

**The Effect of Thalidomide on Oxidation of Midazolam, S-mephenytoin
and Cyclosporine A Mediated by Human P450 Enzymes**

Torrie Sleeper
Senior Comprehensive Paper
Department of Chemistry
The Catholic University of America
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Abstract

Enantiomers of thalidomide have completely different physiological effects; the (R)-enantiomer is effective as a sedative while the (S)-enantiomer is a teratogen. Current studies have been focusing on the different immunomodulatory and anti-angiogenic properties of thalidomide despite the devastating teratogenic effects of the (S)-isomer. Studies have been conducted in order to increase the understanding of the effects thalidomide has on drug metabolizing enzymes, particularly on cytochrome P450 enzymes found in the human liver microsomes, in order to increase understanding of its potential drug interactions. It has been found that thalidomide inhibited and enhanced certain enzyme substrate interactions with P450 enzymes. Particular examples include thalidomide's inhibitions of P450 2C19-dependent S-mephenytoin 4'-hydroxylation at high concentrations. In addition, midazolam 4-hydroxylation activities with P450 3A showed suppression due to exposure to thalidomide, but there was an enhancement in the presence of thalidomide with 1'-hydroxylation and total midazolam oxidation. There was also an increase in midazolam hydroxylation in liver microsomal samples with CYP3A5*1 allele when in the presence of thalidomide. A comparable increased rate of cyclosporine A clearance in the presence of thalimomide was observed for P450 3A5 as well as for liver microsomes that expressed P450 3A5 when exposed to thalidomide. Docking studies with a P450 3A5 homology model revealed close interactions between thalidomide and the heme of P450 3A5. Based on these results, a heterotropic cooperativity model has been proposed for the interaction of thalidomide with P450 3A5 to explain enhancement of midazolam and cyclosporine A metabolism.

I. Background of Thalidomide

More than half of all pregnant women experience morning sickness.¹ With this alarming statistic, a company based in Germany called Grunenthal developed a drug for expecting mothers in the late 1950s called Thalidomide.² Soon after its introduction, over 10,000 babies were born with severe birth defects. These malformities included stunted or missing limbs, deformed eyes and ears, and the absence of a lung.³ A majority of births were still-born or had infants dying shortly after birth. This devastation created a huge scare and sparked decades of research in order to figure out what caused these severe birth defects and how to prevent this from occurring again. However, after years of study, in 2006, thalidomide was approved by the FDA due to its effective responses against multiple myeloma with its anti-inflammatory and antiangiogenic properties. Thalidomide is playing a significant role in cancer research particularly due to drug interactions seen with enzyme to substrate binding.

II. Chemistry of A-phtalimidoglutarimide

A-phtalimidoglutarimide, more commonly known as thalidomide, is a derivative of glutamic acid. Thalidomide contains a phtalimide ring linked to a glutaramide ring via an asymmetric carbon atom as shown in Figure 1.⁴

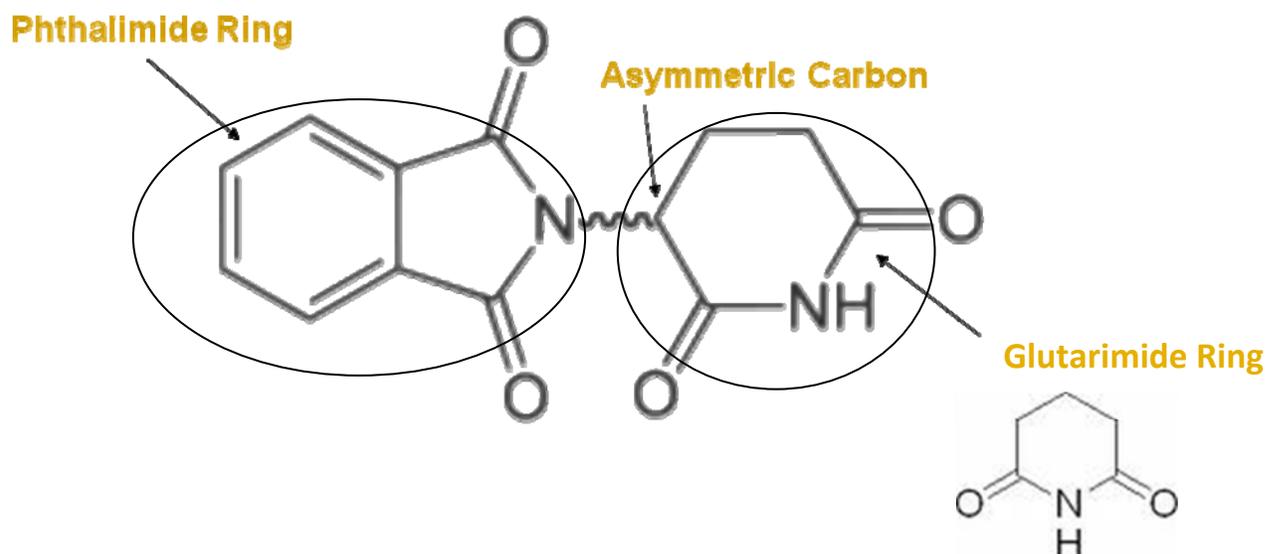
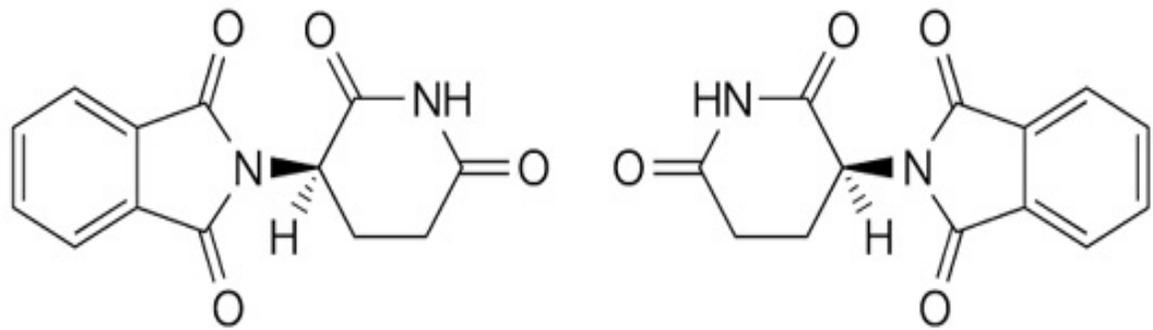


