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Expert Review

Antiplatelet Drug Resistance and Drug-Drug Interactions: Role of Cytochrome P450 3A4

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Abstract. Antiplatelet therapy provided pivotal advances in the treatment of cardiovascular disease. Aspirin and thienopyridine, clopidogrel, is currently the treatment of choice in acute coronary syndromes and the prevention of thrombosis after coronary stent implantation. Despite the efficacy of this dual antiplatelet therapy in reduction of adverse coronary events in patients with acute coronary syndromes, complications persist in a subgroup of these patients. Emerging causes of aspirin and clopidogrel resistance may translate to increase risk for recurrent myocardial infarction, stroke, or cardiac related mortality. However, the mechanism of antiplatelet drug resistance remains incompletely characterized, and a sensitive and specific assay of aspirin and clopidogrel effect that reliably predicts treatment failure has not emerged. To date, evidence supporting antiplatelet drug resistance are pharmacokinetic response variability, drug-drug interaction through competitive inhibition a specific enzymatic pathway, genetic variability, and variability in the induction of enzymatic pathway in metabolic activation of prodrugs, like clopidogrel. Further investigation or guidelines are needed to optimize antiplatelet treatment strategies to identify and treat patients resistant to aspirin and/or clopidogrel.

KEY WORDS: antiplatelet resistant; antiplatelet therapy; aspirin; clopidogrel; platelet.

INTRODUCTION

Platelet activation and aggregation play an important role in the pathogenesis of arterial thrombosis and lead to: acute coronary syndromes (ACS) which include a spectrum of diagnoses that include unstable angina, non- ST-elevation myocardial infarction, and ST-elevation myocardial infarction; and thrombotic complications during and after percutaneous coronary intervention (PCI) (1–3). Acute coronary syndromes are primarily caused by atherosclerotic plaque rupture or fissuring and subsequent occlusive or subocclusive thrombus formation. Disruption of a previously quiescent plaque exposes a thrombogenic surface that stimulates platelet adhesion and activation. Activated platelets then release a variety of vasoactive substances, including thromboxane A_2 (TXA_2) and adenosine diphosphate (ADP) that promote platelet aggregation through autocaine and puvacaine mechanisms. Both aspirin and the thienopyridine, clopidogrel are selective inhibitors of specific pathways that trigger platelet activation leading to platelet aggregation. In combination with aspirin, clopidogrel therapy demonstrated evidenced-based cardiovascular benefits in the risk reduction of non-fatal myocardial infarction ACS (4,5), and the prevention of subacute stent thrombosis in patients undergoing PCI (6). Aspirin irreversibly acetylates cyclooxigenase (COX)-1, resulting in inhibition of TXA_2 generation by platelets and endothelial cells (Fig. 1), while clopidogrel inhibits ADP induced platelet aggregation by irreversibly binding to the specific $P2Y_{12}$ ADP platelet receptor (Fig. 2). Despite effective aspirin and clopidogrel therapy, recurrent arterial thrombotic events manifested as ACS, stroke, and peripheral vascular events occur in 8 to 18% of patients (7). Emerging entities conceptualize the definition of aspirin and clopidogrel 'resistance' using various diagnostic modalities; however a uniformly accepted definition of aspirin and clopidogrel resistance has failed to achieve consensus agreement. Currently, the paradigm of antiplatelet drug resistance is accepted as inter-individual response variability to antiplatelet therapy leading to treatment "failure". A more reliable mechanism-based definition using biochemical end-points correlated with clinical outcomes instead of surrogate platelet aggregation measurements may lead to improvements in therapy (8–10). In this review article, the main determinants of the interindividual variability in response to aspirin and clopidogrel are discussed with emphasis on pharmacokinetic mechanism-based biochemical end-points correlated with clinical outcomes, response variability to clopidogrel, and the hepatic cytochrome P450 3A4 isoenzyme.

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Platelet Activation and Aggregation

Although percutaneous coronary interventions for coronary revascularization continued to improve in the past decade, abrupt coronary vessel closure during the peri- and post-procedural period and restenosis in the months post-procedure remain important limitations. Intracoronary stent implantation has gained substantial success in addressing these limitations. However, despite the technological and therapeutic advances, the incidence of stent thrombosis with the potential complication of ACS including unstable angina and myocardial infarction (with or without ST-segment elevation) is 2–3% within the first month after PCI (6). The initial mechanism associated with stent thrombosis, resulting from iatrogenic vessel injury and implication of a foreign body, and occlusive thrombus resulting from spontaneous erosion of vulnerable atheromatous plaque is the activation of platelets and the extrinsic coagulation cascade(3,11). Vascular endothelial disruption promotes circulating platelet adhesion to the vessel wall through interactions with the exposed glycoprotein constituents of the subendothelium (collagen, von Willebrand factor, and other adhesive proteins such as fibronectin, laminin, and vitronectin) (12–14). Collagen, thrombin, and epinephrine promote the adhered platelets to release the contents of their dense (ADP and serotonin) and alpha-granules (fibrinogen, von Willebrand factor, proinflammatory factors, and prothrombotic factors), which trigger platelet-activating intracellular signals in surrounding platelets (15). Activated platelets in addition to synthesize by and releasing TXA_2 in circulation, also express surface membrane glycoprotein (GP) IIb/IIIa receptors allowing fibrinogen binding and further recruitment of circulating platelets, followed by fibrin-mediated cross-linking between adjacent activated platelets resulting in aggregation and a growing thrombus (2). Activated platelets also express membrane P-selectin that is involved in platelet-monocyte aggregate formation, which in turn induces expression of inflammatory cyotokines. Through the expression of CD40L and P-selectin, platelets also modulate the expression of tissue factor in monocytes. Tissue factor, in turn, produces thrombin, a pivotal platelet activator. Platelet activation also promotes a prothrombotic response in the production of platelet-derived interleukin-1 and platelet factor 4 that are involve in the expression of the inflammatory cytokines, interleukin-6 and interleukin-8, respectively (16–18).

ASPIRIN

Mechanism of Action

Aspirin, or acetylsalicylic acid, exerts its antiplatelet action through the irreversible acetylation of platelet COX-1 at serine residue 530. This enzyme is responsible for conversion of arachidonic acid to eicosanoids, that are precursors of the prostaglandins, TXA_2 and prostacycline, (prostaglandin I_2, Fig. 1). Thromboxane A_2 is a potent vasoconstrictor and platelet agonist that are released when the platelet is activated. Platelet activation leads to activation of phopholipase A_2 (PL A_2) which cleaves membrane phospholipids to release arachidonic acid. Arachidonic acid is converted to TXA_2 by sequential actions of cyclooxygenase-1 and thromboxane synthase present in platelets. Secreted TXA_2 binds to a specific Gq coupled thromboxane receptor and activates and recruits surrounding platelets as a

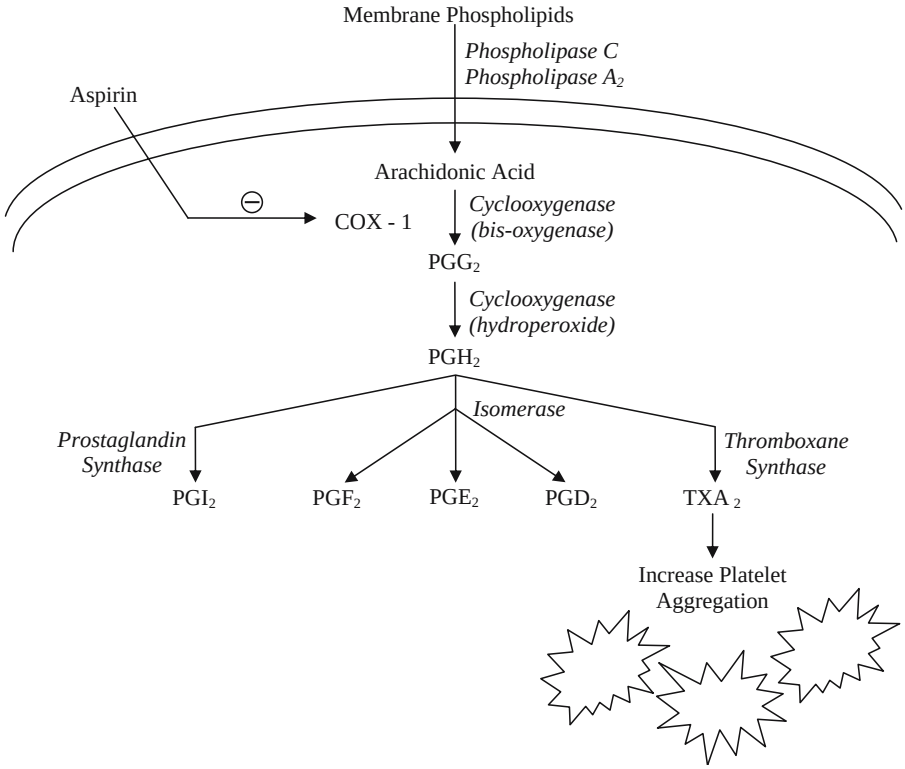

Fig. 1. Aspirin irreversibly acetylate COX-1 resulting in inhibition of TXA_2. TXA_2 = Thromboxane A_2, *COX-1* = Cyclooxygenase-1, *PG* = Prostoglandin, PGI_2 = Prostocyclin.

