Abstract:

METHODS FOR DIAGNOSIS AND TREATMENT OF THROMBOTIC DISORDERS MEDIATED BY CYP2C19*2

Abstract: Genetic polymorphisms in the 2C19*2 gene are useful as biomarkers for diagnosing an increased risk for high platelet reactivity or increased bleeding in a subject suffering from a cardiovascular disorder. In particular, the invention relates to methods of treating and preventing coronary artery disease using P2Y-12 receptor inhibitors. In addition, the present invention relates to kits for use in identifying patients with coronary heart disease with an increased risk for high platelet reactivity or increased bleeding.
METHODS FOR DIAGNOSIS AND TREATMENT OF THROMBOTIC DISORDERS MEDIATED BY CYP2C19*2

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. 119(e) to U.S. Provisional Application No. 61/224,839 filed on July 10, 2009 and U.S. Provisional Application No. 61/321,596 filed on April 7, 2010, which are herein incorporated in their entirety by reference.

FIELD OF THE INVENTION

[0002] This invention relates generally to analytical testing, and more particularly to the analysis of gene expression or hematology profiles as biomarkers for predicting the effectiveness of P2Y12 inhibitors in treating coronary artery disease or other ischemic events. In addition, the present invention relates to methods of treatment of coronary artery disease or ischemic events as well as kits for use in identifying patients with coronary artery disease at risk for high platelet reactivity or increased bleeding.

BACKGROUND OF THE INVENTION


These compounds are prodrugs (nondirect-acting) which are metabolized in vivo by cytochrome P450 (CYP) isoenzymes to an unstable, highly reactive and short-lived intermediate (Savi et al. Thromb Haemost. Nov 2000;84(5):891-896). This intermediate then irreversibly binds to the P2Y12 receptor and prevents adenosine diphosphate (ADP)-induced platelet activation and aggregation.

[0004] Clopidogrel is routinely administered as a daily 75 mg dose along with 81-325 mg aspirin in patients treated with coronary artery stents and after acute coronary syndromes.

**[0005]** Prasugrel, a newer thienopyridine that exhibited better platelet inhibition than clopidogrel in phase II studies, was also associated with lower ischemic event occurrence in a Phase III acute coronary syndrome trial. However, prasugrel therapy is still associated with a recurrent ischemic event rate and an increased bleeding risk. (Gurbel et al. *Curr Opin Investig Drugs* 2008;9:324-36; Wiviott et al. *N Engl J Med.* 2007;357:2001-15). Likewise patient response to prasugrel is also related to cytochrome P450 genetic polymorphisms (Mega et al. *Circulation* May 4, 2009).

**[0006]** In addition, preclinical studies have reported disproportionate effects of the thienopyridines, clopidogrel and prasugrel, on bleeding at high doses. For example, recent preclinical studies performed in rabbits have cautioned against the use of high dose thienopyridines that produce near-to-maximal platelet inhibition, indicating that these doses could significantly increase bleeding complications (Wong et al. *Thromb Haemost.* Jan 2009;101(1):108-115). Particularly, high doses of clopidogrel providing limited incremental benefits in preventing arterial thrombosis have been associated with disproportionate levels of bleeding in both rabbits and rats (Wong et al. *J Cardiovasc Pharmacol.* May 2007;49(5):316-324; Schumacher et al. *7 Pharmacol Exp. Ther.* Jul 2007;322(1):369-377). There was also excessive bleeding reported in patients in the TRITON-TIMI 38 trial (Wiviott et al. *N Engl J Med.* Nov 15 2007;357(20):2001-2015).
While progress has been made in this field, the totality of the available data indicate that significant non-responsiveness, due to cytochrome P450 genetic polymorphisms is undesirable in patients with high risk cardiovascular disease. Likewise, HPR is undesirable in patients with high risk cardiovascular disease, and irreversible, non-direct acting platelet inhibition associated with thienopyridine treatment is a major limitation in patients. Accordingly, new, safer and more effective methods for treating cardiovascular disease that address high on-treatment platelet reactivity (HPR), irreversible, non-direct acting platelet inhibition, and thienopyridine non-responsive patients with cytochrome P450 genetic polymorphisms are needed, especially for high risk patients. There also remains a need for a P2Yi2 inhibitory treatment that differentially affects thrombosis and hemostasis, specifically one that preferentially inhibits arterial thrombosis resulting in a greater therapeutic index and reduced bleeding.

Elinogrel (Portola Pharmaceuticals Inc, South San Francisco, CA) is a new reversible, direct acting P2Y12 receptor inhibitor, that is being developed for several indications, including treatment of thrombosis, see, U.S. Pat. Application No. 11/556,490. The present invention solves these problems by providing methods for determining subjects who are at risk for HPR or developing HPR or excessive bleeding associated with thienopyridine treatment in patients with cytochrome P450 (CYP) gene polymorphisms. The present invention satisfies this and other needs.

BRIEF SUMMARY OF THE INVENTION

The invention provides methods for determining subjects who are at risk for developing HPR or increased bleeding or who would be non-responsive to thienopyridine therapy based upon analysis of biomarkers present in the subject to be treated. In one embodiment, the invention provides for the use of thienopyridine therapy to identify patients at risk for experiencing HPR during thienopyridine therapy for a cardiovascular disorder. In another embodiment, the diagnosis involves the determination of gene expression profiles from the subject to be treated. In another embodiment, the invention provides methods for determining optimal treatment strategies for these patients. The prediction could therefore provide means of safer treatment regimens for the patient by helping the clinician to either (1) alter the dose of the drug, (2) provide additional or alternative concomitant medication or (3) choosing not to prescribe that drug for that patient.

In another embodiment, the invention provides methods for diagnosing an increased risk for high platelet reactivity or increased bleeding in a subject suffering from a
cardiovascular disorder, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) determining whether the subject is at risk for high platelet reactivity or increased bleeding following administration of a irreversible, non-direct acting P2Yn receptor inhibitor.

[0011] In another embodiment, the invention provides methods for diagnosing an increased risk for high platelet reactivity or increased bleeding in a subject suffering from a condition or disorder mediated at least in part by ADP-induced platelet aggregation, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) determining whether the subject is at risk for high platelet reactivity or increased bleeding following administration of a irreversible, non-direct acting P2Y_{12} receptor inhibitor.

[0012] In another embodiment, the invention provides methods of identifying a subject that is susceptible to developing an cardiovascular complication during a course of treatment comprising administering a irreversible, non-direct acting P2Y_{i2} receptor inhibitor (a) providing a biological sample from a subject; (b) determining platelet reactivity in the biological sample; and (c) comparing platelet reactivity in the biological sample to a standard reactivity, wherein 43% or more platelet aggregation compared to the standard indicates that the subject is susceptible to developing a ischemic event during a course of treatment with a irreversible, non-direct acting P2Y_{12} receptor inhibitor.

[0013] In another embodiment, the invention provides methods for preventing or treating thrombosis and thrombosis related conditions in a subject with a P2Y_{12} receptor inhibitor, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) administering a therapeutically effective amount of reversible, direct acting P2Y_{i2} receptor inhibitor to the subject having a CYP2C19*2 polymorphism.

[0014] In another embodiment, the invention provides methods for treating a cardiovascular disease in a patient resistant or non-responsive to a daily dose of irreversible, non-direct acting P2Y_{i2} receptor inhibitor comprising: providing a therapeutic amount of a direct-acting, reversible P2Y_{12} receptor inhibitor; and administering the resistance-surmounting quantity of the direct-acting, reversible P2Y_{12} receptor inhibitor to the patient.

[0015] In another embodiment, the invention provides for the use of reversible, direct acting P2Y_{12} receptor inhibitor in the manufacture of a medicament for the; treatment of thrombotic conditions in a selected patient population non-responsive to irreversible, non-direct acting P2Y_{i2} inhibitor, where in the patient population is selected on the basis of the genotype of the patients at a 2C19*2 genetic locus predictive of antiplatelet activity.
The invention also provides clinical assays, kits and reagents for predicting increased risk of HPR or increased bleeding prior to taking a drug. In one embodiment, the kits contain reagents for determining the gene expression of certain genes, where the expression profile of the genes is a biomarker for the risk of the subject for experiencing HPR or increased bleeding. In another embodiment, the invention provides for a kit for use in diagnosing an increased risk for high platelet reactivity in a subject suffering from a cardiovascular disorder, comprising: (a) a reagent for detecting a genetic polymorphism in CYP2C19*2 that is biomarker of an irreversible, non-direct acting P2Y\textsubscript{i2} receptor inhibitor-mediated high platelet reactivity; (b) a container for the reagent; and (c) a written product on or in the container describing the use of the biomarker in predicting an irreversible, non-direct acting P2Y\textsubscript{i2} receptor inhibitor-mediated high platelet reactivity in subjects.

These and other aspects, objects, features and advantages of the invention will be apparent upon reference to the following detailed description.

BRIEF DESCRIPTION OF FIGURES

[0018] FIG. 1—illustrates the study design. HPR = high platelet reactivity. ASA = aspirin. ADP = Adenosine diphosphate, qd= once daily.

[0019] FIG. 2—illustrates Individual patients' response to elinogrel as measured by 5\textmu M ADP-induced aggregation. The gray line indicates the mean and dotted line represent the 43\% cutoff value used for HPR.

[0020] FIG. 3—illustrates the pharmacodynamic response to a single oral 60 mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (5 \textmu M ADP-induced aggregation).

[0021] FIG. 4—illustrates the pharmacodynamic response to a single oral 60 mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (20 \textmu M ADP-induced aggregation).

[0022] FIG. 5—illustrates the pharmacodynamic response to a single oral 60 mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (10 \textmu M ADP-induced aggregation).
FIG. 6—illustrates the pharmacodynamic response to a single oral 60mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (4 µg/ml collagen-induced aggregation).

FIG. 7—illustrates the pharmacodynamic response to a single oral 60mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (ADP-induced platelet-fibrin clot strength, MA-ADP).

FIG. 8—illustrates the pharmacodynamic response to a single oral 60mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (VerifyNow P2Yi2 assay).

FIG. 9—illustrates the pharmacodynamic response to a single oral 60mg elinogrel dose in patients with high-platelet reactivity as measured by light transmittance aggregometry (vasodilator stimulated phosphoprotein phosphorylation assay, VASP-Platelet Reactivity Index).

FIG. 10—illustrates the pharmacodynamic response to a single oral 60mg elinogrel dose in patients with high-platelet reactivity as measured by real time thrombosis profiler assay.

FIG. 11—illustrates the pharmacodynamic response to single oral 60mg elinogrel dose in patients with high-platelet reactivity. This plot represents the mean plasma concentration achieved at 4, 6 and 24 hrs. Y axis = µg/ml.

FIG. 12—illustrates the effect of Elinogrel on Platelet Reactivity and 2C19*2 Allele Status. 5 µM ADP-induced aggregation in individual patients measured pre- and 4 hours post-elinogrel treatment and CYP2C19*2 allele status. HPR = high platelet reactivity.

FIG. 13—illustrates the response to single oral 60mg elinogrel dose in a patient with high-platelet reactivity (on 75 or 150 mg clopidogrel) as measured by RTTP assay. This plot represents the change in thrombotic profile achieved at 4, 6 and 24 hrs after dosing with elinogrel.

FIG. 14—illustrates the determination of doses of elinogrel, clopidogrel and prasugrel in the mouse providing equivalent inhibition on arterial thrombosis. Doses of elinogrel (7.5, 20 and 60 mg/kg), clopidogrel (1.5, 15 and 50 mg/kg) and prasugrel (1, 3 and 10 mg/kg) were identified as providing similar levels of inhibition on thrombus stability (intermediate (I), high (H) and maximal (M), respectively). N = 5 to 18 per group. **P<0.01, ***P<0.0001 vs V.Ctl (vehicle control). All animals treated with the maximal (M) doses had
patent artery at the end of the observation period (2400 sec). FIG 14 (cont.) - illustrates A) the pharmacokinetic profile of elinogrel following oral dosing in mice. B) PK/time to occlusion (TTO) relationship of elinogrel. Elinogrel significantly inhibited thrombus stability (vascular occlusion) at concentrations > 1 µg/ml in vivo.

FIG. 15A- illustrates Elinogrel, clopidogrel and prasugrel effects on thrombus growth: elinogrel and prasugrel (not clopidogrel) dose-dependently delayed thrombus appearance for first thrombus. B) Thrombotic profiles of maximal doses of elinogrel (Elin. 60), clopidogrel (Clop. 50) and prasugrel (Pras. 10). C) Total amount of platelet deposited at the site of vascular injury throughout the entire experiment. Blood from animals treated with elinogrel was collected at the end of the observation period (42 min after injury) or 2 minutes after vascular injury for determination of plasma concentration (see Supplementary data, Figure 1). N = 5 to 18 per group. *** p<0.0001 vs P2Yi2/-.

FIG. 16— illustrates doses of elinogrel, clopidogrel and prasugrel providing equivalent antithrombotic activities differentially affect bleeding frequency over the 15 min period. Solid lines indicate bleeding period, gap (white bars) period of cessation of blood loss.

FIG. 17— illustrates how clopidogrel and prasugrel increase the volume of blood lost beyond levels attributed to P2Yi2 inhibition. A) Doses of elinogrel, clopidogrel and prasugrel providing equivalent antithrombotic activities differentially affect volume of blood loss. *, p<0.005 vs clopidogrel; †, p<0.005 vs prasugrel; t P<0.0005 vs P2Yi2 ; • P<0.05 vs P2Yi2 . B) Greater therapeutic index (ratio of the fold increase for time to occlusion vs V.Ctl. (vehicle control) and the fold increase in blood loss vs V.Ctl) associated with the use of a reversible, direct acting P2Yi2 antagonist.

