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Metabolic profiling and P450 reaction phenotyping of medroxyprogesterone acetate

Jiang-Wei Zhang, Yong Liu, Jiu-Yang Zhao, Li-Ming Wang, Guang-Bo Ge, Yang Gao, Wei Li, Hong-Tao Liu, Hui-Xin Liu, Yan-Yan Zhang, Jie Sun, Ling Yang

Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China (J.W.Z., Y.L., G.B.G., W.L., H.T.L., H.X.L, Y.Y.Z., L.Y.); The Second Affiliated Hospital of Dalian Medical University, Dalian, China (J.Y.Z., L.M.W., Y.G., J.S.); Graduate School of Chinese Academy of Sciences, Beijing, China (J.W.Z., G.B.G., W.L., H.T.L., H.X.L, Y.Y.Z.)

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Running title: Metabolism of medroxyprogesterone acetate by P450

Address correspondence to: Dr. Ling Yang, Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023 China, Tel: +86-411-84379317, Fax: +86-411-84676961, E-mail: yling@dicp.ac.cn

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Abbreviations:

HLMs, human liver microsomes; HRT, hormone replacement therapy; MPA, medroxyprogesterone acetate; NMR, nuclear magnetic resonance; P450 or CYP, cytochrome P450; PLMs, minipig liver microsomes; RLMs, rat liver microsomes; thioTEPA, triethylenethiophosphoramide.

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Abstract

Medroxyprogesterone acetate (MPA) is one of the most frequently prescribed progestin for conception, hormone replacement therapy, and adjuvant endocrine therapy. MPA has a low oral bioavailability due to extensive metabolism; however, its metabolism was poorly documented. This study was intended to profile the phase I metabolites of MPA and the P450 isoforms involved. After MPA was incubated with human liver microsomes and NADPH-generating system, five main metabolites (namely M-1, M-2, M-3, M-4, and M-5) were isolated by HPLC. Three major metabolites (M-2, M-4 and M-3) were tentatively identified to be 6β-, 2β-, and 1β-hydroxy MPA by LC/MS and ^{1}HNMR. By consecutive metabolism of purified M-2, M-3 and M-4, M-1 and M-5 were proposed to be 2β-, 6β-dihydroxy MPA and 1,2-dehydro MPA, respectively. CYP3A4 was identified to be the isoform primarily involved in the formation of M-2, M-3, and M-4 in studies with specific P450 inhibitors, recombinant P450s, and correlation analysis. Rat and minipig liver microsomes were included evaluating species differences, and the results showed little difference among the species. In human liver microsomes, the K_m values ranged from 10.0 to 11.2 μM, the Vm values ranged from 194 to 437 pmol/min/mg for M-2, M-3, and M-4. In conclusion, CYP3A4 was the major CYP isoform involved in MPA hydroxylation, with 6β-, 2β-, and 1β- being the possible hydroxylation sites. Minipig and rat could be the surrogate models for man in MPA pharmacokinetic studies.

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Introduction

Medroxyprogesterone acetate (17α-acetoxy-6α-methylpregn-4-ene-3,20-dione; MPA, Fig. 1), a synthetic progesterone analog, was used in conception and hormone replacement therapy (HRT) by millions of women worldwide(Ratchanon and Taneepanichskul, 2000). In USA, MPA is the most common HRT progestin in use (Ghatge et al., 2005; Singh, 2007; Otto et al., 2008) and one of the most popular oral conceptive among adolescent girls(Cromer et al., 2004). In large dose, MPA is used in adjuvant endocrine therapy to treat endometrial cancer and breast cancer (Ghatge et al., 2005). MPA was an immunosuppressant, and its potential to treat a number of inflammatory conditions was in clinical trial (Anonymous, 2003).

MPA has a longer half-life than progesterone and can be administrated orally(Ghatge et al., 2005). The oral bioavailability of MPA was low, which was estimated to be 5-15% (Fotherby, 1996). Serum MPA concentration would plateau when oral dose exceeded 1000 mg/d (Etienne et al., 1992). Poor oral bioavailability probably resulted from its low solubility and intense metabolism in the gastrointestinal tract and the liver.

There were considerable reports concerning the adverse effects of MPA, such as irregular bleeding, amenorrhea, weight gain and thrombosis (Chotnopparatpattara and Taneepanichskul, 2000). Our previous findings suggested that there was possibility of drug-drug interactions when MPA was co-administrated with drugs cleared by CYP2C9 (Zhang et al., 2006). Because of the adverse effects, MPA had a high discontinuation rate in contraception (Bonny et al., 2004). Researches from Women's

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Health Initiative Trial indicated that the addition of MPA to conjugated equine estrogens significantly increased the risk of breast cancer (Rossouw et al., 2002; Chlebowski et al., 2003) in HRT. Prescriptions for MPA declined dramatically (Hersh et al., 2004; Wood et al., 2007) thereafter and the reasons for increased breast cancer risk were in intense investigation (Ghatge et al., 2005; Wood et al., 2007; Otto et al., 2008). A recent study suggested that MPA underwent metabolic activation to reactive species that were genotoxic (Siddique et al., 2006). This process was both rat liver S9 (P450) and NADPH dependent (Siddique et al., 2006). It's not clear how the metabolites were activated into reactive species; therefore, it is important to study the metabolic pathway of MPA.

The aim of present study is to identify the main metabolites of MPA in human liver microsomes (HLMs) and the main CYP isoforms involved. Metabolism of MPA was also conducted with minipig liver microsomes (PLMs) and rat liver microsomes (RLMs) to compare the species differences.

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Materials and methods

Chemicals

D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, sulfaphenazole, quinidine, clomethiazole, furafylline, 8-methoxypsoralen, omeprazole, and MPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). S-mephenytoin was purchased from Toronto Research Chemicals Inc. (North York, Canada). Montelukast sodium was obtained from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylenethiophosphoramide (thioTEPA) was purchased from Acros Organics (Geel, Belgium). All other reagents were of HPLC grade or of analytical grade. cDNA-expressed recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 derived from baculovirus infected insect cells coexpressing NADPH-P450 reductase was obtained from BD Gentest Corp. (Woburn, MA, USA). cDNA-expressed CYP2C8 and CYP2C19 in *Escherichia coli* coexpressing NADPH-P450 reductase were purchased from New England Biolabs (Beijing) Ltd (Beijing, China).

Preparation and characterization of liver microsomes

Human livers were obtained from autopsy samples (n=9, male Chinese, ages from 27 to 48) from Dalian Medical University, with the approval of the ethics committee of Dalian Medical University. The medication history of the donors was not known. Research involving human subjects was done under full compliance with government policies and the Helsinki Declaration.

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Procedures involving animals complied with the Laboratory Animal Management Principles of China. Sprague-Dawley rats (n=10, male, weight 180-220 g) were purchased from Dalian Medical University. The animals had free access to tap water and pellet diet. The rats were euthanized by decapitation, and livers were rapidly excised and pooled for preparation of microsomes.

