synthesis of these compounds involves multiple steps with low yield. This is at least in part due to: 1) instability of the AM at room temperature. 2) existence of multiple chiral centers. In the case of the AM of clopidogrel, it contains two chiral centers at C4 and C7 in addition to a double bond at C3-16, resulting in a combination of eight stereoisomers. Only 7S and *cis* double configuration of the AM possesses antiplatelet activity. However, the efficiency of converting *trans* to *cis* configuration is very low (see, e.g., Asai, F., Sugidachi, K., Ikeda, T., Iwabuchi, H., Kuroki, Y., Inoue, T., Iwamura, R., and Shinbakawa, N. (2003) Cyclic amino compounds. (Office, U. P. a. T. ed., Sankyo Company, Limited, US).

It has been previously shown that metabolism of thienopyridine compounds in liver microsomes (LM) produces the AM and its mixed disulfide conjugates (see, e.g., Zhang, H., et al., (2014) Thromb. Haemost. 112, 1304-1311). However use of liver microsomes for large scale synthesis is prohibitory expense due to low product yield and high cost of LM. In fact there is no viable method to produce large quantities of mixed disulfide conjugate of thienopyridine compounds for drug development and other applications.

Accordingly, improved methods for producing large quantities of mixed disulfide conjugate of thienopyridine compounds for drug development and other applications are needed.

The present invention addresses this need.

SUMMARY

Clopidogrel (Plavix), ticlopidine (Ticlid) and prasugrel (Effient) belong to a class of thienopyridinyl compounds widely used as antiplatelet agents to prevent heart attack and stroke. However, several serious drawbacks have been associated with these drugs including variable response, toxicity and increased risk of bleeding. These drawbacks are closely related to the fact that they are all prodrugs that require oxidative bioactivation by polymorphic cytochromes P450 enzymes (P450s).

To overcome drawbacks associated with thienopyridine compounds (Clopidogrel (Plavix), ticlopidine (Ticlid) and prasugrel (Effient)), the present disclosure provides mixed disulfide conjugates of thienopyridine compounds. It is contemplated that such mixed disulfide conjugates of thienopyridine compounds of the present disclosure are capable of producing active thienopyridine metabolites (e.g., active thienopyridine metabolites capable of antiplatelet activity) in the presence of endogenous glutathione (GSH) without the need for bioactivation by P450s. This approach not only bypasses the oxidative bioactivation process by P450s, but circumvents many of the drawbacks associated with thienopyridinyl drugs. For

example, it is contemplated that the mixed disulfide conjugates of thienopyridine compounds of the present disclosure improve dosing consistency because production of the active metabolite from the conjugates is predictable. In addition, it is contemplated that use of the mixed disulfide conjugates of thienopyridine compounds of the present disclosure as antiplatelet agents reduce the toxicity as toxic reactive metabolites will not be produced by the thiol-exchange reaction. In addition, the therapeutic onset time for the mixed disulfide conjugates of thienopyridine compounds of the present disclosure is shortened, which greatly benefits patients who experience acute cardiovascular events. For example, the standard regimen for clopidogrel requires continuously dosing patients for 3-5 days as only a small percentage of the ingested drug are converted to the active metabolite. In contrast, it is contemplated that the mixed disulfide conjugates of thienopyridine compounds of the present disclosure will release the active metabolites with high yields in less than 30 min. In addition, it is contemplated that the mixed disulfide conjugates of thienopyridine compounds of the present disclosure will have superior stability over the active metabolites and therefore can be used to quantitatively generate the active metabolites for basic and clinical research in vitro.

Accordingly, the present disclosure relates to a process for efficiently synthesizing highly optically active mixed disulfide conjugates of thienopyridine compounds with genetically engineered biocatalysts, i.e., a one-step process for preparing highly optically active mixed disulfide conjugates of thienopyridine compounds by mixing 2-oxo thienopyridine and heterocyclic thiols in the presence of reducing reagent NADPH or NADH and a genetically engineered variant of cytochrome P450 BM3 or CYP102A1 as a catalyst. The operation of the process is simple, and the raw materials and reagents are readily available. The methods selectively produce the cis stereoisomer of the conjugates at a higher yield than prior art methods.

