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Patrick M Dansette, Jasleen K Sodhi, Marie Delarosa, Jason S Halladay, James P Driscoll, et al.. Inhibitory Effect of Trapping Agents of Reactive Intermediates of Sulfur-containing Drugs Against Major Human Cytochrome P450. 20th International Conference on Cytochrome P450 (ICCP 20), Aug 2017, Dusseldorf, Germany. . hal-03539866

HAL Id: hal-03539866

<https://hal.science/hal-03539866>

Submitted on 22 Jan 2022

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Inhibitory Effects of Trapping Agents of Sulfur Drug Reactive Intermediates Against Major Human Cytochrome P450.

Patrick Dansette¹, Jasleen K. Sodhi², Erlic Marie Delarosa², Jason S. Halladay², James P. Driscoll², Teresa Mulder², and S. Cyrus Khojasteh²

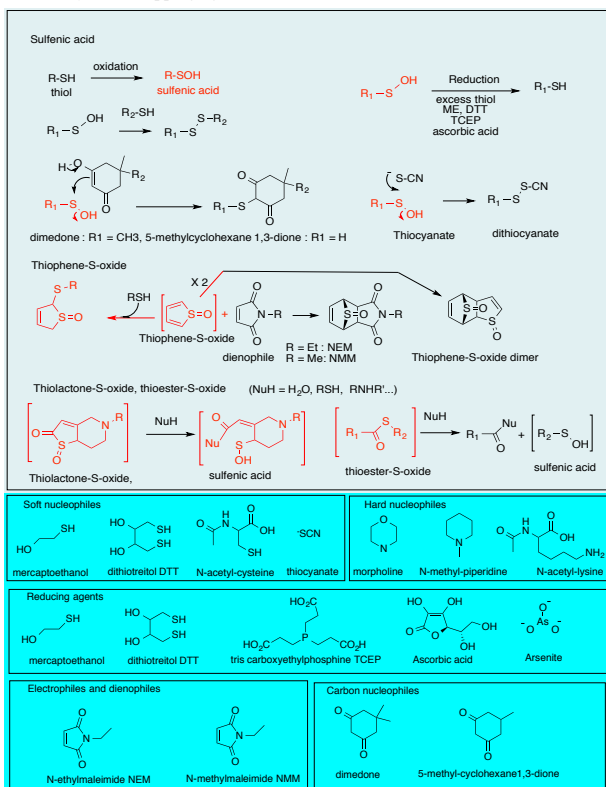
¹ Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS UMR 8601, Université Paris Descartes, Sorbonne Paris, Paris CX06, France; patrick.dansette@parisdescartes.fr
² Department of Drug Metabolism and Pharmacokinetics, Genentech, Inc., South San Francisco, CA 94080, USA; dona.teresa@gene.com; khojasteh.cyrus@gene.com

ABSTRACT:

The formation of reactive species from the metabolism of xenobiotics has been linked to toxicity. Reactive species can covalently bind to trapping agents in *in vitro* incubations of compound with human liver microsomes (HLM) fortified with NADPH, resulting in stable conjugates, thereby facilitating analytical detection and providing evidence of short-lived reactive metabolites. Since reactive metabolites are typically generated by cytochrome P450 (CYP) oxidation, it is important to ensure high concentrations of trapping agents are not inhibiting the activities of CYP isoforms. Here we assessed the inhibitory properties of fourteen trapping agents against the major human CYP isoforms (CYP1A2, 2C9, 2C19, 2D6 and 3A). Based on our findings, eleven trapping agents displayed inhibition, three of which had IC₅₀ values less than 1 mM (2-mercaptoethanol, *N*-methylmaleimide and *N*-ethylmaleimide (NEM)). Three trapping agents (dimedone, *N*-acetyl-lysine and arsenite) did not inhibit CYP isoforms at concentrations tested.

