USE OF CELL LINES TO PRODUCE ACTIVE THERAPEUTIC PROTEINS

RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application serial number 60/510,509, filed on October 10, 2003, which is hereby incorporated in its entirety by reference.

GOVERNMENT GRANTS

This invention was made in part with United States government support under grant number 70-NANB7H3070 awarded by Advanced Technology Program of the United States Department of Commerce and with support from a NIH Small Business Innovative Research grant (Grant number: R 1 43 GM66480-01). The U.S. government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the use of cell lines, particularly virally-immortalized normal human cell lines, to produce proteins, especially therapeutic proteins, including therapeutic plasma proteins (TPP), that are capable of being expressed in active form by

hepatocytes and to the use of proteins, therapeutic proteins, and especially plasma proteins, produced by hepatocytes for the treatment of diseases and conditions affecting the liver and other organs.

BACKGROUND OF THE INVENTION

The safe and efficient production of novel therapeutic proteins represents an expanding market of the biopharmaceutical industry that is fueled by the recent completion of the Human Genome Project and by rapid technological advances in the field of proteomics. Paulaus, A., *The reengineering of drug development in the genomics and proteomics era.* Am Clinical lab, 2001. **5**: p. 55-57.

Although many of these therapeutic proteins are mass-produced by recombinant technology in Chinese Hamster Ovary (CHO) cells and other non human cell types, there are occasions where the commercialization of complex heterologous proteins is better accomplished by using the native form of the therapeutically effective protein. This is particularly true when such proteins are the products of multiple genes and the resulting proteins are highly processed post-translationally. It follows that this may be accomplished by isolating the native form of the protein or a recombinant form of the protein that is expressed and processed in the human producer cell.

In addition, the production of therapeutic plasma proteins (TPP) by cell-based systems would avoid the hazards of blood-derived products, the most notable of which is viral contamination. Although, when processed correctly, blood-derived products are virtually free of transmitting viral infections, a perceived risk exists for the manufacturer, user, and patient. Indeed, the recent discovery of new strains of human immunodeficiency virus and the agents responsible for the transmissible spongiform encephalopathies, such as mad cow disease, exemplify the everlasting concern for blood-derived products. Collinge, J., et al., *Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD.* Nature, 1996. **383**(6602): p. 685-90.

Limitations of Recombinant Proteins as Therapeutic Drugs.

Currently, many proteins that have been approved for clinical and therapeutic use, with the exception of monoclonal antibodies, are mass-produced by recombinant protein technology. Although these products have been proven safe and effective, not all behave identically to their native counterparts. For example, recombinant factors blood clotting factors (rF) VIII and IX are more rapidly cleared following infusion than their plasma

derived counterparts. Shapiro, A., E. Berntorp, and M. Morfini, *Incremental recovery assessment and effects of weight and age in previously untreated patients treated with recombinant factor IX.* Blood, 2000. 96 (suppl 1): p. 265a.

Recent findings suggest that this is the result of incomplete or inappropriate post-translational modification. The rapid clearance of the β-domain deleted form of Factor VIII, which is used in the United States, is due to differences in phospholipid binding. In contrast, differences in sulfation at tyrosine 155 and phosphorylation of serine 158 of Factor IX result in more rapid clearance of the clotting factor. White, C.G.I., A. Beebe, and B. Nielsen, *Recombinant factor IX.* Thromb Haemost, 1997. **78**: p. 261-265.

Clinically, more rapid clearance of these clotting factors means potentially more frequent and higher dosages depending upon the patient population. Although one strategy to circumvent these shortcomings is to use plasma-derived proteins, there are also perceived risks, as mentioned above, associated with this approach.

Significant Unmet Need for Therapeutic Proteins

Hemophilia A (Factor VIII deficiency) and hemophilia B (Factor IX deficiency) are bleeding disorders that are inherited as X- linked recessive traits. Thus, both affect males almost exclusively. Both hemophilia A and hemophilia B are heterogeneous conditions with variable degrees of clinical expression. Hemophilia A is far more common, occurring in 1 in 5000 to 10,000 males in the United States. Soucie, J.M., B. Evatt, and D. Jackson, *Hemophilia Occurrence in the United States.* American Journal of Hematology, 1998. **59**: p. 288-294.

