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Identification of the metabolites of ivermectin in humans

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Abstract

BACKGROUND AND PURPOSE Mass drug administration of ivermectin has been proposed as a possible malaria elimination tool. Ivermectin exhibits a mosquito-lethal effect well beyond its biological half-life, suggesting the presence of active slowly eliminated metabolites. EXPERIMENTAL APPROACH Human liver microsomes, primary human hepatocytes, and whole blood from healthy volunteers given oral ivermectin were used to identify ivermectin metabolites by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry. The molecular structures of metabolites were determined by mass spectrometry and verified by nuclear magnetic resonance. Pure cytochrome P450 enzyme isoforms were used to elucidate the metabolic pathways. KEY RESULTS Thirteen different metabolites (M1-M13) were identified after incubation of ivermectin with human liver microsomes. Three (M1, M3, and M6) were the dominant metabolites found in microsomes, hepatocytes, and blood from volunteers after oral ivermectin administration. The chemical structure defined by LC-MS/MS and NMR indicated that M1 is 3"-O-demethyl ivermectin, M3 is 4-hydroxymethyl ivermectin, and M6 is 3"-O-demethyl, 4-hydroxymethyl ivermectin. Metabolic pathway evaluations with characterized cytochrome P450 enzymes showed that M1 was produced by CYP3A4 and CYP3A5, and that M3 and M6 were produced by CYP3A4. CONCLUSIONS AND IMPLICATIONS Demethylated and hydroxylated ivermectin are the main human metabolites in vivo. Further study to characterize their pharmacokinetic properties and mosquito-lethal activity is now needed.

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Running title: Identification of ivermectin metabolites

Phornpimon Tipthara[1], Kevin C Kobylinski[2], Markus Godejohann[3], Borimas Hanboonkunupakarn[1,4], Alison Roth[5,6], John H Adams[5], Nicholas J. White[1,7], Podjanee Jittmala[1,8], Nicholas P.J. Day[1,7], Joel Tarning[1,7]

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Authorship contributions: P.T., K.C.K. and J.T. designed the research. P.T. performed MS experiments. P.T. analysed data and wrote the first draft of the manuscript. M.G. performed NMR experiments. P.J. and B.H. performed the clinical trial. A.R. and J.H.A. performed the hepatocytes experiments. All authors contributed to the organization of the study and the final draft of the manuscript.

Abbreviations

IVM, ivermectin; MDA, mass drug administration; HLM, human liver microsomes; PHH, primary human hepatocyte; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; CYP450, cytochrome P450; UHPLC QTOF MS, ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; NTDs, neglected tropical diseases; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation

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EXPERIMENTAL APPROACH

Human liver microsomes, primary human hepatocytes, and whole blood from healthy volunteers given oral ivermectin were used to identify ivermectin metabolites by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry. The molecular structures of metabolites were determined

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by mass spectrometry and verified by nuclear magnetic resonance. Pure cytochrome P450 enzyme isoforms were used to elucidate the metabolic pathways.

KEY RESULTS

Thirteen different metabolites (M1-M13) were identified after incubation of ivermectin with human liver microsomes. Three (M1, M3, and M6) were the dominant metabolites found in microsomes, hepatocytes, and blood from volunteers after oral ivermectin administration. The chemical structure defined by LC-MS/MS and NMR indicated that M1 is 3"-*O* -demethyl ivermectin, M3 is 4-hydroxymethyl ivermectin, and M6 is 3"-*O* -demethyl, 4-hydroxymethyl ivermectin. Metabolic pathway evaluations with characterized cytochrome P450 enzymes showed that M1 was produced by CYP3A4 and CYP3A5, and that M3 and M6 were produced by CYP3A4.

CONCLUSIONS AND IMPLICATIONS

Demethylated and hydroxylated ivermectin are the main human metabolites*in vivo* . Further study to characterize their pharmacokinetic properties and mosquito-lethal activity is now needed.

Keywords: Ivermectin, Metabolism, LC-MS/MS, Malaria

Main text:

Introduction

Ivermectin (IVM) is an antiparasitic and endectocidic drug used for decades in animal health and for treating onchocerciasis, lymphatic filariasis, scabies, and strongyloidiasis in humans (Gonzalez et al., 2012). IVM also has some antiviral activity including against SARS-CoV-2*in vitro* (Caly et al., 2020).

Malaria is a mosquito-borne disease transmitted by *Anopheles*mosquitoes during blood feeding. Numerous studies have reported the mosquito-lethal effect of ivermectin (Chaccour et al., 2013; Kobylinski et al., 2020; Smit et al., 2018) and the ability to inhibit sporogony of*Plasmodium* in the mosquito (Kobylinski et al., 2012; Kobylinski et al., 2017; Pinilla et al., 2018). Mass drug administration (MDA) of IVM has been suggested as a possible vector control tool to aid malaria elimination as it has been shown to reduce *Plasmodium*transmission by mosquitoes (Alout et al., 2014) and reduce transmission to humans (Foy et al., 2019). A recent clinical trial in Thailand showed that mosquito lethal effects persisted well beyond the detectable presence of the parent compound which suggests that IVM may have active metabolites that are more slowly eliminated than the parent compound (Kobylinski et al., 2020).