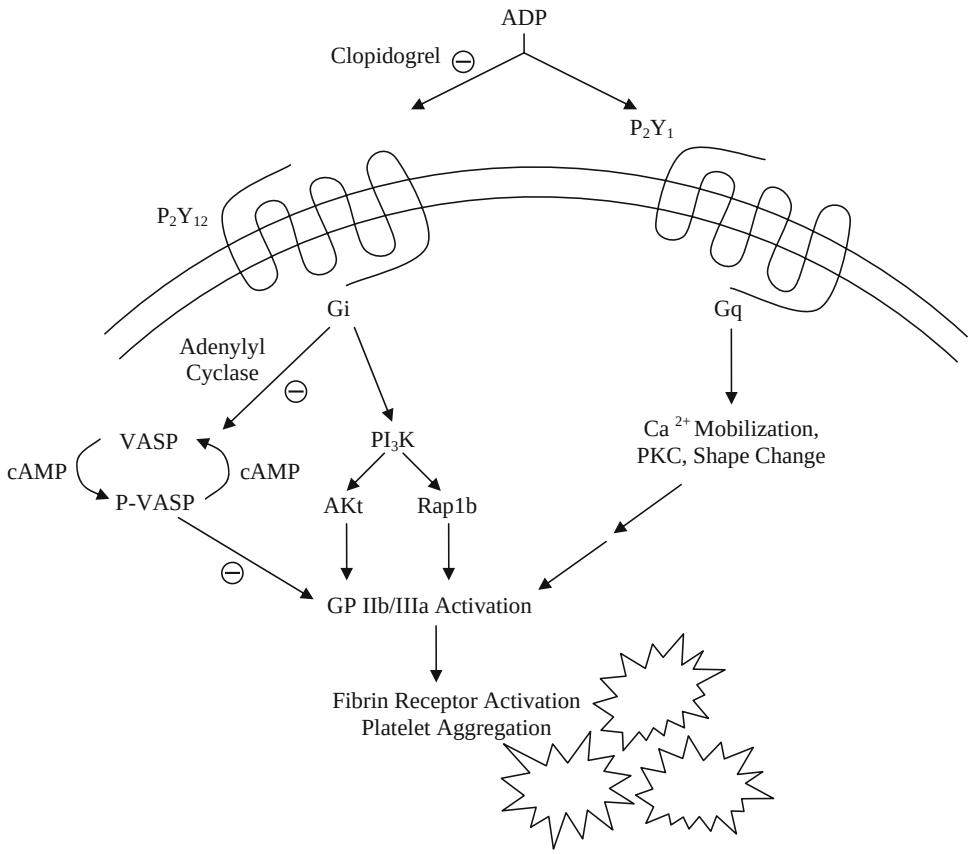

Fig. 2. Intracellular signaling events downstream of the P2Y and $P2Y_{12}$ receptors following activation of ADP-binding receptors. $P2Y_{12}$ is coupled to Gi pathway that contribute to platelet aggregation. $P2Y_{12}$ receptor mediated inhibition of adenylyl cyclase is indirectly responsible for platelet aggregation. *PI3K* Phosphoinositide-3-kinase, *AKt* Serine–threonine kinase, *Rap1b* GTP binding proteins.

positive feedback mechanism. Production of TXA_2 has been shown to be completely inhibited in human volunteers by low dose (100 mg) aspirin (19).

Prostacyclin is a vasodilator and platelet inhibitor synthesized by platelets and vascular endothelium. While aspirin permanently inhibits the rate limiting step of TXA_2 production for the entire life span (7–10 days) of the anucleated platelets by COX-1 acetylation, the inhibited COX-1 in vascular endothelial cells is replaced with functional enzymes, and maintains the synthesis of prostacyclin. Therefore, aspirin inhibits platelets by both reducing the amount of available TXA_2 and by increasing the amount of prostacycline relative to TXA_2 (10). In addition, prostacylin is also produced by the enzymatic activity of COX-2, however is 50–100 times less affected than COX-1. Also the inhibitory effect of aspirin on COX-2 is transient, with a duration of action of 3 h as compared to the lifetime of COX-1.

Platelet Function Assessment

Thorough assessment of the complex biological function of platelets in response to agonists, as well as a uniform quantitative and qualitative measure of the antithrombotic efficacy of aspirin using surrogate laboratory modalities remains uncertain. Despite the complexity of platelet function and the uncertainty whether laboratory measurements of platelet function can encompass aspirin's antiplatelet effect, assessment of the antithrombotic efficacy of aspirin has primarily focused on platelet aggregation and platelet flow cytometry measurements to define pre-treatment responsiveness and post-treatment platelet reactivity to antiplatelet agents (20–22).

Platelet aggregation is traditionally measured in platelet-rich plasma (PRP) against control platelet-poor plasma using an optical aggregometer. The aggregation response is stimulated by the addition of a platelet agonist (epinephrine, ADP, or AA, TRAP, collagen) and analyzed on a 0–100% scale, according to the degree of light transmission. As platelets bind via fibrinogen, light transmission increases. The primary limitations of this assay are labor-intensive preparation of PRP, user variability, and the results may vary with changes in platelet count and agonist used (23). Alternatively, whole blood aggregometry eliminates the process of centrifugation to prepare PRP and measures the platelet aggregation response using electrical impedance in place of optical density. However, measurement time is longer than the time required for measurement by optical aggregometry and the results of whole blood aggregometry have not closely correlated with optical aggregometry (23).

Point-of-care platelet function tests have been developed to monitor the pharmacological effects of the rapidly developing antiplatelet agents in an attempt to optimize therapeutic efficacy and minimize adverse effects. The platelet function analyzer (PFA)-100 system (Dade-Behring, Deerfield, Illionois)

assesses platelet function in whole blood by measuring the time required for closure of a microscopic aperture cut into a membrane coated with collagen and epinephrine (normal reference range 98–185 s) or collagen and ADP (77–133 s) resulting from formation of a platelet plug from adhesion and aggregation of platelets (24,25). The PFA-100 measures the *ex vivo* stimulation of shear stress on platelets. Although non specific, in testing 126 subjects who took 325 mg of aspirin revealed that the PFA-100 system was able to detect 71.7% of aspirin-induced defects with a positive predictive value of 97.8% (26). The ease of operation of the instrument makes it a useful tool to use in screening patients for selected platelet-related homeostasis defects. A major limitation of the PFA-100 is that patients receiving intravenous GP IIb/IIIa inhibitors with profound GP IIb/IIIa inhibition will often have aperture closure times greater than 300 s, making interpretations difficult to delineate (27).

The Rapid Platelet Function Assay (Accumetrics, San Diego, California, USA) is an automated tubidimetric whole blood assay designed to assess platelet function based on the ability to activated platelets to bind fibrinogen. Washed fibrinogen coated polystyrene beads agglutinate in whole blood in proportion to the number of unblocked platelet $P2Y_{12}$ or GP IIb/IIIa receptors (28). As activated platelets bind and agglutinate fibrinogen-coated beads, there is an increase in light transmittance. The assay is rapid and less labor intensive, however limited by the need for comparison measurements with baseline pre-antiplatelet treatment measurements. Thus without a pre-dose baseline measurement for a patient, absolute inhibition levels cannot be established.

Platelet flow cytometry determines platelet function by measuring platelet surface receptor expression using a minute sample of whole blood. Platelet membrane glycoproteins remain in a resting state until agonists (ATP, ADP, thrombin, collagen, *etc.*) induce platelet activation. The internal pool then becomes surface-expressed as functional receptors. Functionally expressed membrane glycoproteins will then bind to fibrinogen, leading to platelet aggregation. Commercially available fluorescent monoclonal antibodies are available for specific platelet membrane glycoproteins that will identify these molecules on the surface of the platelets, allowing the quantification of activated platelets. Monclonal fluorescent labeled platelets are then passed through a focused laser beam at 1,000 to 10,000 cells per second. Mean intensity of immunofluorescence is used as an index of antibody binding and receptor surface expression that is compared with controls for non-specific binding and background florescence. The platelet flow cytometry assay is limited by the expense of monoclonal antibodies and instrumentation, and the need for highly trained laboratory personnel to prepare the samples.

The Thromboelastograph (TEG) Platelet Mapping Assay (Haemoscope Corp, Niles, Illinois) relies on the measurement of clot strength to enable a quantitative analysis of platelet function. The assay uses heparin as an anticoagulant to eliminate thrombin activity in the sample. Reptilase and factor XIIIa (activator F) are used to generate a cross-linked fibrin clot to isolate the fibrin contribution to the clot strength (29). The contribution of the $P2Y_{12}$ receptor or COX pathways to clot formation can thus be measured by the addition of an appropriate agonist, ADP or arachidonic acid. In the case of platelet function assessment for aspirin resistant, arachidonic acid is added to activator F to measure the degree of thromboxane A_2-induced platelet aggregation. Thromboelastography has been shown to correlate with optical aggregometry in detecting aspirin resistance (30). Limitations include in inter-user variability in obtaining the results of various agonists-induced clot strength measurements.

Platelet activation can also be measured by the release of arachidonic acid metabolites. Quantifying the activity of aspirin can be performed by measuring the levels of the product of COX-1 enzyme action. Urinary levels of 11-dehydro TXB_2, a stable metabolite of TXA_2 have been used to study the extent of aspirin-mediated inhibition of thromboxane generation (31). Because variability exists in the assay and methods of measurement among testing laboratories, a standardized enzyme immunoassay for urinary 11-dehydro TXB_2 has been developed (Aspirin Works and Corgenix Medical Corp., Denver, Colorado, USA) using controls from the Canadian patients enrolled in the Heart Outcomes Prevention Evaluation (HOPE) Study (32). This assay is limited in the interpretation of persistent elevation in urinary levels of 11-dehydro TXB_2. It is unclear whether uninhibited platelet COX-1 activity COX-1 independent sources of TXA_2, or both are responsible for elevated levels of urinary 11-dehydro TXB_2 in selected patients treated with aspirin.

Definitions of Aspirin Resistance

The Antithrombotic Trialists' Collaboration confirms that aspirin therapy in patients with atherosclerotic vascular disease reduces non-fatal myocardial infarction by one third, non-fatal stroke by one quarter, and vascular mortality by on sixth (7). No other pharmacologic agent can challenge the risk-benefit or cost-benefit ratios of aspirin therapy. Aspirin is the mandatory treatment for secondary prevention of cardiovascular events. Thus, emerging evidence of aspirin resistance in recent literature has significant clinical implications. Aspirin resistance has been defined either as the failure of aspirin to prevent individuals from clinical thrombotic complications or as the failure to produce an expected response on a laboratory measurement of platelet activation or aggregation (33).

