Unnatural endo type-B PPAPs as novel compounds with activity against _Mycobacterium tuberculosis_.

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ABSTRACT: Before the emergence of SARS-CoV-2, tuberculosis (TB) was the leading cause of infectious disease mortality worldwide. Like all antibiotic-exposed bacteria _Mycobacterium tuberculosis_ ( _Mtb_ ) developed multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains which require new antibiotics with novel mechanism of actions. Hyperforin, a natural type-A polyphenylated polycyclic acylphloroglucinol (PPAP) isolated from St. John’s wort, is known for its antibacterial, antidepressant and antimycobacterial activity. However, hyperforin is not stable and easily degradable in light, heat and oxidizes. Here we report photo- and benchstable type-B PPAPs with structural similarity to hyperforin and enhanced antimycobacterial activity. We tested a panel of PPAPs and identified our previously reported molecule PPAP22 as lead compound. Converting PPAP22 into the corresponding sodium salt, PPAP53, enhanced the solubility dramatically. We show that PPAP53 inhibits the growth of virulent, extracellular _Mtb_. Strikingly, the activity is more pronounced intracellular _Mtb_ residing in human primary macrophages without damaging the host cell or lung cells. Importantly PPAP53 was also highly active against drug-resistant _Mtb_. Additionally, we analysed the _in vitro_ properties of PPAP53 in terms of CYP-induction and PXR interaction. Taken together we introduce type-B PPAPs are a new class of antimycobacterial compounds, with remarkable activity and favourable physical properties.

INTRODUCTION

Tuberculosis (TB) remains a major global health concern with increasing morbidity and mortality rates¹. One-fourth of the world’s population is infected with TB, from which approximately 10 million people developed active TB in 2020 requiring multi-drug treatment ². An emerging problem is the increase in infections with multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, which require new antibiotics with novel mechanisms of action³-⁶. However, the process of discovering new anti-tuberculosis agents is complex and time consuming. For example, in 2012, for the first time in 40 years, only one novel antibiotic, bedaquiline, was approved by US FDA for the treatment of tuberculosis⁶. Treatment of drug-resistant TB is harsh, and patients require long-term therapy with drugs that are less effective and more toxic than first-line drugs. The most frequent resistance is directed against the first-line drug INH, exceeding 10% of the strains isolated in Germany⁶. The
The emergence of INH-resistant \( Mtb \) strains highlights the urgent need for new, effective drugs with novel mechanisms of action. In recent years, only few novel antibiotics, such as bedaquiline and delamanid, have been approved for clinical use. However, cases of resistance to these antibiotics have already been reported\(^9\)-\(^11\). Hyperforin is the active substance of \textit{Hypericum perforatum}, commonly known as St. John’s wort. It is known to have a broad spectrum of biological properties like antidepressant\(^{21}\), antitumor\(^{22}\), anti-inflammatory\(^{23}\), antibacterial\(^{24}\), antifungal\(^{25}\) and antiviral/antiretroviral\(^{26}\) properties. Additionally, this natural product has antimycobacterial activity against drug-resistant \( Mtb \). Compared with established antimycobacterial drugs like rifampicin- and ciprofloxacin-based drugs hyperforin contains a six-membered ring with incorporated acetylacetone moiety that can undergo keto-enol tautomerization (Figure 1A and 1B). However, hyperforin is not stable and easily degradable by light, heat and it oxidizes in contact with air like other type-A PPAPs. These properties prevent a detailed investigation of the mode of action, or target identification. On the contrary, type-B PPAPs are bench- and light-stable making them more suitable for studies of effectivity, physicochemical properties and mechanism of action. Type-A and type-B PPAPs differ structurally in the position of the exocyclic acyl-group, while maintaining the same core structure (Figure 1C). Here, we investigated the possibility that type-B PPAPs combine potent antimycobacterial activity, stability and favorable chemical properties without inducing toxicity to primary human cells.

**RESULTS**

Recently we reported a synthetic route to easily access a large library of endo type-B PPAPs\(^{28}\), which were tested for antibiotic activity\(^{29}\). The strong antibacterial activity against MRSA (meticillin-resistant staphylococcus aureus) and the possibility to synthesize new compound libraries rapidly provided a platform to investigate the interaction of unnatural type-B PPAPs with \( Mtb \).

We expanded a set of previously published unnatural type-B PPAPs with new compounds containing alternating aliphatic and aromatic sidechains and evaluated the activity against virulent \( Mtb \). PPAPs containing one (PPAP32), two (PPAP13) or three (PPAP38) aromatic moieties were synthesized to study the influence of steric demanding scaffolds, while alternating aliph (PPAP22) and prenyl (PPAP19, PPAP23) sidechains enhance flexibility (Figure 2). All compounds were designed to match the structural
and lipophilic properties of the corresponding natural type-APPAP hyperforin.