Ivermectin is a semisynthetic compound derived from avermectin (B_1 series), a natural fermentation product of the soil bacterium *Streptomyces avermilitis.* The regiospecific hydrogenation of the avermectin B_1 at the 22,23-double bond produces the 22,23-single bond derivative called 22,23-dihydroavermectin B_1 or ivermectin (William C. Campbell, 1989). Ivermectin is a mixture containing at least 90% of 22,23-dihydroavermectin B_{1a}(H_2B_{1a} or ivermectin B_{1a}or IVM-B_{1a}) and less than 10% of 22,23-dihydroavermectin B_{1b}(H_2B_{1b} or ivermectin B_{1b}or IVM-B_{1b}). Both show the same antiparasitic activity (W. C. Campbell, 1985). The chemical structures shown in **Figure 1** indicate the alkyl side chain difference between IVM-B_{1a} and IVM-B_{1b} at C25.

There are several studies of IVM metabolites produced in non-human vertebrates (Chiu et al., 1988; Chiu et al., 1986; Chiu et al., 1984; Chiu et al., 1987; Miwa et al., 1982). The major metabolite found in rats, cattle, and sheep is the 24-hydroxymethyl derivative (Chiu et al., 1988; Miwa et al., 1982) while only trace levels are found in pigs. The 3"-*O* -demethyl derivative is the major metabolite present in pigs (Chiu et al., 1984). A previous *in vitro* study using human liver microsomes found nine IVM metabolites, mostly hydroxylated and demethylated compounds including the two listed above (Zeng et al., 1998).

In this study, we aimed to identify the common metabolites of humans through *in vitro* and *in vivo* experiments. Pooled human liver microsomes and primary human hepatocytes were exposed to IVM and

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metabolite fractions were collected for ultra-high performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry (UHPLC-Q-TOF-MS/MS) analysis. Human whole blood, collected from healthy volunteers after a single oral dose of IVM (400 µg/kg), was used to identify metabolites produced *in vivo* . The structure of IVM metabolites were defined by LC-MS/MS and verified by NMR. The metabolic pathways that generated these metabolites were characterized by incubation of IVM with purified human cytochrome P450 (CYP) enzymes, followed by LC-MS/MS analysis.

Methods

Pooled human liver microsomes

Pooled human liver microsomes (containing 20 mg/mL of protein) were thawed on ice. Microsome reactions were performed in microcentrifuge tubes by adding 183 µL of 0.1 M potassium phosphate buffer (pH 7.4), 2 µL of 1.0 mM IVM (prepared in acetonitrile 80% (v/v)), and 5 µL of microsomes. The tube was vortexed briefly and incubated at 37°C for 5 min in a shaking water bath. The reaction was initiated by adding 10 µL of 20 mM NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) prepared in 100 mM potassium phosphate buffer pH 7.4. Total reaction volume per tube was 200 µL with the final concentration of 10 µM IVM and 1 mM NADPH. Each tube was vortexed briefly and a baseline sample (0 min control) was collected by aliquoting 100 µL of the metabolite fraction mixture described above to a separate tube with 100 µL of pre-chilled acetonitrile, which was kept on ice until centrifugation. Two separate negative control tubes were prepared, one without NADPH (negative co-factor control) but with an extra 10 µL of buffer and a second tube without ivermectin substrate (negative ivermectin control) with an extra 2 µL of buffer. All remaining reactions, including negative co-factor control, and negative ivermectin control, were incubated at 37°C for 60 min with gentle shaking. After 60 min of incubation, all tubes were removed from the water bath and cold acetonitrile was added immediately to make a final 1:1 (v/v) ratio. All tubes (0 and 60 min reactions, negative co-factor and negative IVM controls) were vortexed briefly again and centrifuged at 10,000$\times g$for 15 minutes at 4°C. The supernatant was collected and kept frozen at -80°C until LC-MS/MS analysis. The reactions described above were performed using IVM, pure IVM-B_{1a} and pure IVM-B_{1b} as substrates (biological triplicate incubations for each substrate).

For NMR analysis, sixty tubes were prepared each with 10 µL of 1.0 mM ivermectin, 915 µL of 100 mM potassium phosphate buffer (pH 7.4), 25 µL of microsomes, and 50 µL of 20 mM NADPH for a final volume of 1 mL. Reactions were stopped at 60 min with ice-cold acetonitrile and centrifuged immediately as describe above. The supernatant was collected, evaporated in speed vacuum, and kept frozen at -80°C until NMR analysis.

