 μg/ml bovine insulin (Sigma-Aldrich), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 10% charcoal dextran–stripped fetal calf serum (CD-FCS). Cells were washed once before treatment with stimulating medium (seeding medium with CD-FCS reduced to 1%) and treated for 24 h by compounds at indicated concentrations (0.1% DMSO final concentration).

**qPCR-Based Array**

First Strand cDNA Synthesis Kit and Human Drug Metabolism RT² Profiler PCR Arrays were purchased from SABiosciences (Frederick, MD). Both reverse transcription and quantitative PCR (qPCR) (Stratagene Mx3005P QPCR System) were performed following manufacturer’s instructions. Five endogenous control genes—β-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB)—displayed on the PCR array were used for normalization. Cycle threshold (Ct) was normalized to the average Ct of these five endogenous controls. The comparative Ct method was used to calculate the relative quantification of gene expression (Livak and Schmittgen, 2001). The following formula was used to calculate the relative amount of the transcripts in the walrycin A- and vehicle (DMSO)-treated sample, both of which were normalized to the endogenous controls: ΔΔCt = ΔCt (walrycin A) – ΔCt (DMSO). ΔCt is the log2 difference in Ct between the target gene and the average Ct of the five endogenous controls. The fold change for walrycin A-treated sample is expressed relative to the control (DMSO) sample: 2^−ΔΔCt.

**Cell Transfection**

IHH cells were seeded 24 h before transfection in six-well plates (10^6 cells per well) in maintenance medium (Schippers et al., 1997). Cells were transfected using 0.1 nmol of ON-TARGETplus SMARTpool hPXR small interfering RNA (siRNA) or nontargeting siRNA using the Dharmafect1 transfection reagent according to the manufacturer’s protocol (ThermoFisher Scientific, Lafayette, CO). After a 24-h incubation, cells were washed with seeding medium and incubated further for 24 h. Cells were then washed with stimulating medium and treated as described above.

**RNA Extraction, Reverse Transcription, and Real-Time qPCR**

At indicated times, cells were washed with 1× PBS and total RNA was extracted using the Extract-all reagent (EuroBio, Courtaboeuf, France) according to the manufacturer’s protocol. One microgram of total RNA was reverse transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. A 1:20 dilution of complementary DNA was then amplified by real-time qPCR using Brilliant II Fast SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA) and specific primers in a Stratagene Mx3005P QPCR System (Agilent Technologies). Gene expression levels were normalized using the RPLP0 housekeeping gene expression level as internal control. Fold induction were expressed as the ratio of the gene induced expression level to that of the basal level arbitrarily set to one.

**Western Blotting**

At indicated times, transfected cells were washed with 1× PBS followed by total protein extraction using 100 μl of Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s protocol. Western blotting was performed using 40 μg of total proteins. hPXR protein expression was monitored using an anti-hPXR mouse monoclonal antibody (Perseus Proteomics Inc, Tokyo, Japan) and a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). The
immune complexes were detected by chemiluminescence using Pierce ECL plus Western Blotting Substrate (ThermoFisher Scientific) according to the manufacturer’s protocol and visualized with a G:Box gel dock system (Syngene, Cambridge, U.K.). HSP90 protein was used as internal standard for equal loading using a rabbit anti-HSP90 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and an HRP-coupled goat anti-rabbit as secondary antibody (Sigma-Aldrich).

**Limited Proteolysis Assay**

*In vitro* translation of hPXR was performed using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) and the [35S]-containing Protein Labeling Mix Easy Tag (Perkin Elmer, Waltham, MA), in the presence of vehicle (0.1% DMSO), 50μM walrycin A, 50μM rifampicin, or 50μM 6MNol following the supplier’s instructions. Limited proteolysis of the radiolabeled receptor (5 μl of TnT mix) was carried out using increasing concentrations of chymotrypsin ranging from 1 to 5 μg/ml. After a 10-min incubation, digestion was stopped by the addition of 6X Laemmli buffer. Samples were boiled for 5 min and separated by 12% SDS-PAGE (polyacrylamide gel electrophoresis). After gel drying, radiolabeled digestion products were visualized using a STORM PhosphorImager (GE Healthcare, Orsay, France).

