R1-C.2: Compatibilities & Simulants: Explosive Polymer Interactions

I. PARTICIPANTS INVOLVED FROM JULY 1, 2019 TO JUNE 30, 2020

Faculty/Staff			
Name	**Title**	**Institution**	**Email**
Jimmie Oxley	Co-PI	URI	joxley@chm.uri.edu
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Name	**Degree Pursued**	**Institution**	**Month/Year of Graduation**
Michelle Gonsalves	PhD	URI	12/2020
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Jeff Canaria	PhD	URI	5/2021
Victoria Stubbs	MS	URI	8/2020

II. PROJECT DESCRIPTION

A. Project Overview

This project has examined the interaction of explosives with polymers with the aim at revealing new swabs. In the process we have catalyzed research in other groups that remain ongoing. We hope for the same success with our exploration of the biological effects of the peroxide explosives. We have succeeded in identifying a biomarker indicating triacetone triperoxide (TATP) exposure. It may take some time before forensic labs become aware of this useful evidence of illicit explosive handling.

SCHMOO was developed to sequester hazardous materials and preserves the evidentiary value. This it does very well. However, first responders appear more concerned with destroying the hazardous material than preserving evidence.

B. State of the Art and Technical Approach

TATP is a volatile but powerful explosive that appeals to terrorists due to its ease of synthesis from household items. For this reason, bomb squads, canine (K9) units, and scientists must work with this material to mitigate this threat. However, no information on the metabolism of TATP is available.

In vitro experiments using human liver microsomes and recombinant enzymes were performed on TATP and TATP-OH (hydroxy-TATP) for metabolite identification and enzyme phenotyping. Enzyme kinetics for TATP hydroxylation were also investigated. Urine from laboratory personnel collected before and after working with TATP was analyzed for TATP and its metabolites.

While experiments with flavin-containing monooxygenases (FMOs) were inconclusive, those with recombinant cytochrome P450s (CYPs) strongly suggested that CYP2B6 was the principle enzyme responsible for TATP hydroxylation. TATP-O-glucuronide was also identified, and incubations with

recombinant uridine diphosphoglucuronosyl transferases (UGTs) indicated that UGT2B7 catalyzes this reaction. Michaelis-Menten kinetics were determined for TATP hydroxylation, with $K_{M\,=}$ 1.4 μM and $v_{max\,=}$ 8.7 nmol/min/nmol CYP2B6. TATP-O-glucuronide was present in the urine of all three volunteers after being exposed to TATP vapors showing good in vivo correlation to in vitro data. TATP and TATP-OH were not observed.

Since scientists working to characterize and detect TATP to prevent terrorist attacks are constantly exposed to this volatile compound, attention should be paid to its metabolism. This paper is the first to elucidate any metabolism and excretion of TATP in humans. Also, TATP-O-glucuronide can be exploited as a marker of exposure to TATP in urine. (See R1-C.2 Appendix)

C. Major Contributions

- Extensive TATP characterization—safe-scent aids, gentle destruction patented and licensed (Years 1–4)
- The limitations of certain oxidizers in terms of terrorist use (Years 1–6)
- Baseline information about HMTD chemical properties and reactivity (Years 1–7)
- Identifying the hazards of humidity to HMTD (Years 2–3)
- Formation mechanism of HMTD initiated (Years 2–3)
- Gentle destruction methods for HMTD (Years 3–4)
- Safe-scent aids for HMTD (Years 3–4)
- Revealing modes by which peroxide explosive signature can be masked by solvent (Years 3–4)
- Canine training aids for TATP and HMTD (Years 3–4)
- Determining best practices in analyzing peroxide explosives (Years 5–6)
- An extremely reliable standard for HMTD quantification work has been developed (Year 5)
- Examination of ETN, tracking synthesis routes, and attribution (Years 6–7)
- Creating new method of “neutralizing” any small-scale (1-lb. scale) hazard SCHMOO & SCHMMO 2 (Years 6–7)
- Examination of toxicity issues for canines (Year 5) and humans (Year 6), with publication (Years 6–7)
- Development of a new method for “injecting” nonvolatile explosives (e.g., chlorate) into IMS (Year 7)

D. Milestones

A major milestone was the examination of the six-carbon sugars. This study is close to complete. We expect to submit a paper by the end of the summer.

HMTD transformations is just now complete, slowed by COVID-19.

The book chapter(s) are still in progress. Cover and foreword have been submitted.

E. Final Results at Project Completion (Year 7)

- Thirty-five papers on HME (homemade explosives, including TATP, HMTD, ETN, AN, and UN), one full patent (safe-scent training aids), two full patents in process (SCHMOO and ADI), and one provisional patent (sheet pyrotechnics) in process.
- Nine PhD, three master's degree students, and numerous BS students have graduated, and many have entered the homeland security enterprise.
- Safe-scent explosive training aids are on the market and have recently been suggested for authentication aids for TSA.
- SCHMOO has received a great deal of free publicity, and we expect to find a vendor for it shortly.
- Ambient desorption ionization (ADI) with an additional task order from DHS S&T is being integrated into the Smiths 6000 ETD.
- Explosive database has over a thousand subscribers.

It is difficult to separate these projects by project number. The basic chemical characteristic had to be established in R1-A.1 before any work could progress on other projects.

III. RELEVANCE AND TRANSITION

A. Relevance of Research to the DHS Enterprise

Characterization of HMEs is an ongoing research effort within DHS, involving vendors and associated researchers. It impacts the entire HSE. In many cases, our methods of analysis lead the way for other members of the HSE. Our studies on the extreme sensitivity of HMTD to moisture and acidity may have prevented mishandling in a number of laboratories. Many vendors of explosives detection instrumentation have requested access to the explosives database, or asked for help in working with various materials characterized in this project. The characterization of these materials is published on our database (URI Explosives Database), which is subscribed to by over a thousand people, about a quarter of which are from US government agencies. Furthermore, our work is cited in the DHS HME Safety Protocols Handbook, and we were invited to participate in the DHS Chemical Security Analysis Center & Explosives Division 1st Inter-agency Explosives Terrorism Risk Assessment Working Group. We have worked directly with ten vendors of explosive detection instrumentation.

B. Status of Transition at Project End

Safe-scent aids have been licensed to Detectachem. ADI is moving forward with Smiths Detection. Many papers on HME are available in the open literature.

C. Transition Pathway and Future Opportunities

The ADI-Smiths project is ongoing with a task order through Northeastern.

D. Customer Connections

The connections to DHS (central), TSL, and TSA are strong. To date the FBI is the major agency outside of DHS which is aware of the details of this project.

IV. PROJECT ACCOMPLISHMENTS AND DOCUMENTATION

A. Education and Workforce Development Activities

1. Course, Seminar, and/or Workshop Development
   a. Since June 2019 we have held seven classes with 105 attendees. The new class was CTH.
   b. Dr. Oxley gave an invited lecture at the International Pyrotechnic Symposium in Tours, France, summer 2019.
2. Student Internship, Job, and/or Research Opportunities
   a. Five graduate students who were supported by ALERT and graduated are now at Signature Science supporting TSL (two students), the Navy Research Lab (two students), and Los Alamos National Laboratory (one student).
3. Interactions and Outreach to K–12, Community College, and/or Minority Serving Institution Students or Faculty
   a. We ran two two-week workshops introducing high school students to chemical analysis. This program will end with the end of ALERT.
4. Training to Professionals or Others
   a. Since June 2019 we have held seven classes with 105 attendees. The new class was CTH.

B. Peer Reviewed Journal Articles

1. Bezemer, K., McLennan, L., van Duin, L., Kuijpers, C.J., Koeberg, M., van den Elshout, J., van der Heijden, A., Busby, T., Yevdokimov, A.V., Schoenmakers, P., Smith, J.L., Oxley, J.C., & van Asten, A. "Chemical Attribution of the Home-Made Explosive ETN—Part I: Liquid Chromatography–Mass Spectrometry Analysis of Partially Nitrated Erythritol Impurities." *Forensic Science International*, 307(110102), December 2019. https://doi.org/10.1016/j.forsciint.2019.110102.
2. Rettinger, R.C, Porter, M., Canaria, J., Smith, J.L., & Oxley, J.C. "Fuel-Oxidizer Mixtures: A Lab and Field Study." *Journal of Energetic Materials*, *38*(2), 23 October 2019, pp. 170–190. https://doi.org/10.1080/07370652.2019.1679282.

