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(57) **Abrégé/Abstract:**

The invention pertains to dog P-glycoproteins and related P-glycoproteins which include dog-specific amino acids, as well as nucleic acids which encode those polypeptides. The present invention also includes fragments and biologically functional variants of the dog P-glycoprotein. The invention further relates to methods of using such dog P-glycoprotein nucleic acids and polypeptides, especially in methods for determining bioavailability of drugs and for screening for inhibitors of dog PGP. Also included are dog PGP inhibitors which inhibit dog PGP activity by inhibiting the expression or function of dog PGP.

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(54) Title: P-GLYCOPROTEINS AND USES THEREOF

(57) Abstract: The invention pertains to dog P-glycoproteins and related P-glycoproteins which include dog-specific amino acids, as well as nucleic acids which encode those polypeptides. The present invention also includes fragments and biologically functional variants of the dog P-glycoprotein. The invention further relates to methods of using such dog P-glycoprotein nucleic acids and polypeptides, especially in methods for determining bioavailability of drugs and for screening for inhibitors of dog PGP. Also included are dog PGP inhibitors which inhibit dog PGP activity by inhibiting the expression or function of dog PGP.



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P-GLYCOPROTEINS AND USES THEREOF

Field of the Invention

The invention pertains to P-glycoproteins of dog (*Canis familiaris*).

Background of the Invention

P-glycoprotein (PGP; also known as multidrug transporter, MDR1) is a member of the ABC transporter superfamily and is expressed in the human intestine, liver and other tissues. This enzyme serves as an efflux pump exporting small molecules across the cell membrane.

It has been known for several years that high level expression of PGP is a mechanism for tumor resistance to cancer chemotherapy. Intestinal expression of PGP may affect the oral bioavailability of drug molecules that are substrates for this transporter. PGP can efficiently efflux drugs back into the intestinal lumen and thus reduce the amount of drug that enters into circulation.

The measurement of interaction with PGP can provide a better understanding of the reasons why particular drugs demonstrate low or high bioavailability. Interaction with PGP can be studied using either direct assays of drug transport in polarized cell systems or with indirect assays such as drug-stimulated ATPase activity and inhibition of the transport of fluorescent substrates.

Therefore there is a need for additional PGP polypeptides, preferably which are closely related to the human PGP, for use in the foregoing drug assays.

Summary of the Invention

Nucleic acids encoding P-glycoprotein of dog (*Canis familiaris*) have now been identified, isolated, cloned and sequenced. This PGP is closely related (has a high degree of identity) to the human PGP. The invention provides isolated nucleic acid molecules, unique fragments of those molecules, expression vectors containing the foregoing, and host cells transfected with those molecules. The invention also provides isolated polypeptides and inhibitors of the foregoing nucleic acids and polypeptides which reduce drug transport. The PGP nucleic acids and polypeptides are useful in assays for evaluating bioavailability of drugs, as well as for the optimization or discovery of drugs. In addition, the foregoing can be used in the diagnosis or treatment of conditions characterized by PGP activity and can be used

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in methods in which it is therapeutically useful to increase or decrease PGP activity.

According to one aspect of the invention, an isolated nucleic acid molecule is provided which is selected from the group consisting of (a) nucleic acid molecules that code for the amino acid sequence of SEQ ID NO:2, (b) allelic variants of (a), wherein the allelic
5 variants exclude SEQ ID NO:3 and SEQ ID NO:5, and (c) complements of (a) or (b).

Preferred allelic variants include nucleic acid molecules that encode an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:27, particularly nucleotide sequences selected from the group consisting of SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26. In preferred embodiments the isolated nucleic acid molecule
10 codes for SEQ ID NO:2, or comprises the nucleotide sequence of SEQ ID NO:1. In particularly preferred embodiments, the nucleic acid molecules comprise or consist of the coding region of the aforementioned nucleotide sequences.

According to other aspects of the invention, isolated P-glycoprotein polypeptides or fragments thereof are provided. The polypeptides include at least one amino acid of a dog P-glycoprotein selected from the group consisting of amino acids 25, 192, 197, 212, 288, 329,
15 532, 696, 1273 and 1355 of SEQ ID NO:2; amino acid 25 of SEQ ID NO:23; and amino acids 25 and 1148 of SEQ ID NO:25. Other isolated P-glycoprotein polypeptides or fragments thereof include at least one amino acid of a dog P-glycoprotein selected from the group consisting of amino acids 3, 6, 8, 10, 12, 14-26, 36, 38, 48, 52, 56, 64, 74, 78, 84-92, 94, 96,
20 98, 99, 101, 103, 104, 106, 108, 112, 115, 147, 187, 197, 199, 233, 288, 321, 326, 347, 397, 450, 454, 455, 467, 472, 520, 633, 637, 643, 644, 650, 657, 658, 661, 666, 667, 674-677, 679, 685, 689, 691, 693, 694, 703, 707, 717, 731, 736, 740, 744, 745, 756, 759, 763, 853, 914, 920, 942, 943, 946, 968-970, 972, 974, 983, 1005, 1010, 1017, 1025, 1026, 1029, 1040, 1095, 1098, 1105, 1144, 1148, 1149, 1158, 1162, 1165, 1168, 1170, 1252 and 1279 of SEQ ID
25 NO:2; and amino acid 329 of SEQ ID NO:27, wherein the P-glycoprotein is identical to a human P-glycoprotein except for the at least one amino acid of a dog P-glycoprotein. In certain embodiments, the human P-glycoprotein is selected from the group of SEQ ID NO:7 and SEQ ID NO:8. Still other isolated P-glycoprotein polypeptides or fragments thereof include at least one amino acid of a dog P-glycoprotein that is different from a cynomologous
30 P-glycoprotein, wherein the P-glycoprotein is identical to a cynomologous monkey P-glycoprotein except for the at least one amino acid of a dog P-glycoprotein. Yet other polypeptides include combinations of the foregoing dog, human and cynomologous PGP

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polypeptides. In preferred embodiments, the isolated P-glycoprotein polypeptides or fragments thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, fragments of SEQ ID NO:2, fragments of SEQ ID NO:23, fragments of SEQ ID NO:25 and fragments of SEQ ID NO:27.

5 According to still other embodiments of the invention, isolated nucleic acid molecules are provided which encode the foregoing isolated P-glycoprotein polypeptides or fragments thereof. Also included are expression vectors comprising the foregoing isolated nucleic acid molecules operably linked to a promoter, as well as host cells transformed or transfected with the expression vectors.

10 In another aspect of the invention, agents which selectively bind the isolated PGP polypeptides are provided. Preferably the agent does not bind a human or dog P-glycoprotein, except those provided herein. In certain embodiments, the agent is a polypeptide preferably one selected from the group consisting of monoclonal antibodies, polyclonal antibodies, Fab antibody fragments, F(ab)₂ antibody fragments and antibody fragments including a CDR3
15 region. Also provided are agents which selectively bind the foregoing isolated nucleic acid molecules, preferably antisense nucleic acid molecules which selectively bind to the isolated nucleic acid molecule.

According to another aspect of the invention, methods for predicting the bioavailability of a compound are provided. The methods include measuring the
20 transmembrane transport of a test compound by a first P-glycoprotein, comparing the transmembrane transport of the test compound by the first P-glycoprotein and a second P-glycoprotein to predict the bioavailability of the test compound, wherein the relative amount or rate of transport by the first P-glycoprotein and the second P-glycoprotein is predictive of bioavailability of the test compound. In certain embodiments the first P-
25 glycoprotein is selected from the group consisting of dog P-glycoproteins and primate P-glycoproteins, preferably one of the foregoing polypeptides. In other embodiments the second P-glycoprotein is a human P-glycoprotein.

In still other aspects of the invention, methods for inhibiting P-glycoprotein transporter activity in a mammalian cell are provided. The methods include contacting the
30 mammalian cell with an amount of one of the foregoing agents effective to inhibit P-glycoprotein transporter activity in the mammalian cell.

Also included in the invention are methods for increasing bioavailability of a drug in a

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subject. The methods include administering to a subject in need of such treatment one of the foregoing agents in an amount effective to increasing bioavailability of a drug. The inhibitor can be administered prior to administering the drug, or concurrently with the drug.

Also provided are methods for increasing P-glycoprotein transporter activity in a cell.

5 These methods include contacting the cell with a molecule selected from the group consisting of the foregoing nucleic acid molecules, in an amount effective to increase P-glycoprotein transporter activity in the cell. The cell can be contacted under conditions whereby the P-glycoprotein is expressed.

According to yet another aspect of the invention, methods for identifying lead
10 compounds for a pharmacological agent useful in the treatment of disease associated with P-glycoprotein transporter activity are provided. The methods include providing a cell or other membrane-encapsulated space comprising a P-glycoprotein as provided herein; contacting the cell or other membrane-encapsulated space with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a
15 first amount of P-glycoprotein transporter activity; and determining a second amount of P-glycoprotein transporter activity as a measure of the effect of the pharmacological agent on the P-glycoprotein transporter activity, wherein a second amount of P-glycoprotein transporter activity which is less than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces P-glycoprotein transporter
20 activity and wherein a second amount of P-glycoprotein transporter activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases P-glycoprotein transporter activity. The methods can further include a step of loading the cell or other membrane-encapsulated space with a detectable compound, wherein the compound is detected as a measure of the P-glycoprotein
25 transporter activity.

Also included are methods for identifying compounds which selectively bind a P-glycoprotein. The methods include contacting a P-glycoprotein provided herein with a compound, and determining the binding of the compound to the P-glycoprotein. The methods can further include determining the effect of the compound on the P-glycoprotein transporter
30 activity of the P-glycoprotein or determining the effect of the compound on the ATPase activity of the P-glycoprotein.

Additional methods provided according to the invention include methods for

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determining ATPase activity of a P-glycoprotein. The methods include contacting a host cell as provided above, or a membrane fraction thereof, with a test drug, and measuring ATPase activity of the P-glycoprotein. In certain embodiments, the step of measuring ATPase activity is performed at least twice at different times. Also provided methods for determining

5 transmembrane transport of a compound by a P-glycoprotein. The methods include contacting a host cell provided above, or a membrane fraction thereof, with a test drug, and measuring transport of the test drug under sink conditions in at least one direction of transport selected from the group consisting of the apical to basolateral direction and the basolateral to apical direction. In certain embodiments the step of measuring transport of the test drug is

10 performed at least twice at different times.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence encoding dog P-glycoprotein (Genotype C).

15 SEQ ID NO:2 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence encoding a prior art dog P-glycoprotein (GenBank accession number AF045016).

20 SEQ ID NO:4 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:3.

SEQ ID NO:5 is the nucleotide sequence encoding another prior art dog P-glycoprotein (GenBank accession number AF092810).

SEQ ID NO:6 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:5.

25 SEQ ID NO:7 is the amino acid sequence of a prior art human P-glycoprotein, having Genbank accession number M14758.

SEQ ID NO:8 is the amino acid sequence of a second prior art human P-glycoprotein, having Genbank accession numbers AF016535 or NM_000927.

30 SEQ ID NO:9 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:10 is the nucleotide sequence of a PCR primer homologous to dog PGP2.

SEQ ID NO:11 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

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SEQ ID NO:12 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:13 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

5 SEQ ID NO:14 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:15 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:16 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:17 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

10 SEQ ID NO:18 is the nucleotide sequence of a PCR primer homologous to vector.

SEQ ID NO:19 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:20 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:21 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

15 SEQ ID NO:22 is the nucleotide sequence encoding dog P-glycoprotein (Genotype A).

SEQ ID NO:23 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:22.

SEQ ID NO:24 is the nucleotide sequence encoding dog P-glycoprotein (Genotype B).

20 SEQ ID NO:25 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:24.

SEQ ID NO:26 is the nucleotide sequence encoding dog P-glycoprotein (Genotype D).

SEQ ID NO:27 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:25.

25 SEQ ID NO:28 is the nucleotide sequence of the cDNA synthesis primer from the Marathon cDNA Amplification Kit.

SEQ ID NO:29 is the nucleotide sequence of the cDNA Adapter Primer 1 from the Marathon cDNA Amplification Kit.

SEQ ID NO:30 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:31 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

30 SEQ ID NO:32 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

Detailed Description of the Invention

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The present invention in one aspect involves the identification of novel cDNAs encoding dog P-glycoproteins, referred to herein as dog PGP. The nucleotide sequence of a dog PGP (termed "Genotype C") is presented as SEQ ID NO:1, and the amino acid sequence of this dog PGP is presented as SEQ ID NO:2.

