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DMD #056119

Short Communication

Bedaquiline metabolism: enzymes and novel metabolites

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Running title: Bedaquiline metabolism

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Abbreviations: BDQ, bedaquiline; MDR-TB, multi-drug resistant tuberculosis; P450, cytochrome P450; UPLC-QTOFMS, ultra performance liquid chromatography coupled to time-of-flight mass spectrometry; OPLS-DA, orthogonal partial least-squares discriminant analysis

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Abstract

Bedaquiline is a recently approved drug for treatment of multi-drug resistant tuberculosis. Adverse cardiac and hepatic drug reactions of bedaquiline have been noted in clinical practice. The current study investigated bedaquiline metabolism in human hepatocytes using a metabolomic approach. Bedaquiline *N*-demethylation *via* cytochrome P450 3A4 (CYP3A4) was confirmed as the major pathway in bedaquiline metabolism. Besides CYP3A4, we found that both CYP2C8 and CYP2C19 contributed to bedaquiline *N*-demethylation. The K_m values of CYP2C8, CYP2C19 and CYP3A4 in bedaquiline *N*-demethylation were 13.1, 21.3 and 8.5 μM, respectively. We also identified a novel metabolic pathway of bedaquiline that produced an aldehyde intermediate. In summary, this study extended our knowledge of bedaquiline metabolism, which can be applied to predict and prevent drug-drug interactions and adverse drug reactions associated with bedaquiline.

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Introduction

Multi-drug resistant tuberculosis (MDR-TB) is defined as tuberculosis (TB) with resistance to at least isoniazid and rifampicin, the two most powerful first-line anti-TB drugs (Chang and Yew, 2013). There were ~0.31 million incident cases of MDR-TB among TB patients in 2011. Treatment of MDR-TB requires second line anti-TB agents given over a period of 18-24 months (Mukherjee et al., 2004). Significant side effects and high patient death rate were observed during the long-term treatment against MDR-TB (WHO, 2013). Thus, there is an urgent need to develop shorter and less toxic regimen to reduce the side effects and mortality associated with MDR-TB chemotherapy.

Bedaquiline (BDQ) was recently approved by the United States Food and Drug Administration for the treatment of MDR-TB (FDA, 2012b). BDQ is a diarylquinoline with a novel mechanism of action specifically inhibiting mycobacterial ATP synthase (Cole and Alzari, 2005). In a randomized phase 2 placebo-controlled clinical trial among patients with MDR-TB, 48% of patients received BDQ in combination with standard MDR regimen showed conversion to negative culture in 8 weeks, compared with 9% in the placebo group that received the standard MDR regimen (Diacon et al., 2009). After 24 weeks, the proportion of patients that reached treatment success was 78.8% in BDQ group and 57.6% in placebo group (Diacon et al., 2012). However, BDQ carries a boxed warning indicating that this drug can cause QT prolongation, which could lead to an abnormal and potentially fatal heart rhythm (FDA, 2012a). In addition, the rate of drug-related hepatic disorders of BDQ group (8.8%) was higher than that of placebo group (1.9%) (FDA, 2012a).

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Illustration of BDQ metabolism will provide valuable information to predict and prevent drug-drug interactions and adverse drug reactions associated with BDQ. CYP3A4 is involved in BDQ metabolism, leading to the formation of a dominant but less active *N*-desmethyl metabolite (Janssen Pharmacutical Companies, 2012). BDQ and its metabolites, including *N*-desmethyl BDQ, *N*-didesmethyl BDQ and two hydroxyl metabolites, are mainly excreted via feces (Cuyckens et al., 2008; Meermann et al., 2012). However, limited information is available for bioactivation pathways of BDQ that may be related to BDQ toxicity. In addition, it is unknown whether CYP3A4 is the only P450 that contributes to BDQ metabolism. The current study was designed to explore novel metabolic pathways of BDQ metabolism using a metabolomic approach. The involvement of P450s in BDQ *N*-demethylation, the dominant pathway in BDQ metabolism, was also investigated using recombinant P450s. We identified a novel pathway in BDQ metabolism, which produces an aldehyde intermediate. We also found that CYP2C8 and 2C19, together with CYP3A4, are involved in BDQ *N*-demethylation.