The concept of therapeutic resistance originated when various laboratory measurements of platelet aggregation, platelet reactivity, platelet activation, and bleeding time demonstrated variability in antithrombotic responses to aspirin therapy in patients (31,34–36). To date, there is no uniformity in either the laboratory methodologies to detect aspirin resistance or the criteria to define aspirin resistance. The Working Group on Aspirin Resistance, International Society on Thrombosis and Homeostasis, has written a position statement that summarizes the limitations of the current methods of assessing aspirin responsiveness (37). Because the target of aspirin therapy is inhibition of COX-1, methods that directly indicate the activity of this enzyme would best assess whether aspirin resistance is present in a given patient (33). The earlier studies by Gum *et al.* reported a 5% prevalence of aspirin resistance in patients with stable coronary artery disease using >20% arachidonic acid-induced platelet aggregation and >70% ADP-induced aggregation as criteria for defining aspirin resistance (20). Other investigators have used various modalities such as bleeding time, agonist induced platelet aggregation (cationic propyl gallate, ADP, epineph-

rine, and collagen), and point-of-care assays to assess aspirin responsiveness (31,34,35,38–40). However the prevalence of aspirin resistance may in fact be overestimated when patient non-compliance to aspirin therapy is excluded, and COX-1 specific laboratory analyses are used as assays. Tantry *et al.* by using arachidonic acid-induced COX-1 specific platelet aggregation and thromboelastography platelet mapping reported a 0.4% (1/223) incidence of aspirin resistance in patients undergoing PCI (30), and Schwartz *et al.* reported 0.5% incidence of aspirin resistance in patients with history of myocardial infarction (41) as compared to previously reported 20–35% (39,40).

Since thromboxane A_2 is metabolized to thromboxane B_2 (TXB_2) which is excreted in the urine as 11-dehydro thromboxane B_2, the urinary TXB_2 concentration is used to more reliably reflect *in vivo* thromboxane production and the pharmacokinetic efficacy of aspirin therapy and aspirin resistance (31,42). Indeed, in a matched-control substudy of the Canadian Heart Outcomes Prevention Evaluation Study (HOPE) using the surrogate marker, urinary 11-dehdro TXB_2,was compared in 488 patients treated with aspirin who had myocardial infarction, stroke, or cardiovascular death during 5 years of follow-up and to 488 patients treated aspirin who did not have an event. Urinary concentrations of 11-dehydro TXB_2 predicted the risk of myocardial infarction or cardiovascular death, and potentially identify patients who are aspirin resistant that may benefit from additional antiplatelet therapies (19).

Proposed Mechanisms of Aspirin Resistance

The potential mechanism of aspirin resistance is multifactorial, and has been proposed to be in part due to drug bioavailability, redundant platelet activation pathways and receptors, increased COX-2, activity with increased prostaglandin-like compound (isoprostane) activity (43,44), drug-drug interactions, platelet alloantigen 2 (Pl^{A2}) polymorphism of platelet GP IIIa, and a COX-1 polymorhism (45,46).

Drug Bioavailability

From a bioavailability perspective, compliance with the prescribed therapeutic regimen and absorption contributes to individual variability in aspirin responsiveness, but should be distinguished from purported mechanisms of pharmacodynamic resistance. Evidence showed that approximately 3% of aspirin resistant patients are found to be non-compliant with aspirin therapy (30,47), and in-hospital treatment of these non-compliant patients with 325 mg aspirin showed that they were indeed sensitive to aspirin (30). The pharmacokinetic profile of aspirin shows that the absorption of soluble aspirin is largely unaffected by food and the bioavailability was increased in the fed state (48).

Redundant Platelet Activation Pathways

In vivo pathways involving non-TXA_2 dependent agonists such as thrombin, ADP, epinephrine, and collagen bypass the aspirin mediated inhibitory effect leading to platelet activation and thrombosis (49). One such pathway is a catecholamine-induced platelet aggregation that might not be adequately inhibited by aspirin, as such, the antiplatelet effects of aspirin is overcome during exercise or mental stress (50). Also, there is evidence to support that aspirin nonresponders may produce platelets that are oversensitive to collagen (51).

Increased Cyclooxygenase-2 Activity

Alternative pathways for TXA_2 synthesis and the identification of isoprostanes produced from altered expression of COX-2 has been proposed as a mechanism of clinical aspirin resistance. Cyclooxygenase-2 is expressed only in response to inflammatory stimuli, and is present in only a small fraction of platelets. Cyclooxygenase-2 is upregulated 10–20 fold by inflammatory stimuli in platelets, monocytes, macrophages, and vascular epithelium with resultant aspirin-insensitive TXA_2 biosynthesis (52). In addition, COX-2 is present in newly formed platelets and may account for detectable levels of TXA_2 synthesis that promotes platelet aggregation during periods of increased platelet turnover (52).

Drug-Drug Interactions

Potential drug-drug interactions have been described with aspirin. Aspirin and other nonsteroidal anti-inflammatory agents (NSAIDs), like ibuprofen, inhibit arachidonic acid metabolism by inactivating the COX–1 enzyme system (53). Normally, arachidonic acid gains access to its catalytic site in

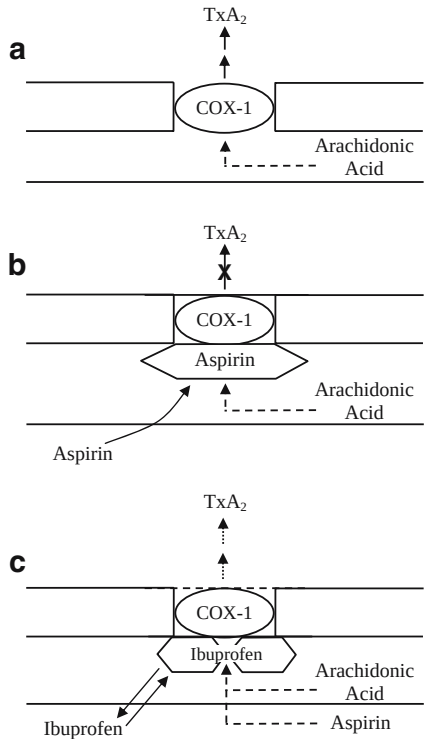

Fig. 3. Effect of aspirin alone and ibuprofen plus aspirin in platelet COX-1. (a) Arachidonic acid binds to COX-1 and converts to TXA_2. (b) Aspirin blocks the access of arachidonic acid and inhibits the production of TXA_2. (c) Prior reversible occupancy of ibuprofen inhibits aspirin from permanently binding to COX-1. COX-1 Cyclooxygenase-1. TXA_2=Thromboxane A_2.

the platelet through a hydrophobic channel within COX-1 (Fig. 3a) and is ultimately converted to TXA_2, which promotes vasoconstriction and platelet aggregation (Fig. 1). However, aspirin differs by irreversibly acetylating a serine residue at position 530 within the channel, blocking access of arachidonic acid to its catalytic site (Fig. 3b), while other NSAIDs are reversible inhibitors of the catalytic site (Fig. 3c), inhibiting platelet aggregation only during part of the dosing interval. Aspirin and ibuprofen (treatment for arthritic pain) co-administration has been associated with increased risk of cardiovascular mortality in one retrospective study (54–56). The mechanism proposed is that ibuprofen inhibits the access of aspirin to the COX-1 acetylation site in platelets resulting in antagonizing irreversible platelet inhibition (Fig. 3c). Aspirin given 2 h before a daily dose of ibuprofen successfully inhibits platelet aggregation (53), however, ibuprofen administered three times daily competitively prevents aspirin from accessing its target serine and inhibiting platelet aggregation.

Clinical controversy emerged in the administration of COX-2 inhibitors (Coxibs) for chronic pain to minimize significant gastric toxicity (a complication frequently associated with COX-1 inhibitors) in patients at risk for cardiovascular disease (57,58). Cyclooxygenase-2 inhibitors selectively decrease prostacyclin production without effectively inhibiting TXA_2 production (Fig. 4), and as such, are not acceptable substitutes for aspirin in patients requiring antiplatelet therapy for cardioprotection. Rofecoxib, was reported to increase the risk of thrombotic cardiovascular events, and was withdrawn from the market after the 25 mg dose was found to double the risk of MI or stroke compared with placebo (3.5 *versus* 1.9%) after 18 months of therapy in a randomized trial that tested the utility of rofecoxib in preventing recurrent colorectal polyps in patients with no significant history for cardiovascular disease. The risk could be greater in patients at risk for or with known atherosclerotic vascular disease. Although the other COX-2 drugs on the market, celecoxib and valdecoxib, have not yet been shown to increase cardiovascular events, neither one has been tested long-term in patients with cardiovascular disease.

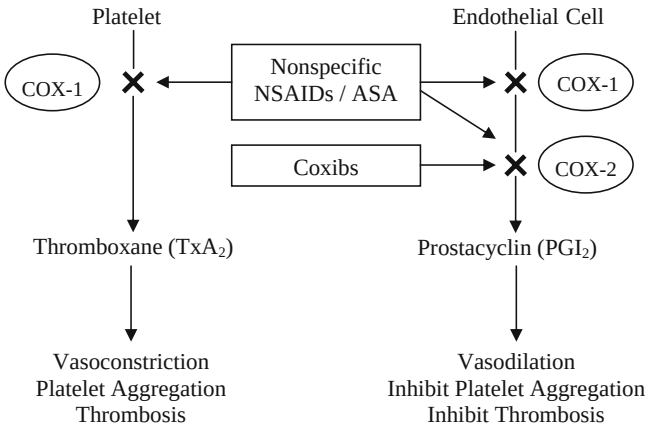

Fig. 4. COX-1 inhibitors decreases TXA_2 production. COX-2 inhibitors inhibit only PGI_2 production and leaves TXA_2 production unchecked. *ASA* Aspirin, *NSAIDs* Nonsteroidal anti-inflammatory drugs, *Coxibs* Specific COX-2 inhibitors.

