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ORIGINAL ARTICLE

Cytochrome P450 1A2 is the most important enzyme for hepatic metabolism of the metamizole metabolite 4-methylaminoantipyrine

Fabio Bachmann[1,2,3] | Henriette E. Meyer zu Schwabedissen[4] | Urs Duthaler[1,2,3] | Stephan Krähenbühl[1,2]

[1]Division of Clinical Pharmacology & Toxicology, University Hospital, Basel, Switzerland

[2]Department of Clinical Research, University of Basel, Switzerland

[3]Department of Biomedicine, University of Basel, Switzerland

[4]Biopharmacy, Department of Pharmaceutical Sciences, University of Basel, Switzerland

Correspondence
Stephan Krähenbühl, MD, PhD, Clinical Pharmacology & Toxicology, University Hospital, 4031, Basel, Switzerland.
Email: stephan.kraehenbuehl@usb.ch

Funding information
Division of Clinical Pharmacology and Toxicology of the University Hospital of Basel, Switzerland

Aims: Metamizole (dipyrone) is a prodrug not detectable in serum or urine after oral ingestion. The primary metabolite, 4-methylaminoantipyrine (4-MAA), can be N-demethylated to 4-aminoantipyrine (4-AA) or oxidized to 4-formylaminoantipyrine (4-FAA) by cytochrome P450 (CYP)-dependent reactions. We aimed to identify the CYPs involved in 4-MAA metabolism and to quantify the effect of CYP inhibition on 4-MAA metabolism.

Methods: We investigated the metabolism of 4-MAA in vitro using CYP expressing supersomes and the pharmacokinetics of metamizole in the presence of CYP inhibitors in male subjects.

Results: The experiments in supersomes revealed CYP1A2 as the major CYP for 4-MAA N-demethylation and 4-FAA formation with CYP2C19 and CYP2D6 contributing to N-demethylation. In the clinical study, we investigated the influence of ciprofloxacin (CYP1A2 inhibitor), fluconazole (CYP2C19 inhibitor) and the combination ciprofloxacin/fluconazole on the pharmacokinetics of metamizole in $n = 12$ male subjects in a randomized, placebo-controlled, double-blind study. The geometric mean ratios for the area under the concentration–time curve of 4-MAA after/before treatment were 1.17 (90% CI 1.09–1.25) for fluconazole, 1.51 (90% CI 1.42–1.60) for ciprofloxacin and 1.92 (90% CI 1.81–2.03) for ciprofloxacin/fluconazole. Fluconazole increased the half-life of 4-MAA from 3.22 hours by 0.47 hours (95% CI 0.13–0.81, $P < .05$), ciprofloxacin by 0.69 hours (95% CI 0.44–0.94, $P < .001$) and fluconazole/ciprofloxacin by 2.85 hours (95% CI 2.48–3.22, $P < .001$).

Conclusion: CYP1A2 is the major CYP for the conversion of 4-MAA to 4-AA and 4-FAA. The increase in 4-MAA exposure by the inhibition of CYP1A2 and by the combination CYP1A2/CYP2C19 may be relevant for dose-dependent adverse reactions of 4-MAA.

KEYWORDS
4-formylaminoantipyrine, 4-methylaminantipyrine, CYP1A2, CYP2C19, metamizole

The authors confirm that the Principal Investigator for this paper is Stephan Krähenbühl and that he had direct clinical responsibility for the study participants

1 | INTRODUCTION

Metamizole (dipyrone) is an analgesic with antipyretic and spasmolytic properties, which has been in use for almost 100 years. Despite several investigations concerning the analgesic effect of metamizole, the analgesic mechanism is currently not completely clear. In a well-designed study, Pierre *et al.* showed that the 2 major metabolites of metamizole, 4-methylaminoantipyrine (4-MAA) and 4-aminoantipyrine (4-AA), inhibit cyclooxygenase **(COX)-1** and **COX-2** by interfering with the Fe^{3+} ion in the haem of the COXs.[1] However, since metamizole showed weaker anti-inflammatory effects than COX-inhibitors in rats,[2] additional, COX-independent mechanisms, may also be involved.[3–7] Similar to the analgesic activity, the spasmolytic effect of metamizole is clinically evident and experimentally demonstrated, but the mechanisms are not entirely established. Several possibilities have been proposed, among them opening of ATP-sensitive potassium channels[8] and inhibition of G protein-coupled receptor mediated constriction of vascular smooth muscle cells.[9]

Metamizole is associated with neutropenia and agranulocytosis, which is the reason why the drug is currently on the market in only a few countries such as Switzerland, Germany and Spain as well as countries in South America, Far East and Africa. The estimates of the frequency of metamizole-associated agranulocytosis vary considerably, ranging from 1:1439 of patients treated in Sweden[10] to 0.46–1.63 cases per 10^6 daily doses (corresponding to approximately 1:10^5 patients treated considering a median treatment duration of 10 days).[11,12] In a review of the available literature, the mortality associated with ingestion of metamizole was 0.25 per 10^6 prescriptions, which was in the same range as for paracetamol but 20 times lower than for diclofenac.[13] The mechanism for metamizole-associated agranulocytosis is currently not known, but appears not to be HLA-associated according to a recent genome-wide association study.[14]

Metamizole is a prodrug, which is converted to 4-MAA already pre-systemically in the intestinal tract and/or in the liver. 4-MAA has a high oral bioavailability (>80%) and is the principal metabolite in plasma.[15,16] As shown in Figure 1, 4-MAA can be formylated to 4-formylaminoantipyrine (4-FAA) or demethylated to 4-aminoantipyrine (4-AA), which can be acetylated to 4-acetylaminoantipyrine (4-AAA). Less than 5% of orally administered metamizole is excreted in the urine as 4-MAA, the rest is excreted as 4-AAA, 4-FAA and 4-AA, as well as additional, quantitatively less important metabolites.[16–18]

Although the 4 main metabolites of metamizole have been well-described, only the enzyme responsible for the formation of 4-AAA, an N-acetyltransferase[16,19,20] eventually recognized as N-acetyltransferase type 2 (NAT2),[21] has been unequivocally identified. In contrast, the enzymes performing the demethylation and formylation of 4-MAA are so far not known with certainty. Experiments with N,N-dimethyl-4-aminoantipyrine (4-DMAA), which carries 2 instead of 1 methyl group at the amino position of 4-aminoantipyrine, revealed that 4-DMAA can be converted to 4-AA by rat and rabbit liver microsomes, suggesting a cytochrome P450 (CYP)-mediated reaction.[22–25] La Du *et al.* showed that 4-MAA can be demethylated by isolated rabbit microsomes in a reaction using NADPH, Mg^{2+} and oxygen and producing formaldehyde, but this reaction accounted for <50% of 4-MAA degradation.[25] Twenty years after the publication of La Du *et al.*, Noda *et al.* demonstrated that the oxidative conversion of 4-MAA to 4-FAA accounted for most of the microsomal activity that had not been identified by La Du *et al.*[26,27] In support of these findings, Geisslinger *et al.* verified that 4-MAA could be converted to 4-AA at a slow rate by human liver microsomes.[28] This reaction could be inhibited by ketoconazole, suggesting the involvement of **CYP3A4**. In addition, patients with liver cirrhosis have a prolonged half-life of 4-MAA, supporting the notion that 4-MAA is metabolized by the liver.[20] In a recent in vitro study, we could confirm that different hepatic CYPs are involved in the N-demethylation of 4-MAA but we also found demethylation activity by myeloperoxidase in neutrophil granulocytes, suggesting that a portion of 4-MAA might be extrahepatically metabolized to 4-AA.[29]

Considering the uncertainties regarding N-demethylation of 4-MAA, the aim of the current study was to investigate the metabolism of 4-MAA in vitro using human recombinant CYP isoforms and in humans using established CYP inhibitors. The in vitro experiments were used to identify the most efficient CYPs regarding 4-MAA demethylation, whose contribution was subsequently investigated in vivo. The information in humans could also be used to estimate the clinical significance of potential interactions with the CYPs involved in the metabolism of 4-MAA.