Primary human hepatocytes

Primary human hepatocytes were seeded on 384-well plates as described previously to stimulate re-acquisition of *in vivo* physiologic activity (10). At day 3 post seed, ivermectin (10 µM) was added to each well. There were no media changes and no subsequent addition of ivermectin. 40 µL of media was collected from five individual wells at 24 hours and pooled in a microcentrifuge tube (total volume 200 µL). 40 µL of media was mixed with cold acetonitrile (160 µL), vortexed for 10 min at ambient temperature, and centrifuged at 10,000$\times g$ for 15 minutes at 4°C (triplicate extractions). The supernatant was collected, evaporated in speed vacuum, and kept frozen at -80°C until LC-MS/MS analysis.

Healthy volunteer samples

Venous blood was collected from three healthy Thai volunteers given a single dose of IVM (400 µg/kg) (NCT02568098) (Kobylinski et al., 2020). The blood samples were collected in sodium heparin tubes at 24 hours post IVM ingestion and kept frozen at -80°C until LC-MS/MS analysis. For metabolite extraction, whole blood samples were thawed at ambient temperature, vortexed briefly, and centrifuged at 3000$\times g$ for 5 min at 20°C. 50 µL of plasma was transferred into a new microcentrifuge tube and mixed with 200 µL of cold acetonitrile. Tubes were vortexed for 10 minutes at ambient temperature and centrifuged at 1100$\times g$ for 5 min at 20°C. The supernatant was collected, evaporated in speed vacuum, and kept at -80°C freezer

until LC-MS/MS analysis.

cDNA-expressed cytochrome P450 enzyme

The metabolism of ivermectin was studied *in vitro* using cDNA-expressed human cytochrome P450 enzymes CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 (1,000 pmol/mL). In separate tubes for each CYP isoform, the following reagents were added: 166 µL of 0.1 M potassium phosphate buffer (pH 7.4), 2 µL of 1.0 mM ivermectin (prepared in acetonitrile 80% (vol/vol)), and 20 µL of CYP enzyme. A separate tube was prepared for the negative control (no CYP enzyme) by adding 20 µL of insect cell microsomes. A second negative control was prepared to evaluate the effect of reductase by adding 20 µL of human P450 oxidoreductase +b5. The tubes were vortexed briefly and incubated at 37°C for 5 min in a shaking water bath. The reactions were initiated by adding 12 µL of NADPH regenerating system solution (pre-mixed 10 µL of solution A and 2 µL of solution B). Each tube was vortexed briefly and a baseline sample (0 min control) was collected by aliquoting 100 µL of the incubation to a separate tube with 100 µL of pre-chilled acetonitrile, which was held on ice until centrifugation. All tubes (reactions and controls) were incubated at 37°C for 60 min with gentle shaking. After 60 min of incubation, all tubes were removed from the water bath and cold acetonitrile was added immediately at a 1:1 ratio (v/v). Tubes were vortexed briefly and centrifuged at 10,000×g for 15 minutes at 4°C. The clear supernatant was collected into new microcentrifuge tubes and kept frozen at -80°C until LC-MS/MS analysis. The incubations and reactions described above were performed in triplicate.

UHPLC-QTOF MS

The LC-MS/MS system used was an ultra-high performance liquid chromatography (Agilent 1260 Quaternary pump, Agilent 1260 High Performance autosampler, and Agilent 1290 Thermostatted Column Compartment SL, Agilent Technologies) coupled to a quadrupole time of flight mass spectrometer (Q-TOF-MS) (TripleTOF 5600^{+}, Sciex) with an electrospray ionization (ESI) using a DuoSpray ion source. Mobile phase system for UHPLC was water containing 10 mM ammonium acetate and 0.1% formic acid (mobile phase A) and acetonitrile:water at a 95:5 ratio (v/v) containing 10 mM ammonium acetate and 0.1% formic acid (mobile phase B). Human liver microsome extracts were transferred directly to a LC vial for injection. Evaporated supernatant from primary human hepatocytes and whole blood samples were reconstituted in 20 and 50 µL, respectively, of mobile phase at starting gradient (mobile phase A:mobile phase B at ratio 60:40 (v/v)) and transferred to a LC vial for injection. LC vials were kept in the auto-sampler at 6°C during analysis. 5 µL of sample was injected on to a C18 reversed phase column (Acquity UPLC HSS T3, 2.1×100mm, 1.8 µM, Waters), protected by a pre-column (Acquity UPLC HSS T3, 2.1×5mm, 1.8 µM, Waters), for separation by UHPLC at a flow rate of 0.3 mL/min at 40°C. The UHPLC elution gradient was started at 40% mobile phase B for 2 min (0-2.0 min), 40-80%B for 2 min (2.0-4.0 min), 80-100% B for 5 min (4.0-9.0 min), 100%B for 5 min (9.0-14.0 min), 100-40%B for 0.1 min (14.0-14.1), and 40%B for 3.9 min (14.1-18.0 min). The UHPLC-QTOF-MS system, mass ion chromatogram and mass spectra were acquired by Analyst™ Software version 1.7 (SCIEX). The QTOF MS was operated in ESI positive mode at ion source gas 1 (GS1) of 40 psi, ion source gas 2 (GS2) of 40 psi, curtain gas (CUR) of 30 psi, ion spray voltage floating (ISVF) of 4500 V, source temperature (TEM) at 350°C, and declustering potential (DP) of 120 V. Data were acquired in the informative dependent acquisition (IDA) mode composed of a TOF-MS scan and 10 dependent product ion scans in the high sensitivity mode with dynamic background subtraction. Mass range of TOF-MS scan was at m/z 100-1,000 and product ion scan was at m/z 50-1,000. IVM standard solution (100 ng/mL) was injected before and after batch analysis for validating the system performance.