Pending:

1. Bezemer, K., McLennan, L., van Duin, L., Kuijpers, C.J., Koeberg, M., van den Elshout, J., van der Heijden, A., Busby, T., Yevdokimov, A.V., Schoenmakers, P., Smith, J.L., Oxley, J.C., & van Asten, A. "Chemical Attribution of the Home-Made Explosive ETN—Part II: Use of Isotopic Ratio." *Forensic Science International*, in preparation.
2. Gonsalves, M.D., Colizza, K., Smith, J., & Oxley, J.C. "In Vitro Metabolism and Enzyme Phenotyping of Triacetone Triperoxide (TATP) in Humans." *Forensic Toxicology*, submitted.

C. Peer Reviewed Conference Proceedings

1. Oxley, J.C., Smith, J.L., Colizza, K., & Gonsalves, M. "In Vitro Metabolism of TATP." *NTREM*, April 2020, meeting proceedings, meeting canceled.

D. Seminars

1. Oxley, J.C. "Evaluation of Explosive Characteristics via Energy-Resolved MS." *ISADE*, April 2020 canceled.
2. Yevdokimov, A.V. "A Novel Approach to IMS Sampling and Analysis." Student Award Winner, *ISADE*, April 2020 canceled.

E. Poster Sessions

1. Gonsalves, M. "Metabolism of TATP." *ISADE*, April 2020 canceled.

F. Interviews and/or News Articles

1. CBS News. "'Innovative Checkpoint' and 'digital dog nose': TSA tests new security technology." *CBS News*, 25 November 2019. https://www.cbsnews.com/news/tsa-testing-advanced-airport-security-technology-digital-dog-nose-innovation-checkpoint/.

G. Other

1. Jimmie Oxley is an on-call American Chemical Society (ACS) expert. Consulted on July 2019 script for Gunpowder & Moon Smell and January 2020 on Explosive Vapor Detection.

H. New and Existing Courses Developed and Student Enrollment

New or Existing	Type	Title	Description	Enrollment
Existing	Short course	Explosive Stability	Analysis and safety of explosives	3
Existing	Short course	Propellants	Propellants	12
Existing	Short course	Fundamentals of Explosives	Fundamentals of explosives	26
Existing	Short course	Explosive Components	Device design	17
Existing	Short course	Explosive Components	Device design	17
Existing	Short course	Dynamic Diagnostics	Instrumentation & analysis	17
New	Short course	CTH for China Lake	Sandia computer code	13

I. Technology Transfer/Patents

1. Patent Applications Filed (Including Provisional Patents)
   a. Oxley, J.C., Smith, J.L., Yevdokimav, A.V., & Colizza, K. "Apparatus and Methods for Explosive Trace Detection Sample Preparation and Introduction into an Ionizing Detection System." Provisional patent 62/816,253, 11 March, 2019.
   b. Oxley, J.C., Smith, J.L, Ichiyama, R., & Kagan, G. "Safe Control of Hazardous Materials or Others Onsite." US 62/837,520, April 2019.
   c. Oxley, J.C., Smith, J.L., Kominia, A., Busby, T., & Stubbs, V. "Plasticized Flexible Pyrotechnic Material and Methods of Using the Same." Provisional Patent 62/993,992, 24 April 2020.

J. Requests for Assistance/Advice

1. From DHS
    a. Oxley is part of the DHS-formed Inter-Agency Explosive Terrorism Risk Assessment Working Group (IExTRAWG). In addition to group meetings, a representative was sent to URI for two days in August 2018, so that we could finalize the metric for selecting threat materials.
    b. On call for a variety of TSA TSS-E personnel.
2. From Federal/State/Local Government
    a. The URI bomb dog and his trainer rely on our lab for advice and explosives.

R1-C.2. APPENDIX

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In Vitro and *In Vivo* Studies of Triacetone Triperoxide (TATP) Metabolism in Humans

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ABSTRACT

Purpose Triacetone triperoxide (TATP) is a volatile but powerful explosive that appeals to terrorists due to its ease of synthesis from household items. For this reason, bomb squad, canine (K9) units, and scientists must work with this material to mitigate this threat. However, no information on the metabolism of TATP is available.

Methods *In vitro* experiments using human liver microsomes (HLM) and recombinant enzymes were performed on TATP and TATP-OH for metabolite identification and enzyme phenotyping. Enzyme kinetics for TATP hydroxylation were also investigated. Urine from laboratory personnel collected before and after working with TATP was analyzed for TATP and its metabolites.

Results While experiments with flavin-containing monooxygenases (FMOs) were inconclusive, those with recombinant cytochrome P450s (CYPs) strongly suggested that CYP2B6 was the principle enzyme responsible for TATP hydroxylation. TATP-O-glucuronide was also identified and incubations with recombinant uridine diphosphoglucuronosyl transferases (UGTs) indicated that UGT2B7 catalyzes this reaction. Michaelis-Menten kinetics were determined for TATP hydroxylation, with K_M = 1.4 μM and v_{max} = 8.7 nmol/min/nmol CYP2B6. TATP-O-glucuronide was present in the urine of all three volunteers after being exposed to TATP vapors showing good *in vivo* correlation to *in vitro* data. TATP and TATP-OH were not observed.

Conclusions Since scientists working to characterize and detect TATP to prevent terrorist attacks are constantly exposed to this volatile compound, attention should be paid to its metabolism. This paper is the first to elucidate any metabolism and excretion of TATP in humans. Also, TATP-O-glucuronide can be exploited as a marker of exposure to TATP in urine.

KEYWORDS energetic material, endoperoxide, human metabolism, TATP exposure, CYP2B6 hydroxylation, UGT2B7 glucuronidation.

INTRODUCTION

Triacetone triperoxide (3,3,6,6,9,9-hexamethyl-1,2,4,5,7,8-hexoxonane, TATP) is a homemade (HME) explosive, easily synthesized from household items.(1) For this reason, TATP has often been used by terrorists,(2) necessitating its research by bomb squad, canine (K9) units, and scientists.(3) In addition to being extremely hazardous, this peroxide explosive is highly volatile, with partial pressure of 4–7 Pa at 20°C.(4,5) Personnel exposed to TATP will most likely absorb it through inhalation and/or dermal absorption. However, no information on the human absorption, distribution, metabolism, excretion and toxicity (ADMET) of TATP is available. Therefore, this paper will investigate the *in vitro* metabolism of TATP and the *in vivo* excretion through urine analysis.

The toxicity of most military explosives has been well characterized.(6) The biotransformation of trinitrotoluene (TNT), for example, has been thoroughly investigated. It is metabolized by cytochrome P450 reductase, forming nitroso intermediates, and yielding 4-hydroxylamino-2,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. These primary metabolites are further reduced by cytochrome P450 to 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene.(7,8) *In vivo* studies of Chinese ammunition factory workers found metabolites such as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene in urine and bound to the hemoglobin in blood.(8,9) TATP has been studied for almost two decades, but its metabolism and toxicity are still unknown. TATP characterization is problematic since it is an extremely sensitive explosive, difficult to analyze and, due to its high volatility, difficult to concentrate in biological samples.(4)

Most xenobiotics are metabolized by cytochrome P450 (CYP) which is a family of heme-containing enzymes found in all tissues, particularly the liver endoplasmic reticulum (microsomes). CYPs catalyze phase I oxidative reactions (among others) in the presence of oxygen and a reducing agent (usually reduced nicotinamide adenine dinucleotide phosphate, NADPH). NADPH provides electrons to the CYP heme via cytochrome P450 reductase. This oxidation generally produces more polar metabolites that are either excreted in the urine or undergo phase II biotransformation, further increasing their hydrophilicity.(10) One of the most common phase II reactions is glucuronidation, which is catalyzed by uridine diphosphoglucuronosyl transferase (UGT) in the presence of the cofactor uridine diphosphate-glucuronic acid (UDPGA). In this reaction, glucuronic acid is conjugated onto an electron-rich nucleophilic heteroatom, frequently added to the substrate by phase I metabolism. Glucuronide metabolites increase the topical polar surface area (TPSA) and are ionized at physiological pH, thus, increasing the aqueous solubility of the compound for excretion.(10)

TATP is a cyclic peroxide, a motif shared with the anti-malarial drug, artemisinin. The endoperoxide functionality of artemisinin is thought to be crucial for its anti-malarial activity.(11) In the presence of ferrous ions, artemisinin undergoes homolytic peroxide cleavage to yield an oxygen radical that may be lethal to malaria parasite, *Plasmodium falciparum*. Biotransformation studies indicate that artemisinin is primarily metabolized by CYP2B6 to deoxy-artemisinin, deoxy-dihydroartemisinin, dihydroartemisinin and 'crystal-7'.(12,13) Similarly, we have previously shown that TATP is metabolized *in vitro* by canine CYP2B11, another CYP2B subfamily enzyme.(14) Artemisinin is further metabolized by glucuronidation, particularly by UGT1A9 and UGT2B7, to dihydroartemisinin-glucuronide, the principal metabolite found in urine, suggesting endoperoxides, like TATP, may be glucuronidated and excreted in urine. (15)

Laboratory personnel who work on synthesizing, characterizing and detecting TATP are inevitably exposed to this volatile compound. Even the small-size samples they work with can result in buildup of TATP in a confined space. Furthermore, bomb-sniffing dogs and their handlers are purposely exposed to these vapors for the sake of training. Our previous study revealed TATP metabolism in dog liver microsomes (DLM).(14) Now we evaluate its biotransformation in HLM and recombinant enzymes, identifying phase I and phase II metabolites, estimating enzyme kinetics and detecting urinary metabolites excreted from scientists exposed to TATP in their work environment.