FIG. 18— illustrates how clopidogrel and prasugrel increase the volume of blood lost in P2Yi2' mice. Effects of maximum doses of elinogrel, clopidogrel and prasugrel on blood loss measurement in P2Yi2' mice. Clopidogrel and prasugrel increased volume of blood loss in P2Yi2' mice.

FIG. 19— illustrates differential effects of elinogrel, clopidogrel and prasugrel on bleeding time frequency vs genetic targeting (micropuncture model in mesenteric veins). A) Schematic of the platelet deposition at site of micropuncture in mesenteric veins. B) Bleeding frequency. C) Reduction in micropuncture lumen of WT-treated animals or P2Yi2' mice. Thienopyridine-treated mice have a delayed occlusion of the vascular wound compared with P2Yi2' mice, while elinogrel-treated animals displayed a faster rate of closure than 20 either
thienopyridine-treated or P2Y12−/− mice that paralleled an increased amount of platelet deposition (D).

[0037] FIG. 20—illustrates differential effects of ADP concentration on anti-aggregatory activity of reversible (elinogrel) vs irreversible (clopidogrel and prasugrel) antagonists of P2Y12. A) Percent inhibition of platelet aggregation of the doses providing maximum levels of inhibition of thrombosis vs vehicle control-treated group. Elinogrel failed at inhibiting platelet aggregation induced by 10 μM ADP while it maximally inhibited aggregation induced by 1 μM ADP. B) Left panel: extent of platelet aggregation (10 μM ADP) is predictive of time to occlusion for the intermediate, high and maximal doses of irreversible inhibitors of P2Y12 (clopidogrel, green square and prasugrel, pink square) n = 0.9, but not when data of the three doses of all three antagonists are combined (elinogrel, black square) (r² = 0.45). Right panel: Extent of platelet aggregation (10 μM ADP) is predictive of blood loss for all classes of P2Y12 antagonists. The greater therapeutic index of elinogrel directly related to its mechanism of action. Differential gradient of ADP concentration between arterial thrombosis and primary hemostasis. High shear platelet thrombosis (1500/sec, <1 mJ/V) Folie BJ, Mcintire LV. Biophys J. 1989. 56:1 121-1 141. Low shear venous thrombosis (100/sec, ~5 mM), Hemostasis (-10 nM) Born et al. J Physiol 1984. 354:419-429.

[0038] FIGS. 21A-D —illustrate how thienopyridines block vasoconstriction of mesenteric veins. FIG. 21A) illustrates vasoconstriction of mesenteric veins induced by 1 μM α,β metATP is inhibited by 3 days (D3) oral gavage with the maximal doses of clopidogrel (n = 5) and prasugrel (n = 7) but not elinogrel (n = 9), Vehicle control (n = 11) of P2Y12-deficiency (n = 10). FIG. 21B) illustrates how Prasugrel (M, 10 mg/kg, n = 6) but not vehicle control (0.5% methyl cellulose, n = 6) inhibits initial response to 1 μM α,β metATP in platelet-depleted P2Y12−/− mice. FIG. 21C) illustrates Prasugrel dose response on α,β metATP-induced vasoconstriction (I, n = 9; H, n = 9; M, n = 4). FIG. 21D) shows the time-dependent effects of Prasugrel (M, 10 mg/kg) on vasoconstriction 1 (n = 4), 2 (n = 4), 5 (n = 4), 10 (n = 7), 24 (n = 10) and 48 hours (n = 10) following administration of the third dose.

DETAILED DESCRIPTION OF THE INVENTION

[0039] As used herein, the below terms have the following meanings unless specified otherwise:
1. Abbreviations and Definitions

The abbreviations used herein are conventional, unless otherwise defined. The following abbreviations are used: g = gram, H2O = water; h = hour, IC₅₀ = The concentration of an inhibitor that is required for 50% inhibition of an enzyme in vitro, µM = micromolar, µL = microliter, mg = milligram, mm = millimeter, mM = millimolar, mmol = millimole, mL = milliliter, mOD/min = millioptical density units per minute, min = minute, and M = molar.

It is noted here that as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

The term "administering" refers to oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, or the implantation of a slow-release device e.g., a mini-osmotic pump, to a subject. Administration is by any route, including parenteral and transmucosal (e.g., buccal, sublingual, palatal, gingival, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

As used herein, the term "ADP-mediated disease or condition" and the like refers to a disease or condition characterized by less than or greater than normal, ADP activity. A ADP-mediated disease or condition is one in which modulation of ADP results in some effect on the underlying condition or disease (e.g., a ADP inhibitor or antagonist results in some improvement in patient well-being in at least some patients).

As used herein the term "allele" shall mean a particular form of a gene or DNA sequence at a specific chromosomal location (locus).

An "antagonist" or "inhibitor" refers to an agent or molecule that inhibits or binds to, partially or totally blocks stimulation or activity, decreases, closes, prevents, delays activation or enzymatic activity, inactivates, desensitizes, or down regulates the activity of a receptor of the invention. As used herein, "antagonist" also includes a reverse or inverse agonist.

As used herein, the term "blood sample" refers to whole blood taken from a subject, or any fractions of blood including plasma or serum.
As used herein, the term "condition" refers to a disease state for which the compounds, compositions and methods of the present invention are being used against.

As used herein, the term "clinical response" means any or all of the following: a quantitative measure of the response, no response, and adverse response (i.e., side effects).

As used herein, the term "clinical trial" means any research study designed to collect clinical data on responses to a particular treatment, and includes but is not limited to phase I, phase II and phase III clinical trials. Standard methods are used to define the patient population and to enroll subjects.

As used herein, "expression" includes but is not limited to one or more of the following: transcription of the gene into precursor mRNA; splicing and other processing of the precursor mRNA to produce mature mRNA; mRNA stability; translation of the mature mRNA into protein (including codon usage and tRNA availability); and glycosylation and/or other modifications of the translation product, if required for proper expression and function.

As used herein the term "gene" shall mean a segment of DNA that contains all the information for the regulated biosynthesis of an RNA product, including promoters, exons, introns, and other untranslated regions that control expression.

As used herein, the term "genotype" shall mean an unphased 5’ to 3’ sequence of nucleotide pair(s) found at one or more polymorphic sites in a locus on a pair of homologous chromosomes in an individual. As used herein, genotype includes a full-genotype and/or a sub-genotype.

As used herein, the term "high platelet reactivity" or "HPR" can have one of the following technical definitions: a) being greater than or equal to 43% 5mM ADP-induced maximal platelet aggregation; or b) being greater than or equal to the amount of aggregation measured 5 min. after addition of ADP 20 µmol L−1 (see Geisler et al. J. Thrombosis and Haemastasis, 6: 54-61, 2007).

As used herein the term "locus" shall mean a location on a chromosome or DNA molecule corresponding to a gene or a physical or phenotypic feature.

The term "mammal" includes, without limitation, humans, domestic animals (e.g., dogs or cats), farm animals (cows, horses, or pigs), monkeys, rabbits, mice, and laboratory animals.

As used herein, the term "nucleotide pair" shall mean the nucleotides found at a polymorphic site on the two copies of a chromosome from an individual.
"Patient" refers to human and non-human animals, especially mammals. Examples of patients include, but are not limited to, humans, cows, dogs, cats, goats, sheep, pigs and rabbits.

The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of salts derived from pharmaceutically-acceptable inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic, manganous, potassium, sodium, zinc and the like. Salts derived from pharmaceutically-acceptable organic bases include salts of primary, secondary and tertiary amines, including substituted amines, cyclic amines, naturally-occurring amines and the like, such as arginine, betaine, caffeine, choline, NJN'-dibenzylethylenediamine, diethylamine, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethylmorpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine, tripropylamine, tromethamine and the like. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, malonic, benzoic, succinic, suberic, fumaric, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (see, e.g., Berge, S.M. et al., "Pharmaceutical Salts," Journal of Pharmaceutical Science, 66:1-19, 1977). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.
[0059] The neutral forms of the compounds may be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

[0060] The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (\(^3\)H), iodine-125 (\(^{125}\)I) or carbon-14 (\(^{14}\)C). AU isotopic variations of the compounds of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

[0061] The terms "pharmaceutically effective amount", "therapeutically effective amount" or "therapeutically effective dose" refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term "therapeutically effective amount" includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the symptoms of the condition or disorder being treated. The therapeutically effective amount will vary depending on the compound, the disorder or condition and its severity and the age, weight, etc., of the mammal to be treated.

[0062] The term "platelet" refers to a minute, nonnucleated, disklike cell found in the blood plasma of mammals that functions to promote blood clotting.

[0063] As used herein the term "polymorphism" shall mean any sequence variant present at a frequency of >1% in a population. The sequence variant may be present at a frequency significantly greater than 1% such as 5% or 10% or more. Also, the term may be used to refer to the sequence variation observed in an individual at a polymorphic site. Polymorphisms include nucleotide substitutions, insertions, deletions and microsatellites and may, but need not, result in detectable differences in gene expression or protein function.

[0064] As used herein, the term "polymorphic site" shall mean a position within a locus at which at least two alternative sequences are found in a population, the most frequent of which has a frequency of no more than 99%.

[0065] As used herein, the term "polynucleotide" shall mean any RNA or DNA, which may be unmodified or modified RNA or DNA. Polynucleotides include, without limitation, single-
and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons.

[0066] As used herein the term "polypeptide" shall mean any polypeptide comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. Polypeptide refers to both short chains, commonly referred to as peptides, glycopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. Polypeptides include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature.

[0067] As used herein, the term "preventing" refers to the prophylactic treatment of a patient in need thereof. The prophylactic treatment can be accomplished by providing an appropriate dose of a therapeutic agent to a subject at risk of suffering from an ailment, thereby substantially averting onset of the ailment.

[0068] As used herein, a "SNP nucleic acid" is a nucleic acid sequence, which comprises a nucleotide that is variable within an otherwise identical nucleotide sequence between individuals or groups of individuals, thus, existing as alleles. Such SNP nucleic acids are preferably from about 15 to about 500 nucleotides in length. The SNP nucleic acids may be part of a chromosome, or they may be an exact copy of a part of a chromosome, e.g., by amplification of such a part of a chromosome through PCR or through cloning. The SNP nucleic acids are referred to hereafter simply as "SNPs". The SNP probes according to the invention are oligonucleotides that are complementary to a SNP nucleic acid.

[0069] As used herein, the term "treating" refers to providing an appropriate dose of a therapeutic agent to a subject suffering from an ailment.
[0070] As used herein, the term "therapeutically effective amount" refers to an amount of a therapeutic agent that is sufficient to affect the treatment of a subject suffering from an ailment.

[0071] The term "recanalization" refers to the process of restoring flow to or reuniting an interrupted channel of the body, such as a blood vessel.

[0072] The term "restenosis" refers to a re-narrowing or blockage of an artery at the same site where treatment, such as an angioplasty or a stent procedure, has been performed.

[0073] The phrase "selectively" or "specifically" when referring to binding to a receptor, refers to a binding reaction that is determinative of the presence of the receptor, often in a heterogeneous population of receptors and other biologies. Thus, under designated conditions, the compounds bind to a particular receptor at least two times the background and more typically more than 10 to 100 times background. Specific binding of a compound under such conditions requires a compound that is selected for its specificity for a particular receptor. For example, small organic molecules can be screened to obtain only those compounds that specifically or selectively bind to a selected receptor and not with other receptors or proteins. A variety of assay formats may be used to select compounds that are selective for a particular receptor. For example, High-throughput screening assays are routinely used to select compounds that are selective for a particular a receptor.

[0074] The "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (e.g., humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats, mice and the like. In preferred embodiments, the subject is a human.

[0075] The term "thrombosis" refers to the blockage or clotting of a blood vessel caused by a clumping of cells, resulting in the obstruction of blood flow. The term "thrombosis" refers to the clot that is formed within the blood vessel.

[0076] The terms "treat", "treating", "treatment" and grammatical variations thereof as used herein, includes partially or completely delaying, alleviating, mitigating or reducing the intensity of one or more attendant symptoms of a disorder or condition and/or alleviating, mitigating or impeding one or more causes of a disorder or condition. Treatments according to the invention may be applied preventively, prophylactically, pallatively or remedially.

[0077] The term "vessel" refers to any channel for carrying a fluid, such as an artery or vein. For example, a "blood vessel" refers to any of the vessels through which blood
circulates in the body. The lumen of a blood vessel refers to the inner open space or cavity of the blood vessel.

2. Embodiments of the Invention

[0078] The invention advantageously provides a way to determine whether a patient will experience high platelet reactivity during drug treatment, prior to actually taking the drugs. The invention thus provides safer treatment regimens for patients by helping clinicians to (1) provide additional or alternative concomitant medication, (2) alter the dose of the drug or (3) choose not to prescribe that drug for that patient.

[0079] Relevant genetic polymorphisms were identified in a clinical trial of elinogrel, which was conducted in stented subjects with and without HPR. The platelet reactivity in 45 subjects treated with elinogrel was evaluated and additional pharmacokinetic information was collected. Twenty of 45 previously stented stable patients on clopidogrel and aspirin had HPR defined by being greater than or equal to 43% 5mM ADP-induced maximal platelet aggregation. Subjects with and without HPR were flagged and genotyped for CYP2C19*2,3,5,17 and CYP3A5*3.