LC-SPE-NMR/MS

The analyses were performed by in-line instruments of interfacing liquid chromatography with parallel NMR and mass spectrometry. The 60 dried microsome pellets described above were each re-constituted in 300 µL of methanol and sonicated for 5 min. Supernatants were pooled into one 50 mL falcon tube. A second extraction of the microsome residue was performed by adding an additional 300 µL of acetonitrile followed by sonication for 5 min. The supernatant from the second extraction was then transferred to the same 50 mL falcon tube

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described above. The pooled supernatant was evaporated under nitrogen gas to a final volume of 200 µL and transferred to an HPLC vial. The extract (33 µL) was injected into the HPLC (Agilent 1260) at a flow rate of 0.5 mL/min at 25°C on a 250×4.6mm, 5µm Kinetex EVO C18 column (Phenomenex). The mobile phase A was water with 0.1% formic acid-d_2 (CDOOD) and mobile phase B was acetonitrile with 0.1% CDOOD. The elution gradient was started at 50% mobile phase B for 2 min (0.0-2.0 min), 50-100 %B for 33 min (2.0-35.0 min), 100%B for 5 min (35.0-40.0 min). UV detection was done at 240 nm. An MS Bridge interface (Bruker Biospin) was used to split a small portion of the effluent from the HPLC column and direct it to the ion source of a MicrOTOF-QII mass spectrometer (Bruker Daltonik, Bremen, Germany) using an acetonitrile make-up flow of 70 µL/min. The mass spectrometer was operated in positive ionization mode with a scan range at m/z 50 to 1,000. Mass calibration was done with sodium acetate infused at the beginning of the chromatography. The isolated metabolites were trapped post-column on 2×10 mm solid phase extraction cartridges filled with HySphere GP resin using the Prospekt 2 SPE interface from Spark Holland (Emmen, the Netherlands). The peaks of 3 injections (each 33 µL) were combined on individual cartridges (multi trapping). In total 2x3 trappings were performed. The make-up flow rate for the trapping was 1.5 mL/min. After chromatography the cartridges including the metabolites were dried with nitrogen gas and the two cartridges containing the metabolites were eluted with each 300uL of acetonitrile-d_3 (CD_3CN) into 5 mm NMR tubes. In total, the six trappings were finally transferred to the NMR tube for each metabolite resulting in a total volume of 600 µL. The API reference sample was measured with a 500 MHz AVANCE III NMR spectrometer equipped with a nitrogen cooled 5 mm Prodigy TCI (triple resonance inverse configuration of the coils with a cooled carbon channel) cryo probe. Post-column SPE fractions of the metabolites were measured first with the 500 MHz spectrometer and later with an 800 MHz Neo NMR spectrometer equipped with a helium cooled 5 mm TCI cryo probe (Bruker Biospin, Rheinstetten, Germany). SPE fractions were analyzed with the CMC-se software using optimized parameter sets including the Proton-1D, edited hetero nuclear single coherence spectroscopy (HSQC) and hetero nuclear multiple bond coherence spectroscopy (HMBC). In addition, selective HMBC experiments were performed for areas of closely resonating carbon resonances. Ivermectin (5.2 mg) was dissolved in deuterated acetonitrile (1 mL) and transferred to a 5 mm NMR tube and run as a reference compound.