MATERIAL AND METHODS

Chemicals

Optima HPLC grade methanol (MeOH), Optima HPLC grade water (H_2O), Optima HPLC grade acetonitrile (ACN), ACS grade acetone, ACS grade methanol, ACS grade pentane, hydrochloric acid (HCl), ammonium acetate (NH_4OAc), dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$) and reduced glutathione (GSH) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1-aminobenzotriazole (1-ABT), methimazole (MMI), 1-naphthol and hydroxyacetone were purchased from Acros Organics (Morris Plain, NJ, USA). Uridine-5'-diphosphoglucuronic acid (UDPGA), saccharolactone and 2,4-dichloro-phenoxyacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bupropion (BUP), benzydamide (BZD) and alamethicin were purchased from Alfa Aesar (Ward Hill, MA, USA). Oxcarbazepine (OXC) was purchased from European

Pharmacopoeia Reference Standard (Strasbourg, France). Ticlopidine (TIC) was purchased from TCI (Tokyo, Japan). Hydroxybupropion (BUP-OH) was purchased from Cerilliant Corporation (Round Rock, Texas, USA). Deuterated acetone (d_6-acetone) was purchased from Cambridge Isotope Labs (Cambridge, MA, USA). Hydrogen peroxide (50 %) was purchased from Univar (Redmond, WA, USA). Human liver microsomes (HLM), rat liver microsomes (RLM), dog liver microsomes (DLM) and human lung microsomes (HLungM) were purchased from Sekisui XenoTech (Kansas City, KS, USA). Human CYP bactosomes (rCYP) expressed in *Escherichia coli* (*E. coli*) were purchased from Cypex (Dundee, Scotland). Human FMO supersomes (rFMO) and human UGT supersomes (rUGT) expressed in insect cells were purchased from Corning (Woburn, MA, USA).

TATP, Deuterated TATP (d_{18}-TATP) and Hydroxy-TATP (TATP-OH) Synthesis

TATP was synthesized following the literature methods using hydrochloric acid as the catalyst.(1) TATP was purified by recrystallization, first with 80/20 (w/w) methanol/water (MeOH/H_2O) and then with pentane. Deuterated TATP was synthesized as above using d_6-acetone. Hydroxy-TATP was synthesized as above using 2:1:1 hydrogen peroxide (50 wt%): acetone: hydroxyacetone.(14) TATP-OH was purified using a Teledyne Isco CombiFlash RF+ system with attached PurIon S MS system, followed by two cycles of drying and reconstituting in solvent to sublime away the TATP. Separation was performed using a C-18 cartridge combined with a liquid chromatograph (LC) flow of 18 mL/min with 10% MeOH (A) and 90% aqueous 10 mM ammonium acetate (NH4OAc) (B) for 1 minute, before ramping to 35% A/65% B over 1 minute, followed by another ramp to 95% A/5% B over the next 1 minute, holding for 2 minutes, before a 30 second transition to initial conditions, with a hold of 2 minutes.(14)

Instrumentation

Metabolite identification was performed by high performance liquid chromatography coupled to Thermo Scientific Exactive or LTQ Orbitrap XL high resolution mass spectrometer (HPLC-HRMS). A CTC Analytics PAL autosampler was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump, and data collection/analysis was done using Xcalibur software (ver. 2.2 and 2.1, respectively).

Metabolite quantification was performed by high performance liquid chromatography coupled to AB Sciex Q-Trap 5500 triple quadrupole mass spectrometer (HPLC-MS/MS). A CTC Analytics PAL autosampler was used for LC injections, solvent delivery was performed using a Thermo Scientific Accela 1200 quaternary pump and data collection/analysis was done with Analyst software (ver. 1.6.2).

The HPLC method for all TATP derivatives was as follow: sample of 40 µL (Exactive and LTQ Orbitrap XL) or 20µL (Q-Trap 5500) in 50/50 acetonitrile/water (ACN/H_2O) were injected into LC flow of 250 µL/min of 10%A/90% B for introduction onto a Thermo Syncronis C18 column (50 X 2.1 mm, 5 µm). Initial conditions were held for 1 minute before ramping to 35% A/65% B over 1 minute, followed by another ramp to 95% A/5% B over the next 1 minute. This ratio was held for 2 minutes before reverting to initial conditions over 30 seconds, which was held for additional 2 minutes. The Exactive MS tune conditions for atmospheric pressure chemical ionization (APCI) positive mode were as follows: N_2 sheath gas flow rate, 30 arbitrary units (AU); N_2 auxiliary gas flow rate, 30 AU; discharge current, 6 µA; capillary temperature, 220 °C; capillary voltage, 25 V; tube lens voltage, 40 V; skimmer voltage, 14 V; and vaporizer temperature, 220 °C. The Exactive MS tune conditions for electrospray ionization (ESI) negative mode were as follows: N_2 sheath gas flow rate, 30 AU; N_2 auxiliary gas flow rate, 15 AU; spray voltage, -3.4 kV; capillary temperature, 275 °C; capillary voltage, -35 V; tube lens voltage, -150 V; and skimmer voltage, -22 V. The LTQ Orbitrap XL MS tune conditions for ESI-, used for TATP-O-gluc verification, were as follows: N_2 sheath gas flow rate, 30 AU; N_2 auxiliary gas flow rate, 15 AU; spray voltage, -4 kV; capillary temperature, 275 °C; capillary voltage, -15 V; and tube lens voltage, -84 V. The single reaction monitoring settings were as follows: m/z 413.13 with isolation width m/z 1.7, activated by higher-energy collision dissociation at 35 eV. The Q-trap 5500 MS tune and multiple reaction monitoring (MRM) conditions are shown in Table 1. TATP and TATP-OH quantification was done as the area ratio to d_{18}-TATP (internal standard, IS) using a standard curve

ranging 10-20,000ng/mL and 10-500 ng/mL (Fig. S-1) or 500-8,000 ng/mL, respectively. TATP-O-gluc relative quantification was done as the area ratio to 2,4-dichloro-phenoxyacetic acid (IS).

The HPLC method for bupropion (BUP), hydroxybupropion (BUP-OH), benzydamine (BZD), benzydamine N-oxide (BZD-NO), and oxcarbazepine (OXC, IS), was as follow: sample of 10 µL in 50/50 ACN/H_2O were injected into LC flow of 250 µL/min with 30% A/70% B for introduction onto a Thermo Scientific Acclaim Polar Advantage II C18 column (2.1 × 50 mm, 3 µm). Initial conditions were held for 1 minute before instant increase to 95% A/5% B and held for 2.5 minutes, then reversed to initial conditions over 30 seconds, with a hold of 1 minute for BUP, BUP-OH and OXC method or 3 minutes for BZD, BZD-NO and OXC method.

Metabolite Identification

All incubations were performed in triplicates in a Thermo Scientific Digital Heating Shaking Drybath set to body temperature 37 °C and 800 rpm. An incubation mixture containing phosphate buffer (pH 7.4), $MgCl_2$,(16) and NADPH (CYP cofactor(10)) was prepared so that at a final volume of 1 mL, their concentrations were 10 mM, 2 mM, 1 mM, respectively. When the incubation times were relatively long (greater than 15 minutes), it was thought necessary to use closed vessels to avoid loss of the volatile TATP or TATP-OH; as a result, prior to incubation, oxygen gas was bubbled through the buffer to ensure ample oxygen availability.(14) To this mixture, microsomes or recombinant enzymes were added and equilibrated for 3 minutes before the reaction was initiated by adding the substrate. Substrates included TATP in ACN, TATP-OH in MeOH, and BUP and BZD in H_2O. Organic solvents can disrupt metabolism, but the catalytic activity of most CYP enzymes is unaffected by less than 1 % ACN or MeOH.(17) At the end point, an aliquot was transferred to a vial containing equal volume of ice cold ACN and immediately vortex-mixed to quench the reaction. The sample was centrifuged for 5 minutes at 14000 rpm, and the supernatant analyzed by LC-MS.

