Journal of Pharmaceutical Sciences xxx (2019) 1-4

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Note

Evaluation of Cytochrome P450 Selectivity for Hydralazine as an Aldehyde Oxidase Inhibitor for Reaction Phenotyping

Xin Yang, Nathaniel Johnson, Li Di*

Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc, Groton, Connecticut 06340

ARTICLE INFO

Article history:
Received 10 August 2018
Revised 16 October 2018
Accepted 2 November 2018

Keywords:
cytochrome P450 (CYP)
hepatocyte(s)
human liver microsomes
inhibition
metabolism
liquid chromatography-mass spectrometry (LC-MS)
drug metabolizing enzyme(s)
drug design
in vitro model(s)
metabolic clearance

ABSTRACT

Hydralazine has been reported as a selective mechanism-based inactivator of aldehyde oxidase (AO) and it is widely used in the pharmaceutical industry for reaction phenotyping to estimate fraction metabolized by AO and to identify AO substrates. In this study, however, hydralazine was found to inhibit CYP1A2, 2B6, 2D6, and 3A in human suspension hepatocytes under reaction phenotyping assay conditions, at concentrations that chemically knocked out most of the AO activities (≥50 μM). Furthermore, hydralazine is a time-dependent inhibitor of CYP1A2. Based on these findings, precautions need to be taken when using hydralazine as an AO inhibitor for *in vitro* studies because fraction metabolized by AO is likely to be overestimated and the likelihood of false positives in identifying AO substrates increases.

Introduction

Aldehyde oxidase (AO) has gained significant attention in recent years due to a number of clinical drug candidate failures caused by underprediction of human clearance and toxicity.[1-5] With the current high throughput metabolic stability screening paradigm using liver microsomes to identify metabolic liabilities, the impact of AO on drug metabolism is often overlooked in drug discovery, as AO is a cytosolic enzyme and not present in liver microsomes. Screening of AO metabolic stability must, therefore, be run in liver cytosols or hepatocytes. Another challenge to measure AO contribution to clearance is that common drug discovery animal models often do not express AO (e.g., dogs) or have quite variable AO activities (e.g., rats and mice).[5-8] Species differences in AO expression and activity further complicate our ability to translate the impact of AO on drug metabolism and pharmacokinetics from preclinical species to humans. Significant advances have been made over the years to understand AO *in vitro*–*in vivo* extrapolation, species differences, animal models, drug-drug interaction potential, interindividual variability, tissue distribution, and genetic polymorphism.[1-3]

Determination of fraction metabolized by AO ($F_{m,AO}$) is the first step toward building *in vitro*–*in vivo* extrapolation and understanding AO victim drug-drug interaction potential. A selective AO inhibitor is critical for the identification of AO substrates, the evaluation of AO-mediated metabolic pathways, reaction phenotyping, and $F_{m,AO}$ determination. Raloxifene, a selective estrogen receptor modulator, has been shown to be a potent, reversible, and noncompetitive AO inhibitor.[9] However, raloxifene also inhibits many major cytochrome P450s (CYPs) making it unsuitable as a selective AO inhibitor in human hepatocytes for reaction phenotyping studies.[10] Raloxifene may still be used in liver cytosols to differentiate the contribution between AO and XO (xanthine oxidase) because CYPs are not present in cytosols.[11] An alternative selective AO inhibitor is therefore needed for $F_{m,AO}$ determination and for identifying AO substrates in hepatocytes. Hydralazine, an antihypertensive agent, has recently been reported as a selective mechanism-based inactivator of AO, but the detailed mechanism

Abbreviations used: AO, aldehyde oxidase; CYP, cytochrome P450; F_m, fraction metabolized; $F_{m,AO}$, fraction metabolized by aldehyde oxidase; IC_{50}, half maximal inhibitory concentration; IVIVE, *in vitro*–*in vivo* extrapolation; K_i, inhibition constant; K_m, substrate concentration at half the maximum velocity; k_{obs}, inactivation rate; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MBI, mechanism-based inactivator; MRM, multiple reaction monitoring; rpm, revolution per min; [S], substrate concentration; TDI, time-dependent inhibitor; XO, xanthine oxidase.

