Published in final edited form as:

Xenobiotica. 2018 October ; 48(10): 973–983. doi:10.1080/00498254.2017.1386335.

METABOLISM OF MEGESTROL ACETATE IN VITRO AND THE ROLE OF OXIDATIVE METABOLITES

Mr. Larry House,

University of Chicago, Medicine, KCBD, 900 E. 57th St., Chicago, 60637 United States,
lhouse@medicine.bsd.uchicago.edu

Dr. Michael Seminerio,

University of Chicago, Medicine, 900 E. 57th St., Chicago, 60637 United States,
mseminerio1@gmail.com

Mrs. Snezana Mirkov,

Northwestern University, Chicago, United States, snezana.mirkov@northwestern.edu

Ms. Jacqueline Ramirez,

University of Chicago, Medicine, 900 E. 57th St., Chicago, 60637 United States,
jramirez@medicine.bsd.uchicago.edu

Mr. Maxwell Skor,

University of Chicago, Chicago, United States, maxwell Skor@gmail.com

Dr. Joseph Sachleben,

University of Chicago, Chicago, United States, jsachleben@uchicago.edu

Dr. Masis Isikbay,

University of Chicago, Chicago, United States, masis.isikbay@gmail.com

Dr. Hari Singhal,

University of Chicago, Chicago, United States, hari.singhal@roche.com

Dr. Geoffrey Greene,

University of Chicago, Chicago, United States, ggorene@uchicago.edu

Dr. Donald Vander Griend,

University of Chicago, Chicago, United States, dvanderg@surgery.bsd.uchicago.edu

Dr. Suzanne Conzen, and

University of Chicago, Chicago, United States, sconzen@medicine.bsd.uchicago.edu

Dr. Mark J Ratain

University of Chicago, Medicine, Chicago, United States

Abstract

There is limited knowledge regarding the metabolism of megestrol acetate (MA), as it was approved by FDA in 1971, prior to the availability of modern tools for identifying specific drug-

metabolizing enzymes. We determined the cytochrome P450s (P450s) and UDP-glucuronosyltransferases (UGTs) that metabolize MA, identified oxidative metabolites, and determined pharmacologic activity at the progesterone, androgen and glucocorticoid receptors (PR, AR, and GR, respectively). Oxidative metabolites were produced using human liver microsomes (HLMs), and isolated for mass spectral (MS) and nuclear magnetic resonance (NMR) analyses. We screened recombinant P450s using MA at 62 μ M (HLM K_m for metabolite 1; M1) and 28 μ M (HLM K_m for metabolite 2; M2). UGT isoforms were simultaneously incubated with UDPGA, NADPH, CYP3A4 and MA. Metabolites were evaluated for pharmacologic activity on the PR, AR, and GR. CYP3A4 and CYP3A5 are responsible for oxidative metabolism of 62 μ M MA. At 28 μ M substrate concentration, CYP3A4 was the only contributing enzyme. Mass spectral and NMR data suggest metabolism of MA to two alcohols. After oxidation, MA is converted into two secondary glucuronides by UGT2B17 among other UGTs. MA, M1 and M2 had significant pharmacologic activity on the PR while only MA showed activity on the AR and GR.

Keywords

megestrol acetate; metabolite activity; metabolite structure; androgen receptor; progesterone receptor; glucocorticoid receptor; drug interaction

Introduction

Megestrol acetate (MA) was originally synthesized in 1963 and first administered to humans in 1967 (Argiles et al., 2013). While MA was first administered in the United Kingdom for the treatment of breast cancer, it became commercially available in the United States in 1971 for the treatment of endometrial carcinoma. Since its initial use, MA has been utilized in oral contraceptive pills and in the treatment of malignant and non-malignant conditions such as melanoma, various types of cancer (ovary, breast, kidney, prostate), benign prostatic hypertrophy, endometrial hyperplasia (Schacter et al., 1989; Tisdale, 1997), and hot flashes (Warren et al., 2008). In addition, due to its orexigenic (appetite inducing effect partially mediated by neuropeptide Y, a potent central appetite stimulant) and weight gaining effects, MA is commonly used to treat anorexia-cachexia and is the only FDA-approved treatment of cancer and AIDS-related anorexia-cachexia syndrome (Argiles et al., 2013).

MA is a synthetic, substituted progesterone with physiologic activity very similar to that of the natural hormone progesterone. It is believed to act as both a progestational agent and anti-inflammatory/glucocorticoid agent (Yeh et al., 2006). Moreover, MA has been shown to reduce the *in vitro* production of serotonin and cytokines (interleukin-1, interleukin-6, and tumor necrosis factor- α) via peripheral blood mononuclear cells of cancer patients (Mantovani et al., 1998; Argiles et al., 2013). Other studies have demonstrated the effect of MA on neuropeptide Y (McCarthy et al., 1994), progesterone receptors (Nishino et al., 2009), metallothionein and glutathione S-transferase-pi (Pu et al., 1998) levels. These findings have begun to shed light on the precise molecular mechanism of MA and the subsequent link to clinical drug interactions. As MA continues to be administered for various indications, the importance of understanding MA pharmacokinetics and its effects when co-administered with other medications is of high priority.