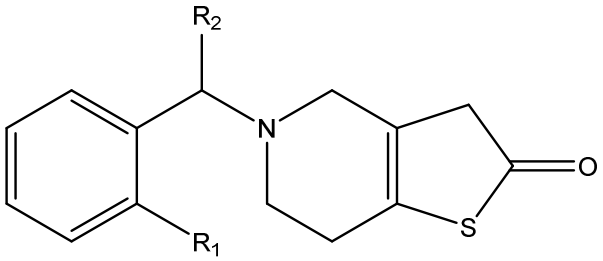
In one aspect, the present disclosure provides a mutant CYP102A1 enzyme capable of catalyzing conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent. In some examples, the catalyzing of conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent results in the generation of mixed disulfide conjugates of thienopyridine compounds. In some examples, the catalyzing of conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent selectively generates cis stereoisomers of the mixed disulfide conjugates of thienopyridine compounds. In some examples, the reducing agent is NADPH or NADH.

In some examples, the enzyme comprises an amino acid sequence having 85% homology with the wild type amino acid sequence for BM3 (SEQ ID NO: 2; Fig. 4B). In some examples, the enzyme comprises an amino acid sequence having 90% homology with the wild type amino acid sequence for BM3 (SEQ ID NO: 2; Fig. 4B). In some examples, the enzyme comprises an amino acid sequence having 95% homology with the wild type amino acid sequence for BM3 (SEQ ID NO: 2; Fig. 4B). In some examples, the enzyme comprises an amino acid sequence having 99% homology with the wild type amino acid sequence for BM3 (SEQ ID NO: 2; Fig. 4B). In some examples, the enzyme comprises an amino acid sequence having one or more of the following amino acid mutations within SEQ ID NO: 2: A82F, L188Q, R47L, F87V, T365N, H116Q, K31T, S56R, A135S, V299D, I458F, P481H, and W1046A. In some examples, the enzyme comprises an amino acid sequence having a specific set of mutations recited in Table 2.

In some examples, the enzyme comprises nucleic acid having at least 85% homology with SEQ ID NO: 1 (wild type cDNA for BM3; Fig. 4A). In some examples, the enzyme comprises nucleic acid having at least 90% homology with SEQ ID NO: 1 (wild type cDNA for BM3; Fig. 4A). In some examples, the enzyme comprises nucleic acid having at least 95% homology with SEQ ID NO: 1 (wild type cDNA for BM3; Fig. 4A). In some examples, the enzyme comprises nucleic acid having at least 99% homology with SEQ ID NO: 1 (wild type cDNA for BM3; Fig. 4A).

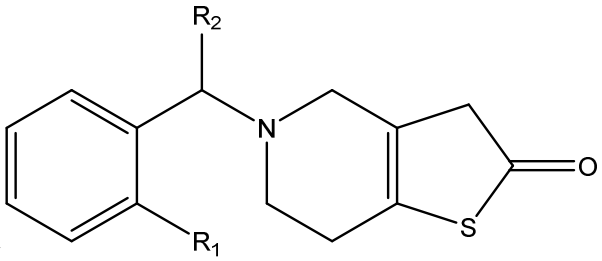
In some examples, the enzyme comprises nucleic acid having at least 85% homology with SEQ ID NO: 3 (a variant BM3 cDNA; Fig. 4C). In some examples, the enzyme comprises nucleic acid having at least 90% homology with SEQ ID NO: 3 (a variant BM3 cDNA; Fig. 4C). In some examples, the enzyme comprises nucleic acid having at least 95% homology with SEQ ID NO: 3 (a variant BM3 cDNA; Fig. 4C). In some examples, the enzyme comprises nucleic acid having at least 99% homology with SEQ ID NO: 3 (a variant BM3 cDNA; Fig. 4C). In some examples, the enzyme comprises nucleic acid having at least 100% homology with SEQ ID NO: 3 (a variant BM3 cDNA; Fig. 4C).