Metabolite identification studies used microsomes (HLM, RLM, DLM) at protein concentrations of 1 mg/mL as part of the incubation mixture. The substrate, TATP, TATP-OH, or d_{18}-TATP (10 µg/mL) was allowed to incubate for several minutes before MS analysis. Negative controls consisted of the incubation mixture excluding either microsomes or NADPH. Positive control used 100 µM BUP, a probe substrate for CYP2B6.(18–20)

Phase II TATP metabolism was examined by two studies. Metabolism by glutathione S-transferase (GST) was probed by equilibrating 5 mM reduced glutathione (GSH, GST cofactor(10)) for 5 minutes in the incubation mixture including HLM before the substrate (TATP) was added. Ticlopidine (TIC, 10 µM) was the substrate for the GST the positive control (Fig. S-7).(21) To examine metabolism by UGT, HLM, buffer, and alamethicin (50 µg/mL in H_2O/MeOH) were equilibrate cold for 15 minutes before saccharolactone (1mg/mL, β-glucuronidase inhibitor(22)), $MgCl_2$, and NADPH were added and the mixture warmed to 37 °C and shaken at 800 rpm. After 3 minutes equilibration, the substrate (TATP or TATP-OH) was added, and in 2 minutes the reaction was started by the addition of 5.5mM UDPGA (UGT cofactor(10)).(23,24) Positive control used 100 µM 1-naphthol, an UGT1A6 substrate (Fig. S-8).(25,26) Alamethicin was employed to replace membrane transporters, in allowing UGT (located in the endoplasmic reticulum lumen) easy access to the UDPGA cofactor.(10)

Enzyme Identification

TATP was incubated as described in the previous section with various enzyme inhibitors in HLM (1 mg/mL). TATP-OH formation was monitored and benchmarked against incubations without inhibitors. Chemical inhibitors, such as 1-aminobenzotriazole (1mM, 1-ABT),(27,28) methimazole (500µM, MMI),(29,30) or TIC (100 µM),(31,32) were pre-equilibrated in the incubation mixture for 30 minutes prior to the addition of the substrate (100µM, TATP or controls). TATP was also tested as a possible CYP2B6 inhibitor; TATP or BUP was pre-equilibration in the incubation mixture before starting the reaction with a known CYP2B6 substrate (BUP) or TATP, respectively. Flavin-containing monooxygenase (FMO) inhibition by heat was also tested.(10,33) In that experiment, HLM was mixed with buffer and pre-heated at 37 °C or 45°C for 5 minutes. After an hour cooling on

ice, the incubation procedure was resumed. BUP, a CYP2B6 substrate,(18–20) and BZD, a FMO substrate,(33) were used as positive and negative control substrates to assess CYP, FMO and CYP2B6 inhibition. Samples not pre-incubated with chemical inhibitors nor heated to 45 °C were used as 100% TATP-OH formation. Inhibition studies were quenched after 15 minutes incubation.

Recombinant CYP and FMO enzymes were employed to identify the isoform responsible for the NADPH dependent metabolism. Human bactosomes expressed in *E. coli* were used for CYP isoform identification; CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (100 pmol CYP/mL) were tested. Human supersomes expressed in insect cells were used for FMO isoform identification; FMO1, FMO3 and FMO5 (100 µg protein/mL) were examined. Both TATP and TATP-OH were tested as the substrate (10 µg/mL) in the incubation mixture with the recombinant enzymes. Negative control incubations were done in *E. coli* control or insect cell control. Positive control incubations were done in HLM (200 pmol CYP/mL), which contains all CYP and FMO enzymes. Recombinant enzymes studies were quenched after 10 minutes incubation.

Recombinant UGT enzymes were used to identify the isoform responsible for phase II metabolism. Human supersomes expressed in insect cells were used for UGT isoform identification; UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 were examined. The incubation mixture was similar to the glucuronidation incubation previously described, except saccharolactone was not added;(34) 500 µg protein/mL was used; and the substrate was 10 µg/mL TATP-OH. Negative control incubations were done in insect cell control. Positive control incubations were done in HLM (1 mg/mL), which contains all UGT enzymes. Glucuronidation with recombinant enzymes were quenched after 2 hours incubation.

Enzyme Kinetics

Kinetics experiments were done to determine the affinity of the enzyme CYP2B6 to the substrate TATP. The human CYP2B6 bactosomes used contained human CYP2B6 and human cytochrome P450 reductase co-expressed in *E. coli*, supplemented with purified human cytochrome b_5. Cytochrome P450 reductase is responsible for the transfer of electrons from NADPH to CYP, a task sometimes extended to cytochrome b_5.(10) The incubation mixture (1 mL) contained 10 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 50 pmol/mL rCYP2B6, 1 mM NADPH, and various concentrations of 0.1 to 20 µM TATP. The reaction was initiated by adding TATP after a 3-minute pre-equilibration and stopped at different end points (up to 5 minutes) to determine rate of TATP hydroxylation. The rate of TATP hydroxylation in lungs was also investigated by incubating TATP (100 µM) in the incubation mixture containing 1 mg/mL HLM or HLungM, instead of rCYP2B6, for up to 10 minutes.

TATP-OH metabolism by CYP2B6 was evaluated by incubating 10 µg/mL TATP-OH in CYP2B6 bactosomes according to the above procedure, with or without NADPH, except the buffer was pre-oxygenated so that the incubation could be performed in a closed vessel. Aliquots were removed and quenched at different time points, up to 30 minutes. TATP-OH depletion by HLM was determined using the same procedure, except 1µM TATP-OH and up to 60 minutes reaction times were used.

Urine Analysis

Laboratory personnel testing TATP are constantly exposed to this volatile compound. Explosive sensitivity experiments, such as drop weight impact tests, are done in a small brick-walled room for explosivity precautions, unlike synthesis reactions which are performed inside a fume hood for coverage protection. Also, testing portable explosive trace detection (ETD) devices that are meant to be used in the field are tested as such, which also contribute to exposure. Urine from laboratory workers was tested for TATP and its metabolites after TATP exposure in the laboratory environment. Urine was collected at the beginning of the work week and 2 hours after performing activities that could lead to high TATP exposure. To determine the longevity of TATP in the body, urine from the day following TATP exposure was also tested. The fresh urine was cleaned and concentrated for analysis using solid-phase extraction (SPE). Restek RDX column was conditioned with 6 mL of MeOH, followed by 6 mL of H_2O, and sample introduction (20–250 mL urine). The sample was washed with two cycles of 3mL 50:50 (v/v)

MeOH:H_2O. Extraction was achieved with two cycles of 1mL ACN. Both the MeOH:H_2O wash and the ACN extraction were tested by LC-MS.

RESULTS

Metabolite Identification

When TATP was incubated in HLM, TATP was depleted and one observable product, hydroxyl-TATP, was formed over time (Fig. 1). Metabolism of TATP in HLM consists of hydroxylation at one methyl group with the peroxide bonds and nine-membered ring structure preserved (Fig. 2).(14) Solaja have reported various monohydroxylated and dihydroxylated products during microsomal incubations with cyclohexylidene and steroidal mixed tetraoxanes where the peroxide bond was also preserved.(11) A TATP-OH standard (Fig. S-2) was chemically synthesized to confirm the metabolite by retention time and mass-to-charge ratio (m/z). TATP-OH, identified as $[TATP\text{-}OH + NH_4]^+$ (m/z 256.1391) by accurate mass spectrometry, increased in incubation samples as time progressed. The hydroxylated metabolite could not be verified with deuterated TATP due to the persistence of a contaminant with the same mass. As previously observed, TATP incubations in different species (dogs and rats) yielded the same metabolite, TATP-OH (Fig. S-3).(14) Other suspected metabolites, including the dihydroxy-species and additional oxidation of the TATP-OH to the aldehyde and carboxylic acid were not observed. Small polar molecules, such as acetone and hydrogen peroxide, the synthetic reagents of TATP, could not be chromatographically separated or are below the lower mass filter limit.