Only PPAP22 showed significant reduction of metabolic activity in \( Mtb \) as determined by \(^3\text{H}\)-uracil uptake (Figure 3). Aromatic substituents at C1, C5 or C7 as well as a substitution of allylic sidechains with prenyl sidechains resulted in complete loss of activity. These results highlight the structural selectivity of this compound class against \( Mtb \).

Exchanging the exocyclic acyl-group at C3 of PPAP22 with a benzyl-group, while keeping allylic sidechains (PPAP32) also led to a loss of antimicrobial activity. Even a smaller change in form of an exocyclic isopropyl group at C3, like PPAP19, inactivated this compound. PPAP22 is very lipophilic and therefore poorly soluble in culture media. To circumvent this obstacle PPAP22 was transformed into the corresponding sodium salt PPAP53. By treating PPAP22 with sodium hydride in THF the corresponding sodium salt precipitated in good yields providing a white powder (Figure 4). PPAP53 showed a moderate effect (IC\(_{50} = 38.8 \mu\text{M}\)) on the metabolism of extracellular \( Mtb \) (Figure 5A). \( Mtb \) is an intracellular pathogen which resides and multiplies in primarily in macrophages. Antimicrobial drugs must therefore gain access to the intracellular compartments in which the bacilli persist. To test the efficacy of PPAP53 against intracellular \( Mtb \), primary human macrophages were infected with virulent \( Mtb \) in the presence or absence of the compound (Figure 5B). The results indicated that PPAP53 reduced the intracellular growth of the bacteria with an IC\(_{50}\) of 6.8 \( \mu\text{M}\).

Figure 4 Transforming PPAP22 into the corresponding sodium salt PPAP53.

PPAP53 was also highly active against an intracellular INH-resistant strain (IC\(_{50}\) of 15.9 \( \mu\text{M}\); Figure 5C).

To exclude that antimicrobial activity was associated with destruction of the macrophages we determined the toxicity of PPAP53 towards primary human cells, such as peripheral blood mononuclear cells (PBMCs) and macrophages (Figure 5D and 5E). PPAP53 at concentrations that induced mycobacterial growth inhibition showed only slight toxicity towards PBMCs and macrophages as determined by resazurin degradation.

In summary, PPAP53 exhibits negligible toxicity towards macrophages, PBMCs, and the lung cancer cell line A549, at concentrations effective against both extra- and intracellular \( Mtb \) (Figure 5F).

HepaRG cells were chosen to evaluate the behavior of PPAP53 in comparison to rifampicin, in order to address relevant endpoints in hepatic drug metabolism related to CYP expression and PXR activation. HepaRG cells offer a high expression of major cytochrome P450 enzymes, phase II enzymes as well as nuclear receptors and by this, maintain a functional metabolic competence comparable to human primary hepatocytes\(^{30-32}\). As shown in Figure 6A and 6B HepG2 exhibit a higher sensitivity against PPAP53 than
HepaRG. In HepaRG **PPAP53** caused cytotoxicity (cell viability < 80%) after treatment with 60 µM, in comparison to HepG2 where cytotoxicity was observed after treatment with 20 µM down to 10 µM after 48 h incubation period. Figure 6C shows the potential of PPAP53 to activate the nuclear receptor PXR. In comparison to the well-known PXR-agonists SR12813 and rifampicin, PPAP53 activates PXR in a concentration-dependent manner but to a substantially lower degree. The different potency of the test compounds to activate PXR was also reflected by the PXR-dependent CYP3A4 reporter gene assays (Figure 6D).

Here again a strong concentration-dependent effect for PPAP53 and rifampicin was detectable. PPAP53 caused only a slight CYP3A4 induction, while 1 µM hyperforin induces CYP3A4 promotor activity on a level comparable to the positive control and the highest tested concentration of rifampicin. In both reporter gene assays rifampicin and hyperforin show similar results to a previous study conducted with these compounds. Subsequently, Cyp3A4 mRNA expression was analyzed (Figure 6E) and revealed a strong upregulation of CYP3A4 by rifampicin and hyperforin, whereas opposite effect was observed for PPAP53. These findings correlate well with

**Figure 5** Toxicology profile of **PPAP53**. Activity of PPAP53 against extracellular Mtb (A), intracellular Mtb in primary human macrophages (B) and extracellular INH-resistant Mtb (C). Macrophages were infected with live bacilli at a multiplicity of infection (MOI) of 5. Afterwards infected cells were treated with the indicated concentrations of compound. Cell lysates were generated on day 0 and day 4 and plated on 7H11 agar plates in serial dilutions. Colony forming units (cfu) were counted after 14 days of incubation. Depicted are the mean values of 3 individual donors. Toxicity of PPAP53 against monocyte derived macrophages (D) and peripheral blood mononuclear cells (E). Cells were treated over night with PPAP53 as indicated, followed by the addition of PrestoBlue reagent. Depicted are the mean values of five individual donors. Errorbars show the standard deviation. Summary of IC_{50} values of **PPAP53** (F). Confocal laser scanning microscopy of human macrophages infected with Mtb and treated with PPAP53 (A). Macrophages were infected with a mcherry expressing Mtb strain at MOI 5. Afterwards cells were treated with PPAP53 (7.5µM). Cells were fixed using paraformaldehyde (pfa), followed by a DAPI nucleus staining. Samples were then mounted and analyzed using a laser scanning microscope. Cell viability of liver cells exposed to **PPAP53**.