5 Three allelic variants of Genotype C dog PGP were isolated ("Genotype A", "Genotype B" and "Genotype D"). The nucleotide and amino acid sequences of Genotype A are presented as SEQ ID NOs:20 and 21, respectively. The amino acid sequence of Genotype A differs from the amino acid sequence of Genotype C at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2. The nucleotide and
10 amino acid sequences of Genotype B are presented as SEQ ID NOs:22 and 23, respectively. The amino acid sequence of Genotype B differs from the amino acid sequence of Genotype C at amino acid 25 (asparagine → lysine) based on a T → A change at nucleotide 91 of SEQ ID NO:2, and at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2. The nucleotide and amino acid sequences of Genotype D are presented
15 as SEQ ID NOs:24 and 25, respectively. The amino acid sequence of Genotype D differs from the amino acid sequence of Genotype C at amino acid 25 (asparagine → lysine) based on a T → A change at nucleotide 91 of SEQ ID NO:2, at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2, at amino acid 329 (serine → threonine) based on a T → A change at nucleotide 1001 of SEQ ID NO:2, and at
20 amino acid 1148 (methionine → valine) based on a A → G change at nucleotide 3458 of SEQ ID NO:2.

Two closely related dog PGP sequence were deposited in GenBank under accession numbers AF045016 (complete cDNA) and AF092810 (partial cDNA). Whereas much of the polypeptides presented herein is identical to the known dog PGPs, the dog PGPs of the
25 invention (SEQ ID NOs:2, 21, 23 and 25) have several single amino acid differences from the prior art sequences, including at least one deletion. These allelic differences in the very highly conserved protein domains of the P-glycoprotein are entirely unexpected.

The invention involves in one aspect dog PGP nucleic acids and polypeptides, as well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent
30 variants, useful analogs and fragments of the foregoing nucleic acids and polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

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The dog PGP nucleic acids and polypeptides of the invention are isolated. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used herein is not a naturally occurring chromosome.

As used herein with respect to polypeptides, "isolated" means separated from its native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may be, but need not be, substantially pure. The term "substantially pure" means that the proteins or polypeptides are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by weight of the preparation. The protein is nonetheless isolated in that it has been separated from the substances with which it may be associated in living systems, i.e. isolated from other proteins.

As used herein a dog PGP nucleic acid refers to an isolated nucleic acid molecule which codes for a dog PGP polypeptide. Such nucleic acid molecules code for dog PGP polypeptides which include the amino acid sequences of SEQ ID NO:2, 23, 25, 27 and fragments thereof. The nucleic acid molecules include the nucleotide sequences of SEQ ID NO:1, and nucleotide sequences which differ from the sequences of SEQ ID NO:1, in codon

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sequence due to the degeneracy of the genetic code. The dog PGP nucleic acids of the invention also include alleles (e.g., SEQ ID Nos: 22, 24, 26) of the foregoing nucleic acids (except those of previously known dog PGP alleles, e.g., SEQ ID NOS:3 and 5), and encoded polypeptides, as well as fragments of the foregoing nucleic acids and polypeptides. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction (PCR). Preferred dog PGP nucleic acids include the nucleic acid sequences of SEQ ID NOS:1, 22, 24, 26 and fragments thereof. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein "dog PGP activity" refers to an ability of a PGP polypeptide to export small molecules across the cell membrane. A molecule which inhibits dog PGP activity (an antagonist) is one which inhibits export of small molecules via PGP and a molecule which increases dog PGP activity (an agonist) is one which increases export of small molecules via PGP. Changes in dog PGP activity can be measured by assays such as those disclosed herein, including efflux of fluorescent compounds from cells.

Alleles of the dog PGP nucleic acids of the invention can be identified by conventional techniques. For example, alleles of dog PGP can be isolated by hybridizing a probe which includes at least a fragment of SEQ ID NO:1 under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for dog PGP polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:1 under stringent conditions (except those of previously known dog PGP alleles, e.g., SEQ ID NOS:3 and 5). The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH₂PO₄(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 - 0.5 x SSC/0.1 x SDS at temperatures up to 68°C.

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There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of
5 dog PGP nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In screening for dog PGP nucleic acids, a Southern blot may be performed using the
10 foregoing stringent conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

The dog PGP nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine
15 residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating dog PGP polypeptide. Similarly, nucleotide sequence triplets
20 which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that
25 differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one
30 activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as transporter activity, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions

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as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having single amino acid changes can be prepared. Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

The invention also provides isolated fragments of SEQ ID NO:1. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween, and are useful e.g. as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 200, 250, 300, 400 or more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the dog PGP polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid

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fragments further can be used as antisense molecules to inhibit the expression of dog PGP nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

The invention also includes functionally equivalent variants of the dog PGP, which
5 include variant nucleic acids and polypeptides which retain one or more of the functional properties of the dog PGP. For example, variants include a fusion protein which includes the extracellular and transmembrane domains of the dog PGP which retains the ability to transport molecules. Still other functionally equivalent variants include truncations, deletions, point mutations, or additions of amino acids to the sequence of SEQ ID NO:1 which retains
10 functions of SEQ ID NOs:2, e.g., the alleles presented herein. Functionally equivalent variants also include a dog PGP which has had a portion of the N-terminus removed or replaced by a similar domain from another P-glycoprotein (e.g. a “domain-swapping” variant). Other functionally equivalent variants will be known to one of ordinary skill in the art, as will methods for preparing such variants. The activity of a functionally equivalent
15 variant can be determined using the methods provided herein, and in references that have described assays using P-glycoproteins of other species. Such variants are useful, *inter alia*, for evaluating bioavailability of drugs, in assays for identification of compounds which bind and/or regulate the transporter function of the dog PGP, and for determining the portions of the dog PGP which are required for transporter activity.

20 Variants which are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing transporter activity.

A dog PGP nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the dog PGP nucleic acid within a eukaryotic or
25 prokaryotic cell. The “gene expression sequence” is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the dog PGP nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not
30 limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, β -actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells

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include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney murine leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined dog PGP nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

The dog PGP nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the dog PGP coding sequence under the influence or control of the gene expression sequence. If it is desired that the dog PGP sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the dog PGP sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the dog PGP sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a dog PGP nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that dog PGP nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The dog PGP nucleic acid molecules and the dog PGP polypeptides (including the dog PGP inhibitors described below) of the invention can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a "vector" is any

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vehicle capable of facilitating: (1) delivery of a dog PGP nucleic acid or polypeptide to a target cell, (2) uptake of a dog PGP nucleic acid or polypeptide by a target cell, or (3) expression of a dog PGP nucleic acid molecule or polypeptide in a target cell. Preferably, the vectors transport the dog PGP nucleic acid or polypeptide into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g. a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a dog PGP nucleic acid or a dog PGP polypeptide) can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are more useful for delivery/uptake of dog PGP nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of dog PGP nucleic acids or dog PGP proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be linked to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; poxviruses; retroviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell

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line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., *"Gene Transfer and Expression, A Laboratory Manual,"* W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. *"Methods in Molecular Biology,"* vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a dog PGP polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 α , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc.*

Acids Res. 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992).

5 In addition to the biological vectors, chemical/physical vectors may be used to deliver a dog PGP nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated dog PGP nucleic acid or polypeptide to a cell.

10 A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size
15 from 0.2 - 4.0 μ can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of
20 biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be
25 useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the dog PGP nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as
30 LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in

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the art and have been described in many publications.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the dog PGP nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a dog PGP nucleic acid into a preselected location within a target cell chromosome).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the dog PGP cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include intestinal cells and liver cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated dog PGP polypeptides which include the amino acid sequence of SEQ ID NO:2, and fragments thereof, encoded by the dog PGP nucleic acids described above. Dog PGP polypeptides also embrace alleles, such as SEQ ID NOs:23, 25 and 27 (except not SEQ ID NOS:3 and 5), functionally equivalent variants and analogs (those non-allelic polypeptides which vary in amino acid sequence from the disclosed dog PGP polypeptides by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides retain dog PGP activity. Non-functional variants also are embraced by the invention; these are useful as antagonists of transporter function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes including as a component of assays.

Fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the dog PGP polypeptide, in particular as a transporter of various molecules. Other functional capabilities which can be retained in a fragment of a dog PGP polypeptide include interaction with antibodies and interaction with other polypeptides (such

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as would be found in a protein complex). Those skilled in the art are well versed in methods for selecting fragments which retain a functional capability of the dog PGP. Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the transporter
5 activity of a dog PGP fragment, one inserts or expresses the fragment in a cell in which molecular transport can be measured. Such methods, which are standard in the art, are described further herein.

The invention embraces variants of the dog PGP polypeptides described above. As used herein, a “variant” of a dog PGP polypeptide is a polypeptide which contains one or
10 more modifications to the primary amino acid sequence of a dog PGP polypeptide. Modifications which create a dog PGP variant can be made to a dog PGP polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a dog PGP polypeptide, such as transport; 2) to enhance a property of a dog PGP polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; 3) to provide a novel
15 activity or property to a dog PGP polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to establish that an amino acid substitution does or does not affect molecular transport activity. Modifications to a dog PGP polypeptide are typically made to the nucleic acid which encodes the dog PGP polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-
20 amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the dog PGP amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein
25 sequence, and can thus “design” a variant dog PGP according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed *de novo*. The method can be applied to a known protein to vary a only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of a dog PGP polypeptide can be proposed and tested to
30 determine whether the variant retains a desired conformation.

Variants include dog PGP polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine

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residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a dog PGP polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

5 Mutations of a nucleic acid which encode a dog PGP polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random
10 mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant dog PGP polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a
15 particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a dog PGP gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of dog PGP polypeptides can be tested by cloning the gene encoding the variant dog PGP polypeptide into a bacterial or mammalian expression vector,
20 introducing the vector into an appropriate host cell, expressing the variant dog PGP polypeptide, and testing for a functional capability of the dog PGP polypeptides as disclosed herein. For example, the variant dog PGP polypeptide can be tested for ability to provide molecular transport (e.g., efflux), as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary
25 skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in dog PGP polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the dog PGP polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino
30 acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as

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are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the dog PGP polypeptides include conservative amino acid substitutions of SEQ ID NO:2 or SEQ ID NO:4. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of dog PGP polypeptide to produce functionally equivalent variants of dog PGP typically are made by alteration of the nucleic acid sequence encoding dog PGP polypeptides (e.g., SEQ ID NO:1). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a dog PGP polypeptide. The activity of functionally equivalent fragments of dog PGP polypeptides can be tested by cloning the gene encoding the altered dog PGP polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered dog PGP polypeptide, and testing for the ability of the dog PGP polypeptide to mediate transmembrane transport of compounds. Peptides which are chemically synthesized can be tested directly for function.

A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated dog PGP molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptide. Those skilled in the art also can readily follow known methods for isolating dog PGP polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

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The invention as described herein has a number of uses, some of which are described elsewhere herein. For example, the invention permits isolation of the dog PGP polypeptide molecules by e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another example, the isolation of the dog PGP gene makes it possible for dog PGP to be used in methods for assaying of molecular transport, such as drug bioavailability studies. These methods involve determining transport of a drug by a first species' PGP (e.g., dog) in comparison to transport of the drug by other species' PGP (e.g. human) as a method for determining or predicting the bioavailability of the drug. Thus the results of whole animal studies on the metabolism of a drug can be evaluated in view of the relative rates or amounts of P-glycoprotein transport of the drug. For example, if a drug administered to a dog has good oral bioavailability and low transport by dog PGP, one can predict that the oral bioavailability of the drug in humans will be good if the transport by human PGP is also low. Conversely, if the transport of the drug by human PGP is high, then the bioavailability of the drug would be predicted to be low.

The invention also embraces agents which bind selectively to the dog PGP nucleic acid molecules or polypeptides as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. The agents include polypeptides which bind to dog PGP, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase dog PGP activity (antagonists and agonists, respectively).

Some of the agents are inhibitors. A dog PGP inhibitor is an agent that inhibits dog PGP mediated transport of molecules across a cell membrane. Efflux assays can be performed to screen and/or determine whether a dog PGP inhibitor has the ability to inhibit dog PGP activity, and whether the inhibition is selective. An exemplary assay of efflux is described below in the Examples.

In one embodiment the dog PGP inhibitor is an antisense oligonucleotide that selectively binds to a dog PGP nucleic acid molecule, to reduce the expression of dog PGP (or other species' PGPs) in a cell. This is desirable in virtually any medical condition wherein a reduction of PGP transporter activity is desirable, e.g., to increase retention of cytotoxic agents in a cell.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified

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oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon SEQ ID NO:1, or upon allelic (except SEQ ID NOS:3 and 5) or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which polypeptides are not expected to bind. Thus, the present invention also provides for antisense oligonucleotides which are complementary to allelic (except SEQ ID NOS:3 and 5) or homologous cDNAs and genomic DNAs corresponding to a dog PGP nucleic acid containing SEQ ID NO:1.