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Material and Method

Chemicals. BDQ was purchased from Adooq Bioscience (Irvine, CA). Hepatocyte maintenance medium, dexamethasone and insulin were obtained from Lonza (Walkersville, MD). Penicillin G/Streptomycin and amphotericin B were obtained from GIBCO Laboratories (Grand Island, NY). Type I (rat-tail) collagen was purchased from BD Biosciences (Bedford, MA). The recombinant human P450s and human liver microsomes were purchased from XenoTech, LLC (Lenexa, KS). Formic acid, β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), dimethyl sulfoxide (DMSO), ethyl acetate (EtOAc) and methoxylamine ($MeONH_2$) were obtained from Sigma Aldrich (St Louis, MO).

BDQ metabolism in human hepatocytes. Fresh isolated human hepatocytes were plated at a cell density of 0.5×10^6 cells/well in 12-well plates previously coated with 0.2 mg/ml type I collagen. The isolated hepatocytes (> 80% viability) were maintained in human maintenance medium supplemented with 100 nM dexamethasone, 100 nM insulin, 100 U/ml penicillin G, 100 µg/ml streptomycin and 10% bovine calf serum and kept at 37°C in a humidified incubator with 95% air/5% CO_2. The hepatocytes were allowed to attach to the plate for 4 hours then the media was replaced with serum-free human maintenance medium. For the metabolism study, human hepatocytes were incubated with 30 µM BDQ for 18 h. The medium was collected and hepatocytes were lysed by adding 200 µl of acetonitrile: H_2O (1:1, *v/v*). Both medium and hepatocytes lysates were extracted by adding 4 fold volume of EtOAc. The mixture was then vortexed for 1 min and centrifuged at 15,000 g for 10 min. The organic phase was transferred to a glass tube and dried under a gentle stream of nitrogen. The residue was reconstituted by 100 µl of acetonitrile: H_2O (1:1, *v/v*). Five µl of the solution was injected onto a system combining ultra

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performance liquid chromatography and time-of-flight mass spectrometry (UPLC-QTOFMS) for metabolite analysis.

BDQ metabolism in recombinant P450s and human liver microsomes. Incubations were carried out in 1×phosphate-buffered saline (pH 7.4), containing cDNA-expressed P450 isozymes and 30 µM BDQ with or without 5 mM of the aldehyde trapping reagent $MeONH_2$ in a final volume of 190 µl. The reactions were initiated by adding 10 µl of 20 mM NADPH and continued for 20 min with gentle shaking. The kinetics of *N*-desmethyl BDQ formation by c-DNA expressed P450s were determined by incubating BDQ at different concentrations up to 100 µM. Incubations were also carried out in human liver microsomes and in presence of $MeONH_2$ (5 mM) to trap aldehyde metabolite of BDQ.

UPLC-QTOFMS analysis. Chromatographic separation of BDQ metabolites was performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters). The flow rate of the mobile phase was 0.40 ml/min and the column temperature was maintained at 50 °C. QTOFMS was operated in positive mode with electrospray ionization. The source and desolvation temperatures were set at 120 and 350 °C, respectively. Nitrogen was applied as the cone gas (10 l/h) and desolvation gas (700 l/h). Argon was applied as the collision gas. The capillary and cone voltages were set at 3.5 kV and 35 V. QTOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine enkephalin in real time. Screening and identification of BDQ metabolites were performed by using MarkerLynx software (Waters) based on accurate mass measurement (mass errors less than 10 ppm). Product ion scans with

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collision energy ramping from 15 to 45 V were used for structural elucidations of BDQ and its metabolites.