Colony-bred Chinese Bama minipig weighing 10 to 12 kg (n=6, male, age 6 months) were obtained from Department of Animal Science, 3rd Military Medical University, China. The animals used are descendants of sows and boars obtained from the original stock at Bama County, GuangXi Province, China. The animals were euthanized by intravenous injection of pentobarbital sodium (150 mg/kg body weight); tissue samples were taken from the left medial lobe of the liver within 5 min after death. Liver samples were pooled together to prepare microsomes.

Liver specimens were stored in liquid nitrogen until preparation of microsomes. Microsomes were prepared from liver tissue by differential ultracentrifugation as described previously (Li et al., 2006). Protein concentration was determined by using bovine serum albumin as standards (Lowry et al., 1951). Total CYP concentration was determined according to Omura and Sato (Omura and Sato, 1964) with the use of molar extinction coefficient 91 $mM^{-1}\cdot cm^{-1}$. Liver microsomes were diluted to 10 mg/mL and were stored at –80 °C. CYP concentration was 0.63, 0.52, and 0.23-0.39 nmol/mg in PLMs, RLMs, and HLMs, respectively.

Incubation system

The incubation mixture, with a total volume of 200 μL, included 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/mL of glucose-6-phosphate dehydrogenase, and 4

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mM $MgCl_2$), liver microsomes (0.3 mg/mL), and MPA (10 μM). MPA were previously dissolved in methanol, with a final methanol concentration below 1% (v/v) in the reaction mixture. After 3-min preincubation at 37°C, the reaction was initiated by adding NADPH-generating system. After incubated for 10 min in a shaking water bath, the reaction was terminated by the addition of methanol (100 μL). The mixture was kept on ice until it was centrifuged at 20,000× *g* for 10 min at 4 °C. Aliquots of supernatants were transferred for HPLC analysis. Control incubations without NADPH or without substrate or without microsomes were included to ensure that metabolites formation were microsomes and NADPH dependent. All incubations were carried out in duplicate, and results were expressed as mean ± SD.

HPLC/MS method

The HPLC system (SHIMADZU, Kyoto, Japan) consisted of a SCL–10A system controller, two LC–10AT pumps, a SIL–10A auto injector, a SPD–$10A_{VP}$ UV detector and a Shim-pack (Shimadzu corporation) C_{18} column (4.6×150 mm, 5 μ) was used to separate MPA and its metabolites. The mobile phases were solvent B, H_2O and solvent A, CH_3OH, with linear gradient from initially 52% A to 80% A over 20 min. The eluent was monitored at 254 nm with a flow rate of 1 ml/min.

LC/MS was used to characterize the structures of MPA metabolites. The HPLC eluent from detector was introduced into the mass spectrometer via a 1:4 split. The mass spectrometer was a TSQ triple quadrupole (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ESI interface. The spray voltage was 4.5 kV and the capillary temperature was 300 ºC. Nitrogen was used as nebulizing and auxiliary gas.

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The nebulizing gas backpressure was set at 40 psi, and auxiliary gas at 20 (arbitrary units). Initially, the mass spectrometer was programmed to perform full scans between m/z 200 and 500 in order to observe the $[M+H]^+$ and $[M-H]^-$ signals.

Metabolite purification

PLMs were used to prepare MPA metabolites since it resemble HLMs in MPA metabolism. The incubation system was scaled up to 500 mL. MPA (200 μM) was incubated with PLMs (1.0 mg/mL) and NADPH-generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/mL of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$) for 60 min at 37 °C. Under these conditions, about 5%, 3%, and 5% of MPA was converted to M-2, M-3, and M-4, respectively. Methanol (250 mL) was added to the reaction mixture to precipitate the protein. After centrifuged at 9000× *g* for 10 min, the supernatant was separated and extracted with ethyl acetate (250 mL ×3). The organic layer was combined and dried in vacuo. Then the residue was dissolved in ethyl acetate (1 mL) and separated by preparative TLC (Silica gel, 20×20 cm, 2 mm, Merck), which was developed by chloroform-acetone (9:1, v/v) (McCamish et al., 1979). MPA and its metabolites were monitored under UV light at 254 nm. The metabolites were isolated, and they were further purified by HPLC. The purity of M-2, M-3, M-4 was about 99%, 97%, and 99% (HPLC), respectively.

NMR spectroscopy

Proton NMR spectra were obtained at 400 MHz on a Bruker AV-400 spectrometer (Bruker, Newark, Germany). Compounds were dissolved in $CDCl_3$ and experiments were conducted at 21 °C. Chemical shifts are reported in ppm with reference to

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tetramethylsilane.

Assay with recombinant CYPs

cDNA-expressed recombinant human CYP isoforms co-expressing NADPH-P450 reductase either from insect cells (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4) or from *Escherichia coli* (CYP2C8, and CYP2C19) was used. The incubations were carried out as described for the human liver microsomal study. To examine the contribution of each CYP isoform, MPA (100 μM) was incubated with each of the recombinant CYPs (40-80 nM) at 37°C for 30 min. HPLC with UV detection was used to monitor possible metabolites. Relative high substrate concentration was selected so that adequate metabolites were generated for the convenience of detection.

Correlation Study

Correlation studies were performed by incubation of MPA (10 μM, near K_m value) with liver microsomes (0.3 mg/mL) from nine individual donors for 10 min. The isoform specific maker reactions were as follows (Zhang et al., 2007): phenacetin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4'-hydroxylation (CYP2C9), S-mephenytoin 4'-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), paclitaxel 3'-p-hydroxylation (CYP3A4) and testosterone 6β-hydroxylation (CYP3A4). The correlation parameter was expressed by the linear regression coefficient (r).

Chemical inhibition study

Chemical inhibition studies were performed by adding different human CYP inhibitors to the incubation mixture of MPA (10 μM) before the addition of

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NADPH-generating system. The serum level of MPA can be as high as 1.7 μM (Ghatge et al., 2005). The selection of a 10-μM concentration was based on the K_m value, and relative high substrate concentration was selected so that adequate metabolites were generated for the convenience of detection. The selective inhibitors of eight major CYPs and their concentrations were as follows (Bjornsson et al., 2003): furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, thioTEPA (50 μM) for CYP2B6 (Rae et al., 2002), montelukast (2 μM) for CYP2C8 (Walsky et al., 2005), sulfaphenazole (10 μM) for CYP2C9, omeprazole (20 μM) for CYP2C19 (Ko et al., 1997), quinidine (10 μM) for CYP2D6, clomethiazole (50 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A4. 8-Methoxypsoralen is known as a mechanism-based inhibitor, so it was preincubated with HLMs, buffer, and NADPH-generating system at 37 °C for 3 min (Koenigs et al., 1997), and the reaction was initiated by the addition of MPA. Furafylline was a potent competitive inhibitor for CYP1A2 (Sesardic et al., 1990); therefore it was used without pre-incubation.