[0080] Of the 5 single nucleotide polymorphisms (SNP) from 2 genes that were genotyped, one in the CYP2C19 gene was associated with HPR (75% vs. 20% p<0.001). CYP2C19 encodes cytochrome P450 2C19 that plays a pivotal role in metabolizing thienopyridines (see also U.S. Patent No. 5,912,120). These results suggest that polymorphisms in CYP2C19, are directly involved with HPR.

[0081] As used herein, a polymorphism in the CYP 2C19*2 genetic locus is "predictive" of a "high" risk of HPR when genetic polymorphism correlates significantly with the development of drug-induced HPR, see, Example 1. Determinations of significance (p values) can be determined by analysis of variance (ANOVA) or Fisher's Exact tests. Associations between CYP 2C19 polymorphisms and HPR had p values of <0.001.

[0082] These results can reasonably be extrapolated to the prediction of HPR in patients following the administration of any thienopyridine, based upon the structural similarity and modes of action of these compounds. Among these thienopyrimidine compounds are clopidogrel, prasugrel and ticagrelor. Moreover, the results can be extrapolated to the prediction of HPR in patients who are being treated for diseases other than coronary artery disease. The method of the invention is applicable to vertebrate subjects, particularly to mammalian subjects, more particularly to human subjects.
As used herein, the administration of an agent or drug to a subject or patient includes self-administration and the administration by another.

The diagnosis of HPR can be accomplished using assays of platelet activity. Serum assays of platelet activity are well-known to those of skill in the medical arts and routine in hospital laboratories and are illustrated in the EXAMPLES.

The maximum tolerated dose (MTD) for a compound is determined using methods and materials known in the medical and pharmacological arts, for example through dose-escalation experiments. One or more patients is first treated with a low dose of the compound, typically 10% of the dose anticipated to be therapeutic based on results of in vitro cell culture experiments. The patients are observed for a period of time to determine the occurrence of toxicity. Toxicity is typically evidenced as the observation of one or more of the following symptoms: vomiting, diarrhoea, peripheral neuropathy, ataxia, neutropaenia, or elevation of liver enzymes. If no toxicity is observed, the dose is increased 2-fold, and the patients are again observed for evidence of toxicity. This cycle is repeated until a dose producing evidence of toxicity is reached. The dose immediately preceding the onset of unacceptable toxicity is taken as the MID. A determination of the MTD for epothilone B is provided above.

Individuals carrying polymorphic CYP 2C19 alleles may be detected at the DNA, the RNA, or the protein level using a variety of techniques that are well known in the art. Strategies for identification and detection are described in e.g. EP 730,663, EP 717,113, and PCT US97/02102. The identification of alleles containing single nucleotide polymorphisms may involve the amplification of DNA from target samples. This can be accomplished by e.g., PCR. See generally PCR Technology: Principles and Applications for DNA Amplification, (ed. Erlich, Freeman Press, New York, N.Y., 1992); PCR Protocols: A Guide to Methods and Applications (eds. Innis, et al., Academic Press, San Diego, Calif., 1990).


[0087] In a particularly preferred embodiment the detection of the CYP 2C19 polymorphism can be accomplished by means of so called TAQMAN® SNP genotyping assays (available from Applied Biosystems, Foster City, CA).

[0088] It is preferred that the individuals included in the clinical population have been graded for the existence of the medical condition of interest. This grading of potential patients could employ a standard physical exam or one or more lab tests. Alternatively, grading of patients could use gene expression pattern for situations where there is a strong correlation between gene expression pattern and disease susceptibility or severity.

[0089] The therapeutic treatment of interest is administered to each individual in the trial population and each individual's response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses and that the investigator will choose the number of responder groups (e.g., low, medium, high) made up by the various responses.
After both the clinical and polymorphism data have been obtained, correlations between individual response and genotype or haplotype content are created. Correlations may be produced in several ways. In one method, individuals are grouped by their genotype or haplotype (or haplotype pair) (also referred to as a polymorphism group), and then the averages and standard deviations of clinical responses exhibited by the members of each polymorphism group are calculated.

Results are analyzed to determine if any observed variation in clinical response between polymorphism groups is statistically significant. Statistical analysis methods which may be used are described in L. D. Fisher & G. vanBelle, Biostatistics: A Methodology for the Health Sciences (Wiley-Interscience, New York, 1993). This analysis may also include a regression calculation of which polymorphic sites in the gene give the most significant contribution to the differences in phenotype.


Correlations may also be analyzed using analysis of variation (ANOVA) techniques to determine how much of the variation in the clinical data is explained by different subsets of the polymorphic sites in the gene. ANOVA is used to test hypotheses about whether a response variable is caused by or correlated with one or more traits or variables that can be measured (Fisher & vanBelle, supra, Ch. 10). For statistical methods for use in the methods of this invention, see: Statistical Methods in Biology, 3rd edition, Bailey N T J, (Cambridge Univ. Press, 1997); Introduction to Computational Biology, Waterman M S (CRC Press, 2000) and Bioinformatics, Baxevanis A D & Ouellette B F F editors (John Wiley & Sons, Inc., 2001).

From the analyses described above, a mathematical model may be readily constructed by the skilled artisan that predicts clinical response as a function of gene expression pattern.
The identification of an association between a clinical response and a genotype or haplotype (or haplotype pair) for the gene may be the basis for designing a diagnostic method to determine those individuals who will or will not respond to the treatment, or alternatively, will respond at a lower level and thus may require more treatment, i.e., a greater dose of a drug. The diagnostic method may take one of several forms: for example, a direct DNA test (i.e., of gene expression pattern), a serological test, or a physical exam measurement. The only requirement is that there be a good correlation between the diagnostic test results and the underlying genotype or haplotype that is in turn correlated with the clinical response. In a preferred embodiment, this diagnostic method uses the predictive haplotyping method described above.

A computer may implement any or all analytical and mathematical operations involved in practicing the methods of the present invention. In addition, the computer may execute a program that generates views (or screens) displayed on a display device and with which the user can interact to view and analyze large amounts of information relating to the gene and its genomic variation, including chromosome location, gene structure, and gene family, gene expression data, polymorphism data, genetic sequence data, and clinical data population data (e.g., data on ethnogeographic origin, clinical responses, genotypes, and haplotypes for one or more populations). The polymorphism data described herein may be stored as part of a relational database (e.g., an instance of an Oracle database or a set of ASCII flat files). These polymorphism data may be stored on the computer's hard drive or may, for example, be stored on a CD-ROM or on one or more other storage devices accessible by the computer. For example, the data may be stored on one or more databases in communication with the computer via a network.

In other embodiments, the invention provides methods, compositions, and kits for haplotyping and/or genotyping the gene in an individual. The compositions contain oligonucleotide probes and primers designed to specifically hybridize to one or more target regions containing, or that are adjacent to, a polymorphic site. The methods and compositions for establishing the genotype or haplotype of an individual at the novel polymorphic sites described herein are useful for studying the effect of the polymorphisms in the etiology of diseases affected by the expression and function of the protein, studying the efficacy of drugs targeting, predicting individual susceptibility to diseases affected by the expression and function of the protein and predicting individual responsiveness to drugs targeting the gene product.

In yet another embodiment, the invention provides a method for identifying an association between a gene expression pattern and a trait. In preferred embodiments, the trait
is susceptibility to a disease, severity of a disease, the staging of a disease or response to a
drug. Such methods have applicability in developing diagnostic tests and therapeutic
treatments for all pharmacogenetic applications where there is the potential for an association
between a genotype and a treatment outcome including efficacy measurements, PK
measurements and side effect measurements.

[0099] The invention also provides a computer system for storing and displaying
polymorphism data determined for the gene. The computer system comprises a computer
processing unit; a display; and a database containing the gene expression pattern data. The
gene expression pattern data may include the gene expression pattern in a reference
population. In a preferred embodiment, the computer system is capable of producing a
display showing gene expression pattern organized according to their evolutionary
relationships.

[0100] In practicing the present invention, many other conventional techniques in
molecular biology, microbiology and recombinant DNA are used. These techniques are well-
known and are explained in, e.g., "Current Protocols in Molecular Biology", VoIs. I-III,
Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); "DNA Cloning: A
Practical Approach", VoIs. I and II, Glover, Ed. (1985); "Oligonucleotide Synthesis", Gait,
Ed. (1984); "Nucleic Acid Hybridization", Hames & Higgins, Eds. (1985); "Transcription
(1986); "Immobilized Cells and Enzymes", IRL Press (1986); Perbal, "A Practical Guide to
Molecular Cloning"; the series, Methods in Enzymol., Academic Press, Inc. (1984); "Gene
Transfer Vectors for Mammalian Cells", Miller and Calos, Eds., Cold Spring Harbor
Laboratory, NY (1987); and Methods in Enzymology, VoIs. 154 and 155, Wu & Grossman,
and Wu, Eds., respectively.

[0101] The standard control levels of the gene expression product, thus determined in the
different control groups, would then be compared with the measured level of an gene
expression product in a given patient. This gene expression product could be the
characteristic mRNA associated with that particular genotype group or the polypeptide gene
expression product of that genotype group. The patient could then be classified or assigned to
a particular genotype group based on how similar the measured levels were compared to the
control levels for a given group.

[0102] As one of skill in the art will understand, there will be a certain degree of
uncertainty involved in making this determination. Therefore, the standard deviations of the
control group levels would be used to make a probabilistic determination and the methods of
this invention would be applicable over a wide range of probability based genotype group
determinations. Thus, for example and not by way of limitation, in one embodiment, if the
measured level of the gene expression product falls within 2.5 standard deviations of the
mean of any of the control groups, then that individual may be assigned to that genotype
group. In another embodiment if the measured level of the gene expression product falls
within 2.0 standard deviations of the mean of any of the control groups then that individual
may be assigned to that genotype group. In still another embodiment, if the measured level of
the gene expression product falls within 1.5 standard deviations of the mean of any of the
control groups then that individual may be assigned to that genotype group. In yet another
embodiment, if the measured level of the gene expression product is 1.0 or less standard
deviations of the mean of any of the control groups levels then that individual may be
assigned to that genotype group.

[0103] Thus this process will allow the determining, with various degrees of probability,
which group a specific patient should be place in and such assignment to a genotype group
would then determine the risk category into which the individual should be placed.

[0104] Methods to detect and measure mRNA levels and levels of polypeptide gene
expression products are well known in the art and include the use of nucleotide microarrays
and polypeptide detection methods involving mass spectrometers and/or antibody detection
detection and quantification techniques. See also, Human Molecular Genetics, 2nd Edition. Tom
Strachan & Andrew, Read (John Wiley and Sons, Inc. Publication, NY, 1999).

[0105] The therapeutic treatment of interest is administered to each individual in the
population and each individual's response to the treatment is measured using one or more
predetermined criteria. It is contemplated that in many cases, the population will exhibit a
range of responses and that the investigator will choose the number of responder groups, e.g.,
low, medium and high, made up by the various responses. In addition, the gene for each
individual in the population is genotyped and/or haplotyped, which may be done before or
after administering the treatment.

Methods of Treatment/Administration

A. Preventing and treating disease conditions characterized by undesired thrombosis

[0106] Methods for preventing or treating thrombosis in a mammal embraced by the
invention include administering a therapeutically effective amount of a reversible, direct
acting P2Y12 receptor inhibitor alone or as part of a pharmaceutical composition of the
invention as described above to a mammal, in particular, a human. A reversible, direct acting P2Y12 receptor inhibitor and pharmaceutical compositions of the invention containing a reversible, direct acting P2Y12 receptor inhibitor of the invention are suitable for use alone or as part of a multi-component treatment regimen for the prevention or treatment of cardiovascular diseases, particularly those related to thrombosis. For example, a compound or pharmaceutical composition of the invention may be used as a drug or therapeutic agent for any thrombosis, particularly a platelet-dependent thrombotic agent, including, but not limited to, coronary heart disease (CHD), acute coronary syndromes (ACS), acute myocardial infarction, unstable angina, chronic stable angina, transient ischemic attacks, strokes, peripheral vascular disease, preeclampsia/eclampsia, deep venous thrombosis, embolism, disseminated intravascular coagulation and thrombotic cytophenic purpura, thrombotic and restenotic complications following invasive procedures, e.g., angioplasty, carotid endarterectomy, post CABG (coronary artery bypass graft) surgery, vascular graft surgery, stent placements and insertion of endovascular devices and protheses, and hypercoagulable states related to genetic predisposition or cancers. In other groups of embodiments, the indication is selected from the group consisting of coronary heart disease (CHD), acute coronary syndromes (ACS), percutaneous coronary intervention (PCI) including angioplasty and/or stent, acute myocardial infarction (AMI), unstable angina (USA), coronary artery disease (CAD), transient ischemic attacks (TIA), stroke, peripheral vascular disease (PVD), Surgeries-coronary bypass, carotid endarterectomy

[0107] Reversible, direct acting P2Y12 receptor inhibitors and pharmaceutical compositions of the invention may also be used as part of a multi-component treatment regimen in combination with other therapeutic or diagnostic agents in the prevention or treatment of thrombosis in a mammal. In certain preferred embodiments, compounds or pharmaceutical compositions of the invention may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice such as anticoagulant agents, thrombolytic agents, or other antithrombotics, including platelet aggregation inhibitors, tissue plasminogen activators, urokinase, prourokinase, streptokinase, heparin, aspirin, or warfarin or antiinflammatories (non-steriodal antiinflammatories, cyclooxygenase II inhibitors). Coadministration may also allow for application of reduced doses of both the anti-platelet and the thrombolytic agents and therefore minimize potential hemorrhagic side-effects. Compounds and pharmaceutical compositions of the invention may also act in a synergistic fashion to prevent reocclusion following a successful thrombolytic therapy and/or reduce the time to reperfusion.
Active Agents

[0108] In one set of embodiments, the reversible, direct acting P2Y<sub>12</sub> receptor inhibitors of the present invention are selected from the class of compounds in the dihydroquinazolinylphenyl thiophenyl sulfonyleurea family. Illustrative examples of suitable dihydroquinazolinylphenyl thiophenyl sulfonyleurea compounds for use in the present invention have the formula (I):

![Formula Image]

wherein:

- R<sub>1</sub> is selected from the group consisting of H, halogen, -OH, -Ci<sub>io</sub>-alkyl and Ci<sub>6</sub>-aikylaniino; and

- X is selected from the group consisting of: F and I.