* *Correspondence to:* Li Di (Telephone: +1 860 715 6172).
E-mail address: Li.Di@pfizer.com (L. Di).

https://doi.org/10.1016/j.xphs.2018.11.007
0022-3549/

of inactivation is not well understood.[12] Although hydralazine appears to be quite selective against various CYPs based on published data using reversible inhibition assay conditions,[10,12] studies on internal drug discovery compounds using the hepatocyte relay reaction phenotyping assay conditions[13] indicate that hydralazine might have an inhibitory effect on CYPs. Other group also found more pronounced CYP2D6 inhibition by hydralazine.[14] To understand the broader applications of hydralazine as a selective AO inhibitor in hepatocytes for reaction phenotyping studies, selectivity against 7 CYPs was evaluated in this study. The results can help to define the potential limitations of using hydralazine for AO reaction phenotyping, $F_{m,AO}$ determination, and AO substrate identification.

Materials and Methods

Materials

All chemicals were obtained from Pfizer Global Material Management (Groton, CT) or purchased from Sigma-Aldrich (St. Louis, MO) unless specified. Cryopreserved human hepatocytes (Lot DCM) consisting of 10 donors with 3 men and 7 women were custom-pooled and prepared by BioreclamationIVT (Baltimore, MD). Human liver microsomes (Lot HLM103) of 50 donors containing 36 men and 14 women were purchased from Xenotech (Kansas City, KS). Polystyrene plates were purchased from Corning (Corning, NY).

CYP Selectivity Study of Hydralazine

Hepatocytes were thawed and resuspended in Williams medium E (WEM GIBCO-BRL, custom formula supplemented with 50 mM HEPES and 26 mM Na_2CO_3). The cells were counted and viability was determined using the trypan blue exclusion method. Hydralazine at final concentrations of 25, 50, 100, and 150 μM was added to suspended hepatocytes at a density of 0.5 million cells/mL in 200 μL total volume and preincubated for 30 min at 37°C to inactivate AO.[13] Media (170 μL) containing hydralazine were removed from the hepatocytes, and fresh media containing substrates were added to the cells with a final cell density of 0.5 million cells/mL, 1 μM substrate concentration, and 200 μL final incubation volume. The preincubation hydralazine concentrations were approximately 7-fold higher than those in the final incubation (3.75, 7.5, 15 and 22.5 μM). The specific substrate reactions were midazolam (3A) to 1′-OH-midazolam, dextromethorphan (2D6) to dextrorphan, diclofenac (2C9) to 4′-OH-diclofenac, paclitaxel (2C8) to 6α-OH-paclitaxel, S-mephenytoin (2C19) to 4′-OH-S-mephenytoin, phenacetin (1A2) to acetaminophen, bupropion (2B6) to OH-bupropion, and zaleplon (AO) to oxozaleplon.[12,13] Metabolite formation was monitored to detect the specific metabolic reaction by each of the enzymes. Detailed mass transitions of the metabolites have been described previously[12,13] and the 6α-OH-paclitaxel MRM transition m/z was 870→286. The plates were put on an orbital shaker (150 rpm; VWR, Radnor, NJ) in a 37°C incubator (95% air/5% CO_2 and 75% relative humidity). At various time points (0, 5, 10, 20, 30, 45, and 60 min), 10 μL of hepatocyte suspension was removed from the incubation and added to 100 μL of cold acetonitrile containing internal standard (10 ng/mL terfenadine). The solution was centrifuged (Eppendorf, Hauppauge, NY) at 3000 rpm for 10 min at room temperature and the supernatant was transferred for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Percentage inhibition was calculated using the initial slope of the metabolite formation time course using peak area ratio from LC-MS/MS (Prism 6 for Windows, Version 6.03; GraphPad Software, Inc., La Jolla, CA).

CYP Time-Dependent Inhibition Study of Hydralazine

The CYP1A2 time-dependent inhibition study by hydralazine was conducted using the "Add Method" described in the literature[15] Hydralazine (50 μM) was incubated with human suspension hepatocytes in 100 μL of 0.5 million cells/mL cell density on an orbital shaker (150 rpm) in an incubator (95% air/5% CO_2 and 75% relative humidity) at 37°C. At various time points within 2 h, 10 μL of phenacetin (1A2 substrate, 50 μM final concentration) or solvent control was added to incubation mixture and continued to incubate for another 5 min. The reactions were quenched with cold acetonitrile containing internal standard, centrifuged, and the supernatant was transferred to a new plate for LC-MS/MS analysis. A similar time-dependent inhibitor (TDI) experiment was also conducted in human liver microsomes at 0.5 mg/mL in the presence of nicotinamide adenine dinucleotide phosphate collecting time points within 1 h with phenacetin or 6 substrate cocktails (1A2, 2C8, 2C9, 2C19, 2D6, and 3A).[16] In all experiments, organic solvent content was <0.1%. Percentage activity remaining was calculated and k_{obs} (inactivation rate) values were obtained using the initial slope based on the Ln (% activity remaining) versus time curve.[17] A parallel line test was performed to evaluate the difference with and without inhibitors (Prism 6 for Windows, Version 6.03; GraphPad Software, Inc.).