Data analysis

Metabolite identification was done by MetabolitePilot^{TM} Software version 2.0 (SCIEX). The MS/MS spectrum of IVM was exported as text files by the PeakView software (SCIEX) and imported to MetabolitePilot^{TM} software (SCIEX) as a reference spectrum for creating the ivermectin library. Raw data files (.wiff) of metabolite sample analyses were imported to MetabolitePilot ^{TM}software and compared against the IVM-library peak finding strategies as described in the supplementary material. For the LC-SPE-NMR/MS system, the HPLC was operated by Hystar 3.2 (Bruker Daltonics), mass spectrum acquired by Microtof control (Bruker Daltonics), and the NMR spectrometer was operated by Topspin 3.5 (Bruker Biospin). The Complete Molecular Confidence – Structure Elucidation (CMC-se) software version 2.6.1 (Bruker Biospin,) was used for structure elucidation.

Materials

Ivermectin (IVM-B_{1a} > 95%, IVM-B_{1b} < 2%) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). IVM-B_{1a} (95% purity) was purchased from Toronto Research Chemical (Toronto, ON, Canada). IVM-B_{1b} (99.27% purity) was purchased from Clearsynth (Mississauga, Ontario, Canada). Pooled human liver microsomes (50 donors, Gibco^{TM}) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cryopreserved primary human hepatocytes (M00995-P, UBV donor) and hepatocyte culture medium (InVitroGroTM CP Medium) were purchased from BioIVT, Inc (Baltimore, MD, U.S.A.). Human cytochrome P450 cDNA-expressed (Supersomes) isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5), NADPH regenerating system, insect cell microsomes (negative control), and human P450 oxidoreductase +b5 were purchased from Corning (Corning, NY, USA). LC-MS grade water and acetonitrile were purchased from J.T Baker (City, PA, USA). LC-MS grade ammonium acetate and formic acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). HPLC grade organic solvent

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for NMR analysis was purchased from Sigma Aldrich (Saint Louis, MO, USA). CD3CN 99.8% and CDOOD 98% was purchased from Deutero GmbH (Kastellaun, Germany). Water for NMR was prepared from a Milli-Q purification system from Merck (Darmstadt, Germany).

Results

In vitro and in vivo metabolite identification by UHPLC-QTOF MS

Thirteen IVM metabolites (M1-M13) were identified from 60 minute -incubated microsomes as shown in the metabolite chromatogram in**Figure 2A.** All metabolites were more polar than the IVM parent compound. M1, M3, M5, and M6 were found in the medium of 24hour-exposed primary human hepatocytes **(Figure 2B)** . M1, M3, and M6 were also found in 24hour-post-treatment volunteer blood **(Figure 2C).**Reference MS/MS spectra of IVM-B_{1a} and IVM-B_{1b} used in the library search were generated from analysis of 100 ng/mL of IVM standard solution. IVM**-** B_{1b} (m/z 878.5) eluted 0.9 min earlier than IVM-B_{1a} (m/z 892.5) as shown in the extract ion chromatogram (**Figure 3A).** Major product ions of IVM-B_{1b} were m/z 293.2, 537.3, and 555.3 (**Figure 3B)** and those of IVM-B_{1a} were m/z 307.2, 551.3, and 569.3 **(Figure 3C)** . The fragment pattern of the ammonium adduct ion of IVM-B_{1a} and IVM-B_{1b} is presented in **Figure 3D.** MS/MS spectra of M1 to M13 (**Figure 4)** were used to define the molecular structure of the metabolites.

Metabolite interpretation

Potential metabolites (M1 to M13) identified from the MetabolitePilot^{TM} software (Sciex) are reported in**Table 1** . The molecular ions that were detected in negative controls (including 0-minute incubations, negative co-factor controls, and negative ivermectin controls) were excluded from the list. Percentage scores were based on three parameters including mass accuracy, mass defect (Sleno, 2012; Zhang et al., 2009), and MS/MS spectrum (for details see supplementary material). Chemical structures and biotransformation sites of the metabolites (M1 to M13) were interpreted based on two fragment ions for IVM-B_{1a}, m/z 307.2 and 551.3. IVM-B_{1a} metabolites without ion m/z 307.2 indicated that biotransformation occurred in the spiroketal. The presence of ion m/z 307.2 without ion m/z 551.3 indicated that biotransformation occurred in the cyclohexene cyclic ether, and metabolites with changed molecular ions with presence of both ions m/z 307.2 and 551.3 indicated that biotransformation occurred in the disaccharide moiety. Identical results were observed for IVM-B_{1b} metabolites, but with ions m/z 293.2 and 537.3. Interpretations of M1 to M13 are detailed in **Table 2.** The 60-min microsome reactions for pure IVM-B_{1a} and IVM-B_{1b} substrates confirmed an identical transformation pattern of both compounds (**Tables S1**).