[0109] In one embodiment, the agent is elinogrel or [4-(6-fluoro-7-methylamino-2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)-phenyl]-5-chloro-thiophen-2-yl-sulfonylurea, in all suitable forms. In one aspect, the invention provides a solid composition, wherein the active agent is [4-(6-fluoro-7-methylamino-2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)-phenyl]-5-chlorothiophen-2-yl-sulfonylurea potassium or sodium salt. Methods for the preparation of compounds of formula (I) are described in US-2007-0123547-A1 and US-2009-0042916-A1.

[0110] In another embodiment, the agent is ticagrelor or [(1S,2S,3R,5S)-3-[7-[(1R,2S)-2-(3,4-Difluorophenyl)cyclopropylamino]-5-(propylthio)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)cyclopentane-1,2-diol, in all suitable forms. Methods for the preparation of compounds of formula (I) are described in US-2007-0123547-A1 and US-2009-0042916-A1.

[0111] The compounds and pharmaceutical compositions of the invention may be utilized in vivo, ordinarily in mammals such as primates, (e.g., humans), sheep, horses, cattle, pigs, dogs, cats, rats and mice, or in vitro. The biological properties, as defined above, of a compound or a pharmaceutical composition of the invention can be readily characterized by
methods that are well known in the art such as, for example, by in vivo studies to evaluate antithrombotic efficacy, and effects on hemostasis and hematological parameters.

[0112] Compounds and pharmaceutical compositions of the invention may be in the form of solutions or suspensions. In the management of thrombotic disorders the compounds or pharmaceutical compositions of the invention may also be in such forms as, for example, tablets, capsules or elixirs for oral administration, suppositories, sterile solutions or suspensions or injectable administration, and the like, or incorporated into shaped articles. Subjects (typically mammalian) in need of treatment using the compounds or pharmaceutical compositions of the invention may be administered dosages that will provide optimal efficacy. The dose and method of administration will vary from subject to subject and be dependent upon such factors as the type of mammal being treated, its sex, weight, diet, concurrent medication, overall clinical condition, the particular compound employed, the specific use for which the compound or pharmaceutical composition is employed, and other factors which those skilled in the medical arts will recognize.

B. Therapeutically effective amount

[0113] Dosage formulations of a reversible, direct acting P2Yi$_2$ receptor inhibitor, or pharmaceutical compositions contain a reversible, direct acting P2Y$_i$$_2$ receptor inhibitor of the invention, to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile membranes such as 0.2 micron membranes, or by other conventional methods. Formulations typically will be stored in a solid form, preferably in a lyophilized form. While the preferred route of administration is orally, the dosage formulations of a reversible, direct acting P2Yi$_2$ receptor inhibitor or pharmaceutical compositions of the invention may also be administered by injection, intravenously (bolus and/or infusion), subcutaneously, intramuscularly, colonically, rectally, nasally, transdermally or intraperitoneally. A variety of dosage forms may be employed as well including, but not limited to, suppositories, implanted pellets or small cylinders, aerosols, oral dosage formulations and topical formulations such as ointments, drops and dermal patches. The reversible, direct acting P2Y$_i$$_2$ receptor inhibitor and pharmaceutical compositions of the invention may also be incorporated into shapes and articles such as implants which may employ inert materials such biodegradable polymers or synthetic silicones as, for example, SILASTIC, silicone rubber or other polymers commercially available. The compounds and pharmaceutical compositions of the invention may also be administered in the form of
liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of lipids, such as cholesterol, stearylamine or phosphatidylcholines.

[0114] Therapeutically effective dosages may be determined by either in vitro or in vivo methods. For each particular compound or pharmaceutical composition of the present invention, individual determinations may be made to determine the optimal dosage required. The range of therapeutically effective dosages will be influenced by the route of administration, the therapeutic objectives and the condition of the patient. For injection by hypodermic needle, it may be assumed the dosage is delivered into the body's fluids. For other routes of administration, the absorption efficiency must be individually determined for each compound by methods well known in pharmacology. Accordingly, it may be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. The determination of effective dosage levels, that is, the dosage levels necessary to achieve the desired result, will be readily determined by one skilled in the art. Typically, applications of compound are commenced at lower dosage levels, with dosage levels being increased until the desired effect is achieved.

[0115] The determination of effective dosage levels, that is, the dosage levels necessary to achieve the desired result, i.e., platelet ADP receptor inhibition, will be readily determined by one skilled in the art. Typically, applications of a compound or pharmaceutical composition of the invention are commenced at lower dosage levels, with dosage levels being increased until the desired effect is achieved. The compounds and compositions of the invention may be administered orally in an effective amount within the dosage range of about 0.01 to 1000 mg/kg in a regimen of single or several divided daily doses. If a pharmaceutically acceptable carrier is used in a pharmaceutical composition of the invention, typically, about 5 to 500 mg of a reversible, direct acting P2Y12 receptor inhibitor is compounded with a pharmaceutically acceptable carrier as called for by accepted pharmaceutical practice including, but not limited to, a physiologically acceptable vehicle, carrier, excipient, binder, preservative, stabilizer, dye, flavor, etc. The amount of active ingredient in these compositions is such that a suitable dosage in the range indicated is obtained.

[0116] In one group of embodiments, the P2Y12 receptor inhibitor is administered at a time interval selected from the group consisting of once per day and twice per day. In another group of embodiments, the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is from at least about 300 to about 450 mg. In another group of embodiments, the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is from at least
about 50 to about 150 mg. In another group of embodiments, the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is from at least about 75 to about 100 mg. In another group of embodiments, wherein the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is at least about 20 to about 100 mg. In another group of embodiments, wherein the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is at least about 45 to about 90 mg. In another group of embodiments, wherein the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is at least about 50 to about 60 mg.

[0117] In another group of embodiments, the invention provides an oral dose of the reversible, direct-acting P2Y12 receptor inhibitor of about 45 mg to about 50 mg to about 60 mg to about 75 mg to about 90 mg to about 100 mg to about 150 mg to about 300 to about 450 mg (see, U.S. Pat. Application No. 61/334068, filed May 12, 2010). In one group of embodiments, these dosage amounts are provided in a single dose.

[0118] In one group of embodiments, the invention provides an intravenous/injectable dose of the reversible, direct-acting P2Y12 receptor inhibitor of between about 0.1 mg to about 1 mg to about 5 mg to about 10 mg to about 15 mg to about 17 mg to about 20 mg to about 23 mg to about 25 mg to about 40 mg to about 50 mg to about 75 mg to about 80 mg to about 100 mg to about 120 mg to about 125 mg to about 150 mg to about 175 mg to about 200 mg and to about 250 mg (see, U.S. Pat. Application No. 61/329725, filed April 30, 2010). In one group of embodiments, these dosage amounts are provided in a single dose.

C. Administration

[0119] Therapeutic compound liquid formulations generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by hypodermic injection needle.

[0120] Typical adjuvants which may be incorporated into tablets, capsules, lozenges and the like are binders such as acacia, corn starch or gelatin, and excipients such as microcrystalline cellulose, disintegrating agents like corn starch or alginic acid, lubricants such as magnesium stearate, sweetening agents such as sucrose or lactose, or flavoring agents. When a dosage form is a capsule, in addition to the above materials it may also contain liquid carriers such as water, saline, or a fatty oil. Other materials of various types may be used as coatings or as modifiers of the physical form of the dosage unit. Sterile compositions for injection can be formulated according to conventional pharmaceutical
practice. For example, dissolution or suspension of the active compound in a vehicle such as an oil or a synthetic fatty vehicle like ethyl oleate, or into a liposome may be desired. Buffers, preservatives, antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

5

D. Kits

[0121] It is to be understood that the methods of the invention described herein generally may further comprise the use of a kit according to the invention. Generally, the diagnostic methods of the invention may be performed ex-vivo, and such ex-vivo methods are specifically contemplated by the present invention. Also, where a method of the invention may include steps that may be practiced on the human or animal body, methods that only comprise those steps which are not practiced on the human or animal body are specifically contemplated by the present invention. In a preferred embodiment, such kit may comprise a DNA sample collecting means.

10 [0122] Thus the invention also encompasses kits for detecting the presence of a polypeptide or nucleic acid corresponding to a marker of the invention in a biological sample, e.g., any body fluid including, but not limited to, serum, plasma, lymph, cystic fluid, urine, stool, csf, acitic fluid or blood and including biopsy samples of body tissue. For example, the kit can comprise a labelled compound or agent capable of detecting a polypeptide or an mRNA encoding a polypeptide corresponding to a marker of the invention in a biological sample and means for determining the amount of the polypeptide or mRNA in the sample, e.g., an antibody which binds the polypeptide or an oligonucleotide probe which binds to DNA or mRNA encoding the polypeptide. Kits can also include instructions for interpreting the results obtained using the kit.

15 [0123] For oligonucleotide-based kits, the kit can comprise, e.g., 1) an oligonucleotide, e.g., a detectably-labelled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to a marker of the invention; or 2) a pair of primers useful for amplifying a nucleic acid molecule corresponding to a marker of the invention.

20 [0124] The kit can also comprise, e.g., a buffering agent, a preservative or a protein-stabilizing agent. The kit can further comprise components necessary for detecting the detectable-label, e.g., an enzyme or a substrate. The kit can also contain a control sample or a series of control samples, which can be assayed and compared to the test sample. Each component of the kit can be enclosed within an individual container and all of the various
containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit.

[0125] The kits of the invention may contain a written product on or in the kit container. The written product describes how to use the reagents contained in the kit to determine whether a patient will experience hepatotoxicity during drug treatment. In several embodiments, the use of the reagents can be according to the methods of the invention. In one embodiment, the reagents are primer pairs for performing PCR analysis of 2C19*2 genetic polymorphisms.

EXAMPLES

[0126] The following examples are offered to illustrate, but not to limit, the claimed invention.

Example 1. Oral Dosing of Elinogrel Overcomes High Platelet Reactivity in Patients Non-responsive to Clopidogrel Therapy and Independent of CYP 2C19*2 Genotype

Patients and Study Design

[0127] Inclusion criteria were clinically stable patients older than 18 years of age who had undergone previous coronary artery stenting and were treated with chronic daily 75 mg clopidogrel and 81 mg aspirin therapy. Fifty patients were screened for HPR (5 patients with HPR had been previously identified). Patients were instructed to take clopidogrel at least 12 hours but no more than 16 hours prior to the initial screening visit (Figure 1). Exclusion criteria were any history of a bleeding diathesis or gastrointestinal bleeding; stroke or transient ischemic attack of any etiology within 30 days of screening, illicit drug or alcohol abuse, consumption of grapefruit or grapefruit juice 48 hours prior to dosing, coagulopathy, major surgery within 6 weeks prior to screening, planned surgical procedure within 30 days of anticipated dosing, enrollment in an investigational drug study within 30 days of screening, a medical or surgical condition which may impair drug absorption or metabolism, platelet count <100,000/mm3, hematocrit <30%, creatinine >2mg/dL, or current use of nonsteroidal anti-inflammatory drugs, anticoagulants, dietary supplements, herbal products or antiplatelet drugs other than aspirin or clopidogrel within 2 weeks of screening. Seven to 14 days after the screening visit patients underwent platelet function testing and were administered a single 60 mg oral dose of elinogrel between 12 and 16 hours after the previous day's dose of clopidogrel. AU subjects were instructed to continue their maintenance aspirin and clopidogrel therapy for the duration of the investigation. Throughout the study, patients
continued their daily therapy of clopidogrel and aspirin and compliance was assessed. Follow-up visits were at 24 hours and 7 days post-dosing with elinogrel.

**Elinogrel Drug Product**

[0128] Elinogrel was supplied by Portola Pharmaceuticals, Inc. as a powder stored at room temperature and was reconstituted with sterile water by an in-hospital pharmacist as a solution containing 2.28 mg/mL. The solution was stored at room temperature to be used within 24 hours of preparation; 30 mL (60 mg) were administered orally to patients together with 100 mL of water.