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the CYP3A4 protein amount analyzed for the same treatment (Figure 6F).

Figure 6. (A) Cell viability of HepaRG cells exposed to solvent control (dark blue), PPAP53 (blue), rifampicin (light blue), hyperforin (green) and 10 µM SR12813 as positive control (PC) (dark green) for 24 h (B). Cell viability of HepG2 cells exposed to PPAP53, rifampicin and hyperforin for 48 h. HepG2 and HepaRG cells were exposed to different concentrations of test compounds, positive control (0.01% Triton X-100) and solvent control (0.5% DMSO). Their cytotoxic potential was assessed using WST-1 assay. (C) Induction of PXR activity after 24 h. HepG2 cells were co-transfected with plasmids expressing GAL4-responsive UAS-driven firefly luciferase, human PXR-LBD fused to GAL4-DBD and Renilla luciferase. (D) Induction of PXR-mediated CYP3A4 promoter activity after 48 h. HepG2 cells were co-transfected with plasmids expressing firefly luciferase under CYP3A4 promoter, human PXR and Renilla luciferase. The transfected cells were exposed to 10 µM of positive control SR12813 or different concentrations of test compounds. After 24 h (C) or 48 h (D), cell lysates were assayed for firefly and Renilla luciferase activity. Firefly luciferase activity was normalized against Renilla luciferase activity and fold induction relative to the solvent control (SC; 0.5 % DMSO) was calculated. (E) Gene expression analysis of CYP3A4. Differentiated HepaRG cells were exposed to PPAP53 and Rifampicin as well as 1 µM Hyperforin for 24 h. Total mRNA was isolated and transcribed into cDNA and subsequently mRNA expression of CYP3A4 was analyzed by real time qPCR. For relative quantification Ct values were normalized to reference genes (ACTB and GAPDH) according to the ΔΔCT method. Log2 fold changes of 2-ΔΔCT values were calculated and mRNA levels were expressed in relation to the solvent control (SC; 0.5 % DMSO). (F) CYP3A4 protein quantification. Differentiated HepaRG cells were exposed to PPAP53, Rifampicin and Hyperforin for 24 h. CYP3A4 protein levels were analyzed via a targeted mass spectrometry-based immunoassay. CYP3A4 protein levels were expressed in relation to the solvent control (SC; 0.5 % DMSO). All data expressed in percent of solvent control (SC) and as means of three independent experiments performed with three replicates each ± SD.

We measured six different drug metabolism activities, each indicative of a distinct CYP isofrom. Compared to hyperforin PPAP53 was approximately 5 to 10-fold less cytotoxic and could be applied up to 20 µM without significantly affecting cell viability. As shown in Figure 7, hyperforin (0.5 µM) and rifampin (10 µM) induced CYP3A4 activity similarly by approximately 8- and 10-fold, respectively, while other CYP activities were less strongly or not induced. By contrast, PPAP53, which was tested at even 20-fold higher concentrations than hyperforin, failed to elicit any significant CYP activity increases. Thus, PPAP53 cannot be considered a CYP enzyme inducer.

Although PPAP53 shares a similar structure to hyperforin, it does not trigger PXR activation significant amount. To better understand this behavior, we compared crystal structures of PXR complexed with hyperforin (PDB ID: 1M13) and rifampicin (PDB ID: 1SKX) to an induced-fit docked model of PPAP53 bound to PXR (Figure 8). The docking result shows PPAP53 binds to the ligand-binding domain (LBD), which is located at the C-terminus of the receptor.

Figure 7 CYP-induction of PPAP53 vs hyperforin vs rifampicin. Cytochrome P450 enzyme induction was determined by incubating differentiated cells with the indicated concentrations of PPAP53 in comparison to hyperforin (0.5 µM) and rifampicin (10 µM) in 0.1 % DMSO-medium for 96 h. CYP enzyme activities were then determined in cell culture supernatants using a liquid chromatog-
raphy with tandem mass spectrometry based substrate cocktail assay. Data are presented as means ± SD of three independent experiments.