Previous studies have demonstrated considerable variation in absorption with patients taking oral MA. It has been suggested that the variability may be due to factors such as enteropathy, achlorhydria, or other factors which alter the gastrointestinal physiology. Furthermore, concurrent medications may influence the variability in pharmacokinetic parameters and patient response (Graham et al., 1994). MA is believed to be completely metabolized in the liver with subsequent metabolites being conjugated via glucuronidation (Bristol-Myers Squibb Canada, 2014). However, these conjugates are hydroxylated indicating phase 1 metabolism. While the metabolites account for only 5–8% of the administered dose, the extent to which they produce a pharmacologic effect remains unknown (Bristol-Myers Squibb Canada, 2014).

While it is known that MA undergoes hydroxylation and glucuronidation yielding three major metabolites (Cooper and Kellie, 1968), these are the only pathways that have been investigated. Furthermore, the specific enzymes involved in these pathways have not yet been clarified. In the present study, we evaluated the metabolism of MA, the cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT) enzyme(s) involved in MA metabolism, and the pharmacologic activity of MA and its oxidative metabolites. The findings presented herein can potentially assist in the identification of clinically significant drug-drug interactions that may occur when drugs that alter P450 or UGT activity are co-administered with MA.

Materials and Methods

Chemicals and reagents

MA and 2,3-diphenyl-1-indenone were purchased from Sigma-Aldrich (St. Louis, MO). Methanol and acetonitrile (both high-performance liquid chromatography (HPLC) grade) were purchased through VWR (West Chester, PA). Nicotinamide adenine dinucleotide phosphate (NADPH) and UDP-glucuronic acid (UDPGA) Regenerating System Solutions A and B were obtained from BD Gentest (Woburn, MA). All other chemicals and reagents used were of the highest grade and commercially available.

HLM and recombinant enzymes

Normal human livers were acquired from the Liver Tissue Cell Distribution System (Pittsburgh, PA; NIH Contract #HHSN276201200017C) and the Cooperative Human Tissue Network with human subjects' approval. Human liver microsomes (HLM) (n=20) were prepared using differential centrifugation methods (Ramírez et al., 2007) and pooled. Microsomes from cDNA-transfected baculovirus-insect cell expressing human P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11, CYP19), UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) and insect cell control microsomes from wild-type baculovirus-infected cells, a common transfection system (Gasser et al., 1999), were purchased from BD Gentest (Woburn, MA).

Oxidation assay with HLMs

Preliminary experiments were performed to determine incubation conditions that both maximized the metabolite production and ensured linearity with respect to microsomal protein concentration and incubation time. A typical reaction mixture contained MA (100 μ M), NADPH Regenerating System Solution A (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂) and 0.12 mg protein of pooled HLM in 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 200 μ l. After pre-incubation for 5 min, the reaction was initiated by adding NADPH Regenerating System Solution B (0.4 U ml⁻¹ glucose 6-phosphate dehydrogenase) and incubated for 25 min at 37° Celsius (C). The reaction was terminated by adding 200 μ l of cold methanol containing internal standard (53.1 μ M 2,3-diphenyl-1-indenone). After removal of the protein by centrifugation at 20,817 g for 15 min, 10 μ l of supernatant was analyzed by HPLC. Control incubations were simultaneously performed in the absence of MA and HLM. Experiments were performed in duplicate or triplicate.

Kinetic analyses in HLMs

Kinetic studies were conducted under linear (optimal) incubation conditions as described above using 8 concentrations of MA ranging from 5–1000 μ M. The experiments were performed in triplicate.

Oxidation assay with recombinant enzymes

Screening experiments with P450 isoforms were performed as described above for HLM using two concentrations of MA (28 μ M; HLM K_m for metabolite 1 (M1) and 62 μ M; HLM K_m for metabolite 2 (M2) and 20 pmol of recombinant P450s. Microsomes prepared from insect cells infected with wild-type baculovirus were used as negative controls. All experiments were performed in duplicate.

Kinetic analyses with CYP3A4

Kinetic studies were conducted as described under *Oxidation assay with HLM* using 20 pmol of CYP3A4 and 8 substrate concentrations ranging from 5–1000 μ M. The experiments were performed in triplicate.

Oxidation-glucuronidation assay with HLMs

MA (100 μ M) was incubated with UDPGA Reaction Mix, Solution B (50 mM Tris-HCl, 8 mM MgCl₂ and 0.025 mg/ml alamethicin) and 0.2 mg of pooled HLM in the final volume of 200 μ l. After 5 min equilibration at 37°C, the reaction was initiated by adding 1 mM NADPH +/- UDPGA Reaction Mix, Solution A (2 mM UDPGA) and incubated for 3 h and 24 h at 37°C. The reaction was terminated as described above and 100 μ l of supernatant was injected into the HPLC. Control incubations were simultaneously performed in the absence of MA and HLM. Experiments were performed in duplicate or triplicate.