In contrast, the incidence of hemophilia B is 0.25 in 10,000 males. Currently, plasma-derived and recombinant Factor VIII and IX concentrates are used for the lifetime treatment of hemophilia. It is estimated that three-quarters of the worldwide hemophilia population receive little or no treatment due to a shortage of this TPP. Thus, there is a clear need for fully functional, naturally-processed blood-clotting factors to overcome the shortcomings of traditional recombinant methodologies and/or the limited availability of blood-derived TPPs.

α-1-antitrypsin (AAT) is a human blood protein whose prime physiological target is neutrophil elastase. Severe AAT deficiency (hereditary emphysema) is thought to affect around 150,000-200,000 individuals in Europe and US. Donohue, T.M., et al., *Synthesis and secretion of plasma proteins by the liver*, in *Hepatology: A Textbook of Liver Disease*, D. Zakim and T.D. Boyer, Editors. 1990, W.B. Sounders Company: Philadelphia. p. 124-137. Many respiratory diseases including AAT congenital deficiency, cystic fibrosis, and chronic obstructive pulmonary disease are characterized by an imbalance of AAT and elastase in the lung. Elastase is a serine protease that hydrolyzes the extracellular matrix protein molecule elastin, among other proteins. An abundance of elastase is thought to contribute to damage of the pulmonary epithelium.

Administration of supplemental AAT is therefore expected to alleviate the deleterious effects of elastase in the lung in these diseases.

Approximately one in 2000 children is born with the CF genetic defect in the Western Hemisphere. Currently, there is only one plasma-derived AAT licensed in the United States, which has been in very limited supply. Many of the diagnosed patients have therefore not had access to AAT treatment. Despite the large body of evidence of the clinical efficacy of AAT to treat general inflammatory conditions, its use has been restricted due to the limited availability of the product. Thus, there is a clear need for fully functional, naturally-processed AAT to overcome the shortcomings of recombinant or blood-derived TPPs.

Sepsis is a disease characterized by an overwhelming systemic response to infection, which can rapidly lead to organ dysfunction and ultimately death. Sepsis can strike anyone and can be triggered by events such as pneumonia, trauma, surgery and burns, or by conditions such as cancer or AIDS. Once triggered, an uncontrolled cascade of coagulation, impaired fibrinolysis (clot breakdown), and inflammation fuels the progression of sepsis. In the United States, sepsis is the leading cause of death in the noncardiac intensive care unit and the 11th leading cause of death overall.

Each year, over 700,000 new cases of sepsis are diagnosed and every day 1400 people worldwide die from severe sepsis. Currently, treatment for sepsis is limited to attempts to manage the underlying infection and supportive therapy if the infection leads to

organ dysfunction. Despite intensive medical care, up to 50% of patients still die from this illness. Rangel-Frausto, M.S., et al., *The natural history of the systemic inflammatory response syndrome (SIRS): a prospective study.* JAMA, 1995. **273**: p. 117-123.

Given the intensive and prolonged care necessary to treat patients with sepsis, the economic burden is profound. For decades, physicians treating patients with severe septic illness have searched for an effective addition to their available therapeutic arsenal (mainly antibiotics) that could reduce the high mortality rate associated with this disease. Many of the attempted therapeutic interventions in human sepsis have been based upon the premise that circulating endotoxin is responsible for the critical clinical manifestations and morbidity of sepsis. Indeed, some investigators have concluded that any adjunctive therapy is destined to fail because once the clinical signs of severe sepsis are present, irreversible organ injury has already occurred. Recently, a promising new class of therapeutic agents based on natural plasma proteins with anti-coagulative activities has appeared on the clinical horizon. In severe sepsis, the coagulation system is activated; an event evidenced by the presence of intravascular thrombi in vessels and tissue and the occurrence of disseminated intravascular coagulation. Large multicenter phase III studies of activated protein C (APC) and antithrombin III (AT-III) in sepsis were completed in early 2001. In late 2001, Eli Lilly began marketing, Xigris, a genetically engineered version of the human activated protein C molecule; however, this drug only reduces the absolute risk of death by six percent. There is a clear need for more effective treatments of this severe sepsis.