**Gene Reporter Assay**

The stable reporter cell line HGPXR stably expressing a GAL4 DBD-hPXR LBD chimeric fusion protein was described previously (Lemaire et al., 2006).

**Methods for Computer-Simulated Ligand Binding (Docking)**

**Protein input files preparation.** Ligand-free PXR (1ILH.pdb and 3HVL.pdb) input files were generated using the protein preparation wizard from the Maestro software (Maestro 8.5, Academic Campaign, http://www.schrodinger.com). The bond orders were assigned and hydrogen atoms were added. The resulting receptor coordinates were saved as a pdb file.

**Ligand input files preparation.** Ligand input structures were generated and 3D optimized with the MarvinSketch Academic Package (MarvinSketch 5.4.1.1, 2011, ChemAxon http://www.chemaxon.com). Ligand structures were saved as mol2 files.

**GOLD and FRED docking protocol.** Docking was performed using chemscore fitness function under standard default settings in the GOLD software (Cambridge Crystallographic Data Centre, http://www.ccdc.cam.ac.uk): search efficiency 200%, population size 100, number of islands 5, number of operations 100,000, niche size 2, migrate 10, mutate 95, cross over 95, and a selection pressure of 1.1. Early termination was allowed if four solutions were within 1.5 angstroms of root mean square deviation. Docking with FRED 2.2.5 (OpenEye Scientific Software, Inc., Santa Fe, NM, www.eyesopen.com, 2011) was performed using Chemgauuss3 scoring for exhaustive search and Chemgauuss3 scoring for optimization.

**Viability Assays**

HepG2 cells were seeded in 96-well plates (10^4 cells per well) in maintenance medium and incubated for 24 h. Cells were treated for 24 h with indicated concentrations of compounds. Cell viability was quantified using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer’s protocol. Absorbance was monitored on MRX spectrophotometer (Thermo Labsysys, Issy Les Moulineaux, France). Results were calculated as follows: (A570−A630)compound/(A570−A630)DMSO. Curve fitting was performed using GraphPad Prism 4.0 software (San Diego, CA). Each concentration was tested in quadruplicate, and data are displayed as means ± SEM.

**Statistical Analysis**

Histograms represent means ± SEM (n = 2–3). Statistical analyses were performed using GraphPad Prism 4.0. Statistical significance was determined using a one-way ANOVA followed by a Dunnett’s multiple comparison post hoc test (p values < 0.01 were considered as significant). For knockdown assays, significant differences were determined using a two-way ANOVA followed by a Bonferroni post hoc test.

**RESULTS**

**Walrycin A Regulates mRNA Expression of Genes Involved in Phase I Drug Metabolism**

Walrycin A harbors potent antibacterial activity against the MRSA strain N315 (Gotoh et al., 2010). Because rifampicin displays a similar efficacy against MRSA (Perliroth et al., 2008) and induces drug-metabolizing enzymes in human hepatocytes at a concentration of ~30–50μM (Rae et al., 2001), we first assessed whether walrycin A could modulate the expression of enzymes involved in drug xenobiotic metabolism pathway at a similar concentration. Because drug metabolism is initiated upon the activation of phase I enzymes, we focused our analysis on this class of enzymes using qPCR macroarrays. Human immortalized hepatocytes (IHH cell line, Schippers et al., 1997) were treated for 24 h by 50μM walrycin A and 0.1% DMSO as a control. The differential messenger RNA (mRNA) expression analysis of 83 phase I enzymes was monitored and analyzed using a fold change cutoff of four. This allowed the identification of 31 upregulated (36.9%), and of only two downregulated (2.4%) genes (Fig. 1B). The expression of 50 genes were not affected (59.5%) (Supplementary fig. S1). Walrycin A-modulated genes could be classified into three main classes of enzymes (alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome p450s). Genes encoding proteins with other functions, such as FMO1, are displayed as “others” (Fig. 1C). Very interestingly, the PXR target genes CYP1A1 and CYP3A4 were strongly upregulated (30.9- and 5.2-fold, respectively) in response to walrycin A. Given that both drug-activated CAR and PXR may equally regulate the expression of genes in human hepatocytes such as CYP1A1 and CYP3A4 (Auerbach et al., 2007; Fauchette et al., 2006), we determined the relative expression of mRNAs coding for these two nuclear receptors in IHH cells. As PXR mRNA was strongly expressed whereas CAR mRNA was not detectable (see Supplementary fig. S2), this indicated that the observed altered gene expression pattern upon walrycin A treatment could result from PXR activation. In addition, this indicated that IHH cells provide a valid experimental model to evaluate specific PXR response upon exposure to xenobiotics.