TATP was investigated for phase II metabolism routes of glutathione and glucuronide conjugation. Incubation in HLM with GSH produced no detectable glutathione metabolite conjugates, indicating that TATP is most likely not a substrate for microsomal GSTs (Fig. S-4). When TATP was incubated with UDPGA, the TATP-OH glucuronic acid metabolite (TATP-O-gluc) was observed (Fig. 2). The m/z 432.1712 for $[TATP\text{-}O\text{-}gluc + NH_4]^+$ was observed at very low levels after 2 and 3 hours of incubation. When the sample was dried and reconstituted in low volume, the intensity of $[TATP\text{-}O\text{-}gluc + NH_4]^+$ increased, but TATP and TATP-OH were evaporated along with the solvent. TATP and TATP-OH are volatile, limiting their use in quantification experiments since sample concentration is not feasible.(4) However, TATP-O-gluc is a non-volatile TATP derivative that is amenable to sample preparation. Formation of TATP-O-gluc over time was monitored as an intensity increase of m/z 432.1712 (Fig. S-5). Even though TATP and TATP-OH generally form ammonia adducts under positive ion APCI, the glucuronide favors negative ion mode ESI. The m/z 413.1301 for $[TATP\text{-}O\text{-}gluc - H]^-$ was easily seen at 1 to 3 hours without sample concentration. The common fragments of glucuronic acid, m/z 175, 113, and 85, were observed in the fragmentation pattern of m/z 413.1301, confirming the presence of a glucuronic acid conjugate (Fig. 3).(35) TATP-O-glucuronide was not observed in negative controls without UDPGA or NADPH, indicating that TATP-OH must be formed and then be further metabolized into TATP-O-gluc. Glucuronide conjugates are highly polar compounds that are easily eliminated by the kidney, suggesting that TATP-O-gluc would likely progress via urinary excretion.(10)

Enzyme Identification

TATP was only metabolized into TATP-OH in HLM in the presence of NADPH, indicating that the metabolism is NADPH-dependent. The predominant microsomal enzymes that require NADPH for activity are CYP and FMO.(10)

The usual roles of CYP are hydroxylation of an aliphatic or aromatic carbons, epoxidation of double bonds, heteroatom oxygenation or dealkylation, oxidative group transfer, cleavage of esters and dehydrogenation reactions.(10) 1-ABT is considered a general mechanism-based inhibitor of CYPs, initiated by metabolism into benzyne, which irreversibly reacts with the CYP heme.(27,28) When CYP activity was inhibited by 1-ABT, TATP-OH formation was also inhibited, with only 3.6% formed, (Table 2), suggesting that CYP is involved in the

hydroxylation of TATP. To support this evidence and to narrow down the CYP isoform catalyzing TATP hydroxylation, TATP was incubated with rCYPs (Fig. 4). The CYPs selected for testing are responsible for the metabolism of 89% of common xenobiotics.(36) TATP-OH was not observed when TATP was incubated with CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. TATP hydroxylation was performed exclusively by CYP2B6, with 5.6 ± 0.3 μM TATP-OH produced in 10 minutes. CYP2B6 has been found to metabolize endoperoxides by hydroxylation, as observed for TATP.(12,13) HLM, which contains CYP2B6, also exhibited TATP hydroxylation (1.5 ± 0.1 μM).

Since dog liver microsomes studies indicated that TATP is metabolized by CYP2B11, it was not surprising that another CYP2B subfamily enzyme, CYP2B6, metabolizes TATP in humans.(14) CYP2B6 metabolism of TATP was further investigated by incubating TATP with TIC, a mechanism-based inhibitor of CYP2B6.(31,32) When CYP2B6 activity was inhibited by TIC, TATP-OH formation was also inhibited by 95% (Table 2), further supporting the primary involvement of CYP2B6 in the metabolism of TATP. BUP hydroxylation is catalyzed by CYP2B6; therefore, it was chosen as a positive control for CYP and CYP2B6 inhibition tests. BUP-OH formation was inhibited by 77 % and 90% when incubated with 1-ABT and TIC, respectively.(37)

FMO catalyzes oxygenation of nucleophilic heteroatoms, such as nitrogen, sulfur, phosphorous and selenium.(33,38) Although FMO involvement in the hydroxylation of the TATP methyl group is unlikely, it seemed prudent to examine this enzyme class. Addition of MMI, an FMO competitive inhibitor,(29,30) caused a decrease in TATP-OH formation by 66% (Table 2), but this is not necessarily direct inhibition of TATP metabolism by FMO since MMI has been reported to reduce CYP2B6 activity by up to 80%.(33,39) While this result was inconclusive about the FMO contributions to TATP metabolism, it could be considered further support for the role of CYP2B6. TATP was incubated with available rFMOs: FMO1, FMO3 and FMO5 (Fig. 4). FMO1 is expressed in adults in the kidneys, and it should not contribute to the liver metabolism of TATP;(33) indeed, none appeared to be involved as no TATP-OH was produced. Since FMO is inactivated by heat,(10,33) TATP was incubated in HLM preheated to 45 °C for 5 minute. Table 2 compares the formation of TATP-OH at 37°C to that at 45°C and to the N-oxidation of the positive control, BZD. Although, there was a decrease in TATP hydroxylation, the inhibitory effect was not as significant compared to the decrease in BZD N-oxidation, which is catalyzed by FMO.

TATP-OH also appears to be metabolized by HLM in an NADPH-dependent manner: therefore, TATP-OH was incubated for 10 minutes with recombinant enzymes (CYP and FMO), to determine which isoform is responsible for this secondary phase I metabolism (Table 3). Although in all incubations some TATP-OH was lost due to sublimation at 37 °C, the only notable loss was with CYP2B6. In that incubation TATP-OH was depleted by 40%, suggesting CYP2B6 metabolizes TATP-OH. TATP-OH depletion in CYP2B6 was faster in the presence of NADPH than its absence, supporting metabolism by CYP2B6 (Fig. S-6). Unfortunately, no subsequent metabolite was identified.

To identify which isoform is responsible for TATP glucuronidation, TATP-OH was incubated with the most clinically relevant rUGTs (Fig. 5).(40) Glucuronidation was not observed with UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9. TATP-O-glucuronide was formed only in UGT2B7 incubations with 0.05 ± 0.03 area count relative to IS produced after 2 hours of incubation. HLM, which contains UGT2B7, also displayed TATP-O-gluc (0.26 ± 0.02 area count relative to IS). Relative quantification of TATP-O-gluc was done by area ratio to the IS because a TATP-O-gluc standard is not available. Endoperoxide glucuronidation by UGT2B7 has been reported, in which urine analysis of patients treated with artesunate, an artemisinin derivative, found dihydroartemisinin-glucuronide to be the principal metabolite excreted.(15)

Enzyme Kinetics

Rate of TATP hydroxylation by CYP2B6 was evaluated by plotting concentration of TATP-OH formed over time. The initial rate of TATP hydroxylation at various TATP concentrations was used to estimate enzyme kinetics using the Michaelis-Menten model: $v = v_{max} * [S] / (K_M + [S])$. Here, [S] is the substrate (TATP) concentration, v_{max} is the maximum formation rate, K_M is the substrate concentration at half of v_{max}, and k_{cat} is the

turnover rate of an enzyme-substrate complex to product and enzyme.(41) The kinetic constants were obtained using nonlinear regression analysis on GraphPad Prism software (ver. 8.2.1). The Michaelis-Menten (Fig. 6) evaluation for TATP hydroxylation by CYP2B6 yielded K_M of 1.4 μM; v_{max} of 8.7 nmol/min/nmol CYP2B6; and k_{cat} of 174 min^{-1}. Other linearized models are in good agreement:

Lineweaver-Burk: $1/v = (K_M/v_{max} * 1/[S]) + 1/v_{max}$; K_M 3.1 μM; v_{max} 11.7 nmol/min/nmol CYP2B6

Eadie-Hofstee: $v = (-K_M * v /[S]) + v_{max}$; K_M 0.54 μM; v_{max} 4.9 nmol/min/nmol CYP2B6

Hanes-Woolf: $[S]/v = [S]/v_{max} + K_M/v_{max}$; K_M 1.2 μM; v_{max} 8.0 nmol/min/nmol CYP2B6

The low K_M indicates TATP has a high affinity for CYP2B6.(42) Table 2 shows that TATP inhibits BUP hydroxylation by CYP2B6, with only 62% BUP-OH formation in 15 minutes. However, in the presence of BUP, TATP-OH formation was enhanced, with 125% formed compared to the reaction uninhibited by BUP (Table 2). CYP2B6 preference for TATP affects the metabolism of BUP, but further testing is needed to establish the specific type of inhibition.

