

An Insight into the Concept and Details of Mechanism-Based Inhibition of CYP450

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Cytochrome P450, family of heme-containing enzymes, catalyzes biotransformation reactions inside the body. The P450-catalyzed reactions result in detoxication of xenobiotics, sometimes, these reactions produce highly reactive metabolites (intermediates) that can react with cellular constituents leading to toxicity or can react with CYP450s resulting in mechanism-based inactivation. Drug-Drug Interactions (DDIs) mostly occur due to inhibition of the P450 enzymes. The P450 inhibition can be classified as reversible (competitive or non-competitive), quasi-irreversible and irreversible (both constitute mechanism-based inactivation). Mechanism-based P450 inactivation or inhibition usually involves biotransformation or bioactivation of the xenobiotic (including drugs) to a reactive metabolite (intermediate), which can either covalently modify an active site amino acid residue (irreversible) and/or may coordinate to the heme prosthetic group (quasi-irreversible). These inhibitory reactions on CYP usually result in toxicological consequences. These critical reasons have compelled most of the drug metabolism groups within pharmaceutical companies to establish various procedures and mechanistic studies to elucidate the mechanism of P450 inactivation and adopt strategies to avoid P450 inactivation/bioactivation liability. In this review, a comprehensive analysis of the biochemical basis, mechanistic, kinetic and structural aspects required for P450 inactivation by xenobiotics is described. The clinical significance and the potential for exploiting mechanism-based inactivators of P450s for therapeutic benefits have also been discussed along with the future prospects.

Introduction

Cytochrome P450 (CYP) is the super-family of metabolizing enzymes, which apart from metabolizing endogenous substances, oxidize xenobiotics (including drugs), increase their polarity and facilitate elimination.¹ The interaction between substrates and CYPs are generally specific, dictated by the electrostatic and steric contributions from residues forming the enzyme catalytic domain. The oxidizing site is the heme centre which absorbs an oxygen molecule and transfers an oxygen atom to the substrate.² Although most of the reactions catalyzed by the cytochrome P450s generally lead to detoxication of xenobiotics by forming more hydrophilic metabolites that can be readily excreted from the body, P450s can also produce reactive intermediates that may react with macromolecules in the cell such as DNA, RNA, and proteins, ultimately leading to toxicity.^{3,4} Chemical structures which can be metabolically transformed to reactive groups and produce potential adverse safety outcomes are considered "structural alerts".⁵ Since, it is widely known that CYP450 catalyse the oxidative transformation of structurally diverse xenobiotics and drugs, the study of inhibition of these versatile enzymes becomes a necessity. In the biotransformation process by the P450s, sometimes compounds or xenobiotics (including

drugs) may be converted into reactive species which can then react with moieties in the active site leading to inactivation of the P450, the process is referred to as "mechanism-based", "suicide", "time-dependent", or "catalysis-dependent" inactivation or inhibition (MBI).⁴⁻⁷

There are three steps in the catalytic cycle of CYP450 which are prone and vulnerable to inhibition and these are as follows: (i) the binding of substrates, (ii) the binding of molecular oxygen subsequent to the first electron transfer, (iii) the catalytic step in which the oxidation of substrate occurs. There may be other steps in the cycle which might get affected or inhibited, but usually, don't lead to CYP450 inhibition. CYP450 inhibitors may be divided into three distinct classes: agents that bind to CYP450 (a) reversibly, (b) quasi-irreversibly by complexing with heme-iron (Metabolite-inhibitor, MI complex formation), and (c) irreversibly to apoprotein (amino acid residues) or to the heme prosthetic group, resulting in destruction and degradation of heme prosthetic group.⁵⁻⁷

The reversible inhibitors (competitive or non-competitive) are those which are interfering in the catalytic cycle prior to the actual oxidative event. Inhibitors which are acting during or subsequent to the oxygen transfer step are generally categorised as quasi-irreversible or irreversible inhibitors, thus, mechanism-based inhibitors (suicide inactivators).

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"Mechanism-Based Inhibition: involves bioactivation of the xenobiotics (or drug) to a reactive intermediate, which covalently modifies an active site amino acid residue and/or coordinates to the heme prosthetic group." The synonyms for MBI are: Suicide inactivation, Time dependent inactivation and catalysis dependent inactivation.^{7,8} Numerous classes of compounds are known to undergo CYP450 catalysed activation to reactive intermediates that irreversibly or quasi-irreversibly inactivate the enzyme responsible for their activation.