INTRODUCTION

The relationship between drug bioactivation *in vivo* and subsequent toxicity is not straightforward; however, instances of drug toxicity directly resulting from reactive metabolite formation have been reported [1,2]. Thus, a major effort during drug discovery is to assess the possibility of reactive metabolite formation in the attempt to minimize the potential of compounds toxicity to humans [3,4]. *In vitro* trapping studies are routinely performed as an integral part of drug discovery, to estimate the bioactivation potential of candidate drugs [5,6]. Trapping agents form stable conjugates with the reactive species allowing their detection [7, 8]. Here we use trapping agents able to trap reactive species derived from sulfur containing drugs. Thus thiophenes can be activated to thiophene-S-oxides which can be trapped by dienophiles (NEM, NMM) [9]. Thioesters and thiolactone can give carbonyl-S-oxides that hydrolyse to sulfenic acids [10]. Some sulfur heterocycles and many thiol derivatives can also lead to sulfenic acids [10] which can be trapped by β-diketone compounds like dimedone or 5-methyl-cyclohexane 1,3-dione or several nucleophiles like thiols giving disulfides, thiocyanate giving dithiocyanate, or reduced to thiol by ascorbic acid or phosphines. Since most of these bioactivation reactions are Cytochrome P450 (CYP) dependent our aim was to access if typical **human CYP are inhibited** by those trapping agents.



Scheme:1 Top: Typical reactive metabolites from sulfur containing drugs; and trapping reactions (in red reactive species). Bottom: trapping agents used.

METHOD

CYP Inhibition : Each trapping agent was incubated with human liver microsomes in six separate reactions with six probe substrates, each one selective for a specific CYP (1A2, 2C9, 2C19, 2D6 and 3A4) in triplicate at various top concentrations of the trapping agent depending on typically used *in vitro* concentrations and limitations in aqueous solubility. The reactions were initiated using NADPH. At the end of each incubation period, the reaction was quenched with 40µL of ACN: AcOH 94:6 containing internal standard. Incubation conditions for each of the six assays have been detailed [13]. Each of the six incubation plates (6 probes) was cassetted into a single plate, which was then centrifuged at 3000 rpm for 10 minutes. 58 µL of Supernatant was diluted into 29 µL of water. The samples (20 µL) were injected using a multiplexing LX-2 Series system from Thermo Scientific coupled to an AB SCIEX Triple Quad 5500 System mass spectrometer. The MRM transitions for the ticlopidine trapping samples were m/z 264→154 (ticlopidine), m/z 405→357 (TSO-NEM) and m/z 559→511 (TSOD).

RESULTS

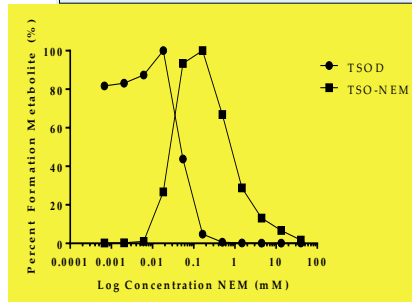
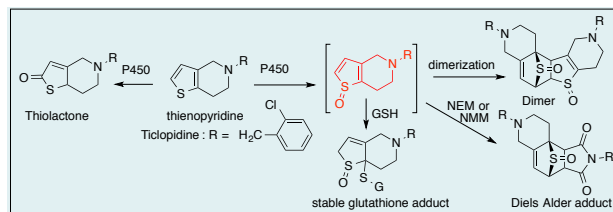
Table 1 shows that 3 trapping agents (dimedone, *N*-acetyl-lysine and arsenite) did not inhibit CYP at the tested concentrations. However 11 trapping agents showed inhibition, 3 of which had IC₅₀ values less than 1 mM (2-mercaptoethanol, *N*-methylmaleimide (NMM) and *N*-ethyl-maleimide (NEM)). Thus IC₅₀ of mercaptoethanol for CYP 2C19 was 0.3 mM, of NEM for CYP 1A2, 0.1 mM, and for CYP3A4 about 0.2 mM. IC₅₀ of NMM versus CYP1A2 was 0.2 mM.