What is already known about this subject

- Metamizole (dipyrone) is a prodrug not detectable in human body fluids.
- Its principal metabolite is 4-methylaminoantipyrine, which is N-demethylated or C-oxidized by cytochrome P450 (CYP)-dependent reactions.
- The CYPs involved in these reactions are currently not known.

What this study adds

- Experiments with supersomes revealed CYP1A2 to be the major CYP for both reactions, with contributions of CYP2C19 and CYP2D6 to N-demethylation.
- These results were confirmed in a clinical study in male human subjects.
- Concomitant treatment with strong CYP1A2 inhibitors may be associated with hypotensive reactions associated with metamizole.

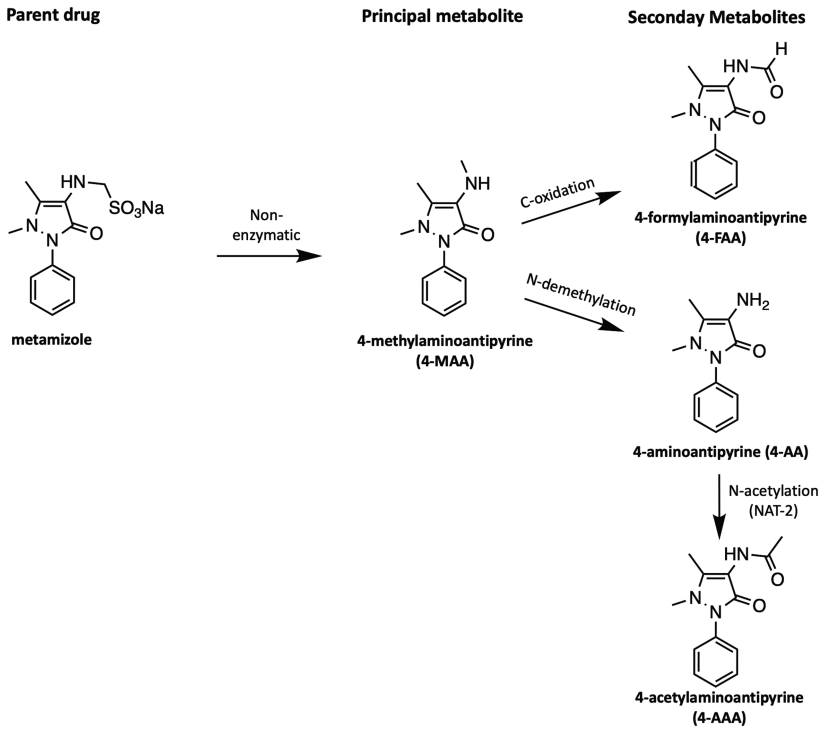

FIGURE 1 Metabolism of metamizole. Metamizole is presystemically cleaved to 4-methyl-aminoantipyrine (4-MAA). 4-MAA is the principal metabolite in plasma and can be converted by CYP-dependent reactions to 4-aminoantipyrine (4-AA) or 4-formylaminoantipyrine (4-FAA). 4-AA can be acetylated by N-acetyltransferase 2 (NAT2) to 4-acetyl-aminoantipyrine (4-AAA). The CYPs involved in the metabolism of 4-MAA have not clearly been identified

2 | METHODS

2.1 | Chemicals and reagents

Dimethylsulfoxide, chlorzoxazone, (+)-N-3-benzylnirvanol, ketoconazole, 4-methylpyrazole hydrochloride, quinidine sulphate, sulfaphenazole, ticlopidine hydrochloride, montelukast, 4-MAA hydrochloride, 4-AA, 4-AAA and 4-FAA were obtained from Sigma-Aldrich (Buchs, Switzerland). Tizanidine hydrochloride, (S)-efavirenz, flurbiprofen, omeprazole, metoprolol, paclitaxel, 6′-hydroxy-chlorzoxazone, 8′-hydroxyefavirenz, 4′-hydroxyflurbiprofen, α-hydroxymetoprolol, 5′-hydroxyomeprazole, 6α-hydroxypaclitaxel, hydroxytizanidine, furafylline, ciprofloxacin hydrochloride, fluconazole, chlorzoxazone-d3, efavirenz-d5, flurbiprofen-d3, metoprolol-d6, midazolam-d6, omeprazole-d3, paclitaxel-d5, tizanidine-d4, 4-MAA-d3, 4-AA-d3, 4-AAA-d3, ciprofloxacin hydrochloride-d8 and fluconazole-d4 were purchased from Toronto Research Chemicals (Toronto, Canada). Midazolam was provided by Roche (Basel, Switzerland) and α-hydroxy-midazolam was acquired from Lipomed (Arlesheim, Switzerland). Corning rhCYP1A2 Supersomes (1 nmol/mL, Lot: 9095001), Corning rhCYP2B6 Supersomes (2 nmol/mL; Lot: 9268001), Corning rhCYP2C8 Supersomes (2 nmol/mL, Lot: 7278001), Corning rhCYP2C9*1 (Arg144) Supersomes (2 nmol/mL, Lot: 9277002), Corning rhCYP2C19 Supersomes (2 nmol/mL, Lot: 9275001), Corning rhCYP2D6*1 Supersomes (1 nmol/mL, Lot: 9274002), Corning rhCYP2E1 Supersomes (2 nmol/mL, lot: 9290001), Corning rhCYP3A4 Supersomes (2 nmol/mL, Lot: 1006003), NADPH regeneration solution A (26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM MgCl 2 in H_2O, Lot: 9288003) and B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, Lot: 8024003) were obtained from Corning Life Sciences B.V. (Amsterdam, The Netherlands). High-performance liquid chromatography (HPLC)-grade methanol, HPLC-grade water and formic acid were purchased from Merck (Darmstadt, Germany).