Mechanism-based (catalysis-dependent) inhibitors are highly enzyme specific and four classes of mechanism-based inhibitors are known depending on the mechanism of inhibition of CYP450 enzyme: (a) agents that are binding covalently to the protein, (b) agents that are binding quasi-irreversibly to the heme iron, (c) agents that are alkylating or arylating the heme porphyrin network, and (d) agents that degrade heme prosthetic to products that themselves modify the protein.⁷⁻¹⁴

Criteria for Mechanism-Based Inhibition

There are 9 criteria which are required to be fulfilled for a drug to be classified as a mechanism-based inhibitor. The most important of all is the presence of cofactors and time dependency⁹.

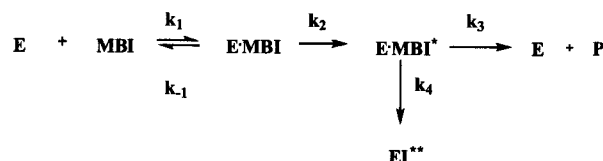
1. *Time Dependency*: The loss of activity exhibited by the enzyme should be time-dependent. Graphically, the plot of enzyme activity remaining after preincubation should give a straight line with respect to preincubation time should give a straight line.
2. *Saturation kinetics*: After reaching a particular concentration of the inhibitor, the rate of inactivation should not increase further, indicating the saturation state.
3. *Presence of cofactors*: Since, the critical step is the bioactivation of the xenobiotics (or drug) to a reactive intermediate, thus, presence of cofactor for metabolism becomes a necessity.
4. *Presence of other substrate or inhibitor*: The presence of other substrate or inhibitor of the enzyme must be avoided as it slows the rate of inactivation of the enzyme by the inhibitor already present.
5. *Catalase or superoxide dismutase*: The inactivation of CYP450 enzyme gets affected by the inclusion of catalase and/or superoxide dismutase. Because, they uncouple P450s leading to production of superoxide and/or hydrogen peroxide that can result in auto inactivation. Therefore, their presence shall be avoided for inactivation of CYP450 to take place.
6. *Irreversible*: the inactivation should be irreversible and activity of CYP450 enzyme should not be regained back by any means (dialysis, etc.).

7. *1:1 stoichiometry*: The enzyme inhibitor must label the enzyme in a manner that results in 1:1 stoichiometry of inactivation.
8. *No scavenger effect*: The rate of inactivation of the enzyme should not be affected by the presence of scavengers such as glutathione (GSH).
9. *No Lag time*: The kinetics for inactivation should not exhibit any lag time.

Kinetics of Mechanism-Based Inhibition

Since, the IC_{50} values of MBIs are time dependent causing serious problems when aiming at ranking different compounds with respect to their inhibitory potential. As a consequence, most studies and ranking schemes related to MBIs rely on the inhibition constant (K_i) and the rate of enzyme inactivation (k_{inact}) rather than on the IC_{50} values (Fig. 1).⁹

Here, K_i is the affinity of the mechanism-based inhibitor for the enzyme.



$$K_i = [(k_{-1} + k_2) / k_1] [(k_3 + k_4) / (k_2 + k_3 + k_4)]$$

$$k_{inact} = k_2 k_4 / (k_2 + k_3 + k_4)$$

Fig. 1. Kinetics of MBI.¹⁵

k_{inact} is the maximal rate constant for MBI.

MBI stands for mechanism-based inactivator,

$E \cdot MBI$ is the Michaelis-Menten enzyme-substrate complex,

$E \cdot MBI^*$ is the reactive intermediate reversibly bound in the active site of the protein, P is the product that leaves the enzyme,

EI^{**} is the inactivated enzyme.

Partition ratio- introduced by Walsh and it is determined by the ratio of k_3/k_4 . It refers to the average number of cycles of metabolism that the enzyme traverses before it is inactivated. Thus, it is a measure of the efficiency of the mechanism-based inactivator.