The Pl^{A2} Polymorphism of Glycoprotein IIIa

Platelets rely on numerous surface GP receptors to perform their complex functions, and many of these receptors are polymorphic. Aspirin resistance may, in part, be explained by genetic differences in the COX-1 gene or the GP IIb/IIIa receptor complex. Polymorphisms of the IIIa subunit have been identified, and the specific alleles $Pl^{A1/A2}$ and $Pl,^{A2/A2}$ are associated with increased thrombin formation and a lower threshold for platelet activation. The Pl^{A2} polymorphism is a single nucleotide, cytosine (Pl^{A1})-to-thymine (Pl^{A2}) polymorphism occurring at the position 1566 in exon 2 of the coding region for GP IIIa located in chromosome 17, resulting in a leucine–proline exchange (59). Allelic Pl^{A2} polymorphism has been attributed to increased surface expression of GP IIb/IIIa receptors and an increased affinity for fibrinogen (60,61). In addition, the presence of the Pl^{A2} allele has been associated with a greater risk of coronary events (62). The antithrombotic effects of aspirin have been proposed to be attenuated among the carriers of the Pl^{A2} polymorphism based on measures of platelet function, particularly in patients homozygous for $Pl^{A2/A2}$ (60,63). Further study is needed to fully characterize the clinical implications of Pl^{A2} polymorphism on the antithrombotic cardiovascular protection of aspirin.

It has been suggested that mutations and/or polymorphisms of the COX-1 gene may, in part, contribute to of aspirin resistance (45,46). In theory, COX-1 polymorphism may result in a reduction of COX activity and decreased production of TXA_2 and plausibly eliminate the need for aspirin. However, if the COX-1 gene is dysfunctional, alternative TXA_2 may be produced by mechanisms of COX-2 induction in monocytes or endothelial cells, rendering patients with this polymorphism aspirin resistant (52,64,65).

CLOPIDOGREL

Mechanism of Action

Clopidogrel, a thienopyridine, is a prodrug that requires metabolic activation by hepatic cytochrome P450 (CYP) 3A4 in order to exert its antiplatelet effect (66). The active metabolite has been identified and irreversibly binds via a disulfide bond to the platelet seven transmembrane adenosine diphosphate (ADP) G-protein coupled $P2Y_{12}$ receptor (67). $P2Y_{12}$ has been reported as a central mediator of the hemostatic response. Inhibition of $P2Y_{12}$ attenuates both the amplification of platelet aggregation and the stability of the thrombus (68).

Platelet Adenosine Diphosphate Receptors

Platelet activation by ADP plays a key role in the development of arterial thrombosis (69,70). Among the variety of soluble agonists that induce platelet aggregation, ADP is a major *in vivo* stimulus. Therefore, drugs like clopidogrel that inhibit ADP-induced platelet function are of considerable interest in the treatment of arterial thrombosis. The dense granules contain high concentrations of ADP that is released when platelets are stimulated by other aggregating agents such as thrombin or collagen. ADP and adenosine triphosphate (ATP) are also released from red blood cells, activated

platelets, and damaged endothelial cells, inducing platelet adhesion and aggregation through activation of the integrin and ATP, GP IIb/IIIa and subsequent binding of fibrinogen (71,72). There are two main purinergic receptor types in the membrane: the guanosine triphosphate (GTP) coupled protein receptors known as G-protein binding sites and the ligand gated ion channel (73–75). The latter receptor is designated $P2X_1$ and the former is designated as P2Y. There are 2 known P2Y receptors; $P2Y_{12}$ and $P2Y_1$ (Fig. 2). The $P2X_1$ receptor mediates extracellular calcium influx and utilizes ATP and not ADP as the agonist (74). As $P2Y_1$ mediates mobilization of intracellular calcium stores, $P2Y_{12}$ mediates adenylyl cyclase inhibition (previously called $P2Y_{AC}$, $P2T_{AC}$, $P2Y_{ADP}$, P2Ycyc) (68).

A detailed description of the intricate intracellular signaling pathways following ADP binding to the P2Y receptors has been previously reviewed (68,76,77). In summary, $P2Y_1$ is coupled to the Gq protein and its intracellular signaling pathways involve the activation of phospholipase C (PLCβ) resulting in diacylglycerol (DAG) and inositol triphosphate (IP3) production. Diacylglycerol activates protein kinase C leading to phosphorylation of myosin light chain kinase. Inositol triphosphate mobilizes intracellular calcium from the dense granules and activates Rap1b, a GTPase, and an important intracellular signaling protein (78). Gαq is also coupled to thrombin PAR1 and PAR4 receptors (76). Recently it has been also discovered that the P2Y1 receptor is linked to another G-protein, G12 that activates the "Rho" protein (76). Thus, the activation of the $P2Y_1$ receptor initiates a series of signaling events that produce intracellular Ca^{2+} mobilization, platelet shape change and aggregation.

The $P2Y_{12}$ receptor is linked to the $G\alpha_{i2}$ protein mediating the inhibition of adenylyl cyclase (AC) and the activation of phosphoinositide-3-kinase (PI3K) (68,76). Phosphoinositide-3-kinase activation subsequently leads to the activation of a serine-threonine protein kinase B (PKB/Akt). In addition, investigators have reported Rap 1b activation downstream from $P2Y_{12}$ (78,79). Usually, intact endothelium secretes prostacyclin that activates adenylyl cyclase and increases platelet cAMP levels. However, activation of the $P2Y_{12}$ receptor is coupled to the inhibition of adenylyl cyclase activity and decreases cAMP levels. A reduction in cAMP affects the activity of cAMP-dependant protein kinases that in turn, affect levels of phosphorylated vasodilator stimulated phoshoprotein (VASP) and eliminate its protective effect on the activation of the GP IIb/IIIa receptor (Fig. 2) (80). Vasodilator stimulated phosphoprotein phosphorylation has been used as a potential marker of $P2Y_{12}$ activity and clopidogrel resistance (81). Thus, activation of the $P2Y_{12}$ receptor results in a complex series of intracellular signaling events that participate in the final activation of the GP IIb/IIIa receptor, the amplification of platelet aggregation, the facilitation of granule release, and the stabilization of the platelet aggregate.

Clinical Application of Clopidogrel

The combination of clopidogrel and aspirin therapy has been shown to decrease recurrent ischemic events after acute coronary syndromes (82) and subacute stent thrombosis after elective percutaneous coronary intervention. (83) In combination with aspirin, clopidogrel is currently the drug of choice to prevent stent thrombosis (84). Stent thrombosis remains an important and potentially lethal clinical problem (incidence—0.9%, mortality—8.9%) (85). The standard clopidogrel regimen administered to prevent stent thrombosis is a 300 mg loading dose followed by a 75 mg daily maintenance dose (86). This dosing regimen was based primarily on studies in normal volunteers and stable patients with coronary artery disease not undergoing stenting. Recent studies have employed a 600 mg clopidogrel loading dose based on superior pharmacodynamic effects as compared to 300 mg (21).

Clopidogrel Metabolism

Evidence Supporting the Importance of Cytochrome P450

Cytochrome P450 (CYP) is a superfamily of mixed function oxidases that catalyze at least one step in the metabolism of most of drugs. More than 60 distinct forms of these enzymes, each encoded by separate genes have been characterized from the human liver (87). The CYP3A subfamily are the most abundant and important drug-metabolizing enzymes. Clopidogrel is a prodrug that requires *in vivo* conversion to an active metabolite to exert its antiplatelet effect. The active metabolite of clopidogrel is an unstable thiol compound, that inhibits platelet aggregation by the formation of a disulfide bridge between the thiol group of the active metabolite and a cysteine residue of the P_2Y_{12} receptor on platelets (67). In rats, clopidogrel requires metabolic activation by hepatic CYP1A2 (88) while an analogue of clopidogrel, CS-747, (Sankyo Co., Ltd. Tokyo Japan) is reported to be activated by human CYP3A4 (89,90).

During the course of evaluating the most effective loading dose of clopidogrel (300 *versus* 450 mg) in inhibiting platelet aggregation following coronary artery angioplasty, it was observed that the effectiveness of clopidogrel was diminished in patients that were taking atorvastatin (91). Of the ten patients who received 450 mg of clopidogrel, four were taking atorvastatin. The average platelet aggregation of patients receiving clopidogrel alone was 42 ± 8% compared to those who received clopidogrel and atorvastatin (68 ± 8%, $p = .03$). Since atorvastatin is metabolized by CYP3A4 (92), and a clopidogrel analogue, CS-747, is activated by human CYP3A4, it was hypothesized that CYP3A4 was responsible for the activation of clopidogrel to its active metabolite. To test this hypothesis, the ability of clopidogrel to affect platelet aggregation was compared prospectively in an additional series of post angioplasty patients taking either atorvastatin, a CYP3A4 inhibitor, or pravastatin, an HMG CoA reductase inhibitor, which is not metabolized by CYP3A4. This study of 44 patients undergoing coronary artery stent implantation treated with clopidogrel demonstrated that the degree of platelet aggregation inhibition achieved 24 h after clopidogrel was significantly attenuated by atorvastatin as compared to the control (77 ± 15 *versus* 34 ± 23%; $p < 0.0001$). In contrast, clopidogrel inhibited platelet aggregation in the control group and patients taking pravastatin (34 ± 23 and 46 ± 18%, respectively; p = ns, Fig. 5) (66). Subsequently, *in vitro* metabolism of clopidogrel was demonstrated primarily by human CYP3A4 (93).