2.2 | In vitro assays

4-MAA (50 μM) or control substrates (CYP1A2: 10 μM tizanidine, CYP2B6: 1 μM efavirenz, CYP2C8: 10 μM paclitaxel, CYP2C9: 1 μM flurbiprofen, CYP2C19: 1 μM omeprazole, CYP2D6: 10 μM metoprolol, CYP2E1: 1 μM chlorzoxazone, CYP3A4: 1 μM midazolam) were pre-incubated for 15 minutes in 100 mM phosphate buffer containing 1.5% BSA, NADPH-regenerating solution A and B (1:20 dilution and 1:100 dilution, respectively) in the presence or absence of specific CYP inhibitors (CYP1A2: 10 μM furaphylline, CYP2B6: 1 μM ticlopidine, 2C8: 20 μM montelukast, CYP2C9: 10 μM sulfaphenazole, CYP2C19: 10 μM (1)-N-3-benzylnirvanol, CYP2D6: 1 μM quinidine, CYP2E1: 20 μM methylpyrazole, CYP3A4: 1 μM ketoconazole). The final volume was 500 μL. The reaction was started by the addition of recombinant supersomes (20 pmol/mL final concentration) and the mixture was incubated on a Thermomixer 5436 (Eppendorf AG, Hamburg, Germany) at 37°C and 600 revolutions/min. After 15 minutes, 30 minutes, 1 hour and 2 hours, a sample (CYP substrates: 50 μL, 4-MAA: 20 μL) was removed and transferred into an autosampler tube containing ice-cold methanol spiked with internal standards (CYP substrates: 150 μL methanol containing 25 ng/mL chlorzoxazone-d3, 50 ng/mL efavirenz-d5, 50 ng/mL flurbiprofen-d3, 5 ng/mL metoprolol-d6, 10 ng/mL midazolam-d6, 10 ng/mL omeprazole-d3, 200 ng/mL paclitaxel-d5 and 10 ng/mL tizanidine-d4; 4-MAA: 400 μL methanol containing 20 ng/mL 4-MAA-

d3, 30 ng/mL 4-AA-d3 and 60 ng/mL 4-AAA-d3). The tubes were vigorously shaken for 30 seconds and stored at −20°C until analysis.

2.3 | Clinical study

We conducted a single centre, phase I study (clinicaltrials.gov, ID: NCT04621253) in 2 successive periods in male Caucasian subjects. The study was approved by the ethics committee EKNZ (EthikkommissionNordwestschweiz/Zentalschweiz) and Swissmedic and conducted in accordance with good clinical practice guidelines and the current version of the Declaration of Helsinki. Due to possible interactions between metamizole and ovulation inhibitors,[30] we recruited only male subjects for the clinical study. The participants were screened for any underlying diseases (physical examination, routine laboratory and electrocardiogram). The use of known CYP inducers (e.g., St. John's Wort) or inhibitors (e.g., grapefruit juice) within 2 weeks before study start was an exclusion criterion as well as excessive caffeine consumption (>8 cups/d), smoking (> 5 cigarettes/d) and use of over-the-counter medication. After signing the informed consent, 12 male subjects were included (mean age: 28.3 y, range 22–39 y, mean body weight: 79.0 kg, range 63–117 kg, mean body mass index: 24.7 kg/m^2, range 21.2–37.3 kg/m^2) into the study. A venous blood sample was drawn to determine routine laboratory parameters and the subjects' CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP2D6 genotype. The first phase of the study was designed as a randomized, double-blind, 3-arm crossover study. The arms were placebo, ciprofloxacin or fluconazole treatment in a random order. Prior to the study day, the subjects were treated for 3 days with either placebo, ciprofloxacin (750 mg twice daily for 3 days) or fluconazole (400 mg loading dose on day −3, followed by 200 mg per day for day −2 and −1). Subjects arrived at the study facility in fasted state with at least 12 hours abstinence of caffeine. The last dose of inhibitor or placebo was taken on the study day (750 mg ciprofloxacin, 200 mg fluconazole, the combination of fluconazole/ciprofloxacin or placebo) 1 hour prior to arrival. After arrival in the study facility, a venous catheter was placed in the nondominant forearm and a blood sample was withdrawn from the catheter to determine the baseline concentrations of the CYP inhibitors. At the same time, participants were treated orally with 1000 mg metamizole (Novalgin tablets 500 mg, Sanofi) and 250 mL of water. After administration of metamizole, blood samples were drawn after 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12 and 24 hours into EDTA coated tubes. The blood samples were centrifuged at 1500 *g* for 10 minutes and the plasma was stored at −20°C until analysis. In the second, open period of the study, 6 randomly chosen participants out of the 12 of the first part of the study gave their consent to participate in the study continuation. These 6 participants were treated with the combination of ciprofloxacin and fluconazole with the same schedule as in the first part of the study. The treatment at the study day was the same as described for the first part of study.

To review compliance of the placebo/inhibitor treatment, pill-counting journals were handed out to the subjects. They had to be filled out and returned at the study day for review as well as the empty blisters.

2.4 | Study drugs

The placebo-controlled study medication was produced under good manufacturing practice conditions by Dr. Hysek Pharmacy, Biel, Switzerland. In short, Ciprofloxacin Helvepharm 750 mg tablets and Fluconazole Helvepharm 200 mg capsules were over-capsuled and placebo capsules were filled with mannitol. The capsules could not be distinguished. To minimize the risk of potential influence of the treatment order, participants were randomly assigned to 1 of 6 treatment sequences (A-B-C, A-C-B, B-A-C, B-C-A, C-A-B, C-B-A) by computed randomization. The study staff did not have access to the randomization schedule until the final analysis of the plasma samples.

Novalgin (500 mg metamizole sodium), Ciprofloxacin Helvepharm (750 mg ciprofloxacin) and Fluconazole Helvepharm (200 mg fluconazole) were purchased through the University Hospital Pharmacy Basel, Switzerland.

2.5 | Genotyping

The genotypes of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP2D6 were assessed as published previously.[31]

2.6 | Bioanalysis

All analytes were quantified with HPLC (Shimadzu, Kyoto, Japan) tandem mass spectrometry (MS; ABSciex, Ontario, Canada). The substrates and the corresponding metabolites (tizanidine, efavirenz, paclitaxel flurbiprofen, omeprazole, metoprolol, chlorzoxazone, midazolam, hydroxytizanidine, 8′-hydroxyefavirenz, 6α-hydroxypaclitaxel, 4′-hydroxyflurbiprofen, 5′-hydroxyomeprazole, α-hydroxymetoprolol, 6′-hydroxychlorzoxazone and 1′-hydroxymidazolam) were analysed according to an earlier publication.[32] The main metabolites of metamizole (4-MAA, 4-AA, 4-FAA, 4-AAA) were quantified with a fully validated method.[33] Quantification of ciprofloxacin and fluconazole was performed by HPLC-MS/MS set in positive mode. The following mass transitions were used: ciprofloxacin: m/z 332.3/314.2, ciprofloxacin-d8: m/z 340.3/322.1, fluconazole: m/z 307.1/238.0, fluconazole-d4: m/z 311.2/242.1. As mobile phases, water (mobile phase A and C) and methanol (mobile phase B), both supplemented with 0.1% formic acid, were applied. Mobile phase C was added prior to the analytical column using a T-union. The analytes were separated using a core-shell C18 column (Kinetex C18, 2.6 μM, 50 mm × 2.1 mm, Phenomenex, CA, USA). The following gradient was used (percentage of mobile phase B): 0–0.5 minutes: 5%, 0.51–1.6 minutes: 5%–60%, 1.61–2.0 minutes: 60%–95%, 2.01–2.5 minutes: 95%, 2.51–3.5 minutes: 5%. The initial flow rate for pump A and B was 0.1 mL/min. Mobile phase C was added at an initial

rate of 0.5 mL/min from the start of the run, decreasing to 0 after 0.5 minutes.