Mechanism-Based Inactivators: Structural Features and Reactive Intermediates

(1) QUASI-IRREVERSIBLE INHIBITORS (MI COMPLEX FORMING AGENTS)

(A) 1,3-BENZDIOXOLE-CONTAINING COMPOUNDS

Compounds containing methylenedioxyphenyl or 1,3-benzodioxole moiety inactivate P450 enzymes via Metabolite-

inhibitor (MI) complex formation with the enzyme as shown in figure 2.¹⁶⁻¹⁸ The examples of some of the drugs in this category are paroxetine,¹⁹⁻²¹ methylenedioxymethamphetamine (ecstasy), lignans of *Piper cubeba* etc.²² Some insecticides such as piperonylbutoxide, obtained from safrole and isosafrole also lead to MBI with CYP450.²³

The mechanism of inhibition of CYP activity remains the same, involving a reactive carbene intermediate which coordinates with the heme-iron resulting in formation of a MI complex (Fig. 2).²⁴ This carbene intermediate forms through abstraction of hydrogen atom from the methylene carbon or by elimination of water molecule from a hydroxymethylene intermediate.

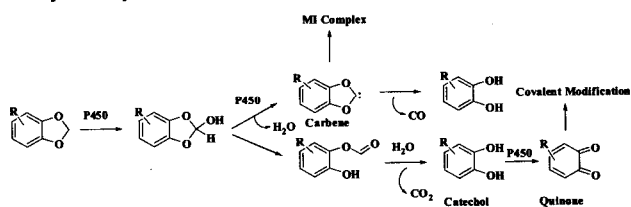


Fig. 2. Mechanism-Based inhibition by carbene reactive intermediate.

(B) HIV PROTEASE INHIBITORS

HIV protease inhibitors occur as both substrates and potent competitive inhibitors of P4503A4. Apart from their P4503A4 competitive inhibitory properties, protease inhibitors such as ritonavir, nelfinavir, amprenavir, lopinavir, and saquinavir are also known to act as mechanism-based inactivators.²⁵⁻²⁷ The detailed study on ritonavir was carried out and it was determined that ritonavir is a type II ligand which gets perfectly docked into the active site cavity of CYOP3A4. The mechanism was found to involve binding of thiazole nitrogen to the heme iron.²⁸ Moreover, the SAR studies revealed that mechanism-based inhibition by ritonavir analogs is due to 5-thiazolyl and 2-(1-methylethyl) thiazolyl groups.²⁹

(C) MACROLIDES

Most of the anti-bacterial agents belonging to macrolides class, containing a tertiary amine group act as MBIs owing to the formation of MI complexes with P450 Fe(II). Macrolides were classified into three different groups according to their affinity for P4503A4; where (i) the first group (e.g. troleandomycin, erythromycin and its prodrugs, and clarithromycin) result in formation of nitroso metabolite,^{29,30} (ii) the second group (e.g. josamycin, flurithromycin, roxithromycin, midecamycin, and miocamycin) rarely result in MI complex and subsequent, mechanism-based inhibition;^{31,32} (iii) the third group (e.g. spiramycin, rokitamycin, dirithromycin and azithromycin) do not give rise to any inactivation of CYPs.³³⁻³⁵

(D) ALIPHATIC AND AROMATIC AMINES

The metabolism of aliphatic and aromatic amines has

been extensively studied and the reactive species involved was believed to be nitrosoalkane obtained via enzymatic oxidation of primary amine substrate followed by hydroxylamine intermediate. Although, primary amine moiety is essential for compound to act as MBI, but secondary and tertiary amines can also sometimes act as inhibitors after undergoing N-dealkylation. Some of the important examples include amine-based Ca^{2+} blockers such as diltiazem, verapamil, etc.

Moreover, antidepressants such as nortriptyline, undergoes N-dealkylation followed by hydroxylamine formation and finally the reactive nitroso species. Certain cyclopropylamines (e.g. N-cyclopropylbenzylamine and related analogs) inactivate P450 activity in a similar fashion. (Fig. 3).³⁶

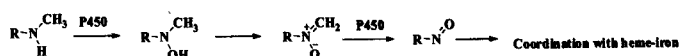


Fig. 3. Mechanism-Based Inhibition by Aliphatic amines.³⁶

(2) IRREVERSIBLE INHIBITORS

The covalent modification of P450 enzymes by reactive metabolites has been extensively studied. The covalent modification occurs mostly with the active-site amino acid residues, which, thereby inactivates the activity of CYP450 enzyme. Also, interaction (covalent binding with heme prosthetic group) can take place, resulting in the destruction of heme.