Data analysis. Mass spectra were acquired by MassLynx 4.1 in centroid format from *m/z* 50 to 1000. Centroid and integrated mass chromatographic data were processed by Markerlynx software to generate a multivariate data matrix. These data were imported to EZinfo software (Umetrics) for orthogonal partial least-squares discriminant analysis (OPLS-DA) on Pareto-scaled data. The corresponding data matrices were then exported in to SIMCA-P+ (Version 13, Umetrics) for multivariate data analysis.

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Results and Discussion

Profiling of BDQ metabolites in human hepatocytes. Metabolomics has proved to be a powerful tool for profiling drug metabolism and bioactivation pathways (Ma et al., 2008; Li et al., 2011). In the current study, an UPLC-QTOFMS-based metabolomic approach was used to profile BDQ metabolism. The results of chemometric analysis on the ions produced by UPLC-QTOFMS assay of control and BDQ treated human hepatocytes are shown in Supplemental Fig.1. The OPLS-DA analysis revealed two clearly separated clusters corresponding to control and BDQ treated groups (Supplemental Fig. 1A). The corresponding S-plot displays ions contributing to the group separation (Supplemental Fig. 1B). The top ranking ions were identified as BDQ metabolites (Fig. 1 and Supplemental Figs. 3-4) based on mass defect shifts and bromide isotopic pattern. Overall, 8 metabolites were identified in human hepatocytes, including 6 previously reported metabolites (M1, M2, M3, M4, M7 and M8) and 2 novel metabolites (M5 and M6). All BDQ metabolites identified in human hepatocytes were also found in the feces of mice treated with BDQ (data not shown). The BDQ metabolites are summarized in Supplemental Table 1.

Identification of novel BDQ metabolites. M6 was found as a novel BDQ metabolite during metabolomic analysis (Supplemental Fig. 1). The retention time of M6 was 8.90 min with protonated ions $[M + H]^+$ at *m/z* = 542 and 544 in a nearly 1: 1 ratio because of the $^{79}Br/^{81}Br$ isotopic pattern (Fig. 1). The MS/MS spectrum of M6 showed major fragment ions at *m/z* 327/329, 310/312, 236/238 and 91 (Fig. 1B and 1C). Compared with the MS/MS spectrum of BDQ (Supplemental Fig. 2), the absence of ions at *m/z* 58, 229 suggest that the dimethylamino

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moiety has been removed. M6 was proposed as a carboxylic acid metabolite (Fig. 1A). In theory, M6 is an oxidized product of an aldehyde, which can be generated in *N*-dealkylation of BDQ. The aldehyde intermediate M5 was confirmed in the incubations with human liver microsomes. $MeONH_2$ was used as a trapping agent that produced M5-oxime as a stable adduct (Fig. 1D). M5-oxime was eluted at 9.52 min with protonated ions $[M + H]^+$ at *m/z* = 555 and 557. Compared with fragment ions of M6 (Fig. 1B and 1C), these same ions were observed in MS/MS analysis of M5-oxime, including m/z 327/329, 310/312, 236/238, 91 (Fig. 1E and 1F). These results suggest that BDQ *N*-dealkylation occurred and produced an aldehyde (M5) (Fig. 1G). Further studies demonstrated that M5 formation is primarily mediated by CYP3A4 (Supplement Fig.5). In general, production of an aldehyde is considered as an unwanted pathway in drug metabolism because aldehydes are more reactive and they may lead to toxicity (Uchida, 2000; O'Brien et al., 2005). Thus, this study provides a clue to investigate adverse drug reactions associated with BDQ in relation to BDQ *N*-dealkylation.