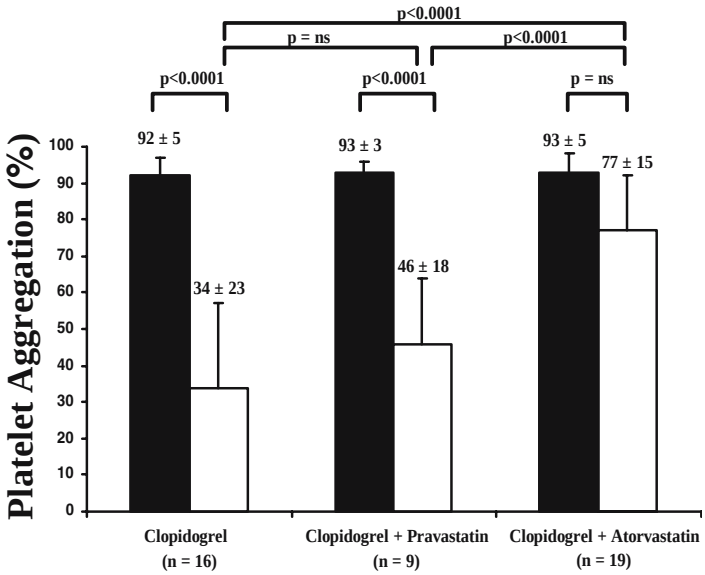

Fig. 5. The effect of atorvastatin and pravastatin on the antiplatelet activity of clopidogrel measured by *ex vivo* platelet aggregation. Atorvastatin attenuated the antiaggregatory effect of clopidogrel. (With permission from Lau, *et al.* (66)).

Another prospective randomized trial was undertaken to demonstrate more conclusively that CYP3A4 is the major form of CYP3A involved in the conversion of clopidogrel to its active metabolite *in vivo*. Troleandomycin (TAO) is a more potent inhibitor of CYP3A4 than erythromycin, and would be expected to diminish the effect of clopidogrel. The ERMBT (Erythromycin Breath Test) allows a mechanistic evaluation of drug-CYP3A interactions *in vivo*. An intravenous dose of [^{14}C-*N*-methyl]-erythromycin (3 μCi, 0.04 mg of erythromycin) is administered and a single breath sample is then collected after 20 min. Quantitation of exhaled $^{14}CO_2$ provides a selective measure of the "instantaneous" hepatic CYP3A4 activity. Because CYP3A4 catalyzes the *N*-demethylation of erythromycin, the ERMBT provides a mean of detecting changes in CYP3A4 activity in response to drug treatment in humans. It is a valid, reproducible, and reliable *in vivo* measurement, frequently employed to examine drug interactions involving CYP3A4. Potent CYP3A4 inhibitors, such as TAO, and inducers, such as rifampin, will, respectively, decrease and increase the percentage of the administered dose that is excreted as $^{14}CO_2$. Baseline platelet aggregation and ERMBT were measured in eight human healthy subjects before any drug administration (0 h) and 2 h following clopidogrel 450 mg oral administration. In addition to the strict exclusion criteria previously described, subjects that did not respond to the initial clopidogrel treatment were dropped from the study. After a 14-day washout period, a single oral dose of troleandomycin (500 mg) was administered. Then, clopidogrel (450 mg) was administered orally 1 h after TAO. The effect of clopidogrel on platelet aggregation and ERMBT were measured at 0 and 3 h. The results demonstrate a near complete inhibition of CYP3A4 activity after TOA was added to clopidogrel, and an attenuated platelet aggregation inhibition after TAO was added to clopidogrel (Fig. 6a and b).

To more definitively determine whether the *in vivo* metabolic activation of clopidogrel requires hepatic CYP3A4A, another prospective randomized trial was undertaken. Erythromycin is an *in vivo* inhibitor of CYP3A4 and would be expected to diminish the antiplatelet effect of clopidogrel. Rifampin is an inducer of CYP3A4 and would be expected to enhance the platelet inhibitory activity of clopidogrel. Therefore, subjects taking clopidogrel were administered erythromycin or rifampin and the ability of these antibiotics to affect the activity of clopidogrel was examined. Subjects were required to refrain from taking any drugs that might affect CYP 3A4 activity. Human volunteers were randomized into two groups. Group 1 (n = 9) received a maintenance dose of 75 mg/day of clopidogrel for 6 days, followed by a washout period of 14 days, followed by 4 days of erythromycin 250 mg QID and finally by both clopidogrel and erythromycin for 6 days. Group 2 (n = 10) was treated the same except they received rifampin 500 mg BID. Platelet aggregation was determined at 0, 6, 20, 24, and 30 days. The results of this study supported the concept that CYP3A4 mediates the metabolic activation of clopidogrel (Fig. 7a and b) (66) and that the rates of metabolic activation of clopidogrel would be limited by the amounts of CYP3A4 expressed in the tissues of an individual. We hypothesized that in subjects not receiving known inducers or inhibitors of CYP3A4, differences in CYP3A4 activity largely account for the observed differences in the response to clopidogrel (94).

Cytochrome P450 Gene Induction by Distinct Nuclear Receptors

Several recent advances have revealed the mechanisms through which xenochemicals induce the expression of hepatic CYP enzymes. Two specific xenobiotic receptor proteins have been identified as playing key roles in CYP gene induction (95). These two distinct "orphan receptors"

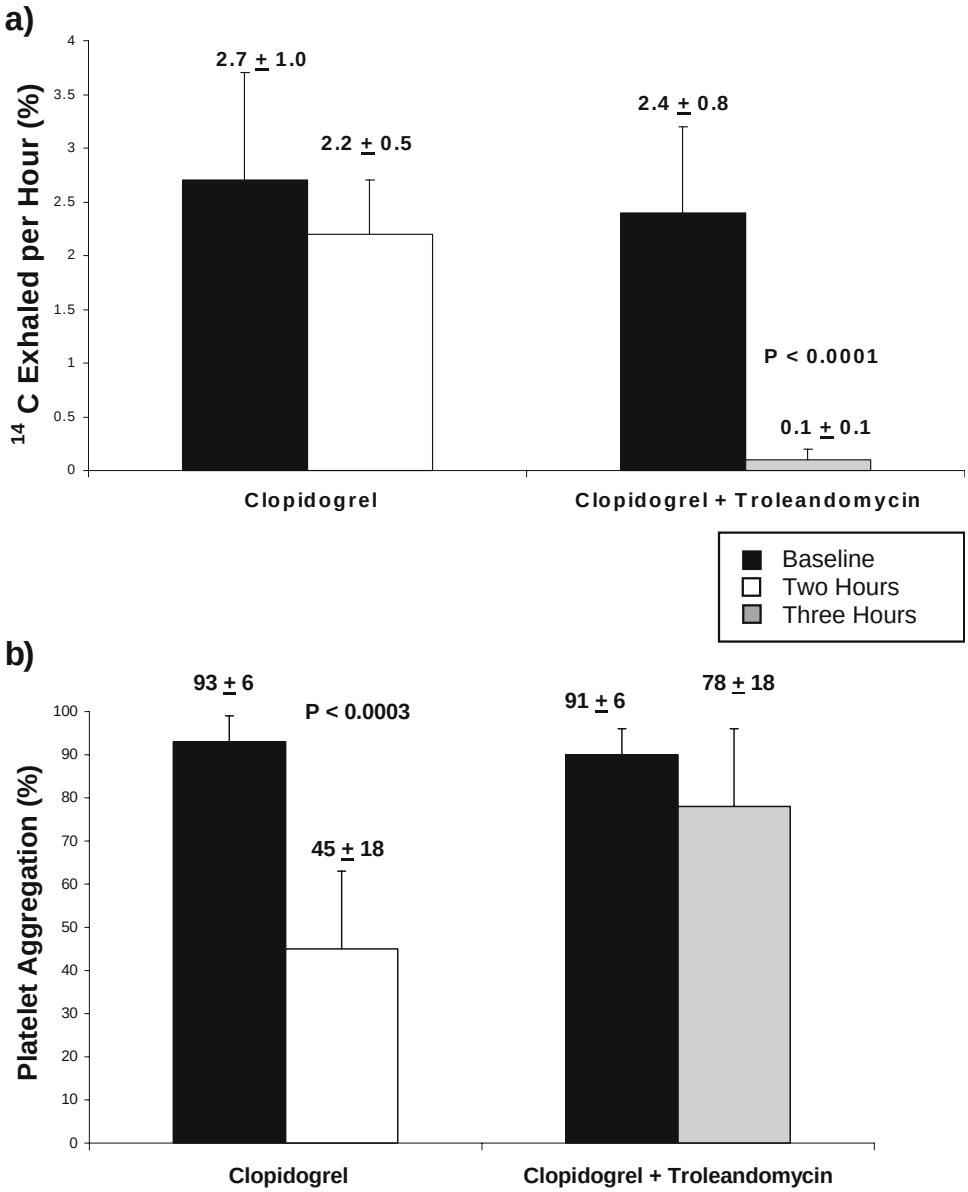

Fig. 6. The effect of troleandomycin on CYP3A4 activity as measured by the ERMBT. (a) Clopidogrel was administered during the time CYP3A4 was known to be inhibited by troleandomycin. (b) The effect of troleandomycin on the antiplatelet activity of clopidogrel in the same patients as Fig. 6a. *CYP3A4* Cytochrome P450 3A4, *ERMBT* erythromycin breath test. (With permission from Lau, *et al.* (94)).

(receptors whose endogenous, physiological ligands are unknown) of the nuclear receptor/steroid receptor superfamily are: 1) the constitutive androstane receptor (CAR), which mediate the induction of CYP2B and CYP1A genes by phenobarbital; and 2) the pregnane X receptor (PXR), which mediates the induction of the CYP3A gene in response to rifampin (96). Phenobarbital caused translocation of CAR to the nucleus where it heterodimerizes with the 9-*cis*-retinoic acid receptor (97). This complex enhances transcription by binding to the phenobarbital response element in the gene promoter regions. Similarly, PXR forms the PXR/PXR_{α} complex that binds to a different response element within CYP3A4 gene promoters (98), and clopidogrel resistance was overcome by rifampin mediated induction of CYP3A4 (94,99). The inducibility of CYP3A4 by rifampin is responsible for the enhanced platelet inhibitory effect of clopidogrel (99). This concept is supported by the findings that mice lacking PXR have abrogated induction of CYP3A in response to the PXR ligands dexamethasone and pregnenolone (98). These mice develop and reproduce normally. However, the CYP3A gene is no longer induced in response to specific rodent CYP inducer. These findings suggest that individuals with PXR nucleotide sequence variations may have no overt or adverse phenotype until challenged with PXR ligands. Furthermore, PXR polymorphisms may impact downstream CYP-mediated drug metabolism.