Figure 1. Thalidomide, IUPAC Name: 2-(2,6-dioxopiperidin-3-yl) isoindoline-1,3-dione

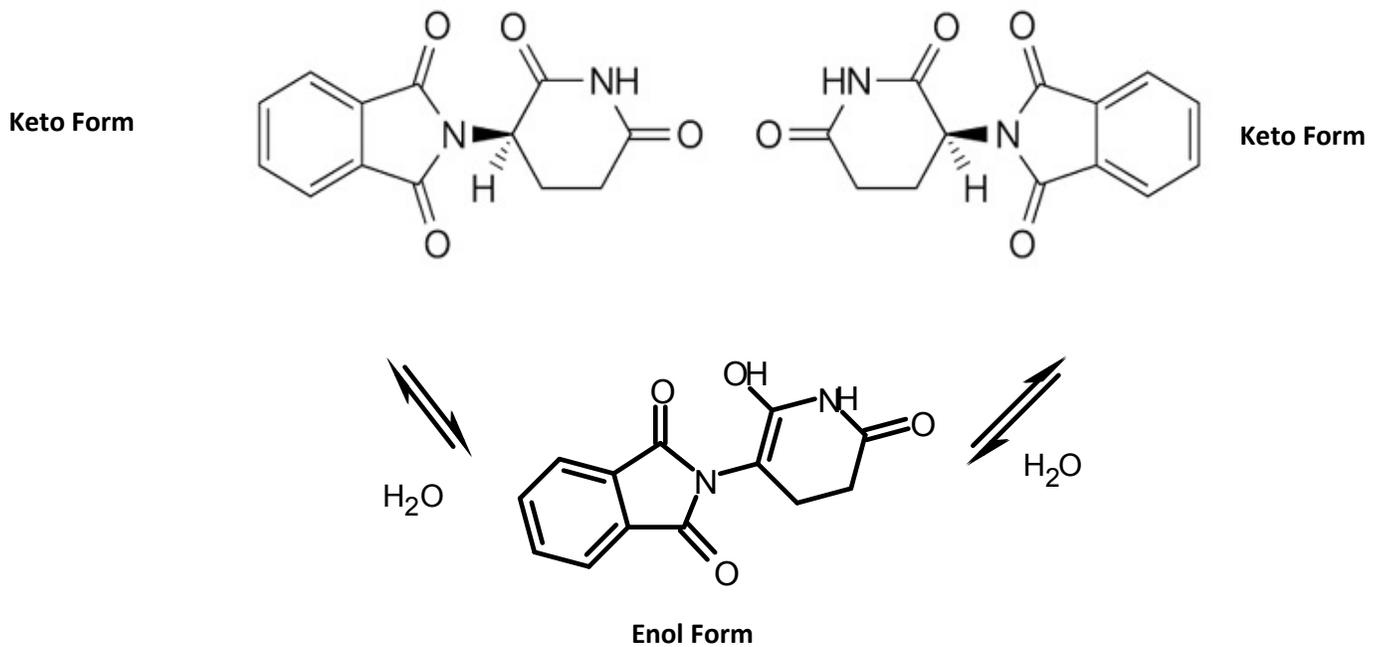
This molecule is insoluble in ether and benzene and is sparingly soluble in water, methanol, ethanol, acetone, and glacial acetic acid. Thalidomide is soluble in dioxane, dimethyl formamide, pyridine, and chloroform.⁵

Stereochemistry

Because it has one chiral center, thalidomide exists as a pair of (R) and (S) enantiomers, as illustrated in Figure 2. The two isomers have completely different physiological effects. The (R) – isomer has the ability to relieve morning sickness in pregnant women. The (S) – isomer caused birth defects which ultimately resulted in aborted fetuses or infants born with severe malformities.⁶

**(R) - Thalidomide****(S) - Thalidomide***Figure 2.* (R) and (S) – isomers of Thalidomide

Treatment with the pure (R) isomer still resulted in birth defects because at physiological pH, the (R) isomer racemizes to produce the other isomer. (Figure 3)

*Figure 3.* Racemization of Thalidomide Enantiomers

Because of safety considerations, in 1961, the FDA forced Grunenthal to pull this drug off the market.⁷

Thalidomide's Physiological Properties

It has been recognized that thalidomide can serve as an immunomodulatory agent. This means the drug inhibits the growth and survival of certain cells, such as myeloma cells.⁸

Thalidomide is a potent inhibitor of tumor necrosis factor α , enhancing the degradation of tumor necrosis factor α messenger RNA. Tumor necrosis factor is a pleiotropic inflammatory cytokine. The cytokine possesses both growth stimulation properties and growth inhibitory properties, as well as self regulatory properties.⁹

