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(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFYING THERAPEUTIC COMPOUNDS

(57) Abstract: By the present invention, enzymes responsible for prodrug activation are identified and utilized for the identification of candidate compounds as prodrugs. The present invention includes methods for identifying a candidate compound as a suitable prodrug as well as methods of screening candidate compounds for suitability as therapeutic agents.



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METHODS AND COMPOSITIONS FOR IDENTIFYING THERAPEUTIC COMPOUNDS

5 The present application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/514,241, filed October 24, 2003, U.S. Provisional Application No. 60/514,299, filed October 24, 2003, U.S. Provisional Application No. 60/513,532, filed October 24, 2003, U.S. Provisional Application No. 60/513,542, filed October 24, 2003, U.S. Provisional Application No. 60/514,894, filed October 29, 2003, and
10 U.S. Provisional Application No. 60/514,925, filed October 29, 2003. Each of the aforementioned applications for which the benefit is claimed under 35 U.S.C. § 119(e) is herein incorporated by reference in its entirety. The present application also claims priority under 35 U.S.C. § 365 (c) and 35 U.S.C. § 120 to, and is a continuation-in-part of international application, PCT/US03/12943, filed April 25, 2003. The present application is
15 also a continuation of, and claims the benefit of, U.S. application entitled "Methods and Compositions for Identifying Therapeutic Compounds", filed October 22, 2004, as attorney docket number 18477.041, which U.S. application is herein incorporated by reference in its entirety.

20 FIELD OF THE INVENTION

 By the present invention, enzymes responsible for prodrug activation are identified and utilized for the identification of candidate compounds as prodrugs. The present invention includes methods for identifying a candidate compound as a suitable prodrug as well as
25 methods of screening candidate compounds for suitability as therapeutic agents.

BACKGROUND

 Improving the delivery of drugs and other agents to target cells and tissues has been
30 the focus of considerable research for many years. Though many attempts have been made to develop effective methods for importing biologically active molecules into cells, both *in vivo* and *in vitro*, none has proved to be entirely satisfactory. Optimizing the association of the drug with its intracellular target, while minimizing intercellular redistribution of the drug, e.g., to neighboring cells, is often difficult or inefficient. Accordingly, a major goal has been
35 to develop methods and compositions for specifically targeting agents to cells and tissues.

Benefits of such treatment include avoiding the general physiological effects of inappropriate delivery of such agents to other cells and tissues, such as uninfected cells.

Intracellular targeting may be achieved by methods and compositions that allow accumulation or retention of biologically active agents inside cells.

5 Assay methods capable of determining the presence, absence or amounts of an infectious agent or presence or absence of a medical condition are of practical utility in the search for inhibitors of such an agent or condition.

 There is a need for d therapeutic agents, e.g., prodrugs, having desired pharmacokinetic properties, including enhanced activity, improved oral bioavailability,
10 greater potency and extended effective half-life *in vivo*. Identified prodrugs will preferably have fewer side effects, less complicated dosing schedules, and be orally active. Such prodrugs may be useful to limit the establishment and progression of a medical condition as well as in diagnostic assays for a medical condition. As such, a need exists for enzymes that facilitate the identification of such prodrugs.

15 There is consensus that the bioactivation of phosphoramidate prodrugs such as nucleotide amidate triesters may follow a general scheme (Valette et al., *J. Med. Chem.*, 39: 1981-1991 (1996); McGuigan et al., *Antivir. Chem. Chemotherapy*, 9: 109-115 (1998), McGuigan et al., *Antivir. Chem. Chemotherapy*, 9:473-479 (1998); Saboulard et al., *Mol. Pharmacol.*, 56: 693-704 (1999); Siddiqui et al., *J. Med. Chem.*, 42:4122-4128 (1999)). See
20 Figure 1. Step A is the hydrolysis of the amino acid-like carboxylic ester. A nucleophilic attack by the carboxylic acid of the phosphorous (Step B) is believed to initiate the formation of a 5-membered cyclic intermediate, which intermediate is quickly hydrolyzed to the monoamidate diester (referred to as the amino acid nucleoside monophosphate, AAM, Metabolite X). AAM compounds such as Metabolite X are considered intracellular depot
25 forms, for example of antiviral nucleoside. Various enzymes as well as non-enzymatic catalysis have been implicated in the hydrolysis of the amide bond of AAM compounds resulting in the formation of the nucleotide. The nucleotide is activated by enzymatic phosphorylation to nucleotide di- and tri-phosphates. Ester hydrolase activity might also be
30 hypothesized to apply to prodrug molecules other than phosphoramidates. However, until now identification of the mechanisms and specificities of ester hydrolase cleavage of prodrugs has been constrained by the limited availability of identifiable ester hydrolase enzymes.

SUMMARY OF THE INVENTION

The present invention includes and provides a method for identifying a candidate compound as a suitable prodrug, comprising: (a) providing a candidate compound having an esterified phosphonate group or an esterified carboxyl group; (b) contacting the candidate
5 compound with an extract that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

10 The present invention includes and provides a method for identifying a candidate compound as a suitable prodrug, comprising: (a) providing a candidate compound having an esterified phosphonate group or an esterified carboxyl group; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds; and (c) identifying the
15 candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention includes and provides a method for identifying a candidate
20 compound as a suitable prodrug, comprising: (a) providing a candidate compound having an esterified phosphonate group; (b) contacting the candidate compound with an extract that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate
25 group of the candidate compound.

The present invention includes and provides a method for identifying a candidate compound as a suitable prodrug, comprising: (a) providing a candidate compound having an esterified carboxyl group; (b) contacting the candidate compound with an extract that
30 comprises GS-7340 ester hydrolase to produce one or more metabolite compounds and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention includes and provides a method for identifying a candidate compound as a suitable prodrug, comprising: (a) providing a candidate compound having an

esterified phosphonate group or an esterified carboxyl group; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells that has GS-7340 ester hydrolase activity, to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite
5 compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention includes and provides a method of screening candidate compounds for suitability as therapeutic agents, comprising: (a) providing a candidate
10 compound identified as a suitable prodrug by providing a candidate compound having an esterified phosphonate group or an esterified carboxyl group, contacting the candidate compound with an extract that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds, and identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of
15 the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound; (b) determining the therapeutic activity of the candidate compound; and (c) determining the intracellular persistence of the candidate compound.

The present invention also includes and provides a method for identifying a candidate
20 compound as a suitable prodrug, comprising: (a) providing a candidate compound formed by substituting an esterified phosphonate or an esterified carboxyl group into a prototype compound believed to have therapeutic activity; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds; and (c) identifying the candidate compound as a
25 suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising: (a) providing a candidate compound having an
30 esterified phosphonate group or an esterified carboxyl group; (b) contacting the candidate compound with an extract that comprises cathepsin A or a fragment thereof to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group

instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising: (a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising: (a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity against human immunodeficiency virus; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising: (a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity against inflammation; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising: (a) providing a candidate compound formed by

substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity against cancer; (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts a scheme for the bioactivation of prodrugs.

Figure 2 depicts an exemplary hydrolase purification.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "cell loading" is the accumulation of a prodrug, prodrug metabolite, or drug molecule inside a cell.

As used herein, an "infectious agent" generally refers to any disease causing organism, including but not limited to, bacteria, viruses, and fungi (including yeast and filamentous fungi).

As used herein, the term "prodrug" refers to any compound that when administered to a biological system generates the drug substance, i.e. active ingredient, as a result of spontaneous chemical reaction(s), enzyme catalyzed chemical reaction(s), photolysis, and/or metabolic chemical reaction(s), i.e., the prodrug forms the drug substance as a prodrug metabolite when administered to a biological system. A prodrug is a covalently modified analog or latent form of a therapeutically-active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, esterified, deesterified, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated, photolyzed, hydrolyzed, or can have other functional group change or conversion involving forming or breaking chemical bonds on the prodrug. In a preferred embodiment, a prodrug has an esterified phosphonate or an esterified carboxyl group.

As used herein, a "pharmaceutically acceptable prodrug" generally refers to a compound that can be metabolized in a subject, for example hydrolyzed or oxidized, by either

enzymatic action or by general acid or base solvolysis, to form an active ingredient. Typical examples of prodrugs of the invention have biologically labile protecting groups on a functional moiety of the compound.

As used herein, a “prototype compound” refers to any candidate compound that is believed to have a therapeutic activity. In general, in the methods of the invention, prototype compounds having known structures and synthesis routes are preferably selected in order to reduce the synthetic burden and development costs.

As used herein, a “subject” is any living organism available to receive treatment for a condition or disease.

A “subject in need of treatment” is any subject, including a human such as a patient, who may benefit from treatment of a disease or condition. Subjects who may benefit from treatment include those who have been diagnosed with a disease or condition, those who are suspected of having a disease or condition, or those who may be susceptible to a disease or condition. Benefits of treatment may include prevention of a disease or condition or amelioration of a disease or condition, including elimination of a disease or condition.

As used herein, samples or subjects that may benefit from treatment include natural or man-made materials such as living organisms; tissue or cell cultures; biological samples such as biological material samples (blood, serum, urine, cerebrospinal fluid, tears, sputum, saliva, tissue samples, and the like); laboratory samples; food, water, or air samples; bioproduct samples such as extracts of cells, particularly recombinant cells synthesizing a desired glycoprotein; and the like. Samples can be contained in any medium including water and organic solvent/water mixtures. Samples include living organisms such as humans, and man made materials such as cell cultures.

As used herein, a “target enzyme” refers to any enzyme whose specific activity is sought to be enhanced.

As used herein, “therapeutic activity” includes the ability of a compound to induce a response when administered to a subject or tested *in vitro*. Therapeutic activity includes treatment, which may be prophylactic or ameliorative. Treatment of a disease or condition can include improvement of a disease or condition by any amount, including elimination of a disease or condition. In order to determine the therapeutic activity, any method by which therapeutic activity of a compound may be evaluated can be used. For example, both *in vivo* and *in vitro* methods can be used, including for example, clinical evaluation, EC₅₀, and IC₅₀ assays, and dose response curves.

As used herein, "GS-7340 ester hydrolase" is also referred to as GS-7340 hydrolase and 7340 hydrolase, for example.

Extract Enzymes and Polypeptides of the Present Invention

5 The present invention includes enzymes or fragments thereof. For example, in an embodiment, the present invention includes GS-7340 hydrolase and fragments and homologs thereof. In another embodiment, the present invention includes cathepsin A and fragments and homologs thereof. In another embodiment, the present invention includes one or more enzymes and fragments thereof with GS-7340 ester hydrolase activity. In another
10 embodiment, the present invention includes an enzyme with cathepsin A-like activity.

In a preferred embodiment, the invention includes an extract comprising one or more enzymes of the present invention or fragments thereof. An extract has typically been extracted, removed, or obtained from any location or source. An extract may be extracted, removed, or obtained by any technique or combination of techniques apparent to the artisan,
5 including those techniques known in the art and those described herein. By way of non-limiting example, an extract may be obtained as described in Example 4.

An extract may comprise any combination of one or more enzymes or fragments thereof and any other components, such as for example, cellular components, buffers or any other component. An extract may be a solution, suspension, emulsion, powder, or any other
10 form apparent to the skilled artisan. In a preferred embodiment, an extract is obtained from human cells. In a highly preferred embodiment, an extract is obtained from human peripheral blood mononuclear cells. An extract may also be prepared synthetically, including for example by recombinant techniques or by peptide synthesis.

In an embodiment, an extract has ester hydrolase activity. In a preferred embodiment,
5 an extract has ester hydrolase activity but has activity in the cleavage of alpha naphthyl acetate (ANA). In another preferred embodiment, an extract has ester hydrolase activity on an esterified carboxylate or an esterified phosphonate but has insignificant activity in the cleavage of alpha naphthyl acetate (ANA).

In another embodiment, an extract of the present invention is an extract having
0 carboxylic ester hydrolase activity. In a preferred embodiment, an extract having carboxylic ester hydrolase activity is an extract of peripheral blood mononuclear cells (PBMCs). In a preferred embodiment, an extract has carboxylic ester hydrolase activity, but has insignificant ester hydrolase activity on the cleavage of alpha naphthyl acetate (ANA). In another preferred embodiment, an extract has carboxylic ester hydrolase activity and is from

PMBCs. In yet another embodiment, the extract from PBMCs having carboxylic ester hydrolase activity comprises GS-7340 ester hydrolase in a cell-free environment.

In a preferred embodiment of the present invention, ester hydrolase activity is measured as described in Example 2B. Varying amounts of extract comprising enzyme activity are incubated with a prodrug substrate. The metabolites that are produced are extracted from each reaction mixture and separated from the parent prodrug substrate using high performance liquid chromatography (HPLC). In an embodiment, one or more metabolite products extracted comprise about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 97%, about 98%, about 99% of the total metabolites produced. Metabolite products are monitored by spectrophotometry. Ester hydrolase activity is expressed as pmoles of Metabolite X produced / minute / volume enzyme sample.

In a preferred embodiment of the present invention, activity in the cleavage of alpha naphthyl acetate is measured as described in Example 3. Varying amounts of extract comprising enzyme activity are incubated with ANA. The cleaved alpha naphthyl product is detected by spectrophotometry. Activity is expressed as pmoles product produced per minute per volume enzyme sample.

Insignificant activity in the cleavage of alpha naphthyl acetate is preferably activity against ANA that is from about 75% less to about 100% less than the ester hydrolase activity against a candidate compound; preferably from about 90% less to about 100% less than the ester hydrolase activity against a candidate compound; or more preferably about 95% less, 96% less, 97% less, 98% less, 99% less, 99.5% less, or about 99.9% less than the ester hydrolase activity against a candidate compound. In a highly preferred embodiment, insignificant activity in the cleavage of alpha naphthyl acetate is no detectable activity against ANA.

In an embodiment, an extract comprises GS-7340 ester hydrolase or a fragment thereof, where the fragment exhibits GS-7340 ester hydrolase activity. GS-7340 ester hydrolase activity includes ester hydrolase activity that is specific, namely it is ester hydrolase activity against a candidate compound and insignificant activity in the cleavage of alpha naphthyl acetate (ANA). GS-7340 ester hydrolase activity includes any activity or combination of activities exhibited by GS-7340 ester hydrolase, such as for example being inhibited by a particular compound or having a particular specific or relative activity against a prodrug substrate. In an embodiment, an extract has GS-7340 ester hydrolase activity. In another embodiment, an extract comprises cathepsin A, or a fragment or a homolog thereof.

In another embodiment, an extract comprises an enzyme with cathepsin A-like activity. In a preferred embodiment of the present invention, cathepsin A-like activity includes ester hydrolase activity that is specific, namely it is ester hydrolase activity against a candidate compound and insignificant activity in the cleavage of alpha naphthyl acetate (ANA). Cathepsin A-like activity includes any activity or combination of activities exhibited by cathepsin A, such as for example being inhibited by a particular compound or having a particular specific or relative activity against a prodrug substrate.

Inhibitors of GS-7340 ester hydrolase may include any composition that inhibits GS-7340 ester hydrolase. Preferred inhibitors of GS-7340 hydrolase include fluorophosphonate/fluorophosphate (diisopropylfluorophosphate (DFP)), isocoumarins such as 3,4 dichloroisocoumarin (3,4-DCI), and peptide carboxyl esters of chloro- and fluoro-methyl ketones (AlaAlaProAla-CMK, AlaAlaProVal-CMK, PheAla-FMK). Many inhibitors of GS-7340 hydrolase may be dissolved in a stock solution, for example where the solution is comprised of the inhibitor in a solvent such as 100% ethanol or aqueous buffer. In a preferred embodiment, inhibitors are dissolved in 100% ethanol. In a further preferred embodiment, inhibition of GS-7340 ester hydrolase is performed in a buffered MES solution as described in Example 12.

In one embodiment, GS-7340 ester hydrolase has a molecular weight of about 50 kDa to about 120 kDa, preferably about 80 kDa to about 110 kDa, more preferably about 70 to about 100 kDa on gel filtration.

In another embodiment, GS-7340 hydrolase has ester hydrolase activity against a candidate compound and insignificant activity in the cleavage of alpha naphthyl acetate (ANA).