LC-MS/MS Conditions

The LC mobile phases were (A) high performance liquid chromatography grade water containing 0.1% formic acid; and (B) acetonitrile containing 0.1% formic acid. The following solvent gradient or equivalent was used: 95%(A)/5%(B) for 0.3 min, 95%(A)/5%(B)-5%(A)/95%(B) from 0.3 to 1.0 min, 5%(A)/95%(B) from 1.0 to 1.7 min, 5%(A)/95%(B)-95%(A)/5%(B) from 1.7 to 2.0 min. A flow rate of 0.5 mL/min was used to elute the compounds from the column (Kinetex C18, 30 × 3 mm, 2.6 μm; Phenomenex, Torrance, CA). A sample aliquot of 3 μL was injected for analysis using a CTC PAL autosampler (Leap Technology, Carrboro, NC). Shimadzu high performance liquid chromatography AD30 pumps (Columbia, MD) connected to an AB Sciex (Foster City, CA) 5500 triple quadrupole mass spectrometer equipped with a TurboIonSpray source using MRM mode was also used. Analyst™ 1.5.2 software (Applied Biosystems, Foster City, CA) was applied to data collection, processing, and analysis.

Results and Discussion

The percentage inhibition data of hydralazine against 7 CYP enzymes and AO at 4 concentrations are summarized in Table 1 with hydralazine removed from media after 30 min preincubation.[13] Hydralazine showed ≥85% inhibition of AO at concentrations ≥50 μM in the suspension human hepatocytes suggesting 50 μM

Table 1
Selectivity of Hydralazine at 4 Concentrations Against CYP Enzymes in Suspension Human Hepatocytes With 30 min Preincubation

Enzymes	Substrate	% Inhibition ± Standard Deviation			
		25 μM	50 μM	100 μM	150 μM
CYP1A2	phenacetin	38 ± 13	56 ± 22	67 ± 15	74 ± 11
CYP2B6	bupropion	15 ± 14	22 ± 14	33 ± 14	36 ± 12
CYP2C8	paclitaxel	5 ± 6	3 ± 4	7 ± 4	6 ± 8
CYP2C9	diclofenac	7 ± 7	9 ± 7	13 ± 4	10 ± 6
CYP2C19	S-mephenytoin	2 ± 5	5 ± 4	7 ± 7	8 ± 7
CYP2D6	dextromethorphan	14 ± 12	31 ± 5	49 ± 11	65 ± 9
CYP3A	midazolam	11 ± 9	25 ± 4	28 ± 14	37 ± 4
AO	zaleplon	71 ± 7	85 ± 4	94 ± 2	93 ± 1

Data were generated from 2-3 runs in different days with duplicates in each day.

hydralazine inhibits most AO activities and can be used for reaction phenotyping studies. At 25 μM, hydralazine had less than 80% AO inhibition, which was too low to knock out most of the AO activity for reaction phenotyping. At concentrations ≥50 μM (concentrations suitable for reaction phenotyping), hydralazine is rather selective against the CYP2C family of enzymes with <20% inhibition (CYP2C8, CYP2C9, and CYP2C19). However, it showed moderate to strong inhibition of CYP1A2 (56%-74%), CYP2B6 (22%-36%), CYP2D6 (31%-65%), and CYP3A (25%-37%). The inhibition is likely to be more pronounced if the media containing inhibitors are not removed after preincubation. Inhibition of CYP2D6 and CYP3A by hydralazine has been previously demonstrated to be ~25% at 50 μM, but inhibition of other CYPs has not yet been reported.[12] The more potent inhibition observed in this study compared to that reported in the literature could potentially be due to lower substrate concentrations (1 μM vs. K_m of 2-40 μM) according to the following equations: $IC_{50}/K_i = 1+[S]/K_m$ for competitive inhibition; $IC_{50}^t/K_I = (1+[S]/K_m)*0.693/(k_{inact}*t)$ for mechanism-based inactivation.[18] Substrate concentration of 1 μM is selected instead of K_m since 1 μM is the typical concentration used in reaction phenotyping studies *in vitro* and it is closer to physiologic exposure concentrations *in vivo* for most drug candidates.