In another aspect, the present disclosure provides methods for synthesizing cis stereoisomers of mixed disulfide conjugates of thienopyridine compounds, comprising mixing a 2-oxo thienopyridine moiety, a heterocyclic thiol moeity, and the described mutant CYP102A1 enzyme in the presence of a reducing reagent. In some examples, the reducing agent is NADPH or NADH. In some examples, the 2-oxo thienopyridine moiety is represented by



; wherein R1 is either Chlorine or Fluorine; wherein R2 is H, COOCH3, or COCHCH2CH2. In some examples, the heterocyclic thiol moiety is represented by R3-SH; wherein R3 is selected from 3-nitropyridine-2-thiol, 2-mercaptopyridine, 2-mercapto-6-methylpyridine, 5-chloropyridine-2-thiol, 2-mercapto-5-trifluoromethyl-pyridine, 3-(trifluoromethyl)pyridine-2-thiol, 2-mercaptopyridine-3-carbonitrile, 4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile, 2-quinolinethiol, 1-amino-3-mercaptoisoquinoline, 6-chloropyridazine-3-thiol, and 2,5-dimethylfuran-3-thiol. In some examples, the mixing occurs at ambient temperature. In some examples, the mixing occurs for a time period between twenty and sixty minutes. In some examples, the mutant CYP102A1 enzyme is comprised within a bacterial cytosolic fraction. In some examples, the amount of mutant CYP102A1 enzyme is between approximately 0.1 and 1 μM. In some examples, the mixing results in the generation of approximately 100 mg of cis stereoisomers of mixed disulfide conjugates of thienopyridine compounds per liter of the 2-oxo thienopyridine moiety, the heterocyclic thiol moeity, the mutant CYP102A1 enzyme, and the reducing agent.

In certain examples, the present disclosure provides a kit comprising a 2-oxo thienopyridine moiety, a heterocyclic thiol moeity, and one or more of the described mutant CYP102A1 enzymes. In one aspect, the present disclosure provides a kit comprising a 2-oxo thienopyridine moiety, a heterocyclic thiol moeity, and the mutant CYP102A1 enzyme as described herein.

In some examples, the kit further comprises a reducing reagent. In some examples, the reducing agent is NADPH or NADH. In some examples, the 2-oxo thienopyridine moiety is represented by 

; wherein R1 is either Chlorine or Fluorine; wherein R2 is H, COOCH3, or COCHCH2CH2. In some examples, the heterocyclic thiol moiety is represented by R3-SH; wherein R3 is selected from 3-

nitropyridine-2-thiol, 2-mercaptopyridine, 2-mercapto-6-methylpyridine, 5-chloropyridine-2-thiol, 2-mercapto-5-trifluoromethyl-pyridine, 3-(trifluoromethyl)pyridine-2-thiol, 2-mercaptopyridine-3-carbonitrile, 4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile, 2-quinolinethiol, 1-amino-3-mercaptoisoquinoline, 6-chloropyridazine-3-thiol, and 2,5-dimethylfuran-3-thiol.

In one aspect, the present disclosure provides a pharmaceutical composition comprising a compound generated with the described method and a pharmaceutically acceptable carrier. In some examples, wherein the pharmaceutical composition is configured for intravenous administration.