The analysis of the samples met the criteria defined by the US Food and Drug Administration (FDA) guidelines for bioanalytical analysis of study samples.[34] The calibration range was linear from 25 to 25 000 ng/mL for 4-MAA, 25 to 10 000 ng/mL for 4-AA, 4-AAA and 4-FAA, and 20 to 10 000 ng/mL for fluconazole and ciprofloxacin. Intra- and interday accuracy was 85.2–114.7% with an imprecision of <13.6% for all analytes measured.

2.7 | Pharmacokinetic analysis

The pharmacokinetic endpoints for the clinical study were the change in the area under the curve (AUC) of 4-MAA, 4-AA, 4-FAA and 4-AAA and of the terminal half-life ($t_{1/2}$) of 4-MAA under the different conditions (placebo, ciprofloxacin, fluconazole, ciprofloxacin+fluconazole). These parameters were determined using noncompartmental methods with PKanalix (version 2019R1, Lixoft SAS, Abtony, France). $AUC_{6h,\ 8h,\ 12h,\ 24h}$ was assessed using the linear log trapezoidal method and $t_{1/2}$ was calculated from the elimination rate constant, which was determined using linear regression of log concentrations and time.

To describe the influence of the inhibitors on the metabolism of metamizole, metabolic ratios were calculated. The impact on the formylation was described as $\frac{AUC_{4-FAA}}{AUC_{4-MAA}}$, the demethylation as $\frac{AUC_{4-AA}+AUC_{4-AAA}}{AUC_{4-MAA}}$, the acetylation as $\frac{AUC_{4-AAA}}{AUC_{4-AA}}$ and the impact on the total metabolism of 4-MAA as $\frac{AUC_{4-AA}+AUC_{4-AAA}+AUC_{4-FAA}}{AUC_{4-MAA}}$.

Both MR and AUCs were assessed at different time points due to the decreasing exposure of the participants to the inhibitors.

2.8 | Statistics

We estimated the number of subjects to be included using an α of .05, a power $(1-\beta)$ of 0.9, a difference between the groups of 30% and a relative standard deviation of the pharmacokinetic determinations of 25%. We compared the means using 1-way repeated measures analysis of variance followed by the Holm–Sidak test for paired samples in case of a significant result. Graphpad Prism (Graphpad Software, La Jolla, California) was used for the statistical analysis. A P value of <.05 was considered as statistically significant (*P < .05, **P < .01, ***P < .001).

3 | RESULTS

3.1 | In vitro metabolism of 4-MAA

To identify the CYPs responsible for the metabolism of 4-MAA, the principal metabolite of metamizole, we started with investigating the metabolism of 4-MAA in vitro using supersomes. To ensure the functionality of the supersomes used, we first studied the metabolism of the specific CYP substrates in the absence or presence of the corresponding inhibitors. The recombinant CYPs investigated were functional as evidenced by the metabolism of the specific substrates and the formation of the respective metabolites, and the reactions could be blocked or impaired by the addition of the respective inhibitors (Figure S1). The incubation of 4-MAA with the same supersomes revealed that CYP1A2, CYP2C19 and CYP2D6 formed 4-AA most efficiently (Figure 2). In comparison, CYP2B6, CYP2C9 and CYP2C8 had a measurable but minor 4-MAA demethylation activity, while CYP2E1 and CYP3A4 exhibited no detectable activity. Similar to the experiments with specific substrates, the formation of 4-AA could be prevented or slowed by the addition of a specific inhibitor. In addition to 4-MAA N-demethylation, the assessment of the formation of 4-FAA showed that the only CYP capable of producing 4-FAA was CYP1A2 (data not shown). To the best of our knowledge, no CYP has so far been identified that catalyses the formation of 4-FAA from 4-MAA.

3.2 | Compliance

Careful review of the pill-counting diaries and control of the empty drug blisters indicated that the participants were compliant to the treatment. As displayed in Figure S2, all participants had residual ciprofloxacin and/or fluconazole plasma concentrations in the morning of the respective study days, also indicating that they were compliant. Surprisingly, when both inhibitors were administered at the same time, the fluconazole plasma concentrations were higher compared to treatment with fluconazole alone, whereas the ciprofloxacin plasma concentrations were in the same range under both conditions.

3.3 | Effect of ciprofloxacin and fluconazole on the plasma concentrations of 4-MAA, 4-AA, 4-AAA and 4-FAA

Treatment with ciprofloxacin increased the plasma concentrations of 4-MAA (Figure 3) and slowed its elimination (Table 1 and Figure S3), confirming that 4-MAA is metabolized by CYP1A2. Accordingly, the $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$ increased by 51 and 66%, respectively (Table S1). The addition of fluconazole further slowed the elimination of 4-MAA and increased the $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$ by 92 and 133%, respectively, compared to placebo. As expected, the formation of 4-AA, 4-FAA and 4-AAA was slowed and decreased by the administration of ciprofloxacin (Figure 3). The addition of fluconazole further slowed and decreased the formation of 4-FAA and 4-AAA, but not of 4-AA.

Similar to ciprofloxacin, also the treatment with fluconazole increased the plasma concentrations of 4-MAA (Figure 3) and slowed its elimination (Table 1 and Figure S3). The increase in the $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$ was 17 and 24%, respectively, approximately 5 times less than the corresponding increase by ciprofloxacin. The addition of ciprofloxacin further slowed the elimination of 4-MAA and increased the $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$ of 4-MAA as described for ciprofloxacin.