In an embodiment, GS-7340 ester hydrolase has an isoelectric point (pI) of about 4.0 to about 6.0, preferably about 4.5 to about 5.5. In an embodiment, GS-7340 hydrolase has an isoelectric point of about 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, or 6.0. In a preferred embodiment of the present invention, pI is measured by elution of bound protein from a Mono P column using a linear pH gradient. *See e.g., Example 11.*

Structural data for selected exemplary candidate compounds is provided in Table 1.

TABLE 1

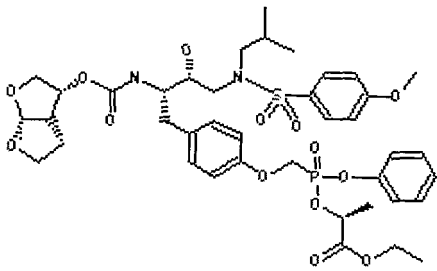
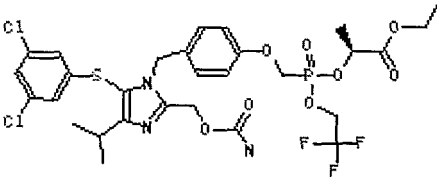
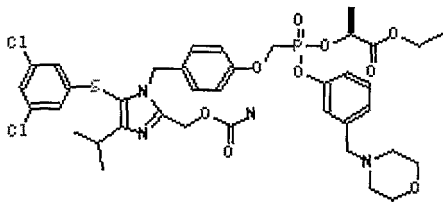
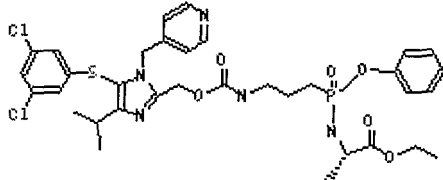
A	
B	
D	
I	

TABLE 1 (cont'd)

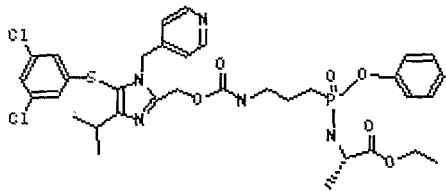
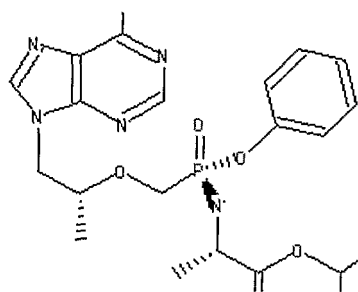
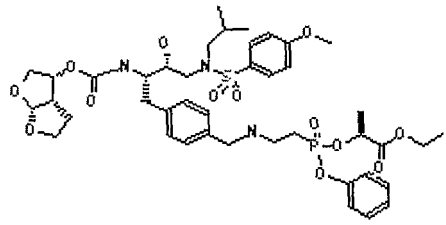
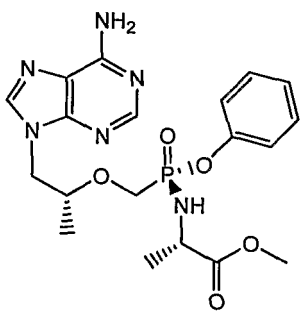
I	
K	
L	
U	

TABLE 1 (cont'd)

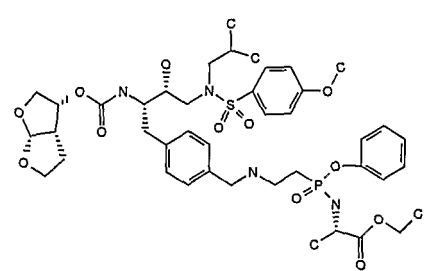
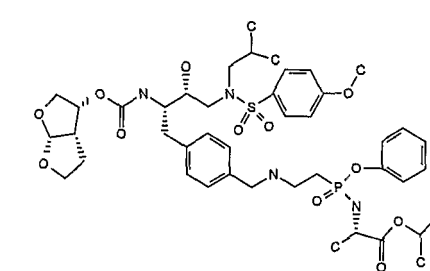
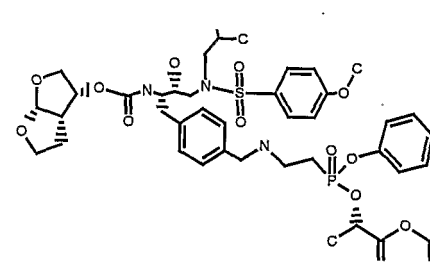
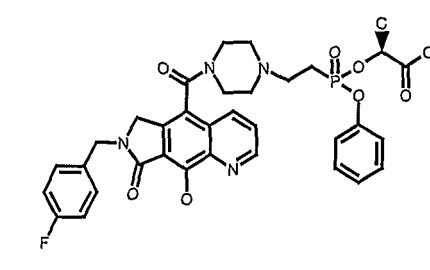
V	
W	
X	
Y	

TABLE 1 (cont'd)

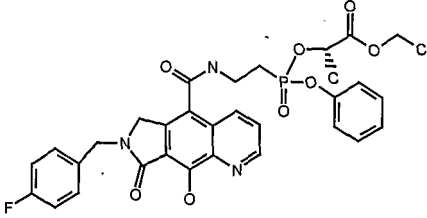
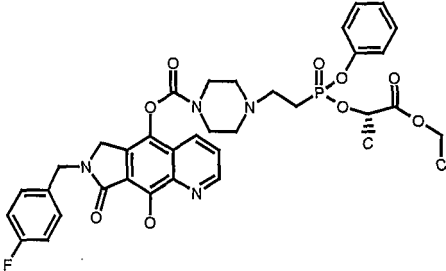
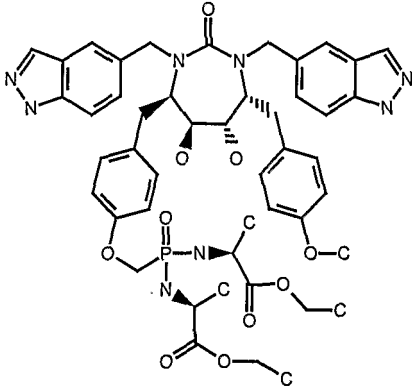
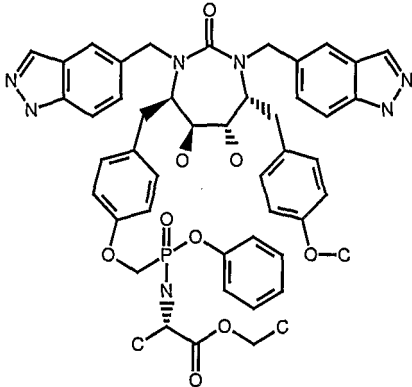
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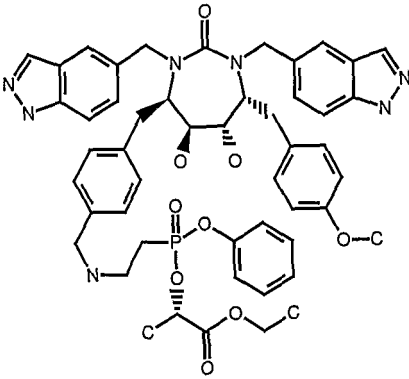
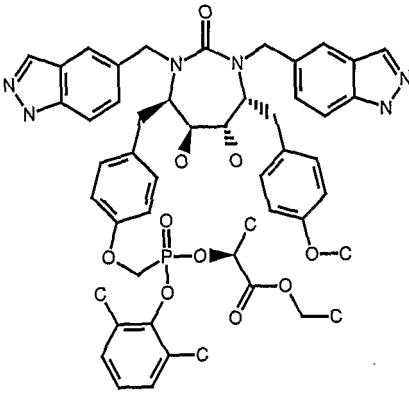
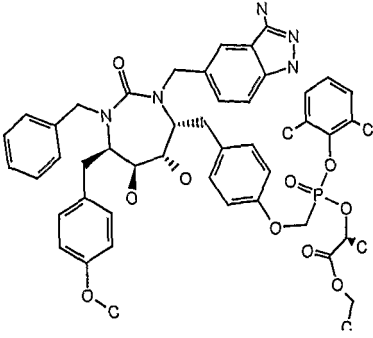
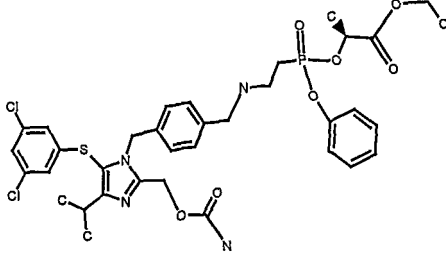
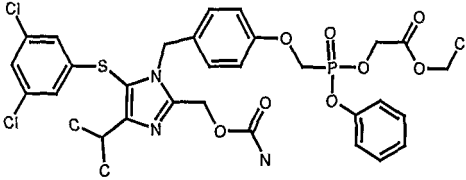
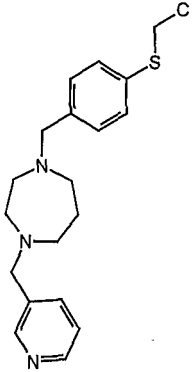
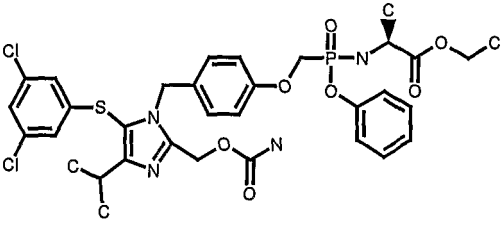
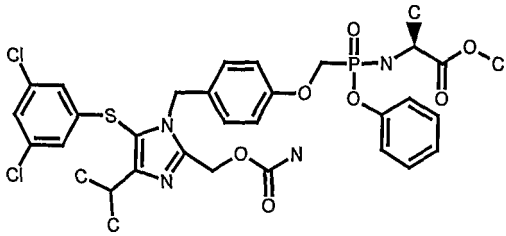
DD	
EE	
FF	
GG	

TABLE 1 (cont'd)

HH	
JJ	
KK	
LL	

In an embodiment of the present invention, an approximately equivalent relative or specific activity refers to a relative or specific activity that is less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1%, above or below the value of the largest of the relative or specific activities in any comparison. In an embodiment of the present invention, a relative or specific activity against one compound that is greater than the relative or specific activity against another compound includes a relative or specific activity that is at least about 20% more, at least about 30% more, at least about 40% more, at least about 50% more, at least about 60% more, at least about 70% more, at least about 80% more, at least about 90% more, at least about 100% more, at least about 200% more, at least about 300% more, at least about 500% more, at least about 700% more, at least about 1000% more, at least about 1500% more, at least about 2000% more, at least about 5000% more, or at least about 10,000% more than its relative or specific activity against another candidate compound.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has an approximately equivalent specific activity against Compound FF and Compound HH.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has an approximately equivalent specific activity against Compound AA and Compound JJ.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound K greater than its specific activity against Compound A.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound U greater than its specific activity against Compound V.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound X greater than its specific activity against Compound Z.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound W greater than its specific activity against Compound EE.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound KK greater than its specific activity against Compound LL.

5 In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound BB greater than its specific activity against Compound Y.

In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound GG greater than its specific activity against Compound I.

10 In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound DD greater than its specific activity against Compound B or Compound D.

15 In an embodiment, where all specific activities are estimated based on relative activities, relative to the conversion of Compound A, GS-7340 hydrolase has a specific activity against Compound CC greater than its specific activity against Compound L.

In an embodiment, where all specific activities are measured relative to the conversion of Compound A, GS-7340 has at least one specific activity, at least 2 specific activities, at least 3 specific activities, at least 4 specific activities, at least 5 specific
20 activities, at least 6 specific activities, at least 7 specific activities, at least 8 specific activities, at least 9 specific activities, at least 10 specific activities, or 11 preferably specific activities selected from the group consisting of (1) an approximately equivalent specific activity against Compound FF and Compound HH; (2) an approximately equivalent specific activity against Compound AA and Compound JJ; (3) a specific activity against Compound
25 K greater than its specific activity against Compound A; (4) a specific activity against Compound U greater than its specific activity against Compound V; (5) a specific activity against Compound X greater than its specific activity against Compound Z; (6) a specific activity against Compound W greater than its specific activity against Compound EE; (7) a specific activity against Compound KK greater than its specific activity against Compound
30 LL; (8) a specific activity against Compound BB greater than its specific activity against Compound Y; (9) a specific activity against Compound GG greater than its specific activity against Compound I; (10) a specific activity against Compound DD greater than its specific activity against Compound B or Compound D; and (11) a specific activity against Compound CC greater than its specific activity against Compound L.

In an embodiment, where relative activity is measured relative to the conversion of Compound A, GS-7340 hydrolase has relative activities against Compound K and Compound U that are approximately equivalent to the relative activities of recombinant cathepsin A against Compound K and Compound U.

5 In an embodiment, GS-7340 hydrolase is inhibited by 50% by DCI at a concentration of about 0.35 to about 0.75 μM , preferably about 0.5 μM . In an embodiment, GS-7340 hydrolase is inhibited by 50% by MeOSuc-Ala-Ala-Pro-Ala-CMK at a concentration of about 100 μM to about 500 μM , preferably about 200 μM to about 400 μM . In an embodiment, GS-7340 hydrolase is inhibited by 50% by MeOSuc-Ala-Ala-Pro-Val-CMK at a concentration of
10 about 75 μM to about 125 μM , preferably about 100 μM . In an embodiment, GS-7340 hydrolase is inhibited by 50% by Biotin-Phe-Ala-FMK at a concentration of about 75 μM to about 125 μM , preferably about 100 μM . In an embodiment, GS-7340 hydrolase is inhibited by 50% by DFP at a concentration of about 2.5 μM to about 7.5 μM , preferably about 5 μM . In an embodiment, GS-7340 hydrolase is inhibited by 50% by at least one, at least two, at
15 least three, at least four, or five compounds selected from the group consisting of DCI at a concentration of about 0.35 to about 0.75 μM , preferably about 0.5 μM ; MeOSuc-Ala-Ala-Pro-Ala-CMK at a concentration of about 100 μM to about 500 μM , preferably about 200 μM to about 400 μM ; MeOSuc-Ala-Ala-Pro-Val-CMK at a concentration of about 75 μM to about 125 μM , preferably about 100 μM ; Biotin-Phe-Ala-FMK at a concentration of about 75 μM to
20 about 125 μM , preferably about 100 μM ; DFP at a concentration of about 2.5 μM to about 7.5 μM , preferably about 5 μM . Inhibition is measured by observing the enzymatic production of metabolite by HPLC assay with and without addition of an inhibitor as described in Example 12.

In an embodiment, GS-7340 hydrolase comprises or consists of SEQ ID NO: 1 or
25 SEQ ID NO: 2 or fragments of either. In an embodiment, GS-7340 hydrolase comprises or consists of SEQ ID NO: 1 and SEQ ID NO: 2 or fragments of either. In an embodiment, GS-7340 hydrolase does not comprise or consist of SEQ ID NO: 1 or fragments thereof. In an embodiment, GS-7340 hydrolase does not comprise or consist of SEQ ID NO: 2 or fragments thereof. In an embodiment, GS-7340 hydrolase does not comprise or consist of any sequence
30 selected from the group consisting of SEQ ID NO: 1 or SEQ ID NO: 2, and fragments of either.