Role of P450s in BDQ *N*-Demethylation. When BDQ is metabolized, *N*-demethylation is the major metabolic pathway and *N*-desmethyl BDQ (M1) is the main circulating metabolite (FDA, 2012a). Compared with BDQ, M1 has a three to six-fold lower anti-mycobacterial activity. However, M1 is more cytotoxic and a stronger inducer of phospholipidosis than BDQ (Mesens et al., 2007). Formation of M1 is mediated primarily by CYP3A4 (Janssen Pharmacutical Companies, 2012). We found that CYP2C8 and CYP2C19 are also involved in BDQ *N*-demethylation (Fig. 2A). The K_m values of CYP2C8, CYP2C19 and CYP3A4 in M1 formation were 13.1, 21.3 and 8.5 μM, respectively (Fig. 2B). These results indicate that CYP3A4 is not the only enzyme in BDQ *N*-demethylation. Thus, when patients receive BDQ together with

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drugs that are CYP3A4 inhibitors, BDQ *N*-demethylation may not be completely blocked, because CYP2C8 and CYP2C19 also contribute to BDQ *N*-demethylation. However, when patients receive BDQ together with drugs that are CYP3A4 inducers, such as ligands of pregnane X receptor, BDQ *N*-demethylation may be extremely accelerated because activation of pregnane X receptor induces the expression of CYP2C8, CYP2C19 and CYP3A4 (Gerbal-Chaloin et al., 2001; Luo et al., 2002).

In summary, BDQ metabolism was investigated in human hepatocytes by using a metabolomics approach. BDQ *N*-dealkylation was identified as a novel metabolic pathway of BDQ that produces an aldehyde intermediate. CYP2C8 and CYP2C19, together with CYP3A4, were involved in BDQ *N*-demethylation. These results can be applied to predict and prevent drug-drug interactions and adverse drug reactions associated with BDQ.

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Authorship contributions

Participated in research design: Liu, Li and Ma

Conducted experiments: Liu, Lu and Liu S.

Contributed the new reagents or analytic tools: Liu, Li, Dorko and Xie

Performed data analysis: Liu and Ma

Wrote or contributed to the writing of manuscript: Liu and Ma

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Footnotes

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Figure legends

Figure 1. **BDQ *N*-dealkylation and formation of an aldehyde and a carboxylic acid metabolite.** (A-C) Identification of a carboxylic acid metabolite of BDQ (M6) in human hepatocytes. (A) Extracted ion chromatograph of M6. (B) and (C) MS/MS spectrum of M6 at *m/z* 542 and 544. (D-F) Trapping of aldehyde (M5) intermediate in BDQ metabolism in human liver microsomes. $MeONH_2$ was used as a trapping agent. (D) Extracted ion chromatograph of M5-oxime. (E) and (F) MS/MS spectrum of M5-oxime at *m/z* 555 and 557. (G) Proposed mechanism for the formation of aldehyde (M5) and carboxylic metabolite (M6) in BDQ metabolism.

Figure 2. **Role of P450s in BDQ *N*-demethylation (M1).** cDNA expressed P450s were used to determine the contribution of individual P450s in M1 formation. The incubation conditions were detailed in the experimental procedures and M1 was determined by UPLC-QTOFMS. (A) Relative contribution of P450s in M1 formation. The data are expressed as mean ± SD (n=3). The data in CYP3A4 group was set as 100%. (B) Enzymatic kinetics of CYP2C8, 2C19 and 3A4 in M1 formation.