Using the Michaelis-Menten parameters, *in vitro* intrinsic clearance ($Cl_{int} = v_{max}/K_M$) was calculated to be 6.13 mL/min/nmol CYP2B6.(10,43,44) Scale-up of the Cl_{int} to yield intrinsic clearance on a per kilogram body weight was done using values of 0.088 nmol CYP2B6/mg microsomal protein, 45 mg microsomal protein/g liver wet weight and 20 g liver wet weight/kg human body weight.(43) Taking that into account, the scale-up Cl_{int} was calculated to be 485 mL/min/kg.

In vivo intrinsic clearance (Cl) is the ability of the liver to metabolize and remove a xenobiotic, assuming normal hepatic blood flow (Q = 21 mL/min/kg(43,45)) and no protein binding.(43) Cl can be extrapolated using the well-stirred model excluding all protein binding as $Cl = Q * Cl_{int} / Q + Cl_{int}$.(43) The *in vivo* intrinsic clearance of TATP was estimated as 20 mL/min/kg. Compared to common drugs, TATP has a moderate clearance.(46)

TATP-OH kinetics was also investigated by substrate depletion. Substrate depletion was plotted as the natural log of substrate percent remaining over time (Fig. 7). Half-life ($t_{1/2}$) was calculated to be 16 minutes, as the natural log 2 divided by the negative slope of the substrate depletion plot. *In vitro* intrinsic clearance (Cl_{int}) can also be estimated using half-life as $Cl_{int} = (0.693/t_{1/2})$ * (incubation volume/mg microsomal protein).(47,48) The *in vitro* intrinsic clearance of TATP-OH was estimated as 0.042 mL/min/mg. Even though we identified two metabolic pathways for TATP-OH, it appears to be cleared slower than TATP.

Lung metabolism

Inhalation is the most probable pathway for systemic exposure since TATP is both volatile and lipophilic. With passive diffusion into the bloodstream being very possible, TATP metabolism in the lung was also investigated. TATP was incubated in lung and liver microsomes for comparison of metabolic rate. The results, shown in Table 4, indicate that TATP hydroxylation in the lungs is negligible. Though CYP2B6 gene and protein are expressed in the lungs, enzyme activity in lungs is minimal compared to the liver, limiting TATP metabolism.(49,50) This suggests that TATP will most likely be distributed through the blood to the liver for metabolism. News reported that traces of TATP was found in the blood samples extracted from the Brussels suicide bombers.(51) This indicates the possibility of using blood tests as forensic evidence for TATP exposure.

Urine Analysis

Laboratory workers, who normally work with TATP on a daily basis, performing tasks like explosive sensitivity testing, were screened for TATP exposure. These laboratory workers volunteered to collect their urine

before and after exposure to TATP vapor. Since the health effects of TATP exposure are unknown, to minimize any additional risks to these workers, this pilot study was performed in duplicates using only three volunteers to establish some reproducibility. TATP and TATP-OH were not observed in the urine of any of the workers. However, TATP-O-gluc was present in all urine samples after TATP exposure (Fig. 8). TATP-O-glucuronide was identified in human urine samples as both $[TATP\text{-}O\text{-}gluc - H]^-$ and $[TATP\text{-}O\text{-}gluc + NH_4]^+$. The presence of TATP-O-gluc in the urine of all three volunteers is summarized in Table 5.

CONCLUSIONS

Because TATP has high volatility it is likely to be absorbed into the body by inhalation; however, no appreciable metabolism in lung was observed in either dog(14) or human microsomes. Therefore, systemic exposure and subsequent liver metabolic clearance was presumed. Across three species, dog, rat, and human, TATP was metabolized in liver microsomes by CYP to TATP-OH. Using recombinant enzymes, we have previously established that CYP2B11 is responsible for this metabolism in dogs.(14) Interestingly, human CYP2B6 appears to be the major phase I enzyme responsible for the same metabolism. Though heat inactivation and specific FMO inhibition appeared to affect TATP hydroxylation, incubations with recombinant FMO suggest FMO was not forming TATP-OH. MMI, an FMO inhibitor, inhibited TATP-OH formation; but, inhibition of BUP under these conditions suggests MMI also inhibits CYP2B6 activity.(39)

When incubated together, TATP and BUP, appear to compete for CYP2B6 metabolism, with BUP hydroxylation being inhibited by 38% in the presence of TATP. Considering CYP2B6 expression in the liver is low and exhibits broad genetic polymorphisms, CYP2B6 activity can be widely affected if TATP affects the metabolism of other compounds, like BUP.(52,53) TATP may be a serious perpetrator for drug-drug interactions (DDI) for compounds cleared by CYP2B6.(54,55)

In vitro clearance of TATP was calculated as 0.54 mL/min/mg protein with *in vivo* extrapolation to 20 mL/min/kg. *In vitro* clearance of TATP-OH was roughly estimated as 0.038 mL/min/mg protein. We also established the clearance of TATP in dogs as 0.36 mL/min/mg protein in our previous study in canine microsomes, which is significantly relevant to K9 units, where the dog and human handler are both exposed to the explosive.(14)

No glutathione adducts of any TATP metabolism products were observed in the microsomal incubations with GSH. However, glucuronidation converted TATP-OH to TATP-O-gluc in HLM with UGT2B7 specifically catalyzing this reaction. Considering glucuronides are often observed as urinary metabolites, the presence of TATP-O-gluc in urine can be exploited as an absolute marker of TATP exposure.

Urine from scientists working to prevent terrorist attacks by synthesizing, characterizing and detecting TATP, who are inevitably exposed to this volatile compound were negatively tested for TATP and TATP-OH, but TATP-O-gluc was present at high levels in their fresh urine. Even though working with TATP falls under the protection of several standard operating procedures (SOPs) to handling explosives, considering the breathing exposure these laboratory workers revealed, implementation of precautionary measures to absorption by inhalation, such as the use of respirators, should be considered. This paper is the first to examine some aspects of TATP ADMET, elucidating the metabolism and excretion of TATP in humans.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.
Ethics approval This study was approved by the University of Rhode Island Institutional Review Board (IRB) for Human Subjects Research (approval number 1920-206).

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TABLES

Table 1: Triple quadrupole mass spectrometer (Q-trap 5500) operating parameters

Parameters	Method 1		Method 2	Method 3			
Source type	APCI+		ESI-	ESI+			
Source temperature °(C)	300		300	260			
Ion spray voltage (V)	N/A		-4500	4500			
Nebulizer current (μA)	0.8		N/A	N/A			
Ion source gas 1 (psi)	50		50	20			
Ion source gas 2 (psi)	2		2	2			
Curtain gas (psi)	28		28	30			
Collision gas (psi)	6		6	5			
Declustering potential (V)	26		-26	30			
Entrance potential (V)	10		-10	10			
Internal standard MRM transitions (m/z)	258 → 80, 46		219 → 161, 125	253 → 208, 180			
Collision energy (V)	11, 27		-13, -27	27, 39			
Collision cell exit potential (V)	14, 20		-36, -20	22, 14			
Analyte	TATP	TATP-OH	TATP-O-gluc	BUP	BUP-OH	BZD	BZD-NO
Analyte MRM transition (m/z)	240 → 74, 43	256 → 75	413 → 113, 87	240 → 184, 166	256 → 238, 139	310 → 86	326 → 102
Collision energy (V)	11, 28	13	-22, -34	17, 23	15, 33	21	19
Collision cell exit potential (V)	10, 11	36	-13, -9	20, 10	12, 16	10	12

Table 2: Average % metabolite formed (triplicates) in 15min incubations with chemical inhibitors or heat

Metabolite Formation in 15min	30 min pre-incubation in HLM					HLM pre-heated for 5min	
	No inhibitor	1-ABT CYP inhibitor	MMI FMO inhibitor	TIC CYP2B6 inhibitor	TATP or BUP	37°C	45°C
TATP-OH (%)	100 ± 5	3.6 ± 0.7	34 ± 2	4.8 ± 0.7	12µ± 21	100 ± 10	69 ± 4
BUP-OH (%)	100 ± 3	23 ± 1	62 ± 2	9.6 ± 0.4	62 ± 2	100 ± 6	99 ± 2
BZD-NO (%)	100 ± 2	97 ± 1	48 ± 1	98 ± 3	N/A	100 ± 2	44 ± 2

Table 3: Average % TATP-OH remaining (triplicates) after 10 min incubation in recombinant enzymes