Oxidation-glucuronidation co-incubations with recombinant enzymes

The co-incubations with recombinant enzymes were performed as described above for HLM using the mixture of 20 pmol recombinant CYP3A4 and 0.1 mg of each recombinant UGT.

Control microsomes prepared from insect cells infected with wild-type baculovirus were used as negative controls. All experiments were performed in duplicate.

Inhibition of HLMS and CYP3A4 with ketoconazole or troleandomycin

Formation of metabolites from MA (28 μ M and 62 μ M) was evaluated in the absence (control) and presence of known CYP3A inhibitors. The inhibitors used were ketoconazole (1 μ M) and troleandomycin (50 μ M). After pre-incubating with ketoconazole (a potent competitive inhibitor), MA (62 μ M), and NADPH for 15 minutes at 37°C, the reaction was initiated by adding HLMS or CYP3A4. After pre-incubating HLMS or CYP3A4 with troleandomycin (a mechanism-based inactivator), and NADPH for 15 minutes at 37°C, the reaction was started by adding MA (62 μ M). The same experiment was performed using a substrate concentration of 28 μ M for HLMS only. Negative controls for all reactions were in the absence of protein. All incubations were performed in triplicate.

HPLC measurement of the formation of MA oxidative metabolites

MA and its oxidative metabolites were quantified by HPLC (Hitachi High Technologies America, San Jose, CA) with slight modification to a previous method (Matin et al. 2002). The eluate was monitored with UV detection at 280 nm. The analytes were separated on a C₁₈ reversed-phase column (μ Bondapak, 3.9 $\times$ 300 mm, 10 μ m, 125Å; Waters, Milford, MA) preceded by a Nova-Pak C₁₈ Guardpak (Waters, Milford, MA). The mobile phase consisted of acetonitrile/water/acetic acid (60/39/1). The flow rate was 1 ml/min with a run time of 25 min. The retention times were: 11.5 min (MA), 5.2 min (M1), 6.7 min (M2), 4.0 (metabolite 3, M3) and 24 min (internal standard). The ratio of the MA metabolite peak areas to the area of the internal standard peak was calculated. The relative formation of M1 and M2 was estimated using a standard curve generated using MA with a concentration range of 5–1000 μ M.

HPLC measurement of the formation of MA glucuronides

The HPLC conditions described above were modified to achieve the detection and separation of MA glucuronides formed by co-incubations of HLM or recombinant enzymes with NADPH and UDPGA in presence of MA. The mobile phase consisted of 2 solvents, A (1% acetic acid in water) and B (1% acetic acid in acetonitrile). A gradient elution was as follows: 0–55 min 75% A 25% B, 55–95 min ramped to 25% A 75% B, 95–105 min 25% A 75% B, and 105–115 min 75% A 25% B. The run time was 115 min. The retention times of MA and its glucuronides were 86.9 min (MA), 27.3 min (MA glucuronide 1, MG1) and 49.9 min (MA glucuronide 2, MG2). MA metabolite production was determined by calculating the ratio of the MA glucuronide peak heights to the peak height of the internal standard. Because authentic standards were unavailable for M1 and M2, the concentrations were based on a standard curve produced from the parent drug. Since the structures are similar, we assumed that the molar extinction coefficients were also similar for both parent drug and metabolites. Quantitative nuclear magnetic resonance (NMR) was not feasible due to the challenge of isolating metabolites in sufficient amounts for analyses.

Oxidative metabolite collection and concentration

The HPLC fractions for the oxidative metabolites of MA were collected and concentrated for mass spectrometry (MS), NMR, and receptor assays. We used the same methods in *Oxidation-glucuronidation assay with HLMs*, but increased the volume by 100X. Briefly, the incubation contained MA (100 μ M) NADPH Regenerating System, Solution A (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂) and 12 mg protein of pooled HLM in 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 20 ml (and aliquoted into 4 ml aliquots). After pre-incubation for 5 min, the reaction was initiated by adding NADPH Regenerating System, Solution B (0.4 U ml⁻¹ glucose 6-phosphate dehydrogenase) and incubated for 25 min at 37°C. The reaction was terminated by adding 4 ml of cold methanol containing internal standard (53.1 μ M 2,3-diphenyl-1-indenone) into each tube. After removal of the protein by centrifugation at 25,314 g for 15 min, each aliquot of supernatant was dried down with nitrogen gas at 37° C and reconstituted in 500 μ l mobile phase. Repeated injections of 50 μ l were introduced into the HPLC system. The relevant peaks for M1 and M2 were collected manually and individually concentrated by drying under nitrogen gas at 37°C.

