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Robert T. Tessman
Children's Mercy Hospital

Whitney M. Nolte
Children's Mercy Hospital

Ashlynn Chipman
Children's Mercy Kansas City

Jennifer Goldman
Children's Mercy Kansas City

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Characterization of Trimethoprim Metabolism and Metabolite Reactivity in Human Liver, Lung, and Skin

Bob Tessman [1], Whitney Nolte [1], Ashlynn Chipman [1,2], Jen Goldman [1]

Children's Mercy

Abstract

Idiosyncratic adverse drug reactions (IADRs) occur in a small but significant percentage of the population, are unpredictable, and frequently cause life-threatening events requiring intensive medical care. The antibiotic, trimethoprim-sulfamethoxazole (TMP-SMX) is generally considered a safe and effective drug but has a relatively high rate of IADRs. Through metabolism, drugs may be bioactivated, yielding reactive metabolites which covalently bind to proteins making them a target for immune-mediated responses, and in some cases, fulminant drug hypersensitivity. Although the liver is the dominant contributor to drug metabolism, other organ systems are known to be metabolically active . Interestingly, TMP-SMX IADRs are associated with serious lung injury and/or mild to serious skin rash. We seek to evaluate TMP metabolites in liver, lung, and skin and to assess their reactivity. Subcellular s9 fractions contain active drug metabolizing enzymes and with the addition of appropriate co-factors, both phase I and II metabolites can be produced in vitro. We discovered differential phase I and II TMP metabolism in liver, lung, and skin. Reaction products were analyzed by Waters Instruments triple quadrupole liquid chromatography/mass spectrometry (LC/MS). The reactivity of tissue-specific metabolites was assessed by incubating s9/TMP reaction products with glutathione, n-acetyl cysteine, and n-acetyl lysine. Potentially trapped metabolites were analyzed using a high-resolution Waters quadrupole/time of flight (q-tof) MS. These data were fed into a peak-picking algorithm (XCMS) to identify unique features against several negative controls. We identified 2 potentially reactive sulfate conjugates of 4-desmethyl trimethoprim. The results indicate that primary metabolites formed in the liver can undergo further bioactivation in lung and skin, the sites of clinical presentation of TMP IADRs. These findings mechanistic insights will advance efforts to understand the etiology of this currently idiopathic medical condition.

Results

Clinical Presentations of TMP ADRs

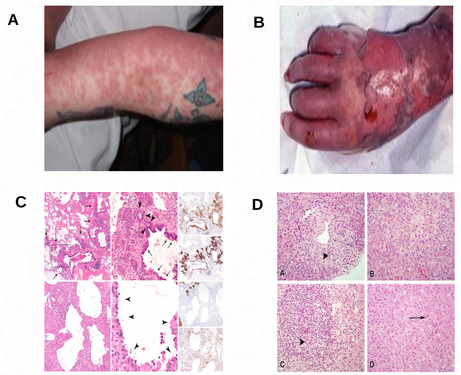

Figure 1. Clinical manifestations of ADRs in liver, lung, and skin. A. Mild skin reaction B. Severe skin reaction C. H&E ADR lung cell death D. H&E ADR liver cell death

Images from: Pirmohamed M. et al. 2011;89(6);896-901. and Ghabril M, et al*Curr Opin Gastroenterol* 2010; 26(3):222-6.

Phase 1 Metabolism: Liver, Lung & Skin

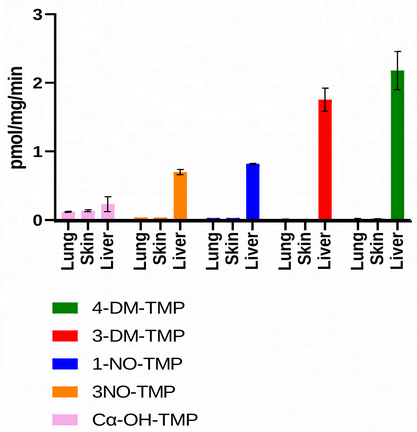

Figure 2. Comparison of TMP phase I metabolite formation in liver, lung, and skin human s9 fractions. Values calculated were adjusted for non-enzymatic/impurity-derived measurements.

Phase 2 Metabolism: Liver, Lung & Skin

Table 1. Liver, lung, and skin form phase II metabolites ex vivo. N-oxide glucuronides below LD in lung and skin

	Sulfation			Glucuronidation		
	Liver	Lung	Skin	Liver	Lung	Skin
4DM-TMP	Yes	Yes	Yes	Yes	Yes	Yes
3DM-TMP	Yes	Yes	Yes	Yes	Yes	Yes
Cα-OH-TMP	No	No	No	No	No	No
1NO-TMP	No	No	No	Yes	No	No
3NO-TMP	No	No	No	Yes	No	No