Confirmation of in vivo metabolites structures (M1 and M3) by LC-SPE-NMR/MS

Microsome metabolites were separated by HPLC followed by MS and UV detection (**Figure 5A**) with subsequent post column trapping of analytes on the SPE cartridges. Molecular formulas of the metabolites were obtained from high resolution mass spectrometry during a preparation run. The final isolation of the metabolites was based on UV response in order to reduce the risk of contamination of the ion source because of the large amount of sample injected on the column. M1 and M3 were separated by HPLC and detected by MS as sodium adducts $[M+Na]^+$ ions (i.e. m/z 883 and m/z 913, respectively) (**Figure 5B**). The NMR spectra confirmed that the demethylation of M1 occurred at C3" (labeled in yellow, **Figure 6A**) and that the hydroxylation of M3 occurred at C4 (labeled in yellow,**Figure 6B**). The evaluation of HSQC and HMBC spectra of IVM, M1, and M3 shows the location of biotransformation (**Table 3, Figure 6, NMR supplemental report**). Chemical shift values for proton and carbon resonances are shown in **Table 3.**

As the sensitivity of the 500MHz instrument was not sufficient to obtain an HMBC spectrum within the period of the nitrogen refill cycle (one week), the isolated metabolites were analyzed using an 800MHz instrument. This provided a complete set of NMR data (within 72 h) for structure elucidation. The HMBC correlations for IVM, M1 and M3 together with the proton spectrum obtained at 800MHz are detailed in the NMR supplement.

Metabolic pathway characterized by pure human CYP enzyme

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IVM metabolites M1, M3, M5, M6, M7, M8, M9, M10, and M12 were all found after 60 minutes of incubation with CYP3A4. IVM metabolite M1 was also produced by CYP3A5. M13 was produced by CYP2C8. None of the other investigated enzymes produced detectable amounts of the identified IVM metabolites. Proposed metabolite pathways are shown in **Figure 7.**

Discussion and conclusions

The peak area of metabolites, relative to IVM, was used to estimate the relative abundance of each metabolite. In 60-min microsomes reactions, the five most abundant metabolites were M3 > M1 > M5 > M6 > M9 **(Table 1).** Metabolites M1, M3, and M6 were found after 24-hour exposure to primary human hepatocytes and in volunteer blood samples taken 24 hours post IVM administration. This is the first report of IVM metabolites identified from human hepatocytes and clinical blood samples.

The IVM demethylation, oxidation, and monosaccharide metabolites identified from microsomes in this study were consistent with those reported previously from human microsomes (Zeng et al., 1998). However, four additional IVM metabolites, including ketone and carboxylic derivatives, were also found in our study. Advancements in UHPLC technology (Churchwell et al., 2005) and state-of-the-art high resolution mass spectrometry (Meyer & Maurer, 2012; Ramanathan et al., 2011; Theodoridis et al., 2012) used here improved the sensitivity for metabolite detection. By using pure CYP enzymes, we observed evidence for two CYP metabolism pathways for IVM; CYP3A5 produced demethylated IVM (M1) and CYP2C8 produced hydroxylated IVM (M13).

The chemical structures of the most abundant metabolites were obtained by NMR for M1 and M3 but not for M6. The metabolic pathway data suggest that M1, M3, and M6 were all produced by CYP3A4, and that M6 is a combination of oxidation and demethylation. Thus, we propose that M6 is 3”-O-demethyl, 4-hydroxymethyl-ivermectin, a further (common) metabolite product of M1 and M3. With two sites of transformation occurring in M6 (demethylation and oxidation), it is more polar and elutes earlier than M1 and M3. Additional reversed phase chromatography data supports the M6 structure based on the elution order. The elution order in this study is also consistent with Zeng et al. 1998.

Interestingly, many low abundance metabolites produced in microsomes were not detected from primary human hepatocytes in culture nor from human volunteer blood after IVM administration. Several factors that could influence the metabolic function of hepatocytes *in vitro* , including initial cell suspension, confluence density of adherent cells, and drug concentration. The lower number of metabolites found in volunteer blood samples compared to microsomes could be because of phospholipids in blood samples. *In vitro* systems are also more efficient in producing metabolites and do not have elimination pathways (such as renal elimination) compared to *in vivo* systems, which could have an impact on detection results. In future studies, we will characterize IVM metabolites produced at later time points from hepatocytes and human blood, when metabolite abundance may possibly be altered compared to the 24-hour time point thus allowing characterization of *in vivo* metabolism over time. To support these efforts, we have performed a clinical trial (NCT03690453) to asses the pharmacokinetic profile of key IVM metabolites in orally treated volunteers over several weeks, characterized and synthesized these metabolites, and evaluated their potential mosquito-lethal and antimalarial effects. A better understanding of IVM metabolite pharmacokinetics can provide further insight into pharmacodynamics and efficacy for NTDs, especially ones requiring multiple administrations such as scabies and strongyloidiasis. Furthermore, these IVM metabolites may inhibit viral replication and should be evaluated against SARS-CoV-2, the causative agent of COVID-19.

