Generation of Presumptive Metabolites of a Novel Synthetic Opioid Using Human Liver Microsomes and Subsequent Analysis by Orbitrap LC-MS/MS

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KEY FINDINGS

Human liver microsome assays identify four major monohydroxylated metabolites of the synthetic opioid 2-methyl AP-237.

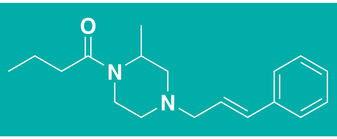

Figure 1. 2-methyl AP-237 structure.

Introduction

The synthetic opioid 2-methyl AP-237 (Cayman Item No. 26485) was originally synthesized and reported in Japan in the 1970s.[1] Related analogs have been used internationally for pain management in the clinical setting but never in the US. This compound has recently appeared for sale on the dark web, and there has been discussion of its use on Reddit and drug user forums. With increased legislation controlling fentanyl and fentanyl analogs, illicit opioid manufacturers may turn to new non-fentanyl opioids.[2] There is mounting evidence that this acylpiperazine opioid is being trafficked and detected in seized materials and drug screens. While compounds like U-47700 are the most prevalent synthetic opioids after fentanyl, new classes continue to emerge from published patents and journal articles. For this reason, we studied 2-methyl AP-237, a member of one of these new opioid classes, to explore what metabolites would arise from incubation with human liver microsomes (HLM). Analysis of these oxidative metabolites of 2-methyl AP-237 by LC-MS/MS can be used to inform what metabolites (and ultimately what corresponding masses) toxicologists may see in a blood or urine screen.

Methods

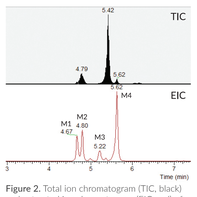

Figure 2. Total ion chromatogram (TIC, black) and extracted ion chromatogram (EIC, red) of HLM incubation assay.

Metabolites of 2-methyl AP-237 were produced using *in vitro* metabolism. 2-methyl AP-237 was prepared as a 50 mM stock solution in DMSO and was further diluted with phosphate buffer (pH 7.4). The compound solution was then incubated for one hour at 37°C with HLM and cofactor NADPH. At one hour, the reaction was quenched with acetonitrile, spun down on a centrifuge, and an aliquot of the mixture was injected onto the UHPLC. Mass fragments were detected using a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer and the data was processed using Thermo Scientific™ Xcalibur™ software.

Additionally, the structures of the metabolites of 2-methyl AP-237 were predicted computationally using Schrödinger's quantitative structure-activity relationship (QSAR) model based on intrinsic reactivity in the presence of CYP3A4. The Schrödinger QSAR model is based on the known reactivity of 384 small molecules and scores empirically by evaluating reactivity of specific functional groups.[3] A computational molecular dynamics simulation was also used to examine the probability of intramolecular hydrogen bonding for various possible hydroxylated metabolites.

Results

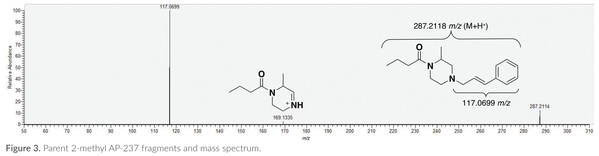

Figure 3. Parent 2-methyl AP-237 fragments and mass spectrum.

Metabolite M1:

The first eluting metabolite (4.67 min) yielded the mass spectrum below with major fragments of 133.0647 *m/z* and 171.1491 *m/z*. The presence of the unmodified acylpiperazine fragment at 171.1491 *m/z* and the presence of the hydroxylated cinnamyl chain narrows down the location of oxidation. Monohydroxylation appears to have occurred on the cinnamyl tail chain at the allylic position or on the benzene ring (133.0647 m/z). Metabolite M1 also shows a small 117.0697 *m/z* fragment, indicating that the hydroxylated metabolite may dehydrate during analysis. The allylic position is highly resonance stabilized and may lead to loss of the hydroxyl group more readily than the oxidation on the ring would. The probable metabolites based on this data are metabolites A, I, J, or K.