DISCUSSION

Treatment against tuberculosis is protracted and tortuous in case of HIV co-infection or multi-/ extensively drug-resistant infections. There is a demand for developing new antibiotics with novel mechanisms of action to tackle those infections to improve and/or shorten the healing process of the patients.

Hyperforin, a natural type-A PPAP, exhibits an effect against different mycobacterial strains. Unfortunately, it is easily degradable and therefore not suitable for detailed analysis of its mode of action or for antituberculosis treatment purposes. Previous work showed that unnatural endo type-B PPAPs might have this potential. We started off with screening a set of different PPAPs ultimately resulting in PPAP53 with the highest potential for future antimycobacterial studies. We were able to show that PPAP53 is not only active against extracellular (active tuberculosis), but also intracellular mycobacteria (latent/persistent tuberculosis) in a low micromolar range.

Furthermore, we were able to show that PPAP53 exhibits an even stronger effect on the metabolism of an isoniazid-resistant Mtb strain. Resistance to the first-line agent isoniazid (INH) is now the most common type of resistance, increasing the risk of multidrug-resistant TB. Recent data indicate that INH-resistant TB patients treated with only first- (EMB)-pyrazinamide (PZA)-levofloxacin (LFX) for 6 months and no addition of injectable agents to the treatment regimen.

The influence of PPAP53 on CYP3A4 expression in human liver cells was investigated using HepaRG cells, a widely distributed model for in vitro experimentation in basic research and drug development. In contrast to HepG2 cells, HepaRG cells express a large panel of liver-specific cytochrome P450 enzymes. Kanebratt and Andersson investigated a panel of 44 genes coding for drug-metabolizing enzymes, transporters and other liver-specific factors and in particular, for CYP3A4 a strong activation was found in HepaRG cells and primary hepatocytes but not in HepG2 cells. This could explain the higher sensitivity of HepG2 against PPAP53, due to the difference in their metabolic capacity.

Regarding the PXR activation, our results consistently demonstrated that rifampicin activates the nuclear receptor by using a GAL4 fusion construct-based assay aimed to monitor binding of a substance to the PXR ligand-binding domain as well as by a classic reporter assay utilizing the PXR responsive human CYP3A4 promoter. Whereas, for PPAP53, a substantially lower activation was demonstrated at nuclear receptor level.

Gene expression analysis confirmed the strong PXR-mediated activation of CYP3A4 transcription after treatment with rifampicin and as previously described also for
hyperforn\textsuperscript{33}. In contrast, PPAP\textsubscript{53} did not lead to a comparable increase in CYP3A4 gene expression, and therefore less CYP3A4 protein level in the cells. The fact that rifampicin induces CYP3A4 on a level comparable to that of the positive control and the lack of concentration dependency are indications that the metabolic capacity of the cells treated with 1 \(\mu\)M rifampicin/1 \(\mu\)M hyperforin is already exhausted as previously described\textsuperscript{33}.

We employed molecular docking to investigate the binding of PPAP\textsubscript{53} to the active site of PXR. Our results indicated that PPAP\textsubscript{53} bind to PXR’s active site in a manner similar to hyperforin and rifampicin. Furthermore, we performed experimental assays to determine the activation of PXR by PPAP\textsubscript{53}, which were consistent with our docking results. However, unlike hyperforin, PPAP\textsubscript{53} did not induce CYP expression.

Using the structure of hyperforin-bound PXR as a guide, the single-site mutation, Phe288-Ala, was found to respond more strongly to hyperforin than wild-type PXR, highlight-
ing the crucial role of hydrophobic interactions within the binding site for PXR activation\textsuperscript{38}. This might be a possible reason for the different PXR activation seen between PPAP\textsubscript{53} containing allylic sidechains and hyperforin containing isoprenyl sidechains. This change also leads to significant difference in the surface area. The calculated Connolly surface of hyperforin is 516\(\text{Å}^2\) while PPAP\textsubscript{53} has a surface area of 359\(\text{Å}^2\) (Figure 9). It was shown that not only the presents of hydrophobic features is essential for PXR activation but more importantly the possibility to occupy large areas of the predominantly hydrophobic binding pocket\textsuperscript{33}. Both compounds show hydrogen bond interactions with Gln285 and His407 and hydrophobic interactions with Tyr306, which are essential for PXR activation.

The differences in surface area, strength of hydrogen bond interactions, and hydrophobic features that allow the ligand to occupy large areas of the binding pocket may explain why hyperforin activates PXR more strongly than PPAP\textsubscript{53}. Understanding the molecular interactions between ligands and PXR can aid in predicting their biological activity and potential for drug-drug interactions.

More detailed experiments should give an insight into the mode of action of PPAP\textsubscript{53} using transcriptomic analysis to check the gene regulation of extracellular \textit{Mtb} and more detailed laser scanning confocal microscopy to investigate, if there is a direct interaction (e.g. membrane disruption of bacteria) or the intracellular localization of PPAP in primary human macrophages. For the last part, fluorochrome or fluorophore modified PPAPs need to be established.