Inter-α-inhibitor proteins (IαIp), natural serine protease inhibitors found in relatively high concentration in plasma have been shown to play roles in inflammation, wound healing and cancer metastasis reviewed by Bost et al. Bost, F., M. Diarra-Mehrpour, and J.P. Martin, *Inter-alpha-trypsin inhibitor proteoglycan family-a group of proteins binding and stabilizing the extracellular matrix.* Eur J Biochem, 1998. **252**: p. 339-346. The major forms of IαIp are inter-α-inhibitor (IαI, containing one light chain peptide called bikunin and two heavy chains) and pre-α-inhibitor PαI, containing one light chain and one heavy chain). In IαI, the two heavy chains are designated H1 and H2. These are cleaved from precursors designated H1P and H2P. These precursors are

encoded by genes designated *ITH1* and *ITH2*. The bikunin subunit is a double-headed, Kunitz-type, protease inhibitor. It is produced by cleavage from a α1m/bikunin precursor known as AMBP and encoded by a gene designated *AMBP*. The properties of the AMBP fusion protein precursor and the cleavage process are discussed further below. In PαI, the light chain is bikunin, and the heavy chain is H3, cleaved from a precursor designated H3P, encoded by a gene designated *ITIH3*. Both IαI and PαI are designated IαIp protein complexes, and that term is used herein to refer generally to either of IαI and PαI, or to both IαI and PαI.

Recently, a monoclonal antibody that recognizes the light chain of human IαIp (MAb 69.31) was developed by scientists at Prothera Biologics (Providence, Rhode Island). Using MAb 69.31 in a competitive ELISA, these investigators demonstrated that plasma IαIp levels were significantly decreased in severe septic patients compared to healthy controls. This decrease correlated with mortality suggesting that IαIp might have predictive value in septic patients. Lim, Y.P., et al., *Inter-trypsin inhibitor: decreased plasma levels in septic patients and its beneficial effects in an experimental sepsis model.* Shock, 2000. 13 (Suppl.): p. 161. *In-vivo* animal studies using a polymicrobial sepsis rat model of cecal ligation and puncture showed that administration of IαIp produced dramatic improvements in survival rates compared to saline controls. *Yang S, et al., Administration of human inter-alpha-inhibitors maintains hemodynamic stability and improves survival during sepsis.* Crit Care Med. 2002 Mar;30(3):617-22. Taken together, the results strongly support the therapeutic potential of IαIp in the management of severe sepsis. Although IαIp can be purified from human serum or plasma, if proven effective there will remain a worldwide shortage of this protein to treat sepsis. There is presently no means to produce or express highly functional, naturally-processed forms of this IαIp (e.g. naturally occurring or recombinant produced). Further the complexity of this protein increases the difficulty of both expressing it in an active, processed form and isolating it in an active state.

There are a number of patents and publications that describe immortalized cell lines: U.S. Patent No. 6,107,043 (Jauregui); U.S. Patent No. 5,665,589 (Harris); U.S. Patent App. No. 2002/0045262 A1 (Prachumsri); and International publication No. WO

99/55853 (Namba). However, to date, among other things, the prior art cell lines do not provide a means to safely, effectively, and cost efficiently perform the protein post-translational modifications, such as glycosylation, that are critical in the production of functional therapeutic plasma protein; produce simultaneously multiple therapeutic plasma proteins, especially Factor VIII protein or Factor IX, as well as IαIp; and maintain the continuous expression of active levels of cytochrome P450 enzyme in a serum-free media.

SUMMARY OF THE INVENTION

One aspect of the present invention is a method of using immortalized human hepatocyte cells to produce a protein comprising the steps of:

(1) providing an immortalized human hepatocyte cell that includes DNA that encodes and can express a protein;

(2) culturing the immortalized hepatocyte cell under conditions in which a gene or genes encoding the protein are expressed so that the protein is produced and processed in the immortalized hepatocyte cell; and

(3) isolating the processed protein from the immortalized hepatocyte cell; wherein the protein is expressed such that the protein is processed and glycosylated, if necessary, so that its *in vivo* function is substantially preserved after its isolation.

The protein can be a protein that is naturally produced by human hepatocytes, or can be a protein that is not naturally produced by human hepatocytes. Preferably the protein is a therapeutic protein. More preferably, the therapeutic protein is a plasma-derived therapeutic protein.

The protein can also be a mutein of a protein that is normally produced by human hepatocytes.

The protein can be a therapeutic protein, such as a plasma protein selected from the group consisting of Factor VIII, Factor IX, human growth hormone (hGH), α-1-antitrypsin, transferrin, and a growth factor, or a mutein of one of these proteins.