Troleandomycin (Anzenbacher et al., 1998), ketoconazole (Li et al., 2006), sulfaphenazole (Kobayashi et al., 2003), and furafylline (Kobayashi et al., 2003) were found to be inhibitors of rat or minipig CYP3A, CYP2C and CYP1A, respectively. Therefore, inhibitory effects of troleandomycin (25 μM), ketoconazole (1 μM), sulfaphenazole (10 μM), and furafylline (10 μM) towards MPA (10 μM) metabolism in RLMs and PLMs were examined. Troleandomycin was preincubated with liver microsomes, buffer, and NADPH-generating system at 37 °C for 10 min, and the reaction was initiated by the addition of MPA.

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Kinetic study

To estimate kinetic parameters, MPA (5-100 μM) was incubated with liver microsomes (0.3 mg/mL) for 10 min. When the substrate concentration was lower than 5 μM, so few metabolites were produced by the liver microsomes that it's difficult to accurately measure the velocities. For recombinant CYP3A4, MPA (2-40 μM) was incubated with CYP3A4 (10 nM) for 10 min for kinetic analysis. The apparent V_m and K_m values were calculated from nonlinear regression analysis of experimental data according to the Michaelis-Menten equation. Preliminary experiments were carried out to make sure that formation of metabolites was in the linear range of both reaction time and the concentration of microsomes.

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Results

Detection of MPA metabolites in different species

After MPA (100 μM) was incubated with HLMs (0.5 mg/mL) and NADPH-generating system for 30 min, at least five main new peaks (M-1 to M-5) were detected by LC-UV (Fig. 2). These new peaks were not observed in the negative controls without NADPH, or without substrate, or without microsomes (data not shown). The formation rate of the main metabolites was in the following order: M-2 > M-4 > M-3 > M-5 ≈ M-1 in HLMs (Table 1). To investigate inter-individual differences, MPA (100 μM) was incubated with HLMs (0.3 mg/mL) from nine donors for 10 min, and the results showed more than 10-fold difference in the biotransformation rates (Fig. 3). Despite rates of metabolism fluctuated in different samples; relative abundance of main metabolites was constant (M-2 > M-4 > M-3 > M-5 ≈ M-1).

To compare species differences, PLMs and RLMs were also included to study MPA metabolism. Similar metabolites profiles were obtained from PLMs and RLMs, where M-2, M-3 and M-4 were the dominant metabolites (Table 1).

Metabolites identification

The positive-ion mode was adopted for structure analysis because it's more sensitive than the negative-ion mode in present investigation. Mass spectra were dominated by $[M+K]^+$. The m/z for the $[M+K]^+$ of M-1, M-2, M-3, M-4, M-5 and MPA were 457.1, 441.1, 441.1, 441.1, 423.1 and 425.1, respectively. Accordingly, the

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molecular weight (MW) was calculated to be 418, 402, 402, 402, and 384 for M-1 to M-5 (Table 1), indicating the incorporation of one (M-2, M-3, and M-4) or two (M-1) oxygen atom, or loss of hydrogen (M-5, Table 1).

The structures of the three major mono-hydroxylated metabolites of MPA, namely M-2, M-3, M-4 were determined by ^{1}HNMR (Table 2). M-2 was the most abundant metabolite; and the most distinctive spectra changes were involved in the C6 region. The 6α-methyl proton signal at 1.07 ppm (3H, d) was replaced by 1.43 ppm (3H, s), indicative of hydroxylation at 6β position, and the 4-proton signal at 5.79 ppm (1H, s) was replaced by 6.04 ppm (1H, s) (Table 2). Moreover, the ^{1}HNMR spectrum of M-2 was in agreement with those reported for 6β-hydroxy-MPA (Fang et al., 1986; Guo et al., 2006), therefore, M-2 was identified as 6β-hydroxy-MPA.

^{1}HNMR spectrum of M-3 was characterized by the simultaneous shift about 0.2 ppm towards the low field for 2α-H, 2β-H, and 6β-H compared with MPA (Table 2). The methyl groups at 6, 18, 19, 21, and 23 were intact, and 19-methyl signal at 1.18 ppm (3H, s) shifted slightly to 1.26 ppm (3H, s). In comparison with the spectra changes between 1β-hydroxytestosterone and testosterone in ^{1}HNMR (Krauser et al., 2004), M-3 was tentatively identified as 1β-hydroxy-MPA. The signal appeared at 4.11 ppm (1H, m) was assigned to 1α proton. Final confirmation of the structure requires comparison with the authentic standard.

M-4 was tentatively identified as 2β-hydroxy-MPA based on the following observation (Table 2): the five methyl groups were intact, and the 2α-proton at 2.35 ppm (1H, m) was replaced by 4.23 ppm (1H, q), and 1α-proton at 1.7 ppm (1H, m)

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was replaced by 2.68 ppm (1H, m). Again final proof of the structure awaits comparison with synthetic standard.

M-1 and M-5 were not separated in large quantity due to low transformation rate. Their structures were inferred indirectly by subsequent metabolism of M-2, M-3 and M-4. Following 10-min incubation with HLMs (0.5 mg/mL) and NADPH-generating system at 37 °C, both purified M-2 and M-4 (about 10 μM) were able to produced M-1, which suggested that M-1 was hydroxylated consecutively at 2β and 6β position, i.e. 2β-, 6β-dihydorxy MPA (MW=418). M-3 (MW=402) was unstable at 37°C (pH 7.4); it slowly converted to M-5 (MW=384), suggesting loss of water. Accordingly, the structure of M-5 is proposed to be 1,2-dehydro MPA.

Chemical inhibition studies

P450 phenotyping and kinetic studies were conducted only for three dominant mono-hydroxylated metabolites, i.e. M-2, M-3, and M-4. Selective inhibitors of the nine major CYPs were used to screen the CYP isoforms responsible for formation of the metabolites in HLMs (Fig. 4). Among tested inhibitors, ketoconazole inhibited MPA metabolism almost completely, with no metabolites detectable. 8-Methoxypsoralen also inhibited formation of three metabolites by about 50%. Inhibitors of other CYP isoforms didn’t show significant inhibition (less than 20% inhibition). Therefore, CYP3A4 might be the major CYP isoform involved in the formation of M-2, M-3, and M-4.

Inhibition of MPA metabolism in RLMs and PLMs by CYP3A, CYP1A, and

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CYP2C inhibitors was also performed. In PLMs, troleandomycin (25 μM) and ketoconazole (1 μM) inhibited the formation of three metabolites (M-2 to M-4) by about 50% and 60%, respectively. In RLMs, troleandomycin and ketoconazole inhibited the formation of three metabolites by about 30% and 60%, respectively. Compared with other two metabolites, M-2 was less sensitive to the inhibitory effect of troleandomycin and ketoconazole. Furafylline and sulfaphenazole exhibited less than 10% inhibition in both RLMs and PLMs for the formation of three metabolites.