In an embodiment, GS-7340 hydrolase has at least one, at least two at least three, at least four, at least five, at least six, or seven characteristics selected from the group consisting

of (1) a molecular weight on gel filtration of about 50 kDa to about 120 kDa, preferably about 80 kDa to about 110 kDa, more preferably about 70 to about 100 kDa; (2) ester hydrolase activity against a candidate compound and insignificant activity in the cleavage of alpha naphthyl acetate (ANA); (3) an isoelectric point (pI) of about an isoelectric point (pI) of about 4.0 to about 6.0, preferably about 4.5 to about 5.5 or an isoelectric point of about 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, or 6.0; (4) inhibition by 50% by at least one, at least two, at least three, at least four, or five compounds selected from the group consisting of at least one, at least two, at least three, at least four, or five compounds selected from the group consisting of DCI at a concentration of about 0.35 to about 0.75 μ m, preferably about 0.5 μ m; MeOSuc-Ala-Ala-Pro-Ala-CMK at a concentration of about 100 μ m to about 500 μ m, preferably about 200 μ m to about 400 μ m; MeOSuc-Ala-Ala-Pro-Val-CMK at a concentration of about 75 μ m to about 125 μ m, preferably about 100 μ m; Biotin-Phe-Ala-FMK at a concentration of about 75 μ m to about 125 μ m, preferably about 100 μ m; DFP at a concentration of about 2.5 μ m to about 7.5 μ m, preferably about 5 μ m; (5) comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and fragments of either; (6) where all specific activities are measured relative to the conversion of Compound A, GS-7340 has at least one specific activity, at least 2 specific activities, at least 3 specific activities, at least 4 specific activities, at least 5 specific activities, at least 6 specific activities, at least 7 specific activities, at least 8 specific activities, at least 9 specific activities, at least 10 specific activities, or 11 preferably specific activities selected from the group consisting of (i) an approximately equivalent specific activity against Compound FF and Compound HH; (ii) an approximately equivalent specific activity against Compound AA and Compound JJ; (iii) a specific activity against Compound K greater than its specific activity against Compound A; (iv) a specific activity against Compound U greater than its specific activity against Compound V; (v) a specific activity against Compound X greater than its specific activity against Compound Z; (vi) a specific activity against Compound W greater than its specific activity against Compound EE; (vii) a specific activity against Compound KK greater than its specific activity against Compound LL; (viii) a specific activity against Compound BB greater than its specific activity against Compound Y; (ix) a specific activity against Compound GG greater than its specific activity against Compound I; (x) a specific activity against Compound DD greater than its specific activity against Compound B or Compound D; and (xi) a specific activity against Compound CC greater than its specific activity against Compound L; and (7) where relative activity is measured relative to the conversion of Compound A, GS-7340 hydrolase has relative

activities against Compound K and Compound U that are approximately equivalent to the relative activities of recombinant cathepsin A against Compound K and Compound U.

In a preferred aspect of the present invention, an extract is a purified extract. A purified extract may contain one or more enzymes or fragments thereof in purified form. A purified form includes any degree or type of purification. Any number of purification steps may be performed. In the context of the present invention, a purified extract may be partially purified, moderately purified, substantially purified, or fully purified. As used herein, a partially purified extract contains at least about 25% less activity on the cleavage of ANA (ANA activity) than the same extract that has not been subjected to any purification. A moderately purified extract contains at least about 50% less ANA activity than the same extract that has not been subjected to any purification. A substantially purified extract contains at least about 90% less ANA activity than the same extract that has not been subjected to any purification. A fully purified extract contains no detectable activity on the cleavage of ANA. In a preferred embodiment, a purified extract is fully purified. An extract that is partially, moderately, substantially, or fully purified may result from any number and combination of purification steps. An extract that is partially, moderately, or substantially purified may preferably be subjected to one or more further purification steps.

In a further preferred embodiment, a purified extract contains GS-7340 ester hydrolase activity. In a preferred embodiment, a purified extract comprises GS-7340 ester hydrolase or a fragment thereof. In another preferred embodiment, a purified extract comprises cathepsin A or a fragment thereof. In another preferred embodiment, a fully purified extract comprises cathepsin A-like activity. In another embodiment, a purified extract contains an enzyme that comprises or consists of one or more sequences selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, and fragments of either.

In another preferred embodiment, a purified extract contains only one enzyme with detectable ester hydrolase activity. In another preferred embodiment, a purified extract contains only one enzyme with detectable GS-7340 ester hydrolase activity. In a preferred embodiment, a purified extract comprises only one enzyme with detectable GS-7340 ester hydrolase activity, and the enzyme is GS-7340 ester hydrolase. In a preferred embodiment, a purified extract comprises only one enzyme with detectable GS-7340 ester hydrolase activity, and the enzyme is cathepsin A or a fragment or a homolog thereof. In an embodiment, an extract contains only one enzyme with detectable GS-7340 activity and the enzyme comprises or consists of a polynucleotide

sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO: 2, and fragments of either.

Fragments, Identity, and Homologs of Enzymes

5 One subset of the enzymes of the present invention is fragments of enzymes. In an embodiment, a fragment of an enzyme may be a polypeptide. As used herein, a polypeptide is any molecule that has three or more amino acid molecules joined by peptide bonds. A polypeptide may contain any additional chemical groups and may be folded into any conformation. In an embodiment, fragment molecules have ester hydrolase activity against a candidate compound. In a preferred embodiment, polypeptide molecules have ester hydrolase activity against a candidate compound and insignificant activity against alpha naphthyl acetate. Fragments of an enzyme may consist of significant polypeptide sequences, or indeed most of the polypeptide sequences of, the enzymes of the present invention. Alternatively, the fragments may comprise smaller polypeptides, for example, having from 10 about 3 to about 150 amino acids and more preferably, about 5 to about 15 amino acids, or about 20 to about 40 amino acids, or about 40 to about 70 amino acids, or about 70 to about 150 amino acids, or about 90 to about 120 amino acids.

In another aspect of the invention, one or more of the enzymes or fragments thereof of the invention share between about 100% and 70% sequence identity with one or more of the polypeptide sequences set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In 20 a further aspect of the invention, one or more of the polypeptide molecules of the invention shares between about 100% and 90% sequence identity with one or more of the polypeptide sequences set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In an aspect of the invention, one or more of the polypeptide molecules of the invention shares between 25 about 100% and 95% sequence identity with one or more of the polypeptide sequences set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In another aspect of the invention, one or more of the polypeptides of the invention shares between about 100% and 99% sequence identity with one or more of the polypeptide sequences set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either.

30 In a preferred embodiment, percent identity calculations are performed using the Megalign program of the LASERGENE bioinformatics computing suite (default parameters, DNASTAR Inc., Madison, Wis.).

Homologs are also included in the present invention. As used herein, a homolog or a fragment thereof is a counterpart molecule or fragment thereof in another species. A

homolog can also be generated by molecular evolution or DNA shuffling techniques, so that the molecule retains at least one functional or structural characteristic of the original polypeptide (see, for example, U.S. Pat. No. 5,811,238).

The compounds of the present invention also include polypeptides that are fused to one another. The compounds of the present invention also include polypeptides that are introduced into host cells.

Conservative Substitutions

By the present invention, an enzyme or fragment thereof may include modifications made by one of ordinary skill in the art. For example, as will be apparent to the skilled art worker, a GS-7340 hydrolase or fragment thereof may be modified such as by conservative amino acid changes within the polypeptide sequences of the invention. For example, it is contemplated that a GS-7340 hydrolase or fragments thereof may be modified by conservative amino acid changes that do not diminish the ester hydrolase activity of the enzyme fragment thereof. Conservative changes that do not significantly diminish ester hydrolase activity may cause less than about a 25% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 15% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 15% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 10% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, preferably less than about a 7% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 5% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 4% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 3% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 2% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, less than about a 1% reduction in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes, or no detectable change in ester hydrolase activity as compared to the enzyme with no conservative amino acid changes.

In an embodiment, a GS-7340 hydrolase or fragment thereof or an enzyme with GS-7340 hydrolase activity comprises or consists of SEQ ID NO: 1, SEQ ID NO: 2 or fragments thereof and conservative substitution is made to SEQ ID NO: 1, SEQ ID NO: 2, or fragments

thereof. Such changes permit optimization of codon usage, for example, if the GS-7340 hydrolase or fragment or other enzyme of the invention or fragment thereof is introduced into a cell or organism. Conservative amino acid changes can be made by substituting one amino acid within one group with another amino acid in the same group. Conservative amino acid changes can also be made by substituting one or more codons with one or more different codons that produce the same amino acids. In this manner, conservative changes are made at the nucleotide level so that the same amino acid is coded for by a different nucleotide sequence. Biologically functional equivalents of the enzymes or fragments thereof of the present invention can have ten or fewer conservative amino acid changes, more preferably seven or fewer conservative amino acid changes, and most preferably five or fewer conservative amino acid changes. The encoding nucleotide sequence will thus have corresponding base substitutions, permitting the nucleotide sequence to encode biologically functional equivalent forms of the enzymes or fragments thereof of the present invention.

It is understood that certain amino acids may be substituted for other amino acids in a polypeptide without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Certain amino acid sequence substitutions can be made in a polypeptide sequence and, of course, its underlying DNA coding sequence and, nevertheless, a polypeptide with like properties can be obtained. It is thus contemplated that various changes may be made in the polypeptide sequence of the enzymes or fragments thereof of the present invention, or corresponding DNA sequences that encode said polypeptides, without appreciable loss of their biological utility or activity. It is understood that codons capable of coding for such amino acid changes are known in the art.

In making changes to polypeptides of the present invention, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, *J. Mol. Biol.* 157, 105-132 (1982)). It is accepted that the relative hydropathic character of amino acids contributes to secondary structure, which in turn defines interaction with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, *J. Mol. Biol.* 157, 105-132 (1982)); these are isoleucine (+4.5), valine (+4.2), leucine (+3.8), phenylalanine (+2.8), cysteine/cystine (+2.5), methionine (+1.9), alanine (+1.8), glycine (-0.4), threonine (-0.7),

serine (-0.8), tryptophan (-0.9), tyrosine (-1.3), proline (-1.6), histidine (-3.2), glutamate (-3.5), glutamine (-3.5), aspartate (-3.5), asparagine (-3.5), lysine (-3.9), and arginine (-4.5).

In making such changes, the substitution of amino acids whose hydrophathic indices are within +/- .2 is preferred, those within +/- .1 are particularly preferred, and those within +/- 0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0), lysine (+3.0), aspartate (+3.0./-1), glutamate (+3.0./-1), serine (+0.3), asparagine (+0.2), glutamine (+0.2), glycine (0), threonine (-0.4), proline (-0.5./-1), alanine (-0.5), histidine (-0.5), cysteine (-1.0), methionine (-1.3), valine (-1.5), leucine (-1.8), isoleucine (-1.8), tyrosine (-2.3), phenylalanine (-2.5), and tryptophan (-3.4).

In making such changes, the substitution of amino acids whose hydrophilicity values are within +/-2 is preferred, those which are within +/-1 are particularly preferred, and those within +/-0.5 are even more particularly preferred.

Nucleic Acid Molecules of the Present Invention

Nucleic acid molecules of the present invention include nucleic acid molecules or fragments thereof that encode an enzyme or fragment thereof of the present invention. In an embodiment, a nucleic acid molecule of the present invention encodes an enzyme or a fragment thereof with GS-7340 ester hydrolase activity. In an embodiment, a nucleic acid molecule of the present invention encodes a GS-7340 ester hydrolase enzyme or a fragment thereof.

In an embodiment, a nucleic acid molecule of the present invention encodes an enzyme that comprises or consists of SEQ ID NO: 1 or a fragment thereof. In another embodiment, a nucleic acid molecule of the present invention encodes an enzyme that comprises or consists of SEQ ID NO: 2 or a fragment thereof. In an embodiment, a nucleic acid molecule encodes an enzyme that does not comprise or consist of any sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, and fragments of either.

In an embodiment, a nucleic acid molecule of the invention encodes cathepsin A or a fragment or a homolog thereof. In an embodiment, a nucleic acid molecule of the invention does not encode any member selected from the group consisting of cathepsin A, fragments thereof, and homologs thereof. In an embodiment, a nucleic acid molecule of the present invention encodes an enzyme or a fragment thereof with cathepsin A-like activity. In an

embodiment, a nucleic acid molecule of the present invention does not encode any member selected from the group consisting of an enzyme with cathepsin A-like activity and a fragment thereof. In an embodiment, a nucleic acid molecule of the invention encodes cathepsin A or a fragment thereof. In another embodiment, a nucleic acid molecule of the invention encodes a homolog of cathepsin A or a fragment thereof. In an embodiment, a nucleic acid molecule of the invention does not encode any member selected from the group consisting of a homolog of cathepsin A and fragments thereof.

Fragments, Identity, and Homology of Nucleic Acid Molecules

One subset of the nucleic acid molecules of the invention is fragment nucleic acids molecules. Fragment nucleic acid molecules may consist of significant portions of, or indeed most of, the nucleic acid molecules of the invention. Alternatively, the fragments may comprise smaller oligonucleotides, for example oligonucleotides having from about 15 to about 400 nucleotide residues and more preferably, about 15 to about 30 nucleotide residues, or about 50 to about 100 nucleotide residues, or about 100 to about 200 nucleotide residues, or about 200 to about 400 nucleotide residues, or about 275 to about 350 nucleotide residues.

With respect to nucleic acid molecules, as used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if the two molecules exhibit complete complementarity. As used herein, molecules are said to exhibit complete complementarity when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be minimally complementary if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional low-stringency conditions. Similarly, the molecules are said to be complementary if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional high-stringency conditions.

Conventional stringency conditions are described by Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989) and by Haymes et al., Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C. (1985). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. Thus, in order for a nucleic acid molecule to

serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

Appropriate stringency conditions, which promote DNA hybridization, for example, 6.0X sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0X SSC at 20-25 °C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0X SSC at 50 °C to a high stringency of about 0.2X SSC at 65 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed.

In a preferred embodiment, a nucleic acid of the invention will specifically hybridize to one or more of the nucleic acid molecules that encodes SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either under moderately stringent conditions, for example at about 2.0X SSC and about 65 °C. In a particularly preferred embodiment, a nucleic acid of the invention will include those nucleic acid molecules that specifically hybridize to one or more of the nucleic acid molecules that encodes the polypeptide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either under high stringency conditions such as 0.2X SSC and about 65 °C.

In another aspect of the invention, one or more of the nucleic acid molecules of the invention shares between about 100% and 70% sequence identity with one or more of the nucleic acid sequences that encode the polypeptide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In a further aspect of the invention, one or more of the nucleic acid molecules of the invention share between about 100% and 90% sequence identity with one or more of the nucleic acid sequences that encode the polypeptide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In an aspect of the invention, one or more of the nucleic acid molecules of the invention share between about 100% and 95% sequence identity with one or more of the nucleic acid sequences that encode the polypeptide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either. In another aspect of the invention, one or more of the nucleic acid molecules of the invention share between about 100% and 99% sequence identity with a one or more of the nucleic acid

sequences that encode the polypeptide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or fragments of either.

A nucleic acid molecule of the invention can also encode a homologous polypeptide or a fragment thereof.

5 In another aspect of the present invention, one or more of the nucleic acid molecules of the present invention differ in nucleic acid sequence from those encoding a enzyme or fragment thereof due to the fact that one or more codons encoding an amino acid has been substituted for by a codon that produces the same amino acid originally encoded. Techniques of conservative substitution that may be employed may be those apparent to the artisan as
10 well as those described, for example, herein *supra*.

The compounds of the present invention also include nucleic acid molecules that are fused to one another. The compounds of the present invention also include nucleic acid molecules that are introduced into host cells.

15 Purification of Extract Enzymes and Fragments Thereof

Following extraction, the enzymes or fragments thereof of the present invention can be separated or purified or both to the desired degree of homogeneity and activity by the techniques known in the art. An extract may be processed by any techniques that enhance one or more characteristics of the extract including for example, quantity, quality, purity,
20 specific activity, or relative activity. Processing may enhance one or more characteristics of the extract while having any effect, including an advantageous effect, a detrimental effect, or no effect, on one or more other characteristics. In a preferred embodiment, an extract is a purified extract. A purified extract includes any extract that has been purified by any known method or combination of methods for purification.

25 A variety of techniques related to polypeptide purification will be apparent to the artisan. Numerous texts including Scope's Protein purification: principles and practice (Springer Verlag, New York (1997)), Harris' Protein purification applications: a practical approach (Oxford, New York (1990)), and Deutscher's "Guide to protein purification" in Methods in Enzymology (Vol. 128, Academic Press, San Diego (1990)) provide guidance
30 regarding protein purification. In addition, in a preferred embodiment, an extract may be purified by any one or more of the procedures in Examples 5-7 and variations thereof.

Separation and purification may involve, for example, multiphase extraction, crystallization from a solvent or solvent mixture, distillation, sublimation, or chromatography.