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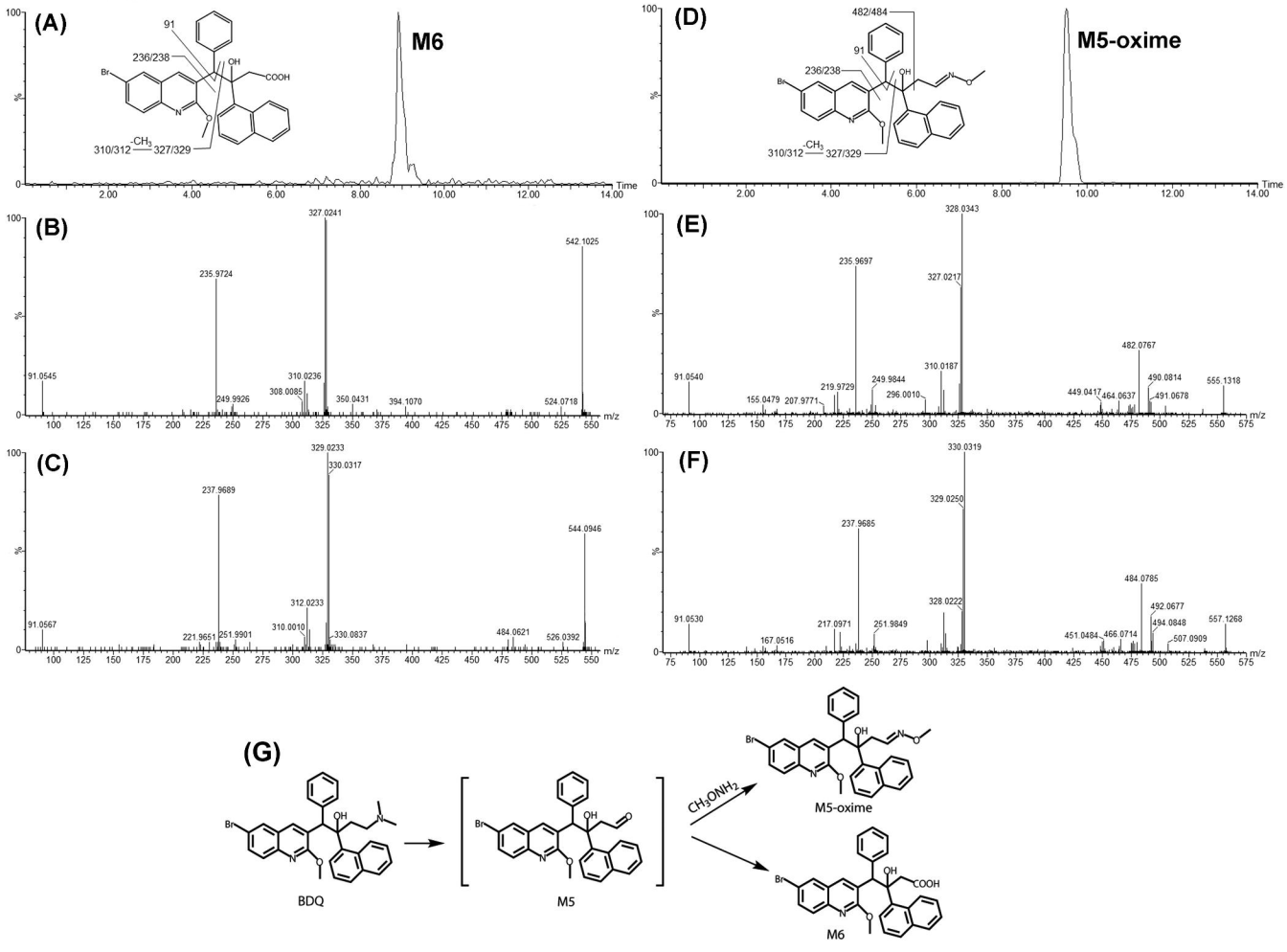
Figure 1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M6
91
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COOH
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310/312
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327/329
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12.00