Assay with recombinant human CYPs

To further verify CYP isoforms involved in the metabolism of MPA, activity of MPA hydroxylation was determined using nine cDNA-expressed CYP isoforms. The there main metabolites (M-2, M-3, and M-4) were formed exclusively by CYP3A4. None of the three metabolites were detected in the incubation with CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 (less than 0.01 pmol/min/pmol CYP). Therefore, all three metabolites were ascribed to CYP3A4.

Correlation Study

Correlation was the highest between the formation of three metabolites (M-2, M-3, and M-4) and testosterone 6β-hydroxylation (CYP3A4, r>0.9, Table 3), paclitaxel 3'-p-hydroxylation (CYP3A4, r>0.9). MPA hydroxylation also correlated with phenacetin O-deethylation (CYP1A2, r=0.6), coumarin 7-hydroxylation (CYP2A6,

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r=0.8), and paclitaxel 6α-hydroxylation (CYP2C8, r=0.8). These correlations probably result from high correlation among individual CYP isoforms (r=0.7 between CYP1A2 and CYP3A4, r=0.9 between CYP2A6 and CYP3A4, and r=0.9 between CYP2C8 and CYP3A4). Moreover, recombinant CYP1A2, CYP2A6, and CYP2C8 failed to metabolize MPA. Therefore, CYP3A4 was the major CYP isoform involved in the formation of M-2, M-3, and M-4 in HLMs.

Kinetic of MPA metabolism

H1 liver sample was selected for kinetic study because it represented moderate CYP3A4 activity. In liver microsomes, formation rates of MPA metabolites were linear up to 0.3-mg/mL microsomal protein and 10-min incubation. Thus, 0.3 mg/mL HLMs and 10 min were adopted in the following kinetic assay. Due to limitation of detection sensitivity and low biotransformation rate, it's difficult to accurately quantify the metabolites in low substrate concentration range; therefore, the range of substrate concentrations for kinetic analysis in liver microsomal assay was 5-100 μM, and 2-40 μM in CYP3A4. In the concentration range tested, MPA hydroxylation obeyed the Michaelis-Menten kinetics, as evidenced by Eadie-Hofstee plot (data not shown). The kinetic parameters, including K_m and V_m for MPA hydroxylation were listed in Table 4 and results of typical kinetic experiments of MPA hydroxylation in HLMs were graphically displayed in Fig. 5. All three metabolites exhibited similar K_m values in HLMs (around 10 μM) and recombinant CYP3A4 (ranged from 6.1 to 11.4 μM), indicating comparable binding affinities towards these hydroxylation sites

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(Table 4). In PLMs and RLMs, however, the K_m values were higher and more diversified (Table 4), reflecting species differences. The intrinsic clearance (V_m/K_m) was highest for M-2 among the three metabolites in the incubation system with HLMs, PLMs, and recombinant CYP3A4; suggesting M-2 was the major metabolism pathway. The V_m/K_m value for M-4 was the highest in PLMs.

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Discussion

The 6α-methyl and 17-acetoxy in MPA makes it more resistant to hepatic metabolism than progesterone (McCamish et al., 1979). These structure modifications improve the pharmacokinetics behavior of MPA by increasing the half life and oral bioavailability. Nevertheless, the slow metabolism property also made metabolic studies more difficult (McCamish et al., 1979). In vitro MPA metabolism study dated back to 1979, McCamish et al isolated three metabolites of MPA in the incubation system of RLMs (McCamish et al., 1979). C6 and C2 were proposed as hydroxylation sites for two metabolites by mass spectra (McCamish et al., 1979). In vivo evidences suggested that hydroxylation is the main reaction involved in MPA metabolism (Helmreich and Huseby, 1962). The preferred hydroxylation sites were 2-, 6- and 21-positions(Sturm et al., 1991), with 6β, 21-dihydroxy-MPA was the main metabolite (Helmreich and Huseby, 1962; Fukushima et al., 1979). While has been extensively studied in vivo, MPA metabolism by modern methodology was relative poorly documented (Dollery, 1998, M17-21; Lobo, 1999; Mimura et al., 2003). For example, the structures of the metabolites were mostly proposed by mass spectra and were not fully characterized. For in vivo studies, some artifacts would be introduced during long hydrolysis and extraction process of urine or stool (Castegnaro and Sala, 1962; Fukushima et al., 1979).

Follow incubation with HLMs, the main MPA metabolites were proposed to be 6β-, 2β-, and 1β- hydroxy MPA by LC/MS and ^{1}HNMR (Table 1 and Table 2). Hydroxylation at 6β- and 2β- positions was in agreement with previous speculations

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(McCamish et al., 1979; Sturm et al., 1991). We also observed a dehydro MPA (M-5), which was observed by McCamish et al. (McCamish et al., 1979). Previous studies suggested that the MPA 21-hydroxylation represented one of the major metabolism routes (Helmreich and Huseby, 1962; Sturm et al., 1991), however, the structure of 21-hydroxy MPA or 6β, 21-dihydroxy-MPA was not confirmed by ^{1}HNMR. The 21-hydroxy MPA was not detected, or at least not a major metabolite in the present study. M-2, M-3, and M-4, three major metabolites were excluded to be 21-hydroxy MPA, because the ^{1}HNMR spectra showed that the 21-proton were intact. M-1 (MW=418) was proposed to be 2β-, 6β-dihydorxy MPA since it was a metabolite of both M-2 (6β-hydorxy MPA) and M-4 (2β-hydorxy MPA). M-5 was a degradation product of M-3 (1β-hydorxy MPA) and mass spectra suggested loss of water (MW 384 versus 402). Therefore, the structure of M-5 was proposed to be 1, 2-dehydro MPA. M-2, M-3 and M-4 are dominant metabolites, which account for more than 85% of the five metabolites. Although M-1 and M-5 are the minor metabolites in vitro, they could be the major metabolites in vivo, where lower substrate concentration and longer biotransformation time were expected.