**Blood and Urine Collection**

[0129] Blood was collected from the antecubital vein with an 18 gauge needle at screening and at predosing 12-16 hours after the previous day’s clopidogrel dose and then at 4 hours, 6 hours, 24 hours, and 7-10 days after dosing with elinogrel. Three Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ) containing 3.2% trisodium citrate were used for LTA (5µM and 20µM ADP and 2mM arachidonic acid), thrombelastography (TEG) and vasodilator-stimulated phosphoprotein (VASP) phosphorylation measurements; two tubes containing a proprietary anticoagulant CT921-78 (factor Xa inhibitor; Portola Pharmaceuticals, San Francisco, CA) were used for LTA (10µM ADP and 4µg/mL collagen) and for the perfusion chamber assay (PCA) to ensure physiologic calcium concentrations; one tube containing lithium heparin (Becton- Dickinson, Franklin Lakes, NJ) was used for TEG PlateletMapping™; and one tube containing 3.2% sodium citrate (Greiner Bio-One Vacuette® North America, Inc., Monroe, NC) was used for VerifyNow™ assay. One tube containing ethylene diamine tetraacetate (1.8mg/ml) was used for elinogrel plasma concentration measurements (Becton-Dickinson, Franklin Lakes, NJ). Urine samples were collected at screening and on the day of dosing prior to administration of elinogrel to test for pregnancy in women of childbearing potential and to assess for amphetamine, methamphetamine, cocaine, opiate and tetrahydrocannabinol (THC) use.

**Light Transmittance Aggregometry**

[0130] Platelet aggregation was assessed as previously described using a Chronolog Lumi-Aggregometer (Model 490-4D) with the Aggrolink software package (Chronolog, Havertown, PA) (Gurbel et al. Circulation 2003;107:2908-13). Maximum aggregation was expressed as the maximum percent change in light transmittance from baseline in platelet rich
plasma, using platelet poor plasma as a reference. Final aggregation was measured at 6 minutes after the addition of an agonist.

**VerifNow™ P2Y_{i2} Assay**

[0131] The VerifyNow™ is a turbidimetric based optical detection assay designed to measure platelet aggregation that is based upon the ability of activated platelets to bind to fibrinogen. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads. The change in optical signal is reported as P2Y_{i2} Reaction Units (PRU).

**Thrombelastograph® (TEG) Hemostasis System with PlateletMapping™**

[0132] The TEG Hemostasis Analyzer with PlateletMapping assay (Haemoscope Corporation, Niles, Illinois) relies on the measurement of platelet-fibrin clot strength to enable a quantitative analysis of platelet function (Gurbel et al. *J Am Coll Cardiol* 2005;46:1820-6). In heparinized blood, reptilase and factor XIIIa are used to generate a cross-linked fibrin clot. The contribution of the P2Y_{i2} receptor to platelet-fibrin clot strength is measured by the addition of ADP and is expressed as MAADP (mm).

**Vasodilator Stimulated Phosphoprotein Phosphorylation**

[0133] VASP phosphorylation was determined in whole blood using a flow cytometric assay [Platelet VASP; Diagnostica Stago (Biocytex), Asnieres, France] as previously described (Gurbel et al. *J Am Coll Cardiol*. 2005;46:1827-32). The platelet reactivity index (PRI) was calculated from the mean fluorescence intensity (MFI) according to the formula:

\[
PRI = [(\text{MFI}_{\text{PGEI}} - \text{MFI}_{\text{PGEI+ADP}}) / \text{MFI}_{\text{PGEI}}] \times 100\%.
\]

**Capillary Perfusion Chamber Preparation and Real Time Thrombosis Profiler fRTTP-2**

[0134] Rectangular capillaries with 0.2 mm x 2 mm sections (Vitrocom, Mountain Lakes, NY) were coated with human type III fibrillar collagen (Chronolog Corp, Havertown, PA) as previously described (Andre et al. *Arterioscler Thromb Vase Biol* 1996;16:56-63). Evaluation of thrombotic deposits was performed at 8 mm from the proximal end of the capillary. Experiments were completed within 1 hour of blood sampling.

[0135] The RTTP-2 consists of an epifluorescence microscope to monitor thrombus formation and a syringe pump (Harvard Apparatus, Holliston, MA) to establish the desired flow and wall shear rate in the capillary perfusion chamber. Platelets were labeled by incubating rhodamine 6G (final concentration 1.25 µg/ml, EMD, Gibbstown, NJ) in the whole blood at 37 °C for 15 min. A high-power light emitting diode with a spectral maximum
at 530 nm and a spectral half width of 35 nm (Luxeon V, Lumileds Lighting, San Jose, CA) excited the dye. Excitation and emission light were filtered with a set of fluorescence filters (31002, Chroma Technologies, Rockingham, VT). A microscope objective images an area of 360 x 270 µm on the internal wall of the capillary onto a Sony XCD X-710 digital camera (resulting magnification ca. 13 x). Images were recorded at a frequency of 1 Hz. A personal computer with custom software was used to control the camera, the syringe pump, to display experimental conditions, and record images.

**Measurement of Thrombosis Kinetics**

[0136] Thrombus size was represented as the measurement of the fluorescence intensity divided by total area. Segmentation, partitioning of an image into non-overlapping regions, was accomplished based on a method proposed by Otsu (Otsu et al. *IEEE Trans. Syst. Man Cybern* 1979;SMC-9:62-66). Watershed algorithm was applied to identify individual thrombi in the image (Gonalez et al. Digital image processing. 2003, Prentice Hall, New Jersey). Once the image was segmented, total object volume, area and perimeter were computed.

Total volume was computed as sum of intensity values of pixels inside the foreground objects. Total area was computed as number of pixels inside the foreground objects. Data were expressed as fluorescence intensity (pixels)/total area (µm$^2$).

**Analysis of Plasma Concentration**

[0137] Blood samples were centrifuged and plasma was harvested and stored at -20°C until analysis. Plasma was extracted with acetonitrile (protein precipitation) and analyzed for elinogrel concentration using liquid chromatography followed by tandem mass spectrometry. The analytical range was 0.500 to 500 ng/mL.

**Genotype Analysis**

[0138] Genotyping of the known common loss of function CYP2C19*2 variant (rs4244285), as well as other functional variants of CYP2C19 [*3 (rs4986893), *5 (rs56337013), *17 (rs12248560)] and CYP3A5*3 (rs776746) was performed using TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA).

**Adverse Events**

[0139] Adverse events (AE) were defined as any untoward medical occurrence in a subject that may or may not have been due to treatment with elinogrel, including any unfavorable or unintended sign, symptom, or disease temporarily associated with the use of the study drug, whether or not it was considered study-drug related. This included any newly occurring event
or previous condition that had increased in severity or frequency since the administration of study drug. Serious adverse events (SAE) were defined as the occurrence of any event regardless of causality that resulted in death, was life-threatening, required inpatient hospitalization, resulted in persistent or significant disability/incapacity, or was an important medical event (defined as an event that may not have resulted in death, was life-threatening, or required hospitalization but may have been considered an SAE when, based upon appropriate medical judgment, may have jeopardized the subject and may have required medical or surgical intervention).

Statistical Analysis and Sample Size Determination

Categorical variables were expressed as absolute numbers and percentages and compared using chi-square test. Continuous variables were expressed as mean +/- SD. Overall comparisons of platelet function measurements were performed by 1 way ANOVA. Comparisons between predosing and post-dosing platelet function measurements were performed by post-hoc Bonferroni multiple comparisons t-test or Kruskal-Wallis ANOVA on ranks followed by Dunn's multiple comparisons method as appropriate. Platelet function measurements at screening in screen failure and treated patients were compared by t-test; p<0.05 was considered significant (SigmaStat software, Point Richmond, CA). The relationship between plasma concentration and the ADP-induced maximum aggregation at the ADP concentrations of 5-, 10- and 20-µM was evaluated using WinNonlin program (version 5.2, Pharsight Corp., Cary, North Carolina). Platelet reactivity measurements were compared in *2 and *17 genotype groups by 1 way ANOVA.

The primary objective was to evaluate the change in 5µM ADP-induced aggregation from pre- to 4 to 6 hours post- clopidogrel treatment in patients with HPR currently on clopidogrel therapy. In order to detect an absolute change (mean of upper tertile minus lower tertile cutpoint) of 20% (52% to 32%) in 5µM ADP-induced platelet aggregation with a standard deviation of 10, a sample size of approximately 10 subjects is required to give a 95% power with an alpha of 0.05 (Bliden et al. JAm Coll Cardiol 2007;49:657-66).

Patients and Demographics

Fifty patients were screened; 5 patients were excluded at screening. Screening platelet function analyses were performed in 45 patients; 20 patients had HPR (Figure 1). Blood sampling was complete for all timepoints except in two patients who did not return for the follow up visit (7-10 days). Compliance with antiplatelet therapy was 100% based on patient confirmation. Demographics are shown in Table 1. Most patients were Caucasian and
cardiovascular risk factors were common. Patients with HPR were more often diabetic, had a
greater prevalence of prior myocardial infarction, coronary intervention, restenosis and
cerebrovascular accident than screen failure patients. Elinogrel was well tolerated in all
patients and there were no adverse events.

Platelet Function Analyses

As defined by the inclusion criteria, patients selected for elinogrel treatment at the
screening visit had higher maximum platelet aggregation induced by 5, 10, and 20µM ADP
than screen failure patients (53±10% vs. 33±14%, 51+13% vs. 37±13%, 63+11% vs. 44+15%
respectively; p<0.001 for all measurements). Arachidonic acid-induced aggregation was
8±19% in elinogrel treated patients indicative of aspirin responsiveness and indicating
general adherence to the chronic treatment regimen. Measurement of ADP-induced platelet
aggregation at the pre-dosing time demonstrated that the HPR phenotype was stable for 7 to
14 days (p = NS vs. screening for all concentrations of ADP). There was a significant change
in platelet function over time after elinogrel administration as measured by all methods (p
<0.006).

ADP-induced Platelet Aggregation (LTA)

The individual patients’ response to elinogrel measured by 5µM ADP-induced
platelet aggregation is shown in Figure 2. The mean absolute decrease in maximum platelet
aggregation in response to 5µM ADP in elinogrel treated patients was -22% at 4 hours post-
dosing. The figure indicates that: (1) the primary endpoint of the study, was achieved at 4 hrs
(mean aggregation fell from the top to the bottom tertile) and was also significantly reduced
at 6 hrs, (2) The majority of patients showed a significant decrease in platelet aggregation at 4
or 6 hrs, relative to their baseline value, and (3) most of the patients had a stable HPR
phenotype that persisted from the screening visit to the predose visit, and was reproduced at
the follow-up visit, despite ongoing dual antplatelet therapy. The antplatelet effect of
elinogrel was rapid. Aggregation significantly fell in response to all concentrations of ADP at
4 hours (the earliest time point evaluated) and 6 hours post-dosing, the earliest time point
evaluated, and the antplatelet effect of elinogrel was reversible within 24 hours (Figures 3-
5). The mean extent of maximum aggregation at 4 hrs with 5 uM ADP met the predetermined
endpoint of dropping from the highest to lowest tertile (< 32% maximum aggregation).
Collagen-induced Aggregation (TTA)

There was a non-significant decrease in collagen aggregation at 4 and 6 hours post-dosing (Figure 6).

Thrombelastography (TEG) Platelet Mapping Assay

[0145] The rapid and reversible antiplatelet effect of elinogrel was also demonstrated by thrombelastography; MAADP decreased at 4 and 6 hours post-dosing and at 24 hours post-dosing did not differ from pre-dosing values (Figure 7).

VerifNow P2Y1 Receptor Assay

[0146] PRU also decreased at 4 and 6 hours post-dosing and the effect was reversible at 24 hours postdosing (Figure 8).

Vasodilator Stimulated Phosphoprotein (VASP)

[0147] VASP PRI significantly decreased from baseline at 4 and 6 hours post-treatment and again the effect was reversible at 24-hours post-dosing (Figure 9).

Real Time Thrombosis Profiler (RTTPV2)

[0148] Concordant with the other platelet function analyses, there was a significant decrease in thrombus size [fluorescence intensity / area (uim)] at 4 and 6 hours post-dosing and the inhibitory effect on thrombus size was reversible at 24 hours (Figure 10).

Pharmacokinetics/Pharmacodynamics Relationship

[0149] The mean (± SD) observed maximum plasma concentration of elinogrel achieved at the median Tmax (4hrs) was 2459 (± 1460) ng/nL following the administration of 60 mg elinogrel (Figure 11). The relationship between plasma concentration and the ADP-induced maximum aggregation could be described by an inhibitory effect Emax model. The ICso (inhibitory constant) increased with increasing ADP concentrations, as expected for a competitive, reversible, direct acting P2Yi2 antagonist. The ICso for the 5-, 10-, and 20 uM ADP-induced aggregation were 2230, 2412, and 5852 ng/mL, respectively. A good correlation between plasma concentrations of elinogrel and pharmacodynamic measurements was observed in all assays, except for collagen induced aggregation.

Genotype Analysis and Relation to Platelet Function

[0150] Seventeen patients with HPR and 19 without HPR consented to genetic screening. The results are shown in Table 2. At least one CYP2C19*2 allele was present in 44%: the *2
allele frequency was higher in HPR patients than in patients without HPR (13/17 (77%) vs. 3/19 (16%), p=0.0004). All patients with HPR who were CYP2C19*2 negative were diabetic and 3/4 were CYP2C19*17 positive. The presence of CYP2C19*17 carriers was numerically less in HPR patients (29% vs. 53%, p = 0.08) relative to patients that did not have HPR. The CYP 2C19*3, and *5 and CYP 3A5*3 alleles were not present in any patient. Platelet reactivity was higher in CYP2C19*2 carriers during clopidogrel and aspirin therapy alone (Table 3). Platelet reactivity fell in CYP2C19*2 carriers and non-carriers after elinogrel administration (Figure 12).