Incubation matrix	% TATP-OH Remaining
HLM	69 ± 5
rCYP control	76 ± 4
rCYP1A2	77 ± 2
rCYP2B6	60 ± 3
rCYP2C9	71 ± 8
rCYP2C19	76 ± 5
rCYP2D6	76 ± 5
rCYP2E1	73 ± 5
rCYP3A4	78 ± 6
rFMO control	69 ± 3
rFMO1	78 ± 12
rFMO3	78 ± 6
rFMO5	72 ± 7

Table 4: Rate of TATP hydroxylation (triplicates) in HLM versus HLungM

Human Microsomes	Rate of TATP-OH Formation (nmol/min/mg)
Liver	0.425 ± 0.06
Lung lot1710142	< LOQ
Lung lot1410246	< LOQ

Table 5: Summary of TATP-O-glucuronide presence in human urine (triplicates). TATP glucuronide is observed as $[TATP\text{-}O\text{-}gluc - H]^-$ (m/z 413.1301) and $[TATP\text{-}O\text{-}gluc + NH_4]^+$ (m/z 432.1712, less sensitive)

Human	m/z 413.1301			m/z 432.1712		
	#1	#2	#3	#1	#2	#3
Before TATP exposure	-	-	-	-	-	-
2 hours after TATP exposure	+	+	+	+	+	+
1 day after TATP exposure[a]	+	+	-	+	-	-

[a] Only one trial performed on next day samples.

LEGENDS TO FIGURES

Fig. 1: TATP biotransformation into TATP-OH monitored over time in HLM. Performed in triplicates

Fig. 2: TATP metabolic pathways in HLM

Fig. 3: Mass spectrum of $[TATP\text{-}O\text{-}gluc - H]^-$ (m/z 413.1301), fragmented with 35 eV using ESI-. Proposed structure are shown(35)

Fig. 4: TATP-OH formation from TATP incubations with recombinant CYP and FMO. Experiments with rCYP or rFMO consisted of 10 µg/mL TATP incubated with phosphate buffer (pH 7.4), 2 mM $MgCl_2$ and 1 mM NADPH. Incubations done in triplicates and quenched at 10 minutes

Fig. 5: TATP-O-glucuronide formation from TATP-OH incubations with recombinant UGT. Experiments with rUGT consisted of 10 µg/mL TATP-OH incubated with phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 50 µg/mL alamethicin, 1 mM NADPH, and 5.5µM UDPGA. Glucuronidation done in triplicates and quenched at 2 hours. Quantification was done using area ratio TATP-O-gluc/IS

Fig. 6: Rate of TATP hydroxylation by CYP2B6 versus TATP concentration. Incubations of various TATP concentrations consisted of 50 pmol CYP2B6/mL human bactosomes with phosphate buffer (pH 7.4), 2mM $MgCl_2$ and 1mM NADPH. Incubations done in triplicates and quenched every minute up to 5 minutes.

Fig. 7: Natural log of TATP-OH % remaining in HLM versus time. TATP-OH (1µM) incubated in 1 mg/mL HLM with pre-oxygenated phosphate buffer (pH 7.4), 2 mM $MgCl_2$ and 1 mM NADPH. Incubations done in closed vials, in triplicates and quenched every 10 minutes up to 1 hour.

Fig. 8: Extracted ion chromatogram of $[TATP\text{-}O\text{-}gluc - H]^-$ (m/z 413.1301) in HLM 2 hours after incubation with TATP, and in human urine, before TATP exposure and 2 hours after TATP exposure.