In certain examples, the present disclosure provides methods of treating, ameliorating, or preventing a cardiovascular disease in a patient comprising administering to said patient a therapeutically effective amount of a compound generated with the described methods. In some examples, the administration is selected from the group consisting of oral administration and intravenous administration. In some examples, the cardiovascular disease is selected from the group consisting of coronary artery disease, peripheral vascular disease, atherothrombosis, and cerebrovascular disease. In some examples, the compound reduces aggregation of platelets. In some examples, the reduces aggregation of said platelets occurs through irreversible binding to $P2Y_{12}$ receptors. In some examples, the reduces aggregation of said platelets occurs through blocking ADP receptors. In some examples, the method further comprises co-administration of at least one agent selected from the group consisting of a HMG-CoA reductase inhibitor, an ACE Inhibitor, a Calcium Channel Blocker, a Platelet Aggregation Inhibitor, a Polyunsaturated Fatty Acid, Fibric Acid Derivative, a Bile Acid Sequestrant, an Antioxidant, a Thrombolytic Agent, and an Antianginal Agent. In one aspect, the present disclosure provides a compound generated with the method described herein for use in therapy. In another aspect, the present disclosure provides a use of a compound generated with the method described herein in the manufacture of a medicament for treating, ameliorating, or preventing a cardiovascular disease in a patient, wherein the medicament is to be administered to said patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: HPLC analyses of products of mixed disulfide conjugate of clopidogrel. The reaction was performed by mixing 2-oxoclopidogrel, heterocyclic thiol, BM3 and NADPH at 25 ^{o}C for 20 min. The reaction was then quenched with equal volume of acetonitrile containing 1% formic acid. Aliquots of 5 µl of the reaction mixture were analyzed by HPLC.

What Is Claimed Is:

1. A mutant CYP102A1 enzyme capable of catalyzing conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent.

2. The mutant CYP102A1 enzyme of claim 1, wherein the catalyzing of conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent results in the generation of mixed disulfide conjugates of thienopyridine compounds.

3. The mutant CYP102A1 enzyme of claim 1, wherein the catalyzing of conjugation between 2-oxo thienopyridine and heterocyclic thiols in the presence of a reducing reagent selectively generates cis stereoisomers of the mixed disulfide conjugates of thienopyridine compounds.

4. The mutant CYP102A1 enzyme of claim 1, wherein the reducing agent is NADPH or NADH.

5. The mutant CYP102A1 enzyme of claim 1, wherein the enzyme comprises one or more of the following amino acid mutations within SEQ ID NO: 2: A82F, L188Q, R47L, F87V, T365N, H116Q, K31T, S56R, A135S, V299D, I458F, P481H, and W1046A.

6. The mutant CYP102A1 enzyme of claim 1, wherein the enzyme comprises an amino acid sequence having a specific set of mutations recited in Table 2.

7. The mutant CYP102A1 enzyme of claim 1, wherein the enzyme comprises nucleic acid having at least 99% homology with SEQ ID NO: 1.

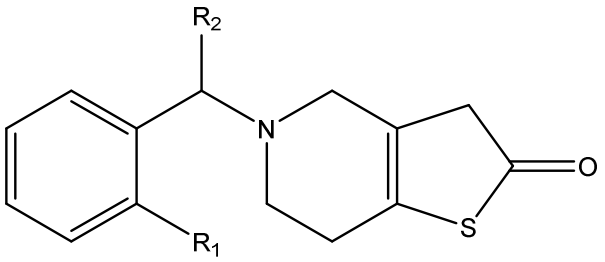
8. The mutant CYP102A1 enzyme of claim 1, wherein the enzyme comprises nucleic acid having at least 100% homology with SEQ ID NO: 3.

9. A method for synthesizing cis stereoisomers of mixed disulfide conjugates of thienopyridine compounds, comprising mixing a 2-oxo thienopyridine moiety, a heterocyclic

thiol moeity, and the mutant CYP102A1 enzyme of claim 1 in the presence of a reducing reagent.

10. The method of claim 9, wherein the reducing agent is NADPH or NADH.

11. The method of claim 9, wherein the 2-oxo thienopyridine moiety is represented by



; wherein R1 is either Chlorine or Fluorine; wherein R2 is H, COOCH3, or COCHCH2CH2.

12. The method of claim 9, wherein the heterocyclic thiol moiety is represented by R3-SH; wherein R3 is selected from 3-nitropyridine-2-thiol, 2-mercaptopyridine, 2-mercapto-6-methylpyridine, 5-chloropyridine-2-thiol, 2-mercapto-5-trifluoromethyl-pyridine, 3-(trifluoromethyl)pyridine-2-thiol, 2-mercaptopyridine-3-carbonitrile, 4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile, 2-quinolinethiol, 1-amino-3-mercaptoisoquinoline, 6-chloropyridazine-3-thiol, and 2,5-dimethylfuran-3-thiol.