The above example demonstrated that HPR accompanying standard maintenance clopidogrel and aspirin therapy in stable patients who had undergone prior coronary stenting is rapidly and reversibly overcome by a single 60mg oral dose of elinogrel. The strong additive antiplatelet effect of elinogrel was demonstrated by concordant results from multiple assays which measure P2Yi2 receptor reactivity. The pharmacodynamic effect followed a concordant pharmacokinetic response. Platelet inhibition occurred within 4 hours of drug administration, the earliest time point evaluated, and corresponded to maximal plasma concentrations of the drug, and platelet function returned to pre-dose levels within 24 hours when drug levels were minimal as indicated by plasma concentrations of 200-300 ng/mL. This corresponds to the time of maximum drug concentration of elinogrel observed in previous studies in healthy subjects (Conley et al. ASH Annual Meeting Abstracts), Nov 2006; 108: 900). This example demonstrates that the CYP2C19*2 allele is strongly associated with HPR observed during clopidogrel and aspirin therapy. However, a single 60 mg oral dose of elinogrel can overcome HPR in patients who are either wild type (CYP2C19*1) or who have at least one CYP2C19*2 allele.

Elinogrel is a direct-acting, non-prodrug, competitive inhibitor of the P2Yi2 receptor available in both oral and parenteral formulations. It has a terminal half-life of approximately 12 hours, is cleared by both renal and hepatic routes and undergoes limited metabolism. When given as an intravenous bolus, immediate and full platelet inhibition of ADP-induced platelet aggregation was observed (Lieu et al. J Thromb Haemost 2007; 5 Supplement 2: P-T-292). Moreover, intravenous bolus doses up to 60 mg administered concurrently with standard therapy were well tolerated in patients undergoing primary angioplasty for ST16 elevated myocardial infarction.

Additional platelet inhibition has been demonstrated in patients treated with chronic maintenance clopidogrel and aspirin therapy by administration of a loading dose of clopidogrel (Kastrati et al. Circulation 2004;110:1916-9). Bonello et al. demonstrated...
additional platelet inhibition by measuring VASP phosphorylation levels in patients with high platelet reactivity who were repeatedly treated with 600mg clopidogrel up to a total dose of 2400mg over three days (Bonello et al. J Am Coll Cardiol 2008;51: 1404-11).

The third generation thienopyridine, prasugrel, has attempted to address the limitations of delayed and variable inhibition by clopidogrel. However, irreversible platelet inhibition often precludes prepercutaneous intervention administration of thienopyridines until the coronary anatomy is known. A greater frequency of bleeding was observed with prasugrel versus clopidogrel, with a 4-fold increase seen in patients undergoing coronary bypass graft surgery as well as increased bleeding during chronic prasugrel administration (Wiviott et al. N Engl J Med 2007; 357:2001-15). However, emerging data with direct-acting, non-thienopyridine, reversible agents such as elinogrel demonstrate that these agents can achieve immediate high level blockade following parenteral bolus administration, and may have a broader therapeutic index than the irreversible thienopyridine inhibitors, in that they have less effect on hemostasis at equivalent levels of antithrombotic activity in preclinical studies (Andre et al. J Thromb Haemost 2007; 5 Supplement 2: O-W-031).

Current observations of a readily reversible pharmacodynamic effect suggest that CABG bleeding may be reduced if performed 24 hours after a single 60 mg dose of elinogrel. The reversibility after an intravenous dose may be even more rapid due to a < 1 hour distribution half-life after an intravenous bolus. Moreover, in patients undergoing surgery, the hiatus in antiplatelet therapy may be shortened considerably with a reversible agent such as elinogrel compared to the present guidelines recommending withholding clopidogrel therapy for 5-7 days (Eagle et al. Circulation 2004; 110: e340-437). Thus, a rapid-acting, reversible agent, such as elinogrel can reduce bleeding risk in both the chronic and acute settings, and allow for easier management of surgical procedures for patients with drug eluting stents who require dual antiplatelet therapy.

The current study used a simple liquid formulation of elinogrel. The formulation used in the ongoing phase II Randomized, Double-Blind, Active-Controlled Trial to Evaluate Intravenous and Oral PRT060128, a Selective and Reversible P2Y12-Receptor Inhibitor, vs. Clopidogrel, as a Novel Antiplatelet Therapy in Patients Undergoing Non-Urgent Percutaneous Coronary Intervention (INNOVATE-PCI) trial is an immediate release tablet. The 60 mg dose in the present study achieved plasma concentrations that are at the low end of the range being studied in INNOVATE-PCI trial (50, 100, 150 mg twice daily). The combination of intravenous and oral elinogrel will allow for a seamless transition from the acute to the chronic setting, and avoids the issues of transitioning from an intravenous
reversible inhibitor such as cangrelor, to a thienopyridine prodrug, where the presence of the competitive reversible inhibitor has been shown to block the ability of the active metabolite of clopidogrel or prasugrel to inhibit platelets by irreversibly binding the P2Yi2 receptor (Dovlatova et al. *J Thromb Haemost* 2008;6: 1153-9; Steinhubl et al. *Thromb Res* 2008;121:527-34).


The other loss of function alleles, CYP2C19*3, and *5 were not present in the patients. In line with its direct inhibition of the P2Yi2 receptor, the antiplatelet effect of elinogrel was observed irrespective of CYP2C19*2 status. The increased function allele, CYP2C19*17 was frequent in this example with patients that did not have HPR, while patients with HPR had a numerically lower prevalence of this allele.

The CYP 2C19*2 allele is strongly associated with HPR observed during conventional dual antiplatelet therapy. Elinogrel rapidly and reversibly overcomes HPR in patients on clopidogrel and aspirin therapy.

The demographics of clinical pharmacogenetic analysis participants is shown in Table 1:
Table. 1 Patients Demographics

<table>
<thead>
<tr>
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<th>Elinogrel Treated Patients (n=20)</th>
<th>Screen Failure Patients (n=25)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>61 ± 12</td>
<td>65 ± 12</td>
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<td>Gender n, (male %)</td>
<td>13 (65)</td>
<td>11 (44)</td>
<td>0.08</td>
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<td>Caucasians n, (%)</td>
<td>14 (70)</td>
<td>18 (72)</td>
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<td>African-Americans n, (%)</td>
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<td>7 (28)</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>32 ± 6</td>
<td>29 ± 6</td>
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<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>128 ± 16</td>
<td>133 ± 74</td>
<td>0.77</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mm Hg)</td>
<td>71 ± 12</td>
<td>74 ± 16</td>
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<tr>
<td>History of Smoking n, (%)</td>
<td>7 (35)</td>
<td>8 (32)</td>
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<td>Current Smokers n, (%)</td>
<td>0 (0)</td>
<td>3 (12)</td>
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<td>Hypertension n, (%)</td>
<td>17 (85)</td>
<td>19 (76)</td>
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<tr>
<td>Hyperlipidemia n, (%)</td>
<td>16 (80)</td>
<td>17 (68)</td>
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<td>Diabetes n, (%)</td>
<td>12 (60)</td>
<td>5 (20)</td>
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<tr>
<td>Myocardial infarction n, (%)</td>
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<td>Coronary artery bypass graft n, (%)</td>
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<td>Percutaneous transluminal coronary angioplasty n, (%)</td>
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<td>Family History of coronary artery disease n, (%)</td>
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<td>Beta-blocker n, (%)</td>
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<td>Hemoglobin g/dL</td>
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<td>Creatinine mg/dL</td>
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Table 2. Patients Genotypes

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1 = presence of one allele, 2 = presence of two alleles, 0 = absence of any allele
Table 3. Pre-elinogrel Platelet Function in Relation to Genotype.

<table>
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<tr>
<th></th>
<th>Patients with CYP2C19*2 Allele (n=17)</th>
<th>Patients without CYP2C19*2 Allele (n=1.9)</th>
<th>p-value</th>
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<tr>
<td>5µM ADP-Induced Platelet Aggregation (%)</td>
<td>52 ± 13</td>
<td>36 ± 14</td>
<td>0.001</td>
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<td>20µM ADP-Induced Platelet Aggregation (%)</td>
<td>61 ± 12</td>
<td>50 ± 13</td>
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<tr>
<td>VASP Assay-Platelet Reactivity Index</td>
<td>72 ± 12</td>
<td>58 ± 20</td>
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<tr>
<td>VerifyNow P2Y12 assay-Platelet Reactivity Units</td>
<td>259 ± 68</td>
<td>214 ± 82</td>
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<tr>
<td>VerifyNow P2Y12 assay-Percent Inhibition</td>
<td>19 ± 19</td>
<td>35 ± 24</td>
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<tr>
<td>TEG-ADP-induced platelet-fibrin clot strength mm</td>
<td>57 ± 8</td>
<td>49 ± 12</td>
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<tr>
<td>TEG-Percent Inhibition</td>
<td>23 ± 9</td>
<td>36 ± 20</td>
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<table>
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<tr>
<th></th>
<th>Patients with CYP2C19*17 Allele (n=15)</th>
<th>Patients without CYP2C19*17 Allele (n=21)</th>
<th>p-value</th>
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<tr>
<td>5µM ADP-Induced Platelet Aggregation (%)</td>
<td>41 ± 19</td>
<td>45 ± 14</td>
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<tr>
<td>20µM ADP-Induced Platelet Aggregation (%)</td>
<td>51 ± 14</td>
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<tr>
<td>VASP Assay-Platelet Reactivity Index</td>
<td>60 ± 23</td>
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<td>VerifyNow P2Y12 assay-Platelet Reactivity Units</td>
<td>206 ± 91</td>
<td>251 ± 65</td>
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<tr>
<td>VerifyNow P2Y12 assay-Percent Inhibition</td>
<td>35 ± 28</td>
<td>24 ± 18</td>
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<tr>
<td>TEG-ADP-induced platelet-fibrin clot strength (mm)</td>
<td>51 ± 12</td>
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<tr>
<td>TEG-Percent Inhibition</td>
<td>35 ± 28</td>
<td>24 ± 18</td>
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0.17 ADP = adenosine diphosphate, VASP = vasodilator stimulated phosphoprotein, TEG = thrombelastography

Example 2: Differences between pharmacological inhibition and gene targeting of the ADP receptor with P2Y_{in} inhibitors.

Methods: Drugs

Clopidogrel was from Sequoia Research Products Ltd. (United Kingdom). Prasugrel was from Albany Molecular Research (USA).

Animals

C57/BL6J mice (Charles River) were used for determination of antithrombotic activities of clopidogrel and prasugrel, determination of their effects on primary hemostasis and ex vivo platelet aggregation studies. P2Y_{i2}-λ mice (on a pure C57/BL6J background (>10 times backcrossed)) were used for thrombosis and hemostasis studies. All experiments were performed by investigators blinded to the different treatments. All procedures conformed to institutional guidelines and to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, Md).

Intravital microscopy

Thrombosis on mouse mesenteric arteries (1000-1300 s-i) was performed and recorded as previously described with minor modifications. Platelets were labeled in situ using rhodamine 6G (0.2 mg/mL) administered through the tail vein 10 minutes before visualization of the arteries. Vessel-wall injury was induced by a 1x1-mm filter paper saturated with a 10% FeCb solution. After 5 minutes, the filter paper was removed and mesenteric arteries rinsed with warmed saline (37°C). Platelet vessel-wall interactions were recorded for 40 additional minutes or until full occlusion occurred and lasted for more than 40 seconds. C57B16J mice were orally gavaged 48, 24 and 2 hours prior to injury with vehicle control (0.5% methylcellulose), clopidogrel (0.25, 1.5, 15 and 50 mg/kg) or prasugrel (0.1, 0.3, 1, 3, 10 mg/kg), or 2 hours prior to injury with clinogrel (0.83, 2.5, 7.5, 20 and 60 mg/kg).
Video analysis

[0163] Thrombosis was analyzed in real time using Simple PCI software 1. The fluorescence intensity was recorded at a rate of 2 Hz for 40 minutes and plotted over time. Time to occlusion and time for appearance of first thrombus were analyzed.

Tail bleeding time measurement

[0164] Male mice (6-8 weeks old) were anesthetized (by subcutaneous injection) with ketamine cocktail (ketamine [40 mg/kg], xylazine [2.5 mg/kg], and acepromazine [0.75 mg/kg], Henry Schein, Melville, NY, USA) 6 minutes prior to tail transection. Mice were then placed in lateral recumbence on a firm dissecting board (Richard-Allan Scientific, Kalamazoo, MI, USA) with the tail straight out. Tails were transected 2 mm from the tip with a number 10 scalpel blade (Bard-Parker; Becton Dickinson, Franklin Lakes, New Jersey, USA) and immediately immersed into a 20-ml scintillation vial (Wheaton Science Products, Millville, NJ, USA) filled with 10 mL normal saline held at 37°C by an unstirred digitally-controlled water bath (VWR International, Buffalo Grove, IL, USA).

[0165] A stopwatch was started immediately upon trans-section to determine time to cessation of bleeding, frequency, and duration of re-bleed for a 15 minute period. C57B16J mice were orally gavaged with vehicle control, clopidogrel (1.5, 15 and 50 mg/kg) or prasugrel (1, 3, 10 mg/kg) 48, 24 and 2 hours prior to tail trans-section or 2 hours prior to trans-section with elinogrel (7.5, 20 and 60 mg/kg). In one set of experiments, P2Y12-/-mice were also orally gavaged (using the same regimen) with maximal doses of clopidogrel, prasugrel, elinogrel and vehicle control.