In one embodiment, chromatography techniques can be applied for purifying enzymes of the present invention. Chromatography can involve any number of methods including, for example: adsorption chromatography; affinity and immunoaffinity chromatography; size exclusion chromatography; ion exchange chromatography; partition chromatography; hydrophobic interaction chromatography (HIC); chromatofocusing; high, medium, and low pressure liquid chromatography; small scale analytical chromatography; simulated moving bed (SMB) and preparative thin or thick layer chromatography, as well as techniques of small scale thin layer chromatography; reverse-phase and normal phase chromatography; and gravity and flash chromatography.

In separating and purifying the enzymes of the present invention, techniques such as column purification, for example using ion exchange resin, may be used. Ion exchange resins contain charged groups. Resins may be acidic or basic in nature. Acid resins are cation exchangers, and basic resins are anion exchangers. Weak or strong cation and anion exchangers may be used. Non-limiting exemplary resins include CM cellulose/sephadex (a weak cation exchanger), SP sephadex (a strong cation exchanger), DE cellulose/sephadex (a weak anion exchanger), and QAE sephadex (a strong anion exchanger).

Techniques such as Q15 Anion Exchange, Concanavalin A (Con A) affinity, Chromatofocusing, HR Anion Exchange, Butyl Sepharose-HIC, Hydroxyapetite, Gel Filtration, Hydrophobic Interaction Chromatography (HIC), and Lentil Lectin are among those techniques contemplated. In an embodiment of the present invention, enzyme purification is achieved by the use of a Q15 Anion Exchange Column, followed by a Butyl Sepharose-HIC column. In an aspect of the present invention, an extract of the present invention is purified by consecutive applications of the enzyme mixture to one or more Q15 Anion Exchange Columns, Butyl Sepharose Columns, and Mono P columns, or a combination thereof. In a preferred embodiment, enzyme purification may be enhanced by Chromatofocusing Chromatography. In a highly preferred embodiment, enzyme purification is achieved by use of a Q15 column, followed by use of a Butyl Sepharose HIC column, followed by a Con A column.

Another class of separation and purification methods useful in the present invention involves treatment of a mixture with a reagent selected to bind to or render otherwise separable a desired product, unreacted starting material, reaction byproduct, or the like. Such reagents include adsorbents or absorbents such as activated carbon, molecular sieves, ion exchange media, or the like. Alternatively, the reagents can be acids in the case of a basic material, bases in the case of an acidic material, binding reagents such as antibodies, binding

proteins, selective chelators such as crown ethers, liquid/liquid ion extraction reagents (LIX), and the like.

In applying the methods of the present invention, selection of appropriate methods of separation and purification depends on the nature of the materials involved, including for example, boiling point, and molecular weight in distillation and sublimation, presence or absence of polar functional groups or pH in chromatography, stability of materials in acidic and basic media in multiphase extraction, and the like. One skilled in the art will apply techniques most likely to achieve the desired separation or purification or both. Exemplary, non-limiting separation and purification methods are provided in the specification, examples, and figures, including at Examples 5-7.

An extract to be purified can be obtained from any source. In an embodiment, an extract to be purified may be generated by recombinant methods. In an alternative embodiment, the extract may be derived from a natural source, such as an organic source found in nature. Preferably, the extract is obtained from a mammalian source. In a highly preferred embodiment, the extract is obtained from peripheral blood mononuclear cells (PBMCs) having carboxylic ester hydrolase activity. An enzyme in an extract may be modified by any one or more chemical procedures. Modification by chemical procedure may include oxidation, reduction, hydrolysis, amidation, esterification phosphorylation, glycosylation, and the like or any other chemical manipulation that is within the comprehension of one of skill in the art.

In a preferred aspect, an extract of the present invention is purified from cellular extract of peripheral blood mononuclear cells (PBMCs) and shows ester hydrolase activity on a candidate compound but has insignificant ester hydrolase activity on the cleavage of alpha naphthyl acetate (ANA). In another preferred aspect, an extract of the present invention can be separated from non-specific esterases capable of cleaving ANA through any chromatography techniques known in the art, including but not limited to those methods exemplified in the specification, examples, and figures. *See e.g.*, Examples 5-7. Preferably, a compound of the present invention can be separated from non-specific esterases capable of cleaving ANA through anion exchange chromatography, hydrophobic interaction chromatography (HIC), and concanavalin chromatography.

In an aspect, purification may remove one or more unwanted components. In an embodiment, purification yields a purified extract that has less than about 95%, less than about 90%, less than about 80%, less than about 70%, less than about 60%, less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%,

less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% of the ester hydrolase activity of an unpurified extract against ANA. An unpurified extract is an extract that has not been subjected to any purification following extraction.

In a preferred aspect, purification produces a purified extract that has less than about 50% of the ester hydrolase activity of an unpurified cellular extract on the cleavage of ANA. More preferably, the purified extract of the present invention has less than about 40%, less than about 30%, less than about 20%, or less than about 10% of the ester hydrolase activity of the unpurified cellular extract on the cleavage of ANA. Even more preferably, the purified compound of the present invention has less than about 5% of the ester hydrolase activity of the unpurified cellular extract on the cleavage of ANA. Still more preferably, the purified compound of the present invention has less than about 4%, less than about 3%, less than about 2% or less than about 1% of the ester hydrolase activity of the unpurified cellular extract on the cleavage of ANA. In a highly preferred embodiment, a compound of the present invention shows no detectable ester hydrolase activity on ANA.

Ester hydrolase activity against any compound, including for example candidate compounds and alpha naphthyl acetate, can be measured by any procedure or combination of procedures available to the artisan. In a preferred embodiment of the present invention, ester hydrolase activity is measured as described in the Examples 2B and 3.

Purification may also enhance the proportion of a particular enzyme as compared with the proportion of that enzyme in the original extract. In an embodiment, concentration of a target enzyme in a purified extract may be compared with concentration of a target enzyme in an unpurified extract. Varying degrees of purification of a target enzyme may be achieved by the methods of the present invention. In an embodiment, after purification, the target enzyme may be about 2-fold to about 10,000-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 50-fold to about 100-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 101-fold to about 400-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 401-fold to about 1500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1700-fold to about 6200-fold more pure

than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1501-fold to about 6500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1000-fold to about 8500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme. In another embodiment, after purification, the target enzyme is more than about 10,000-fold pure than the target enzyme in the original enzyme composition as measured by the increase in concentration of the target enzyme.

In another embodiment, the specific activity of an enzyme may also be enhanced by purification. As such, purity of the target enzyme may be assessed by reference to specific activity of the target enzyme. The specific activity of an enzyme may be increased by any amount. In an embodiment, after purification, the target enzyme may be about 2-fold to about 10,000-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 50-fold to about 100-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 101-fold to about 400-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 401-fold to about 1500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1700-fold to about 6200-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1501-fold to about 6500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme may be about 1000-fold to about 8500-fold more pure than the target enzyme in the original enzyme composition as measured by the increase in specific activity of the target enzyme. In another embodiment, after purification, the target enzyme is more than about 10,000-fold pure than the target enzyme in

the original enzyme composition as measured by the increase in specific activity of the target enzyme.

By the methods of the present invention, purification of an enzyme is preferably achieved while preserving concentration of one or more of the enzymes in an extract. The concentration of one or more enzymes may be preserved by any amount, where the preservation is measured relative to the concentration of the unpurified enzyme extract. In a preferred embodiment, the concentration of enzyme composition preserved is preferably at least about 0.2% of the concentration of enzyme in the unpurified enzyme extract, at least about 0.5% of the concentration of enzyme in the unpurified enzyme extract, at least about 1% of the concentration of enzyme in the unpurified enzyme extract, at least about 2% of the concentration of enzyme in the unpurified enzyme extract, at least about 5% of the concentration of enzyme in the unpurified enzyme extract, at least about 10% of the concentration of enzyme in the unpurified enzyme extract, at least about 15% of the concentration of enzyme in the unpurified enzyme extract, at least about 20% of the concentration of enzyme in the unpurified enzyme extract, at least about 25% of the concentration of enzyme in the unpurified enzyme extract, at least about 30% of the concentration of enzyme in the unpurified enzyme extract, at least about 40% of the concentration of enzyme in the unpurified enzyme extract, at least about 50% of the concentration of enzyme in the unpurified enzyme extract, at least about 60% of the concentration of enzyme in the unpurified enzyme extract, at least about 70% of the concentration of enzyme in the unpurified enzyme extract, at least about 80% of the concentration of enzyme in the unpurified enzyme extract, at least about 90% of the concentration of enzyme in the unpurified enzyme extract, or even more preferably, more than about 90% of the concentration of enzyme in the unpurified enzyme extract.

Candidate Compounds

A candidate compound includes any organic compound that might be a substrate for an ester hydrolytic enzyme. Non-limiting exemplary candidate compounds include esters and amides such as for example, carboxyl esters (*e.g.*, esterified carboxylates), thioesters (*e.g.*, thiocarboxylic acid esters and thioesters of thiophosphonic acids), phosphate esters, sulfate esters, esterified phosphonates, and carboxyamides. Particularly preferred exemplary candidate compounds include those with an esterified carboxylate or an esterified phosphonate. In a preferred embodiment, a candidate compound will be hydrolyzed by an extract that has insignificant activity on alpha naphthyl acetate. Candidate compounds may

also include metabolites of candidate compounds. A metabolite is a compound that has been metabolized *in vivo*. Compounds that have been metabolized include compounds resulting for example from the oxidation, reduction, hydrolysis, amidation, esterification and the like of a candidate compound, primarily due to enzymatic processes. Metabolite structures can be determined in any fashion, including for example by conventional techniques such as MS, NMR, or IR analysis.

In a preferred embodiment, a candidate compound comprises an esterified carboxylate or an esterified phosphonate. In a preferred embodiment, a candidate compound comprising an esterified phosphonate group is monosubstituted with a hydroxyorganic acid linked to the phosphorus atom through an oxygen atom. In a preferred embodiment, the hydroxyorganic acid is in the alpha position.

In another preferred embodiment, a candidate compound is substituted with an amino acid group in which a carboxyl group of the amino acid is esterified. In a preferred embodiment, the amino acid group is in the alpha position. In a preferred embodiment of the present invention, a candidate compound is an amino acid phosphonoamidate, where a carboxyl group of the amino acid is esterified. In another preferred embodiment, a candidate compound is substantially stable against extracellular hydrolysis of the esterified group.

In a preferred embodiment, a candidate compound is a prototype compound. In a highly preferred embodiment, a candidate compound is formed by substituting a prototype compound with an esterified carboxyl or an esterified phosphonate group, where prior to substitution, the prototype compound is believed to have therapeutic activity against human immunodeficiency virus, cancer, or inflammation. Any of a variety of synthetic means apparent to the artisan may be used to substitute a prototype compound with an esterified carboxyl or an esterified phosphonate group.

Human immunodeficiency virus, within the context of the present invention, refers to that which is ordinarily understood in the art. In a preferred embodiment, human immunodeficiency virus is associated with white blood cells. Prototype compounds believed to have therapeutic activity against human immunodeficiency virus are described for example in the Physician's Desk Reference (*See e.g.*, 58th ed., Thomson PDR Pub., (2004) ISBN 1-56363-471-6).

Cancer, within the context of the present invention, refers to that which is ordinarily understood in the art. In a preferred embodiment, cancer is associated with white blood cells. In a more preferred embodiment, cancer is any type of leukemia. Prototype compounds believed to have therapeutic activity against cancer are described for example in the

Physician's Desk Reference (*See e.g.*, 58th ed., Thomson PDR Pub., (2004) ISBN 1-56363-471-6).

Inflammation, within the context of the present disclosure refers to that which is ordinarily understood in the art. In a preferred embodiment, inflammation is inflammation associated with white blood cells. In a more preferred embodiment, inflammation is any form of tissue rejection such as solid organ transplant rejection, asthma, or any type of arthritis, such as preferably rheumatoid arthritis.

A prototype compound may be believed to have therapeutic activity on the basis of any information available to the artisan. For example, a prototype compound may be believed to have therapeutic activity on the basis of information contained in the Physician's Desk Reference. *See supra*. In addition, by way of non-limiting example, a compound may be believed to have therapeutic activity on the basis of experience of a clinician, structure of the compound, structural activity relationship data, EC₅₀, assay data, IC₅₀ assay data, animal or clinical studies, or any other basis, or combination of such bases. In another embodiment, a prototype compound is not a nucleoside and does not contain a nucleoside base.

A therapeutically-active compound is a compound that has therapeutic activity, such as for example the ability of a compound to induce a specified response when administered to a subject or tested *in vitro*. Treatment of a disease or condition can include improvement of a disease or condition by any amount, including prevention, amelioration, and elimination of the disease or condition. Therapeutic activity may be conducted against any disease or condition, including in a preferred embodiment against human immunodeficiency virus, cancer, arthritis or any combination thereof. In order to determine therapeutic activity any method by which therapeutic activity of a compound may be evaluated can be used. For example, both *in vivo* and *in vitro* methods can be used, including for example, clinical evaluation, EC₅₀, and IC₅₀ assays, and dose response curves.

Candidate compounds for use with an assay of the present invention or identified by assays of the present invention as useful pharmacological agents can be pharmacological agents already known in the art or variations thereof or can be compounds previously unknown to have any pharmacological activity. Candidate compounds of the present invention can be naturally occurring or designed in the laboratory. Candidate compounds can comprise a single diastereomer, more than one diastereomer, a single enantiomer, or more than one enantiomer. The ester hydrolase compounds of the present invention can have more or less ester hydrolase activity on one or another diastereomer or one or another enantiomer of a candidate compound. In a preferred embodiment, a candidate compound comprises a

diastereomer, upon which the ester hydrolase activity is higher than the ester hydrolase activity on any other diastereomers of that candidate compound. In another preferred embodiment, a candidate compound comprises an enantiomer, upon which the ester hydrolase activity is higher than the ester hydrolase activity on any other enantiomers of that candidate compound. In another preferred embodiment, a candidate compound comprises a single diastereomer. In another preferred embodiment, a candidate compound comprises a single enantiomer.

Candidate compounds can be isolated, as from microorganisms, animals or plants, for example, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, candidate compounds of the present invention can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries. The other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds and are preferred approaches in the present invention. See Lam, *Anticancer Drug Des.* 12: 145-167 (1997).

Methods for synthesis of molecular libraries are well known in the art (see, for example, DeWitt et al., *Proc. Nat. Acad. Sci. USA* 90: 6909-6913 (1993); Erb et al. *Proc. Natl. Acad. Sci. U.S.A.* 91: 11422 (1994); Zuckermann et al., *J. Med. Chem.* 37: 2678 (1994); Cho et al., *Science* 261: 1303 (1993); Carell et al., *Angew. Chem. Int. Ed. Engl.* 33: 2059 (1994); Carell et al., *Angew. Chem. Int. Ed. Engl.* 33: 2061 (1994); Gallop et al., *J. Med. Chem.* 37: 1233 (1994)). Libraries of compounds can be presented in solution (see, e.g., Houghten, *BioTechniques* 13: 412-421 (1992)), or on beads (Lam, *Nature* 354: 82-84 (1991)), chips (Fodor, *Nature* 364: 555-556 (1993)), bacteria or spores (Ladner et al., U.S. Patent No. 5,223,409), plasmids (Cull et al., *Proc. Natl. Acad. Sci. USA* 89, 1865-1869 1992), or phage (Scott & Smith, *Science* 249: 386-390 (1990); Devlin, *Science* 249: 404-406 (1990)); Cwirla et al., *Proc. Natl. Acad. Sci. USA*, 97: 6378-6382 (1990); Felici, *J. Mol. Biol.* 222: 301-310 (1991); and Ladner et al., U.S. Patent No. 5,223,409).

Methods of the Present Invention

In an embodiment, the present invention provides a method of identifying a candidate compound as a suitable prodrug. A suitable prodrug includes any prodrug that may be

identified by the methods of the present invention. For example, in an embodiment, a suitable prodrug includes any prodrug that may be identified by the production of one or more metabolite compounds. In a preferred embodiment, a suitable prodrug is identified by the production of one or more metabolite compounds that have a phosphonic acid group or a carboxylic acid group instead of an esterified phosphonate group or an esterified carboxyl group present in the candidate compound. A suitable prodrug identified by a method of the present invention may be subjected to any desired use or analysis following identification. For example, a suitable prodrug may be analyzed for toxicity, suitability as a therapeutic agent, effective concentration, or any other characteristic. In an embodiment, a suitable prodrug identified by the present invention may be used to treat a sample or subject.