Identification of MA oxidative metabolites

To determine the mass for oxidative metabolites of MA, HPLC fractions were collected for M1 and M2 and subjected to mass spectrometry (MS) using an API 2000 LC-MS/MS triple quadrupole system (Applied Biosystems, Foster City, CA). M1 and M2 were isolated by HPLC (using the analytical method described above for oxidative metabolites) and separately analyzed by MS after drying down and reconstituting fractions in 100% methanol. Additionally, MA was analyzed. MA and the metabolites were each infused at 20 μ l/min with a syringe pump. The electrospray ionization (ESI) voltage was set at +5500 V with a temperature of 10°C. Positive ion Q1 monitoring was performed. The curtain (CUR) gas, collisionally activated disassociation (CAD) gas, gas 1 (GS1), and gas 2 (GS2) were set at 35, 5, 50, and 70 psi, respectively. The voltages for declustering potential (DP), focusing potential (FP), entrance potential (EP) and cell exit potential (CEP) were set at 80, 400, 7 and 18 volts, respectively.

To determine sites of metabolic change to MA, NMR spectroscopy was employed. NMR spectra were acquired on a Bruker AVANCE III HD NMR spectrometer (Billerica, MA) operating at a proton frequency of 600.13 MHz utilizing a 5 mm inverse triple resonance gradient probe tuned to ¹H, ¹³C, and ¹⁵N. All samples were dissolved in 0.5 mL d-chloroform containing 0.03% (v/v) tetramethylsilane (TMS) (Aldrich, St. Louis, MO) and spectra were acquired at 25°C. A series of one dimensional regular proton spectroscopy (¹H 1D), homonuclear correlation spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), total correlation spectroscopy (TOCSY), and heteronuclear single quantum correlation (¹H,¹³C HSQC) spectra were acquired. Typical ¹H 90° times were 7.5 μ s and the recycle delay was 1 s. In the ¹H 1D spectra, 16 k complex points were acquired over a spectral width of 10 parts per million (ppm) with a resulting acquisition time of 2.7s. In the 2D spectra, 1k complex points were typically taken over 10 ppm in the directly detected dimension (acquisition time 170 ms), while in the indirectly detected dimension

128 complex points were acquired with a 10 ppm spectral width for ^1H in the COSY, NOESY, and TOCSY spectra and 100 ppm for the ^{13}C spectral width in ^1H , ^{13}C HSQC.

Progesterone receptor (PR) activity

T47D breast cancer cells were cultured in charcoal stripped serum medium for 48 h. Subsequently, the cells were treated for 8 h with ethanol (vehicle), 10 nM progesterone agonist R5020, 100 nM MA, 100 nM M1, or 100 nM M2. Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA). Taqman real time PCR assays (Invitrogen, Grand Island, NY) were used to measure the transcript expression of progesterone regulated genes growth regulation by estrogen in breast cancer 1 (*GREB1*), FK506 binding protein 5 (*FKBP5*) and E2F transcription factor1 (*E2F1*). The gene expression under various drug treatments was normalized to gene expression in ethanol-treated cells.

Androgen receptor (AR) activity

The Los Angeles prostate cancer-4 (LAPC4) human prostate carcinoma cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA) and 1% penicillin (100 units/mL)/streptomycin (100 Ag/mL; BioWhittaker/Cambrex, East Rutherford, NJ). Cells were plated in a 24 well dish (2,500 cells/well) and treated for 48 hrs using the following compounds: ethanol (vehicle), 10 μM MDV3100, MA (1.3 μM , 0.13 μM , 2.6 μM , and 0.26 μM), M1 (0.7 μM and 0.07 μM), or M2 (0.3 μM and 0.03 μM). Secreted total prostate-specific antigen (PSA) was measured from collected media using the Elecsys Total PSA Assay (Roche, Indianapolis, IN).

Glucocorticoid receptor (GR) activity

GR activity was performed as previously described (Skor et al., 2013). Briefly, MDA-MB-231 (triple-negative breast cancer) cells were seeded at ~50% confluence and allowed to adhere overnight in Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS (Invitrogen, Grand Island, NY), then cultured in 2.5% CS-FCS for an additional 48 h. Media was removed and equal volumes of either ethanol (vehicle), 0.1 μM dexamethasone (DEX), 1 μM MA, 1 μM M1, or 1 μM M2 diluted in DMEM supplemented with 2.5% CS-FCS was then added. After 4 h of treatment, 100 μl of RNA-Solv Reagent from the EaZy Nucleic Acid Isolation Kit (Omega Biotek, Norcross, GA) was supplemented with 2% 2-mercaptoethanol and added to each well to harvest RNA. Total RNA was extracted using the Qiagen All-Prep DNA/RNA Mini kit. cDNA was then reverse-transcribed from 0.5 μg of total RNA with Quanta reverse transcription reagents (Quanta Biosciences, Gaithersburg, MD) using the GeneAmp PCR System 9700 (Applied BioSystems, Grand Island, NY) per manufacturer's instruction. The cDNA was diluted in PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) and quantitative real-time PCR was carried out in a BioRad PCR System MyIQ (BioRad Life Sciences, Hercules, CA). The following primers were used: serum/glucocorticoid regulated kinase 1 (*SGK1*), 5'-AGGCCATCCTTCTCTGTTT-3' (forward) and 5'-TTCAGTCTCCCCTCAGTCT-3' (reverse); glucocorticoid-induced leucine zipper/Tsc22d3 (*GILZ/TSC22D3*), 5'-ACAGGCCATGGATCTGGTGA-3' (forward) and 5'-CAGCTCTCGGATCTGCTCCTT-3' (reverse); *Actin- β* , 5'-CAGCGGAACCGCTCATTGCCAATGG-3' (forward) and 5'-