Another characteristic of thalidomide is its ability to inhibit angiogenesis.¹⁰ Angiogenesis is the formation of new blood vessels, normally occurring during embryonal growth and wound healing and is important for the proliferation and metastases of most malignant neoplasms.¹¹ Studies have shown that thalidomide inhibits the growth and survival of stromal cells, tumor cells, and cells in the bone marrow.¹² It also has the ability to alter chemical messengers which effect the growth and survival of myeloma cells, alter expression of adhesion molecules and stimulate T-cells.¹³ All of these support further development of thalidomide as a cancer treatment. Lastly, the drug has been responsive against anti-inflammatory activities, particularly in the treatment of erythema nodosum leprosum (ENL) in leprosy.¹⁴

Current Interest in Thalidomide as a Therapy

Recent studies have shown effective methods in cancer treatments, such as when thalidomide is used in conjunction with dexamethasone (thal-dex). This method was recently approved by the FDA in May 2006.¹¹ Clinical trials have shown positive outcomes with patients that have used this drug combination as a treatment option. In fact, results have shown that response rates to thal-dex were superior to standard chemotherapy by reducing the myeloma cell mass of both IgG and IgA types.¹² It is currently being used in patients with relapsed or

refractory (cancer that does not respond to treatment) myeloma, as well as early stage disease. Research has also shown that thalidomide is effective against painful skin diseases related to leprosy, myelodysplastic syndromes, gliomas, kaposi's sarcoma, renal cell carcinoma, advanced breast cancer, and colon cancer.¹⁵

Because thalidomide has potential in therapy, recent studies have focused on the effect of thalidomide on drug metabolism in humans, specifically on the interaction of drugs with P450 in the presence of thalidomide. Recent work focuses on the metabolism by P450 enzymes of three drugs, midazolam, S-mephenytoin and cyclosporine A.

Recent Study Involving Thalidomide

Drug interactions of thalidomide with midazolam and cyclosporine A: heterotropic cooperativity of human cytochrome P450 3A5 which appears in volume 37 of Drug Metabolism and Disposition reports on the reactions of human P450 enzymes with midazolam, S-mephenytoin and cyclosporine A in the presence of varying amounts of thalidomide.¹⁶

Midazolam, chemical name 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo (1,5-a)(1,4)benzodiazepine, is a benzodiazepine derivative and has potent anxiolytic, amnestic, hypnotic, anticonvulsant, skeletal muscle relaxant and sedative properties. It is unique among benzodiazepines due to its water solubility.¹⁷ The structure of midazolam is shown in Figure 4.

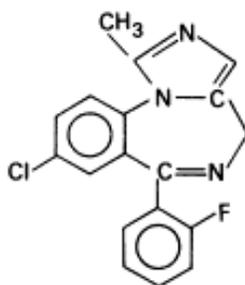


Figure 4: Midazolam

In addition, midazolam is recognized for its use as a probe drug for assessment of in vivo CYP3A(4) activity and widely used in clinical practice for preoperative sedation, induction, and maintenance of anesthesia, and sedation of patients in intensive care units. It is mainly metabolized by CYP3A4.¹⁸ In this study, the 1'-hydroxylation and 4'-hydroxylation of midazolam catalyzed by P450 enzymes was monitored.

S-Mephenytoin has the chemical name (S)-5-ethyl-3-methyl-5-phenylhydantoin and is a specific substrate for cytochrome P450 2C19 (CYP2C19), which is a gene that encodes a member of the cytochrome P450 superfamily of enzymes and is located within a cluster of cytochrome P450 genes on chromosome 10q24.¹⁹ The metabolite of this reaction is 4'-hydroxymephenytoin and the reaction is catalyzed by forms of human liver cytochrome P450.²⁰ S-Mephenytoin (Figure 5a) and S-Mephenytoin 4' - OH (Figure 5b) are shown in Figures 5a and 5b.

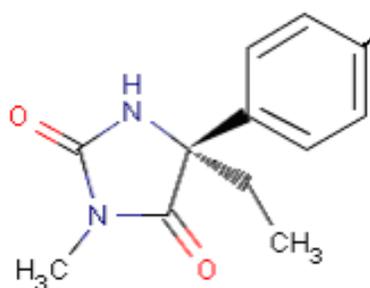


Figure 5a – S-mephenytoin

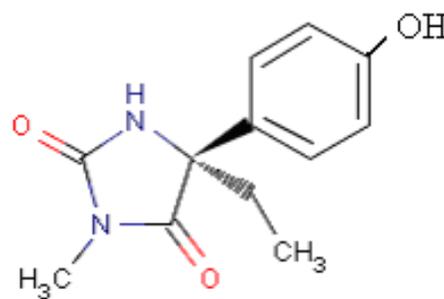


Figure 5b – S-mephenytoin 4' -OH

Cyclosporine A is an immunosuppressive agent and is used for the treatment of kidney, liver, heart, and other organ transplantation; rheumatoid arthritis; and psoriasis. It is a non-polar cyclic polypeptide consisting of 11 amino acids (Figure 6).²¹ Cyclosporin A indirectly elicits an immunosuppressive response via an interaction with immunophilin, a cytoplasmic protein.²²