Any method apparent to the artisan may be used to identify a candidate compound as a suitable prodrug. In an embodiment, identification of a suitable prodrug is made by providing a candidate compound and recognizing the formation of one or more metabolites. Such assays may involve without limitation providing a candidate compound having an esterified phosphonate group or esterified carboxyl group, contacting the candidate compound with an extract capable of catalyzing the hydrolysis of a carboxylic ester to produce one or more metabolite compounds, and identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

In an embodiment, the methods of the present invention include providing a candidate compound. In an embodiment, the methods of the present invention further include contacting a candidate compound with an extract. In a preferred embodiment, the present invention contemplates contacting a candidate compound with an extract comprising GS-7340 ester hydrolase. However, a candidate compound may be contacted with any extract. A candidate compound may be contacted with an extract in any manner that permits the extract to interact with the candidate compound. As an example, a candidate compound may be contacted with an extract by mixing the candidate compound and the extract together in any container such as for example a tube or a vial. In an embodiment, a candidate compound is contacted with an extract *in vitro*. In another embodiment, a candidate compound is contacted with an extract in a cell-free environment. In a further embodiment, a candidate compound is contacted with an extract in cell culture, preferably in a peripheral blood mononuclear cell culture.

In a further embodiment, the methods of the present invention providing a candidate compound identified as a suitable prodrug if the extract has catalyzed the formation of one or more metabolite compounds. Analysis of metabolite compounds may be achieved by any method. For example, methods of analysis described in the specification or any other methods of analysis known to the skilled artisan may be used. Conventional techniques such as NMR, IR, including FT-IR, and titration may be used without limitation to identify a metabolite. The production of a metabolite compound may also be monitored by the use of a radioactive substrate to produce a radio-labeled metabolite as shown in Example 2A. In another aspect, ester hydrolase activity is preferably monitored by the production of a non-radio-labeled metabolite as in Example 2B. In the case of radioactive metabolites, methods including for example scintillation counting may be used to ascertain the specific activity of an ester hydrolase on a candidate compound. In the case on non-radio-labeled metabolites, ester hydrolase activity may be detected by techniques, including, for example, chromatography and mass spectrometry.

Cleavage of a prodrug by ester hydrolase activity can be compared with the EC_{50} of the drug. In a preferred embodiment, a correlation is observed between ester hydrolase activity of a compound of the present invention and EC_{50} of the drug. In a preferred embodiment, cleavage of a prodrug may be used as a predictor of drug activity or cell loading or both.

An enzyme or fragment thereof of the present invention that shows ester hydrolase activity against a candidate compound may be evaluated for activity against any other candidate compounds, including for example candidate compounds of the same drug class or of different drug classes.

The methods of the present invention may be conducted *in vivo* or *in vitro*. In a preferred aspect, the methods of the present invention are conducted using peripheral blood mononuclear cells (PBMCs). Peripheral blood mononuclear cells may be obtained from a patient who is or is not undergoing leukapheresis. In a preferred embodiment, the PBMCs are obtained from a patient who is undergoing leukapheresis.

In another aspect of the present invention, the activity of the ester hydrolase compounds of the present invention may be inhibited by any compound or agent that inhibits ester hydrolase activity. Exemplary inhibitors include fluorophosphonate, fluorophosphonate derivatives, isocoumarins such as 3,4 dichloroisocoumarin, and peptide carboxyl esters of chloro- and fluoro-methyl ketones. Inhibition of ester hydrolase activity may be ascertained

by any techniques available to the artisan. In a preferred embodiment of the present invention, inhibition is measured by IC₅₀ assay as described in Example 12.

In another aspect, the present invention provides methods of screening candidate compounds for suitability as therapeutic agents. Screening for suitability of therapeutic agents may include assessment of one, some or many criteria relating to the compound that may affect the ability of the compound as a therapeutic agent. Factors such as, for example, efficacy, safety, efficiency, retention, localization, tissue selectivity, degradation, or intracellular persistence may be considered. In an embodiment, a method of screening candidate compounds for suitability as therapeutic agents is provided, where the method comprises providing a candidate compound identified as a suitable prodrug, determining the therapeutic activity of the candidate compound, and determining the intracellular persistence of the candidate compound. Intracellular persistence can be measured by any technique apparent to the skilled artisan, such as for example by radioactive tracer, heavy isotope labelling, or LCMS.

In a further preferred embodiment, a method of screening candidate compounds for suitability as therapeutic agents further comprises determining the tissue selectivity of a candidate compound or a metabolite, preferably an acid metabolite, of the candidate compound. Tissue selectivity refers to the propensity of one or more compounds, including for example a candidate compound or one or more metabolite compounds, to accumulate preferentially in one or more cells, tissues, or organs. Tissue selectivity may be evaluated by any of a variety of techniques apparent to the artisan. For example, tissue selectivity may be observed on the basis of a radioactive, fluorescent or other dye tag that has been added to a candidate compound. The accumulation of the tag in particular tissues may then be observed.

In an embodiment, the present invention provides a method of identifying a candidate compound as a suitable prodrug. A suitable prodrug includes any prodrug that may be identified by the methods of the present invention. For example, in an embodiment, a suitable prodrug includes any prodrug that may be identified by the production of one or more metabolite compounds. In a preferred embodiment, a suitable prodrug is identified by the production of one or more metabolite compounds that have a phosphonic acid group or a carboxylic acid group instead of an esterified phosphonate group or an esterified carboxyl group present in the candidate compound. A suitable prodrug identified by a method of the present invention may be subjected to any desired use or analysis following identification. For example, a suitable prodrug may be analyzed for toxicity, suitability as a therapeutic agent, effective

concentration, or any other characteristic. In an embodiment, a suitable prodrug identified by the present invention may be used to treat a sample or subject.

In order to identify a candidate compound as a suitable prodrug, any method may be used. For example, in a preferred embodiment, a candidate compound may be identified as a suitable prodrug by providing a candidate compound having an esterified phosphonate group or esterified carboxyl group, contacting the candidate compound with an extract capable of catalyzing the hydrolysis of a carboxylic ester to produce a metabolite compound, and identifying the candidate compound as a suitable prodrug if the metabolite compound has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

In an embodiment, a method of screening candidate compounds for therapeutic activity may also include determining the therapeutic activity of a candidate compound or any metabolites thereof or both. Therapeutic activity against any disease or condition may be assessed. In a preferred embodiment, therapeutic activity against human immunodeficiency virus (HIV) may be assessed. In another preferred embodiment, determining the therapeutic activity of a candidate compound comprises determining activity against HIV protease, HIV integrase, HIV reverse transcriptase, any other HIV enzyme or any combination of such enzymes.

In another preferred embodiment, determining the therapeutic activity of a candidate compound comprises determining the resistance of HIV to the candidate compound or any metabolites thereof or both. In an embodiment, determining the therapeutic activity of a candidate compound may further include determining the anti-HIV activity of a metabolite of the candidate compound. In a preferred embodiment, a metabolite of a candidate compound is an acid metabolite, particularly preferably a carboxylic acid or phosphonic acid. Methods of determining the therapeutic activity of a candidate compound will be known to the artisan. Such methods may be performed *in vitro* or *in vivo*. Exemplary methods include those such as clinical evaluation, K_i , EC_{50} , CC_{50} , and IC_{50} assays, as well as dose response curves and resistance studies. *See e.g.*, Example 17.

In screening compounds for suitability as therapeutic agents, intracellular persistence of a candidate compound may also be evaluated. Evaluation of intracellular persistence may comprise, for example, evaluation of intracellular residence time or half-life of a compound. In a preferred embodiment, half-life of a compound in human tissue is determined. Half-life may be determined in any tissue. Preferred human tissues for determining half-life of a

compound of the invention include without limitation helper cells, killer cells, lymph nodes, and peripheral blood mononuclear cells. Intracellular persistence, including for example, intracellular residence time of any metabolite compound, preferably an acid metabolite, may also be evaluated. Any technique known to the art worker for determining intracellular persistence may be used in the present invention. By way of non-limiting example, persistence of a compound may be measured by retention of a radiolabeled or dye labelled substance, including for example a candidate compound or any metabolite compound.

A further aspect of the present invention relates to methods of inhibiting the activity of a condition or disease comprising the step of treating a sample or subject believed to have a disease or condition with a prodrug identified by a compound of the invention.

Compositions of the invention act as identifiers for prodrugs that have therapeutic activity against a disease or condition. In a preferred aspect, compositions of the invention act as identifiers for drugs that show therapeutic activity against conditions including for example cancer, inflammation, rheumatoid arthritis, and immunosuppression or any combination thereof. Compositions of the invention may also act as identifiers for drugs that have therapeutic activity against infectious agents. Infectious agents against which the therapeutic agents may be effective include, without limitation, bacteria, viruses, and yeast. In a non-limiting example, the enzymes may be useful to identify inhibitor prodrugs that bind to locations on the surface or in a cavity of HIV protease having a geometry unique to HIV protease.

If desired, after application of an identified prodrug, the amount of an infectious organism or the level or any material indicative of the infection or condition may be observed by any method including direct and indirect methods of detecting such level. Quantitative, semi-quantitative, and qualitative methods of determining such a level are all contemplated. Any method, including but not limited to, observation of the physiological properties of a living organism, are also applicable.

However, in some cases, for example when screening compounds capable of inhibiting HIV protease viruses, the results of enzyme assays may not correlate with cell culture assays. Thus, a cell-based assay should be the primary screening tool for use in the HIV context.

Cells

In an aspect of the present invention, a cell-based system can be used to screen for prodrug compounds. In one aspect, that cell-based system is a non-recombinant cell-based

system. In an aspect of the present invention, the cells are fresh human peripheral blood mononuclear cells obtained from patients undergoing leukapheresis. In another aspect, the cells are fresh human peripheral blood mononuclear cells obtained from patients not undergoing leukapheresis. In a further aspect, a nucleic acid sequence or fragment thereof encoding a GS-7340 hydrolase or fragment thereof, is used in a recombinant cell-based system. In another aspect, a nucleic acid sequence or fragment thereof encoding an enzyme with GS-7340 hydrolase activity is used in a recombinant cell-based system.

Polynucleotides of the present invention encoding GS-7340 ester hydrolase or a fragment thereof may be introduced into a host cell. A host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process an expressed GS-7340 ester hydrolase enzyme or fragment thereof in the desired fashion. Such modifications of GS-7340 ester hydrolase or fragment thereof include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the GS-7340 ester hydrolase or fragment thereof also can be used to facilitate correct insertion, folding and/or function. A variety of host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC 10801 University Boulevard, Manassas, Va. 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein. Additional host cells may be maintained in the laboratory stock or be commercially available. Suitable host strains will be known to one of ordinary skill in the art.

In selecting a host cell, stable expression is generally preferred for long-term, high-yield production of recombinant polypeptides. For example, cell lines which stably express GS-7340 ester hydrolase can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced enzyme sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. *See, for example, Animal Cell Culture*, R.I. Freshney, ed., 1986.

Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler et al., *Cell*

11: 223-32 (1977)) and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22: 817-23 (1980)) genes which can be employed in *ti^r* or *aprt^r* cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate (Wigler et al., *Proc. Natl. Acad. Sci. USA*, 77: 3567-70 (1980)), *npt* confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin et al., *J. Mol. Biol.*, 150: 1-14 (1981)), and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murray, 1992). Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci. USA*, 85: 8047-51 (1988)). Visible markers such as anthocyanins, β -glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes et al., *Methods Mol. Biol.* 55: 121-131 (1995)).

In addition to cell-based systems, a candidate compound can be screened in a non-transgenic or transgenic organism. In a preferred embodiment, the organism is a mouse, rat, dog, cat, rabbit, guinea pig, or monkey.

Mammalian Expression

Enzymes or fragments thereof or polynucleotides encoding enzymes or fragments thereof of the present invention may be expressed in mammalian systems. For example, a number of viral-based expression systems can be used to express enzymes or fragments thereof in mammalian host cells. If an adenovirus is used as an expression vector, sequences encoding enzymes or fragments thereof can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing an enzyme or fragment thereof in infected host cells (Logan & Shenk, *Proc. Natl. Acad. Sci. USA* 81: 3655-3659 (1984)). If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can be used to increase expression in mammalian host cells.

Alternatively, in the present invention, human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are constructed and delivered to cells via conventional delivery methods (e.g., liposomes, polycationic amino polymers, or vesicles).

Specific initiation signals also can be used in the methods of the present invention to achieve more efficient translation of sequences encoding enzymes or fragments thereof. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding an enzyme or fragment thereof, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression of the enzymes or fragments thereof or polynucleotides or fragments thereof of the present invention can be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used (*See Scharf et al., Results Probl. Cell Differ.* 20, 125-162, 1994).

Transgenic Animals

In an embodiment, a nucleic acid molecule encoding a GS-7340 ester hydrolase enzyme or fragment thereof may be introduced into an animal in order to produce a transgenic animal. In another embodiment, a nucleic acid molecule encoding an enzyme or fragment thereof with GS-7340 ester hydrolase activity may be introduced into an animal in order to produce a transgenic animal. Techniques to introduce such nucleic acids are known in the art. In a preferred embodiment, the transgenic animal is a mammal, including for example, a mouse, rat, dog, cat, rabbit, guinea pig, or monkey. Preferred specific tissues for expression in transgenic animals of the invention include liver, spleen, muscle, and blood. It may be preferable to specifically overexpress a GS-7340 ester hydrolase enzyme or any polypeptide of the invention in specific blood cells, for example, PBMCs. Transgenic animals expressing or overexpressing a GS-7340 ester hydrolase may be used for pharmacokinetic analysis and metabolite analysis. In another embodiment of the present invention, the transgenic animals express antisense constructs encoding a GS-7340 ester hydrolase enzyme or any polypeptide of the invention. Such animals may be used to demonstrate the conversion of test compounds in one or more tissues.

The following examples are illustrative and not intended to be limiting in any way.

EXAMPLES

5

Example 1

Metabolism of Nucleotide Phosphoramidates

There is broad consensus that the bioactivation of nucleotide amidate triesters follows a general scheme (Valette et al., *J. Med. Chem.*, 39: 1981-1991 (1996); McGuigan et al.,
10 *Antivir. Chem. Chemotheraphy*, 9: 109-115 (1998), McGuigan et al., *Antivir. Chem. Chemotheraphy*, 9:473-479 (1998); Saboulard et al., *Mol. Pharmacol.*, 56: 693-704 (1999); Siddiqui et al., *J. Med. Chem.*, 42:4122-4128 (1999)). Step A is the hydrolysis of the amino acid-like carboxylic ester. A nucleophilic attack by the carboxylic acid of the phosphorous (Step B) is believed to initiate the formation of the 5-membered cyclic intermediate which in
15 turn is quickly hydrolyzed to the monoamidate diester (referred to as the amino acid nucleoside monophosphate, AAM, or metabolite X', Step C). This compound is considered an intracellular depot form of the antiviral nucleoside. Various enzymes as well as non-enzymatic catalysis have been implicated in Step D, which is the hydrolysis of the amide bond resulting in the formation of the nucleotide. The nucleotide is activated by enzymatic
20 phosphorylation to nucleotide di- and tri-phosphates. See Figure 1.

In the case of Compound K, the efficient conversion of this prodrug to the amino acid nucleoside monophosphate (Metabolite X) is a step leading to the observed accumulation of Metabolite X in peripheral blood mononuclear cells (PBMC). See Figure 1. Cellular
25 accumulation of Metabolite X can be measured as in Examples 2A and 2B. In the case of other drug scaffolds with phosphonate prodrug moieties, cleavage of the carboxylic ester present in the phosphonate can also be expected to be a necessary step for the accumulation of the corresponding Metabolite X product. Purification of GS-7340 ester hydrolase B, which cleaves Compound K and other phosphonate prodrug substrates containing an amino acid-like carboxyl or phosphonate ester to form Metabolite X, is described in the examples
30 that follow.