In one set of embodiments, the antisense oligonucleotides of the invention may be

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composed of “natural” deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include “modified” oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term “modified oligonucleotide” as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamides, carboxymethyl esters and peptides.

The term “modified oligonucleotide” also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding dog PGP polypeptides, together with pharmaceutically acceptable carriers.

Agents which bind dog PGP also include binding peptides and other molecules which bind to the dog PGP polypeptide and complexes containing the dog PGP polypeptide. When the binding molecules are inhibitors, the molecules bind to and inhibit the activity of dog PGP. To determine whether a dog PGP binding agent binds to dog PGP any known binding

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assay may be employed. For example, the binding agent may be immobilized on a surface and then contacted with a labeled dog PGP polypeptide. The amount of dog PGP which interacts with the dog PGP binding agent or the amount which does not bind to the dog PGP binding agent may then be quantitated to determine whether the dog PGP binding agent binds to dog PGP.

The dog PGP binding agents include molecules of numerous size and type that bind selectively or preferentially to dog PGP polypeptides, and complexes of both dog PGP polypeptides and their binding partners. These molecules may be derived from a variety of sources. For example, dog PGP binding agents can be provided by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array.

One then can select phage-bearing inserts which bind to the dog PGP polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the dog PGP polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences.

DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the dog PGP polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more

additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the dog PGP polypeptides. Thus, the dog PGP polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the dog PGP polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of dog PGP and for other purposes that will be apparent to those of ordinary skill in the art.

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Therefore the invention generally provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions associated with aberrant PGP activity and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance transport of molecules through dog PGP. Such methods are adaptable to automated, high throughput screening of compounds. Examples of such methods are described in US patent 5,429,921.

A variety of assays for pharmacological agents are provided, including, labeled *in vitro* protein binding assays, efflux assays using detectable molecules, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a dog PGP. The candidate pharmacological agents can be derived from, for example, combinatorial peptide libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of efflux involves contacting a cell having a dog PGP with a candidate pharmacological agent under conditions whereby the efflux of a detectably labeled molecule can occur. Specific conditions are well known in the art and are described, for example, in Sharom et al., *Biochem. Pharmacol.* 58:571-586, 1999, and references cited therein. A reduction in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the efflux activity of dog PGP. An increase in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the efflux activity of dog PGP.

Dog PGP used in the methods of the invention can be added to an assay mixture as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a dog PGP polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the dog PGP as a preloaded polypeptide or as a nucleic acid (e.g. a cell transfected with an expression vector containing a dog PGP). In the assays described herein, the dog PGP polypeptide can be produced recombinantly, or isolated from biological extracts, but preferably is synthesized *in vitro*. Dog PGP polypeptides encompass chimeric proteins comprising a fusion of a dog PGP polypeptide with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, or enhancing stability of the dog PGP polypeptide under assay conditions. A polypeptide fused to a dog PGP polypeptide or fragment thereof may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

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The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

Therefore, a source of candidate agents are libraries of molecules based on known P-glycoprotein inhibitors, in which the structure of the inhibitor is changed at one or more positions of the molecule to contain more or fewer chemical moieties or different chemical moieties. The structural changes made to the molecules in creating the libraries of analog

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inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on existing P-glycoprotein inhibitors.

5 A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease
10 inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the dog PGP mediates the efflux of a control amount of a compound such as a drug. For determining the binding of a candidate pharmaceutical agent to a dog PGP, the mixture is incubated under conditions
15 which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and
20 typically are between 1 minute and 10 hours.

After incubation, the level of efflux or the level of specific binding between the dog PGP polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a
25 variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize signal to noise ratios, primarily to minimize background binding, as well as for ease of
30 separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead,

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particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as a transmembrane transport assay. The transport of a directly or indirectly detectable product, e.g., a fluorescent molecule such as calcein AM or rhodamine 123, is preferred. For cell free binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc). or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a dog PGP polypeptide or the candidate pharmacological agent.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The dog PGP binding agent may also be an antibody or a functionally active antibody fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining dog PGP binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments $F(ab')_2$, and Fab. $F(ab')_2$, and Fab fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

Monoclonal antibodies may be made by any of the methods known in the art utilizing

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dog PGP, or a fragment thereof, as an immunogen. Alternatively the antibody may be a polyclonal antibody specific for dog PGP which inhibits dog PGP activity. The preparation and use of polyclonal antibodies is also known to one of ordinary skill in the art.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

In general, intact antibodies are said to contain "Fc" and "Fab" regions. The Fc regions are involved in complement activation and are not involved in antigen binding. An antibody from which the Fc' region has been enzymatically cleaved, or which has been produced without the Fc' region, designated an "F(ab')₂" fragment, retains both of the antigen binding sites of the intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an

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“Fab” fragment, retains one of the antigen binding sites of the intact antibody. Fab' fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain, denoted “Fd.” The Fd fragments are the major determinants of antibody specificity (a single Fd fragment may be associated with up to ten different light chains
5 without altering antibody specificity). Isolated Fd fragments retain the ability to specifically bind to antigen epitopes.

The sequences of the antigen-binding Fab' portion of the anti-dog PGP monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing
10 methods that are routine in the art. It is well established that non-CDR regions of a mammalian antibody may be replaced with corresponding regions of non-specific or hetero-specific antibodies while retaining the epitope specificity of the original antibody. This technique is useful for the development and use of “humanized” antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a
15 functional antibody. Techniques to humanize antibodies are particularly useful when non-human animal (e.g., murine) antibodies which inhibit dog PGP activity are identified. These non-human animal antibodies can be humanized for use in the treatment of a human subject in the methods according to the invention. Examples of methods for humanizing a murine antibody are provided in U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and
20 5,859,205. Other antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as “chimeric” antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, and Fab fragments of an anti-dog PGP monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3
25 regions of an anti-dog PGP antibody have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-dog PGP antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by
30 homologous human or non-human sequences.

According to the invention dog PGP inhibitors also include “dominant negative” polypeptides derived from SEQ ID NOs:2. A dominant negative polypeptide is an inactive

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variant of a polypeptide, which, by interacting with the cellular machinery, displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand.

The end result of the expression of a dominant negative dog PGP polypeptide of the invention in a cell is a reduction in PGP activity such as molecular transport. One of ordinary skill in the art can assess the potential for a dominant negative variant of a dog PGP polypeptide, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a dog PGP polypeptide, one of ordinary skill in the art can modify the sequence of the dog PGP polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989.

The skilled artisan then can test the population of mutagenized polypeptides for diminution in dog PGP activity and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a dog PGP polypeptide will be apparent to one of ordinary skill in the art.

Each of the compositions of the invention is useful for a variety of therapeutic and non-therapeutic purposes. For example, the dog PGP nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. A suitable oligonucleotide or set of oligonucleotides, which is capable of hybridizing under stringent hybridization conditions to the desired sequence, a variant or fragment thereof, or an anti-sense complement of such an oligonucleotide or set of oligonucleotides, can be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the desired sequence, variant or fragment thereof by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY (1989). To facilitate the detection of a desired nucleic acid sequence, or variant or fragment thereof, whether for

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cloning purposes or for the mere detection of the presence of the sequence, the above-described probes may be labeled with a detectable group. Such a detectable group may be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and, in general, many labels useful in such methods can be applied to the present invention. Particularly useful are radioactive labels. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. If single stranded, the oligonucleotide may be radioactively labeled using kinase reactions. Alternatively, oligonucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., et al., *Proc. Natl. Acad. Sci. (USA)* 80:4045 (1983); Renz, M. et al., *Nucl. Acids Res.* 12:3435 (1984); and Renz, M., *EMBO J.* 6:817 (1983).

Additionally, complements of the dog PGP nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a dog PGP "knockout" phenotype. The administration of antisense RNA probes to block gene expression is discussed in Lichtenstein, C., *Nature* 333:801-802 (1988).

Alternatively, the dog PGP nucleic acid of the invention can be used to prepare a non-human transgenic animal. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of dog PGP knockout and transgenic animals as models for the study of disorders involving transport of molecules across cell membranes. A variety of methods known to one of ordinary skill in the art are available for the production of transgenic animals associated with this invention.

Inactivation or replacement of the endogenous PGP/MDR1 gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-

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human mammals having a PGP^{-/-} knockout phenotype may be made transgenic for the dog PGP and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the dog PGP. In this manner, such therapeutic drugs can be identified.

5 Additionally, a normal or mutant version of dog PGP can be inserted into the germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of dog PGP. These animals are useful in studies to define the role and function of dog PGP in cells.

 The compositions of the invention are also useful for therapeutic purposes.

10 Accordingly the invention encompasses a method for inhibiting dog PGP activity in a mammalian cell. The invention further provides methods for reducing or increasing dog PGP activity in a cell. In one embodiment, the method involves contacting the mammalian cell with an amount of a dog PGP nucleic acid or polypeptide effective to inhibit molecular transport out of the mammalian cell. Such methods are useful *in vitro* for the purpose of, for
15 example, elucidating the mechanisms involved in drug resistance and reduced drug bioavailability.

 The invention also encompasses a method for increasing PGP expression in a cell or subject. The amount of dog PGP can be increased in such cell or subject by contacting the cell with, or administering to the subject, a PGP nucleic acid or a PGP polypeptide of the
20 invention to the subject in an amount effective to increase transmembrane transport in the cell or the subject. An increase in PGP activity can be measured by the assays described herein, e.g., assays of transmembrane transport.

 The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further
25 doses, produces the desired response. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. It is preferred generally that a maximum
30 dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any

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other reasons.

Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The dog PGP inhibitors or dog PGP nucleic acids and polypeptides useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

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The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being
5 treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, intradermal, or parenteral routes. The term "parenteral" includes
10 subcutaneous, intravenous, intrathecal, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes
15 one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active
20 compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the dog PGP inhibitor or dog PGP nucleic acids and polypeptides, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be
25 formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition,
30 sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier

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formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems such as the biological/chemical vectors is discussed above. Such systems can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Example 1: Isolation of dog P-glycoprotein

cDNA libraries were prepared using dog (*Canis familiaris*) mRNA according to standard procedures. The libraries were screened for P-glycoprotein clones using a human P-glycoprotein DNA probe. Clones were isolated, purified and sequenced in accordance with standard procedures, as described below.

Liver and lung tissue was excised from Beagle dog tissue samples and flash frozen in liquid nitrogen.

A 890bp fragment of dog PGP1 was PCR'd out of a lambda gt11 vector cDNA library custom made by Stratagene. PCR was done using reagents from Clontech. Primers were custom designed, one specific for sequence homologous to human PGP1, the other homologous to dog PGP2.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dp1216F	5'-gaa ctg tga ttg cgt ttg gag gac-3'	24mer	9	human PGP1
Dp2133R	5'-ttc agg gcc gcc tgt acc tct g-3'	22mer	10	dog PGP2

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PCR was performed with a Perkin Elmer 9700 thermocycler. PCR was done at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 40°C for 20 seconds and 72°C for 2 minutes and 30 seconds, and then incubated at 72°C for 3 minutes. The PCR product was run on a 1% agarose gel, and stained with EtBr. The DNA band at 917bp was cut out,
 5 and purified using the QIAquick Gel Extraction kit from Qiagen Inc.

The DNA was then ligated to the vector pCR2.1 using the Original TA Cloning kit from Invitrogen. INValphaF' cells were transformed with the ligation retain, a colony was selected, grown up in 100mL of L broth with ampicillin. DNA purification was done using the Plasmid Midi purification kit from Qiagen. The insert was sequenced using an ABI 377
 10 sequencer. This sequence corresponds to an 890bp fragment of dog PGP, from base 816 to base 1706 in the open reading frame.

Five other fragments were isolated, cloned and sequenced using the same method as stated above, with any differences in procedure stated below.

RNA extraction from dog lung tissue was performed using Nucliobond RNA Maxi
 15 Prep. purification kit from Clontech. cDNA was synthesized from the RNA preparation using Superscript Preamplification System for First Strand cDNA Synthesis from Life Technologies.

Fragment 1: cDNA source-dog lung cDNA library prep.

Primer name	sequence	size	SEQ NO:	homology
Dm1766F	5'-ccc cac aga tgg cat ggt ctg t-3'	22mer	11	dog PGP1
Dp2769R	5'-cgc ttg gtg agg atc tct cca gc-3'	23mer	12	human PGP1

This corresponds to a 981bp fragment of dog PGP from base 1364 to base 2345 in the open reading frame.