In conclusion, we report for the first time, novel IVM metabolites from human liver microsomes and IVM metabolites from primary human hepatocytes and from human blood after oral IVM dosing. Importantly, we identify that the two major IVM metabolites in humans are 3”-*O* -demethylation IVM and 4-hydroxymethyl IVM.

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Table 1. Identification of ivermectin metabolites from human liver microsomes, primary human hepatocytes, and volunteer blood.

Met ID	Molecular ion	Formula	Neutral Mass	m/z	Charge	ppm	R
	Parent - B1a [M+NH4]+	C48H74O14	874.5083	892.5421	1	0.5	13
	Parent - B1b [M+NH4]+	C47H72O14	860.492	878.5258	1	-0.3	13
M1	Loss of CH2 [M+NH4]+	C47H72O14	860.4922	878.526	1	0	12
M2	Loss of C7H12O3 [M+NH4]+	C41H62O11	730.4283	748.4621	1	-1.2	12
M3	Oxidation [M+NH4]+	C48H74O15	890.5033	908.5371	1	0.6	11
M4	Ketone Formation [M+NH4]+	C48H72O15	888.4862	906.5201	1	-1	11
M5	Oxidation [M+NH4]+	C48H74O15	890.503	908.5368	1	0.2	10
M6	Demethylation and Oxidation [M+NH4]+	C47H72O15	876.4868	894.5206	1	-0.4	10
M7	Demethylation and Methylene to Ketone [M+NH4]+	C47H70O15	874.4708	892.5046	1	-0.8	10
M8	Demethylation to Carboxylic Acid [M+NH4]+	C48H72O16	904.4808	922.5146	1	-1.3	9.
M9	Demethylation and Oxidation [M+NH4]+	C47H72O15	876.487	894.5208	1	-0.2	9.
M10	Demethylation to Carboxylic Acid [M+NH4]+	C48H72O16	904.4819	922.5157	1	-0.1	9.
M11	Loss of C7H12O3+Oxidation [M+NH4]+	C41H62O12	746.4225	764.4563	1	-2.2	9.
M12	Di-Oxidation [M+NH4]+	C48H74O16	906.4976	924.5314	1	-0.1	9.
M13	Oxidation [M+NH4]+	C48H74O15	890.5027	908.5365	1	-0.1	8.

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HLM = human liver microsomes, PHH = primary human hepatocytes.

Table 2. Molecular structure of metabolite predicted by accurate mass and mass fragmentation pattern.

Metabolite derivative	**Biotransformation**	**Metabolite**	**Molecular ion (m/z)**
Hydroxylate	Demethylation (R-CH3 to R-H)	M1	878.5
	Oxidation (+O)	M3	908.5
		M5	908.5
		M13	908.5
	Demethylation (R-CH3 to R-H) and oxidation (+O)	M6	894.5
		M9	894.5
	Dioxidation (+2O)	M12	924.5
Monosaccharide	Loss of monosaccharide	M2	748.5
		M11	764.5
Ketone	Ketone formation (R-CH_2-R' to R-CO-R')	M4	906.5
		M7	892.5
Carboxylate	Oxidation and ketone formation (+O-CH2+CO)	M10	922.5
		M8	922.5

Table 3. Chemical shift values for proton and carbon resonances obtained from HSQC and HMBC spectra of IVM, M1 and M3.

Position	**IVM**	**IVM**	**M1**	**M1**	**M3**	**M3**
	H shift [ppm]	C shift [ppm]	H shift [ppm]	C shift [ppm]	H shift [ppm]	C shift [ppm]
1	-	171.91	-	171.98	-	171.72
2	3.16	45.61	3.19	45.67	3.25	45.53
3	5.42	118.65	5.42	118.60	5.70	118.64
4	-	136.50	-	136.69	-	**139.78**
5	4.19	67.47	4.20	67.43	**4.41**	**65.22**
6	3.78	80.37	3.79	80.31	3.81	80.48
7	-	80.48	-	80.47	-	80.79
8	-	140.36	-	140.37	-	140.28
9	5.84	120.29	5.84	120.33	5.86	120.55
10	5.89	125.16	5.89	125.15	5.90	125.18
11	5.81	137.39	5.80	137.56	5.81	137.68
12	2.63	39.44	2.63	39.52	2.64	39.55
13	3.96	81.74	3.96	81.72	3.96	39.55
14	-	134.84	-	134.81	-	134.85