TCACCCCTGTGCCCATCTACGA-3' (reverse). Relative quantification of gene expression was calculated according to the standard curve method, as described by Applied Biosystems User Bulletin 2, October 2001, based on the C_t approach. A ratio of GR target gene expression to *Actin- β* expression was calculated.

Data analysis

Apparent kinetic parameters were estimated by fitting oxidation rates vs. MA concentrations by nonlinear regression using the Enzyme Kinetics Module from SigmaPlot 12.3 (Systat Software, Inc., San Jose, CA). Goodness of fit to kinetic models was assessed by inspection of Michaelis-Menten and Eadie-Hofstee plots, r^2 and the Akaike Information Criterion AICc. Differences between groups were analyzed by Student's t -test analyzed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

Results

Oxidation of MA by HLM

In the presence of NADPH, HLMs metabolized MA to two major oxidative metabolites (M1 and M2) and a minor one (M3). Based on the *in vitro* intrinsic clearance (CL_{int}) (Table 1), the drug is preferentially metabolized to M1 ($CL_{int}=51 \mu\text{l}/\text{min}/\text{mg}$ protein) over M2 ($CL_{int}=31 \mu\text{l}/\text{min}/\text{mg}$ protein). The metabolites were not observed in the incubations when MA and HLM were omitted from the reaction. Formation to M3 was not characterized due to its very low production. Oxidation of MA by HLM followed substrate inhibition kinetics with Eadie-Hofstee plots (*inset*) (Figure 1A and Figure 1B for M1 and M2, respectively). The kinetic parameters for M1 and M2 formation by HLM are shown in Table 1.

Oxidation of MA by recombinant enzymes

Figure 2 shows the results of a screening experiment to identify those P450s involved in MA oxidative metabolism. Formation rates (pmol/min/pmol P450) for M1 by CYP3A4 and CYP3A5 were 4.5 ± 0.3 (28 μM MA), 9.6 ± 0.3 (62 μM MA) and 0.6 ± 0.1 (28 μM), 1.0 ± 0.1 (62 μM), respectively, while the formation rates (pmol/min/pmol P450) for M2 by CYP3A4 were 2.6 ± 0.04 (28 μM MA) and 4.9 ± 0.1 (62 μM MA) (Figure 2A and 2B). Formation of M2 is exclusively catalyzed by CYP3A4 (Figure 2B). Oxidative metabolites were not detected in the insect control microsomes. Oxidation of MA by CYP3A4 followed substrate inhibition kinetics for M1 (Figure 1C), and Hill kinetics for M2 (Figure 1D). Eadie-Hofstee plots (*inset*) are also shown in the respective figures. The kinetic parameters for production of M1 and M2 by CYP3A4 are shown in Table 1.

Inhibition of HLMs and CYP3A4 with ketoconazole and troleandomycin

We tested the effect of CYP3A-specific chemical inhibitors on MA metabolism in HLMs and CYP3A4 and observed >93% inhibition in all experiments compared to controls. When utilizing a substrate concentration of 62 μM , we observed 100% and 94.4% inhibition of M1 for ketoconazole and troleandomycin, respectively, and 100% inhibition of M2 for both inhibitors in HLMs. At a substrate concentration of 28 μM , we saw 96.2% and 93.2% inhibition of M1 for ketoconazole and troleandomycin, respectively, and 100% inhibition of

M2 for both inhibitors in HLMs. At a substrate concentration of 62 μ M, we observed 98.6% and 97.4% inhibition of M1 for ketoconazole and troleandomycin, respectively, and 100% inhibition of M2 for both inhibitors in CYP3A4.