TDI of hydralazine was also investigated for 6 CYP enzymes (1A2, 2C8, 2C9, 2C19, 2D6, and 3A) using a cocktail approach up to 300 μM[16] in human liver microsomes supplemented with nicotinamide adenine dinucleotide phosphate. Hydralazine was identified as a TDI for CYP1A2 from the initial screen, but the other CYPs (2C8, 2C9, 2C19, 2D6, and 3A) were not (data not shown). This suggests that hydralazine inhibition of other CYPs is reversible. The TDI of hydralazine for CYP1A2 was further studied in both human hepatocytes and human liver microsomes at 50 μM, a lowest concentration knocks down most AO activities and is suitable for reaction phenotyping. The results are shown in Figure 1. The k_{obs} values for CYP1A2 inactivation of hydralazine at 50 μM are 0.0072 min^{-1} in hepatocytes and 0.012 min^{-1} in liver microsomes. The parallel line test shows that the slopes are significantly different for 50 μM hydralazine versus solvent control ($p < 0.0001$ for both hepatocytes and microsomes). The reason that previous studies[10,12] did not observe TDI may be due to substrate protection caused by high substrate concentration under the assay conditions.[19-22] These data indicate that hydralazine is a TDI of CYP1A2 at the effective AO inhibitory concentration (50 μM) in both hepatocytes and microsomes, and that the inactivation might be CYP-mediated. A structurally similar analog of hydralazine, dihydralazine, has been reported to be a mechanism-based inactivator of CYP1A2 and CYP3A4.[23] The mechanism of inactivation has been proposed to be covalent modification of CYPs. The chemically reactive metabolite(s) of dihydralazine generated by CYP1A2 and CYP3A4 through metabolic activations bind and inactivate the enzyme themselves.[23] It has also been postulated that covalent binding of a reactive metabolite of dihydralazine to CYP1A2 might be involved in the formation of antiliver microsome antibodies in dihydralazine hepatitis.[23] CYP1A2 inactivation by hydralazine might have similar mechanisms as dihydralazine due to structural similarity. Whether covalent modification of CYP1A2 by hydralazine has any relevance to its immune responses and hepatotoxicity like dihydralazine remains to be seen. Although there are no reports in the literature that hydralazine is a CYP1A2 substrate, *in silico* prediction with StarDrop™ 6.5[24-26] shows that CYP1A2 has high probability of being involved in hydralazine metabolism. Radicals generated by other mechanisms (metal ions, peroxidases, and synthase)[27] might also be potential mechanisms of inactivation. Hydralazine was one of the first oral antihypertensive medications introduced into the clinic in the late 1950s, but was officially approved for use in the United States in 1984.[28] Hydralazine has been linked to several forms of acute liver injury as well as a lupus-like syndrome.[28] After oral administration in humans, hydralazine undergoes extensive first-pass metabolism by N-acetyltransferases.[29,30] For intravenous dosing, hydralazine combines with endogenous aldehydes and ketones to form hydrazine metabolites that are equipotent as hydralazine.[29] The acute liver injury appears to be due to hydralazine metabolism to an immunologic adduct that can result in an immunoallergic hepatitis or a more delayed lupus- and an autoimmune hepatitis-like syndrome.[28] Because hydralazine is structurally similar to dihydralazine, it is possible that protein adduct formation due to covalent modification of CYP1A2 might also link to its immune responses and hepatic toxicity.