Alternatively, the protein can be a protein selected from the group consisting of albumin, transcobalamin II, C-reactive protein, fibronectin, ceruloplasmin, and other

proteins having structural, enzymatic, or transport activities, or a mutein of one of these proteins.

The protein can be one that is expressed by a gene that occurs naturally in the hepatocytes, in which case expression of the naturally-occurring gene encoding the protein is enhanced by introduction of a high-level promoter into the hepatocytes.

Alternatively, expression is enhanced by introducing multiple copies of the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed via the use of one or more recombinant vectors that include: (1) the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed; and (2) at least one control element affecting the transcription of the gene, the control element being operably linked to the gene.

In one alternative, the expressed protein is secreted from the cell into the surrounding culture medium.

The protein can be glycosylated or post-translationally processed.

The protein can be expressed in a form wherein it is fused to a cleavable tag.

The protein comprises at least two different subunits. In this case, the immortalized hepatocyte cell can be transformed or transfected with at least two vectors, each vector including: (1) DNA including at least one gene that encodes at least one subunit of the protein; and at least one control element operably linked to the DNA encoding at least one gene that encodes the subunit of the protein.

Another aspect of the invention is a method of treating a disease or condition comprising the steps of:

(a) providing an active protein produced by the method described above; and

(b) administering the active protein to a patient suffering from the disease or condition in a therapeutically effective quantity to treat the disease or condition.

Preferrably, the protein produced by the methods of the present invention are formulated in a pharmaceutical composition for delivery to the patient suffering from the disease or condition.

Another aspect of the invention is a method of using eukaryotic cells, other than human hepatocytes, to produce an IαIp protein complex comprising the steps of:

(1) providing a eukaryotic cell, other than a human hepatocyte, that includes DNA that encodes and can express proteins forming an IαIp protein complex, the eukaryotic cell having been transformed or transfected with at least one vector that includes: (a) DNA including at least one gene for a precursor of a protein that is part of an IαIp protein complex; and (b) at least one control element operably linked to the DNA encoding at least one precursor gene in order to enhance expression of the precursor gene;

(2) culturing the transformed or transfected eukaryotic cell under conditions in which genes encoding proteins forming an IαIp complex are expressed so that an IαIp complex is produced; and

(3) isolating the expressed IαIp protein complex from the transformed or transfected eukaryotic cell.

Another aspect of the invention is an immortalized human hepatocyte cell that includes DNA that encodes and can express a protein, the immortalized human hepatocyte cell having been transformed or transfected with at least one vector that includes: (1) DNA including at least one gene encoding a protein; and (2) at least one control element operably linked to the DNA encoding the protein in order to enhance expression of the protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows immunostaining of the Ea1C-35 immortalized hepatocyte cell line for large T antigen that confirms the integration of SV40DNA into genomic DNA of the immortalized cell.

Figure 1b shows immunostaining of cultured Fa2N-4 cells that demonstrates that the proliferating cells continue to express albumin.

Figure 1c shows the morphology of the immortalized cells showing well-defined nucleoli and granulated cytoplasm, which are characteristic features of normal primary hepatocytes.

Figure 2 shows an immunoblot showing induction of CYP3A4 consequent of treatment of Fa2N-4 and EA1C-35 with Rifampin (RIF), beta-naphthoflavone (BNF) and phenobarbital (PB). C is the untreated control. It should be noted that the upper band is nonspecific and that BNF, a CYP1A inducer does not induce CYP3A4 protein expression.

Figure 3 shows the following lanes: 1) Human Plasma; 2) Empty; 3) Culture Medium (Control); 4) Primary human hepatocytes (72 hr culture); 5) Ea1C-35 monolayer, 72 hrs culture; 6) Ea1C-35, roller bottle, 7-day culture; 7) Ea1C-35 roller bottle /14-day culture; 8) Fa2N-4 monolayer /72 hrs culture; 9) Fa2N-4 roller bottle /7-day culture; 10) Fa2N-4, roller bottle /14-day culture.

Figure 4 is a flowchart showing basic procedures for conducting enzyme induction studies in primary cultures of human hepatocytes and Fa2N-4 cells.

Figure 5 is photomicrographs showing the morphology of human hepatocytes (left panel) and Fa2N-4 cells (right panel) at the light microscopy level.