**Dose-Dependent Activation of CYP3A4 by Walrycin A Is PXR Dependent**

We further assessed the contribution of PXR to the observed alteration of the gene expression pattern. IHH cells were treated in parallel for 24 h with the PXR reference agonist rifampicin or walrycin A or the walrycin A structural analog 6MNol. As expected, rifampicin induced CYP3A4 mRNA expression in a dose-dependent manner (Fig. 2A) with an EC50 ~1μM.
reaching a plateau starting at 10μM (fourfold maximal induction). Interestingly, CYP3A4 mRNA expression was also enhanced in a dose-dependent manner by walrycin A, with a maximal induction reached at 100μM and an EC_{50}~30μM (Fig. 2B). 6MNol was inactive in this assay. Taken together, these results indicate that walrycin A activates signaling pathway(s) controlling CYP3A4 mRNA expression.

We then hypothesized that walrycin A-induced CYP3A4 mRNA expression was PXR dependent. To assess this possibility, PXR knockdown was performed in IHH cells using siRNAs (Figs. 2C and D). Both PXR mRNA and protein were significantly downregulated upon anti-PXR siRNA treatment (Fig. 2C), whereas a nonspecific siRNA did not affect PXR expression. CYP3A4 mRNA was induced upon rifampicin treatment, whereas PXR knockdown significantly blunted this response (Fig. 2D). Importantly, walrycin A-induced CYP3A4 mRNA expression was decreased upon PXR knockdown but not affected by the nonspecific siRNA (Fig. 2D). Thus, induction of CYP3A4 expression in response to walrycin A is a PXR-dependent process.

Walrycin A Interacts With PXR and Modifies Its Conformation

As we demonstrated that the induction of CYP3A4 by walrycin A is PXR dependent, we next hypothesized that walrycin A could directly interact with PXR. To assess this possibility, limited proteolysis experiments were performed to probe potential structural modifications of the PXR polypeptide upon walrycin A binding. Full-length PXR was translated in vitro using 35S-labeled methionine in the presence of either DMSO (negative control), rifampicin (positive control), walrycin A or the related compound 6MNol. Unliganded and liganded PXR were then submitted to limited proteolysis using increasing concentrations of
chymotrypsin ranging from 0.5 to 5 μg/ml (Fig. 3). Full-
length radiolabeled PXR protein was more amenable to
degradation by chymotrypsin in the presence of rifampicin (black bars) compared with negative control (1% DMSO), leading to several proteolysis resistant peptides (Fig. 3). The PXR proteolysis pattern in the presence of walrycin A was totally different to that induced by DMSO, suggesting that walrycin A, like rifampicin, interacts with PXR and modifies its conformation, hence exposing chymotrypsin cleavage sequences. The digestion pattern generated in presence of 6MNol was comparable to that observed in the presence of DMSO, indicating that this compound is unlikely to bind PXR. These results are in line with the (lack of) effect of these compounds on PXR-mediated induction of CYP3A4 expression.