Under linear conditions, i.e. 0.3 mg/mL HLMs and 10-min incubation, formation of M-1 and M-5 was negligible, therefore, kinetic and P450 phenotyping studies were conducted only for the dominant mono-hydroxylated metabolites, i.e. M-2, M-3, and M-4. CYP3A4 was identified as the main CYP isoform involved in MPA metabolism in HLMs by the following observation: 1) ketoconazole inhibited the formation of M-2, M-3 and M-4 almost completely; 2) only CYP3A4 was able to catalyze MPA to

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M-2, M-3 and M-4; 3) the formation of M-2, M-3 and M-4 correlated with testosterone 6β-hydroxylation. This result was in agreement with study by monitoring the parent drug disappearance (Kobayashi et al., 2000). In HLMs, 8-methoxypsoralen inhibited the metabolites formation by about 50%. However, 8-methoxypsoralen also inhibited the formation of MPA metabolites to a similar extent when MPA was incubated with recombinant CYP3A4 (data not shown). Therefore, the inhibitory effect of 8-methoxypsoralen towards MPA metabolism in HLMs resulted from its inhibitory effect towards CYP3A4. The contribution of CYP2A6 to the formation of three metabolites of MPA was negligible because none of the metabolites was detected when MPA was incubated with recombinant CYP2A6. Accordingly, the metabolic pathway of MPA was proposed in Fig. 6.

The spectra of metabolites were in general rather similar among liver microsomes from human, rat and minipig. In PLMs or RLMs incubation system, M-2 was also the most abundant metabolite, followed by M-4 and then by M-3 (Table 1). Ketoconazole, a CYP3A inhibitor, inhibited the metabolites formation in both PLMs and RLMs incubation system, which indicated the possible involvement of CYP3A in MPA metabolism. The involvement of CYP3A in MPA metabolism has also been confirmed in rats based on parent drug disappearance (Mimura et al., 2003). These results suggested that rat and minipig could be surrogate models for man in MPA metabolism study.

Although MPA itself rather than its metabolites was regarded as the active form (Ghatge et al., 2005), the biological activities of its metabolites were not fully

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characterized. Structure of M-5 is of interest because it resembles quinoids that might eventually produce reactive oxygen species that are genotoxic (Siddique et al., 2006). However, M-5 was not separated in adequate amount for toxicological analysis due to low yield, and such a hypothesis worth further investigation.

In conclusion, three main hydroxylation site of MPA were proposed to be 6β, 2β, and 1β position, which lead to five major metabolites. The main metabolites were generated by CYP3A in human. PLMs and RLMs metabolized MPA in a similar way to HLMs. Clarification of MPA metabolites and the involving CYP isoforms is helpful to the studies of pharmacological and toxicological property of metabolites.

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Footnotes

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Author for correspondence: Dr. & Prof. Ling Yang, Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, NO.457 Zhongshan Road, Dalian, 116023 China, E-mail: yling@dicp.ac.cn

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Figure legends

Fig. 1. Structure of MPA. Arrows denote proposed hydroxylation sites for its main metabolites.

Fig. 2. Representative HPLC profiles of MPA and its metabolites (M-1 to M-5). MPA (100 μM) was incubated with HLMs (0.5 mg/mL) at 37 °C for 30 min with NADPH-generating system as described in Materials and Methods.

Fig. 3. Metabolism of MPA in nine different human liver samples (H1 to H9). MPA (100 μM) was incubated with liver microsomes (0.3 mg/mL) for 10 min. Mean ± SD of duplicate incubations.

Fig. 4. Inhibition of MPA metabolism by selective P450 inhibitors in HLMs. The selective inhibitors of eight major CYPs and their concentrations were as follows: furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, thioTEPA (50 μM) for CYP2B6, montelukast (2 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, omeprazole (20 μM) for CYP2C19, quinidine (10 μM) for CYP2D6, clomethiazole (50 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A4. Mean ± SD of duplicate incubations.

Fig. 5. Michaelis-Menten plots of MPA metabolism in H1 liver microsomes. MPA (5.0-100 μM) was incubated with HLMs (0.3 mg/mL) at 37 °C for 10 min with NADPH-generating system.

Fig. 6. Proposed metabolic pathway of MPA in HLMs.

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Table 1. Formation rates of different MPA metabolites (M-1 to M-5) in different species. MPA (100 μM) was incubated with liver microsomes (0.3 mg/mL) for 10 min. The rates of formation are expressed in pmol/mg/min. Results represent average of duplicate incubations.

Metabolite	M-1	M-2	M-3	M-4	M-5
t_R(min)	13.4	18.7	20.1	22.1	23.4
t_R(%)	51	71	76	84	89
MW	418	402	402	402	384
HLMs	54	391	172	227	52
PLMs	33	264	168	218	63
RLMs	51	321	86	300	28

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Table 2. ^{1}HNMR data for MPA and its metabolite (M-2, M-3, and M-4).

	δ(ppm)			
Proton	MPA	M-2	M-3	M-4
18-CH_3	0.67, s	0.71, s	0.68, s	0.68, s
6-CH_3	1.07, d	**1.43, s**	1.08, d	1.06, d
19-CH_3	1.18, s	**1.40, s**	1.26, s	1.17, s
21-CH_3	2.03, s	2.05, s	2.03, s	2.04, s
23-CH_3	2.09, s	2.10, s	2.10, s	2.10, s
6β	2.42, m	N/A	**2.58, m**	2.48, m
1α	–	–	**4.11, m**	**2.68, m**
2α	2.35, m	2.40, m	**2.56, m**	**4.23, m**
2β	2.43, m	2.53, m	**2.61, m**	N/A
16β	2.93, m	2.95, m	2.93, m	2.94, m
4-H	5.79, s	**6.04, s**	5.84, s	5.81, s

N/A, no proton attached; –, shift was not assigned.

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Table 3. Correlation (r) values between formation rates of MPA metabolites (M-2, M-3, and M-4) and P450 isoform specific activities. MPA (10 μM) was incubated with liver microsomes (0.3 mg/mL) for 10 min.

P450s	P450 isoform specific reactions	M-2	M-3	M-4
CYP1A2[a]	Phenacetin O-deethylation	0.62	0.61	0.61
CYP2A6[a]	Coumarin 7-hydroxylation	0.82	0.82	0.83
CYP2C8[a]	Paclitaxel 6α-hydroxylation	0.82	0.83	0.83
CYP2C9	Diclofenac 4'-hydroxylation	-0.11	-0.11	-0.08
CYP2C19	S-Mephenytoin 4'-hydroxylation	0.14	0.14	0.13
CYP2D6	Dextromethorphan O-demethylation	0.31	0.32	0.35
CYP2E1	Chlorzoxazone 6-hydroxylation	0.37	0.39	0.42
CYP3A4	Testosterone 6β-hydroxylation	0.97	0.98	0.98
CYP3A4	Paclitaxel 3'-p-hydroxylation	0.99	0.99	0.98

[a]No metabolite formation was observed by recombinant CYP1A2, CYP2A6, and CYP2C8.

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Table 4. Kinetic parameters of MPA metabolism in HLMs, RLMs, PLMs and recombinant CYP3A4.