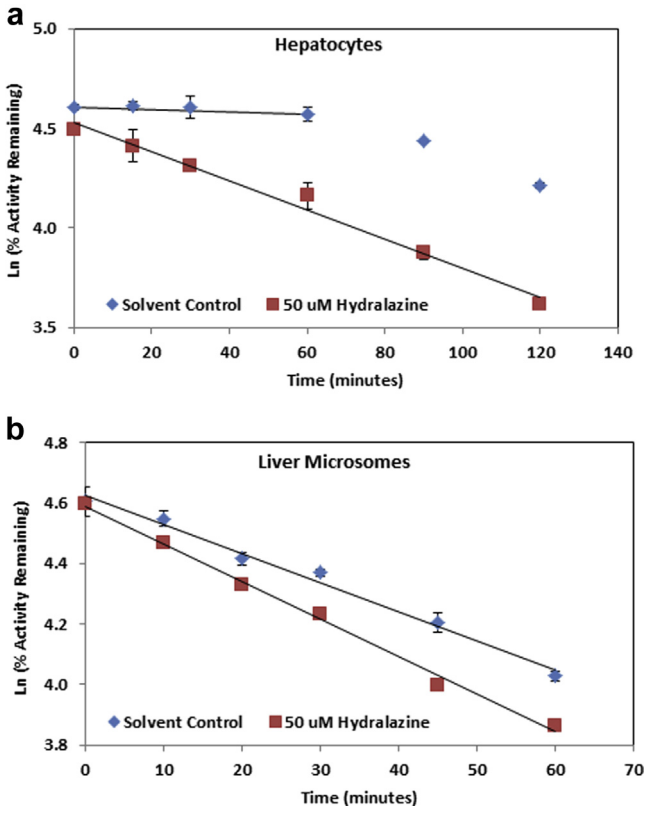

Figure 1. Time-dependent inhibition of CYP1A2 by hydralazine at 50 μM in human suspension hepatocytes and human liver microsomes.

When evaluating enzyme selectivity, it is best to consider using conditions that closely mimic those in the reaction phenotyping assays, including substrate concentration. At high substrate concentrations, assays are less sensitive in detecting inhibition due to concentration dependency of reversible inhibition and substrate protection effects of irreversible inhibition. Both reversible and time-dependent inhibition need to be evaluated for selectivity of an inhibitor. Selectivity can also be substrate-dependent and, therefore, monitoring the selectivity for the actual drug discovery compound during reaction phenotyping experiments can also be helpful to inform F_m estimation. At the concentrations that knock out most AO activity (≥50 μM), hydralazine shows significant inhibition of a number of CYPs. Therefore, precautions need to be made when using hydralazine as an inhibitor to identify AO substrates and for reaction phenotyping to estimate $F_{m,AO}$ in hepatocyte systems *in vitro*.

Acknowledgments

The authors thank Karen Atkinson for editing the article and Amit Kalgutkar for useful discussion.