Fragment 2: cDNA source dog - lung cDNA library prep.

Primer name	sequence	size	SEQ NO:	homology
Dm2037F	5'-aga aac aga gaa tcg cca ttg ctc-3'	24mer	13	dog PGP1
Dp3793R	5'-gct gca gtc aaa cag gat ggg ct-3'	23mer	14	human PGP1

This corresponds to a 1728bp fragment of dog PGP from base 1635 to base 3362 in the open reading frame.

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Fragment 3: cDNA source dog - lung cDNA prep

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm3411F	5'-agt tca ttt gct cct gac tat gcc-3'	24mer	15	dog PGP1
Dp4214R	5'-gat gcc ttt ctg ggc cag cag c-3'	22mer	16	human PGP1

5 This corresponds to a 779bp fragment of dog PGP from base 3004 to base 3783 in the open reading frame.

An RNA preparation from dog liver tissue was performed using the same method as used previously. cDNA was made from it using the SMART RACE cDNA Amplification Kit
 10 from Clontech using SMART II oligonucleotide. PCR conditions were changed to 94°C for 5 minutes, followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes. Five cycles of 94°C for 30 seconds, 70°C for 45 seconds and 72°C for 2 minutes were performed next. The final 30 cycles were at 94°C for 30 seconds, 68°C for 45 seconds and 72°C for 2 minutes. The PCR was concluded with 72°C for 7 minutes.

15

Fragment 4: cDNA source - dog liver cDNA (SMART RACE) prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm3612FL	5'-gag gtg aag aag ggc cag acg ctg gcc ctc-3'	30mer	17	dog PGP1
RACE	5'-cta ata cga ctc act ata ggg caa gca gtg gta aca acg cag agt -3'	18		vector

20 This corresponds to a 3' end fragment of dog PGP from base 3212 to base 4264 in the open reading frame.

An RNA preparation from dog liver tissue was performed using the same method as used previously. cDNA was made from it using the SMART RACE cDNA Amplification Kit
 25 from Clontech using the gene specific Primer DM1680RL 5'-cgc agc cac tgt tcc caa cca gcg cca ct-3', 29mer, SEQ ID NO:19, dog PGP1.

Fragment 5: cDNA source - dog liver cDNA (SMART RACE with internal primer) prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dp409F	5'-gga gcg cga ggt cgg gat gga tc- 3'	23mer	20	human PGP1
Dm1355RL	5'-gga gag gac caa gga ggt ccc ata cca gaa a-3'	31mer	21	dog PGP1

This corresponds to an ATG fragment of dog PGP from base -15 to base 945 in the open

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reading frame.

Assembly of Clones

1Kb fragment (890bp) and 981bp fragment

5 DNA preps were made of each fragment. Both fragments were cut with NcoI restriction enzyme. The 1Kb fragment was also cut with EcoRI. The 981bp fragment was also cut with BamHI. Each fragment was purified, ligated together, then the ends were cut, the cut molecule was gel purified and ligated into pUC19 cut with EcoRI and BamHI.

10 1Kb fragment (890bp)/981bp fragment and 1728bp fragment

These fragments were assembled as above except that both fragments were cut with BamHI and the 1728bp fragment was also cut with XbaI.

1Kb fragment (890bp)/981bp fragment/1728bp fragment and ATG fragment

15 3'end and 779bp fragment

1Kb fragment (890bp)/981bp fragment/1728bp fragment ATG fragment and
3'end/779bp fragment

All of these fragments were assembled as above except that, in some cases, the fragments were cut with different restriction enzymes according to their nucleotide sequences.

20

The nucleotide sequence of a dog P-glycoprotein is presented as SEQ ID NO:1. The coding sequence consists of nucleotides 17-3859, producing a polypeptide of 1281 amino acids (SEQ ID NO:2).

25 **Example 2: Activity of dog P-glycoprotein**

Materials and Methods

Dog PGP cDNA (SEQ ID NO:1) is introduced into a clonal population of LLC-PK1 cells in a vector that confers resistance to hygromycin B. LLC-PK1 cells are obtained from the American Type Culture Collections and are propagated in Medium 199 supplemented to
30 7% with fetal bovine serum. LLC-PK1 cells are recloned prior to transfection in order to assure homogeneity of the cell population. Briefly, dog PGP cDNA is incorporated into the p222CMV vector. This vector is derived from the p220.2 episomal vector system based on

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the OriP sequences for Epstein Barr virus and the EBNA-1 gene product (Sugden et al., *Mol. Cell Biol.* 5:410-413, 1985; Yates et al., *Nature (Lond.)* 313: 812-815, 1985). The PGP cDNA is under the control of the cytomegalovirus (CMV) immediate early promoter. The vector confers resistance to hygromycin B. Cells (in 0.4 mL) and DNA (10 to 20 µg) were transfected by electroporation using a BTX Electro cell manipulator model 600 using a 2 mm gap cell, 100V, 2500 µF capacitance and 72 ohm resistance. After electroporation, the cells are plated in multiwell plates (48 well, Corning Costar) at 10% of confluence. One to two days after transfection hygromycin B is introduced at a final concentration of 400 to 600 µg/ml. Cells are refed every 2 to 4 days and are propagated in 400 to 600 µg/ml hygromycin B for 6 to 8 days at which point the bulk of the wild type cells are detached. The hygromycin B is reduced to 100 µg/ml and maintained in this concentration of hygromycin B. After 14 to 18 days the wells are inspected and wells containing single colonies are trypsinized and scaled up to bulk cultures. Expression of PGP is measured by the polarization of vinblastine (0.1 µM) transport in Transwells™.

LLC-PK1 cell based transport studies are conducted in 24 well Transwells™ (Corning Costar, Catalog number 3415). Transwells™ are prepared by the addition of 0.6 mL media to the basolateral space and 0.1 mL media to the apical space. Cells are seeded at 4×10^4 cells per insert (typically in 0.05 mL to 0.15 mL), refed with fresh media every 2 to 4 days and used for transport studies 4 to 8 days post seeding. Transport assays are conducted in Hank's balanced saline (HBSS) buffered with 10 mM HEPES (pH 7 to 7.2). Cell monolayers are rinsed with HBSS prior to use in transport assays. Transport is measured under sink conditions in both the apical to basolateral (A to B) and basolateral to apical (B to A) directions. At least duplicate monolayers are used per determination. At the desired time points, samples are withdrawn from the receiver chamber (apical or basolateral chambers). Quantitation of the amount of compound transported is by liquid scintillation counting (vinblastine) or HPLC with UV or mass spectrometric detection.

Dog PGP cDNA is expressed in insect cells using a baculovirus vector. Membranes are prepared according to the method of (Sarkadi et al., *J. Biol. Chem.* 267: 4854-4858, 1992) and stored at -80°C until use. ATPase assays are conducted in 96 well microtiter plates. The assays are conducted using a modification of the methods of (Sarkadi et al., 1992 and Druekes et al., *Anal. Biochem.* 230: 173-177, 1995).

A detailed method for each well of a 96 well plate is contained below:

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A 0.06 ml reaction mixture containing 40 μ g membranes, 20 μ M Verapamil (positive control) or test drug, and 3-5 mM MgATP, in buffer containing 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide, is incubated at 37°C for 20 min. An identical reaction mixture containing 100 μ M sodium orthovanadate is assayed in parallel.

5 Orthovanadate inhibits PGP by trapping MgADP in the nucleotide binding site. Thus, ATPase activity measured in the presence of orthovanadate represents non-PGP ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. The reaction is stopped by the addition of 30 μ l of 10 % SDS + Antifoam A. Two additional reaction mixtures (+ and - orthovanadate) but without
10 MgATP, are also prepared and incubated with the others, and then supplemented with SDS and MgATP, to represent time = 0 min of reaction. The incubations are followed with addition of 200 μ l of 35 mM ammonium molybdate in 15 mM zinc acetate:10 % ascorbic acid (1:4) and incubated for an additional 20 min at 37°C. The liberation of inorganic phosphate is detected by its absorbance at 800 nm and quantitated by comparing the absorbance to a
15 phosphate standard curve.

Ligand binding assays and assays for measuring inhibition of fluorescent dye uptake are preformed as described by Sharom et al. (*Biochem. Pharmacol.* 58:571-586, 1999).

I. Stable PGP Expression in LLC-PK1 Cells.

20 Functional expression of dog PGP is measured by the polarization of transport of vinblastine. Control cells typically demonstrate a B to A / A to B ratio of between 1 and 3. PGP transfected cells demonstrate a much higher ratio. The expression of cDNA-derived dog is stable.

II. Activation of ATPase Activity in PGP Membranes.

25 The stimulation of ATPase assay provides a rapid measure of the concentration dependence of any interaction of a drug with PGP. The liberated inorganic phosphate is measured by a simple spectrophotometric assay performed in a microtiter plate format. The testing of multiple drug concentrations allows estimation of the affinity of the drug for PGP
30 and whether saturation of the response was observed.

III. Drug Transport Across Cell Monolayers.

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The ATPase assay does not directly measure drug transport. In order to examine the concordance between activation of ATPase and actual transport, the rates of transport of the drugs are measured in control LLC-PK1 and dog PGP cell monolayers. For each drug concentration, four measurements are made:

5	A:	A to B	Control cells
	B:	B to A	Control cells
	C:	A to B	PGP cells
	D:	B to A	PGP cells

The polarization of transport is calculated in control cells (B/A) and PGP cells (D/C).

10 The intrinsic activity (IA) of PGP is calculated as the sum of the amount PGP facilitated B to A transport in PGP cells relative to control cells (D minus B) and the amount that PGP impeded A to B transport in PGP cells relative to control cells (A minus C). The intrinsic clearance of PGP is calculated from a plot of the concentration dependence data by either calculating the slope of the line under non-saturating conditions or from the calculated
15 apparent Km and Vmax values when saturation is observed. Intrinsic clearance is expressed as mL/m²/min.

The ATPase data provides useful concentration response data. For example, the apparent Km values for some compounds are in good agreement between the ATPase and transport systems. However, other drugs activate ATPase activity but transport by PGP is not
20 detectable. At the least, ATPase assay can identify a concentration range below which the response to transport by PGP was linear with respect to drug concentration. This should allow simplification of the experimental design for measuring the intrinsic clearance of PGP, an important consideration if large numbers of compounds are to be tested.

25 *IV. Bioavailability*

Bioavailability studies are performed by performing one or more of the assays described above with two or more different PGP types. The different PGP types can be different species (e.g., dog and human, cynomologous monkey and human, dog and cynomologous monkey, etc.) or can be different alleles of the same species. The results of
30 these assays are compared to determine or estimate the bioavailability of a drug in individuals of the different species or in individuals that express different PGP alleles. The results of one determination also may be compared to a previously determined value of, e.g., ATPase or

transport, as an historical control.

Protocols and Procedures

Isolation of RNA

5 Frozen beagle liver tissue was ground up using a mortar and pestle. Pulverized tissue (300 mg) was transferred to a dounce homogenizer. TRIzol Reagent (3mL; Cat. # 15596 from Life Technologies) was added and the tissue was homogenized then incubated for 15 minutes at room temperature. Aliquots (1mL) of the solution were transferred to three microfuge tubes and 200 μ L of chloroform were added to each tube. Each tube was vortexed at a high
10 setting for 15 seconds, then incubated at room temperature for 3 minutes before being centrifuged at 10 000 g for 15 minutes at 4°C. The upper "aqueous" phase was transferred to a new microfuge tube while the remaining lower and inter phases were discarded. 500 μ L of Isopropanol was added to each of the new tubes and mixed by inverting the tube several times. Each tube was then incubated at room temperature for 10 minutes before being
15 centrifuged at 10 000 g for 10 more minutes at 4°C. A small white pellet was observed and the supernatant was discarded. 1mL of 75% ethanol was added to each tube. The tubes were vortexed briefly then centrifuged at 10 000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet dried for 5 minutes at room temperature. 50 μ L of DEPC treated sterile deionized water was added to each tube. Each pellet was resuspended and the contents
20 of each tube were pooled.