**CONCLUSION**

In conclusion, we have successfully developed a water-soluble, photo- and bench-stable type-B PPAP, PPAP\textsubscript{53}, with potent antimycobacterial activity against extracellular and intracellular \textit{Mtb}. Furthermore, PPAP\textsubscript{53} does not exhibit toxicity towards human macrophages, PBMCs, or lung tissue at concentrations effective against \textit{Mtb}. More importantly, PPAP\textsubscript{53} shows even stronger activity against INH-resistant \textit{Mtb} strains. Future research will focus on identifying the mode of action of PPAP\textsubscript{53}. Notably, unlike rifampicin and other TB drugs, PPAP\textsubscript{53} does not induce cytochrome P450 including CYP3A4. Additionally the activation of other CYP-isoforms by PPAP\textsubscript{53} was tested negative. PXR activation experiments showed a slight activation of PXR, which was confirmed by docking studies. Differences between hyperforin, a strong PXR activator, and PPAP\textsubscript{53} were evaluated, with hyperforin having over 40% greater surface area than PPAP\textsubscript{53}, allowing it to occupy larger areas within the predominantly hydrophobic binding pocket of PXR presumably leading to stronger receptor activation. Overall, these findings suggest that PPAP\textsubscript{53} represents a promising candidate for further development as a novel antimycobacterial compound.

**MATERIAL AND METHODS**

Bacteria
Mtb H37Rv (ATCC 27294), INH resistant Mtb (ATCC 35822) and Mtb-mcherry (provided by Dr. O. Neyrolles, IPBS, Toulouse, France) were grown in suspension with constant gentle rotation in roller bottles (Corning, Corning, NY, United States) containing Middlebrook 7H9 broth (BD Biosciences, Franklin Lakes, NJ, United States) supplemented with 1% glycerol (Roth, Karlsruhe, Germany), 0.05% Tween 80 (Sigma-Aldrich, Steinheim, Germany), and 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (BD Biosciences, OADC). Aliquots from logarithmically growing cultures were frozen at −80°C in 7H9 broth with 20% glycerol, and representative vials were thawed and enumerated for viable colony forming units (CFU) on Middlebrook 7H11 plates (BD Biosciences). Staining of bacterial suspensions with fluorochromic substrates differentiating between live and dead bacteria (BacLight, Invitrogen, Carlsbad, CA, United States) revealed a viability of the bacteria >90%. Thawed aliquots were sonicated in a water bath for 10 min at 40 kHz and 110 W before use.

Generation of macrophages

Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Paque™ Plus; GE Healthcare) of buffy coat preparations from anonymous donors (purchased from the Institute of Transfusion Medicine, Ulm University). Monocytes were isolated from PBMCs by adherence on tissue culture treated plastic flasks. Monocyte-derived macrophages were generated by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) in Macrophage-Serum Free Medium (M-SFM; Gibco, ThermoFisher Scientific, Schwerte, Germany) for 6 days. Phenotypic characterization by flow cytometry demonstrated that macrophages expressed CD68 (anti-CD68-FITC, clone Y1/82A; BD Biosciences, Franklin Lakes, NJ, United States) and MHCII (anti-HLA-DR-PerCP, clone L243; BD Biosciences) as described.

3H-Uracil Proliferation Assay

As a correlate of mycobacterial metabolism, we measured the rate of RNA synthesis by determining the uptake of radioactively labeled 5.6-3H-Uracil (ART-0282, Biotrend, Cologne, Germany). About 2 × 10⁵ sonicated Mtb were incubated with PPAPs in a 96-well plate in 7H9-medium containing Middlebrook 7H9 broth (BD Biosciences, Franklin Lakes, NJ, United States) supplemented with 1% glycerol (Roth, Karlsruhe, Germany), 0.05% Tween 80 (Sigma-Aldrich, Steinheim, Germany), and 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (BD Biosciences, OADC). The final volume was 100 µl and all samples were set up in triplicates. After 72 h, 3H-Uracil (0.3 µCi/well) was added and cultures were incubated for additional 18 h. Mtb were inactivated by 4% PFA for 20 min, followed by a transfer onto glass fiber filters (Printed Filtermat A, PerkinElmer, Waltham, MA, United States) using a 96-well-cell harvester (Inotech, Nabburg, Germany). Fiber filters were sealed at 75°C with a wax plate containing the scintillation liquid (MeltiLex, Perkin Elmer). Incorporation of 3H-Uracil by the bacilli was measured using a β-counter (Sense Beta, Hidex, Turku, Finland). Antimicrobial activity (%) was calculated as follows: 100 - [(cpm of the treated sample) / (cpm of the un-treated sample)] × 100.