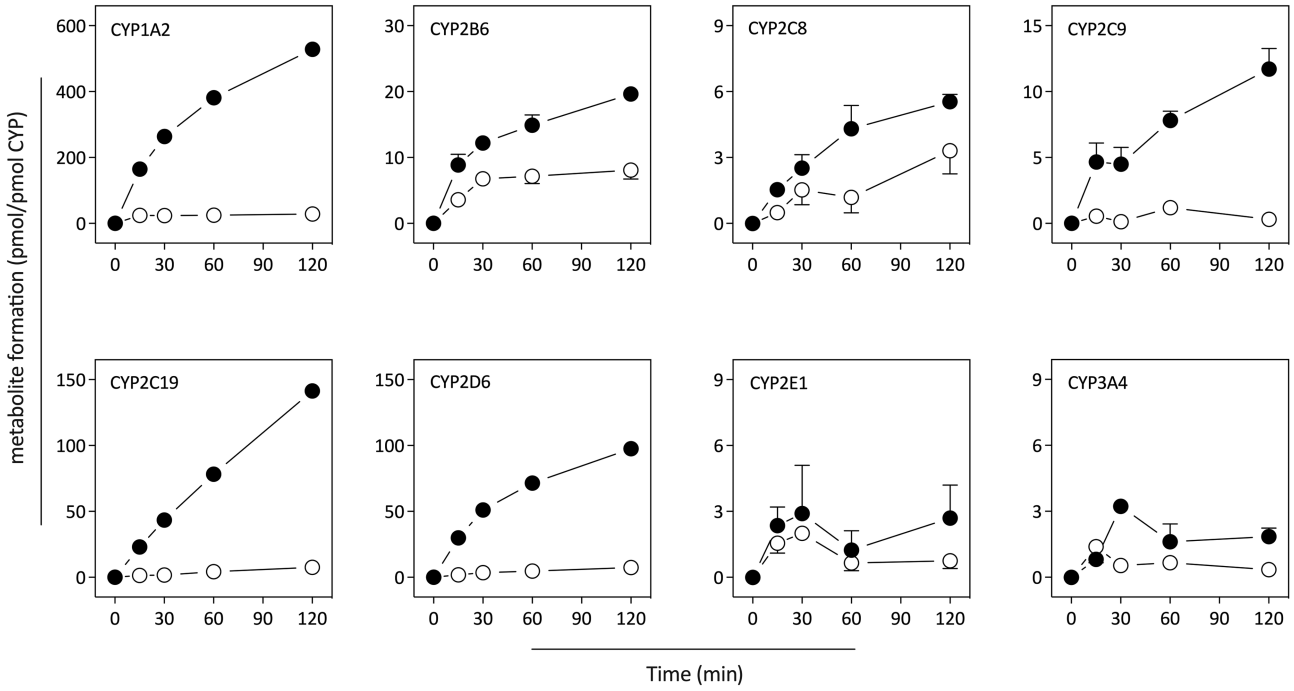

FIGURE 2 In vitro metabolism of 4-methyl-aminoantipyrine (4-MAA). The N-demethylation of 4-MAA to 4-aminoantipyrine (4-AA) was investigated in vitro. The reactions were performed in the absence (closed circles) and presence of the specific inhibitors furaphylline (CYP1A2), ticlopidine (CYP2B6), montelukast (CYP2C8), sulfaphenazole (CYP2C9),(1)-N-3-benzylnirvanol (CYP2C19), quinidine (CYP2D6), methylpyrazole (CYP2E1) and ketoconazole (CYP3A4) (open circles). The results are displayed mean ± standard error of the mean of 6 independent measurements

Fluconazole retarded and decreased the formation of 4-AA, 4-FAA and 4-AAA slightly with effects on the AUC only up to 8 hours after ingestion of metamizole. The effect of ciprofloxacin, fluconazole and the combination ciprofloxacin/fluconazole on the $AUC_{0\text{-}12h}$ of the 4 metamizole metabolites is shown in Figure S4. The figure shows that the inhibition of the metabolism of 4-MAA is much stronger for ciprofloxacin compared to fluconazole and that the reduction in the $AUC_{0\text{-}12h}$ by ciprofloxacin or fluconazole is more accentuated for 4-FAA than for 4-AA and 4-AAA.

3.4 | Quantification of the effect of fluconazole and ciprofloxacin on the metabolism of 4-MAA

As shown in Figure 1, the metabolism of 4-MAA is complex. 4-MAA can be converted to 4-AA and 4-FAA, and 4-AA can be metabolized further to 4-AAA. Figure 4 shows the effect of ciprofloxacin, fluconazole and the combination ciprofloxacin/fluconazole on the plasma concentration of 4-MAA, 4-AA, 4-AAA and 4-FAA at different time points after ingestion of metamizole compared to placebo. The effect of fluconazole and ciprofloxacin on the increase in the plasma concentration of 4-MAA grows with time, reaching approximately 300% for ciprofloxacin/fluconazole at 12 hours. In contrast to 4-MAA, the reduction in the plasma concentration of 4-AA, 4-AAA and 4-FAA by fluconazole and ciprofloxacin decreases with time. At 12 hours after the administration of metamizole, the effect fluconazole, ciprofloxacin and their combination on the plasma concentration 4-AAA and 4-FAA is minor or completely absent. This may be due to accumulation of 4-MAA, which is the substrate for the formation of these metabolites. The figure also demonstrates that the effects of fluconazole and ciprofloxacin on the increase in the plasma concentration of 4-MAA are additive.

An additional possibility to express the inhibition of the metabolism of 4-MAA by ciprofloxacin and fluconazole is by calculating the metabolic ratio, which also considers the increase in the AUC of the substrate (in this case 4-MAA) and not only the decrease in the formation of the metabolites (Table S2). The reduction in the MR was strongest at 6 hours and decreased with time, similar to the effect on the AUC of 4-MAA. The reduction in the MR was more accentuated for ciprofloxacin than fluconazole and strongest for the combination ciprofloxacin/fluconazole. The strongest reduction was observed for the couple 4-FAA/4-MAA at 6 hours, reaching 63% for ciprofloxacin, 24% for fluconazole and 79% for the combination ciprofloxacin/fluconazole. At 12 hours, the corresponding values were 55, 22 and 75% for ciprofloxacin, fluconazole and ciprofloxacin/fluconazole, respectively. In comparison, the effect on the couple 4-AAA/4-AA was much weaker, reaching statistical significance only for ciprofloxacin. This reflects the fact that the formation of 4-AAA from 4-AA is dependent on NAT2 and not on CYPs.[35]

3.5 | Effect of the genotype on 4-MAA metabolism

The participants were genotyped for CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP2D6 (Table S3). In Figure 5, the (4-AA+4-AAA