Walrycin A Activates PXR Through Its Ligand-Binding Domain

Because protease-resistant fragments observed in limited proteolysis assays can be attributed to structural alterations...
occurring in the LBD of nuclear receptors (Benkoussa et al., 1997), our limited proteolysis assays strongly suggested that walrycin A modulates PXR activity through interaction with the PXR LBD. To verify this hypothesis, a luciferase gene reporter assay was performed using a modified HeLa cell line (HGPXR cells) in which a chimeric GAL4 DBD-hPXR LBD fusion protein is stably expressed (Lemaire et al., 2006, 2007). HeLa cells were treated with concentrations of walrycin A ranging from 0.5 to 500 μM, and the luciferase activity was monitored (Fig. 4A). This system was not sensitive to walrycin A concentrations below 5 μM. Nonetheless, a dose-dependent increase in the luciferase activity was observed starting at 5 μM and reached a maximum at approximately 150 μM, with a calculated EC50 ~10 μM. To evaluate PXR selectivity for walrycin A or 6MNol, an independent experiment was performed in the same system using 150 μM of walrycin A or of 6MNol. As shown in Figure 4B, walrycin A significantly enhanced luciferase transactivation when used at 150 μM when compared with vehicle (DMSO), up to a level close to 80% of the maximal response obtained with the reference PXR ligand SR12813 (Moore et al., 2000), whereas 6MNol was again inactive. Taken together, these results show that walrycin A activates PXR through its LBD and is likely to be a novel ligand for this nuclear receptor.

FIG. 3. Walrycin A induces conformational changes in the human PXR receptor polypeptide. In vitro translation of hPXR was carried out in the presence of 0.1% DMSO, 50 μM 6MNol, walrycin A, or rifampicin. Upon completion of protein synthesis, limited proteolysis assay was performed as described in the Materials and Methods section using varying concentrations of chymotrypsin (up to 5 μg/ml). Radiolabeled peptides were detected using a PhosphorImager system. Arrow plus bar indicates the short fragments corresponding to LBD of PXR partially protected from digestion in presence of rifampicin or walrycin A.

FIG. 4. Walrycin A activates PXR through its ligand-binding domain. HeLa cell line in which a chimeric GAL4 DBD-PXR LBD fusion protein was stably expressed was treated with DMSO (negative control), SR12813 (positive control), walrycin A, or 6MNol. (A) Cells were treated with increasing concentrations of walrycin A ranging from 0.5 to 200 μM. (B) Cells were treated with 150 μM of walrycin A or 6MNol. All experiments were performed in duplicates and results were plotted compared with maximal luciferase activity obtained using 1 μM of SR12813 (100% of transactivation).

The Walrycin A Oxidation Product Russig’s Blue Is Efficiently Docked Into the PXR LBD

Having shown that walrycin A can alter PXR conformation in vitro and activates PXR through its LBD in cellular assays, we next analyzed the ability of walrycin A, of the closely related molecule 6MNol, and of Russig’s blue, an oxidation product of walrycin A (Shoji et al., 2010), to fit in the ligand-binding pocket of PXR by in silico docking experiments. Docking was first implemented using PXR LBD coordinates extracted from the 3hvl.pdb file, corresponding to a human PXR LBD crystal obtained in the presence of a short SRC-1 coactivator peptide and of the bound synthetic PXR agonist SR12813 (Watkins et al., 2001). As shown in Figure 5A, SR12813 was efficiently docked by our procedure with solutions mostly superimposable to the 3HVL coordinates.
Walrycin A and 6MNol docked perpendicular to the phenyl ring of SR12813, leaving most of the ligand-binding pocket empty (Fig. 5A). In contrast, Russig’s blue positioned similarly to SR12813 (Fig. 5B). Ranking ligand fit in 3HVL showed that Russig’s blue has the highest score compared with walrycin A and 6MNol, using either GOLD or FRED softwares (Fig. 5B). Similar conclusions were drawn when using other PXR LBD coordinates (1ILH), corresponding to the human PXR LBD cocrystallized with SR12813 but without a coactivator peptide (data not shown). These data suggest that walrycin A may dock into the PXR LBD in its oxidized form Russig’s blue.

Walrycin A and Hepatic Cells Viability

Rifampicin and walrycin A share common bactericidal properties, raising the possibility of a combined antibacterial therapy. We therefore investigated the effect of walrycin A alone or in combination with rifampicin on the viability of human hepatoma cell lines IHH and HepG2. Cell viability assays were performed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) as a substrate for mitochondrial reductase. To assess walrycin A toxicity, cells were independently exposed for 24 h to walrycin A and 6MNol concentrations increasing from 2 μM up to 5 mM, whereas rifampicin concentrations were from 0.5 μM to 1 mM. Walrycin A concentrations up to 156 μM did not affect HepG2 viability (Fig. 6A). Walrycin A IC_{50} was calculated to be 240 μM, indicating that this compound impacted cell viability at a concentration lower than that of rifampicin (IC_{50} ~1 mM, Fig. 6A and Nakajima et al., 2011) and 6MNol (IC_{50} ~1.7 mM, data not shown). Similar results were obtained using IHH cell line (IC_{50} walrycin A ~350 μM, IC_{50} rifampicin ~4.5 mM, IC_{50} 6MNol ~1.8 mM, data not shown).