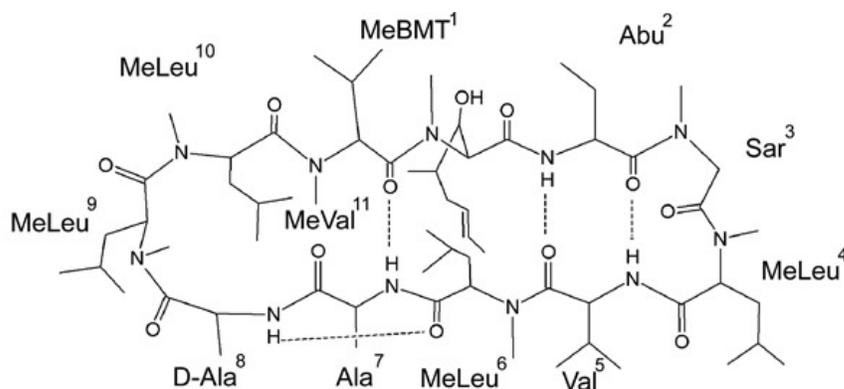


Figure 6. Cyclosporine A

Cytochrome P450 is a very large and diverse superfamily of heme proteins found in all domains of life. They are a group of heme thiolate monooxygenases which are responsible for the oxidative metabolism of many endogenous and xenobiotic compounds. They are a major determinant of the metabolism of many drugs including important anticancer drugs that sometimes have a profound impact on therapeutic efficacy and result in toxic side effects.²³ Figure 7 shows the structure of P450 3A4 obtained by a crystal structure through x-ray diffraction by Williams, P.A, et al.²⁴ P450 3A4 is the major P450 enzyme in human liver. Recent studies have observed drug oxidation by P450 3A5 in Asian, African America and White populations.²⁵ P450 2C19 metabolizes thalidomide in human liver and small intestine and is involved in the metabolism of toxins, steroid hormones, carcinogens, in addition to more than 50% of the drugs used today.²⁶ Thus, P450 3A4, P450 3A5, and P450 2C19 were investigated in this work.

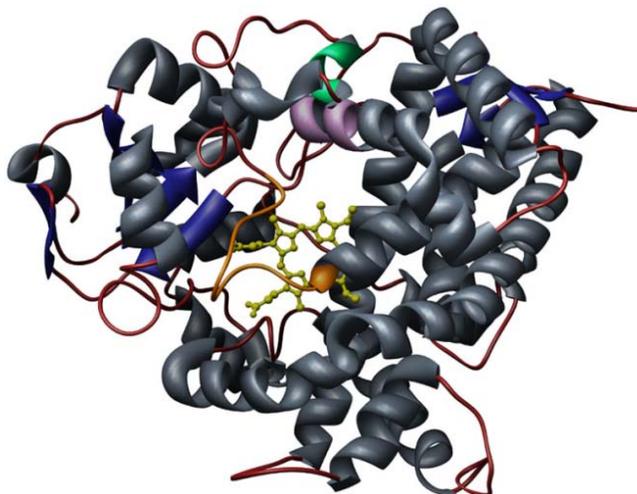


Figure 7. Crystal Structure of CYP3A4

II. The Effect of Thalidomide on the Oxidation of Several Substrates by P450 Enzymes

In this study, *Drug Interactions of Thalidomide with Midazolam and Cyclosporine A: Heterotropic Cooperativity of Human Cytochrome P450 3A5* by Okada et al.¹⁶ the interactions between thalidomide and the P450 enzyme were observed by studying the effect of thalidomide on the oxidation of the substrates catalyzed by the human P450 enzyme, specifically P450 3A4, P450 3A5 and P450 2C19.¹⁶

Experimental

A. Kinetic Studies

Experimental protocol was to incubate the enzyme (P450) with the substrate with varying quantities of thalidomide at varying reaction times. After a specific time, the reaction was quenched and the mixture was worked up. Then the concentrations of the products of hydroxylation were determined by high-performance liquid chromatography. Kinetic analysis was performed using nonlinear regression analysis and the Michaelis-Menten equation (Equation 1).

$$v = \frac{V_{\max} [S]}{[S] + K_M} \quad (\text{Equation 1})$$

where V_{\max} represents maximum velocity, K_M is the substrate concentration required for an enzyme to reach one-half its maximum velocity, and $[S]$ represents substrate concentration.

Inhibition was analyzed by the modification of Michaelis-Menten equation given in Equation 2:

$$v = \frac{V_{\max} [S]}{[S] + K_{\text{obs}}} \quad (\text{Equation 2})$$

In equation 2, the observed K_{obs} is defined as in Equation 3.

$$K_{\text{obs}} = K_M (1 + [I]/K_i) \quad (\text{Equation 3})$$

where $[I]$ is the concentration of thalidomide and K_i is the inhibition constant. This describes the case where the reaction undergoes competitive inhibition.

Substrate inhibition was observed for 1'-hydroxylation of midazolam. The substrate inhibition constant K_s was determined by fitting the rate data to Equation 4:

$$v = \frac{V_{\max} \cdot [S]}{K_{\text{obs}} + [S] + [S]^2/K_s} \quad (\text{Equation 4})$$

Enhancement by thalidomide was analyzed by fitting the data to an equation analogous to that for inhibition (Equation 2):

$$v = \frac{V_{\max} [S]}{[S] + K_{\text{obs}}} \quad (\text{Equation 2})$$

where K_{obs} is defined in terms of thalidomide concentration $[E]$ and K_e as shown in Equation 5:

$$K_{\text{obs}} = \frac{K_M}{1 + [E]/K_e} \quad (\text{Equation 5})$$

Figure 8 shows the effects of enhancement and inhibition on the substrate saturation curve for a reaction following these kinetics.

For an inhibition reaction (line A) a larger K_M is observed, consistent with the definition of $K_{\text{obs}} = K_M (1 + [I]/K_i)$. By analogy, an enhanced reaction would show a smaller K_{obs}

($K_{\text{obs}} = \frac{K_M}{1 + [E]/K_e}$) (line C). This treatment of the enhancement of thalidomide is an oversimplified case because Equations 2 and 5 assume Michaelis Menten type behavior.