MA metabolite characterization

A proposed pathway and structures are shown in Figure 3 for the major oxidative metabolites. MS results showed that the mass of MA increased from 384 Da to 400 Da in both M1 and M2 HPLC fractions. This suggests that MA has been oxidized by addition of a single ^{16}O . Comparison of NMR spectra of MA and M1 shows that the methyl attached to C-6 has been converted to an alcohol. 1D NMR Spectra were also run of MA, M1, and M2 (Figure 4A, 4B, and 4C respectively). In this spectrum it is clear that the methyl peaks due to positions 18, 19, 21, and 23 do not move, indicating that they are unchanged; however, the peak due to methyl 24 disappears and a new peak at $\sim 4.30\text{ppm}$ appears. This shift is consistent with a CH_2OH attached to a double bonded carbon. In addition, the peaks due to 4 and 7 shift to higher ppm also consistent with oxidation of 24. The ^{13}C HSQC of M2 is overlaid with the assigned ^{13}C HSQC of MA (Supplemental Figure 1). The peaks due to the germinal protons at positions 1 and 2 disappear, while those from protons at positions 11 and 9 shift slightly. The methyl group at position 19 also shifts. This suggests that the OH has been added at either position 1 or 2. The identity of the altered site was assigned using a combination of COSY, NOESY, and ^{13}C heteronuclear multiple bond correlation (HMBC) experiments. New peaks, marked with arrows show the existence of a CHOH (4.16 ppm, 68.62 ppm) and a new environment for a CH_2 (1.99 ppm, 39.64 ppm) (2.17 ppm, 39.64 ppm). Methyl 19 moves to (1.19ppm, 21.35ppm). COSY spectra show that the new CHOH peak at 4.16 ppm is coupled to the new peaks at 1.99 ppm and 2.17 ppm indicating that they are due to hydrogens on neighboring carbon atoms (Supplemental Figure 2). NOESY spectra also show crosspeaks between these peaks. In addition, the NOESY spectrum shows cross peaks between the new CH_2 peaks at 1.99 ppm and 2.17 ppm and the peaks due to position 11 (Supplemental Figure 3). NOESY spectra also show crosspeaks between methyl 19 and H2 and H7. The assignment of methyl 19 was confirmed by the HMBC spectrum. The hypothesized structures of M1 and M2 are shown in Figure 1.

Glucuronidation of MA by HLM

Preliminary experiments revealed that MA does not form any glucuronides by HLM when incubated in the presence of UDPGA alone (data not shown). The conjugated metabolites were also absent from the incubations where HLMs were incubated with NADPH only. When co-incubations including both NADPH and UDPGA cofactors were performed for 24 hrs, two glucuronides were detected. Not enough glucuronide was produced at 3 hrs.

Glucuronidation of MA by recombinant enzymes

Our experiments showed that CYP3A4 is the major isoform responsible for MA oxidation. To determine which UGTs are contributing to its glucuronidation, UGT isoforms were screened in the presence of CYP3A4 and UDPGA + NADPH. Either one or two glucuronides were detected with multiple isoforms. Both glucuronides (MG1 and MG2) were formed by UGT2B17, UGT1A9, UGT1A8 and UGT1A3 (Figure 5A and 5B). Other

isoforms involved in MG1 formation were UGT2B15, UGT2B10, UGT2B7 and UGT1A1. Glucuronides were absent in incubations containing insect control microsomes.

MA activity on the PR

MA oxidative metabolites are active on the PR (Figure 6). R5020 (progesterone agonist, 10 nM) had an average mRNA fold change of 2.2 ± 0.1 , 9.5 ± 0.3 , and 4.8 ± 0.4 for *GREB1* (Figure 6A), *FKBP5* (Figure 6B) and *E2F1* (Figure 6C), respectively. In the same order, MA (100 nM) had an average mRNA fold change of 2.5 ± 0.8 , 8.9 ± 2.4 and 4.8 ± 1.1 , respectively; while M1 (100 nM) had an average mRNA fold change of 2.0 ± 0.3 , 4.7 ± 0.8 , and 2.0 ± 0.4 , respectively; and M2 (100 nM) had an average mRNA fold change of 2.3 ± 0.1 , 7.5 ± 0.2 , and 3.6 ± 0.2 , respectively. All values are represented as the mean of triplicate determination $\pm$ S.D.

MA activity on the AR

PSA is a canonical target gene for the AR and was utilized in this study to gauge the activity of MA on the AR. We used MDV3100 (a potent AR antagonist, 10 μ M) as a positive control which resulted in a decrease in PSA levels (1.1 ± 0.3 ng/ml) compared to vehicle (2.4 ± 0.2 ng/ml). MA displayed some antagonistic effects also (PSA = 1.8 ± 0.01 ng/ml, 1.8 ± 0.3 ng/ml, 1.6 ± 0.1 ng/ml and 1.7 ± 0.1 ng/ml at 1.3 μ M (Figure 7), 0.13 μ M, 2.6 μ M (Figure 7) and 0.26 μ M, respectively. However, both metabolites demonstrated no effect at the AR (M1: 2.3 ± 0.3 ng/ml at 0.7 μ M (Figure 7) and 2.5 ± 0.3 ng/ml at 0.07 μ M; M2: 2.6 ± 0.1 ng/ml at 0.3 μ M (Figure 7) and 2.8 ± 0.4 ng/ml at 0.03 μ M. All values are represented as the mean of triplicate determination $\pm$ S.D.

MA activity on the GR

Dexamethasone (0.1 μ M), the positive control, had an average mRNA fold change of 5.6 (Figure 8A) and 18.5 (Figure 8B) for *SGK1* and *GILZ*, respectively. MA (1 μ M), M1 (1 μ M) and M2 (1 μ M) produced an average mRNA fold change of 9.0, 2.4 and 3.1 (Figure 8A) for *SGK1*, and 21.2, 2.3 and 3.4 for *GILZ* (Figure 8B).