The potential synergy between walrycin A and rifampicin was then assessed in the MTS assay. HepG2 cells were exposed to increasing concentrations of rifampicin (from 3 to 100 μM) with or without 50 μM walrycin A for 24 or 48 h (Supplementary fig. S4). Highest rifampicin and walrycin A concentrations (100 and 50 μM, respectively) did not impact cell viability after the 24-h exposure, whereas the combined walrycin A/rifampicin treatment significantly decreased cell viability by approximately 35% (Fig. 6B, left panel). After a 48-h exposure, HepG2 cell viability was significantly decreased upon rifampicin (100 μM) treatment when compared with walrycin A (50 μM) treatment, corroborating data from Singh et al. (2011). The combined walrycin A/rifampicin exposure for 48 h significantly decreased cell viability by approximately 50% when compared with rifampicin alone (Fig. 6B, right panel). Similar results were obtained by exposing HepG2 cells to increasing concentrations of walrycin A (from 3 to 100 μM) together with rifampicin 10 μM (Supplementary fig. S4). Taken together, these results indicated that rifampicin and walrycin A synergistically impacted human hepatoma cell viability in a dose- and time-dependent manner.

DISCUSSION

Bacterial infections, despite intense efforts to thwart them, remain a major public health problem not only in developing but also in developed countries, as highlighted by the reemergence of nosocomial infections. MRSA is a widespread nosocomial pathogen (Diekema et al., 2001) resistant to β-lactam antibiotics, cephalosporins, and to the last resort antibiotic vancomycin, to which 40% of MRSA-infected patients are resistant (Jeffres et al., 2006; Rello et al., 1994). Therefore, given the high-mortality rates caused by these drug-resistant bacteria and the difficulty to develop novel potent and specific antibiotics targeting these bacterial pathogens, combined antibactericidal treatments are currently used to kill increasingly common antibiotic-resistant strains. Rifampicin harbors valuable properties in combination with first-line antibiotics, and the combination of rifampicin and vancomycin...
is an effective treatment against nosocomial MRSA-induced pneumoniae (Jung et al., 2010). The novel antibacterial compound walrycin A is effective against the MRSA N315 strain (Gotoh et al., 2010; Kuroda et al., 2001), suggesting that it could be successfully used in combination with other antibiotics such as rifampicin, because its high minimal inhibitory concentration on MRSA (734 μM) precludes its use as a standalone therapy.

However, drug-drug interactions induced upon antibiotics (co)administration may lead to increased cytotoxicity as exemplified by liver damage and to reduced efficiency due to rapid drug inactivation resulting from the activation of the nuclear xenobiotic sensors CAR and PXR (Singh et al., 2011). Evaluating the activity of walrycin A on hepatic functions is therefore critical to evaluate its potential usefulness as an anti-MRSA drug.

The structure of walrycin A is based on a naphthalene scaffold, conferring hydrophobic properties, substituted by alcohol and methoxy functions in position 1 and 4, respectively. As described by Gotoh et al. (2010), walrycin A targets the WalK/WalR two-component signal transduction system of Gram (+) bacteria, indicating that this compound gets effectively through the bacterial peptidoglycan wall and cytoplasmic membrane of prokaryotes. Therefore, walrycin A could pass through the eukaryote plasma membrane to activate various signaling pathways, including those controlled by PXR. Indeed, walrycin A was found to induce the expression of several PXR target genes such as the drug metabolism phase I enzymes CYP1A1/1A2/2B6/2C8/2C19/3A4/11A1/11B1/11B2 (di Masi et al., 2009) and CYP4A11 (Siest et al., 2008). Walrycin A also deregulated the expression of several enzymes not identified as PXR target genes, suggesting that walrycin A modulates other nonidentified signaling pathways.