References

1. Barr JT, Choughule K, Jones JP. Enzyme kinetics, inhibition, and regioselectivity of aldehyde oxidase. In: *Methods in Molecular Biology (New York, NY, United States) 1113(Enzyme Kinetics in Drug Metabolism)*. New York, NY: Springer; 2014:167-186.
2. Rashidi M-R, Soltani S. An overview of aldehyde oxidase: an enzyme of emerging importance in novel drug discovery. *Expert Opin Drug Discov*. 2017;12(3):305-316.
3. Mota C, Coelho C, Leimkuhler S, et al. Critical overview on the structure and metabolism of human aldehyde oxidase and its role in pharmacokinetics. *Coord Chem Rev*. 2018;368:35-59.
4. Hutzler JM, Obach RS, Dalvie D, Zientek MA. Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin Drug Metab Toxicol*. 2013;9(2):153-168.
5. Di L. The role of drug metabolizing enzymes in clearance. *Expert Opin Drug Metab Toxicol*. 2014;10(3):379-393.
6. Garattini E, Terao M. The role of aldehyde oxidase in drug metabolism. *Expert Opin Drug Metab Toxicol*. 2012;8(4):487-503.
7. Dalvie D, Xiang C, Kang P, Zhou S. Interspecies variation in the metabolism of zoniporide by aldehyde oxidase. *Xenobiotica*. 2013;43(5):399-408.
8. Choughule KV, Barr JT, Jones JP. Evaluation of rhesus monkey and Guinea pig hepatic cytosol fractions as models for human aldehyde oxidase. *Drug Metab Dispos*. 2013;41(10):1852-1858.
9. Obach RS. Potent inhibition of human liver aldehyde oxidase by raloxifene. *Drug Metab Dispos*. 2004;32(1):89-97.
10. Nirogi R, Kandikere V, Palacharla RC, et al. Identification of a suitable and selective inhibitor towards aldehyde oxidase catalyzed reactions. *Xenobiotica*. 2014;44(3):197-204.
11. Morrison RD, Blobaum AL, Byers FW, et al. The role of aldehyde oxidase and xanthine oxidase in the biotransformation of a novel negative allosteric modulator of metabotropic glutamate receptor subtype 5. *Drug Metab Dispos*. 2012;40(9):1834-1845.
12. Strelevitz TJ, Orozco CC, Obach RS. Hydralazine as a selective probe inactivator of aldehyde oxidase in human hepatocytes: estimation of the contribution of aldehyde oxidase to metabolic clearance. *Drug Metab Dispos*. 2012;40(7):1441-1448.
13. Yang X, Atkinson K, Di L. Novel cytochrome P450 reaction phenotyping for low-clearance compounds using the hepatocyte relay method. *Drug Metab Dispos*. 2016;44(3):460-465.
14. Zientek MA, Youdim K. Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab Dispos*. 2015;43(1):163-181, 119.
15. Chen Y, Liu L, Monshouwer M, Fretland AJ. Determination of time-dependent inactivation of CYP3A4 in cryopreserved human hepatocytes and assessment of human drug-drug interactions. *Drug Metab Dispos*. 2011;39(11):2085-2092.
16. Chen ZH, Zhang SX, Long N, et al. An improved substrate cocktail for assessing direct inhibition and time-dependent inhibition of multiple cytochrome P450s. *Acta Pharmacol Sin*. 2016;37(5):708-718.
17. Yates P, Eng H, Di L, Obach RS. Statistical methods for analysis of time-dependent inhibition of cytochrome P450 enzymes. *Drug Metab Dispos*. 2012;40(12):2289-2296.
18. Maurer TS, Tabrizi-Fard MA, Fung H-L. Impact of mechanism-based enzyme inactivation on inhibitor potency: implications for rational drug discovery. *J Pharm Sci*. 2000;89(11):1404-1414.
19. Yu X-C, Liang C, Strobel HW. Kinetics of substrate reaction in the course of hydroperoxide-mediated inactivation of cytochrome P450 1A1. *Biochemistry*. 1996;35(20):6289-6296.
20. Lin H-L, Kent UM, Hollenberg PF. Mechanism-based inactivation of cytochrome P450 3A4 by 17α-ethynylestradiol: evidence for heme destruction and covalent binding to protein. *J Pharmacol Exp Ther*. 2002;301(1):160-167.
21. Chun J, Kent UM, Moss RM, Sayre LM, Hollenberg PF. Mechanism-based inactivation of cytochromes P450 2B1 and P450 2B6 by 2-phenyl-2-(1-piperidinyl) propane. *Drug Metab Dispos*. 2000;28(8):905-911.
22. Kent UM, Bend JR, Chamberlin BA, Gage DA, Hollenberg PF. Mechanism-based inactivation of cytochrome P450 2B1 by N-benzyl-1-aminobenzotriazole. *Chem Res Toxicol*. 1997;10(5):600-608.
23. Masubuchi Y, Horie T. Mechanism-based inactivation of cytochrome P450s 1A2 and 3A4 by dihydralazine in human liver microsomes. *Chem Res Toxicol*. 1999;12(10):1028-1032.
24. StarDrop. Cambridge, UK: Optibrium. Available at: www.optibrium.com/stardrop. Accessed December 17, 2018.
25. Tyzack JD, Hunt PA, Segall MD. Predicting regioselectivity and lability of cytochrome P450 metabolism using quantum mechanical simulations. *J Chem Inf Model*. 2016;56(11):2180-2193.
26. Hunt PA, Segall MD, Tyzack JD. WhichP450: a multi-class categorical model to predict the major metabolising CYP450 isoform for a compound. *J Comput Aided Mol Des*. 2018;32(4):537-546.
27. Sinha BK, Mason RP. Biotransformation of hydrazine dervatives in the mechanism of toxicity. *J Drug Metab Toxicol*. 2014;5(2):8.
28. National Institutes of Health. Drug record: hydralazine. Washington, DC: NIH. Available at: https://livertox.nih.gov/Hydralazine.htm. Accessed December 17, 2018.
29. Drugbank. Hydralazine. Drugbank; 2007. Available at: https://www.drugbank.ca/drugs/DB01275. Accessed December 17, 2018.
30. Allen CE, Doll MA, Hein DW. N-acetyltransferase 2 genotype-dependent N-acetylation of hydralazine in human hepatocytes. *Drug Metab Dispos*. 2017;45(12):1276-1281.