Clearly, this is not simple Michaelis Menten behavior because in the data from Figure 11, discussed later, V_{max} increases in the presence of thalidomide.¹⁶

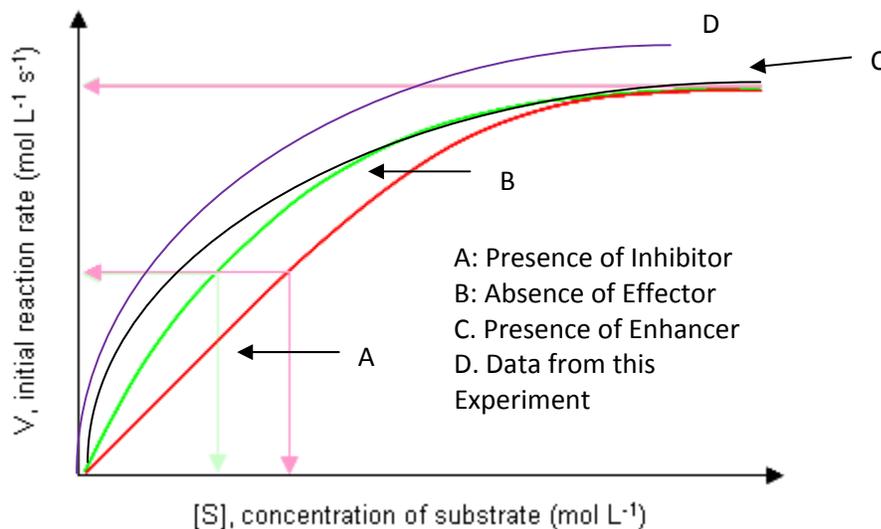


Figure 8. Saturation Curve for Reactions Following Michaelis-Menten Kinetics

Docking Study

A homology model of P450 3A5 was created using the known sequence of P450 3A5 and crystal structure of P450 3A4. Docking studies were carried out in order to determine possible interactions of thalidomide with P450 3A4 and P450 3A5 by MOE-Dock software. This is based on the simulated annealing method which calculates the electrostatic energies and Van der Waals energies between the target and ligand by simulated annealing. It is by calculating the docking energy values that the sum of the electrostatic, Van der Waals energies and the flexibility of the ligand itself are observed. A low docking energy indicates high binding ability.²⁷

Results and Discussion

P450 2C19 S-mephenytoin 4'-hydroxylation activity was suppressed by thalidomide in a concentration-dependent manner. A 50% inhibition was observed when the concentration of thalidomide was at 270 μ M as seen in Figure 9(A). Similar inhibitory effects were observed with thalidomide on S-mephenytoin hydroxylase activity in liver microsomal preparations (9B). Results in Figure 9 are presented as means and ranges of duplicate determinations.¹⁶

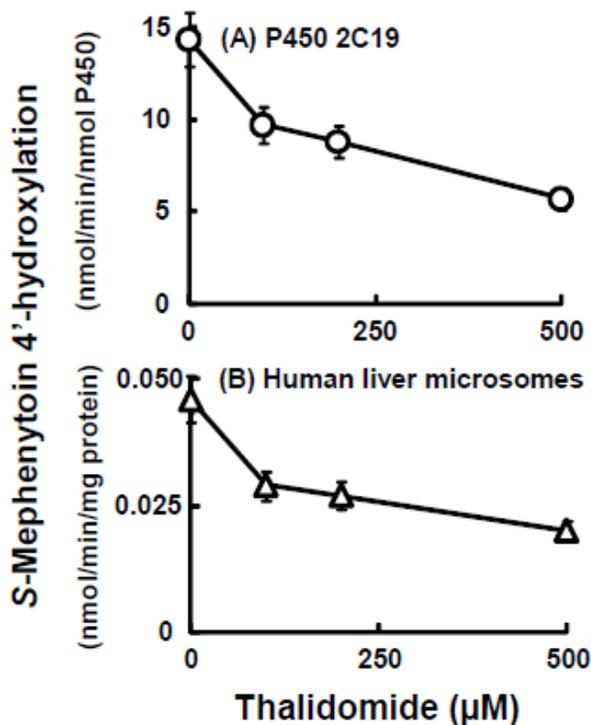


Figure 9. Effects of thalidomide on S-mephenytoin 4'-hydroxylation activity by recombinant P450 2C19 (A) and human liver microsomes (B).

Thalidomide's ability to enhance activities of human P450 enzymes was observed in thalidomide's enhancement of P450 3A mediated 1' and 4 – hydroxylation of midazolam activities, shown in Figure 10.¹⁶

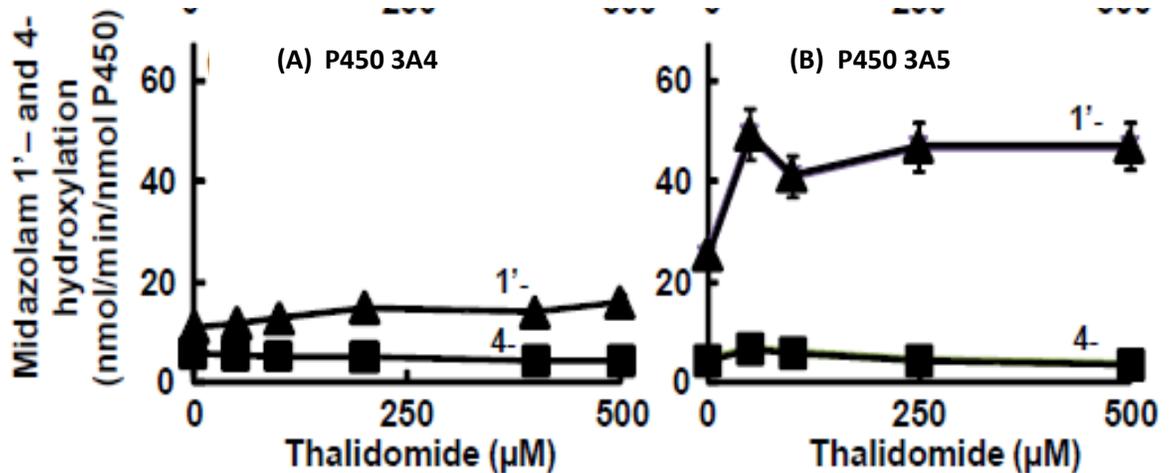


Figure 10. Effect of Thalidomide on P450 3A4 (A) and P450 3A5 (B) Mediated Midazolam 1' – and 4' Hydroxylation