The functional groups mostly involved in this type of irreversible inhibition are as follows:

(A) EPOXIDE RING

There are several therapeutic drugs which contain unsaturated portion, which after epoxidation, results in inhibitory activity of CYP450 enzyme. The furan ring, containing agents are said to be involved in MBI activity, due to metabolism to furan electrophilic epoxide. The HIV-protease inhibitor, L-754,394 gets bound covalently to the apoprotein which strongly implicates the involvement of epoxide and/or the aldehyde species as shown in Fig. 4 derived from the bioactivation of L-754,394 to be responsible for covalent binding to P4503A4 leading to its inactivation.¹⁰

Similarly, 4-ipomeanol generates the epoxide intermediate, which after alkylating the protein leads to irreversible inactivation. A similar mechanism for inhibition is found in 8-methoxypsoralen. Other rings such as thiophene (tienilic acid),³⁷ phenyl and substituted phenyl (raloxifene)³⁸ also undergo epoxidation, thereby resulting in mechanism-based inhibition.

(B) QUINONE IMINE AND QUINONE-METHIDE

Various drug molecules are known to form quinone-imine as the reactive intermediate that covalently binds to CYP

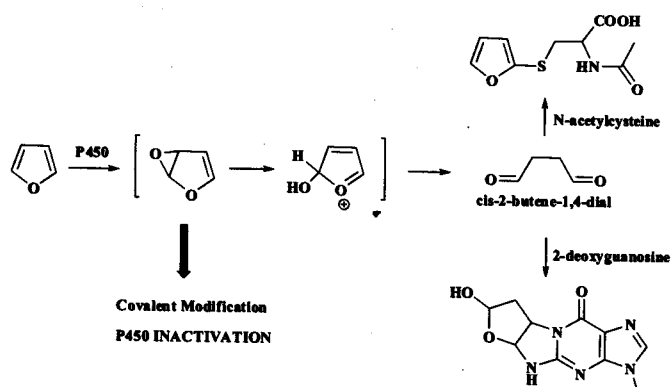


Fig. 4. Mechanism-Based Inhibition by epoxide reactive intermediate.¹⁰

enzyme, resulting in its inhibition. There are several drugs such as nefazodone, a tricyclic antidepressant, which undergoes aromatic hydroxylation, followed by the reactive quinone-imine metabolite. Similarly, diclofenac, a nonsteroidal antiinflammatory drug, undergoes initial hydroxylation on either the dichlorophenyl ring or on the phenylacetic acid portion of the molecule, resulting in 4'-hydroxydiclofenac and 5-hydroxydiclofenac respectively. Thereafter, oxidation of the p-aminophenol moiety occurs resulting in quinone-imine intermediate that can interact covalently with amino acid residues resulting in irreversible inhibition. Raloxifene also undergoes two-electron oxidation resulting in reactive quinone species or epoxide. Several N-substituted morpholino derivative, potassium channel opener drugs, also lead to mechanism-based inactivation of CYP3A4, via the formation of reactive quinone-imine or imine-methide intermediates. This is preceded by the initial aromatic hydroxylation ortho to the morpholine ring (or para to benzylamine methine).^{10,39,40}

Consequences of Mechanism-Based Inactivation of P450s: DDIs

Most of the inactivators lead to pharmacokinetic-based drug interactions via irreversible inactivation of P450 enzymes that metabolize drugs.⁴¹ In case of reversible inhibitors, the extent of DDI diminishes as the clearance of inhibitor occurs from the body, whereas recovery from mechanism-based inactivation is rigorous and often, requires *de novo* synthesis of P450 enzyme.^{42,43} Some of the agents known to cause DDI in humans via mechanism-based inactivation are listed in Table 1.¹⁰

A drug-drug interaction occurs when a substance (including drugs) affects the activity of a drug, i.e. the effects are increased or decreased, or they result in a new effect that neither can produce on its own.⁴⁴ Most of the drug interactions occur via certain changes in the biotransformation process mediated by CYPs and they can in-turn be the consequence of P450 inactivation.⁴⁵ The

Table1. Drugs leading to DDI in humans via Mechanism-Based Inactivation of cytochrome P450 enzymes.¹⁰