Tail blood loss measurement

[0166] Blood loss was assessed when collection of blood for bleed time determination had concluded. To measure blood loss volume, any blood collected as described above following tail transection was frozen at -80 °C overnight. After thawing the following day, 10 ml of deionized water was added to further induce hemolysis. Aliquots of each sample were analyzed via spectrophotometry (Spectramax Plus384, Molecular Devices, Sunnyvale, CA, USA) and diluted further (1/5, 1/10, or 1/20) if necessary. Resulting OD490nm values (% T) were compared against a previously-determined standard curve to estimate the blood volume lost.
Micropuncture-induced primary hemostasis in vivo in mesenteric veins

[0167] The in vivo primary hemostasis model was performed via micropuncture (using a 27 G needle) of mesenteric veins according to the model developed by van Gestel and colleagues (van Gestel et al. Microcirculation. Apr-May 2007;14(3):193-205). Briefly, 3-4 week-old anesthetized P2Yi2/- and WT mice previously orally gavaged (48, 24 and 2 hours prior to injury) with either 0.5% methylcellulose, clopidogrel (50 mg/kg), prasugrel (10 mg/kg) or elinogrel (60 mg/kg 2 hours prior to micropuncture) were injected with R6G (0.2 mg/ml) to fluorescently label platelets in situ. Bleeding time and platelet recruitment at site of micropuncture was recorded and quantified in real time using intravital microscopy.

Ex vivo platelet aggregation

[0168] C57B16J mice were orally gavaged 48, 24 and 3 hours prior to blood collection with 0.5% methylcellulose, clopidogrel (1.5, 15 and 50 mg/kg) or prasugrel (1, 3, 10 mg/kg) or 3 hours prior to blood collection (elinogrel, 7.5, 20 and 60 mg/kg). Measurements were performed using a 4-channel Chronolog lumiaggregometer using Aggrolink software. Blood samples (0.6 ml per anesthetized mouse) were obtained via intracardiac puncture (on 3.2% trisodium citrate lvol TSC : 9 vol of blood), centrifuged at 200xg for 10 minutes and platelet rich plasma collected. For each dose, PRP of three animals was pooled for aggregation measurements. Experiments for each dose were repeated on a minimum of 3 sets of three animals. Aliquots (250 µl) of PRP were placed in cuvettes containing magnetic stirrer bars, warmed at 37°C, and stirred for 1 minute to obtain a stable baseline. Aggregation in PRP was induced using ADP (Chronolog) at 1 and 10 µM final concentration and change in light transmittance was recorded for an additional 4 minutes.

Statistical analysis

[0169] Analysis was performed using the standard Student's t test. Statistical analysis of the blood loss study comparing doses of clopidogrel, prasugrel and elinogrel to P2Yi2-/- mice was performed using the two-tailed unpaired t-test. The nonlinear curve fitting calculations for time to occlusion vs plasma concentration was performed using Prism software. All values are expressed as mean ± SEM.
Determination of clopidogrel, prasugrel and elinogrel doses reproducing the thrombotic profile of P2Yi2-/-mice.

[0170] Antithrombotic activities of clopidogrel, prasugrel and elinogrel were evaluated in the FeCb thrombosis model and compared to the phenotype of P2Yi2-/- mice. Maximal (M) doses of clopidogrel (50 mg/kg), prasugrel (10 mg/kg) and elinogrel (60 mg/kg) were identified that shared the phenotypic characteristics associated with the genetic modulation of P2Yi2 (destabilization of growing arterial thrombi preventing vascular occlusion; Figure 14A). Some subtle differences were observed with clopidogrel when compared with prasugrel, elinogrel and P2Yi2-/- mice as it did not reach similar levels of inhibition on the initial rate of thrombus growth (p<0.0001 vs P2Yi2-/- for time for appearance of first thrombus; Figure 15A). Similarly, while preventing vascular occlusion, there was more fluorescent platelets deposited throughout the 40 min observation period (p=0.0023 vs P2Yi2-/-mice) in presence of clopidogrel. Prasugrel (10 mg/kg) and elinogrel (60 mg/kg) had a thrombosis phenotype identical to that of P2Yi2-/-mice (Figure 15). Doses of elinogrel (7.5 and 20 mg/kg), clopidogrel (1.5 and 15 mg/kg) and prasugrel (1 and 3 mg/kg) were identified as providing similar levels of inhibition on thrombus stability and classified intermediate (I) and high (H), respectively.

Pharmacological modulation by thienopyridines or elinogrel differentially affects blood loss vs gene targeting

[0171] The effects on bleeding frequency, (Figure 16) and blood loss (Figure 17) were studied the three doses of elinogrel, clopidogrel and prasugrel providing intermediate high and maximal levels of inhibition of arterial thrombosis and compared their effects to the phenotype of P2Yi2-/- mice. Most of the animals treated with placebo (n = 40) stopped bleeding before 2 min but subsequently displayed multiple periods of rebleed and cessation of blood loss. For the intermediate doses, 7 out of 10 (clopidogrel), 10 out of 10 (prasugrel) and 2 out of 10 animals (elinogrel) had continuous bleeding that lasted for 15 min (Figure 16). For the high doses, all animals administered clopidogrel, 9 out of 10 (prasugrel) and 3 out of 10 (elinogrel) displayed continuous bleeding. For the maximal doses, all animals treated with clopidogrel and prasugrel as well as P2Yi2-/- mice but only 4 out of 10 animals treated with elinogrel had continuous bleeding (Figure 16).

[0172] Blood loss measurement revealed differences between the three P2Yi2 antagonists and P2Yi2-/- mice. The volume of blood loss was significantly lower with elinogrel (20 and 60
mg/kg) than that of its corresponding clopidogrel counterpart doses (*, p<0.005; Figure 17A), and significantly lower when compared with its three equivalent prasugrel doses (+, p<0.005; Figure 17A). Maximal elinogrel dose (mean+sem plasma [c] = 3972+746 ng/ml) was associated with lower blood loss (152+62 µl; P=0.0519), while maximal clopidogrel and prasugrel doses significantly increased volume of blood loss when compared to P2Yi2-/-mice (clopidogrel, 551+43 µl; prasugrel, 561+73 µl; P2Yi2-/-mice, 293+38 µl, fP<0.0005). The ratio of fold increase in time to occlusion for P2Yi2 inhibition vs vehicle control over fold increase in volume of blood loss for P2Yi2 inhibition vs vehicle control indicated that for all doses, elinogrel possessed a better therapeutic index than thienopyridines while maximal doses of clopidogrel and prasugrel had lower therapeutic index than the knockout animals (Figure 17B).

**Clopidogrel and prasugrel increase the volume of blood loss in P2Yi2-/-mice (Figure 18)**

Since the maximal doses of clopidogrel and prasugrel extended blood loss beyond the level of the P2Yi2-/- mouse, their effects on measurement of blood loss in the knockout animals was evaluated. Oral administration of clopidogrel and prasugrel significantly increased the volume of blood loss in P2Yi2-/-mice (612.6+71, 802+49 µl vs 293.5+38 µl in P2Yi2-/-mice, P<0.001). In contrast, elinogrel did not significantly increase the volume of blood loss in P2Yi2-/-mice (441+66 vs 328+54 µl, respectively; P=0.2124).

**Reduced (thienopyridines) and increased (elinogrel) platelet deposition in a primary hemostasis model versus P2Yn -/- mice**

The micropuncture model is characterized by a rapid obliteration of the vascular wound by fluorescent platelets in the ~ 20 seconds that followed injury in all animals, independently of the treatment or genotype (See Figure 19A), followed by a reopening of the wound. As found in tail trans-section model, the maximal dose of elinogrel had less effect on the frequency of bleeding than its corresponding thienopyridines treatment doses or than the loss of P2Yi2 via gene targeting (Figure 19B). Measurement of the lumen of the vascular wound revealed differences between thienopyridine-treated and P2Y12-/- mice, and between elinogrel-treated and P2Yi2-/-mice (Figure 19C). The diameter of the lumen of thienopyridine-treated mice decreased at a slower pace, while elinogrel-treated animals had a faster rate of occlusion than P2Yi2-/-mice. These data paralleled the differences observed in the amount of platelets deposited at sites of micropuncture during the initial response (up to 5 min post-micropuncture; Figure 19D).
Mechanism of action-based differences differentially affects the thrombosis to bleeding ratio of elinogrel

[0175] At the dose recapitulating P2Yi2-deficiency phenotype on arterial thrombosis, elinogrel demonstrated lower effects on primary hemostasis in the tail trans-section model. Data obtained for the micropuncture model indicated that the amount of platelets deposited at sites of vascular wound was greater in the elinogrel-treated group than in the P2Yi2-deficient animals suggesting that the competitive, direct nature of elinogrel could account for this difference. The exogenous ADP concentration affects elinogrel and thienopyridine inhibition of platelet aggregation was then determined. The maximal dose of elinogrel failed at inhibiting platelet aggregation induced by 10 μM ADP (11% inhibition vs vehicle control) while maximally inhibiting aggregation induced by 1 μM ADP (Figure 20A). Levels of inhibition by thienopyridines agents were less affected by the exogenous concentration of ADP. Linear regression analysis showed that extent of aggregation (10 μM ADP) correlated with time to occlusion when clopidogrel and prasugrel were computed (n = 0.90) but no longer did when elinogrel data were included (n = 0.45; Figure 20B left panel). On the other hand, measure of ADP-induced platelet aggregation was more predictive of the volume of blood loss in the tail trans-section model for the two classes of P2Yi2 antagonists (Figure 20B, right panel).

In vivo vasoconstriction studies in mesenteric veins

[0176] Mesenteric veins (100-190 μm diameter) from 3 to 4 week-old anesthetized P2Yi2−/− or WT mice were observed using bright-field inverted microscopy. In a first set of experiments, mice were orally gavaged with 0.5% methylcellulose, clopidogrel (50 mg/kg), prasugrel (10 mg/kg) or elinogrel (60 mg/kg) for 3 consecutive days. Veins were stimulated by a superfusion of α,β metATP (Sigma-Aldrich; 10 μl of a 1 μM solution; a non-hydrolyzable P2X1 agonist not degraded by CD39) 2 hours after last oral gavage. Challenge and evaluation of the vascular tone were performed by an investigator blinded to the treatment regimen. In a second set of experiments, P2Yi2+/− mice were orally gavaged with prasugrel (10 mg/kg) for three consecutive days. Sixteen hours prior to superfusion of α,β metATP, P2Yi2−/− animals were injected with antiGPIIbα antibodies (2 mg/kg; Emfret laboratories) for platelet depletion. In a third set of experiments, WT mice were orally gavaged with prasugrel (10 mg/kg) for 3 consecutive days and their mesenteric veins
challenged 1, 2, 5, 10, 24 and 48 hrs after the last oral gavage. The diameter of the mesenteric veins was monitored for 5 minutes post challenge.

[0177] FIGS. 21A-D—illustrate how thienopyridines block vasoconstriction of mesenteric veins. FIG. 21A) illustrates vasoconstriction of mesenteric veins induced by 1 μM α,β metATP is inhibited by 3 days (D3) oral gavage with the maximal doses of clopidogrel (n = 5) and prasugrel (n = 7) but not elinogrel (n = 9). Vehicle control (n = 11) of P2Y12-deficiency (n = 10). FIG. 21B) illustrates how Prasugrel (M, 10 mg/kg, n = 6) but not vehicle control (0.5 % methyl cellulose, n = 6) inhibits initial response to 1 μM α,β metATP in platelet-depleted P2Y12-/- mice. FIG. 21C) illustrates Prasugrel dose response on α,β metATP-induced vasoconstriction (I, n = 9; H, n = 9; M, n = 4). FIG. 21D) shows the time-dependent effects of Prasugrel (M, 10 mg/kg) on vasoconstriction 1 (n = 4), 2 (n = 4), 5 (n = 4), 10 (n = 7), 24 (n = 10) and 48 hours (n = 10) following administration of the third dose.

[0178] The present invention directly assessed P2Yi2 contribution to primary hemostasis via comparison of the effects of genetic targeting to those attributed to irreversible (clopidogrel, prasugrel) and reversible (elinogrel) pharmacological inhibitors. The present invention revealed novel and unexpected findings: first, thienopyridine agents in the mouse increase bleeding beyond the levels attributed to P2Yi2 inhibition; second, the competitive, reversible nature of elinogrel allows for a subtle dissociation between effects on arterial thrombosis and primary hemostasis.