In Figure 11, 1'- and 4'-hydroxylation activities are plotted against substrate concentrations in the presence of various concentrations of thalidomide.¹⁶ Thalidomide only had a small effect on the midazolam hydroxylation activities of P450 3A4 (Figure 11A). On the other hand, in the presence of thalidomide, midazolam 1'-hydroxylation activities of P450 3A5 at low substrate concentrations were enhanced (Figure 11B). In the presence of thalidomide, midazolam 4'-hydroxylation was suppressed (Figure 11B). An additional enzyme source, human liver microsomes, was used, as illustrated in Figure 11C & D. There is a minimum effect associated with the midazolam hydroxylation activities of liver microsomal sample HL-3 genotyped as *CYP3A5*3/*3*. There is an increase in activity observed due to low concentrations of thalidomide in Figure 11D associated with midazolam 1'-hydroxylation activities in liver microsomal sample HL-1, genotyped as *CYP3A5*1/*3*. The results are presented as means of duplicate determinations. These results are tabulated in Table 1 with values of K_e , K_i , K_M , and K_S . The kinetic parameters were calculated by a non-linear regression analysis of the kinetic data using Equations 2, 4, and 5.¹⁶

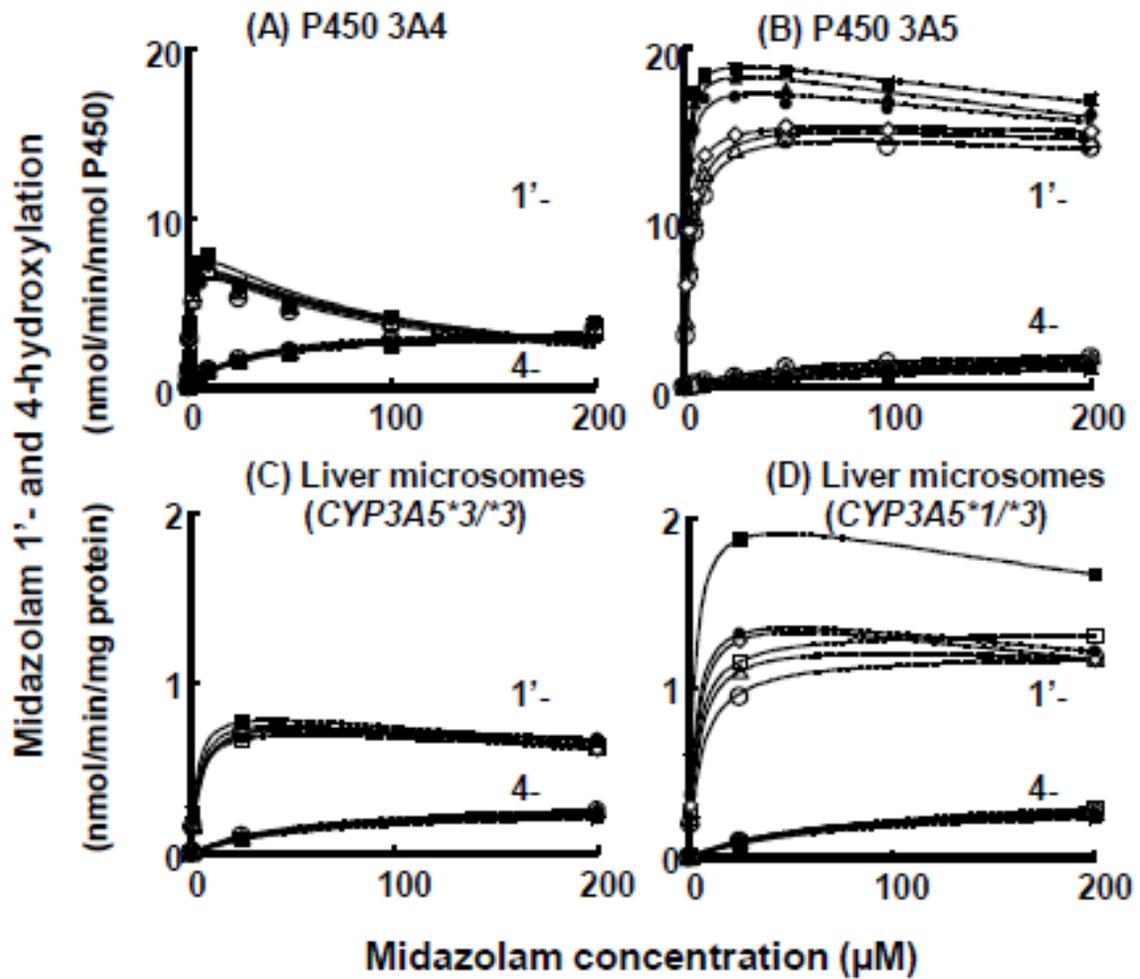


Figure 11. Effects of substrate concentrations (0.25-200 μM) on midazolam 1'- and 4-hydroxylation activity of recombinant P450 3A4 (A) and P450 3A5 (B) and human liver microsomes (C,D) in the absence (\circ) or the presence of 10 (Δ), 25 (\square), 50 (\diamond), 100 (\bullet), 250 (\blacktriangle), and 500 μM thalidomide.