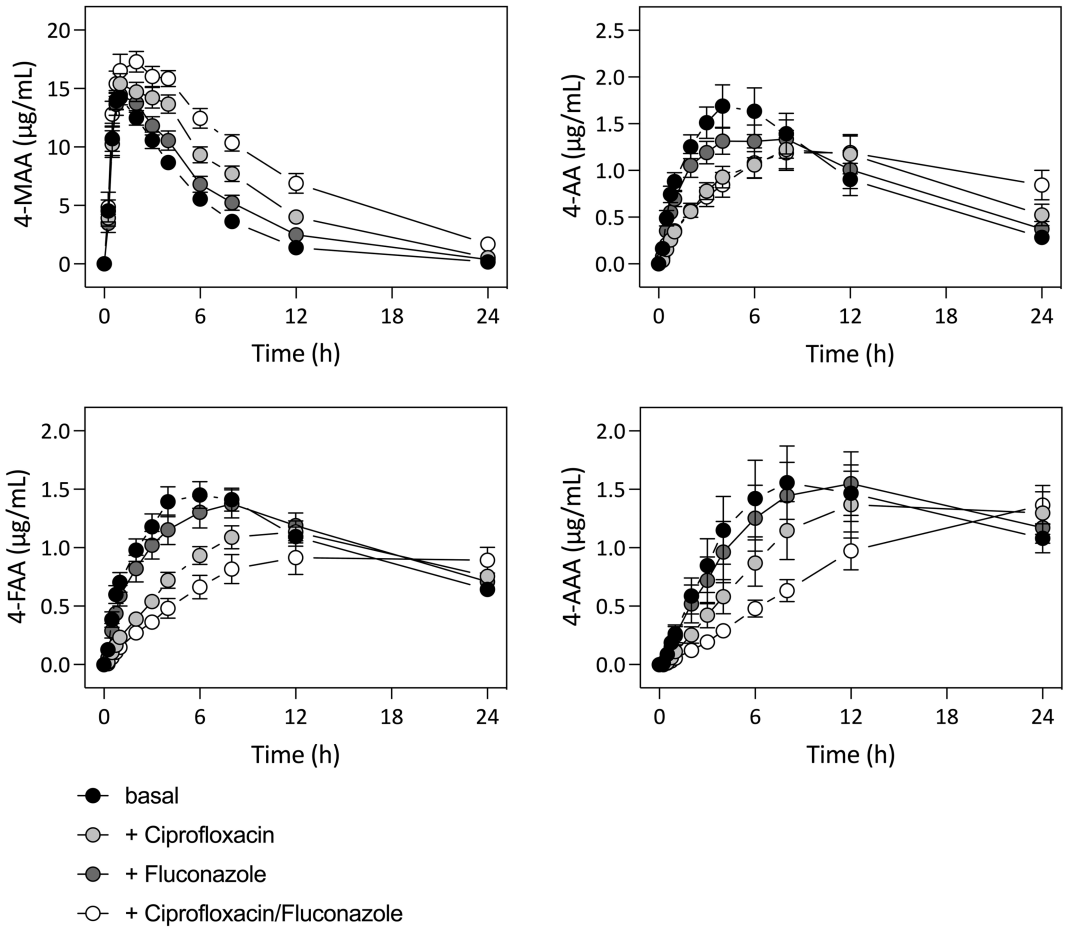

FIGURE 3 Effect of fluconazole, ciprofloxacin and the combination ciprofloxacin/fluconazole on the plasma concentrations of 4-methyl-aminoantipyrine (4-MAA), 4-aminoantyprine (4-AA), 4-formylaminoantipyrine (4-FAA) and 4-acetyl-aminoantipyrine (4-AAA). Participants were pretreated with placebo ($n = 12$), ciprofloxacin ($n = 12$; 750 mg twice daily for 3 days), fluconazole ($n = 12$, 400-mg loading dose on day −3, followed by 200 mg for day −2 and −1) or the combination ciprofloxacin/fluconazole ($n = 6$; ciprofloxacin and fluconazole as above) before oral administration of 1 g metamizole. The last dose of the inhibitors was administered in the morning of the study day. 4-MAA, 4-AA, 4-FAA and 4-AAA plasma concentrations were determined by LC–MS/MS. The results are displayed as mean ± standard error of the mean

+ 4-FAA)/4-MAA MR at 12 hours of the placebo arm is plotted according to the respective genotype of the participants. For CYP2B6 and CYP2D6, there was no visible association between genotype and MR. However, for CYP1A2, 2C9 and 2C19, the MR, which reflects the metabolic activity, correlated with the expected enzymatic activity of the respective genotype.

4 | DISCUSSION

The aim of the current study was to find out which CYPs are involved in the metabolism of 4-MAA and whether inhibition of the CYPs involved leads to clinically relevant drug interactions. The in vitro experiments with supersomes revealed that CYP1A2 is the most important enzyme for 4-MAA metabolism and that CYP2C19 and CYP2D6 contribute to 4-MAA N-demethylation. Our in vivo data confirmed these findings and showed that the effects of ciprofloxacin and fluconazole regarding inhibition of the formation of 4-AA and 4-FAA are additive.

Both our in vitro and in vivo experiments showed that CYP1A2 is the dominant enzyme for the demethylation of 4-MAA to 4-AA but also for the conversion of 4-MAA to 4-FAA. So far, it has been demonstrated that both reactions are catalysed by hepatic microsomes[27–29] but the CYPs involved had not been clearly identified and verified in a clinical study. The results of the current study are in agreement with those in a recent study where we investigated the effect of metamizole on the activity of different CYPs.[31] In this study, we found that metamizole inhibits the conversion of caffeine to paraxanthine, which is catalysed by CYP1A2, probably in a competitive fashion. The results of the current study support this interpretation. However, the findings in the current study disagree with the reports of Geisslinger *et al.*[28] and of Bachmann *et al.*,[29] which were both performed with human microsomes as the enzyme source. Geisslinger *et al.* showed that the demethylation of 4-MAA to 4-AA

TABLE 1 Pharmacokinetic parameters of the main metabolites of metamizole. Participants were pretreated with placebo, fluconazole, ciprofloxacin and the combination fluconazole/ciprofloxacin before assessing the metabolism of metamizole. Values are given as the geometric mean with the 95% confidence interval in parentheses. The elimination half-lives of 4-aminoantipyrine, 4-formylaminoantipyrine and 4-acetylaminoantipyrine could not be calculated due to limited sampling

	Placebo ($n = 12$)	Fluconazole ($n = 12$)	Ciprofloxacin ($n = 12$)	Fluconazole/ ciprofloxacin ($n = 6$)
4-methyl-aminoantipyrine				
AUC_{12h} (μg/mL h)	75.0 (65.2–86.4)	88.1** (72.9–106)	113*** (98.9–130)	144* (125–167)
C_{max} (μg/mL)	15.2 (13.8–16.7)	15.4 (13.7–17.2)	16.4* (14.8–18.2)	17.7 (15.2–20.6)
$T_{1/2}$ (h)	3.22 (2.84–3.64)	3.69* (3.03–4.49)	3.91*** (3.43–4.45)	6.07* (5.37–6.86)
4-aminoantipyrine				
AUC_{12h} (μg/mL h)	13.7 (9.73–19.2)	12.1* (8.73–16.8)	9.94*** (7.15–13.8)	10.4* (6.72–15.9)
C_{max} (μg/mL)	1.62 (1.21–2.17)	1.38* (1.04–1.83)	1.16*** (0.814–1.64)	1.18* (0.737–1.88)
4-formylaminoantipyrine				
AUC_{12h} (μg/mL h)	13.7 (11.3–16.7)	12.6 (10.1–15.6)	9.23*** (7.55–11.3)	6.68* (4.48–9.98)
C_{max} (μg/mL)	1.47 (1.22–1.77)	1.35 (1.11–1.65)	1.13*** (0.946–1.36)	1.03 (0.831–1.28)
4-acetylaminoantipyrine				
AUC_{12h} (μg/mL h)	11.4 (7.74–16.8)	10.5 (7.00–15.6)	7.66*** (5.03–11.7)	5.30* (3.63–7.74)
C_{max} (μg/mL)	1.41 (0.989–2.02)	1.41 (1.01–1.97)	1.36 (0.968–1.90)	1.32 (1.00–1.74)

AUC_{12h}, area under the concentration–time curve up to 12 hours; C_{max}; maximum plasma concentration; $T_{1/2}$, elimination half-life
***$P < .001$, **$P < .01$, *$P < .05$ *vs*. placebo

by human hepatic microsomes could partially be inhibited by ketoconazole, suggesting a major contribution of CYP3A4.[28] Geisslinger *et al*. used a ketoconazole concentration of 10 μM in their study, a concentration far below the K_i of ketoconazole for CYP1A2 and 2C19,[36] excluding inhibition of CYP1A2 and CYPC19 as an explanation for their findings. Bachmann *et al*. investigated the conversion of 4-MAA to 4-AA by human liver microsomes using specific CYP inhibitors and identified CYP2B6, 2C8, 2C9 and 3A4 as the most important contributors to 4-MAA demethylation.[29] The discrepancies between the current study and the studies of Geisslinger *et al*. and Bachmann *et al*. may be due to the different enzyme sources used, human recombinant CYPs expressed in supersomes versus human liver microsomes. The discrepancy is relevant, indicating that results obtained in in vitro studies should be confirmed in another in vitro system or, preferably, by a clinical study. The fact that we obtained almost identical results for the in vitro and in vivo investigations in the current study supports the notion that CYP1A2 is the most important CYP for 4-MAA N-demethylation and conversion to 4-FAA.