Discussion

This paper provides novel evidence of the involvement of CYP3A4 and UGT2B17 in the metabolism of MA, and structural characterization of the oxidative metabolites of MA formed in humans *in vitro*. In addition, we have shown that MA is first metabolized into two alcohols (M1 and M2) which have activity on the PR. These oxidative metabolites undergo glucuronidation for elimination.

The primary involvement of CYP3A4 in MA metabolism shown herein has also been demonstrated in previous studies using a similar progesterone derived progestin, medroxyprogesterone acetate (MPA) as the substrate (Kobayashi et al., 2000; Zhang et al., 2008). Atypical kinetics are likely attributed to homotropy, as has been reported in the CYP3A family previously (Korzekwa et al., 1998; Houston and Galetin, 2005; Emoto and Iwasaki, 2006).

Since we now know that M1 and M2 are the hydroxylated compounds of MA at C24 and C2, respectively, we hypothesize that conjugation occurs at these hydroxyl groups (proposed structure shown in Figure 3) based on the *in vivo* study showing glucuronidation at these two sites (Cooper and Kellie, 1968). A third glucuronide formed by conjugation with both C24 and C2 in most, but not all, women studied (Cooper and Kellie, 1968) were probably not observed in our incubations as it may be the product of M3. In the oxidation assay, M3 had a shorter retention time than M1, M2 and MA. This suggests that M3 is the most polar metabolite which would be in agreement with the presence of two hydroxyl groups. If this is the case and since its production was very low compared to the single hydroxylated metabolites, it is likely that there was not enough M3 formed to be glucuronidated, or that the production of a third glucuronide was too low to be measured with our assay.

We have shown that MA, M1 and M2 show pharmacologic activity on the PR, while MA additionally shows activity on AR and GR. It is known that MPA activates expression of PR in T47D breast cancer cell lines (Ghatge et al., 2005) and our data are consistent with these findings. In previous studies, short-lived induction of PR target genes has been associated with an increased cell cycle progression rate and potential unregulated cell proliferation in PR-positive T47D breast cancer cells (Yin et al., 2012; Musgrove et al., 1991). The antagonistic effects of MA on the canonical target gene for AR (PSA) have also been observed by MPA in breast cancer cells (Bentel et al., 1999). The increased mRNA gene expressions of the anti-inflammatory genes *GILZ* and *SGK-1* on the GR by MA agrees with previous studies of MPA (Koubovec et al., 2005; Govender et al., 2014). *GILZ* and *SGK-1* induction has been associated with cytoprotection of cardiomyocytes (Aguilar et al., 2013), and inhibition of apoptosis in breast cancer cell lines (Mikosz et al., 2001), respectively. A direct association between MA administration and these effects remains to be investigated.

Because MA is metabolized by CYP3A4 and UGT2B17, there is a high probability for DDIs. CYP3A4 proteins play a major role in drug metabolism and are thought to metabolize roughly 50% of drugs (Hustert et al., 2001). Some drugs known to be potent CYP3A inhibitors including antifungals such as itraconazole (Lohitnavy et al., 2015), or antihypertensive drugs like diltazem (Varhe et al., 1996), may cause adverse reactions in patients also taking MA (demonstrated herein). Since MA is given to treat cancer patients for related anorexia-cachexia, there is additional potential for DDIs with certain chemotherapies. Several chemotherapeutic drugs such as etoposide (Zhuo et al., 2004), ifosfamide (Huang et al., 2000), tamoxifen (Desta et al., 2004), and vinblastine (Zhou-Pan et al., 1993) are known CYP3A4 substrates and can potentially act as competitive inhibitors. We hypothesize that patients receiving MA concomitantly with substrates of UGT2B17, such as 4-methylumbelliferone (Benitez et al., 2013) or ibuprofen (Turgeon et al., 2003), may experience adverse reactions. Further studies need to be performed *in vitro* to investigate this potential DDI.

Overall, the results presented herein characterize the metabolism of MA and demonstrate pharmacologic activity of MA and its oxidative metabolites. Based on our findings, careful consideration should be made when prescribing MA with other CYP3A4 substrates. In addition, as UGT2B17 is polymorphic and oxidative metabolites of MA are pharmacologically active, these findings may be clinically relevant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Nonstandard abbreviations:

^1H, ^{13}C HSQC	heteronuclear single quantum correlation
^1H 1D	one dimensional regular proton spectroscopy
AR	androgen receptor
C	Celsius
CAD	collisionally activated disassociation
CEP	cell exit potential
CL_{int}	intrinsic clearance
COSY	homonuclear correlation spectroscopy
CUR	curtain
DEX	dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DP	declustering potential
EP	entrance potential
ESI	electrospray ionization
E2F1	E2F transcription factor1
GREB1	growth regulation by estrogen in breast cancer 1
FCS	fetal calf serum
FKBP5	FK506 binding protein 5
FP	focusing potential
GILZ/TSC22D3	glucocorticoid-induced leucine zipper/Tsc22d3
GR	glucocorticoid receptor
GS1	gas 1
GS2	gas2
HPLC	high-performance liquid chromatography
HLM	human liver microsomes

HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence spectroscopy
IMDM	Iscove's Modified Dulbecco's Medium
LAPC4	Los Angeles prostate cancer-4
M1	metabolite 1
M2	metabolite 2
M3	metabolite 3
MA	megestrol acetate
MG1	megestrol acetate glucuronide 1
MG2	megestrol acetate glucuronide 2
MPA	medroxyprogesterone acetate
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NOESY	nuclear overhauser enhancement spectroscopy
NMR	nuclear magnetic resonance
P450	cytochrome P450
ppm	part per million
PR	progesterone receptor
PSA	prostate-specific antigen
SGK1	serum/glucocorticoid regulated kinase 1
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy
UDPGA	UDP-glucuronic acid
UGT	UDP-glucuronosyltransferase

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Figure 1.

Kinetic plots for formation of M1 and M2 by HLM (A and B) and CYP3A4 (C and D). MA (5–1000 μM) was incubated with 0.12 mg HLMs or 20 pmol CYP3A4 in the presence of NADPH (final volume, 200 μl) at 37°C for 25 minutes. The velocities (pmol/min/mg protein or pmol/min/pmol P450) vs. MA concentration fit either a substrate inhibition model (A,B,C) or Hill model (D). Data represent the mean of triplicate determination $\pm$ SD.

Figure 2. MA oxidation to (A) M1 and (B) M2 by recombinant P450s using 28 μM and 62 μM substrate. MA was incubated with a panel of 12 recombinant human P450s in the presence of NADPH (final volume, 200 μl) at 37°C for 25 minutes. Data represent the mean of triplicate determination ± SD.

Figure 3.

Proposed pathways and structures of MA undergoing phase 1 metabolism into M1 and M2 based on NMR analysis. MG1 and MG2 are hypothesized structures after undergoing phase 2 metabolism.

Figure 4.
1D ^1H NMR spectra for MA (A), M1 (B) and M2 (C).

Figure 5.
MA glucuronidation to (A) MG1 and (B) MG2 by recombinant UGTs using 100 μ M substrate. MA was incubated with a panel of 13 recombinant human UGTs in the presence of UDPGA (final volume, 200 μ l) at 37°C for 24 hours. Data represent the mean of triplicate determination $\pm$ SD.

Figure 6.

The fold change in (A) *GREB1*, (B) *FKBP5*, and (C) *E2F1* mRNA after treating T47D breast cancer cells for 8 hrs with ethanol, 10 nM progesterone agonist (R5020), 100 nM MA, 100 nM M1 or 100 nM M2. Gene expression was measured by Taqman real time PCR assays. Data represent the mean of triplicate determination $\pm$ SE. The significance of differences was assessed by Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle).

Figure 7.

Total PSA levels after LAPC4 human prostate carcinoma cells were treated for 48 hrs with ethanol (vehicle), 10 µM MDV3100, 1.3 µM MA, 2.6 µM MA, 0.7 µM M1 or 0.3 µM M2. PSA was measured by Elecsys Total PSA Assay. Data represent the mean of triplicate determination $\pm$ SE. The significance of differences was assessed by Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle).

Figure 8.

The fold change in (A) *SGK1* and (B) *GILZ* mRNA after treating MDA-MB-231 triple-negative breast cancer cells for 4 hrs with ethanol, 0.1 μ M dexamethasone, 1 μ M MA, 1 μ M M1 or 1 μ M M2. Real time PCR assays measured glucocorticoid gene expression of both genes. Data represent the mean of triplicate determination.

Table 1.

Kinetic parameters for MA metabolism in pooled HLMs and CYP3A4.

Metabolite	Enzyme	Kinetics model	K_m or S_{50} ^a	V_{max} ^b	CL_{int} ^c	K_i or n ^d
M1	HLM	Substrate inhibition	62 ± 14	3,133 ± 398	51	1,213 ± 418
M1	CYP3A4	Substrate inhibition	182 ± 85	42 ± 15	0.2	388 ± 207
M2	HLM	Substrate inhibition	28 ± 4	865 ± 58	31	1660 ± 401
M2	CYP3A4	Hill	33 ± 3	7 ± 0.3	0.2	2 ± 0.4

^a K_m and S_{50} , apparent substrate concentration at half-maximal velocity (μM)

^b V_{max} , maximal velocity (pmol/min/mg protein) for HLM or (pmol/min/pmol P450) for CYP3A4

^c CL_{int} , intrinsic clearance (μl/min/mg protein)

^d K_i , inhibition constant (μM) for data fitted using the substrate inhibition model or n for data fitted using the Hill equation. Values are expressed as Means ± S.E.