Example 2**Ester Hydrolase Assay****A. Rate of Production of [¹⁴C] Metabolite X:**

The enzymatic production of Metabolite X from Compound K is monitored using the following ester hydrolase assay. Varying amounts of peripheral blood mononuclear cell (PBMC) extracts, column fractions or pools are incubated with [¹⁴C] Compound K at 37°C for 10 – 90 min. The production of [¹⁴C] Metabolite X is monitored by measuring the amount of radioactivity retained on an anion exchange resin (DE-81). High performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis of the reaction mixture and radioactivity retained on the filter confirm that only [¹⁴C]-Metabolite X binds the DE-81 filter. Under the assay conditions, the more hydrophobic [¹⁴C] Compound K is not retained on the DE-81 membrane. The final reaction conditions are 25 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.5, 100 mM NaCl, 1 mM DTT, 30 µM [¹⁴C] GS-7340 substrate, 0.1% NP40 and varying amounts of GS-7340 ester hydrolase enzyme in a final volume of 60 µl. The reaction mixture is incubated at 37°C and at 10, 30 and 90 minutes. 17µl of the reaction mixture is spotted onto a DE-81 filter. The filter is washed with 25 mM Tris, pH 7.5 100 mM NaCl, dried at room temperature, and placed in vials containing 5 ml of scintillation fluid. [¹⁴C]-Metabolite X present on the filters is determined using a scintillation counter (LS 6500, Beckman, Fullerton, CA). Activity is expressed as pmoles of Metabolite X produced per minute per volume enzyme sample. GS-7340 ester hydrolase specific activity is expressed as pmoles of Metabolite X produced / minute / µg protein.

B. Rate of Production of Metabolite X:

The enzymatic production of Metabolite X from non-radioactive Compound K and other prodrug compounds is also monitored using the following HPLC assay. Varying amounts of peripheral blood mononuclear cell (PBMC) extracts, column fractions or pools are incubated with non-radioactive compound substrates at 37°C for 10 – 90 min. Metabolite X is extracted from the reaction mixture and separated from the parent prodrug using HPLC. Reaction mixtures contain 25 mM MesNa (pH 6.5), 100 mM NaCl, 1 mM DTT, 0.1% NP-40, 30 µM substrate and varying amounts of enzyme in a final volume of 100µl. The enzymatic reaction is performed at 37°C for 10 –90 minutes and stopped by adding 180 µl of ice cold methanol. Samples are incubated at -20°C for 30 min, and centrifuged 13,000 RPM for 30 min at 4°C. The supernatant is transferred to a 96-well plate and evaporated under vacuum

using a speedvac. The precipitate is dissolved in 100 μ l of buffer A (25 mM potassium phosphate, pH 6.0, 5 mM TBAB). Bound substrate and the Metabolite X products are monitored at 260 nm, and resolved using a 20 column volume (CV) gradient of buffer B (25 mM potassium phosphate, pH 6.0, 5 mM TBAB, 60% acetonitrile (CH_3CN)). Metabolite X product (identified by the retention time of synthesized Metabolite X and mass spectrometry) consistently elutes earlier than the prodrug substrate and is quantitated by integration of peak area. Activity is expressed as pmoles of Metabolite X produced / minute / volume enzyme sample. GS-7340 ester hydrolase specific activity is expressed as pmoles of Metabolite X produced / minute / μ g protein.

Example 3

Non-Specific Esterase Assay

Non-specific ester hydrolase activity is monitored by monitoring the enzymatic cleavage of alpha naphthyl acetate (ANA) (Mastropaolo and Yourno, *Anal. Biochem.* 115:188-193 (1981)). This substrate has been used for both the measurement of esterase enzyme activity and *in situ* staining of esterases in tissue samples (Yourno and Mastropaolo, *Blood* 58:939-946 (1981); Yourno et al., *Blood* 60:24-29 (1982); Yourno et al., *J. Histochem. Cytochem.* 34:727-733 (1986)). The method described is a modification of the assay described by Mattes and Mattes, *Toxicol. Appl. Pharmacol.* 114: 71-76 (1992). Varying amounts of peripheral blood mononuclear cell (PBMC) extracts, column fractions, or pools are incubated with ANA at 37°C for 20 min. The final reaction conditions are: 10 mM sodium phosphate, pH 6.5, 97 μ M ANA and varying amounts of GS-7340 ester hydrolase enzyme in a final volume of 150 μ l. The reaction mixture is incubated at 37°C for 20 minutes, and the reaction is stopped by the addition of 20 μ l of 10 mM Blue salt RR in 10% sodium dodecyl sulfate (SDS). The alpha naphthyl-Blue salt RR product is detected by reading absorbance at 405 nm. Activity is expressed as pmoles product produced per minute per volume enzyme sample.

Example 4

Isolation of GS-7340 Ester Hydrolase

Extraction of GS-7340 Ester Hydrolase from Human PBMCs

Fresh human PBMCs are obtained from patients undergoing leukapheresis; cells are shipped in plasma and processed within 26 h of draw. PBMCs are harvested by centrifugation at 1200 X g for 5 minutes and washed three times by resuspension in RBC

lysis buffer (155 mM NH_4Cl , 1 mM EDTA, 10mM KHCO_3). Washed cells (29×10^9) are suspended in 150 ml of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 20 mM CaCl_2 , 1 mM DTT and 1% NP40) and incubated on ice for 20 minutes. The PBMC crude extract is centrifuged at 1000 X g for 30 min to remove unlysed cells and the supernatant is centrifuged at 100,000 X g for 1h. The 100,000 X g supernatant (PBMC Extract: P0) is harvested (165ml) and the pellets (1000 X g and 100,000 X g pellets) are resuspended in 10 mM Tris, pH 7.4, 150 mM NaCl, 20 mM CaCl_2 , 1 mM DTT and assayed for GS-7340 ester hydrolase activity. Assays show that less than 2% of the GS-7340 ester hydrolase enzymatic activity is present in the pellets. The cell extract is snap frozen in liquid nitrogen and stored at -70°C . Stability of GS-7340 hydrolase activity at -70°C after freezing and thawing has been demonstrated.

Example 5

Anion Exchange Chromatography

The PBMC Extract (30×10^9 cells, 75 – 85 ml) is diluted (1:10, vol: vol) with 25 mM Tris, pH 7.5, 10% glycerol, 1 mM DTT (Q15 Buffer A) and loaded onto an anion exchange column (2.5 cm X 8.0 cm, Source Q15 (Amersham Biosciences, Piscataway, NJ)), previously equilibrated with Q15 Buffer A. Bound protein is eluted with a linear NaCl gradient (30 column volumes (CV)) to 0.5M NaCl. Eluting protein is detected by monitoring absorbance at 280 nm. Fractions (12.0 ml) are collected and assayed for both GS-7340 ester hydrolase and ANA esterase activity. GS-7340 ester hydrolase activity elutes as a single major peak at 50 – 75 mM NaCl. Recovery of total GS-7340 ester hydrolase activity in the eluted fractions is 50 – 65% of total activity loaded. Significant ANA esterase activity (30-40% of total activity loaded) is detected in the column flow through; however, approximately 30% elutes in two peaks at 70 – 100 mM NaCl. Fractions containing GS-7340 ester hydrolase activity (Q15 pool) are pooled, snap frozen in liquid nitrogen and stored at -70°C .

Example 6

Hydrophobic Interaction (HIC) Chromatography

The Q15 pool is defrosted and diluted (1:1, vol: vol) with 25 mM Tris, pH 8.0, 0.5 M $(\text{NH}_4)_2\text{SO}_4$, 1 mM DTT, 10% glycerol BS-HIC Buffer A). 1 M $(\text{NH}_4)_2\text{SO}_4$ is added to yield a final concentration of 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in the sample. The sample (300 ml / 10×10^9 cells) is loaded onto a Butyl Sepharose HIC column (5 ml HiTrap, Amersham Biosciences, Piscataway, NJ) previously equilibrated with BS-HIC Buffer A. Bound protein is eluted with

a linear gradient (15 CV) decreasing to 25 mM Tris, pH 8.0, 1 mM DTT, 10% glycerol. Eluting protein is detected by monitoring absorbance at 280 nm. Fractions (4.0 ml) are collected and assayed for both GS-7340 ester hydrolase and ANA esterase activity. GS-7340 ester hydrolase activity elutes as a single major peak at 200 – 75 mM (NH₄)₂SO₄. Recovery of total GS-7340 ester hydrolase activity in the eluted fractions is about 50 – 70% of total activity loaded. Significant ANA esterase activity (85% of total activity loaded) is detected in the column flow through; however, approximately 10-15% elutes in a peak at 450 – 300 mM (NH₄)₂SO₄. Fractions containing GS-7340 ester hydrolase activity (BS-HIC pool) are pooled, snap frozen in liquid nitrogen and stored at –70°C.

Example 7

Concanavalin A (Con A) Affinity Chromatography

The BS-HIC pool (40 ml / 10 X 10⁹ cells) is defrosted and diluted (1:1, vol:vol) with Con A Buffer A (20 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂). The sample containing the GS-7340 ester hydrolase activity is loaded onto a Con A column (1.0 ml), previously equilibrated with Con A Buffer A. Bound protein is eluted with Con A Buffer B (Con A Buffer A + 1 M methyl- α 1-manno-pyraniside). Eluting protein is detected by monitoring absorbance at 280 nm. Fractions (0.5 ml) are collected and assayed for GS-7340 ester hydrolase activity. Recovery of total GS-7340 ester hydrolase activity in the eluted fractions is 25-30% of total activity loaded. Fractions containing GS-7340 ester hydrolase activity (Con A pool) are snap frozen in liquid nitrogen and stored at –70°C.

Example 8

Summary of GS-7340 Ester Hydrolase Purification

The following table summarizes the purification of GS-7340 ester hydrolase. Protein is measured by a Coomassie Blue stain colorimetric assay (Bradford Protein Assay, BioRad, Hercules, CA). A 1732 – 6200 fold purification is achieved from the PBMC extracts. Overall recovery of GS-7340 ester hydrolase from PBMC extracts is approximately 5-6%.

Table 2: Purification Summary of GS-7340 Ester Hydrolase

Sample name	PBMC	Protein concentration (mg/ml)	Volume (ml)	Protein (mg)	% Recovery
P0 PBMC	30 X 10 ⁹	5.0	200	1000	
Q15 Pool		0.116 – 0.167	300	35 - 50	~50

Sample name	PBMC	Protein concentration (mg/ml)	Volume (ml)	Protein (mg)	% Recovery
BS-HIC Pool		0.02 – 0.035	100	2.0 – 3.5	~50
ConA Pool		0.0015 – 0.0025	10	0.015 – 0.025	~25
					~6
Estimated Cathepsin A in ConA Pool				0.003 – 0.005	

Example 9

SDS-PAGE Resolution and Identification of Proteins in Con A Pool

The Con A pool containing purified GS-7340 hydrolase is concentrated to approximately 60 μ l (10 kDa MWCO Viva cell, Viva Science, Carlsbad, CA). Aliquots (10 μ l and 50 μ l) are loaded in adjacent lanes onto an SDS-PAGE gel (4-20% acrylamide gradient, NuPAGE; Invitrogen, Inc, Carlsbad, CA) and proteins are resolved according to the manufacturer's instructions. Protein bands in the 10 μ l lane are visualized using silver staining, and the adjacent areas of the gel in the unstained lane (50 μ l aliquot), corresponding to a stained protein band, are excised. In-gel digestion of proteins is performed in 50 mM ammonium bicarbonate containing excess trypsin at 37°C overnight. The resultant peptides are purified by passage through a C₁₈ ZipTip and analyzed by positive ESI-mass spectrometry (ESI-MS) using a Sciex Q-Star / Pulsar mass spectrometer (ABI Biotechnologies, Foster City, CA). Samples are introduced using a nanospray needle and data is collected in the MCA mode. Peptides are sequenced using MS/MS fragmentation. Proteins are identified from the generated sequences by blast analysis of the NCBI nr protein / peptide database. All of the visualized proteins are identified using this technique. A prominent protein band migrating with an apparent molecular weight of 29 kDa yields the sequence I/L F P E Y K. This band represents approximately 5–20 % of the total protein present in the Con A Pool and is identified as human protective protein for beta-galactosidase: Cathepsin (NCBI accession # GI:12653639) (SEQ ID NO: 1--- proform, SEQ ID NO: 2--- mature form). The identity of GS-7340 hydrolase as cathepsin A is confirmed by comparing the biochemical characteristics of the native enzymatic activity to those of recombinant cathepsin A (Cat # 1049-SE, R&D Systems, Minneapolis, MN), and comparing the relative rates of cleavage of the carboxylic ester bond present in different nucleotide phosphoramidate produgs (structure activity relationship, SAR) using the native enzyme and recombinant cathepsin A.

Example 10**High Resolution Gel Filtration Chromatography**

The BS-HIC pool (5ml / 1.25×10^9 cells) is defrosted, concentrated to 0.05 ml using a 5 kDa molecular weight cutoff concentrator (20 ml Vivaspin concentrator, Viva Science, Carlsbad, CA), and loaded onto a high resolution Gel Filtration column (8 mm X 300 mm, KW 802.5; Shodex, Thomas Instrument Co., Oceanside, CA), previously equilibrated with 25 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 20 mM CaCl_2 , 1 mM DTT (KW 802.5 column buffer). Eluting protein is detected by monitoring absorbance at 280 nm. Fractions (0.5 ml) are collected and assayed for GS-7340 ester hydrolase activity. GS-7340 ester hydrolase activity elutes as a single major peak in fractions corresponding to an apparent molecular weight on gel filtration of 70 – 100 kDa. Recovery of total GS-7340 ester hydrolase activity in the eluted fractions is greater than 75% of total activity loaded. Fractions containing GS-7340 ester hydrolase activity (KW 802.5 pool) are pooled, snap frozen in liquid nitrogen and stored at -70°C .

Example 11**Determination of the Isoelectric point (pI) of GS-7340 Ester Hydrolase**

The isoelectric point (pI) of a protein is defined as the pH at which the protein has no net ionic charge. Chromatofocusing is a chromatographic procedure in which a negatively charged protein is bound to a hydrophilic column with a net positive ionic charge. The protein is loaded at a pH 1 to 2 pH units higher than its estimated pI, and the bound protein is eluted by generating a decreasing pH gradient using a pH 3.0 to 4.0 buffer. The proteins are eluted at a pH corresponding to pI.

An aliquot of the BS HIC pool (20 ml, 5×10^9 cells) is concentrated to 4.0 ml and prepared for chromatofocusing chromatography by exchanging buffer using a desalting column. 1.0 ml aliquots of the concentrated BS-HIC pool are loaded onto a 5.0 ml desalting column (5.0 ml HiTrap, Amersham Biosciences, Piscataway, NJ) previously equilibrated with 25 mM ethanolamine, pH 7.8 (pH'd with iminodiacetic acid), 10% glycerol (Mono P Buffer A). The desalted GS-7340 ester hydrolase activity is loaded onto a chromatofocusing column (5 mm X 5 mm HR Mono P, Amersham Biosciences, Piscataway, NJ) previously equilibrated with Mono P Buffer A. Bound protein is eluted with a 20 CV gradient to pH 3.6 with 10 ml / 100 ml Polybuffer 74 (Amersham Biosciences, Piscataway, NJ) and the pH adjusted to 4.0 with iminodiacetic acid. A linear pH gradient is produced from pH 7.8 to pH 3.6. Eluting protein is detected by monitoring absorbance at 280 nm. Fractions (0.5 ml) are

collected and assayed for GS-7340 ester hydrolase activity. GS-7340 ester hydrolase activity elutes as a single major peak at pH 5.5 to 4.5. Recovery of total GS-7340 ester hydrolase activity in the eluted fractions is 65 -70% of total activity loaded. Fractions containing GS-7340 ester hydrolase activity (KW 802.5 pool) are pooled, snap frozen in liquid nitrogen and stored at -70°C .