Optical density of the resuspended pellet was analyzed using a Hitachi U3010 spectrophotometer to determine the quantity of RNA that was isolated. The RNA was then diluted using DEPC treated sterile deionized water to a concentration of 0.55 μ g/ μ L. 2 mL of this RNA was split evenly into 2 microfuge tubes. Next, 100 μ L of 5 M NaCl was added to
25 each tube. Both tubes were heated to 65°C for 15 minutes, then put on ice for 5 minutes. 500 μ L of Oligo (dT) Cellulose Suspension from the MessageMaker mRNA Isolation System (Cat. # 10551-018 from Life Technologies) were added to both tubes. The tubes were then incubated at 37°C for 10 minutes. The contents of the tubes were transferred to the Filter Syringe provided with the MessageMaker mRNA Isolation System. After expelling the liquid
30 contents, the pellet in the syringe was washed using Wash Buffer 1 and Wash Buffer 2 according to the manufacturer's instructions. The RNA pellet in the syringe was resuspended in 1 mL of DEPC treated sterile deionized water (65°C), followed by the addition of 2.5 μ L of

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50 µg/mL glycogen and 100 µL of 7.5M ammonium acetate and mixing by inversion. The contents of the tube were then transferred to a two microfuge tubes and 1 mL of ethanol was added to each tube. The contents of both tubes were mixed by inversion, followed by incubation at -20°C overnight.

5 The tubes were centrifuged at 10 000 g for 30 minutes at 4°C. The supernatant was discarded and 200 µL of 75% ethanol was added to each tube. The tubes were vortexed for 10 seconds followed by centrifugation at 10 000 g for 15 minutes at 4°C. The supernatant was discarded and all remaining liquid was carefully pipetted off. The pellet was resuspended in 10 µL of DEPC treated sterile deionized water.

Preparation of cDNA

CDNA was prepared using the Marathon cDNA Amplification Kit (Cat. # K1802-1 from Clontech Laboratories, Inc., Palo Alto, CA) as follows. Four microliters of the resuspended mRNA were transferred to a new microfuge tube, and 1 µL of one of the following primers at 10 µM concentration was added: Dm1680RL cgc agc cac tgt tcc caa cca gcg cca ct (SEQ ID NO:19; custom designed, synthesized by Operon Technologies, Inc.) or cDNA Synthesis Primer, nnt ttt ttt ttt ttt ttt ttt ttt ttc gcc ggc gac tta aga tct t (SEQ ID NO:28), from the Marathon cDNA Amplification Kit. Tubes were incubated at 70°C for 2 minutes then placed on ice for 5 minutes. 2 µL of 5x First-Strand Buffer, 1 µL of 10mM dNTP Mix and 1 µL of 20 U/µL AMV Reverse Transcriptase were added to each tube and gently mixed. The tubes were incubated at 42°C for 1 hour, then placed on ice. 2 µL of each single stranded cDNA were used as template in PCR (see below, "PCR conditions"). 48.4 µL of DEPC treated deionized water, 16 µL of 5x Second-Strand Buffer, 1.6 µL of 10mM dNTP Mix and 4 µL of 20x Second-Strand Enzyme Cocktail were added to the remaining cDNA sample. The tube was incubated at 16°C for 1.5 hours. 2uL of 5U/uL T4 DNA Polymerase was added to the tube, which was then incubated at 16°C for an additional 45 minutes. 20x EDTA/Glycogen Mix (4 µL) was added followed by 430 µL of Buffer PN from the QIAquick Nucleotide Removal Kit (Cat. # 28304 from QIAGEN Inc., Valencia, CA). The sample was then loaded into a QIAquick Spin Column and centrifuged at 10 000 g for 1 minute. The collection tube was emptied and 750 µL of PE Buffer (QIAGEN) were added to the QIAquick Spin Column which was again centrifuged at 10 000 g for 1 minute. The collection tube was once again emptied and the QIAquick Spin Column was centrifuged at 10 000 g for 1 minute

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to dry the column membrane. 50 μ L of sterile deionized water heated to 50°C was added to the QIAquick Spin Column and incubated at room temperature for 1 minute before being centrifuged at 10 000 g for 1 minute into a clean microfuge tube.

To precipitate nucleic acid, 10.8 μ L 7.5M ammonium acetate and 144 μ L ethanol were added to the tube before it was centrifuged at 10 000 g for 20 minutes. The supernatant was removed, then 300 μ L of 75% ethanol was added. The tube was vortexed and centrifuged at 10 000 g for 20 minutes. The supernatant was removed and the sample was resuspended in 12 μ L of sterile deionized water. 5 μ L of this cDNA were placed in a clean microfuge tube. 2 μ L of 10 μ M Marathon cDNA Adapter, 2 μ L 5x DNA Ligation Buffer and 1 μ L of 400U/ μ L T4 DNA Ligase, from the Marathon cDNA Amplification Kit, were added to the cDNA and mixed gently. This mixture was incubated over night at 16°C. The ligase was heat inactivated at 70°C for 5 minutes. PCR was done using this sample as a template.

PCR conditions

PCR was performed using reagents supplied with the Clontech Advantage 2 kit (Clontech, Palo Alto, CA). Briefly, 2 μ L of cDNA was placed in a 200 μ L MicroAmp reaction tube (Cat. # N801-0540, PE Biosystems), then 34 μ L of sterile deionized water, 5 μ L of 10x Clontech Advantage 2 Polymerase Mix (Cat. # 4700-1 Clontech Laboratories, Inc.), 3 μ L of 10 mM Advantage UltraPure PCR Deoxynucleotide Mix (Cat. # 8430-1 Clontech Laboratories, Inc.) and 2 μ L each of a forward and reverse primer at 10mM concentration were added. Primers were either Marathon cDNA Adapter Primer 1, cca tcc taa tac gac tca ctg tag ggc (SEQ ID NO:29), from the Marathon cDNA Amplification Kit or various custom gene specific primers made by Operon Technologies Inc. Custom primers include (all listed as 5'-3'): Dm409FL: gga gcg cga ggt cgg gat gga tc (SEQ ID NO:20), Dm1455RL: gca aat gct tca atg ctt ggg gat gcc tgt cca a (SEQ ID NO:30), Dm2037F: aga aac aga gaa tcg cca ttg ctc (SEQ ID NO:13), Dm3920RL: gag ctg ggt tcc ttt gtc tcc tac tct ggt gtt (SEQ ID NO:31), Dp1216F: gaa ctg tga ttg cgt ttg gag gac (SEQ ID NO:9), and Dp2587R: gca aat gct ggt tgc agg cct cc (SEQ ID NO:32).

Thermocycling was done using a Perkin Elmer 9700 at 94°C for 30 seconds followed by 2 cycles of 94°C for 5 seconds and 72°C for 4 minutes, followed by 16 cycles of 94°C for 5 seconds and 68°C for 4 minutes.

Four fragments were acquired by PCR for each Genotype. Fragment 1 was generated

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using primers Marathon cDNA Adapter Primer 1 and Dm1455RL. Fragment 2 was generated using primers Dp1216F and Dp2587R. Fragment 3 was generated using primers Dm2037F and Dm3920RL. Fragment 4 was generated using primers Dp3612FL and Marathon cDNA Adapter Primer 1.

5

Post PCR sample processing

Electrophoresis was performed on all PCR products using 10x Gel-loading Buffer IV (*Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, page 6.12), on a 1% agarose gel in 0.5x TBE buffer (Sambrook, et al., page B-23) containing EtBr. Fragments of gel containing the PCR products were cut out and placed in 2 mL microfuge tubes. The mass of each fragment was determined and 3 μ L/mg of Buffer QX1 from QIAquick Gel Extraction Kit (Cat. # 28704 from QIAGEN Inc.) was added. Each tube was then incubated for 10 minutes at 50°C with mixture every two minutes. 1 μ L/mg of isopropanol was added to each tube and mixed. The resulting solution was transferred to a QIAquick spin column from the QIAquick Gel Extraction Kit. These columns were centrifuged for 1 minute at 10 000 g at room temperature. 750 μ L of Buffer PE from the QIAquick Gel Extraction Kit were added to each column followed by centrifugation for 1 minute at 10 000 g at room temperature. Each column was centrifuged again for 1 minute at 10 000 g at room temperature to remove any residual buffer. 30 μ L of sterile deionized water at 50°C were added to each column and incubated for 1 minute at room temperature. Each column was then placed in a microfuge tube and centrifuged for 1 minute at 10 000 g.

Next, 6 μ L of purified PCR product was transferred to a clean microfuge tube and 1 μ L of 10x Ligation Buffer, 2 μ L of pCR 2.1 vector and 1 μ L of T4 DNA Ligase (all from the Original TA Cloning Kit (Cat. # K2000-J10) Invitrogen, Carlsbad, CA) were added to the microfuge tube and mixed. The reaction was incubated over night at 16°C.

One 50 μ L vial of frozen One Shot competent cells (Invitrogen) was thawed on ice for each ligation reaction. 3.5 μ L of ligation reaction mixture was added to the competent cells and gently mixed. The cells incubated on ice for 30 minutes, followed by a 30 second "heat shock" at 42°C. After cooling on ice again, 250 μ L of SOC medium (Invitrogen) was added to each vial and the vials were put directly into a 37°C water bath for 1 hour. 50 μ L and 200 μ L aliquots of each transformation reaction were plated on Luria-Bertani Medium (Sambrook

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et al., page A-1) containing 1% agarose, 1 mg/mL glucose, 50 ng/mL ampicillin and 1.6 ng/mL X-gal. The inoculated plates were then incubated at 37°C overnight. White colored colonies were selected and used to inoculate separate 100 mL cultures of L-B Medium containing 50 ng/mL ampicillin which were incubated at 37°C overnight while being stirred at 100 rpm.

Plasmid DNA was prepared using QIAGEN Midi Plasmid Kit (Cat. # 12143 from QIAGEN Inc.), according to the manufacturer's instructions, as follows. Each 100 mL culture was aliquoted into two 50 mL conical vials and centrifuged at 3 000 g for 10 minutes at 4°C. The supernatant was poured off and 4 mL of Buffer P1 were used to resuspend the pellet in one of the two vials. This suspension was transferred to the other tube and used to resuspend that pellet. Buffer P2 (4 mL) was added, mixed and the suspension was incubated at room temperature for 5 minutes. Buffer P3 (4 mL) was added, mixed and the suspension was incubated at 4°C for 15 minutes. The samples were centrifuged at 3 000 g for 15 minutes at 4°C. All of the supernatant was transferred to a QIAgen-tip 100 that had been previously treated with 4 mL of Buffer QBT. The supernatant was allowed to drain through the column. Buffer QC (10 mL) was added to the column and allowed to drain. Another 10 mL of Buffer QC was added and allowed to drain. Finally 4 mL of Buffer QF was added to the column and allowed to drain into a clean 15 mL conical vial. 2.8 mL of isopropanol was added and mixed with this solution. This was aliquoted into microfuge tubes, incubated at 4°C for 10 minutes and centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was removed, then 1 mL of 70% ethanol was added to each microfuge tube. Each tube was vortexed briefly, then centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was removed and 100 µL of sterile 1x TE buffer (Sambrook et al., page B-20) were added.

Restriction digests were performed on the purified DNA. Enzymes used to cut fragments out were: fragment 1, KpnI (Cat. # R0142S from New England Biolabs, Inc., Beverly, MA) and NsiI (Cat. # R0127S from New England Biolabs, Inc.); fragment 2, NsiI and HindIII (Cat. # R0104S from New England Biolabs, Inc.); fragment 3, HindIII and PstI (Cat. # V0279S from New England Biolabs, Inc.); and fragment 4, PstI and NotI (Cat. # R0189S from New England Biolabs, Inc.). All restriction digests were performed at 37°C in digestion buffers recommended by New England Biolabs.

Electrophoresis was performed on all restriction digest products using 10x gel-loading buffer IV (Sambrook et al., page 6.12), on a 1% agarose gel in 0.5x TBE buffer (Sambrook et

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al., page B-23) containing EtBr. Fragments of gel containing the restriction digest products were cut out and placed in 2 mL microfuge tubes. DNA fragments were extracted from the gel slices using a QIAquick Gel Extraction Kit (Cat. # 28704 from QIAGEN Inc.) according to the manufacturer's instructions. The mass of each fragment was determined and 3 μ L/mg of Buffer QX1 was added. Each tube was then incubated for 10 minutes at 50°C with mixture every two minutes. One volume of isopropanol was added to each tube and mixed. The resulting solution was transferred to a QIAquick spin column and centrifuged for 1 minute at 10 000 g at room temperature. Buffer PE (750 μ L) was added to each column followed by centrifugation for 1 minute at 10 000 g at room temperature. Each column was centrifuged again for 1 minute at 10 000 g at room temperature to remove any residual buffer. 30 μ L of sterile deionized water at 50°C was added to each column and incubated for 1 minute at room temperature. Each column was then placed in a microfuge tube and centrifuged for 1 minute at 10 000 g.