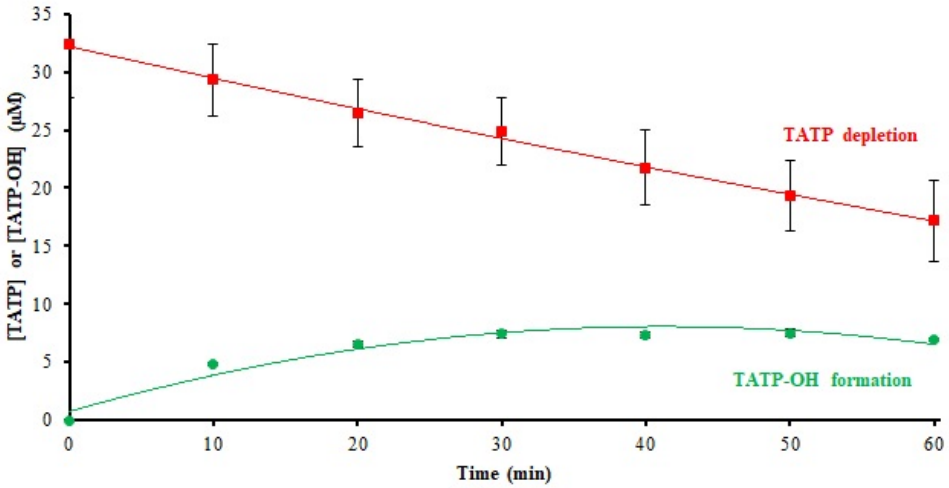

Figure 1

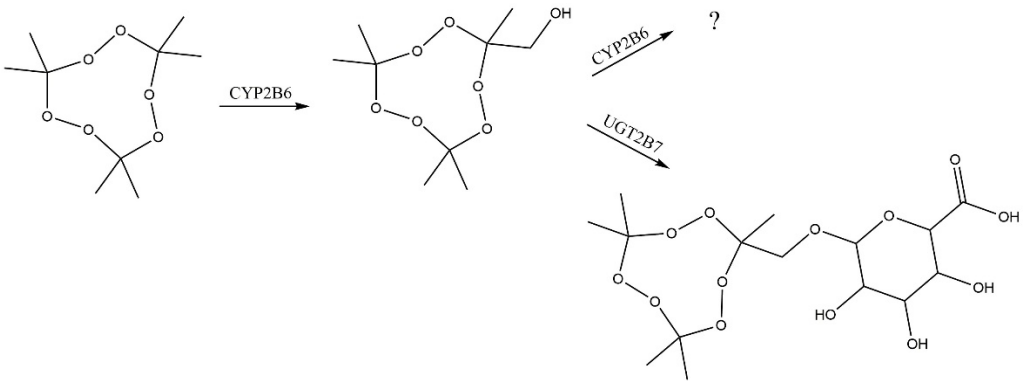

Figure 2

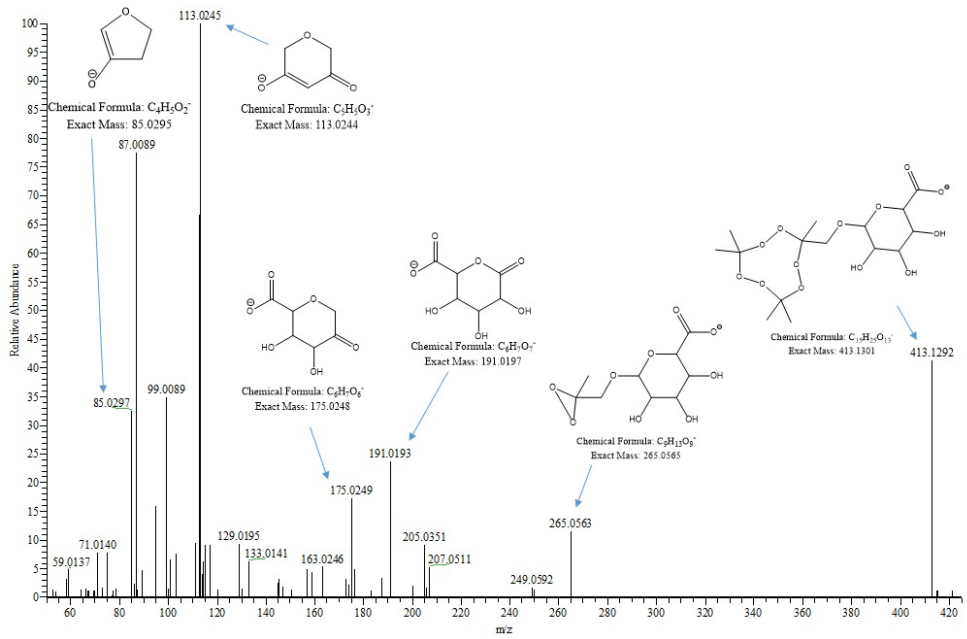

Figure 3

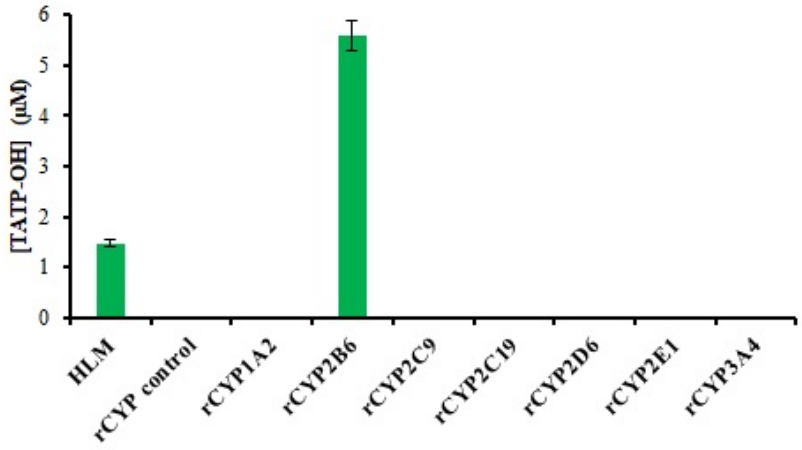

Figure 4

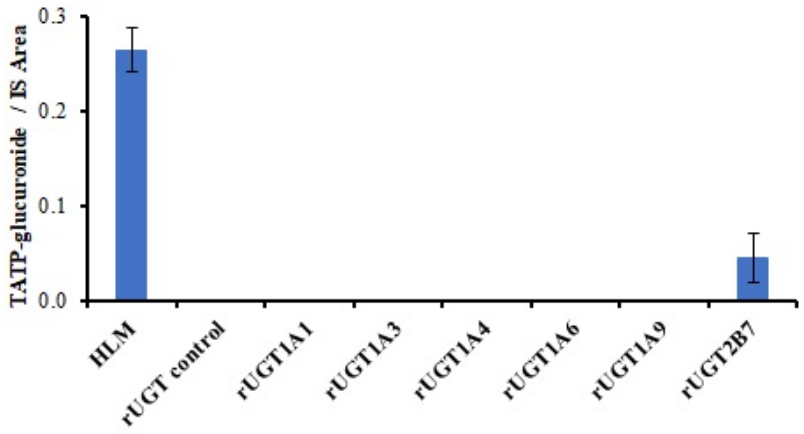

Figure 5

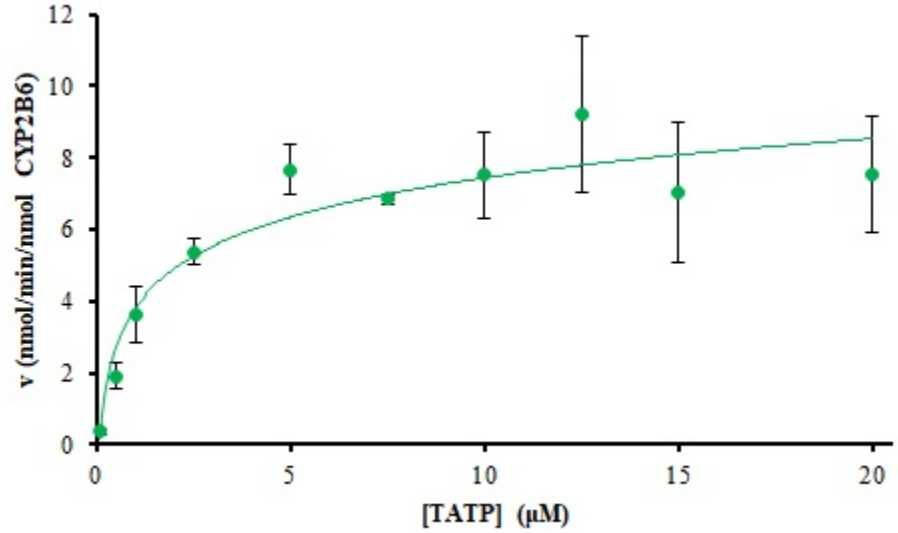

Figure 6

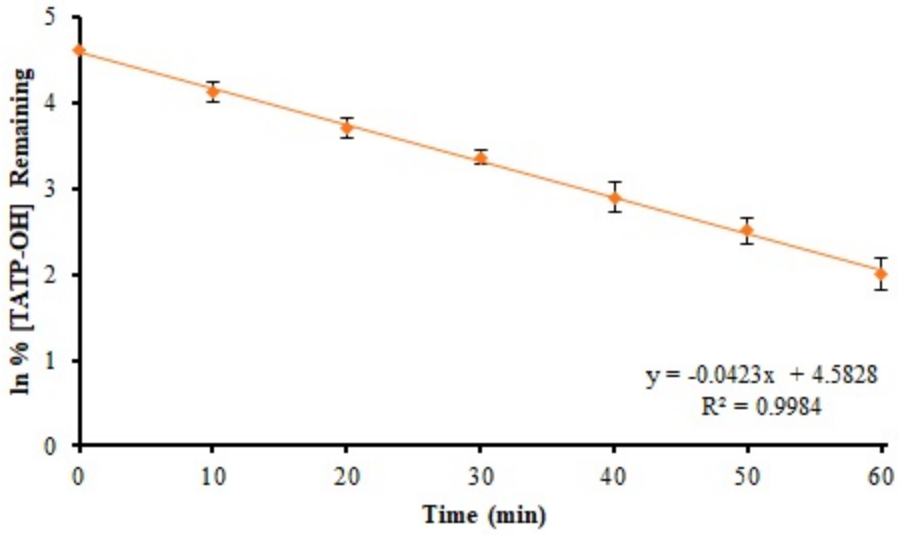

Figure 7

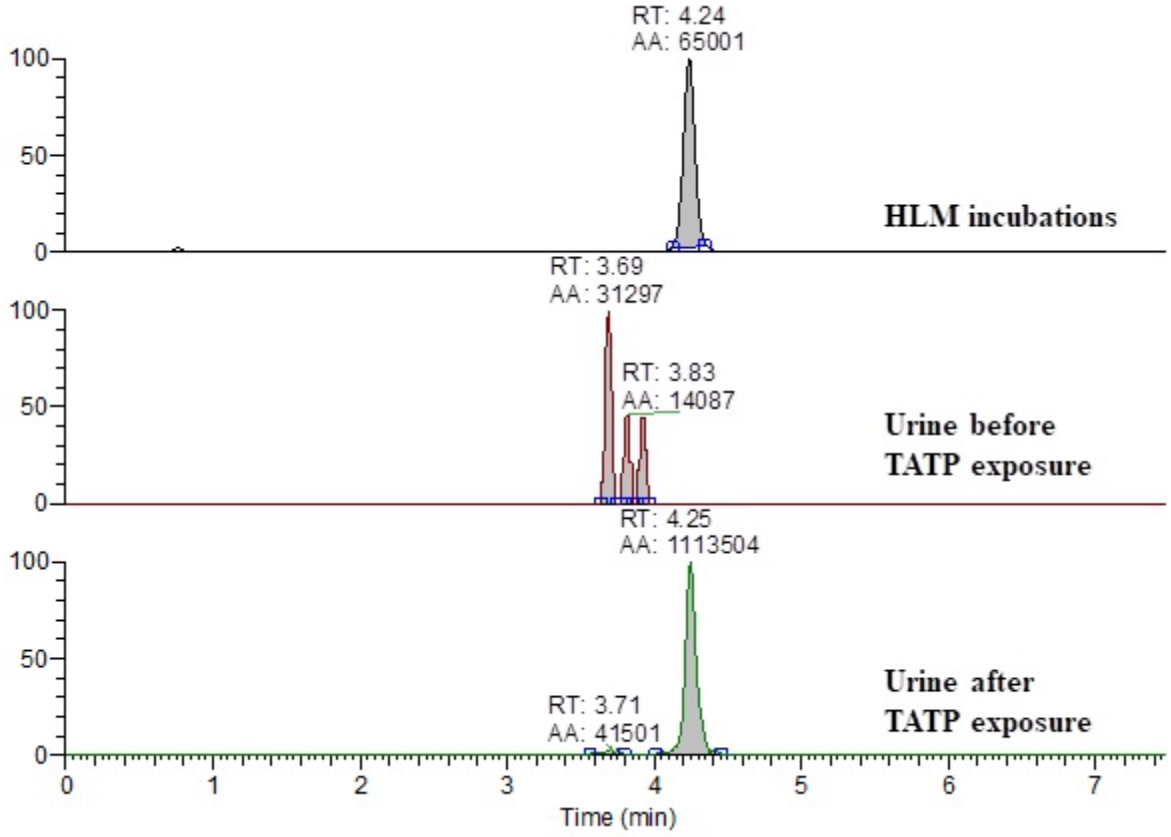

Figure 8