Example 12

Inhibition of GS-7340 Ester Hydrolases by Serine Hydrolase Inhibitors

Fluorophosphonate (Diisopropylfluorophosphate (DFP)) derivatives, isocoumarins such as 3,4 dichloroisocoumarin (3,4-DCI) and peptide carboxyl esters of chloro- and fluoromethyl ketones (AlaAlaProAla-CMK, AlaAlaProVal-CMK, PheAla-FMK) are known effective inhibitors of serine hydrolases (Powers and Harper, *Inhibitors of Serine Proteases in Protease Inhibitors* 55-152 (Barrett and Salvesen, eds., Elsevier, 1986); Delbaere and Brayer, *J. Mol. Biol.* 183: 89-103 (1985); Bullock et al., *J. Mol. Biol.* 255: 714-725 (1996); Yongsheng et al., *Proc. Natl. Acad. Sci. USA* 96: 14694-14699 (1999); Kam et al., *Bioconjug. Chem.* 4: 560-567 (1993)). Inhibition of the enzymatic production of Metabolite X from Compound K is monitored using the following ester hydrolase inhibition assay. Varying amounts of purified GS-7340 ester hydrolase and control enzymes (human leukocyte elastase (huLE), porcine liver carboxylesterase (PLCE)) are incubated with [^{14}C] Compound K in the presence and absence of varying amounts of known serine hydrolase inhibitors at 37°C for 10 – 90 min. The production of [^{14}C] Metabolite X is monitored by measuring the amount of radioactivity retained on an anion exchange resin (DE-81). The final reaction conditions are: 25 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.5, 100 mM NaCl, 1 mM DTT, 30 μM [^{14}C] GS-7340 substrate, 0.1% NP40, and varying amounts of enzyme and inhibitors (1.0 μM – 1 mM) in a final volume of 60 μl . The reaction mixture is incubated at 37°C for 10, 30 and 90 minutes, and 17 μl of the reaction mixture is spotted onto a DE-81 filter at each time point. The filter is processed and the amount of [^{14}C]-Metabolite X present is determined as described above. Activity is expressed as pmoles Metabolite X produced / minute / volume enzyme sample. Inhibition of ester hydrolase and control hydrolases is expressed as percent activity present at a given concentration of inhibitor compared to hydrolase activity in the absence of the inhibitor. The results of the inhibition experiments are shown in Table 3.

Table 3: Inhibition of GS-7340 Ester Hydrolase and Control Enzymes by Serine Hydrolase Inhibitors

Inhibitor	IC ₅₀ (μM)		
	GS-7340 Ester Hydrolase	PLCE	huLE
3,4-dichloroisocoumarin	0.5	250	3.0
MeOSuC-Ala-Ala-Pro-Ala-CMK	200-400	>1000	60
MeOSuc-Ala-Ala-Pro-Val-CMK	100	>1000	4.0
Biotin-Phe-Ala-FMK	100	>1000	100
DFP	5	0.05	-

Example 13

5 **Relative Cleavage Rates of Nucleotide Phosphoramidate Prodrugs by Native 7340 Hydrolase and Recombinant Cathepsin A**

The relative rates of cleavage (relative to cleavage of Compound K) of the carboxylic ester bond present in different nucleotide phosphoramidate prodrugs (structure activity relationship or SAR) are determined using the native enzyme and recombinant cathepsin A.

- 10 Reaction mixtures contain 25mM MesNa (pH 6.5), 100 mM NaCl, 1 mM DTT, 0.1% NP-40, 30 μM substrate and varying amounts of either GS-7340 hydrolase or recombinant cathepsin A, in a final volume of 100μl. The production of Metabolite X product is monitored using the HPLC method described in Example 2A. Relative activity is expressed as pmoles Metabolite X produced / minute / volume enzyme sample relative to the cleavage of
- 15 Compound K. GS-7340 ester hydrolase and recombinant cathepsin A demonstrate parallel activity against numerous prodrug substrates.

Example 14

20 **Relative Activity of Native 7340 Hydrolase, 9005 Hydrolase A and 9005 Hydrolase B against Phosphonate Prodrug Substrates**

The relative activities of several ester hydrolase enzymes, including native 7340 hydrolase, 9005 hydrolase A and 9005 hydrolase B against several phosphonate prodrug substrates (structure activity relationship, SAR) are determined. The SAR of 7340 hydrolase

against phosphonate prodrugs is distinct from the SAR observed for other ester hydrolases, including both 9005 hydrolase A and 9005 hydrolase B.

Example 15

5 Labeling of GS-7340 Hydrolase and Recombinant Cathepsin A with [¹⁴C] Compound K and [³H] DFP

Labeling of GS-7340 hydrolase (5 µg/ml), recombinant cathepsin A (Cat A) (10 µg/mL) and concanavalin A (75 µg/ml) with [¹⁴C] Compound K and [³H] DFP is performed with both native and denatured enzymes. 100 µL reactions are prepared containing 25 mM
10 Mes-Na buffer, pH 6.5, 100 mM NaCl, 0.1% NP40, and 1 mM DTT. Denatured enzymes are prepared by incubation at 80°C for 3 minutes. Denatured enzymes, native enzymes and concanavalin A (60 µl each) are pre-incubated separately for 2 minutes at 37 °C and reacted with either [¹⁴C] Compound K (1.5 mM, 0.04 mCi/ml, 37°C /2min) or [³H] DFP (25 µM, 0.1 mCi/ml, 37°C /30 min). Labeling is quenched with 0.3% SDS, and proteins are precipitated
15 with ice cold TCA (10% final concentration). Proteins are harvested by centrifugation at 13000 X g. TCA precipitated proteins are harvested by centrifugation at 13000 X g for 30 minutes at 4 °C and washed twice with 700 µl of ice cold 100% acetone. Proteins are dissolved in SDS-PAGE sample buffer (NuPAGE, Novex, La Jolla, CA) and further denatured for 10 min at 70 °C prior to electrophoresis. The gels are fixed in an
20 isopropanol:water:acetic acid solution (25:65:10), soaked in Amplify Fluorographic Reagent (GE Health Care., Piscataway, NJ), dried under vacuum at 80 °C, and exposed to pre-flashed X-ray film (HyperFilm, MP, GE Health Care, Piscataway, NJ) for two weeks. Radioactively labeled bands are visualized after developing the film according to the manufacturer's instructions.

25 The general enzymatic mechanism of serine hydrolases (including cathepsin A) involves the formation of a stable acyl-enzyme complex between the serine residue in the active site of the enzyme and the substrate (Satoh, T and Hosakawa, M 1998). Denaturing of the enzyme with SDS/TCA during the course of the reaction should allow capture of this acyl-enzyme complex. SDS-PAGE analysis of each reaction mixture shows the presence of a
30 single [¹⁴C]-Compound K-labeled band at 29 kDa. The substrate fails to label both heat denatured GS-7340 hydrolase and cathepsin A, as well as concanavalin A protein (15 µg loaded).

Labeling with [³H]-DFP is an additional tool for visualizing catalytic subunits of serine hydrolases. (Lundqvist, H., Dahlgren, C., *Inflamm. Res.*, "The serine protease inhibitor

diisopropylfluorophosphate inhibits neutrophil NADPH-oxidase activity induced by the calcium ionophore ionomycin and serum opsonised yeast particles”, 44(12):517-517 (1995)). Unlike transient formation of an acyl-enzyme complex, DFP forms a stable covalent bond with the hydroxy group in serine in the active site of these enzymes. [³H]-DFP specifically labels the 29 kDa catalytic subunits of both GS-7340 hydrolase and cathepsin A. Denatured enzymes fail to react with the inhibitor. These data provide strong evidence that the purified hydrolase fraction contains a single protein capable of hydrolyzing Compound K. Furthermore, the labeled 29 kDa band co-migrates with the catalytic subunit of cathepsin A. Western blot analysis using polyclonal goat antibodies against human cathepsin A (R&D Systems, Minneapolis, MN)) confirm that cathepsin A is present in all pools of fractions showing GS-7340 hydrolase activity, and that the antibody reacts with a band which co-migrates with the 29 kDa catalytic subunits labeled using [¹⁴C]-Compound K and [³H]-DFP.

Example 16

Metabolism of Acyclic Nucleotide Phosphonate Prodrugs in Cat A+/- Fibroblasts

Human primary fibroblasts from patients exhibiting galactosialidosis are deficient in the expression of cathepsin A (Cat A-) due to mutations in the sequence that prevent proper folding. Cat A- (GM05076, GM02348) and Cat A+ (GM00409, GM5400 and GM05757B) fibroblasts (Coriell Institute for Medical Research, Camden, NJ) are cultivated in minimum essential (Eagle-Earle) medium supplemented with 2mM L-glutamine, 0.1 mM non-essential amino acids, 1 x vitamins and 15% fetal bovine serum (FBS). HEL fibroblasts (Cat A +) (ATCC, Manassas, VA 20108) are maintained in MEM (Eagle) medium with 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and 10% FBS. Cells are split 1:3 (vol:vol) when the cellular density reaches 80% confluence.

Cells are seeded into T75 flasks at an initial cell density of 4000-12000 cells per cm² in cultivation media. The next day the medium is replenished and cell cultures are pre-incubated for 1.5 hour at 37°C. ¹⁴C-labeled Compound K is added to a final concentration of 10 μM (0.521μCi/ml) and fibroblasts are further incubated at 37 °C for 15 – 240 min. At selected time points (0, 30, 60, 120, and 240 min) media is removed and cells are washed 2x with ice cold PBS (1x). Fibroblasts are detached from the flask by incubation for 3 min at 37°C using 1.5 ml of 1x Trypsin-EDTA PBS solution (0.5g/ml). Trypsin reaction is stopped by adding 5 ml of cultivation media and cells are harvested by centrifugation for 5 min at 1500 RPM (Beckman GPR, 4 °C). Cells are washed with 8 ml of PBS, harvested and extracted in 1 ml of 80% MetOH.

Measurement of Cell Associated [C^{14}]-Metabolites

Cell extracts (80% MetOH suspensions) are incubated at -20°C overnight. Supernatants containing solubilized C^{14} -metabolites are recovered by centrifugation (13000 g), evaporated in a speed-vac, and resolubilized in 200 μl of water. Aliquots (40 μl) are analyzed to determine the amount of radioactivity (C^{14} -metabolites) present in the cells using scintillation reader (Beckman LS 6500). The calibration curve for radioactivity (cpm) vs pmol of [^{14}C] Compound K and the number of cells present in 40 μl of lysate is used to calculate the total amount of metabolites (in pmols) present in 10^6 cells. The cell volume measurements are used to determine metabolite concentration (μM).

Cell Volume Measurement

Fibroblasts are washed with PBS and detached by incubation with Trypsin (0.5g/ml in PBS, 1 μM EDTA) for 5 minutes at 37°C . The cells are incubated for 30 minutes in the culture medium to regain their spherical shape. Pictures of cells are taken and their diameters measured. Cell diameter is determined using a hemacytometer. Cell volume is calculated using the mean diameter obtained from 150 cells. The concentration of metabolites in Cat A+ control fibroblasts is about 6-9 times greater than the concentration of metabolites observed in fibroblasts from Cat A- (galactosialidosis) patients.

Example 17

Characterization of Exemplary Anti-HIV Compounds

HIV-1 Protease Enzyme Assay (K_i):

The assay is based on the fluorimetric detection of synthetic hexapeptide substrate cleavage by HIV-1 protease in a defined reaction buffer as initially described by Toth and Marshall, *Int. J. Peptide Protein Res.* 36: 544 (1990).

The substrate used is (2-aminobenzoyl)Thr-Ile-Nle-(p-nitro)Phe-Gln-Arg substrate (Catalog No. H-2992) from Bachem California, Inc. (Torrance, CA). Recombinant HIV-1 protease expressed in *E. coli* is also obtained from Bachem California, Inc. (Torrance, CA, Catalog No. H9040). The reaction is conducted in a reaction buffer (RB) containing: 100 mM ammonium acetate, pH 5.3; 1 M sodium chloride; 1 mM ethylenediaminetetraacetic acid; 1 mM dithiothreitol (DTT); and 10% dimethylsulfoxide. In order to determine the inhibition constant (K_i), the following assay is conducted. A series of solutions containing identical amount of the enzyme (1 to 2.5 nM) and different concentrations of a tested inhibitor is prepared in the reaction buffer. The solutions are transferred (190 μL each) into a white 96-

well plate. The reactions are preincubated for 15 minutes at 37°C. The substrate is solubilized in 100% dimethylsulfoxide at a concentration of 800 μ M. The reaction is started by adding 10 μ L of 800 μ M substrate into each well to a final substrate concentration of 40 μ M. The real-time reaction kinetics are measured at 37°C by using a Gemini 96-well plate fluorimeter (Molecular Devices, Sunnyvale, CA) at $\lambda(\text{Ex}) = 330$ nm and $\lambda(\text{Em}) = 420$ nm. The initial velocities of the reactions are determined with different inhibitor concentrations. The K_i value (in picomolar concentration units) is calculated by using EnzFitter program (Biosoft, Cambridge, U.K.) according to an algorithm for tight-binding competitive inhibition described by Ermolieff et al., *Biochemistry* 36: 12364 (1997).

Anti-HIV-1 Cell Culture Assay (EC_{50}):

The following anti-HIV-1 assay is based on quantification of the HIV-1-associated cytopathic effect by a colorimetric detection of the viability of virus-infected cells in the presence or absence of tested inhibitors. The HIV-1-induced cell death is determined using a metabolic substrate 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), which substrate is converted only by intact cells into a product with specific absorption characteristics as described by Weislow et al., *J. Natl. Cancer Inst.* 81: 577 (1989).

In order to determine the EC_{50} , the following assay conditions are used. MT2 cells are maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum and antibiotics. The MT2 cells are infected with the wild-type HIV-1 strain IIIB (Advanced Biotechnologies, Columbia, MD) for 3 hours at 37°C using the virus inoculum corresponding to a multiplicity of infection equal to 0.01. A set of solutions is prepared to contain various concentrations of the tested inhibitor by making 5-fold serial dilutions in 96-well plate (100 μ L/well).

Infected cells are distributed into the 96-well plate (20,000 cells in 100 μ L/well). Samples with untreated infected and untreated mock-infected control cells are included. The cells are incubated for 5 days at 37°C. 6 mL of XTT solution per assay plate is prepared at a concentration of 2mg/mL in a phosphate-buffered saline, pH 7.4. The solution is heated in water-bath for 5 min at 55°C. 50 μ L of N-methylphenazonium methasulfate (5 μ g/mL) per 6 mL of XTT solution is added. 100 μ L media is removed from each well on the assay plate. 100 μ L of the XTT substrate solution is added to each well and the assay plate is incubated at

37°C for 45 to 60 min in a CO₂ incubator. In order to inactivate the virus, 20 µL of 2% Triton X-100 is added to each well.

Absorbance is read at 450 nm and the background absorbance at 650 nm is subtracted. The percentage absorbance is plotted relative to untreated control and the EC₅₀ value is estimated as drug concentration resulting in a 50% protection of the infected cells.

Cytotoxicity Cell Culture Assay (CC₅₀):

The following cytotoxicity assay is based on the evaluation of cytotoxic effect of tested compounds using a metabolic substrate 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) as described by Weislow et al., *J. Natl. Cancer Inst.* 81: 577 (1989). MT2 cells are maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum and antibiotics. A set of solutions is prepared to contain various concentrations of the tested inhibitor by making 5-fold serial dilutions in 96-well plate (100 µL /well). Cells are distributed into the 96-well plate (20,000 cells in 100 µL/well). Samples with untreated cells are included as a control. Cells are incubated for 5 days at 37°C. Sufficient XTT solution for 6mL solution per assay plate is prepared in the dark at a concentration of 2mg/mL in a phosphate-buffered saline, pH 7.4. The solution is heated in a water-bath at 55°C for 5 min. 50 µL of N-methylphenazonium methasulfate (5 µg/mL) is added per 6 mL of XTT solution. 100 µL media is removed from each well on the assay plate and 100 µL of the XTT substrate solution is added to each well. The assay plate is incubated at 37°C for 45 to 60 min in a CO₂ incubator. 20 µL of 2% Triton X-100 is added to each well to stop the metabolic conversion of XTT. The absorbance at 450 nm is read and the background at 650 nm is subtracted. The absorbance is considered as directly proportional to the cell growth. The percentage absorbance relative to untreated control is plotted and the CC₅₀ value is estimated as drug concentration resulting in a 50% inhibition of the cell growth.