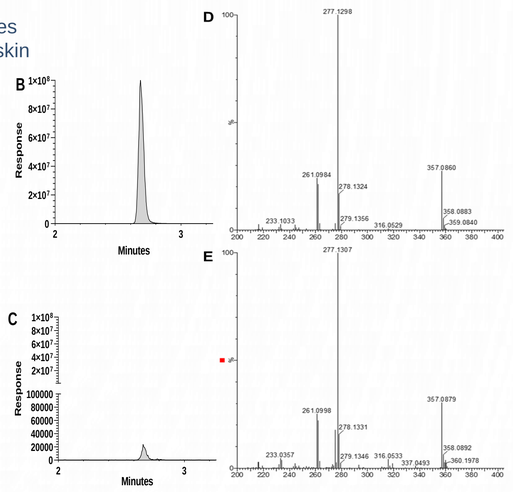

Figure 3. Determination of phase II metabolites in liver, lung, and skin. A. Vetting process to confirm formation of phase II metabolites B. s9 3DM-TMP glucuronide full reaction C. s9 3DM_TMP glucuronide negative control D. MS/MS fragmentation of 3DM sulfate in patient urine E. MS/MS fragmentation *ex vivo* 3DM-TMP sulfate

Methods

TMP phase I metabolite formation in liver, lung, and skin: Liver, lung, and skin s9s (0.5, 2.0, and 2.0 ug/uL respectively) were incubated at 37°for up to 6 hours with 250uM TMP and 1mM NADPH regenerating system in 50 mM phosphate buffer pH 7.4. Reactions were quenched with 4:1 cold acetonitrile, vortexed, centrifuged at 16K g for 10 minutes to pellet protein. Reaction supernatant was analyzed with metabolite analytical standards. No s9/NADPH were used as negative controls.

TMP phase II metabolite formation in liver, lung, and skin: s9s (0.5, 2.0, and 2.0 ug/uL respectively) were incubated at 37°for 2 hours with 50uM TMP and 1mM UDPGA (glucuronidation) or PAPS (sulfation) in 50 mM phosphate buffer pH 7.4. Reactions were quenched with 4:1 cold acetonitrile, vortexed, centrifuged at 16K g for 10 minutes to pellet protein. Reaction supernatant was analyzed using MRM transitions based upon the addition/loss of the known masses of glucuronic acid and sulfate.

Reactive metabolite trapping assays were performed by incubation of 4DM-TMP sulfate analytical standard with 10uM GSH for 12 hours in 50mM ammonium bicarbonate pH 7.4. No GSH and no 4DM-TMP sulfate reactions were used as negative controls. Reactions were analyzed using high resolution MS1 scans from 100 Da-1000Da. Data files from full scans were centroided and then processed by a pick-picking algorithm (XCMS, Scripps). Peaks were selected if they were 3X greater in amplitude and t test scores were <0.05 as compared to negative controls. Peaks were then manually inspected for signal: noise and peak shape over negative controls. Peaks that passed manual curation were then subjected to MS/MS scans to assist in structure elucidation.

Adduct Metabolism

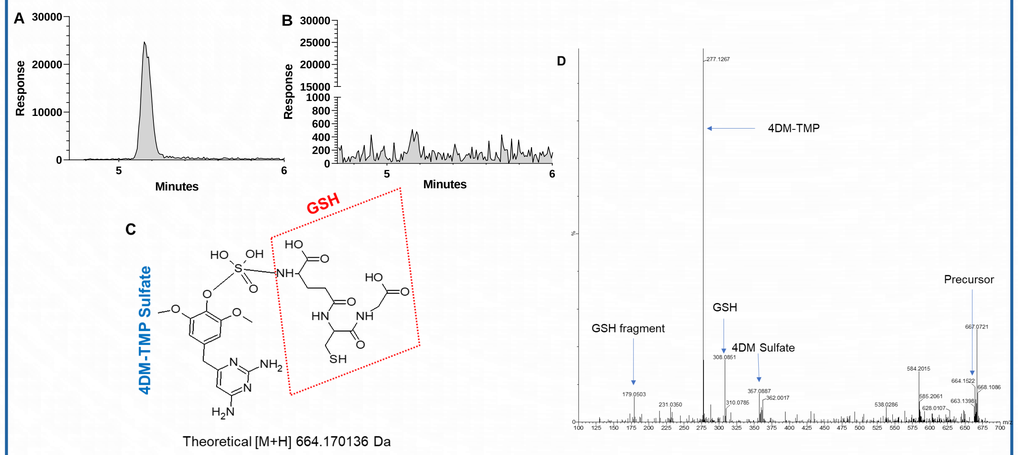

Figure 4. 4DM-TMP sulfate GSH adduct formation. A. XCMS detected peak at 664.16. B. No 4DM-TMP sulfate negative control. C. Proposed 664.16 adduct structure D. MS/MS fragmentation of 664.16 XCMS hit

Conclusions

- **Phase 1 Metabolism: Liver s9 fractions produce phase I metabolites as seen in HLMs. No significant phase I metabolite production in lung or skin**
- **Phase 2 Metabolism: We have demonstrated that phase 2 metabolites were produced by all 3 tissue types with some differences in phase II metabolism products among tissues**
- **The phase II metabolite, 4DM-TMP sulfate was trapped by GSH demonstrating a possible protein reactivity**

Future Directions

- **Characterization of the specific lung and skin phase I & II enzymes involved in the production of TMP metabolites**
- **Further structural elucidation of GSH and NAL 4DM-TMP adducts**
- **Additional trapping experiments for phase I/II metabolites including additional small molecule nucleophiles and proteins**

Acknowledgements

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[1] Children's Mercy Hospital Kansas City, MO [2] Kansas City University