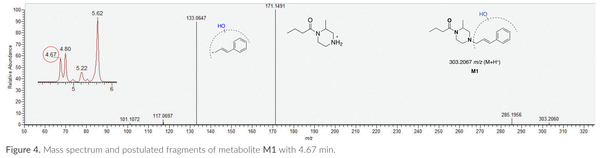

Figure 4. Mass spectrum and postulated fragments of metabolite M1 with 4.67 min.

Metabolites M2, M3, and M4:

The second, third, and fourth eluting metabolites had retention times of 4.80 min, 5.22 min, and 5.62 min, respectively. All three metabolites yielded the same fragments of 117.0698 *m/z* (or 117.0699 *m/z*) and 303.2061 *m/z* (or 303.2062 *m/z*), indicating that monohydroxylation occurred on the piperazine ring or acyl chain. M2 yielded one minor additional fragment of 185.1285 *m/z*, further indicating that the position of hydroxylation is likely on the piperazine ring (metabolites B, C, G, or H) or acyl chain (metabolites D, E, or F). Metabolites M3 and M4 gave similar spectra, also indicating oxidation on the piperazine ring or acyl chain. Metabolite M4 appears to be the major metabolite based on the EIC.

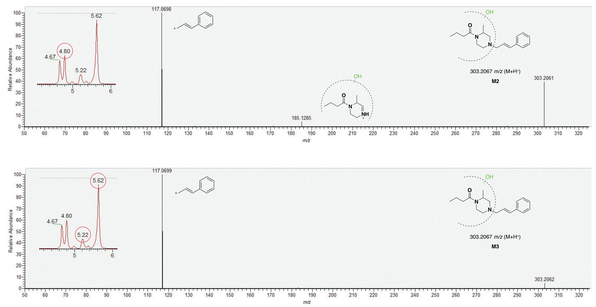

Figure 5. Mass spectrum and postulated fragments of Metabolite M2 (top, 4.80 min) and Metabolites M3 and M4 (bottom, 5.22 min and 5.62 min, respectively).

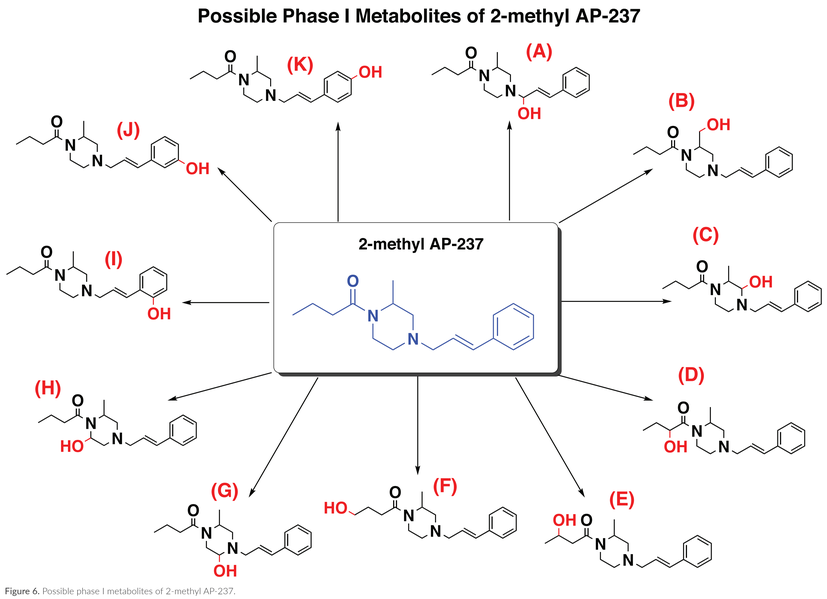

Figure 6. Possible phase I metabolites of 2-methyl AP-237.