Resistance Evaluation (I50V and I84V/L90M fold change):

The assay is based on the determination of a difference in the susceptibility to a particular HIV protease inhibitor between the wild-type HIV-1 strain and a mutant HIV-1 strain containing specific drug resistance-associated mutation(s) in the viral protease gene. The absolute susceptibility of each virus (EC₅₀) to a particular tested compound is measured by using the XTT-based cytopathic assay as described above. The degree of resistance to a tested compound is calculated as fold difference in EC₅₀ between the wild type and a specific mutant virus. This represents a standard approach for HIV drug resistance evaluation as

documented in various publications (e.g., Maguire et al., *Antimicrob. Agents Chemother.* 46: 731 (2002); Gong et al., *Antimicrob. Agents Chemother.* 44: 2319 (2000); Vandamme and De Clercq, in *Antiviral Therapy* 243 (E. De Clercq, ed.), ASM Press, Washington, DC (2001)).

5 HIV-1 strains used for resistance evaluation:

Two strains of mutant viruses containing I50V mutation in the protease gene are used in the resistance assays: one with M46I/I47V/I50V mutations (designated I50V #1) and the other with L10I/M46I/I50V (designated I50V #2) mutations in the viral protease gene. A third virus with I84V/L90M mutations is also employed in the resistance assays. Mutants

10 I50V #1 and I84V/L90M are constructed by a homologous recombination between three overlapping DNA fragments: 1. linearized plasmid containing wild-type HIV-1 proviral DNA (strain HXB2D) with the protease and reverse transcriptase genes deleted; 2. DNA fragment generated by PCR amplification containing reverse transcriptase gene from HXB2D strain (wild-type); 3. DNA fragment of mutated viral protease gene that has been generated by PCR

15 amplification. An approach similar to that described by Shi and Mellors, in *Antimicrob. Agents Chemother.* 41: 2781-85 (1997) is used for the construction of mutant viruses from the generated DNA fragments. Mixture of DNA fragments is delivered into Sup-T1 cells by using a standard electroporation technique. The cells are cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics until the recombinant virus

20 emerges (usually 10 to 15 days following the electroporation). Cell culture supernatant containing the recombinant virus is harvested and stored in aliquots. After verification of protease gene sequence and determination of the infectious virus titer, the viral stock is used for drug resistance studies. Mutant I50V #2 is an amprenavir-resistant HIV-1 strain selected *in vitro* from the wild-type IIIB strain in the presence of increasing concentration of

25 amprenavir over a period of > 9 months using an approach similar to that described by Partaledis et al., *J. Virol.* 69: 5228-5235 (1995). Virus capable of growing in the presence of 5 μ M amprenavir is harvested from the supernatant of infected cells and used for resistance assays following the titration and protease gene sequencing.

30

All publications and patent applications cited are herein incorporated by reference in their entireties. Although certain embodiments are described in detail, one of ordinary skill in the art will clearly understand that many modifications are possible without departing from

the spirit and scope of the teachings herein. All such modifications are intended to be encompassed within the present invention.

What is claimed is:

1. A method for identifying a candidate compound as a suitable prodrug, comprising:
 - (a) providing a candidate compound having an esterified phosphonate group or an esterified carboxyl group;
 - (b) contacting the candidate compound with an extract that comprises GS-7340 ester hydrolase to produce one or more metabolite compounds; and
 - (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.
2. The method of claim 1, wherein said extract is obtained from peripheral blood mononuclear cells.
3. The method of claim 1, wherein said extract is a fully purified extract.
4. The method of claim 1, wherein said extract comprises an enzyme comprising SEQ ID NO: 1 or fragment thereof.
5. The method of claim 1, wherein said providing step comprises providing a candidate compound formed by substituting a prototype compound.
6. The method of claim 1, further comprising (d) determining the intracellular persistence of the candidate compound.
7. The method of claim 1, further comprising (d) determining the intracellular persistence of at least one of the one or more metabolite compounds.
8. The method of claim 3, further comprising (d) determining the tissue selectivity of the candidate compound.
9. The method of claim 3, further comprising (d) determining the tissue selectivity of at least one of the one or more metabolite compounds.

10. A method of screening candidate compounds for suitability as therapeutic agents, comprising:
- 5 (a) providing a candidate compound identified by providing the candidate compound having an esterified phosphonate group or an esterified carboxyl group, contacting the candidate compound with an extract that has GS-7340 ester hydrolase activity to produce one or more metabolite compounds, and identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified
- 10 carboxyl group of the candidate compound; and
- (b) determining the therapeutic activity of the candidate compound; and
- (c) determining the intracellular persistence of the candidate compound.
11. The method of claim 10, wherein said step (b) comprises determining the activity of
- 15 the candidate compound against HIV protease.
12. The method of claim 10, wherein said step (b) comprises determining the ability of the candidate compound to inhibit HIV.
13. The method of claim 12, wherein said step (b) comprises determining the ability of
- 20 the candidate compound to inhibit HIV protease.
14. The method of claim 12, wherein said step (b) comprises determining the ability of the candidate compound to inhibit HIV integrase.
- 25 15. The method of claim 12, wherein said step (b) comprises determining the ability of the candidate compound to inhibit HIV reverse transcriptase.
16. The method of claim 12, wherein said step (b) is performed by *in vitro* assay.
- 30 17. A method for identifying a candidate compound as a suitable prodrug, comprising:
- (a) providing a candidate compound formed by substituting a prototype compound believed to have therapeutic activity with an esterified phosphonate or an esterified carboxyl group;

- (b) contacting the candidate compound with an extract that has GS-7340 ester hydrolase activity to produce one or more metabolite compounds; and
- (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.
18. The method of claim 17, wherein said extract of peripheral blood mononuclear cells comprises an enzyme comprising SEQ ID NO: 1 or fragment thereof.
19. The method of claim 17, wherein said contacting step comprises contacting the candidate compound with the extract in a cell-free environment.
20. The method of claim 17, wherein said contacting step comprises contacting the candidate compound with the extract *in vitro*.
21. The method of claim 17, wherein said contacting step comprises contacting the candidate compound with the extract in cell culture.
22. The method of claim 17, wherein said contacting step comprises contacting the candidate compound with the extract in a culture of peripheral blood mononuclear cells.
23. The method of claim 17, wherein said therapeutic activity is therapeutic activity against HIV.
24. The method of claim 17, wherein said therapeutic activity is therapeutic activity against cancer.
25. The method of claim 17, wherein said therapeutic activity is therapeutic activity against inflammation.
26. A method for identifying a candidate compound as a suitable prodrug, comprising:
- (a) providing a candidate compound having an esterified phosphonate group or an

esterified carboxyl group;

(b) contacting the candidate compound with an extract that comprises cathepsin A or a fragment thereof to produce one or more metabolite compounds; and

(c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

27. A method for identifying a candidate compound as a suitable prodrug comprising:

(a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity;

(b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and

(c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

28. The method of claim 27, wherein the prototype compound is not a nucleoside and does not contain a nucleoside base.

29. The method of claim 27, further comprising (d) determining the intracellular persistence of the candidate compound.

30. The method of claim 27, further comprising (d) determining the intracellular persistence of at least one of the one or more metabolite compounds.

31. The method of claim 27, further comprising (d) determining the intracellular persistence of the candidate compound and at least one of the one or more metabolite compounds.

32. The method of claim 27, further comprising (d) determining the tissue selectivity of the candidate compound.
33. The method of claim 27, further comprising (d) determining the tissue selectivity of at least one of the one or more metabolite compounds.
34. The method of claim 27, further comprising (d) determining the tissue selectivity of the candidate compound and at least one of the one or more metabolite compounds.
35. A method for identifying a candidate compound as a suitable prodrug comprising:
- (a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity against human immunodeficiency virus;
 - (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and
 - (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.
36. The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising:
- (a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound believed to have therapeutic activity against inflammation;
 - (b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and
 - (c) identifying the candidate compound as a suitable prodrug if at least one of the one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

37. The present invention also includes and provides a method for identifying a candidate compound as a suitable prodrug comprising:

(a) providing a candidate compound formed by substituting an esterified phosphonate group or an esterified carboxyl group into a prototype compound
5 believed to have therapeutic activity against cancer;

(b) contacting the candidate compound with an extract of peripheral blood mononuclear cells having carboxylic acid ester hydrolase activity to produce one or more metabolite compounds; and

(c) identifying the candidate compound as a suitable prodrug if at least one of the
10 one or more metabolite compounds has a phosphonic acid group instead of the esterified phosphonate group of the candidate compound, or a carboxylic acid group instead of the esterified carboxyl group of the candidate compound.

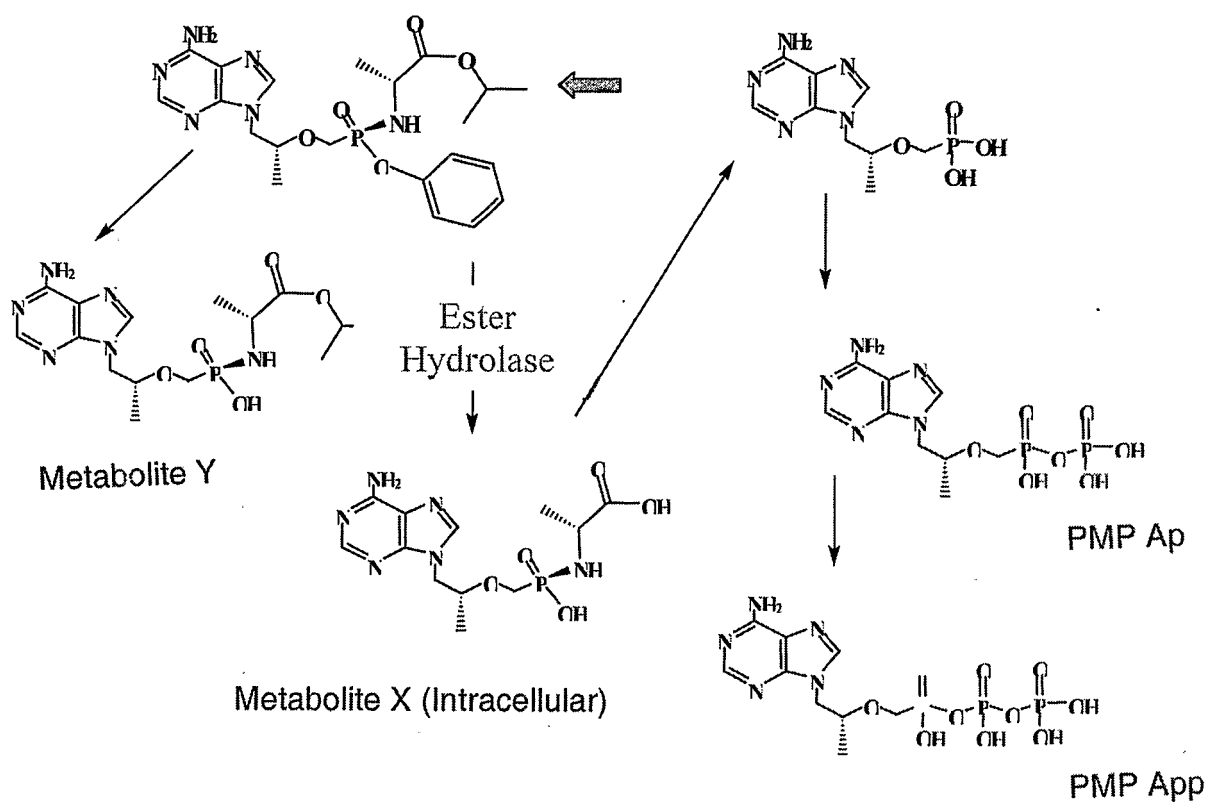
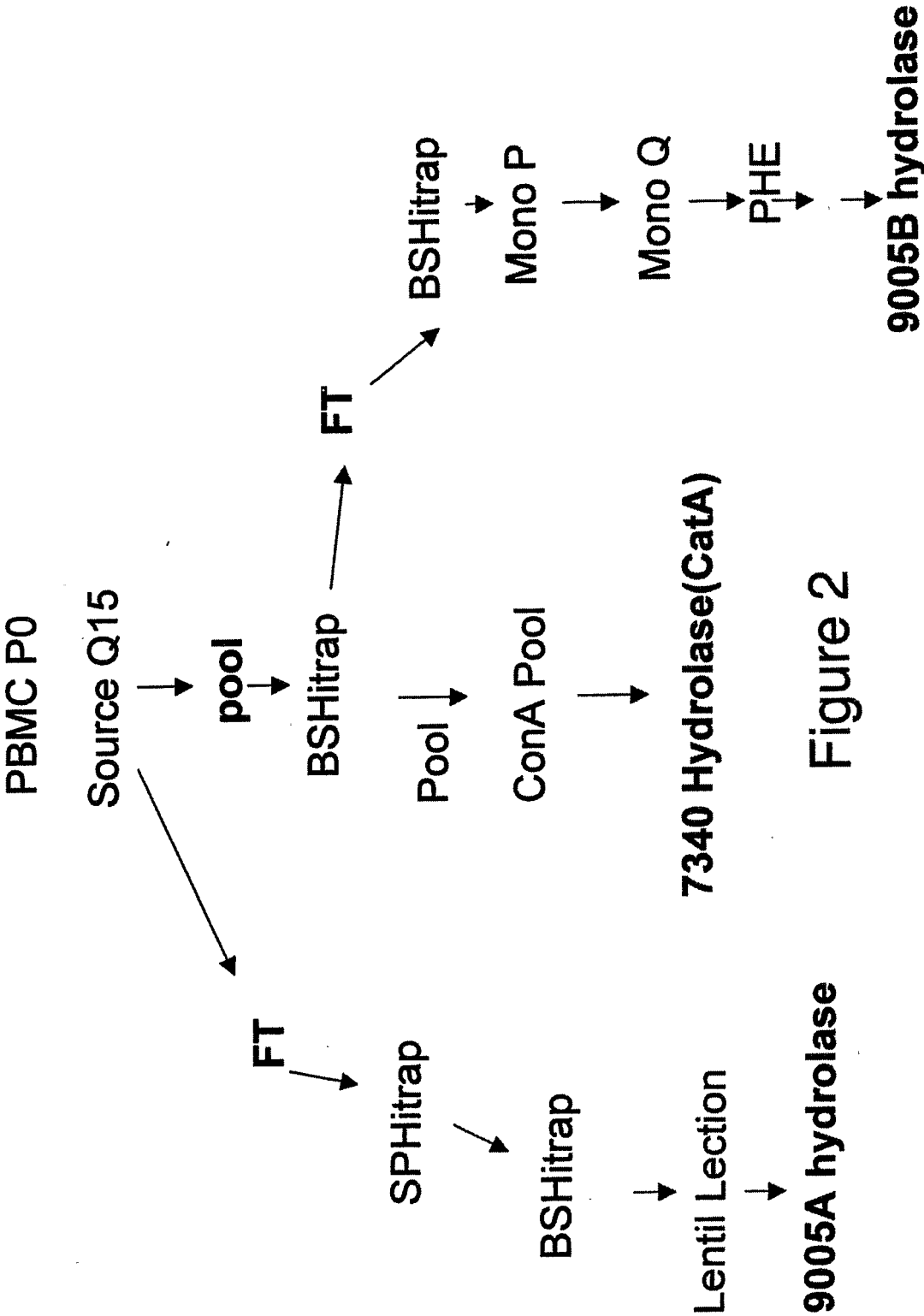


Figure 1

Exemplary Hydrolase Purification



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专利名称(译)	用于鉴定治疗化合物的方法和组合物		
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[标]申请(专利权)人(译)	吉利德科学公司		
申请(专利权)人(译)	GILEAD SCIENCES , INC.		
当前申请(专利权)人(译)	GILEAD SCIENCES , INC.		
发明人	MCDERMOTT, M., C/O GILEAD SCIENCES, INC.		
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摘要(译)

通过本发明，鉴定了负责前体药物活化的酶，并将其用于鉴定候选化合物作为前药。本发明包括鉴定候选化合物作为合适的前药的方法，以及筛选候选化合物作为治疗剂的适合性的方法。