Clopidogrel Resistance

Currently, clopidogrel is administered to the vast majority of patients without any assessment of platelet inhibition. Moreover, there is no uniform consensus about the preferred methodology to measure clopidogrel-induced platelet inhibition. Variable platelet aggregation inhibition after clopidogrel occurs among individuals: nonresponders to clopidogrel have been identified (22,86,99,100). The concept of clopidogrel resistance and response variability has long been recognized, during investigations of platelet reactivity following elective coronary stent implantation (84) and have recently been published (86). A concern for the development of stent thrombosis exists in those patients who have high platelet reactivity despite ongoing therapy with antiplatelet agents (86). Indeed, increased rates of subacute coronary stent thrombosis (101) and recurrent ischemic events after primary PCI (102) have been noted in clopidogrel nonresponders. However, further work is needed in defining clopidogrel resistance and its correlation with adverse clinical events before it can be considered clinically relevant.

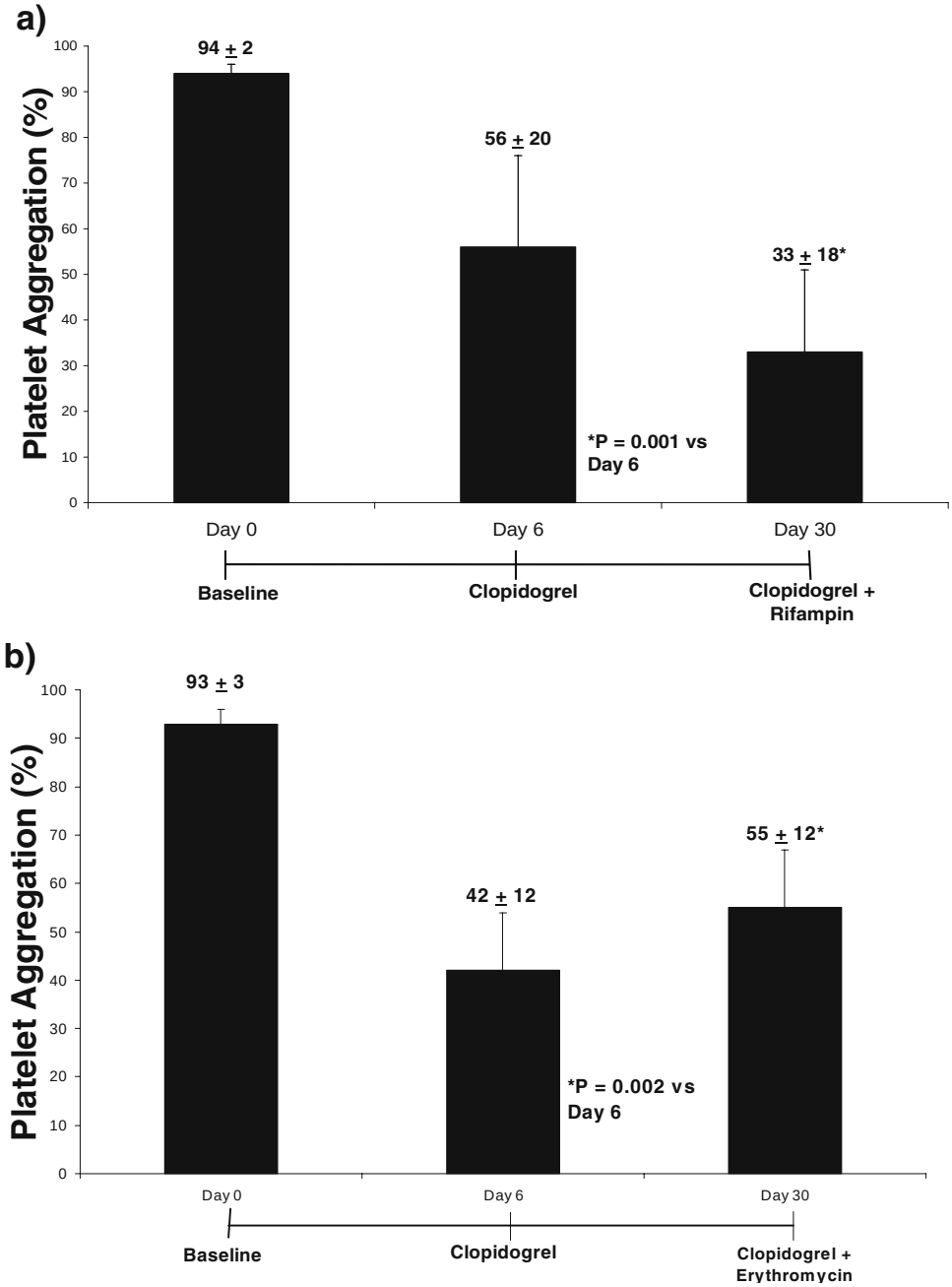

Fig. 7. (a) The antiaggregatory effect of clopidogrel was enhanced when coadministered with the CYP3A4 inducer, rifampin. (b) Clopidogrel was less active when coadministered with the CYP3A4 inhibitor, erythromycin. (With permission from Lau, *et al.* (94)).

Response Variability and Resistance: Methodology, Definition and Incidence

The methodology most commonly reported in the literature to measure clopidogrel resistance is conventional light transmittance (optical) aggregometry. In this method, platelet-rich plasma is prepared from blood usually anticoagulated with citrate. The sample is then stimulated with various concentrations of ADP ranging from 5 to 20 μM. In addition, whole blood flow cytometry has been used to measure the expression of activation-dependent markers, such as p-selectin and the active conformation of GP IIb/IIIa after stimulation with ADP. In patients with ADP receptor blockade, the rise in p-selectin following stimulation with maximal doses of ADP will be attenuated. Early investigations in patients undergoing elective coronary stenting demonstrating modest inhibition of 5 μM ADP-induced platelet aggregation following chronic clopidogrel and aspirin therapy (37 ± 14% inhibition of baseline aggregation) (103) have led to subsequent evidence demonstrating the effects of pre-stent clopidogrel loading response variability and frank drug resistance (86). In addition to the term, "resistant", other terms have been used to describe a patient with ineffective or undesirable platelet inhibition by clopidogrel, including "hyporesponsive", "non-responsive" and "semi-responsive" (83,102,104).

One definition for resistance is an absolute change in aggregation (ΔA) less than or equal to 10%. Therefore, ΔA = Baseline aggregation (%) minus post-drug aggregation (%). In other definitions, ΔA is normalized in each patient with their baseline aggregation where non-responders were those with a relative change in aggregation less than 10% (83). Most recent study patients were administered a regimen of 300 mg clopidogrel loading dose in the catheterization laboratory immediately after successful stenting followed by a 75 mg daily maintenance dose (86). Then serial assessed ADP-induced platelet aggregation using light transmittance aggregometry was performed. Surface receptor expression of p-selectin and the active conformation of GP IIb/IIIa were measured by flow cytometry using labeled monoclonal antibodies. Platelets were studied at baseline and serially for the following 30 days. In this study we observed high rates of non-responsiveness to clopidogrel. At both 24 h and 5 days post-stenting the incidence of clopidogrel resistance was ~30%. Similar high resistance rates were also measured by ADP-stimulated p-selectin expression. Since this initial report others have followed supporting the conclusion that clopidogrel resistance is indeed a real phenomenon (77,81,83,99,102,104). Most of these studies have used platelet aggregation in response to various concentrations of ADP as the method to measure clopidogrel responsiveness.

There is controversy regarding the optimal threshold for the definition of clopidogrel resistance, and the argument that the current measurement of platelet aggregation or receptor expression following stimulation by ADP is only a surrogate reflection of clopidogrel-induced inactivation of the $P2Y_{12}$ receptor since ADP activates both $P2Y_1$ and $P2Y_{12}$. The acceptance of this novel concept of clopidogrel resistance and potential clinical implication was acknowledged in the 2003 European Society of Cardiology Congress by dedicating an entire session on this important subject.

Clinical Characteristics of Clopidogrel Response Variability

The Optimal Representation of Response Variability

Evidence-based studies support that the representation of clopidogrel response variability in a given study population to be by a histogram that records incidence on the *y*-axis and ΔA (deciles) on the *x*-axis (105). Using this method one can easily project a "fingerprint" of the response variability of the entire population and immediately observe the incidence of drug resistance. Studies have well demonstrated that the inhibitory response to clopidogrel follows a normal distribution (86,106).

Durability of Platelet Inhibition by Clopidogrel in Stented Patients

Gurbel and Bliden (107) have demonstrated that patients who were early responders at 24 h post-loading in general, remained responsive over 30 days. Approximately one-half of early non-responders crossed over to become responders by 30 days. The mechanism(s) underlying these findings remain unclear but may involve induction of hepatic CYP3A4 via the nuclear pregnane X receptor (PXR) response element by clopidogrel (99). This phenomenon may, in part,explain the early enhancement of platelet reactivity by stenting that subsequently wanes as time accrues following the procedure (84). The stability of platelet inhibition beyond 30 days is entirely unknown and may be critical especially in the use of drug-eluting stents *versus* bare metal stents where prolonged platelet inhibition is desired due to impaired vessel wall healing.

Relation of Pre-treatment Reactivity to Post-treatment Reactivity

Another important finding that significantly contributes to an optimal clopidogrel treatment strategy is that pretreatment platelet reactivity strongly affects the overall inhibitory response from clopidogrel. A study of 96 consecutive patients treated with a 300 mg loading dose have demonstrated that those patients in the highest pretreatment tertile for aggregation and platelet surface P-selectin expression also remained the most reactive after treatment. This relationship is particularly evident in the early (within 24 h) post procedural period, as such, patients with high pretreatment reactivity may be at the greatest risk of ischemic events particularly if they are also clopidogrel resistant (86).