Metabolite	HLMs			PLMs			RLMs			CYP3A4		
	V_m	K_m	V_m/K_m	V_m	K_m	V_m/K_m	V_m	K_m	V_m/K_m	V_m	K_m	V_m/K_m
M-2	437	11.2	39.2	349	41.0	8.5	453	46.5	9.8	21.1	6.1	3.5
M-3	194	10.1	19.2	184	17.2	10.7	97	21.4	4.5	9.6	11.4	0.8
M-4	253	10.0	25.5	261	26.8	9.8	330	8.6	38.5	13.4	6.4	2.1

K_m values were in μM; V_m values were in pmol/mg/min for liver microsomes, or in min^{-1} for CYP3A4. The range of substrate concentrations was 5-100 μM for liver microsomes, or 2-40 μM for CYP3A4.

FIG1

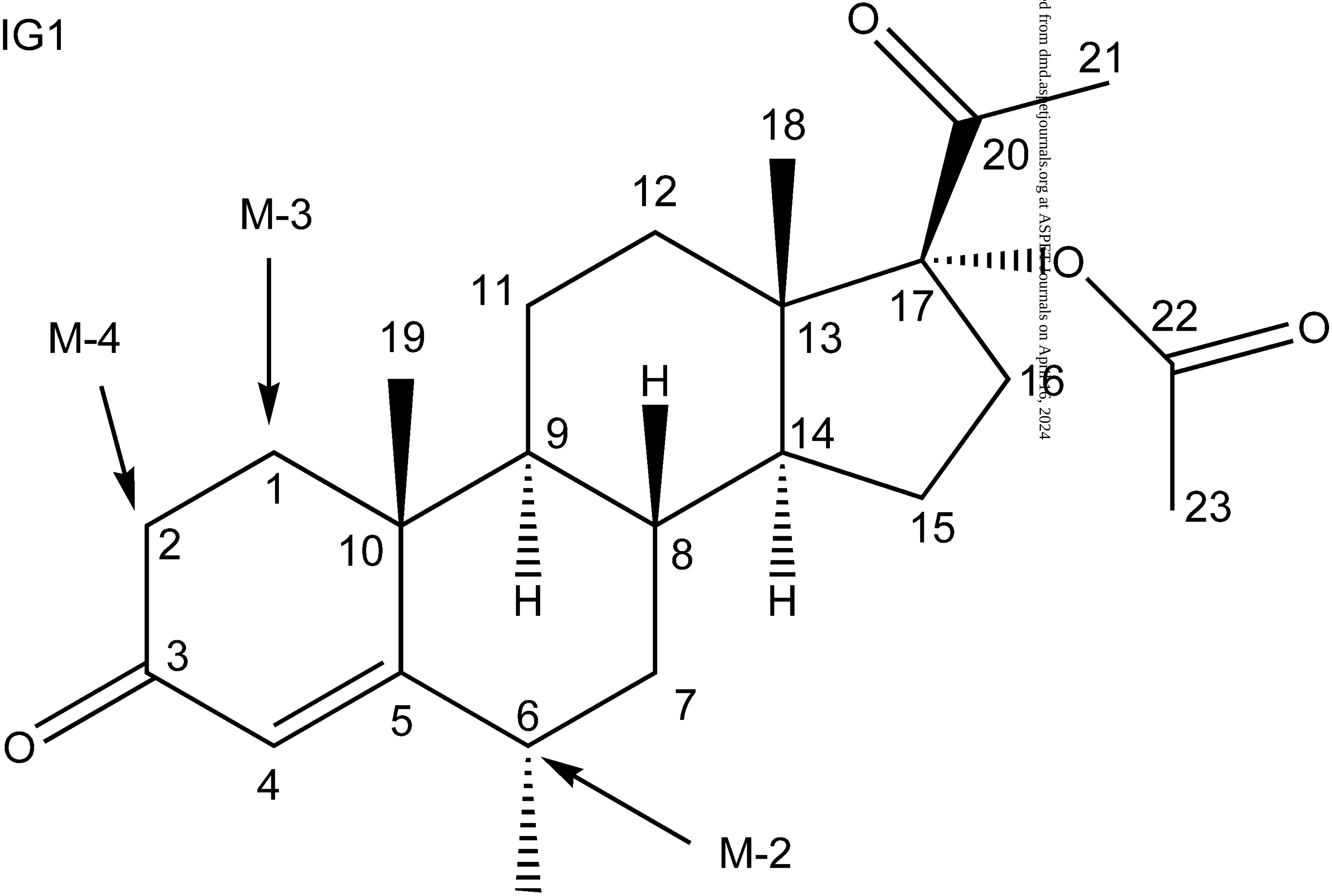

M-3
M-4
M-2
O
O
O
O
H
H
H
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

FIG2

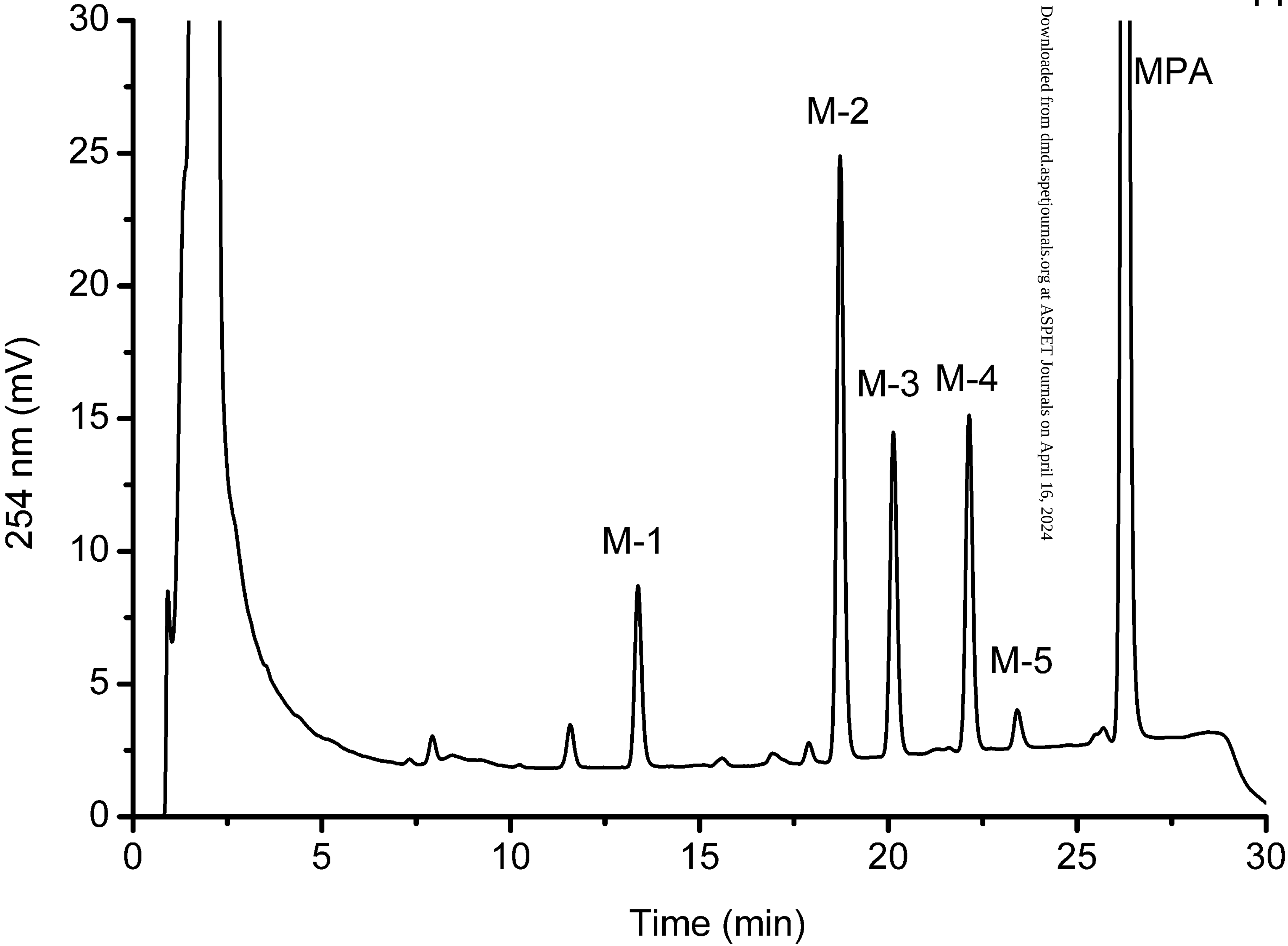
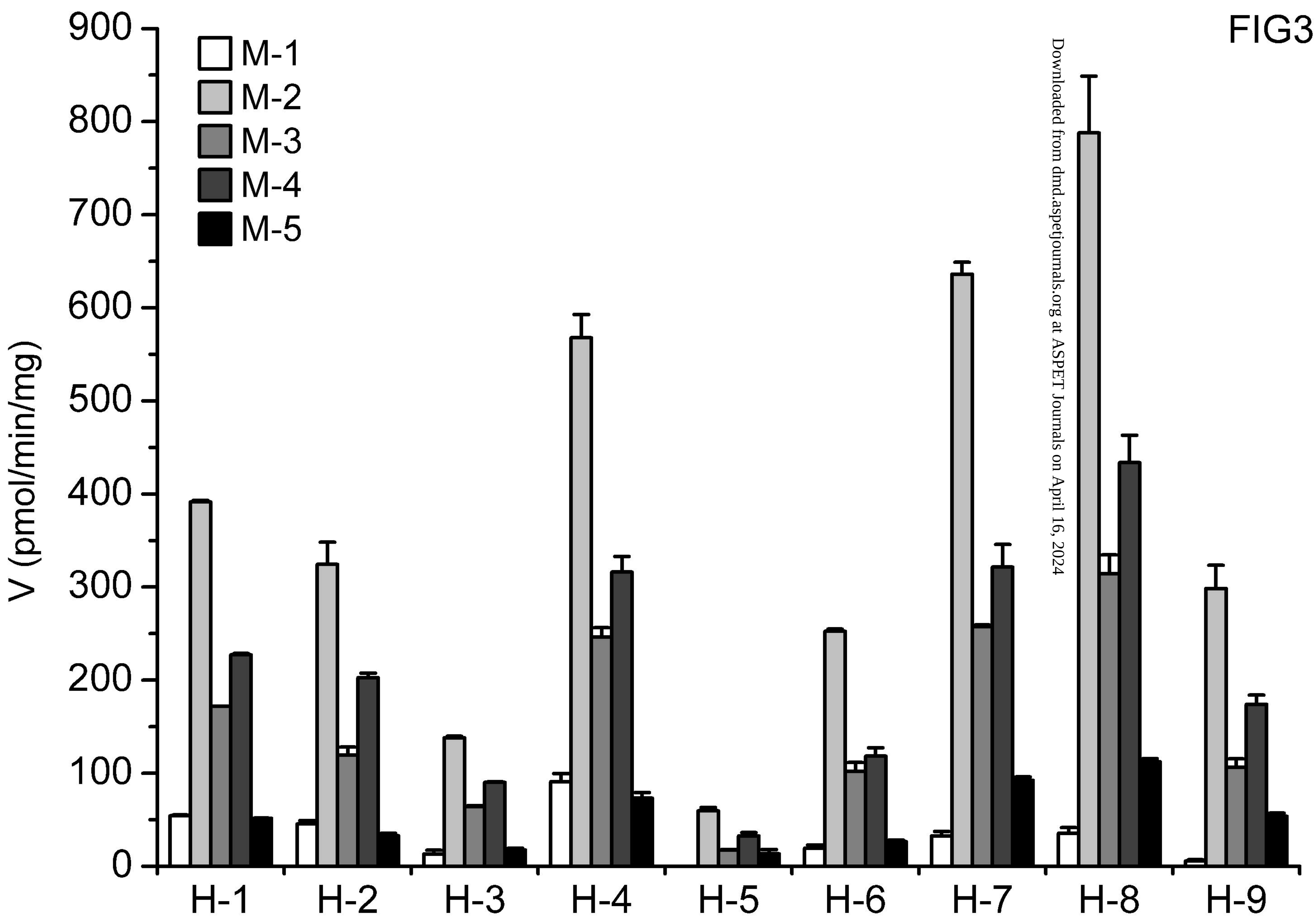
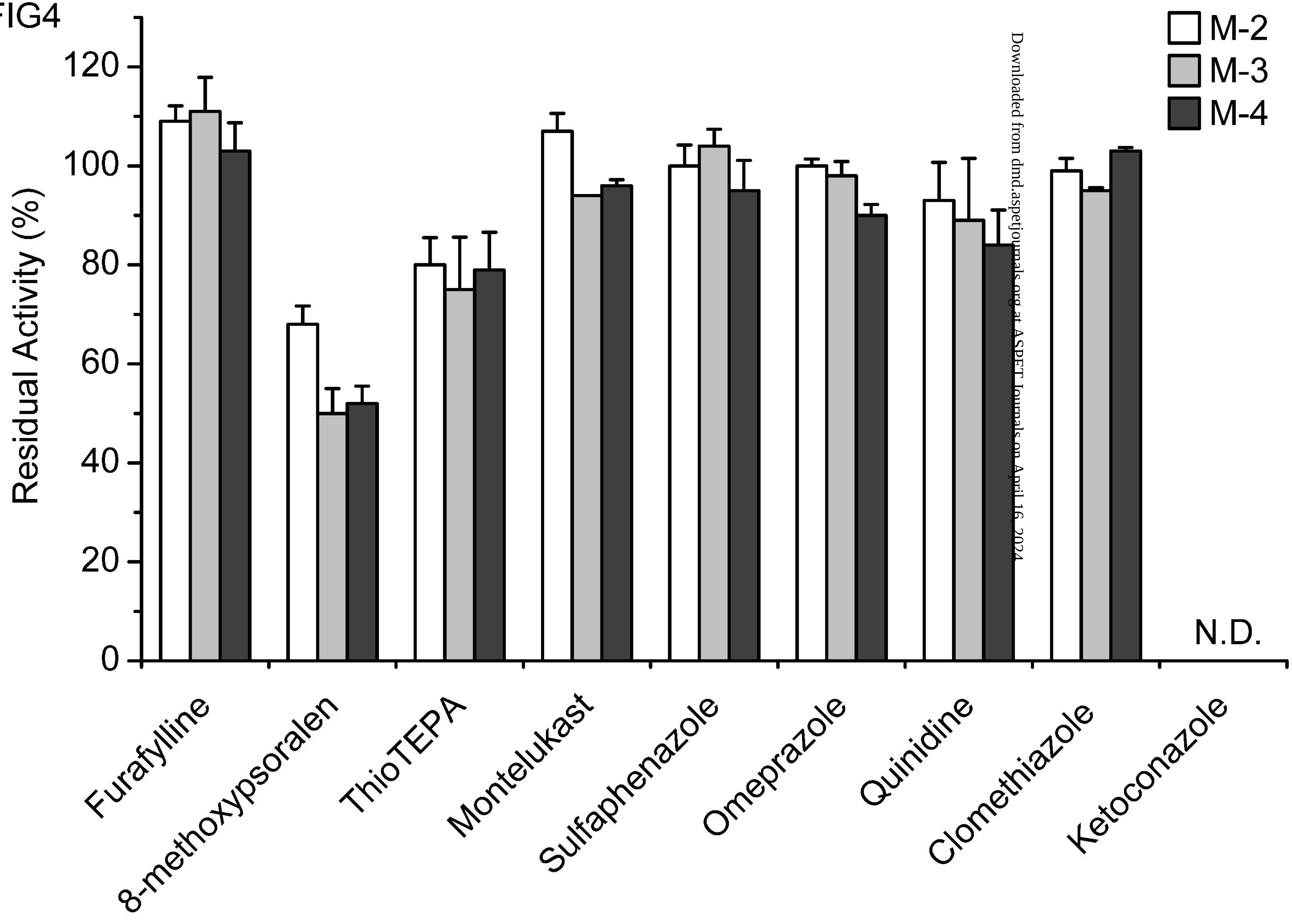