Two microliters of each fragment was added to a microfuge tube as well as 1 μ L of 10x Ligation Buffer and 1 μ L of T4 DNA Ligase (Cat. # M0202S from New England Biolabs, Inc.). This reaction was incubated at 16 °C overnight. Restriction enzymes KpnI and NotI (1 μ L each) were added to the reaction and incubated at 37°C for 1 hour. Electrophoresis was performed using 10x Gel-loading Buffer IV on a 1% agarose gel in 0.5x TBE buffer containing EtBr. The fragment of gel containing the assembled product was cut out and the DNA fragment was extracted using a QIAquick Gel Extraction Kit as described above.

The fragment was ligated into a vector using an Original TA Cloning Kit, as follows: 6 μ L of purified DNA were transferred to a clean microfuge tube and 1 μ L of 10x Ligation Buffer, 2 μ L of pCR 2.1 vector (previously digested with KpnI and NotI), and 1 μ L of T4 DNA Ligase were added and mixed. The reaction was incubated over night at 16°C. The ligation reaction mixture (3.5 μ L) was used to transform One Shot competent cells as described above, 50 μ L and 200 μ L aliquots of the transformation reaction were plated on Luria-Bertani Medium containing 1% agarose, 1mg/mL Glucose, 50ng/mL Ampicillin and 1.6ng/mL X-gal and grown as described above. White colored colonies were selected and grown as described above. Plasmid DNA was isolated and purified using a QIAGEN Midi Plasmid Kit as described above. DNA was resuspended in 100 μ L of sterile 1x TE buffer. This DNA was then sequenced using ABI 100 DNA sequencer (from ABI) by Tufts University Core Facility.

Identification of Four Genotypes of Dog P-glycoprotein

The dog P-glycoprotein identified in Example 1 is now termed genotype C.

Sequencing of the DNA isolated from beagle liver as described in this example permitted the
5 identification of three additional allelic variants of dog P-glycoprotein, termed genotypes A, B
and D. the allelic differences in DNA sequence are set forth in Table 1 below.

Table 1: Nucleotide and Amino Acid Differences Between Dog P-glycoprotein Genotypes
(Allelic Variants)

SEQ ID NO:		Genotype	Nucleotide position				Amino acid position			
DNA	Protein		91	607	1001	3458	25	197	329	1148
22	23	A	T	A	T	A	N	Q	S	M
24	25	B	A	A	T	A	K	Q	S	M
1	2	C	T	C	T	A	N	H	S	M
26	27	D	A	A	A	G	K	Q	T	V

Genotype A has a different nucleotide at base number 607 relative to SEQ ID NO:2.
This is a base change from C to A in the DNA sequence which causes an amino acid change
from Histidine to Glutamine at amino acid 197. Genotype B has different nucleotides at base
15 number 91 and 607 relative to SEQ ID NO:2. The base change at position 91 is from T to A
and causes an amino acid change from Asparagine to Lysine at amino acid 25. Base change
at position 607 is identical to that in Genotype A. Genotype D has different nucleotides at
base number 91, 607, 1001 and 3458 relative to SEQ ID NO:2. Base changes at position 91
and 607 are identical to that in Genotype B. The base change at position 1001 is from T to A
20 and causes an amino acid change from Serine to Threonine at amino acid 329. The base
change at position 3458 is from A to G and causes an amino acid change from Methionine to
Valine at amino acid 1148.

A previously identified dog P-glycoprotein (SEQ ID NOs:3 and 4; GenBank
Accession number AF045016), has 13 nucleotide differences from the genotype C dog
25 P-glycoprotein nucleotide sequence (SEQ ID NO:1). There is a 3 base deletion at base 89 to
base 91 (relative to SEQ ID NO:1) leaving out AAT which causes a deletion of amino acid 25
Asparagine. There is a base change from A to G at position 590 which causes an amino acid
change from Isoleucine to Valine at amino acid 192. There is a base change from C to A at
position 607 which causing an amino acid change from Histidine to Glutamine at amino acid

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197. There is a base change from G to C at position 651 which causes an amino acid change from Arginine to Proline at amino acid 212. There is a base change from G to A at position 878 which causes an amino acid change from Glycine to Arginine at amino acid 288. There is, a base change from T to A at position 1001 which causes an amino acid change from
5 Serine to Threonine at amino acid 329. There is a base change from A to G at position 1012 which does not cause an amino acid change. This is a silent point mutation at Glutamine, amino acid 332. There is a base change from A to G at position 1611 which causes an amino acid change from Glutamine to Arginine at amino acid 532. There is a base change from A to T at position 2098 which does not cause an amino acid change. This is a silent point mutation
10 at Valine, amino acid 694. There is a base change from C to T at position 2102 which causes an amino acid change from Proline to Serine at amino acid 696. There is a base change from C to T at position 3808 which does not cause an amino acid change. This is a silent point mutation at Alanine, amino acid 1264. There is a base change from G to A at position 3833 which causes an amino acid change from Valine to Isoleucine at amino acid 1273. There is a
15 base change from C to T at position 4080 which causes an amino acid change from Threonine to Isoleucine at amino acid 1355.

Each of the foregoing patents, patent applications and references is hereby incorporated by reference. While the invention has been described with respect to certain
20 embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is:

SEQUENCE LISTING

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 10 <130> G0307/7013WO
 <150> US 60/156,510
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 15 <160> 32
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 25 <220>
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 30 1 5 10
 aag aac ttc tgg aaa atg ggc aaa aaa agt aaa aaa aat gag aag aaa 100
 Lys Asn Phe Trp Lys Met Gly Lys Lys Ser Lys Lys Asn Glu Lys Lys
 15 20 25
 35 gaa aag aaa cca act gtc agc acg ttt gca atg ttt cgc tat tca aat 148
 Glu Lys Lys Pro Thr Val Ser Thr Phe Ala Met Phe Arg Tyr Ser Asn
 30 35 40
 40 tgg ctt gat agg ttg tat atg ttg gtg ggg aca atg gct gcc atc atc 196
 Trp Leu Asp Arg Leu Tyr Met Leu Val Gly Thr Met Ala Ala Ile Ile
 45 50 55 60
 45 cat gga gct gca ctc cct ctc atg atg ctg gtt ttt gga aac atg aca 244
 His Gly Ala Ala Leu Pro Leu Met Met Leu Val Phe Gly Asn Met Thr
 65 70 75
 gat agc ttt gca aat gca gga att tca aga aac aaa act ttt cca gtt 292
 Asp Ser Phe Ala Asn Ala Gly Ile Ser Arg Asn Lys Thr Phe Pro Val
 50 80 85 90
 ata att aat gaa agt att acg aac aat aca caa cat ttc atc aac cat 340
 Ile Ile Asn Glu Ser Ile Thr Asn Asn Thr Gln His Phe Ile Asn His
 95 100 105
 55 ctg gag gag gaa atg acc acg tat gcc tat tat tac agt ggg atc ggt 388
 Leu Glu Glu Glu Met Thr Thr Tyr Ala Tyr Tyr Tyr Ser Gly Ile Gly
 110 115 120
 60 gct ggc gtg ctg gtg gct gct tac atc cag gtt tca ttc tgg tgc ctg 436

	Ala	Gly	Val	Leu	Val	Ala	Ala	Tyr	Ile	Gln	Val	Ser	Phe	Trp	Cys	Leu	
	125					130					135					140	
5	gca	gca	gga	aga	cag	ata	ctc	aaa	att	aga	aaa	caa	ttt	ttt	cat	gct	484
	Ala	Ala	Gly	Arg	Gln	Ile	Leu	Lys	Ile	Arg	Lys	Gln	Phe	Phe	His	Ala	
					145					150					155		
10	atc	atg	cga	cag	gag	att	ggc	tgg	ttt	gac	gtg	cat	gac	gtt	ggg	gag	532
	Ile	Met	Arg	Gln	Glu	Ile	Gly	Trp	Phe	Asp	Val	His	Asp	Val	Gly	Glu	
				160					165					170			
15	ctt	aac	acc	cgg	ctc	aca	gac	gat	gtc	tcc	aaa	atc	aat	gaa	gga	att	580
	Leu	Asn	Thr	Arg	Leu	Thr	Asp	Asp	Val	Ser	Lys	Ile	Asn	Glu	Gly	Ile	
			175					180					185				
	ggc	gac	aaa	att	gga	atg	ttc	ttt	cac	tca	ata	gca	aca	ttt	ttc	acc	628
	Gly	Asp	Lys	Ile	Gly	Met	Phe	Phe	His	Ser	Ile	Ala	Thr	Phe	Phe	Thr	
		190					195					200					
20	ggt	ttt	ata	gtg	ggg	ttt	aca	cgt	ggt	tgg	aag	cta	acc	ctt	gtg	att	676
	Gly	Phe	Ile	Val	Gly	Phe	Thr	Arg	Gly	Trp	Lys	Leu	Thr	Leu	Val	Ile	
	205					210					215					220	
25	ttg	gcc	atc	agc	cct	gtt	ctt	gga	ctt	tca	gcc	gcc	atc	tgg	gca	aag	724
	Leu	Ala	Ile	Ser	Pro	Val	Leu	Gly	Leu	Ser	Ala	Ala	Ile	Trp	Ala	Lys	
					225					230					235		
30	ata	cta	tct	tca	ttt	act	gat	aaa	gaa	ctc	ttg	gcc	tat	gca	aaa	gct	772
	Ile	Leu	Ser	Ser	Phe	Thr	Asp	Lys	Glu	Leu	Leu	Ala	Tyr	Ala	Lys	Ala	
				240					245					250			
35	gga	gca	gta	gct	gaa	gaa	gtc	tta	gca	gca	atc	aga	act	gtg	att	gcc	820
	Gly	Ala	Val	Ala	Glu	Glu	Val	Leu	Ala	Ala	Ile	Arg	Thr	Val	Ile	Ala	
			255				260						265				
	ttt	gga	gga	caa	aag	aaa	gaa	ctt	gaa	agg	tac	aac	aaa	aat	tta	gaa	868
	Phe	Gly	Gly	Gln	Lys	Lys	Glu	Leu	Glu	Arg	Tyr	Asn	Lys	Asn	Leu	Glu	
		270					275					280					
40	gaa	gct	aaa	gga	att	ggg	ata	aag	aaa	gct	atc	acg	gcc	aac	att	tct	916
	Glu	Ala	Lys	Gly	Ile	Gly	Ile	Lys	Lys	Ala	Ile	Thr	Ala	Asn	Ile	Ser	
	285					290					295					300	
45	att	ggt	gcc	gct	ttc	tta	ttg	atc	tat	gca	tca	tat	gct	ctg	gct	ttc	964
	Ile	Gly	Ala	Ala	Phe	Leu	Leu	Ile	Tyr	Ala	Ser	Tyr	Ala	Leu	Ala	Phe	
					305					310					315		
50	tgg	tat	ggg	acc	tcc	ttg	gtc	ctc	tcc	agt	gaa	tat	tct	att	gga	caa	1012
	Trp	Tyr	Gly	Thr	Ser	Leu	Val	Leu	Ser	Ser	Glu	Tyr	Ser	Ile	Gly	Gln	
				320					325					330			
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 Leu Pro Leu Met Met Leu Val Phe Gly Asn Met Thr Asp Ser Phe Ala
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 Gln Ile Leu Lys Ile Arg Lys Gln Phe Phe His Ala Ile Met Arg Gln
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 Gly Phe Thr Arg Gly Trp Lys Leu Thr Leu Val Ile Leu Ala Ile Ser
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Lys Asn Phe Trp Lys Met Gly Lys Lys Ser Lys Lys Asn Glu Lys Lys
15 20 25

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gaa aag aaa cca act gtc agc acg ttt gca atg ttt cgc tat tca aat 148
Glu Lys Lys Pro Thr Val Ser Thr Phe Ala Met Phe Arg Tyr Ser Asn
30 35 40

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tgg ctt gat agg ttg tat atg ttg gtg ggg aca atg gct gcc atc atc 196
Trp Leu Asp Arg Leu Tyr Met Leu Val Gly Thr Met Ala Ala Ile Ile
45 50 55 60

45
cat gga gct gca ctc cct ctc atg atg ctg gtt ttt gga aac atg aca 244
His Gly Ala Ala Leu Pro Leu Met Met Leu Val Phe Gly Asn Met Thr
65 70 75

50
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Asp Ser Phe Ala Asn Ala Gly Ile Ser Arg Asn Lys Thr Phe Pro Val
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Ile Ile Asn Glu Ser Ile Thr Asn Asn Thr Gln His Phe Ile Asn His
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Leu Glu Glu Glu Met Thr Thr Tyr Ala Tyr Tyr Tyr Ser Gly Ile Gly
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gct ggc gtg ctg gtg gct gct tac atc cag gtt tca ttc tgg tgc ctg 436
Ala Gly Val Leu Val Ala Ala Tyr Ile Gln Val Ser Phe Trp Cys Leu