The FDA defines strong, moderate and weak enzyme inhibitors as drugs that increase the AUC of a sensitive substrate by ≥5-fold, ≥2 to <5-fold and ≥1.25 to <2-fold, respectively.[37] The FDA lists ciprofloxacin as a strong inhibitor of CYP1A2 and fluconazole as a strong inhibitor of CYP2C19 and as a moderate inhibitor of CYP2C9 and CYP3A4. In the current study, ciprofloxacin increased the AUC of 4-MAA time-dependently by a factor of 1.31, 1.56 and 1.51 and fluconazole by a factor of 1.10, 1.32 and 1.17 at 6, 8 and 12 hours, respectively (Table S1). According to the FDA, the inhibition of 4-MAA metabolism by ciprofloxacin and fluconazole was therefore weak. The reason for an only weak inhibition in the presence of strong inhibitors could be the existence of alternative metabolic pathways. Therefore, we also assessed the combined application of ciprofloxacin and fluconazole, which increased the effect on the AUC of 4-MAA, resulting in AUC ratios of 1.54, 1.90 and 1.92 at 6, 8 and 12 hours, respectively. The combined application of ciprofloxacin and fluconazole showed that the effects of ciprofloxacin and fluconazole on the AUCs of the metamizole metabolites are additive, excluding a mutual compensation between CYP1A2 and CYP2C19. However, Volz and Kellner have investigated the pharmacokinetics of orally administered ^{14}C-labelled metamizole in male subjects.[17] They detected 7 metabolites in serum and could identify 4 of them as 4-MAA, 4-AA, 4-FAA and 4-AAA. Forty-eight hours after administration, 90% of the radioactivity had been excreted renally, but the 4 metabolites accounted only for approximately 60% of the excreted radioactivity. The study therefore indicated the existence of additional metabolic pathways that may compensate for the inhibition of CYP1A2 and CYP2C19.

Based on the results in the supersomes, compensation by CYP2D6 is an obvious possibility, which we did not study in our clinical trial. However, the activity of CYP2D6 in supersomes was not higher than for CYP2C19, and, in contrast to CYP1A2 and CYP2C19, the CYP2D6 genotype showed no correlation with the metabolic activity, rendering a major contribution of CYP2D6 to the metabolism of 4-MAA unlikely.

An additional possibility is the contribution of myeloperoxidase in granulocytes, as proposed in the report of Bachmann *et al*.[29] The results of the current study do not exclude this possibility but suggest that the contribution of this pathway would probably be less than the contribution by the described hepatic metabolism. If the extrahepatic metabolism were dominant, inhibition of the (in that case minor) hepatic pathway would not be expected to increase the AUC of 4-MAA and to decrease the formation of 4-AA. The inhibition of the (minor) hepatic pathway should be compensated by the dominant extrahepatic pathway. In the current study, pretreatment with

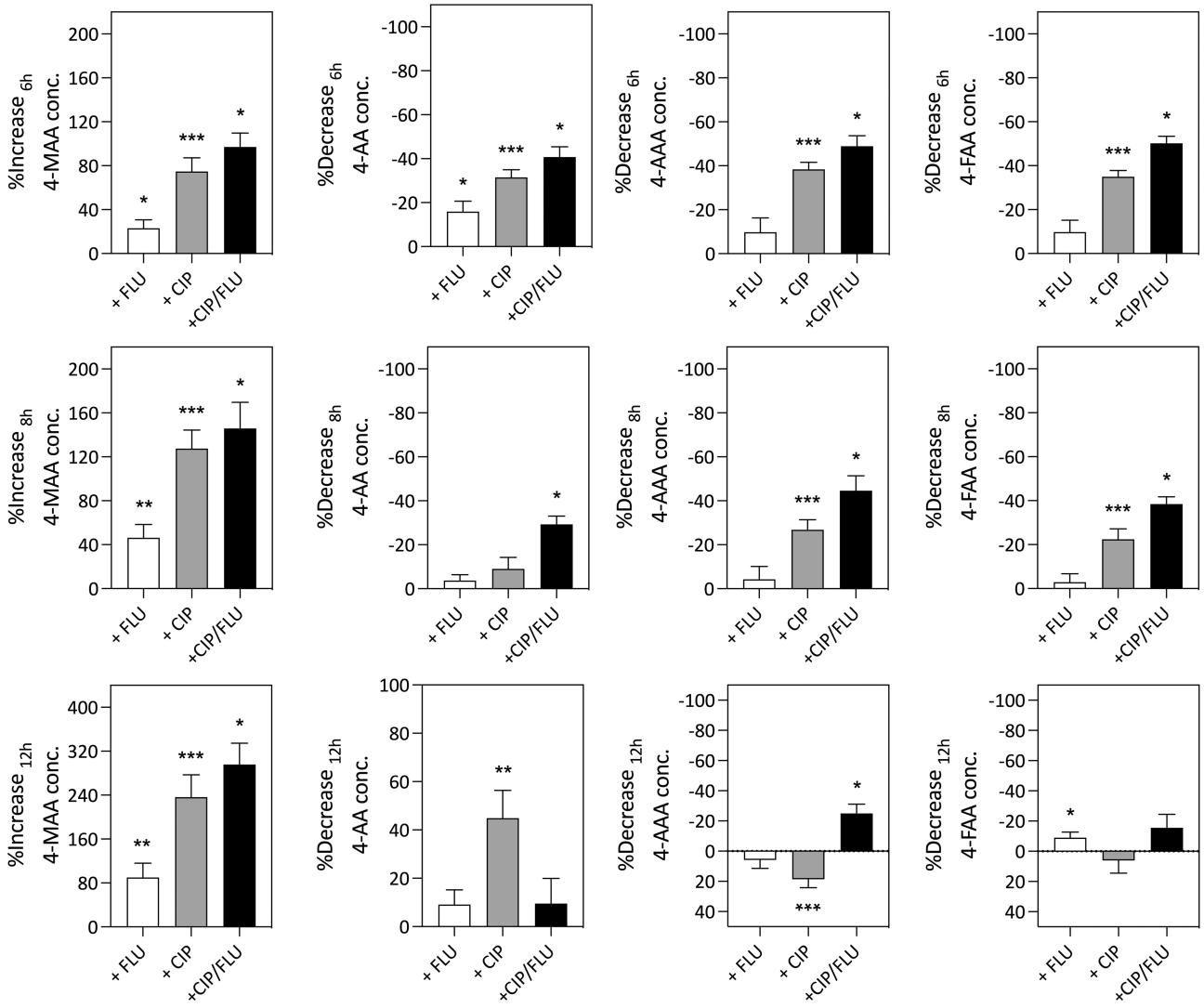

FIGURE 4 Effect of fluconazole (FLU), ciprofloxacin (CIP) and the combination fluconazole/ciprofloxacin (CIP/FLU) on the plasma concentration of the metamizole metabolites at 6, 8 and 12 h after ingestion of metamizole. The graph displays the increase (4-MAA) or decrease (4-AA, 4-AAA, 4-FAA) of the plasma concentration as a percentage compared to placebo. $n = 12$ participants for placebo, fluconazole and ciprofloxacin and $n = 6$ for the combination fluconazole/ciprofloxacin. Results are displayed as mean ± standard error of the mean. $^{***}P < .001$, $^{**}P < .01$ and $^{*}P < .05$ *vs.* placebo

ciprofloxacin/fluconazole increased the $AUC_{0\text{-}12h}$ of 4-MAA by 92% and decreased the $AUC_{0\text{-}12h}$ of 4-AA by 24%, rendering the existence of a dominant extrahepatic pathway unlikely.