Quantification of intracellular growth

Macrophages were infected in six-well plates with single-cell suspensions of Mtb at a multiplicity of infection 5 (MOI 5) in macrophage-serum free medium. After 2 h, macrophages were washed thoroughly to remove extracellular Mtb and harvested using EDTA (1 mM). The rate of infection and cellular viability were determined using Auramine-Rhodamine (Merck) and Annexin V staining (BD, Franklin Lakes, NJ, United States) as previously described. The rate of infected macrophages was donor-dependent and ranged between 25 and 40%. About 2 × 10⁵ infected macrophages were seeded in 24-well plates and incubated with PPAP53 using the concentrations 2.5µM, 7.5µM and 25µM for 4 days. To enumerate the number of viable bacilli, infected macrophages were lysed with 0.3% saponin (Sigma-Aldrich). Cell lysates were vigorously resuspended, transferred in screw cap tubes and sonicated for 10 min. Afterward, serial dilutions (1:10; 1:100; and 1:1000) of the sonicates were plated on 7H11 agar plates (BD) and incubated for 4 weeks. The number of viable bacilli was calculated using the concentrations 2.5µM, 7.5µM and 25µM for 4 days. To enumerate the number of viable bacilli, infected macrophages were lysed with 0.3% saponin (Sigma-Aldrich). Cell lysates were vigorously resuspended, transferred in screw cap tubes and sonicated for 10 min. Afterward, serial dilutions (1:10; 1:100; and 1:1000) of the sonicates were plated on 7H11 agar plates (BD) and incubated for 14 days before counting the CFU.

Toxicity of PPAP53 against primary human cells

About 1 × 10⁵ macrophages or 5 × 10⁵ PBMCs were incubated with PPAP53 for 18 h in a 96-well plate, followed by addition of 10% PrestoBlue™ (Thermo Fisher) for 20 min. The non-fluorescent resazurin-based PrestoBlue is reduced to fluorescent resorufin by mitochondrial enzymes of viable cells. The fluorescence intensity (FI) was measured at λex 560 nm and λem 600 nm using Infinite 200 PRO (Tecan, Männedorf, Suisse) plate reader. Cell viability (%) was calculated using the following formula where the ratio of the FIs of the sample and the untreated control is calculated after subtracting the background FI introduced by the volume of cell culture medium: 100 - ([FI (sample)-FI (background)] / [FI (untreated control)-FI (background)]) × 100).

Visualization of the effect of PPAP53 on intracellular Mtb

About 1 × 10⁵ infected macrophages were seeded in 200 µl M-SFM in an eight-chamber slide (Thermo Fisher) and incubated with PPAP53 (7.5µM). After overnight incubation, cells were fixed (4% PFA, Sigma-Aldrich). Cell nuclei were stained with DAPI (1:200, Sigma-Aldrich) diluted in 1% BSA and 0.1% Triton X-100 in PBS (all Sigma-Aldrich). Images were acquired using the inverted laser scanning confocal microscope LSM 710 (Zeiss, Oberkochen, Germany). Image processing was performed using ImageJ software (v 1.52c). All images displayed in this study are processed for brightness/contrast.

Toxicity of PPAP53 against the adenocarcinomic human alveolar basal epithelial cell line A549.

A549 cells were cultivated at 37°C with 5% CO₂ in Dulbecco’s modified eagle medium (Fisher Scientific GmbH, Schwerte, Germany) supplemented with 10% FBS (Fisher Scientific GmbH, Schwerte, Germany) and 1% non-essential
Statistical analysis
All statistical analyses were performed using GraphPad Prism v8.2.1 (GraphPad Software).

For the calculation of the approximate IC₅₀ values of PPAP22 and PPAP53 we used the exponential equation of the trendline of the inverted corresponding graphs.

Chemicals
Rifampicin (CAS 13292-46-1) and SR12813 (CAS 126411-39-0) were purchased from Sigma-Aldrich (Taufenkirchen, Germany) on the highest available purity. Compounds were dissolved in dimethyl sulfoxide (DMSO; AppliChem, Aidenbach, Germany) and 50 µM hydrocortisone (Sigma-Aldrich, Taufenkirchen, Germany) on the highest available purity. Compounds were purchased from AppliChem (Darmstadt, Germany). Triton X-100 (CAS 9036-19-5) was purchased from AppliChem (Darmstadt, Germany) and dissolved in PBS. Solved compounds were stored at -20°C. Standard laboratory chemicals were obtained from Sigma-Aldrich or Merck.