QSAR Modeling:

A quantitative structure-activity relationship (QSAR) model was used to identify which sites on 2-methyl AP-237 will potentially be hydroxylated during phase I metabolism.[3] The most probable sites for metabolic oxidation were found to be the allylic position (tα) on the cinnamyl chain, illustrated with a red circle, and the sites on the piperazine ring (2, 3, 5, and 6), illustrated with orange circles. With the first eluting metabolite M1 (4.67 min) hydroxylation at the allylic position (tα), in red, is the most probable based on the 133.0647 *m/z* fragment and the QSAR model. Metabolites M2, M3, and M4 all have fragments of 117.0698 *m/z* and 303.2061 *m/z*, which indicate oxidation at either the acyl chain or piperazine ring. The QSAR model predicted the most probable sites for oxidation were on the piperazine ring.

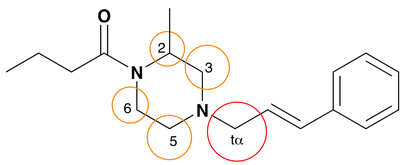

Figure 7. QSAR prediction of 2-methyl AP-237 sites of oxidation (larger circle size indicates greater probability of oxidation at that site).

Intramolecular Hydrogen Bonding Molecular Dynamics Simulation:

The most abundant metabolite M4 appeared to be less polar than the parent compound because it had a longer retention time (5.62 min). A molecular dynamics (MD) simulation was used to predict the probability of intramolecular H-bonding for hydroxylated metabolites at positions (2, 3, 5, and 6) on the piperazine and on acyl chain positions (α, β, and γ).[4] It was postulated that intramolecular hydrogen bonding may reduce the polarity of the unknown hydroxylated metabolites relative to the parent 2-methyl AP-237. In addition to being one of the predicted oxidative sites by the QSAR model, monohydroxylation at the 6 position is most probable due to an intermolecular H-bond, which could decrease the polarity of the compound in solution compared to 2-methyl AP-237. This data identifies metabolite H as the most probable metabolite M4 of 2-methyl AP-237.

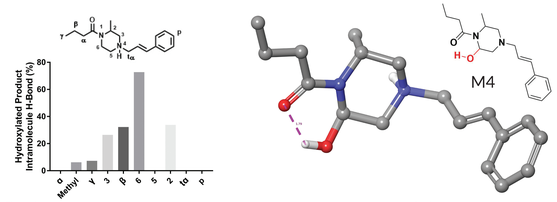

Figure 8. Strength of intramolecular hydrogen bonding for hydroxylated metabolites at various positions (left). Line drawing and ball-and-stick model of 6-hydroxy metabolite showing the intramolecular hydrogen bond potential with the acyl group carbonyl (right).

Conclusions

- Incubating of 2-methyl AP-237 with HLM resulted in four monohydroxylated phase I metabolites (M1, M2, M3, and M4).
- One of the metabolites is hydroxylated on the cinnamyl chain region; the other three are hydroxylated at either the acyl chain or on the piperazine ring.
- QSAR predictions and molecular dynamics simulations helped further narrow down the most likely site for hydroxylation to the piperazine ring carbon with C6-OH (M4, major metabolite by EIC abundance).
- Biological sample screens that detect the presence of fragments with 117.0698 *m/z*, 133.0647 *m/z*, or 303.2061 *m/z* may contain the novel synthetic opioid 2-methyl AP-237.

Future Directions

- Analyze emerging novel psychoactives using HLM assays, including non-opioid drug classes.
- Report presumptive metabolites and their masses to support the toxicology community.
- Use HLM assays to inform what reference standards to provide.

References

1. Nishimura, N., Kiuchi, M., Kanetake, Y., *et al.* Clinical evaluation of a new analgesic agent Ap-237. *Masui* **19(6)**, 653-656 (1970).
2. Steward, K. Identifying novel opioid agonists from metabolites. https://www.technologynetworks.com/applied-sciences/blog/identifying-novel-opioid-agonists-from-metabolites-322453 (2019).
3. Feng, S. and Friesner, R.A. Prediction of cytochrome P450-mediated metabolism using a combination of QSAR-derived reactivity and induced fit docking. *arXiv e-link* **1811.09366**, (2018).
4. Bowers, K.J., Chow, D.E., Xu, H., *et al.* Scalable algorithms for molecular dynamics simulations on commodity clusters. *Proceedings of the 2006 ACM/IEEE Conference on Supercomputing* (2006).