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Table 1. Effect of thalidomide on midazolam 1'- and 4-hydroxylation activities of P450 3A4 and 3A5 and human liver microsomes with the kinetic parameter K_m .

| Enzyme | Midazolam | | Midazolam | | Thalidomide | | Goodness of fit, r^2 |
|----------------------------------|---------------|-----------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------|
| | hydroxylation | V_{max} | K_m , μM | K_m , μM | K_i , μM | K_m , μM | |
| P450 3A4 | 1'- | 8.6 \pm 0.3 | 1.8 \pm 0.2 | 87 \pm 10 | — | 850 \pm 370 | 0.98 |
| | 4- | 3.4 \pm 0.1 | 25 \pm 2 | — | 1500 \pm 620 | — | 0.99 |
| P450 3A5 | 1'- | 18 \pm 1 | 5.5 \pm 0.5 | 1100 \pm 200 | — | 40 \pm 7 | 0.98 |
| | 4- | 2.2 \pm 0.1 | 59 \pm 6 | — | 160 \pm 20 | — | 0.99 |
| nmol/min/nmol P450 | | | | | | | |
| nmol/min/mg protein | | | | | | | |
| HL-3 microsomes (CYP3A5*3/*3) | 1'- | 0.83 \pm 0.02 | 3.9 \pm 0.3 | 700 \pm 100 | — | 770 \pm 300 | 0.98 |
| | 4- | 0.30 \pm 0.01 | 52 \pm 4 | — | 850 \pm 240 | — | 0.98 |
| HL-1 microsomes (CYP3A5*1/*3) | 1'- | 1.7 \pm 0.1 | 12 \pm 3 | 670 \pm 290 | — | 62 \pm 24 | 0.94 |
| | 4- | 0.35 \pm 0.01 | 66 \pm 5 | — | 1100 \pm 300 | — | 0.94 |

Kinetic parameters were calculated with non-linear regression analysis with SE values. K_m , effector constant; K_i , inhibition constant;

K_m , Michaelis constant, K_i , substrate inhibition constant.

It is important to note that there was a reduction observed when calculating the apparent K_m values (K_{Obs}) for midazolam 1'-hydroxylation activities of P450 3A5 by means of the Michaelis-Menten equation. There was a change from 5.5 μM to 3.0, 1.8, 1.2, 0.97, and 0.77 μM in the presence of 10, 50, 100, 250, and 500 μM thalidomide, displayed in Figure 11B, consistent with the prediction made from Equation 5, that the observed K_M should be decreased as a result of enhancement of the reaction by thalidomide.

To confirm the enhancing effects of thalidomide on liver microsomal P450 3A5, the oxidation of cyclosporine A was examined as illustrated in Figure 12.¹⁶ When P450 3A4-catalyzed cyclosporine A oxidation was exposed to thalidomide, it had no effect, shown in Figure 12A. However, when P450 3A5-catalyzed cyclosporine A oxidation was exposed to thalidomide, it was enhanced as shown in Figure 12B. There was a comparable enhancement in cyclosporine A oxidation with thalidomide as observed in liver microsomes expressing both P450 3A5 and 3A4 (Figure 12D). However, there was no enhancement in other liver microsomes predominately expressing P450 3A4 (Figure 12C).

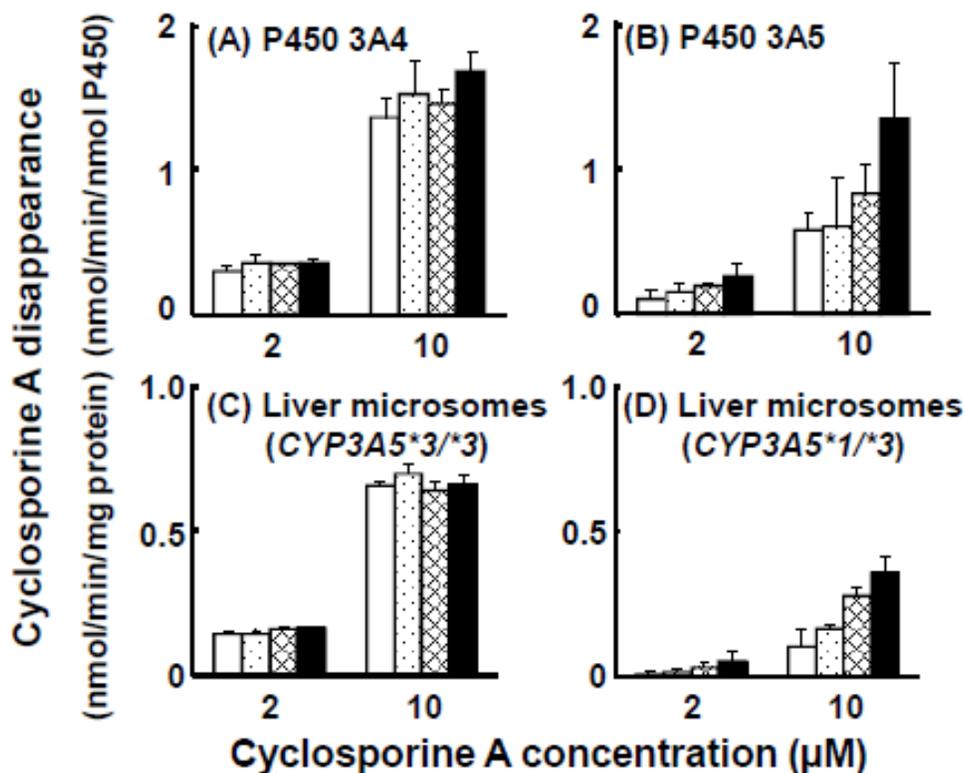


Figure 12. Enhanced cyclosporine oxidation activity with recombinant P450 3A4 (A) and 3A5 (B) and human liver microsomes (C,D) in the absence (□) or the presence of 2 (▨), 10 (▩), and 30 μM (■) thalidomide.