Cultivation of HepG2 and HepaRG cells
HepaRG cells (Biopredic International, Sant Grégoire, France) were cultivated in William’s E medium with 2 mM glutamine (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FBS (FBS Good Forte EU approved; PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), 0.05% human insulin (PAN-Biotech, Aidenbach, Germany) and 50 µM hydrocortisone-hemisuccinate (Sigma-Aldrich, Taufenkirchen, Germany) as previously described. According to manufacturer’s instructions cells were seeded (96-well plates: 9’000 cells/well, 12-well plates 100’000 cells/well). Cells were maintained for 2 weeks in culture medium for proliferation. For differentiation, cells were afterwards cultured for two more weeks in the above-described culture medium, additionally containing 1.7% DMSO. 48 h prior to incubation, HepaRG cells were adapted to treatment medium containing lower concentrations of DMSO (0.5%) and FBS (2%) to enable the cells to lower their CYP-expression. Otherwise, no additional CYP-induction will be visible.

After adaption, treatment with the respective test compounds and controls diluted in treatment medium with a final DMSO concentration of 0.5% was performed. HepaRG cells were used for CYP3A4 expression analysis. Due to poor transfection efficiency of HepaRG cells, reporter gene assays were performed in HepG2 cell.

HepG2 cells (ATCC, Middlesex, UK) were cultivated in DMEM High Glucose medium (Pan-Biotech, Aidenbach, Germany) supplemented with 10% FBS (FBS Superior; Sigma-Aldrich, Taufenkirchen, Germany) and 100 U/mL penicillin and 100 µg/mL streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany). Cells were seeded at a confluence of about 80% - 90% in 96-well plates for experiments.

Cell viability analysis
Cytotoxic effects of test compounds were analyzed using the cell proliferation reagent WST-1 (Immunoservice, Hamburg, Germany). HepG2 cells were seeded in 96-well plates (20’000 cells/well) and were treated with the test compounds 24 h and 48 h after seeding. HepaRG were seeded in 96-well plates (9’000 cells/well) and after 28 days of differentiation and 48 h prior incubation cells were adapted to treatment medium, as described above and afterwards incubated with test compounds for 24 h. All test compounds and controls are diluted in culture medium with a final solvent concentration of 0.5% DMSO. Triton X-100 (0.01%) served as a positive control. One hour before incubation ends, 10 µL WST-1 reagent was added to each well containing 100 µL medium, and plates were incubated one more hour. Afterwards, absorbance was measured at 450 nm with a reference wavelength of 620 nm using the Tecan plate reader Infinite M200 Pro (Tecan group, Männedorf, Switzerland). Values of the reference wavelength were subtracted from absorbance values, and data were corrected for background absorbance by subtracting the values from wells incubated without cells. Data were referred to solvent control, which was set to 100%. At least three independent, biological replicates with three technical replicates per condition were performed. For all following assays, PPAP53, rifampicin and hyperforin were tested only in non-toxic concentrations.

Dual luciferase reporter assay
Assays and plasmids were described in detail previously. In brief, dual luciferase reporter assays were conducted to measure the activation of human pregnane X receptor (PXR) in two ways. Firstly, via a GAL4-based transactivation assay, for which the LBD of PXR had been fused to the DBD of GAL4 (pGAL4-hPXR-LBD), coupled with a GAL4-responsive UAS-driven firefly luciferase reporter (pGAL4-(UAS)5-TK-LUC). Agonistic binding of a test compound to the LBD of PXR leads to the activation of the fusion protein, which binds to the UAS and initiates transcription of the firefly luciferase gene. Secondly, as a classic luciferase reporter assay providing firefly luciferase expression under the control of PXR-regulated sequences from the human CYP3A4 promoter (pGL4.14-XP2-CYP3A4) in combination with a PXR expression plasmid (pSG5-hPXR).
Additionally, cells were always transfected with a plasmid constitutively expressing the reporter gene Renilla luciferase (pcDNA3-RLuc) as internal control for normalization. Plasmid concentrations and positive controls are listed in Table 1 (Supporting Information).

To perform dual luciferase reporter assays 20’000 and 18’000 HepG2 cells were seeded in 96-well plates for the PXR- and the PXR-CYP3A4 assays, respectively. Cells were transiently transfected 24 h after seeding using TransIT-LT1 (Mirus Bio LCC, Madison, WI, USA) according to manufacturer’s protocol. After 4-6 h, cells were incubated with test compounds or controls diluted in culture medium containing 0.5% DMSO. As positive controls, the well-known PXR agonists SR12813 were used. After 24 h (PXR) or 48 h (PXR-CYP3A4) of incubation, cells were lysed with 50 µL lysis buffer (100 nM potassium phosphate with 0.2% (v/v) Triton X-100, pH 7.8). Five µL cell lysate was investigated for firefly and Renilla luciferase activities. The dual-luciferase assay was performed as previously described. Luminescence was measured with an Infinite M200 Pro plate reader (Tecan group, Männedorf, Switzerland). The firefly signal was normalized to the Renilla signal and was expressed relative to the solvent control (containing 0.5% DMSO). Three independent, biological replicates were performed with six technical replicates per condition.

