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# Mechanism of activation of clopidogrel into its active thiol metabolite: P450-catalyzed opening of the 2-oxo-clopidogrel thiolactone: S-oxidation or Baeyer-Villiger oxidation?

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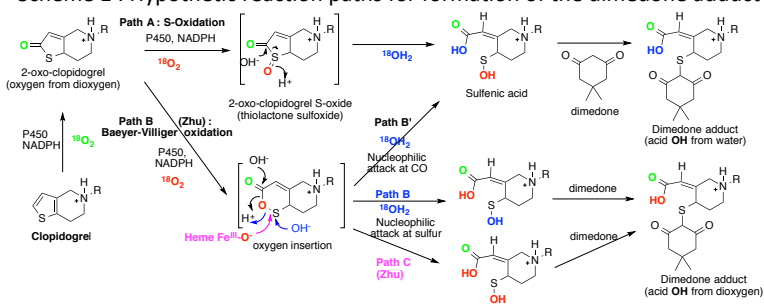
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**Introduction:** Clopidogrel and ticlopidine are activated to the thiol active metabolite, that inhibits receptor P2Y12, in two oxidative steps and a reduction: First P450 catalysed oxidation into thiolactones : 2-oxo-clopidogrel and 2-oxo-ticlopidine. Second, P450-catalyzed opening into a **sulfenic acid (1)**. We proposed that the thiolactone is oxidized to a **thiolactone S-oxide (Path A)** that hydrolyzes to a **sulfenic acid**, reacting with glutathione to give a mixed disulfide, further reduced (enzymatically or chemically) to the active thiol. Alternatively in presence of dimedone the sulfenic acid is trapped as a stable dimedone adduct (1, 2) or in presence of thiols into mixed disulfides that are reduced by excess thiol into the active thiol and its isomers (1). In presence of ascorbic acid, TCEP or DTT, the sulfenic acid is reduced into the active thiol (2).

A recent paper by Zhu (3) challenges our hypothesis and propose an alternative **Baeyer-Villiger oxidation** of the thiolactone (**Path B**) through a 3-oxo-1,2-oxathiolane, followed by nucleophilic attack by the heme-Fe-O<sup>-</sup> (**Path C**) to afford the same **sulfenic acid intermediate**.

Scheme 1 : Hypothetic reaction paths for formation of the dimedone adduct



The acid group OH comes from water in Path A and B'. It comes from dioxygen in Path B. In path C the Sulfenic OH comes from dioxygen

The aim of our study is to use as end product the **sulfenic acid dimedone adduct** to analyze incorporation of <sup>18</sup>O from <sup>18</sup>O water or <sup>18</sup>O dioxygen, and compare it to the incorporation of <sup>18</sup>O in other co-metabolites in incubations of clopidogrel (and 2-oxo-clopidogrel and 2-oxo-ticlopidine) with rat liver microsomes (RLM), to try to decide between these two hypothetical pathways. The <sup>18</sup>O incorporation should be different for each pathway as shown in Scheme 1. Thus starting from clopidogrel the carbonyle (CO) of the thiolactone (2-oxo-clopidogrel) and that of the dimedone adduct comes from dioxygen.

The OH of the carboxylic (COOH) group comes from water in **Path A** and **B'**, from dioxygen **Path B**. In the hypothesis of Zhu (**Path C**) both the acid OH and the sulfenic OH come from dioxygen.

**Methods :** Clopidogrel, 2-oxoclopidogrel and 2-oxoticlopidine (100 μM) were incubated with phenobarbital treated rat liver microsomes (1) in presence of 1mM NADPH and 1 mM dimedone, either in normal pH 7.4 phosphate buffer or in buffered <sup>18</sup>O water or in presence of <sup>18</sup>O dioxygen. After 30 min the reaction was stopped with half volume CH<sub>3</sub>CN/AcOH 9/1 and centrifuged. The supernatant was analyzed by HPLC/MS (Thermo Surveyor and a LCQ advantage) as described (1) using MS and MS/MS.

**Results :** Clopidogrel was metabolized in addition to the dimedone adduct into a number of other metabolites as shown in Scheme 2 : 2-oxo-clopidogrel, 7a-hydroxy-2-oxo-clopidogrel, clopidogrel S-oxide dimer, clopidogrel sesquioxide and clopidogrel acid.