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91
236/238
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-CH3
327/329
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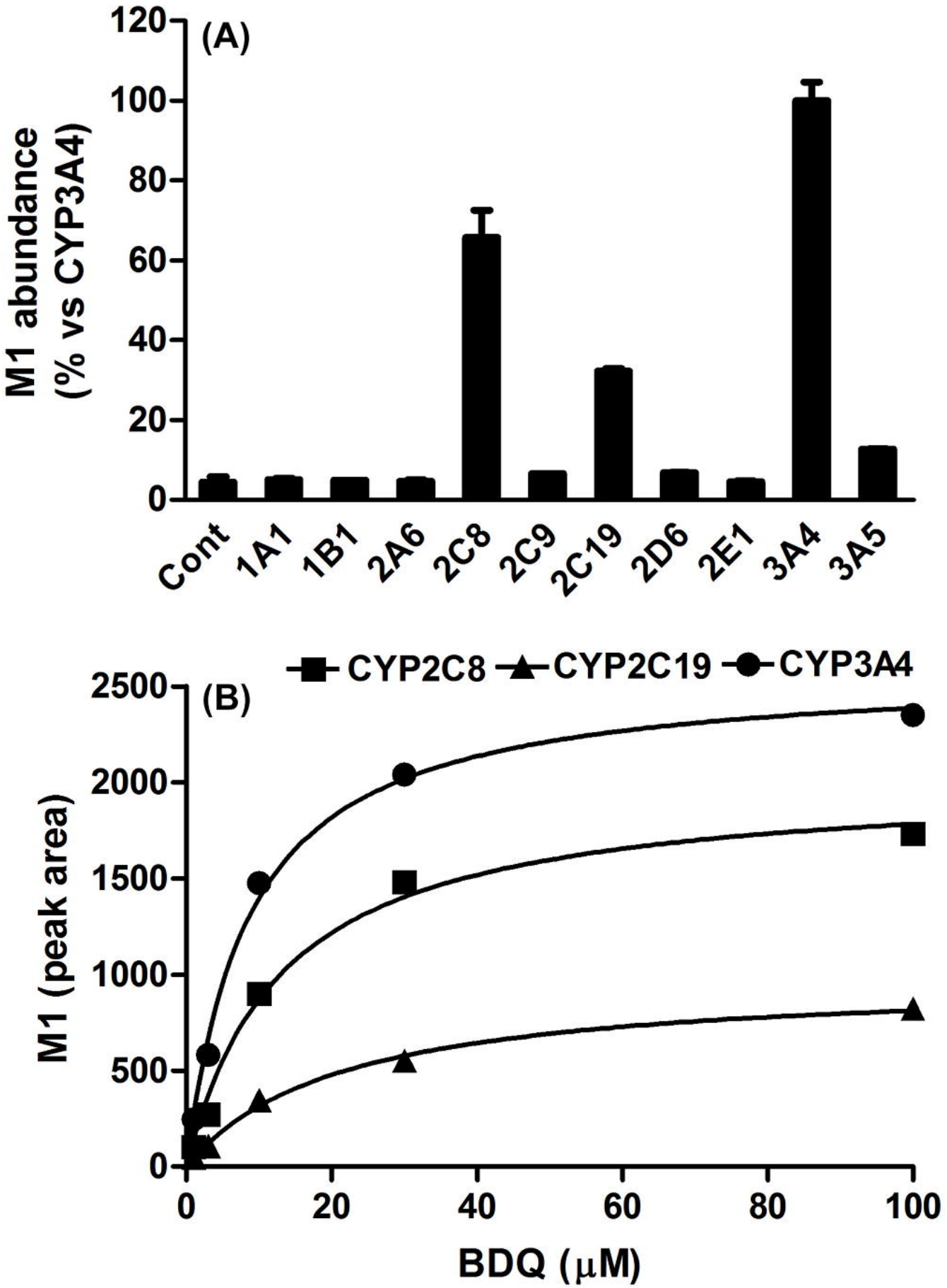
M6

Figure 2


(A)
M1 abundance (% vs CYP3A4)
120
100
80
60
40
20
0
Cont
1A1
1B1
2A6
2C8
2C9
2C19
2D6
2E1
3A4
3A5
(B)
CYP2C8
CYP2C19
CYP3A4
M1 (peak area)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
BDQ (μM)