Gene expression analysis
HepaRG cells were seeded in 12-wells plates (100’000 cells/wells) and cultivated as described above. After differentiation of the cells followed by 48 h adaption to treatment medium, cells were incubated for 24 h with test compounds. Cells were washed twice with ice-cold PBS and harvested with 350 µL RLT buffer (RNeasy Mini Kit, Qiagen, Hilden Germany) containing 3.5 µL β-mercaptoethanol. Total RNA was extracted following the manufacturer’s instructions with minor modifications as described previously. RNA quality and quantity were measured with a Tecan Infinite M200 Pro plate reader (Tecan group, Männedorf, Switzerland). Reverse transcription of 0.5 µg RNA was done with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative real-time RT-PCR was performed using 20 ng cDNA, the Maxima SYBR Green/ROX qPCR Master Mix (Thermofisher Scientific, Waltham, Massachusetts, USA) and primers (5 µM; see Table 2 in Supporting Information). Primer design was performed by using the free available software tool Primer3 (University of Massachusetts Medical School, U.S.A.). Primers were designed intron-spanning and checked for mispriming, hairpins and dimers using NetPrimer (University of Massachusetts Medical School, U.S.A.) and the ensemble database (www.ensembl.org). Primers were purchased from Eurofins Genomics Germany GmbH (Ebersberg, Germany).

Gene expression was analyzed on an ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling conditions; 10 min (95°C) initial denaturation, 40 cycles of denaturation (15 s at 95°C) and annealing/elongation (1 min 60°C), a final elongation (15 min at 60°C) plus dissociation curve analysis. Expression levels of the target gene were normalized to the geometric mean of the reference genes ACTB and GAPDH. RNA from three independent, biological replicates was used. Relative gene expression was calculated using the ΔΔCT method. Three independent, biological replicates were performed.

CYP3A4 protein quantification
HepaRG cells were seeded in 12-wells plates (100’000 cells/wells) and cultivated as described above. After differentiation of the cells followed by 48 h adaption to treatment medium, cells were incubated for 24 h with test compounds. Cells were washed twice with ice-cold PBS, harvested and lysed as described previously. The CYP3A4 protein quantification was conducted according to the methodology mentioned previously. Briefly, prior to analysis, the bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA) was used to determine the total protein amount and 15 µL lysate (2-12 µg protein) was proteolyzed using trypsin (Pierce Trypsin Protease, MS-grade; Thermo Scientific, Waltham, MA, USA). Surrogate peptides and internal standard peptides were precipitated using triple X proteomics (TXP) antibodies. Peptides were then eluted and quantified using a modified version of the previously described LC-MS methods. There, 6 min parallel reaction monitoring (PRM) methods are described (UltiMate 3000 RSLCnano and PRM–QExactive Plus; Thermo Scientific, Waltham, MA, USA). Raw data were processed using TraceFinder 4.1 (Thermo Scientific, Waltham, MA, USA). Peptide amounts were calculated by forming the ratios of the integrated peaks of the endogenous peptides and the isotope-labeled standards. Protein quantities were normalized and reported as fmol per µg extracted protein.

CYP induction
HepaRG cells (batch HPR101007, passage no. 12) were obtained from Biopredic International (Rennes, France) and cultured as described. For differentiation HepaRG cells were maintained in 2% DMSO-medium in 96-well plates for two to three weeks as described. For chemical treatments the medium was exchanged every 24 h including the chemicals. Viability of differentiated HepaRG cells maintained for 96 h at different concentrations of hyperforin or PPAP53 in 2% DMSO-medium was determined in comparison to vehicle by alamarBlue Cell Viability assay (Thermofisher, Waltham, USA). Cytochrome P450 (CYP) enzyme induction was determined by incubating differentiated cells with the indicated concentrations of PPAP53 in comparison to hyperforin (0.5 µM) and rifampicin (10 µM) in 0.1 % DMSO-medium for 96 h. CYP enzyme activities were then determined in cell culture supernatants using a liquid chromatography with tandem mass spectrometry based substrate cocktail assay as previously described.

Molecular Modeling
Induced fit docking and ligand modeling of the interaction of PPAP53 and PXR was performed using Maestro (Schrödinger Release 2022-4: Maestro, Schrödinger, LLC, New York, NY, 2021.). The PXR crystal structure (PDB code 1M13) was prepared for docking by removing H₂O and hyperforin from the PDB file. In Maestro, original hydrogens
were removed and replaced, bond orders were assigned, and the structure was minimized. A grid was prepared around the active site centered at the position of hyperforin. All ligands were prepared for docking in Maestro using the ligand preparation function. Ligands were docked to the active-site grid using Induced Fit Glide Docking with post-docking minimization. Connolly surface was calculated with Maestro. Visualization was processed with ChimeraX.52

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