Position	**IVM**	**IVM**	**M1**	**M1**	**M3**	**M3**
15	5.21	118.89	5.21	118.98	5.22	118.94
16 (a/b)	2.31/2.24	33.74	2.31/2.25	33.56	2.32/2.25	33.68
17	3.73	67.42	3.74	67.40	3.74	67.35
18 (a/b)	1.9/0.79	36.50	1.91/0.80	36.51	1.91/0.80	36.55
19	5.04	68.55	5.07	68.44	5.09	68.60
20 (a/b)	2.09/1.25	41.46	2.09/1.26	41.40	2.10/1.27	41.42
21	-	97.43	-	97.41	-	97.46
22 (a/b)	1.61/1.52	35.50	1.60/1.52	35.66	1.61/1.52	35.72
23 (a/b)	1.53/1.52	27.99	1.53/1.52	27.90	1.53/1.52	27.99
24	1.54	31.10	1.54	31.07	1.54	31.09
25	3.29	76.19	3.30	76.28	3.30	76.23
26 (a/b)	4.60/4.61	67.54	4.61/4.62	67.62	4.63/4.62	67.66
CH3/CH2 (a/b) (4)	1.81	18.79	1.82	18.75	**4.16/4.15**	**62.53**
CH3 (12)	1.16	19.68	1.16	19.62	1.17	19.63
CH3 (14)	1.53	14.13	1.53	14.04	1.54	14.12
CH3 (24)	0.81	16.72	0.82	16.63	0.82	16.72
27	1.62	35.22	1.62	35.17	1.63	35.22
CH3 (27)	0.97	11.74	0.89	11.62	0.89	11.63
28 (a/b)	1.56/1.40	27.03	1.56/1.41	27.03	1.57/1.41	27.01
CH3 (28)	0.89	11.69	0.97	11.65	0.98	11.67
1´	4.78	94.89	4.79	94.81	4.79	94.90
2´ (a/b)	2.30/1.51	34.31	2.30/1.53	34.07	2.29/1.53	34.22
3´	3.62	79.34	3.62	79.45	3.62	79.30
4´	3.18	80.13	3.18	80.15	3.19	80.25
5´	3.83	67.14	3.83	67.05	3.84	67.15
CH3 (3´)	3.37	55.74	3.37	55.69	3.38	55.57
CH3 (5´)	1.23	17.95	1.24	17.92	1.24	17.98
1´´	5.34	98.04	5.31	97.95	5.34	98.08
2´´ (a/b)	2.26/1.46	34.55	**2.07/1.59**	**38.02**	2.27/1.47	34.69
3´´	3.33	78.05	**3.64**	**68.43**	3.35	78.06
4´´	3.01	76.01	**2.92**	**77.70**	3.02	76.14
5´´	3.66	68.42	3.67	68.44	3.68	68.45
CH3 (3´´)	3.38	56.09	**-**	**-**	3.38	56.27
CH3 (5´´)	1.20	17.11	1.20	17.01	1.20	17.08

For labeling of positions refer to Figure 1. Differences from shift values for M1 and M3 in comparison to IVM are marked in bold letters.

Figure legends

Figure 1. Molecular structure of ivermectin.

Ivermectin consists of a spiroketal unit (C17-C28), cyclohexene cyclic ether unit (C2-C8), and a disaccharide unit at C13. A secondary butyl sidechain at C25 give rise to the major component (ivermectin B_{1a}) and an isopropyl sidechain give rise to the minor component (<10%; ivermectin B_{1b}).

Figure 2. Metabolite chromatogram.

The chromatograms show the (A) metabolites identified using pooled human liver microsomes incubated with ivermectin for 60 min, (B) metabolites identified in the media fraction of primary human hepatocytes

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exposed to ivermectin for 24 hours, and (C) metabolites identified in human volunteer blood 24 hours after ivermectin administration.

Figure 3. Extracted ion chromatogram and MS/MS spectra .

The figure shows (A) extracted ion chromatogram of ivermectin B_{1a} and B_{1b}, (B) MS/MS spectrum of ivermectin B_{1b}, (C) MS/MS spectrum of ivermectin B_{1a}, and (D) major fragment pattern of ivermectin.

Figure 4. MS/MS spectra of metabolite M1 to M13.

Fragment ion peaks of metabolites that matched library peaks show in orange.

Figure 5. Metabolite purification and detection for NMR analysis.

Panel (A) is an extracted ion chromatogram of IVM, M1, and M3 during the preparation chromatography, and UV chromatogram at 240nm for the trapping experiment (the dotted lines indicate the start and end of the trapping procedure, (B) is a mass spectrum from the peaks in the extracted ion chromatogram showed that the molecules form stable sodium adducts.

Figure 6. NMR spectra zoomed in at the signal of IVM (left) comparison with metabolite M1 (middle) and M3 (right).

Structure of the IVM was labeled in yellow at signals showing most significant differences in the spectra of the metabolites. The cursor in HSQC spectrum indicates signals missing due to changes in the metabolites. Panel A) the shift of the –CH group in the metabolite M1 (m/z 883) at the position labeled in yellow and B) the missing $–CH_3$ group of the metabolite M3 (m/z 913) at position labeled in yellow. The changes in chemical shift values are listed in Table 3.

Figure 7. Proposed metabolic pathway of ivermectin metabolites.

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