Table 1. Inhibition of Trapping Agents Against the Major CYP Isoforms

Class	Trapping Agent	Typically Used in <i>In Vitro</i> Concentration ¹ (mM)	Highest Tested Concentration (mM)	IC ₅₀ (mM) (95% Confidence Interval)						
				CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A ₄ ²	CYP3A ₄ ²	
Nucleophiles	Morpholine	10	100	>100	7.9	14	13	16	8.1	
	<i>N</i> -Methylpiperidine	10	100	>50 ^h	(6.0 – 10.1)	(9.9)	(11 – 16)	(15 – 18)	(11 – 14)	(7.3 – 11.5)
	5-Methyl-1,3-cyclohexanedione (MCD)	4	10	>50 ^h	(8.0 – 12.1)	(6.7)	(13 – 28)	(5.7 – 7.4)	(12 – 16)	(11.1 – 14)
	2-Mercaptoethanol	1-4	5	>5	(2.9 – 4.3)	(5.4 – 8.3)	(3.1)	>10	(3.7 – 8.1)	>10
	Thiocyanate (SCN)	4	100	>100	18	20	49	43	(34 – 54)	(35 – 61)
	2-Mercaptoethanol	20	100	42	(26 – 67)	2.0	(1.3 – 3.1)	(42 – 59)	7.3	(6.3 – 9.4)
	<i>N</i> -Acetyl-cysteine1	5	50	9.3	(1.8 – 2.3)	>50	(0.30 – 0.36)	(22 – 27)	16	(8.3 – 12.7)
	<i>N</i> -Acetyl-lysine1	5	50	9.3	(7.8 – 11.0)	>50	(15 – 22)	(20 – 25)	15	(13 – 18)
	<i>N</i> -Acetyl-lysine1	5	50	9.3	>50	>50	>50	>50	>50	>50
	<i>N</i> -Acetyl-lysine : <i>N</i> -Acetyl-cysteine	5 : 5	50 : 50	3.3	(3.7 – 5.1)	41	(19 – 96)	(12 – 16)	(11 – 17)	(11 – 15)
	Arsenite	20	10	>10	>10	>10	>10	>10	>10	>10
	Reducing Agents	Tris(2-carboxyethyl) phosphine (TCEP)	4	100	3.4	(1.2 – 9.9)	32	7.3	>100	8.9
Dithiotreitol (DTT)		4	100	16	(13 – 19)	7.2	3.4	3.3	3.4	(2.7 – 4.0)
<i>N</i> -Ethylmaleimide (NEM)		4	40	0.12	(0.01 – 0.14)	3.2 ^a	(0.77 – 14.7)	(1.2 – 7.1)	>40	0.14
<i>N</i> -Methylmaleimide		4	20	0.20	(0.16 – 0.26)	8.2 ^a	(0.87 – 51.0)	(1.87 – 3.5)	>20	3.2
Radical Scavenger	Ascorbic Acid	3	50	16	(13 – 20)	>50	16	12	2.7	(1.6 – 4.9)
										(8 – 16)

Trapping of Ticlopidine S-oxide

As an illustration of these concepts, Ticlopidine was studied. Ticlopidine is metabolized by human and rat liver microsomes into many metabolites, in particular a reactive thiophene-S-oxide which spontaneously dimerizes by Diels Alder cycloaddition. This S-oxide can be trapped with dienophiles like NMM or NEM at the expense of dimer formation [9]. It can also be trapped by GSH [9,11,12]



Ticlopidine (1 µM) incubated in absence of trap and in presence of NADPH produced the S-oxide dimer (TSOD). In presence of increasing NEM concentration TSO-NEM increased until 0.16 mM NEM then plateaued and then decreased. This decrease is most probably due to inhibition of CYP by NEM as shown in the table 1.

CONCLUSION

Trapping agents are useful to indicate the formation of reactive metabolites. In case of cytochrome P450 dependent reactions, some trapping agents can also inhibit the enzyme. Thus it is necessary to avoid too high concentration. The present results give guidelines on the non inhibitory concentrations which can be used routinely. Then optimisation for each trapping agent should be carried out.

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