Mechanisms of Clopidogrel Resistance

Consensus agreement has accepted one major mechanism of clopidogrel resistance may be the failure of clopidogrel, a prodrug, to be metabolically activated by hepatic cytochrome P450 (CYP) 3A4 (100). This mechanism is in part due to variability in the phenotypic expression of the CYP3A4 isoenzyme secondary to nucleotide polymorphisms at the site of nuclear pregnane X receptor (PXR) regulating the induction of the CYP3A4 isoenzyme (99). Other potential mechanisms include underdosing, drug–drug interaction, impaired gastrointestinal absorption of the prodrug, $P2Y_{12}$ polymorphisms, and downstream intracellular signaling variability (77).

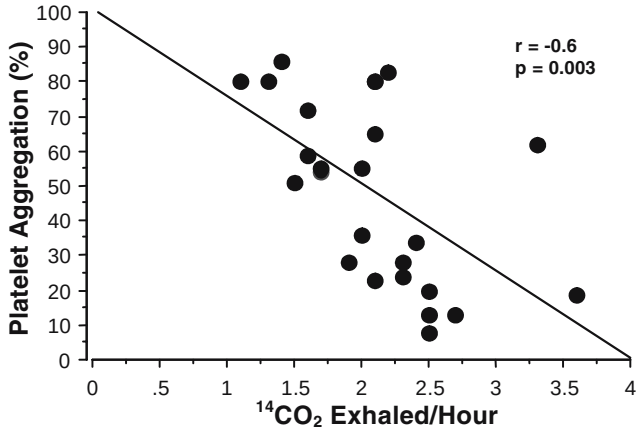

Fig. 8. Significant negative correlation was demonstrated in the comparison of platelet aggregation (%) 4 h after clopidogrel 450 mg versus erythromycin breath test 2 h after clopidogrel 450 mg. (With permission from Lau, *et al.* (99)).

Variability in the Phenotypic Expression of Hepatic CYP3A4

A recent study supports the important role of CYP3A4 activity in relation to the antiplatelet effect induced by clopidogrel (99). The results demonstrated that variability response of clopidogrel exists in healthy volunteers and in patients undergoing coronary stenting and showed that post-clopidogrel platelet aggregation strongly correlated with the *in vivo* activity of the CYP3A4 pathway ($p = 0.003$), measured by the erythromycin breath test in volunteer subjects as an instantaneous marker of CYP3A4 activity in comparison to point-of-care *ex vivo* platelet aggregometry after clopidogrel 450 mg (Fig. 8).

One mechanism that can explain the variability in the phenotypic expression of CYP3A4 is PXR nucleotide polymorphisms. In collaboration with Dr. Erin Schuetz (St. Jude Children's Research Center, Memphis, TN), the genetic alterations involved in PXR polymorphisms leading to impaired clopidogrel response was examined. Our data in 30 healthy subjects demonstrates that individuals heterozygous for a 6 base pair (bp) deletion located in the intron 1 of PXR (108) (Fig. 9) correlated with clopidogrel non-response, while those homozygous (with no 6bp deletion) correlated with responders (Abstract submitted to American Heart Association Scientific Sessions 2006 Chicago, Illinois). Current investigation is underway to determine whether individuals heterozygous for the variant PXR 6bp deletion, who are clopidogrel non- and low-responders, can convert to responders after the administration of a potent CYP3A4 inducer, St. John's wort (109).

Underdosing of Clopidogrel

Controversy exists regarding the optimal clopidogrel loading and maintenance dose regimens. Studies have demonstrated that a 600 mg load dose of clopidogrel produces superior inhibition as compared to 300 mg (21,110). A recent large clinical trial, also conducted in elective patients, compared a 600 mg clopidogrel loading dose to the same loading dose combined with abciximab, an intravenous GP IIb/IIIa inhibitor (111). The finding of equivalent primary endpoint rates in both arms lends further support to use of the 600 mg clopidogrel loading dose in elective stenting. Furthermore, all of the above data are evidence that a large percentage of clopidogrel resistance may be due to insufficient production of the active clopidogrel metabolite. A portion of these patients may experience improved inhibition by receiving larger doses to provide higher concentration of substrate for the metabolic activation by CYP3A4 isoenzyme. Furthermore, since clopidogrel is an inducer of CYP3A4 (112), it would be speculated that higher loading doses lead to enhanced phenotypic expression of CYP3A4 to activate clopidogrel.

Drug-Drug Inhibitory Interaction

Controversy abounds in the literature with respect to clopidogrel-atorvastatin interactions (66). Clopidogrel and

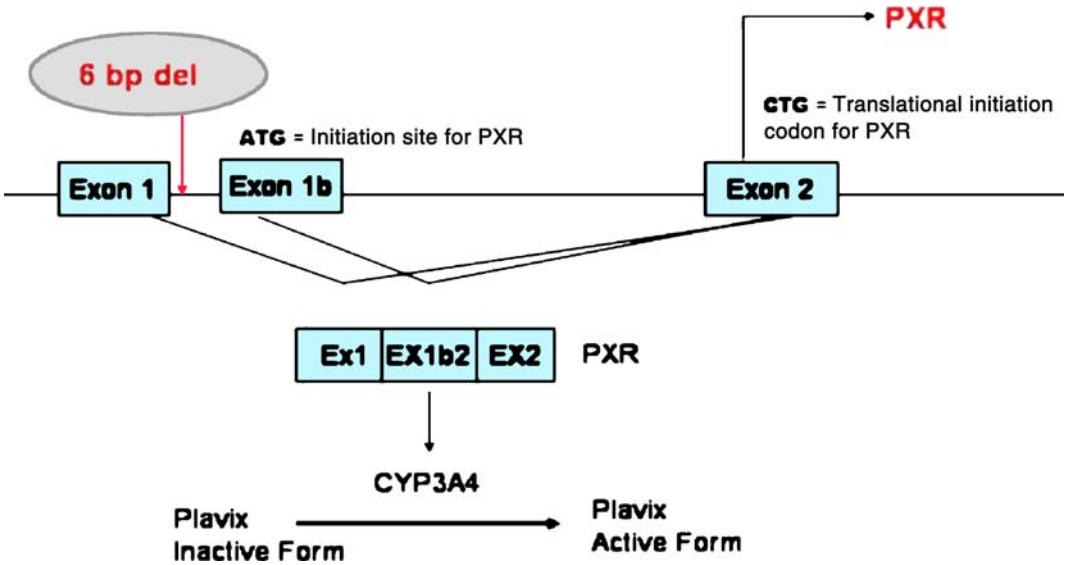

Fig. 9. A 6 base pair (bp) deletion (del) in the pregnane X receptor (PXR) gene, located in intron 1 of PXR leading to variability in genetic expression of PXR, with resultant CYP3A4-mediated variability in clopidogrel (Plavix) response. ATG-Initation codon for PXR, CTG-Translational initation codon for PXR.

atorvastatin are competitive substrates of the CYP3A4 isoenzyme (93). Because clopidogrel has low bioavailability and a lower affinity for the CYP3A4 substrate binding site as compare to atorvastatin, and because atorvastatin has a long half-life, atorvastatin competitively inhibits the ability of clopidogrel to be metabolized to its active form in a dose-dependent manner. The retrospective and prospective studies (113,114) reporting no relation of statin use to clopidogrel effect may be explained by: 1) the low doses of atorvastatin used in the studies; and 2) under power of the sample size of the non-CYP3A4 metabolized statins when compared to the CYP3A4 metabolized statins to demonstrate statistical significance. There is evidence that this drug–drug interaction may indeed be associated with increased adverse cardiac events. Although not statistically significant because of sample size, Weinberger *et al.* reported a 26% increased odds ratio for mortality in patients treated with for an acute coronary syndrome prescribed atorvastatin *versus* other statin therapies (115). A recent preliminary report by Brophy *et al.* described a significant two-fold increased relative risk for the combined end point of death, hospitalization for myocardial infarction or unstable angina, repeat revascularization, or stroke in patients after percutaneous coronary intervention treated with co-administration of atorvastatin and clopidogrel *versus* clopidogrel without atorvastatin (116). In perspective, CYP3A4 metabolizes as much as one half of the drugs that physicians prescribe. Thus, only an adequately designed, prospective, randomized clinical trial can definitively demonstrate whether co-administration with atorvastatin inhibits the therapeutic benefit expected with clopidogrel therapy.

Drug-Drug Enhancing Interaction

St. John's wort is a popular herbal product that originates from the flowers of the perennial plant Hypericum perforatum, native to Europe and Asia. The herb contains naphthodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), phenylpropanes, flavonol derivatives, biflavones, proanthocyanidins, xanthones, and amino acids (117). St. John's wort constituent hyperforin is a potent ligand for PXR (118). St. John's wort induces CYP3A4 activity, and as such, could increase the efficacy of drugs that are dependent on CYP3A4 for their metabolic activation (112).

At a cellular level, treatment of primary human hepatocytes with hyperforin results in significant induction of CYP3A4 expression (119). A recent study defined the crystalline structure of hyperforin in complex with the ligand binding domain of human PXR. Hyperforin was demonstrated to induce conformational changes in PXR's ligand binding pocket. Dose response analysis showed that hyperforin is a more potent PXR activator than the known CYP3A4 inducer, rifampin (119). In addition, the concentration of hyperforin required to activate PXR is well below those achieved in humans after St. John's wort supplementation (120). Thus, it is very likely that St. John's wort activates PXR in humans. Indeed, Roby *et al.* showed in 13 healthy volunteers that St. John's wort supplements (300 mg 3×/day, 14 days) effectively induce CYP3A4 activity (121). It is therefore likely that this induction of CYP3A4 was mediated by a PXR-dependent mechanism, though this causation has never been tested experimentally.