FIG3



900
800
700
600
500
400
300
200
100
0
V (pmol/min/mg)
M-1
M-2
M-3
M-4
M-5
H-1
H-2
H-3
H-4
H-5
H-6
H-7
H-8
H-9

FIG4



M-2
M-3
M-4
Residual Activity (%)
0
20
40
60
80
100
120
N.D.
Furafylline
8-methoxypsoralen
ThioTEPA
Montelukast
Sulfaphenazole
Omeprazole
Quinidine
Clomethiazole
Ketoconazole

FIG5

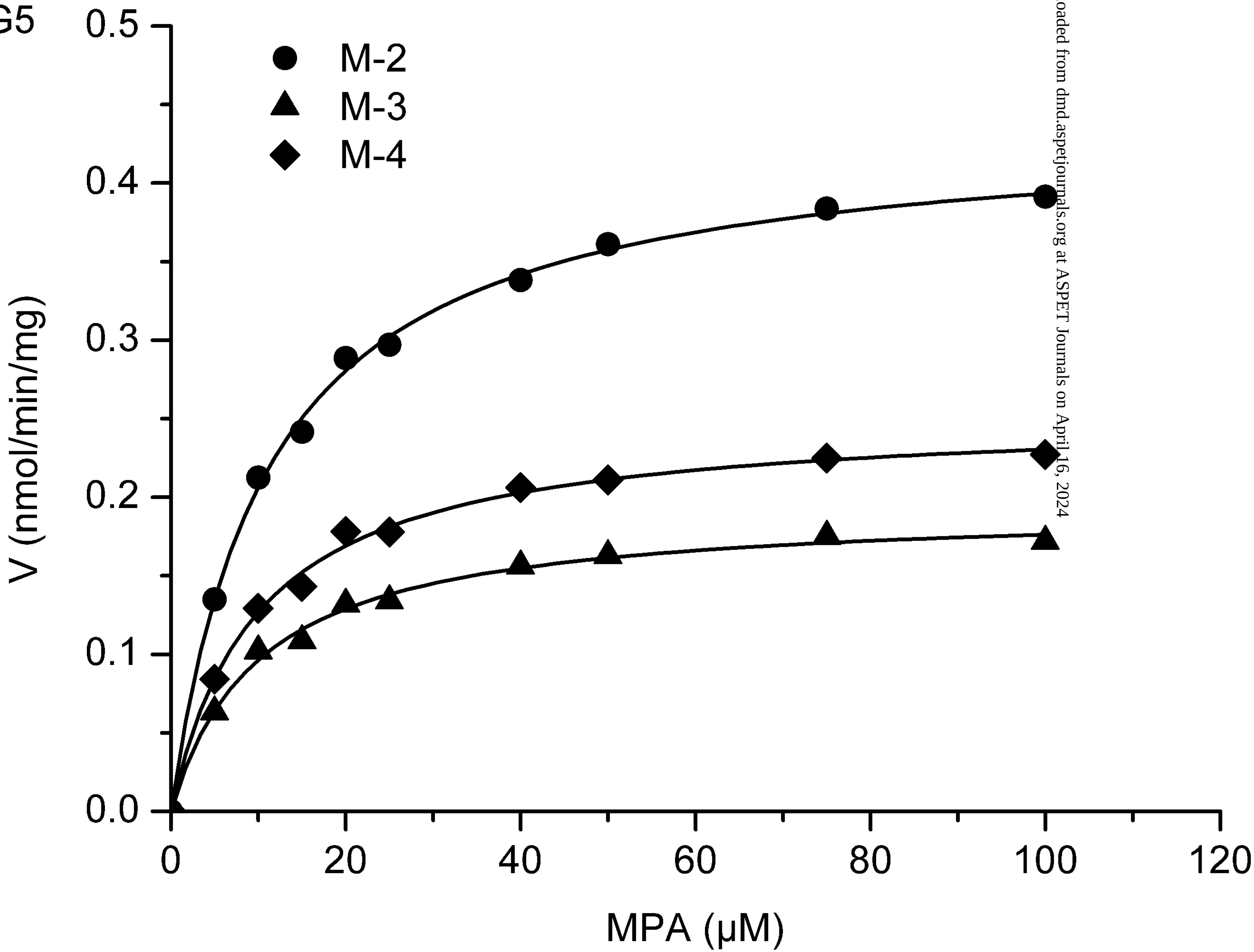


FIG6