13. The method of claim 9, wherein the mixing occurs at ambient temperature.

14. The method of claim 9, wherein the mixing occurs for a time period between twenty and sixty minutes.

15. The method of claim 9, wherein the mutant CYP102A1 enzyme is comprised within a bacterial cytosolic fraction.

16. The method of claim 9, wherein the amount of mutant CYP102A1 enzyme is between approximately 0.1 and 1 μM.

17. The method of claim 9, wherein the mixing results in the generation of approximately 100 mg of cis stereoisomers of mixed disulfide conjugates of thienopyridine compounds per

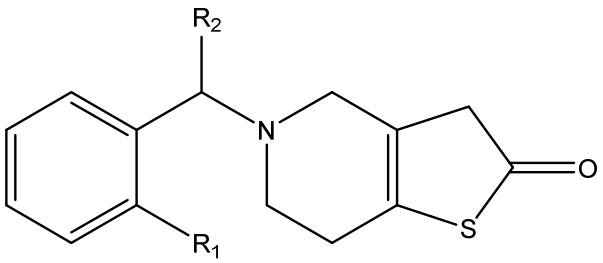
liter of the 2-oxo thienopyridine moiety, the heterocyclic thiol moeity, the mutant CYP102A1 enzyme, and the reducing agent.

18. A kit comprising a 2-oxo thienopyridine moiety, a heterocyclic thiol moeity, and the mutant CYP102A1 enzyme of claim 1.

19. The kit of claim 18, further comprising a reducing reagent.

20. The kit of claim 19, wherein the reducing agent is NADPH or NADH.

21. The method of claim 18, wherein the 2-oxo thienopyridine moiety is represented by



; wherein R1 is either Chlorine or Fluorine; wherein R2 is H, COOCH3, or COCHCH2CH2.

22. The method of claim 18, wherein the heterocyclic thiol moiety is represented by R3-SH; wherein R3 is selected from 3-nitropyridine-2-thiol, 2-mercaptopyridine, 2-mercapto-6-methylpyridine, 5-chloropyridine-2-thiol, 2-mercapto-5-trifluoromethyl-pyridine, 3-(trifluoromethyl)pyridine-2-thiol, 2-mercaptopyridine-3-carbonitrile, 4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile, 2-quinolinethiol, 1-amino-3-mercaptoisoquinoline, 6-chloropyridazine-3-thiol, and 2,5-dimethylfuran-3-thiol.

23. A pharmaceutical composition comprising a compound generated with the method of claim 9 and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23, wherein said pharmaceutical composition is configured for intravenous administration.

25. A compound generated with the method of claim 9 for use in therapy.

26. Use of a compound generated with the method of claim 9 in the manufacture of a medicament for treating, ameliorating, or preventing a cardiovascular disease in a patient, wherein the medicament is to be administered to said patient.

27. The use of claim 26, wherein said administration is selected from the group consisting of oral administration and intravenous administration.

28. The use of claim 26, wherein said cardiovascular disease is selected from the group consisting of coronary artery disease, peripheral vascular disease, atherothrombosis, and cerebrovascular disease.

29. The use of claim 26, wherein said compound reduces aggregation of platelets.

30. The use of claim 29, wherein said reduces aggregation of said platelets occurs through irreversible binding to $P2Y_{12}$ receptors.

31. The use of claim 29, wherein said reduces aggregation of said platelets occurs through blocking ADP receptors.

32. The use of claim 26, wherein the medicament is to be co-administered with at least one agent selected from the group consisting of a HMG-CoA reductase inhibitor, an ACE Inhibitor, a Calcium Channel Blocker, a Platelet Aggregation Inhibitor, a Polyunsaturated Fatty Acid, Fibric Acid Derivative, a Bile Acid Sequestrant, an Antioxidant, a Thrombolytic Agent, and an Antianginal Agent.