	125		130		135		140	
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15	ctt aac acc cgg ctc Leu Asn Thr Arg Leu	aca gac gat gtc tcc aaa atc aat gaa gga att Thr Asp Asp Val Ser Lys Ile Asn Glu Gly Ile	175	180	185	580		
20	ggc gac aaa att gga Gly Asp Lys Ile Gly	atg ttc ttt caa tca ata gca aca ttt ttc acc Met Phe Phe Gln Ser Ile Ala Thr Phe Phe Thr	190	195	200	628		
25	ggt ttt ata gtg ggg Gly Phe Ile Val Gly	ttt aca cgt ggt tgg aag cta acc ctt gtg att Phe Thr Arg Gly Trp Lys Leu Thr Leu Val Ile	205	210	215	220	676	
30	ttg gcc atc agc cct Leu Ala Ile Ser Pro	gtt ctt gga ctt tca gcc gcc atc tgg gca aag Val Leu Gly Leu Ser Ala Ala Ile Trp Ala Lys	225	230	235	724		
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40	gga gca gta gct gaa Gly Ala Val Ala Glu	gaa gtc tta gca gca atc aga act gtg att gcc Glu Glu Val Leu Ala Ala Ile Arg Thr Val Ile Ala	255	260	265	820		
45	ttt gga gga caa aag Phe Gly Gly Gln Lys	aaa gaa ctt gaa agg tac aac aaa aat tta gaa Lys Glu Leu Glu Arg Tyr Asn Lys Asn Leu Glu	270	275	280	868		
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55	att ggt gcc gct ttc Ile Gly Ala Ala Phe	tta ttg atc tat gca tca tat gct ctg gct ttc Leu Leu Ile Tyr Ala Ser Tyr Ala Leu Ala Phe	305	310	315	964		
60	tgg tat ggg acc tcc Trp Tyr Gly Thr Ser	ttg gtc ctc tcc agt gaa tat tct att gga caa Leu Val Leu Ser Ser Glu Tyr Ser Ile Gly Gln	320	325	330	1012		
65	gta ctc act gtc ttc Val Leu Thr Val Phe	ttt tct gta tta att ggg gct ttt agt att gga Phe Phe Ser Val Leu Ile Gly Ala Phe Ser Ile Gly	335	340	345	1060		
70	cag gca tcc cca agc Gln Ala Ser Pro Ser	att gaa gca ttt gca aac gca aga gga gca gct Ile Glu Ala Phe Ala Asn Ala Arg Gly Ala Ala	350	355	360	1108		
75	tat gaa atc ttc aag Tyr Glu Ile Phe Lys	ata att gac aat aaa cca agc att gac agc tat Ile Ile Asp Asn Lys Pro Ser Ile Asp Ser Tyr	365	370	375	1156		

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5	tcg Ser	aag Lys	agt Ser	gga Gly	cat His 385	aaa Lys	cca Pro	gat Asp	aat Asn	att Ile 390	aag Lys	gga Gly	aat Asn	ttg Leu	gaa Glu 395	ttc Phe	1204
10	aaa Lys	aat Asn	gtt Val	cac His 400	ttc Phe	agt Ser	tac Tyr	cct Pro	tct Ser 405	cga Arg	aaa Lys	gaa Glu	gtt Val	aag Lys 410	atc Ile	tta Leu	1252
	aag Lys	ggt Gly	ctc Leu 415	aac Asn	ctg Leu	aag Lys	gtt Val	cag Gln 420	agt Ser	ggg Gly	cag Gln	aca Thr	gtg Val 425	gcg Ala	ctg Leu	gtt Val	1300
15	ggg Gly	aac Asn 430	agt Ser	ggc Gly	tgc Cys	ggg Gly	aag Lys 435	agc Ser	acg Thr	acc Thr	gtg Val	cag Gln	ctg Leu	atg Met	cag Gln	agg Arg	1348
20	ctc Leu 445	tat Tyr	gac Asp	ccc Pro	aca Thr	gat Asp 450	ggc Gly	atg Met	gtc Val	tgt Cys	att Ile 455	gat Asp	gga Gly	cag Gln	gac Asp	att Ile 460	1396
25	agg Arg	acc Thr	ata Ile	aat Asn	gta Val 465	agg Arg	cat His	ctt Leu	cgg Arg	gaa Glu 470	att Ile	act Thr	ggt Gly	gtg Val	gtg Val	agt Ser 475	1444
30	cag Gln	gag Glu	cct Pro	gtg Val 480	ttg Leu	ttt Phe	gcc Ala	acc Thr	acg Thr	ata Ile	gct Ala	gaa Glu	aac Asn	att Ile 490	cgc Arg	tat Tyr	1492
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35	gcc Ala	aat Asn 510	gcc Ala	tat Tyr	gat Asp	ttt Phe	atc Ile 515	atg Met	aaa Lys	cta Leu	cct Pro	aat Asn 520	aaa Lys	ttt Phe	gac Asp	act Thr	1588
40	ctg Leu 525	gtt Val	gga Gly	gag Glu	aga Arg	ggg Gly 530	gcc Ala	cag Gln	ctg Leu	agt Ser	ggt Gly 535	gga Gly	cag Gln	aaa Lys	cag Gln	aga Arg 540	1636
45	atc Ile	gcc Ala	att Ile	gct Ala	cgg Arg 545	gcc Ala	ctg Leu	gtt Val	cgc Arg	aac Asn 550	ccc Pro	aag Lys	att Ile	ctt Leu	ctg Leu 555	ctg Leu	1684
50	gat Asp	gag Glu	gca Ala	acg Thr 560	tca Ser	gct Ala	ctg Leu	gac Asp	act Thr	gaa Glu	agt Ser	gaa Glu	gca Ala	gtg Val 570	gtt Val	cag Gln	1732
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55	cat His	cgt Arg	ttg Leu	tct Ser	aca Thr	gtt Val	cgt Arg 595	aat Asn	gcc Ala	gat Asp	gtc Val	att Ile	gct Ala	ggg Gly	ttt Phe	gat Asp	1828
60	gat Asp	gga Gly	gtc Val	att Ile	gtg Val	gag Glu	aaa Lys	gga Gly	aat Asn	cat His	gat Asp	gaa Glu	ctc Leu	atg Met	aaa Lys	gag Glu	1876

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10	att gag tta gaa aat gcc act ggt gaa tcc aaa agt gaa agt gat gcc Ile Glu Leu Glu Asn Ala Thr Gly Glu Ser Lys Ser Glu Ser Asp Ala	640		645		650	1972	
15	ttg gaa atg tct cca aaa gat tca ggg tcc agt tta ata aaa aga aga Leu Glu Met Ser Pro Lys Asp Ser Gly Ser Ser Leu Ile Lys Arg Arg	655		660		665	2020	
20	tca act cgc agg agt ata cat gca cca caa ggc caa gac aga aag ctt Ser Thr Arg Arg Ser Ile His Ala Pro Gln Gly Gln Asp Arg Lys Leu	670		675		680	2068	
25	ggg aca aaa gag gac ttg aat gag aat gta cct cca gtt tcc ttc tgg Gly Thr Lys Glu Asp Leu Asn Glu Asn Val Pro Pro Val Ser Phe Trp	685		690		695	2116	
30	agg att ctg aag ctg aac tca act gaa tgg cct tat ttt gtg gtt ggt Arg Ile Leu Lys Leu Asn Ser Thr Glu Trp Pro Tyr Phe Val Val Gly	705		710		715	2164	
35	ata ttt tgt gct att ata aac gga ggc ctg caa cca gca ttt tca ata Ile Phe Cys Ala Ile Ile Asn Gly Gly Leu Gln Pro Ala Phe Ser Ile	720		725		730	2212	
40	ata ttt tca agg att ata ggg atc ttt acc cga gat gag gat cct gaa Ile Phe Ser Arg Ile Ile Gly Ile Phe Thr Arg Asp Glu Asp Pro Glu	735		740		745	2260	
45	aca aaa cga cag aat agt aac atg ttt tct gta ttg ttt cta gtc ctt Thr Lys Arg Gln Asn Ser Asn Met Phe Ser Val Leu Phe Leu Val Leu	750		755		760	2308	
50	gga att att tct ttt att aca ttt ttc ctc cag ggc ttc aca ttt ggc Gly Ile Ile Ser Phe Ile Thr Phe Phe Leu Gln Gly Phe Thr Phe Gly	765		770		775	2356	
55	aaa gct ggg gag atc ctc act aag cgg ctt cga tac atg gtt ttc aga Lys Ala Gly Glu Ile Leu Thr Lys Arg Leu Arg Tyr Met Val Phe Arg	785		790		795	2404	
60	tcc atg ctg aga cag gat gtc agc tgg ttt gat gac cct aaa aac acc Ser Met Leu Arg Gln Asp Val Ser Trp Phe Asp Asp Pro Lys Asn Thr	800		805		810	2452	
65	act gga gca ttg aca acc agg ctt gcc aat gat gcg gct caa gtt aaa Thr Gly Ala Leu Thr Thr Arg Leu Ala Asn Asp Ala Ala Gln Val Lys	815		820		825	2500	
70	ggg gct ata ggt tcc agg ctt gct gtc att acc cag aat ata gca aat Gly Ala Ile Gly Ser Arg Leu Ala Val Ile Thr Gln Asn Ile Ala Asn	830		835		840	2548	
75	ctt ggg aca ggc att att ata tcc tta atc tat ggt tgg caa tta aca Leu Gly Thr Gly Ile Ile Ile Ser Leu Ile Tyr Gly Trp Gln Leu Thr						2596	

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5	ctt tta ctc tta gca att gta ccc atc att gca ata gca gga gtt gtt Leu Leu Leu Leu Ala Ile Val Pro Ile Ile Ala Ile Ala Gly Val Val	865	870	875	2644
10	gaa atg aaa atg ttg tct gga caa gca ctg aaa gat aag aaa gag cta Glu Met Lys Met Leu Ser Gly Gln Ala Leu Lys Asp Lys Lys Glu Leu	880	885	890	2692
15	gaa gga gct ggg aag att gct aca gaa gcc atc gaa aac ttc cga act Glu Gly Ala Gly Lys Ile Ala Thr Glu Ala Ile Glu Asn Phe Arg Thr	895	900	905	2740
20	gtt gtt tct ttg act cgg gag cag aag ttt gaa tac atg tat gca cag Val Val Ser Leu Thr Arg Glu Gln Lys Phe Glu Tyr Met Tyr Ala Gln	910	915	920	2788
25	agt ttg caa gta cca tac aga aac tct ttg agg aaa gca cac atc ttc Ser Leu Gln Val Pro Tyr Arg Asn Ser Leu Arg Lys Ala His Ile Phe	925	930	935	2836
30	ggg gtc tca ttt tct atc acc cag gca atg atg tat ttt tcc tat gct Gly Val Ser Phe Ser Ile Thr Gln Ala Met Met Tyr Phe Ser Tyr Ala	945	950	955	2884
35	ggc tgt ttc cgg ttt ggt gcc tac ttg gtg gca aat gag ttc atg aac Gly Cys Phe Arg Phe Gly Ala Tyr Leu Val Ala Asn Glu Phe Met Asn	960	965	970	2932
40	ttt cag gat gtt ctt ttg gta ttc tca gct att gtc ttt ggt gcc atg Phe Gln Asp Val Leu Leu Val Phe Ser Ala Ile Val Phe Gly Ala Met	975	980	985	2980
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50	gta tca gca gcc cac gtc atc atg atc att gaa aaa agc cct ctg att Val Ser Ala Ala His Val Ile Met Ile Ile Glu Lys Ser Pro Leu Ile	1005	1010	1015	3076
55	gac agc tac agc cct cac ggc ctc aag cca aat acg ttg gaa gga aat Asp Ser Tyr Ser Pro His Gly Leu Lys Pro Asn Thr Leu Glu Gly Asn	1025	1030	1035	3124
60	gtg aca ttt aat gag gtc gtg ttc aac tat ccc act cga cca gac atc Val Thr Phe Asn Glu Val Val Phe Asn Tyr Pro Thr Arg Pro Asp Ile	1040	1045	1050	3172
65	ccc gtg ctc cag ggg ctg agc ctc gag gtg aag aag ggc cag acg ctg Pro Val Leu Gln Gly Leu Ser Leu Glu Val Lys Lys Gly Gln Thr Leu	1055	1060	1065	3220
70	gcc ctc gta ggt agc agt ggc tgt ggg aag agc aca gtt gtt cag ctc Ala Leu Val Gly Ser Ser Gly Cys Gly Lys Ser Thr Val Val Gln Leu	1070	1075	1080	3268
75	cta gag cgc ttc tat gac ccc ttg gct ggt tca gtg cta att gat ggc Leu Glu Arg Phe Tyr Asp Pro Leu Ala Gly Ser Val Leu Ile Asp Gly				3316