Even if the interaction with CYP1A2 and CYP2C19 inhibitors is quantitatively small, this does not mean that this interaction is clinically negligible. Important adverse reactions of metamizole are hypotensive events, skin eruptions, myelotoxicity possibly leading to agranulocytosis and hepatic injury.[38] While hepatic injuries and skin toxicities are mainly dose-independent, immunological reactions,[38,39] hypotensive reactions are dose-dependent. Regarding myelotoxicity, a recent genome-wide association study failed to reveal an HLA association and suggested that impaired antioxidative defence mechanisms in granulocyte precursors could be a risk factor for neutropenia and agranulocytosis.[14] In vitro studies using HL60 cells support the existence of toxicological mechanisms due to the formation of reactive metabolites from MAA under certain conditions.[40,41] An increase in the plasma concentration of 4-MAA by drug interactions and/or enzyme polymorphisms could therefore enhance the risk for hypotensive events and could aggravate myelotoxicity in patients at risk.

The current study has some limitations. Since we did not recruit females and could recruit only Caucasians, the results are valid only for male Caucasians. In addition, we obtained blood samples only for 24 hours after dosing metamizole, which precluded the exact determination of the AUC and half-lives of the metabolites of MAA. However, the aim of the study was to investigate the effect of CYP inhibition on the metabolism of 4-MAA. As suggested from the half-lives provided in Table 1 and as shown in Figure 3A, 4-MAA was eliminated after 24 hours by >90% also in the presence of the inhibitors, enabling us

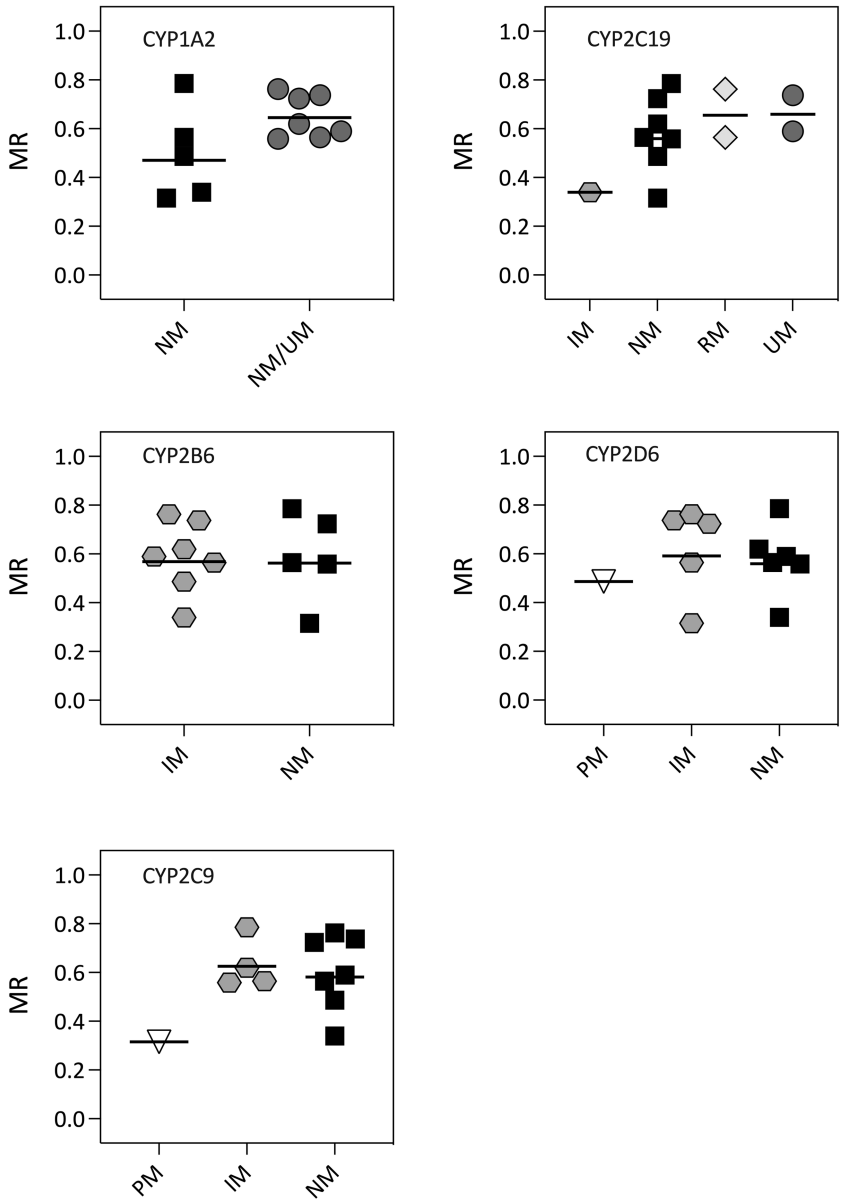

FIGURE 5 Effect of the cytochrome P450 (CYP) genotype on the metabolic ratio (MR) of 4-MAA. The MR was calculated as ($AUC_{12h\ 4\text{-}AA}$ + $AUC_{12h\ 4\text{-}AAA}$ + $AUC_{12h\ 4\text{-}FAA}$)/$AUC_{12h\ 4\text{-}MAA}$ and the results displayed according to the genotype of the different CYPs assessed. An increase in the MR reflects an increase in the activity of a specific CYP. Individual data are plotted. IM, intermediate metabolizer; NM, normal metabolizer; PM, poor metabolizer; RM, rapid metabolizer; UM, ultrarapid metabolizer

to obtain reliable pharmacokinetic data for 4-MAA and to reach the aims of the study. Finally, we failed to provide an additional dose of ciprofloxacin 12 hours after the morning dose at the study day, which may have compromised the inhibitory effect of ciprofloxacin over 24 hours. The dosing of the drugs used in the current study is complicated since the typical dose intervals are different (6–8 h for metamizole, 12 h for ciprofloxacin and 24 h for fluconazole). We therefore decided to concentrate on the 12-hour interval after dosing, during which CYP inhibition by ciprofloxacin and fluconazole is maintained (Figure 3) and which provides enough information regarding the inhibition of CYP1A2 and/or CYP2C19 on the metabolism of 4-MAA.

In conclusion, we provide evidence that CYP1A2 is the major CYP for the conversion of 4-MAA to 4-AA and 4-FAA. CYP1A2 inhibition increases the 4-MAA exposure by a factor of approximately 1.5, which could be relevant for dose-dependent adverse reactions.

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CONTRIBUTOR

FB helped to design the study, carried out the clinical part of the study, performed the analysis in the laboratory and the pharmacokinetic calculations and prepared the figures. HEMS performed the pharmacogenomic analysis. UD supervised the analytical part of the study and helped in data interpretation and figure preparation. SK helped in the study design, the clinical part of the study and in data interpretation and wrote the manuscript. All authors commented on the manuscript draft and read the final version of the manuscript.

COMPETING INTERESTS

S.K. gave talks in symposia sponsored by Sanofi. All other authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Fabio Bachmann https://orcid.org/0000-0003-2789-4377

Henriette E. Meyer zu Schwabedissen https://orcid.org/0000-0003-0458-4579

Urs Duthaler https://orcid.org/0000-0002-7811-3932

Stephan Krähenbühl https://orcid.org/0000-0001-8347-4145

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SUPPORTING INFORMATION

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