The PXR/CYP3A4 pathway is involved in approximately 60% of reported drug-drug interactions (Evans, 2005). CYP3A4 mRNA expression was enhanced in a PXR-dependent manner upon walrycin A treatment. Owing to its structure, walrycin A can potentially activate PXR through direct binding or by triggering posttranslational modifications of this nuclear receptor. To sort out these nonexclusive hypotheses, we investigated whether walrycin A is a direct activator of PXR. Using in vitro limited proteolysis and gene reporter assays, we demonstrated that walrycin A likely acts through the PXR LBD. However, in silico docking of walrycin A into the PXR ligand–binding pocket revealed that the walrycin A oxidation product, Russig’s blue, is more likely to behave as a bona fide PXR ligand. This is consistent with the fact that walrycin A spontaneously oxidizes in various aqueous solutions (Shoji et al., 2010 and Salbert, unpublished data), and with the strict stereoselectivity of PXR activation, on which the structurally related 6MNol is inactive and unable to convert into a spectrally detectable compound (Salbert, unpublished data).

Activation of PXR is known to be species specific, and several reports document the irrelevance of rodent models for assessing the ability of xenobiotics to regulate human PXR activity (Jones et al., 2000; LeCluyse, 2001; Ma et al., 2007). Importantly, the expression of mouse Cyp3a family members is not altered upon administration of rifampicin (Ma et al., 2007) in contrast to dexamethasone, a known mouse PXR activator (Scheer et al., 2010). Our in vitro transactivation assays established that walrycin A is an activator of human but not of mouse PXR (Supplementary figs. S3A and S3B). In line with these results, orally administrated walrycin A (200 mg/kg) for 8 days to C57Bl6 mice neither induced the expression of Cyp3a11, the mouse ortholog of human CYP3A4, nor caused significant macroscopic liver damage (data not shown). This clearly suggested that walrycin A is unable to activate mouse PXR. Our data and others (Ma et al., 2007) thus underline the need for humanized PXR mouse models to study the in vivo effects of walrycin A and other antibiotics rather than wild-type mice.
CAR also plays a major role in drug biotransformation pathways by regulating the expression of, among others, CYP2B, CYP2C, CYP3A, UGTs, GSTs, and membrane transporters MRP2 and MRP4 (di Masi et al., 2009; Omiecinski et al., 2011). It is therefore important to investigate the effects of walrycin A on CAR activity. Our data indicate that walrycin A behaves, like many CAR modulators, as an inverse agonist of CAR1 (Supplementary fig. S5). Up to 15 CAR isoforms are expressed in human liver (Lamba et al., 2004) and some compounds, such as clotrimazol, display opposite modulatory properties on CAR1 and CAR3 isoforms (Auerbach et al., 2005; Moore et al., 2000). This highly complex signaling system, which does not occur in IHH and HepG2 hepatoma cell lines, does not allow an easy prediction of biological outcomes following CAR activation in the liver, and certainly deserves further investigation.

In addition to its PXR and CAR modulatory effects, walrycin A exhibited weak cytotoxic properties on human hepatoma cells but synergistically increased rifampicin toxicity and vice versa. This suggests that a combination of rifampicin and walrycin A could induce deleterious hepatic effects in vivo. This drawback has also been reported for the vancomycin/ rifampicin combined treatment (Jung et al., 2010), which is likely due at least in part to drug-drug interactions. Finally, we noted that some walrycin A-exposed mice exhibited a marked splenomegaly, in agreement with a study in rats (Eastman Kodak Co., Environmental Protection Agency, and Office of Toxic Substances. (1992)).

Finally, to the best of our knowledge, walrycin A is not yet validated as a therapeutically usable drug and there is therefore no information on human exposure levels. Nevertheless, as this compound and/or its derivative Russig’s blue activate PXR and CAR, hepatic effects of a simultaneous exposure to walrycin A and to other drugs should be considered in the future. As walrycin A is also a widely used compound in chemical synthesis (Talaat and Nelson, 1986), the effects of a fortuitous acute or chronic exposition should be monitored in workers exposed to this compound, especially since combined, unwanted exposure to other environmental pollutants such as phthalates and bisphenol A, also known to activate human PXR and CAR (DeKeyser et al., 2011), can dangerously impact health (Howdeshell et al., 2007).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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