To determine whether the antiplatelet effect of clopidogrel can be enhanced by administration of St. John's wort, we designed a clinical study to determine whether St. John's wort, a potent inducer of CYP3A4, can effectively enhance clopidogrel efficacy in non- and low-responder volunteers. We recruited ten healthy known clopidogrel non-response subjects (relative decrease in platelet aggregation<30% at 0, 2, 4, and 6 h after clopidogrel 300 mg). After a 14 day washout period, subjects were treated with commercially available St. John's wort product Kira (Lichterwer Pharma, Berlin, Germany) 300 mg, 3 times/day for 14 days. Platelet aggregation was again measured at 0, 2, 4, and 6 h after clopidogrel treatment. With each subject acting as his/her own control, the platelet aggregation profile of Plavix was compared before and after St. John's wort supplementation. Results show that St. Johns

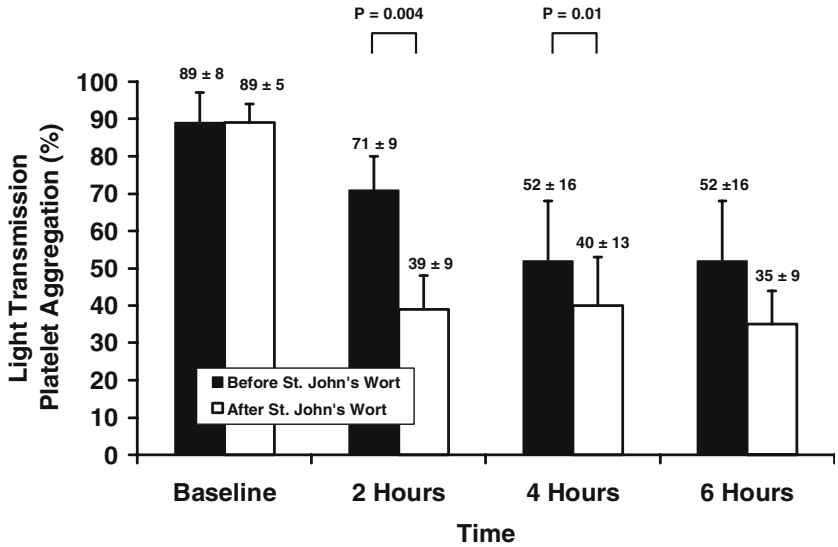

Fig. 10. After St. John's wort supplementation for 14 days, clopidogrel more significantly inhibited platelet aggregation at 2 and 4 h (n = 10), relative to the antiplatelet activity of clopidogrel before St. John's wort supplementation.

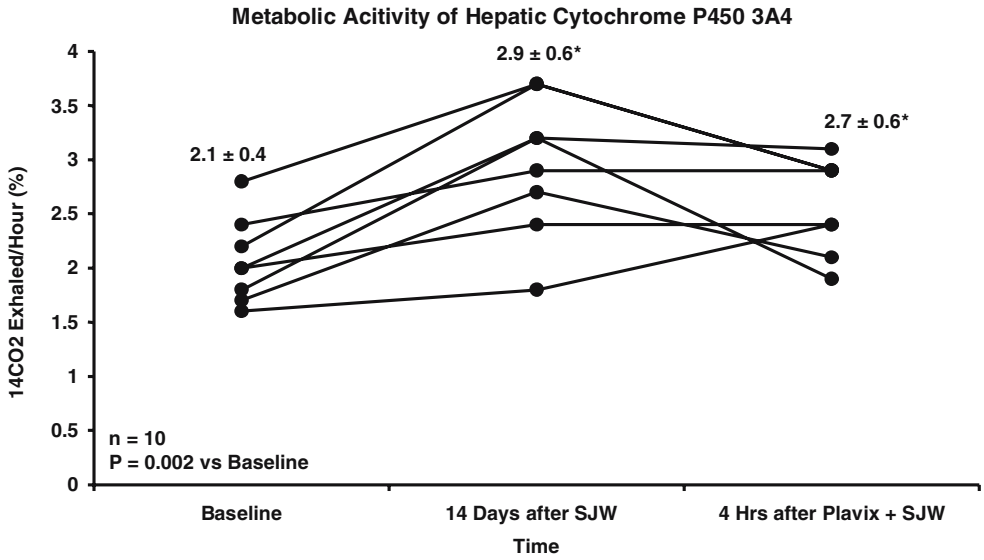

Fig. 11. Metabolic activity of CYP3A4 measured using ERMBT before clopidogrel and St. John's wort, after 14 days of St. John's wort, and after clopidogrel and St. John's wort at 4 h. Results demonstrated St. John's wort significant increase the hepatic CYP3A4 metabolic activity. *CYP3A4* Cytochrome P450 3A4, *ERMBT* erythromycin breath test.

wort supplementation improved the effect of clopidogrel, detected at 2 and 4 h after clopidogrel administration, with the effect waning by 6 h (Fig. 10) (109).

To assess whether supplementation with St. John's wort also improved CYP3A4 activity, we measured metabolic activity of CYP3A4 for the same ten volunteer subjects using ERMBT—before clopidogrel and St. John's wort (Day 0), after St. John's wort (Day 14), and 4 h after clopidogrel administration in St. John's wort-supplemented subjects. Results demonstrated (Fig. 11) that St. John's wort significantly induced CYP3A4 enzymatic activity *versus* baseline values (2.1 ± 0.4 at baseline *versus* 3.0 ± 0.6% $^{14}CO_2$ exhaled/hour after St. John's wort, $p = 0.002$). In addition, data collected 4 h after clopidogrel administration shows that elevated CYP3A4 activity was sustained. These results demonstrate that this herb-drug interaction can convert clopidogrel non-responders to clopidogrel responders (109).

Clopidogrel Bioavailability

Previous studies have demonstrated that net absorption of clopidogrel after a single oral dose of 75 mg was not significantly modified by food or by antacid ingestion (122). Evidence support a dose dependent absorption of clopidogrel and its active metabolite. Where single doses of clopidogrel higher than 300 mg resulted in significantly enhanced suppression of platelet function, however single doses of clopidogrel higher than 600 mg (900 mg) were not associated with an additional significant suppression of platelet function (123). Whether the mechanism of a dose dependent absorption of clopidogrel is in part due to the metabolic activation of clopidogrel by intestinal epithelial CYP3A4 isoenzyme leading to response variability is currently not well defined (124). Furthermore, patients with coronary artery disease receiving concomitant HMG-CoA reductase inhibitors (statins) that inhibits CYP3A4 appears to have equal effective inhibition of the multidrug transport P-glycoprotein (MDR1 gene) production which is also regulated by PXR (125–127), as such, it is currently unclear if clopidogrel absorption is mediated via this transporter but if so, its inhibition could also contribute to clopidogrel resistance.

$P2Y_{12}$ ADP Receptor Polymorphism

Polymorphisms of the $P2Y_{12}$ and GP IIb/IIIa receptors may also play a role in the occurrence of clopidogrel resistance. Recently, it has been shown that specific sequence variations of the G_i-coupled ADP receptor gene $P2Y_{12}$ are associated with greater aggregation and potentially a reduced response to $P2Y_{12}$ inhibitors (128). Three single nucleotide polymorphisms and one nucleotide insertion in total linkage disequilibrium in the $P2Y_{12}$ receptor gene, which determined two haplotypes designated H1 and H2 were identified. The H2 haplotype was associated with maximal platelet aggregation in response to ADP. Downregulation of the platelet cAMP concentration induced by ADP was also demonstrated to be more marked in the haplotype (H2) carrier for the $P2Y_{12}$ genetic polymorphism (128).

It has also been shown that a specific polymorphism of the GP IIb/IIIa receptor (Pl^{A2A2}) was associated with greater expression of the receptor after stimulation with ADP (60). However, whether the Pl^{A2A2} polymorphism would show a higher incidence of clopidogrel resistance is not known.

These findings were recently challenged by Angiolillo *et al.* who demonstrated that similar nucleotide polymorphisms of the $P2Y_{12}$ receptor gene did not modulate platelet response to clopidogrel either in the early or long-term treatment phases of patients (129).

Proposed Managements for Clopidogrel Resistance

One approach to managing clopidogrel resistance may involve administration of higher loading and maintenance doses of clopidogrel. The mechanism is providing more prodrug as a

substrate for its metabolic activation by CYP3A4 isoenzyme. This treatment however may be limited by increased potential side effects of clopidogrel.

Another approach may be to utilize alternative more potent thienopyridines such as prasugrel (CS-747) that can achieve a more rapid onset and higher level of inhibition of platelet aggregation (130,131). This is due to the fact that in addition to a CYP3A4 dependent metabolic activation of prasugrel, prasugrel is metabolized by CYP1A2 and CYP2B6. This results in the effective generation of active metabolite and superior and consistent platelet inhibition even in the presence of CYP3A4 inhibitors. A recent Phase 2 clinical trial in a preparation of a Phase 3 trial has demonstrated that prasugrel and clopidogrel both resulted in low risk of bleeding (132). Non-thienopyridine $P2Y_{12}$ inhibitors such as AR-C6931MX have also been proposed as an alternative management for clopidogrel resistance (133).

Agents such as St. John's wort that induces the phenotypic expression of CYP3A4 mediated by activation of the proximal PXR promoter site can potentially be utilized to enhance the antiplatelet effect of clopidogrel in clopidogrel non-responders (109). Prospective randomized clinical trials are needed to determine the efficacy and safety of St. John's wort.

Future Perspective

Aspirin and clopidogrel are currently the treatment of choice in acute coronary syndrome and prevention of thrombosis after coronary stent implantation. Recent evidence-based consensus agreement acknowledged aspirin and/or clopidogrel resistance that can be linked to adverse cardiovascular events. While new alternative treatment strategies to overcome aspirin and clopidogrel resistance are currently being sought, further investigations should focus on the development of a standardized methodological approach to detect inter-individual aspirin and/or clopidogrel resistance to tailor individual antiplatelet therapy.

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