The results show that the oxygen atoms of the first oxidation for 2-oxo-clopidogrel (thiolactone, one O), clopidogrel S-oxide dimer (two O) came from dioxygen. Clopidogrel sesquioxide, (oxidized dimer), incorporated **three O** atoms from dioxygen. **Two O** were incorporated into 7a-hydroxy-2-oxo-clopidogrel (one in the CO and one in the 7a-OH). Clopidogrel acid incorporated **O** from water. These metabolites

Scheme 2 : Other microsomal metabolites of clopidogrel

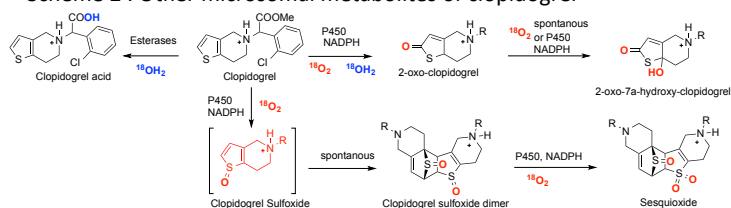
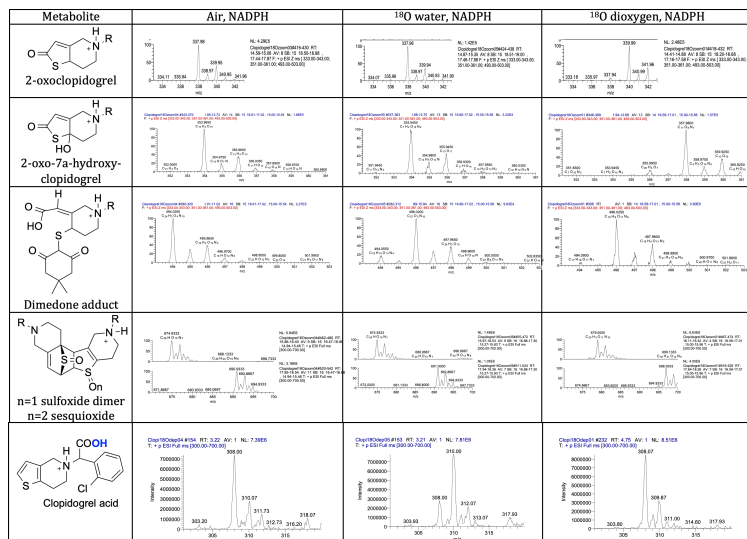


Table: Mass spectra of clopidogrel metabolites from incubations with RLM in presence of air or air and <sup>18</sup>O water or <sup>18</sup>O dioxygen and NADPH.



Metabolite	Air, NADPH	<sup>18</sup> O water, NADPH	<sup>18</sup> O dioxygen, NADPH
2-oxo-clopidogrel	m/z = 338	m/z = 338	m/z = 340 (+2), one <sup>18</sup> O from dioxygen
2-oxo-7a-hydroxyclopidogrel	m/z = 354	m/z = 354	m/z = 358 (+4), two <sup>18</sup> O
Dimedone adduct	m/z = 494	m/z = 496 (+2), one <sup>18</sup> O from water	m/z = 496 (+2), one <sup>18</sup> O from dioxygen
Sulfoxide dimer	m/z = 675	m/z = 675	m/z = 679 (+4), two <sup>18</sup> O from dioxygen
Sesquioxide	m/z = 691	m/z = 691	m/z = 697 (+6), three <sup>18</sup> O from dioxygen
Clopidogrel acid	m/z = 308	m/z = 310 (+2), one <sup>18</sup> O from water	m/z = 308 (trace +2)

allow to verify the incubation conditions (if <sup>18</sup>O water or dioxygen were present in the incubation).

However the **dimedone adduct** derived from clopidogrel incorporated only one **O from dioxygen** in the CO of the COOH coming from its thiolactone precursor and only traces of a second oxygen. The OH of the COOH came from **water**.

Thus the above results agree with Path A or B'. The fact that OH of the carboxylic acid comes from **water** is not compatible with path B nor C. However Path B and C is not totally excluded because of the slight incorporation of a second oxygen into the dimedone adduct (3-8%).

Similar results were obtained for 2-oxo-ticlopidine: The **7a-OH** of 7a-hydroxy-2-oxo-ticlopidine came from <sup>18</sup>O **dioxygen**. The **carboxyle-OH** of the dimedone adduct came from <sup>18</sup>O **water** and not from **dioxygen**.

Also 2-oxo-prasugrel gave similar results (4).

**Discussion :** The results obtained show that only **Path A** or **B'** are compatible with the oxygen incorporation found. The slight <sup>18</sup>O incorporation from dioxygen into the dimedone adducts (< 8%) could be due to some exchange or to **Path C** proposed by Pr Zhu. Thus a Baeyer-Villiger oxidation cannot be fully excluded. Path A and B' (nucleophilic attack at CO) is also compatible with the fact that the reactive intermediate acts as a bis-electrophile (5). Path B and C are not!

Diels-Alder adducts with dienophiles of 2-hydroxy-thiophene S-oxides, tautomer of the thiolactone sulfoxides, support **Path A** (7).

Analogous reactive intermediates, a thiolactone Sulfoxide or a 3-oxo-1,2-oxathiolane (a carboxylic-sulfenic anhydride), were proposed by Latham and Walsh in the inactivation by thiolactones of a Baeyer-Villiger flavo-protein : cyclohexanone mono-oxygenase (6).

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