The results of the study of cyclosporine A oxidation confirm the hypothesis that thalidomide preferentially binds to P450 3A5. Docking simulations of thalidomide into P450 3A4 and P450 3A5 models were performed to further investigate the preference of thalidomide for interacting with P450 3A5. It was observed that thalidomide could closely “dock” to the P450 3A5 heme region. It is suggested that this interaction results in an activation of P450 3A5 in a type of heterotropic cooperativity. In the P450 3A4 docking simulation, thalidomide was found far from the heme of P450 3A4, which is in contrast to the P450 3A5 simulation where the glutarimide ring of thalidomide was found in close proximity to the center of the heme of P450 3A5 (Figure 13).¹⁶

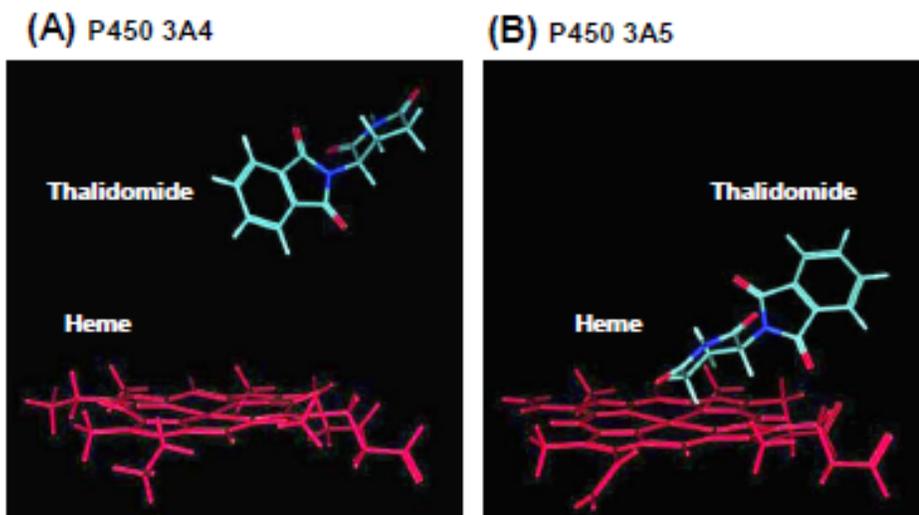


Figure 13. Docking Simulation of Thalidomide into a Reported Structure of P450 3A (A) and a homology Model of P450 3A5 (B).

It is proposed that when thalidomide docks near the heme iron and/or suitable substrate binding areas, the projected substrate pocket of P450 3A5 would be affected causing a more accessible active site. In the case of P450 3A4, no enhancement due to a cooperativity effect is proposed (Figure 14).¹⁶

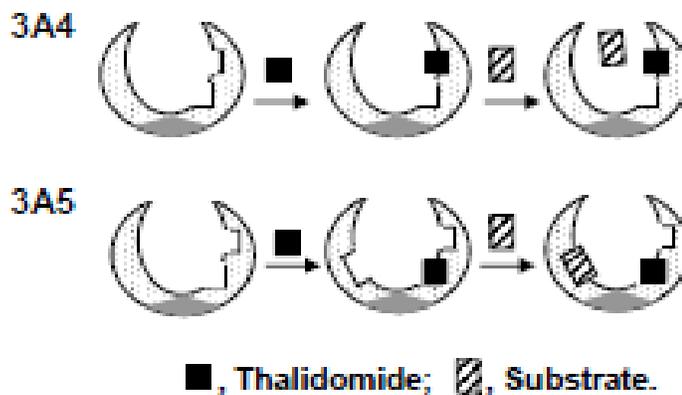


Figure 14. Proposed Working Models for Heterotropic Binding to P450 3A4 and 3A5.

The difference in reactivity proposed in Figure 14 for P450 3A4 and P450 3A5 could be derived from the differences in amino acid residues observed at positions 210 through 214 on the F-helix of P450 3A4 far from the heme of the P450 3A4 catalytic center listed in Table 2.²⁸ Amino acid differences at positions Leu210, Asp214, and Ser239 in P450 3A4 and Phe 210, Gly214, and Cys239 in P450 3A5 might produce more flexibility and higher lipophilicity, which may account for the differences between the interaction of thalidomide with P450 3A4 and P450 3A5. As a result, a thalidomide molecule causes a shift in P450 3A5 into a conformation so that the binding site is in close proximity to the heme, which leads to the stimulation of the cyclosporine and midazolam oxidation activation.

| | <u><i>F-helix</i></u> | | | | | <u>SRS-3</u> |
|-----------------|-----------------------|------------|-------------------|------------|-------------------|---------------------|
| Position | 210 | 211 | 212 | 213 | 214 | 239 |
| 3A4: | Leu | Leu | Arg | Phe | Asp | Ser |
| 3A5: | <u>Phe</u> | Leu | <u>Lys</u> | Phe | <u>Gly</u> | <u>Cys</u> |

Table 2. SRS-3, Substrate Recognition Site-3.

Conclusion

The results of an investigation of the effects of thalidomide on P450 mediated oxidation of midazolam, S-mephenytonin and cyclosporine A suggest that thalidomide interacts with P450 enzymes to inhibit or enhance oxidation of substrates. Thalidomide enhancement of midazolam hydroxylation and cyclosporine A clearance by P450 3A5 can be explained by a heterotropic cooperativity mechanism. Simulations of the docking of thalidomide with P450 3A4 and P450 3A5 models support the hypothesis of heterotropic cooperativity for the reactions of P450 3A5. Differences in the amino acid residue sequence at position 210 through 214 on the F-helix of the enzymes may account for the difference between the binding of thalidomide to P450 3A4 and to P450 3A5.

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