[0179] Previous studies performed in rats and rabbits have also reported that clopidogrel produced an unexpectedly large amount of bleeding compared to the inhibition of platelet aggregation or thrombosis (Wong et al. Thromb Haemost. Jan 2009;101(1): 108-1 15; Wong et al. J Cardiovasc Pharmacol. May 2007;49(5):3 16-324; Schumacher et al. J Pharmacol Exp. Ther. Jul 2007;322(l):369-377). The data obtained in mice show that doses of thienopyridines that reproduced the phenotype of P2Yi2-/- mice on thrombosis were accompanied by volumes of blood loss that were beyond the levels attributed to gene targeting. Since these maximal doses of clopidogrel and prasugrel achieved equivalent inhibition of thrombosis as the P2Yi2-deficient mice, it is unlikely that a compensatory mechanism in the P2Yi2-deficient mice is responsible for improved hemostasis in these animals, relative to those treated with thienopyridines. When dosed into P2Yi2-deficient animals both clopidogrel and prasugrel further increased the volume of blood loss. In addition, thienopyridine doses showing only intermediate effects on arterial thrombosis induced levels of blood loss similar (clopidogrel) to or greater (prasugrel) than that of the
P2Y<sub>i2</sub>-null mouse. The results of this invention indicate that clopidogrel and prasugrel (whose active metabolites share high structural similarities), can both act on molecular targets other than P2Y<sub>i2</sub>. A similar observation was made in the micropuncture model applied to mesenteric veins. In this model, fewer platelets were deposited in the thienopyridinatreated groups when compared to P2Y<sub>i2</sub>-/-mice suggesting a platelet P2Y<sub>i2</sub>-independent effect under these low shear conditions (although one should not exclude a possible direct or indirect effect of the drugs on the vasomotor properties of the vascular wall). Thus, two different models of hemostasis in the mouse suggest that part of the bleeding associated with high doses of clopidogrel and prasugrel is unrelated to their primary function (P2Y<sub>i2</sub>-inhibition).

[0180] As opposed to the thienopyridines, elinogrel was accompanied by less bleeding when compared with P2Y<sub>i2</sub>-/-mice. This is likely attributable to the competitive, reversible, nature of elinogrel. An illustration of the impact the competitive and reversible nature of elinogrel has on platelet biology was highlighted in ex vivo platelet aggregation studies, in which 10 µM ADP overcame the inhibitory effects of the maximal dose of elinogrel which totally prevented arterial occlusion. Various observations come together to substantiate localized areas of greatly varying ADP concentrations within the blood vessel and growing thrombus as a whole. Born and Kratzer reported that damaged cells in the severed vessel wall are the primary source of ADP and ATP (Born et al. J Physiol Sep 1984;354:419-429), thus creating an ADP concentration gradient across the blood vessel lumen in such a way that highest concentrations are found closest to the vessel wall, i.e. the site of hemostasis or initiation of arterial thrombosis. Since ADP concentrations can exceed 5 µM under low shear environment, a greater competition between ADP and elinogrel is expected to occur and to reduce the effect of the reversible inhibitor on the activation level and pro-coagulant activity of the platelets (Leon et al. Blood. Jan 15 2004;103(2):594-600; van der Meijden et al. Thromb Haemost. Jun 2005;93(6): 1128-1 136). Increased amount of platelets deposited at the wound of mesenteric veins of mice treated with elinogrel were found vs P2Y<sub>i2</sub>-/-mice. Conversely, lower amounts of ADP are expected to be released by platelet-platelet interactions under arterial shear rates.

[0181] Moake and collaborators have shown that only 4-5 % (300-400 nM) of the ADP platelet content was released upon high shear stress-induced platelet aggregation in presence of large vWF multimers (Moake et al. Blood. May 1988;71(5):1366-1374) and the mathematical model proposed by Folie and McIntire estimated the ADP concentration present in the vicinity of a thrombus forming at 1500s-i to be around 1 µM (Folie et al.
The present studies show that ADP concentrations as low as 300 nM were sufficient to confer stability to aspirinated human arterial thrombi in a perfusion chamber assay, probably via activation of GP IIb-IIIa (Kamae et al. *Thromb Haemost.* Jun 2006;4(6):1379-1387; Goto et al. *J Am Coll Cardiol.* Jan 3 2006;47(1):155-162; Cosemans et al. *Blood.* Nov 1 2006;108(9):3045-3052). Finally, these results also indicate that the value of pharmacodynamic assays utilizing exogenous and non-physiological concentrations of ADP at predicting antithrombotic activity of antiplatelet drugs may not apply to reversible antagonists.

In summary, these results indicate that the two thienopyridines, clopidogrel and prasugrel affect hemostasis beyond the levels attributed to the inhibition of P2Y12 in the mouse. In the same models, direct-acting, competitive, reversible antagonism of P2Y12 preferentially affected thrombosis over hemostasis.

The present invention provides a number of embodiments. It is apparent that the examples may be altered to provide other embodiments of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the appended claims rather than by the specific embodiments, which have been represented by way of example.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety to the same extent as if each reference was individually incorporated by reference. From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.
WHAT IS CLAIMED IS:

1. A method for diagnosing an increased risk for high platelet reactivity or increased bleeding in a subject suffering from a cardiovascular disorder, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) determining whether the subject is at risk for high platelet reactivity or increased bleeding following administration of a irreversible, non-direct P2Y₁₂ receptor inhibitor.

2. A method for diagnosing an increased risk for high platelet reactivity or increased bleeding in a subject suffering from a condition or disorder mediated at least in part by ADP-induced platelet aggregation, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) determining whether the subject is at risk for high platelet reactivity or increased bleeding following administration of a irreversible, non-direct acting P2Y₁₂ receptor inhibitor.

3. The method of claims 1 or 2 wherein the diagnosing occurs prior to the administration of a therapeutic agent to the patient.

4. The method of claims 1 or 2 wherein the diagnosing occurs during the course of drug treatment.

5. A method of identifying a subject that is susceptible to developing an cardiovascular complication during a course of treatment comprising administering a irreversible, non-direct P2Y₁₂ receptor inhibitor (a) providing a biological sample from a subject; (b) determining platelet reactivity in the biological sample; and (c) comparing platelet reactivity in the biological sample to a standard reactivity, wherein 43% or more platelet aggregation compared to the standard indicates that the subject is susceptible to developing a ischemic event during a course of treatment with a irreversible, non-direct acting P2Y₁₂ receptor inhibitor.

6. The method in accordance with claim 5, wherein said ischemic event is selected from the group consisting of coronary heart disease (CHD), acute coronary syndromes (ACS), myocardial infarction, stable or unstable angina, acute reocclusion after percutaneous coronary intervention, and/or stenting, restenosis, peripheral vessel ballon angioplasty and/or stenting, thrombotic stroke, transient ischemic attack, reversible ischemic neurological deficit and intermittent claudication.
7. The method in accordance with claim 5, wherein said ischemic event is selected from the group consisting of coronary heart disease (CHD), acute coronary syndromes (ACS), percutaneous coronary intervention (PCI) including angioplasty and/or stent, acute myocardial infarction (AMI), unstable angina (USA), coronary artery disease (CAD), transient ischemic attacks (TIA), stroke, peripheral vascular disease (PVD), Surgeries-coronary bypass, carotid endarectomy.

8. An improved method for preventing or treating thrombosis and thrombosis related conditions in a subject with a P2Y_12 receptor inhibitor, comprising (a) obtaining the genotype of a subject to be treated for CYP2C19*2, (b) administering a therapeutically effective amount of reversible, direct acting P2Y_12 receptor inhibitor to the subject having a CYP2C19*2 polymorphism.

9. A method for treating a cardiovascular disease in a patient resistant or non-responsive to a daily dose of irreversible, non-direct acting P2Y_12 receptor inhibitor comprising: providing a therapeutic amount of a direct acting, reversible P2Y_12 receptor inhibitor; and administering the resistance-surmounting quantity of the direct acting, reversible P2Y_12 receptor inhibitor to the patient.

10. The method of claim 9, wherein the P2Y_12 receptor inhibitor is administered at a time interval selected from the group consisting of once per day and twice per day.

11. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y_12 receptor inhibitor is from at least about 300 to about 450 mg.

12. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y_12 receptor inhibitor is from at least about 50 to about 150 mg.

13. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y_12 receptor inhibitor is from at least about 75 to about 100 mg.

14. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y_12 receptor inhibitor is at least about 20 to about 100 mg.

15. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y_12 receptor inhibitor is at least about 45 to about 90 mg.
16. The method of claim 9, wherein the therapeutic amount of the reversible, direct-acting P2Y12 receptor inhibitor is at least about 50 to about 60 mg.

17. The method of claim 9, wherein the cardiovascular disease is selected from the group consisting of coronary heart disease (CHD), acute coronary syndromes (ACS), acute myocardial infarction, unstable angina, chronic stable angina, transient ischemic attacks, strokes, peripheral vascular disease, preeclampsia/eclampsia, deep venous thrombosis, embolism, disseminated intravascular coagulation and thrombotic cytopenic purpura, thrombotic and retenotic complications following invasive procedures resulting from angioplasty, carotid endarterectomy, post CABG (coronary artery bypass graft) surgery, vascular gram surgery, stent, in-stent thrombosis, and insertion of endovascular devices and prostheses, and hypercoagulable states related to genetic predisposition or cancers.

18. Use of reversible, direct acting P2Yi2 receptor inhibitor in the manufacture of a medicament for the treatment of thrombotic conditions in a selected patient population non-responsive to an irreversible, non-direct acting P2Yi2 inhibitor, where in the patient population is selected on the basis of the genotype of the patients at a 2Cl 9*2 genetic locus predictive of high platelet reactivity or increased bleeding.

19. The method of any one of claims 8 to 17, wherein the reversible, direct acting P2Yi2 receptor inhibitor is elinogrel.

20. The method of any one of claims 8 to 17, wherein the reversible, direct acting P2Yi2 receptor inhibitor is elinogrel, potassium salt.

21. The method of any one of claims 8 to 17, wherein the reversible, direct acting P2Yi2 receptor inhibitor is elinogrel, potassium salt, form A.

22. The method of any one of claims 8 to 17, wherein the reversible, direct acting P2Yi2 receptor inhibitor is elinogrel, sodium salt.

23. The method of any one of claims 8 to 17, wherein the reversible, direct acting P2Yi2 receptor inhibitor is ticagrelor.

24. A kit for use in diagnosing an increased risk for high platelet reactivity in a subject suffering from a cardiovascular disorder, comprising: (a) a reagent for detecting a
genetic polymorphism in CYP2C19*2 that is biomarker of an irreversible, non-direct acting
P2Y12 receptor inhibitor-mediated high platelet reactivity; (b) a container for the reagent; and
(c) a written product on or in the container describing the use of the biomarker in predicting
an irreversible, non-direct acting P2Y12 receptor inhibitor-mediated high platelet reactivity in
subjects.

25. The method of any of the preceding claims, wherein the irreversible,
non-direct acting P2Y12 receptor inhibitor is a thienopyridine selected from the group
consisting of clopidogrel, prasugrel and ticlopidene.
Figure 1

Screening
- 50 stable outpatients with prior coronary stenting
- clopidogrel 75 mg qd, 81 mg ASA qd

(n=5) eliminated due to exclusion criteria

(n=45) Screening platelet aggregation

(n=25) eliminated due to < 43%
5 μM ADP-induced platelet aggregation

20 patients with HPR

Continue clopidogrel and ASA (7-14 days)

Pre-dosing pharmacokinetic and pharmacodynamic measurements

Single 60 mg elinogrel dose

Pharmacodynamic Measurements:
4h, 6h, 24h and 7-10 days post-dosing

Pharmacokinetic Measurements:
4h, 6h, and 24h post-dosing
Figure 12

Post-dose (4 hrs)

Predose

5 uM ADP-induced Platelet Aggregation (%)

p=0.0001

HPR Cutpoint
Figure 17

Blood Volume Loss
Figure 19

A

B

C

D

- WT
- WT + Pras. (10)
- WT + Clop. (50)
- WT + Elin. (60)
- WT + V.Ctl.
- P2Y12 KO

Time (min)

Fluorescence Intensity (arbitrary units)

Time (sec)

- WT + Pras. (10)
- WT + Clop. (50)
- WT + Elin. (60)
- WT + V.Ctl.
- P2Y12 KO

Lumen (μm²)

Time (sec)
Figure 20

A

Inhibition (%)

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<td>Elinogrel (60)</td>
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<td>Clopidogrel (50)</td>
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<td>Prasugrel (10)</td>
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<td>(mg/kg)</td>
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B

Extent of aggregation (%)

- Elinogrel
- Clopidogrel
- Prasugrel
- Vehicle Control

Time to occlusion (sec)

\[ r^2 = 0.45 \]  
\[ r^2 = 0.9 \]

Blood Loss (μl)

\[ r^2 = 0.78 \]
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/216 A61K31/517 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

6. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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D. See patent family annex

Special categories of cited documents

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search

6 October 2010

Date of mailing of the international search report

23/11/2010

Authorized officer

Bradbrook, Derek
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| X         | SIMON TABASSOME ET AL: "Genetic Determinants of Response to Clopidogrel and Cardiovascular Events."
<p>| Y         | --/-- | 8-23, 25 |</p>
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<td>&amp; 56TH ANNUAL SCIENTIFIC SESSION OF THE AMERICAN-COLLEGE-OF-CARDIOLOGY; NEW ORLEANS, LA, USA; MARCH 24 -27, 2007 ISSN: 0735-1097 abstract</td>
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<td>LIEU ET AL: &quot;Initial intravenous experience with PRT060128 (PRT128), an orally-available, direct-acting, and reversible P2Y12 inhibitor&quot;</td>
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<td>HULOT JEAN-SEBASTIEN ET AL: &quot;Genetic or Acquired CYP2C19 Enzyme Deficit is Associated With an Excess of Cardiovascular Risk in Clopidogrel-treated Patients&quot; CIRCULATION, vol. 120, no. 18, Suppl. 2, November 2009 (2009-11), page S1101, XP002603687 &amp; 82ND SCIENTIFIC SESSION OF THE AMERICAN-HEART-ASSOCIATION; ORLANDO, FL, USA; NOVEMBER 14-18, 2009 ISSN: 0009-7322 abstract</td>
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