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5	aaa gag ata aag cac ctg aat gtc cag tgg ctc cga gca cac ctg ggc Lys Glu Ile Lys His Leu Asn Val Gln Trp Leu Arg Ala His Leu Gly	1105	1110	1115	3364
10	atc gtg tct cag gag ccc atc ctg ttt gac tgc agc att gcc gag aac Ile Val Ser Gln Glu Pro Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn	1120	1125	1130	3412
15	att gcc tat gga gac aac agc cgg gtc gta tca cat gaa gag att atg Ile Ala Tyr Gly Asp Asn Ser Arg Val Val Ser His Glu Glu Ile Met	1135	1140	1145	3460
20	cag gca gcc aag gag gcc aac ata cac cac ttc atc gag aca ctc cct Gln Ala Ala Lys Glu Ala Asn Ile His His Phe Ile Glu Thr Leu Pro	1150	1155	1160	3508
25	gag aaa tac aac acc aga gta gga gac aaa gga acc cag ctc tct ggt Glu Lys Tyr Asn Thr Arg Val Gly Asp Lys Gly Thr Gln Leu Ser Gly	1165	1170	1175	3556
30	ggc cag aaa cag cgc att gcc ata gct cgc gct ctt gtt aga cag cct Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Gln Pro	1185	1190	1195	3604
35	cat att ttg ctt ttg gat gaa gct aca tca gct ctg gat aca gaa agt His Ile Leu Leu Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser	1200	1205	1210	3652
40	gaa aag gtt gtc caa gaa gcc ctg gac aaa gcc aga gaa ggc cgc acc Glu Lys Val Val Gln Glu Ala Leu Asp Lys Ala Arg Glu Gly Arg Thr	1215	1220	1225	3700
45	tgc att gtg atc gcc cac cgc ttg tcc acc atc cag aat gca gat tta Cys Ile Val Ile Ala His Arg Leu Ser Thr Ile Gln Asn Ala Asp Leu	1230	1235	1240	3748
50	ata gtg gtg ttt cag aat ggc aaa gtc aag gag cat ggc aca cat caa Ile Val Val Phe Gln Asn Gly Lys Val Lys Glu His Gly Thr His Gln	1245	1250	1255	3796
55	cag ctg ctg gcc cag aaa ggc atc tat ttt tcc atg gtc agt gtc cag Gln Leu Leu Ala Gln Lys Gly Ile Tyr Phe Ser Met Val Ser Val Gln	1265	1270	1275	3844
60	gct gga gca aag cgc tagtgaactg tggccatatg agctgttaaa tatttttttaa Ala Gly Ala Lys Arg	1280			3899
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5	ctt tta ctc tta gca att gta ccc atc att gca ata gca gga gtt gtt	2644
	Leu Leu Leu Leu Ala Ile Val Pro Ile Ile Ala Ile Ala Gly Val Val	
	865 870 875	
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40	tgc att gtg atc gcc cac cgc ttg tcc acc atc cag aat gca gat tta	3748
	Cys Ile Val Ile Ala His Arg Leu Ser Thr Ile Gln Asn Ala Asp Leu	
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				20					25					30		
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		530					535					540				
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	Asn	Ala	Thr	Gly	Glu	Ser	Lys	Ser	Glu	Ser	Asp	Ala	Leu	Glu	Met	Ser
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30	Pro	Lys	Asp	Ser	Gly	Ser	Ser	Leu	Ile	Lys	Arg	Arg	Ser	Thr	Arg	Arg
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40	Ile	Ile	Gly	Ile	Phe	Thr	Arg	Asp	Glu	Asp	Pro	Glu	Thr	Lys	Arg	Gln
			740						745					750		
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		755						760					765			
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		770					775					780				
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		835						840					845			
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55	Ala	Ile	Val	Pro	Ile	Ile	Ala	Ile	Ala	Gly	Val	Val	Glu	Met	Lys	Met
	865					870					875					880
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60	Lys	Ile	Ala	Thr	Glu	Ala	Ile	Glu	Asn	Phe	Arg	Thr	Val	Val	Ser	Leu
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 5 Ser Ile Thr Gln Ala Met Met Tyr Phe Ser Tyr Ala Gly Cys Phe Arg
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60

5 <210> 29
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Claims

1. An isolated nucleic acid molecule selected from the group consisting of
 - (a) nucleic acid molecules that code for the amino acid sequence of SEQ ID NO:2,
 - (b) allelic variants of (a), wherein the allelic variants exclude SEQ ID NO:3 and SEQ
- 5 ID NO:5, and
 - (c) complements of (a) or (b).
2. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule codes for SEQ ID NO:2.
- 10 3. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid comprises the nucleotide sequence of SEQ ID NO:1.
4. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid
- 15 comprises the coding region of SEQ ID NO:1.
5. The isolated nucleic acid molecule of claim 1, wherein the allelic variants are nucleic acid molecules that code for an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:27.
- 20 6. The isolated nucleic acid molecule of claim 5, wherein the allelic variant codes for SEQ ID NO:23.
7. The isolated nucleic acid molecule of claim 5, wherein the allelic variant codes for
- 25 SEQ ID NO:25.
8. The isolated nucleic acid molecule of claim 5, wherein the allelic variant codes for SEQ ID NO:27.
- 30 9. The isolated nucleic acid molecule of claim 5, wherein the nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26.

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10. The isolated nucleic acid molecule of claim 9, wherein the nucleotide sequence comprises SEQ ID NO:22.

5 11. The isolated nucleic acid molecule of claim 9, wherein the nucleotide sequence comprises SEQ ID NO:24.

12. The isolated nucleic acid molecule of claim 9, wherein the nucleotide sequence comprises SEQ ID NO:26.

10

13. The isolated nucleic acid molecule of claim 5, wherein the nucleic acid molecule comprises a coding region of a nucleotide sequence selected from the group consisting of SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26.

15 14. An isolated P-glycoprotein polypeptide or fragment thereof which comprises at least one amino acid of a dog P-glycoprotein selected from the group consisting of positions 25, 192, 197, 212, 288, 329, 532, 696, 1273 and 1355 of SEQ ID NO:2; position 25 of SEQ ID NO:25; and positions 25 and 1148 of SEQ ID NO:27.

20 15. An isolated P-glycoprotein polypeptide or fragment thereof which comprises at least one amino acid of a dog P-glycoprotein selected from the group consisting of positions 3, 6, 8, 10, 12, 14-26, 36, 38, 48, 52, 56, 64, 74, 78, 84-92, 94, 96, 98, 99, 101, 103, 104, 106, 108, 112, 115, 147, 187, 197, 199, 233, 288, 321, 326, 347, 397, 450, 454, 455, 467, 472, 520, 633, 637, 643, 644, 650, 657, 658, 661, 666, 667, 674-677, 679, 685, 689, 691, 693, 694, 703, 25 707, 717, 731, 736, 740, 744, 745, 756, 759, 763, 853, 914, 920, 942, 943, 946, 968-970, 972, 974, 983, 1005, 1010, 1017, 1025, 1026, 1029, 1040, 1095, 1098, 1105, 1144, 1148, 1149, 1158, 1162, 1165, 1168, 1170, 1252 and 1279 of SEQ ID NO:2; and position 329 of SEQ ID NO:27, wherein the P-glycoprotein is identical to a human P-glycoprotein except for the at least one amino acid of a dog P-glycoprotein.

30

16. The isolated P-glycoprotein polypeptide or fragment thereof of claim 15, wherein the human P-glycoprotein is selected from the group of SEQ ID NO:7 and SEQ ID NO:8.

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17. The isolated P-glycoprotein polypeptide or fragment thereof of any of claims 14-16, wherein the amino acid sequence of the polypeptide or fragment thereof is an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:23, SEQ ID
5 NO:25, SEQ ID NO:27, fragments of SEQ ID NO:2, fragments of SEQ ID NO:23, fragments of SEQ ID NO:25 and fragments of SEQ ID NO:27.

18. An isolated nucleic acid molecule which encodes the isolated P-glycoprotein polypeptide or fragment thereof of any of claims 14-17.

10 19. An expression vector comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter.

15 20. An expression vector comprising the isolated nucleic acid molecule of claim 18 operably linked to a promoter.

21. A host cell transformed or transfected with the expression vector of claim 19.

20 22. A host cell transformed or transfected with the expression vector of claim 20.

23. An agent which selectively binds the isolated polypeptide of claim 14.

24. The method of claim 23, wherein the agent does not bind a human or dog P-glycoprotein.

25 25. The agent of claim 23, wherein the agent is a polypeptide.

26. The agent of claim 25, wherein the polypeptide is selected from the group consisting of monoclonal antibodies, polyclonal antibodies, Fab antibody fragments, F(ab)₂ antibody
30 fragments and antibody fragments including a CDR3 region.

27. An agent which selectively binds the isolated nucleic acid molecule of claim 1 or

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claim 18.

28. The agent of claim 27, wherein the agent is an antisense nucleic acid which selectively binds to the isolated nucleic acid molecule.

5

29. A method for predicting the bioavailability of a compound, comprising measuring the transmembrane transport of a test compound by a first P-glycoprotein, comparing the transmembrane transport of the test compound by the first P-glycoprotein and a second P-glycoprotein to predict the bioavailability of the test compound, wherein the relative amount or rate of transport by the first P-glycoprotein and the second P-glycoprotein is predictive of bioavailability of the test compound.

10

30. The method of claim 29, wherein the first P-glycoprotein is selected from the group consisting of dog P-glycoproteins and primate P-glycoproteins.

15

31. The method of claim 29, wherein the first P-glycoprotein is the polypeptide of claims 14-17.

32. The method of claim 29, wherein the second P-glycoprotein is a human P-glycoprotein.

20

33. A method for inhibiting P-glycoprotein transporter activity in a mammalian cell comprising

contacting the mammalian cell with an amount of the agent of claim 27 effective to inhibit P-glycoprotein transporter activity in the mammalian cell.

25

34. A method for increasing bioavailability of a drug in a subject comprising administering to a subject in need of such treatment the agent of claim 27 in an amount effective to increasing bioavailability of a drug.

30

35. The method of claim 34, wherein the inhibitor is administered prior to administering the drug.

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36. The method of claim 34, wherein the inhibitor is administered concurrently with the drug.

5 37. A method for increasing P-glycoprotein transporter activity in a cell comprising contacting the cell with a molecule selected from the group consisting of the nucleic acid molecule of claim 1 and the nucleic acid molecule of claim 18, in an amount effective to increase P-glycoprotein transporter activity in the cell.

10 38. A method for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with P-glycoprotein transporter activity comprising providing a cell or other membrane-encapsulated space comprising a P-glycoprotein as claimed in claim 14 or 15;

15 contacting the cell or other membrane-encapsulated space with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of P-glycoprotein transporter activity; determining a second amount of P-glycoprotein transporter activity as a measure of the effect of the pharmacological agent on the P-glycoprotein transporter activity, wherein a second amount of P-glycoprotein transporter activity which is less than the first amount
20 indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces P-glycoprotein transporter activity and wherein a second amount of P-glycoprotein transporter activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases P-glycoprotein transporter activity.

25 39. The method of claim 38, further comprising the step of loading the cell or other membrane-encapsulated space with a detectable compound, wherein the compound is detected as a measure of the P-glycoprotein transporter activity.

30 40. A method for identifying compounds which selectively bind a P-glycoprotein comprising, contacting the P-glycoprotein claimed in claim 14 or 15 with a compound,

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determining the binding of the compound to the P-glycoprotein.

41. The method of claim 40 further comprising determining the effect of the compound on the P-glycoprotein transporter activity of the P-glycoprotein.

5

42. The method of claim 40 further comprising determining the effect of the compound on the ATPase activity of the P-glycoprotein.

43. A method for determining ATPase activity of a P-glycoprotein comprising
10 contacting the host cell of claim 21 or 22, or a membrane fraction thereof, with
a test drug, and
measuring ATPase activity of the P-glycoprotein.

44. The method of claim 43, wherein the step of measuring ATPase activity is performed
15 at least twice at different times.

45. A method for determining transmembrane transport of a compound by a P-
glycoprotein, comprising
contacting the host cell of claim 21 or 22, or a membrane fraction thereof, with
20 a test drug, and
measuring transport of the test drug under sink conditions in at least one direction of
transport selected from the group consisting of the apical to basolateral direction and the
basolateral to apical direction.

25 46. The method of claim 45, wherein the step of measuring transport of the test drug is
performed at least twice at different times.

AMENDED SHEET

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