CYP isoforms	Drugs
1A2	Enoxacin, Zileuton, Furaflavone
2D6	Paroxetine, Cimetidine
2B6	Clopidogrel, ThioTEPA
2E1	Disulfiram
2C8	Gemfibrozil
3A4	Clarithromycin, Diltiazem, Verapamil Erythromycin, Fluoxetine, Nelfinavir, Ritonavir, Saquinavir
2C9	Tienilic acid, Suprofen
2C19	Ticlopidine

intake of terfenadine along with erythromycin is generally avoided, because of the potential drug-drug interaction.⁴⁶ The plasma levels of terfenadine increase due to the decrease in its metabolism, thereby resulting in cardiac arrest.⁴⁷ Sometimes, inactivation of P450 results in a decreased metabolism of prodrugs, such as cyclophosphamide, that have to metabolise to show therapeutic activity.⁴⁸ Some specific contents in juices and dietary components have also been shown to exhibit mechanism-based inactivation of P450s. Example: seville orange juice increases the bioavailability of felodipine owing to the presence of bergamottin, CYP 3A4 inhibitor.⁴⁹ Similarly, grape juice and tea inhibit hydroxylation in flurbiprofen.⁵⁰

Beneficial implications of Mechanism-Based Inactivation

The deleterious effects of Mechanism-Based Inhibition are already seen (DDIs), but on the other hand, these mechanism-based inactivators are also beneficial therapeutically. Resveratrol, an inhibitor of CYP1A1 was found to decrease the metabolism of carcinogen benzo[a]pyrene. This results in abrogation of damaging effects of benzo[a]pyrene on the lung tissue. Thus, resveratrol counteracts the deleterious effects of benzo[a]pyrene, and eliminates the harmful molecules from the body.^{51,52} Similarly, a co-formulation of ritonavir and lopinavir was designed keeping in mind the improved therapeutic activity against HIV-1 protease. This was because of the improved pharmacokinetic properties.^{53,54}

With the same view, a scientific group proposed the inactivation of P450 2A6 as a novel therapeutic approach for smoking cessation. It is known that CYP 2A6 is responsible for the metabolism of nicotine. Therefore, in their study, it was shown that pharmacokinetics for nicotine was altered ($t_{1/2}$ increased) as a result of inhibition of CYP 2A6. Hence, it was considered that inhibition of nicotine metabolism would reduce craving of a person to smoke and thereby, decreasing the possibility of release of pro-carcinogens.^{53,54} Also, Ritonavir, a mechanism-based inhibitor of CYP450 increases the concentration of triazolam, a triazolo-benzodiazepine hypnotic drug, thereby enhancing its sedative effect.⁵⁵

Conclusions and Future Prospects

Many clinically important pharmacokinetic drug-drug interactions result from the impairment of metabolic clearance via mechanism-based inhibition of P450s. Thus, a strategy shall be devised during the early stages of drug discovery to avoid the phenomena of mechanism-based inactivation of P450. This could be possibly achieved by excluding certain functional groups that are liable to CYP450-mediated biotransformation and thereafter, CYP450 inactivation. However, this would accompany certain disadvantages and flaws i.e. limitation of compounds within an area of interest, which would promptly result in neglect of good and biologically active (no inhibitory effect) drug(s). Therefore, a more critical and advanced practical approach involving the efforts of computational chemists, synthetic chemist, medicinal chemists, pharmacologists and metabolism experts is necessary. Thereafter, various consequential studies together with assessment of bioactivation pathways would provide useful information to medicinal chemists and could be utilized accordingly to minimise or curb mechanism-based inhibition. Alternatively, other series of compounds which do not lead to mechanism-based inhibition could be utilized as a suitable replacement for further optimization of ADMET properties.

The prospect of a new drug candidate leading to DDI or toxicity due to P450 inactivation depends on several factors, some of which are: overall disposition (extent of metabolism leading to P450 inactivation relative to latent metabolic or non-metabolic fate); daily dose, therapeutic regimen (acute versus chronic) and the intended target population; and these are the factors that need to be taken into consideration when making a final decision to develop a drug candidate that is bio-processed to reactive intermediates.

Since, the mechanism of agents acting as MBIs is uncertain and the atomic level details are missing, various computational tools can be utilized accordingly to answer a few questions. The use of Modeling together with quantum mechanical (QM), quantum-mechanics/ molecular

mechanics (QM/MM) can be applied, to arrive at interesting and fruitful conclusions. Therefore, the knowledge and understanding of Mechanism-Based Inhibition becomes an essential requirement and necessity in the process of Drug Discovery and Development.

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