Figure 6 is a set of graphs showing the induction of CYP enzymes by omeprazole and rifampin in Fa2N-4 cells (CYP1A2, CYP3A4, CYP2B6, and CYP2C9; DMSO control).

Figure 7 is a graph depicting the reproducibility of CYP2B6 induction in rifampin-treated Fa2N-4 cells in 6-, 12- and 24-well plates.

Figure 8 is a graph depicting the reproducibility of CYP1A2 and CYP3A4 induction across multiple cell passages.

Figure 9 is a summary graph depicting that the induction of CYP2B6 by rifampin is the same in 6-, 12- and 24-well plates.

Figure 10 is a graph depicting the effect of cell culture format on the induction of CYP1A2 by omeprazole and the induction of CYP3A4 by rifampin.

Figure 11 is a graph showing the time course of CYP1A2 and CYP3A4 induction in Fa2N-4 cells.

Figure 12 is a graph showing the concentration-response curves for CYP1A2 induction by omeprazole and for CYP3A4 induction by rifampin in Fa2N-4.

Figure 13 is a graph showing that compounds shown previously to activate PXR and induce CYP3A4 in human hepatocytes induce CYP3A4 activity in Fa2N-4 cells, whereas Ah receptor agonists do not.

Figure 14 is a graph showing the range of CYP3A4 induction in primary cultures of human hepatocytes.

Figure 15 is a graph showing the effect of enzyme inducers on CYP1A2 and CYP3A4 activity in Fa2N-4 cells (left panel, CYP1A2; right panel, CYP3A4).

Figure 16 is a graph showing results of the use of the immortalized hepatocytes in toxicity studies.

Figure 17 is a phase contrast image of confluent Fa2N-4 cells plated in 96-well Biocoat Type I collagen plates in MPE media at 200X magnification.

Figure 18 is graphs depicting the induction of CYP1A2, CYP2C9, CYP3A4, UGT1A, and MDR1 transcripts in Fa2N-4 cells.

Figure 19 is graphs showing the measurement of induction by cytochrome-450 enzyme activity in Fa2N-4 cells ((A): Measurement of CYP3A4 activity; (B): Measurement of CYP2C9 activity; (C): Measurement of CYP1A2 activity).

Figure 20 is a graph showing EC50 plots for Fa2N-4 cells using increased CYP3A4 transcript values (panel (A)) and increased CYP3A4 enzyme activity (panel (B)).

Figure 21 is a graph showing response of multiple passages of Fa2N-4 cells to a CYP3A4 inducer with a weak response (50 μM dexamethasone) and a CYP3A4 inducer that exhibits a strong response (10 μM rifampin).

Figure 22 is a graph showing induction of CYP3A4 transcript in Fa2N-4 cells after 48 hour exposure to 10 μM rifampin (closed bars) is shown in comparison with vehicle (open bars).

Figure 23 is an autoradiograph of a gel (gel 1) the lanes for which are shown in Table 9 in Example 10.

Figure 24 is an autoradiograph of a gel (gel 2) the lanes for which are shown in Table 9 in Example 10.

Figure 25 is an autoradiograph of a gel (gel 3) the lanes for which are shown in Table 9 in Example 10.

Figure 26 is an autoradiograph of a gel (gel 4) the lanes for which are shown in Table 9 in Example 10.

Figure 27 is an autoradiograph of a gel (gel 5) the lanes for which are shown in Table 10 in Example 10.

Figure 28 is an autoradiograph of a gel (gel 6) the lanes for which are shown in Table 10 in Example 10.

Figure 29 is an autoradiograph of a gel (gel 7) the lanes for which are shown in Table 10 in Example 10.

Figure 30 is an autoradiograph of a gel (gel 8) the lanes for which are shown in Table 10 in Example 10.

Figure 31 is a picture of gels after two-dimensional electrophoretic analysis of the secreted proteins of the Fa2N-4 and Ea1C-35 cell lines ((A): Fa2N-4; (B): Ea1C-35; (C): Western blot using anti-Factor IX antibody for Ea1C-35 to detect secreted Factor IX).

Figure 32 is an autoradiograph of a gel (gel 8) the lanes for which are shown in Table 11 in Example 11.

Figure 33 shows a photograph of an ELISA (plate 1) containing a colorimetric enzyme immunoassay for the quantitative determination of secreted hGH utilizing the sandwich ELISA principle; the key for this plate is shown in Table 18.

Figure 34 shows a photograph of an ELISA (plate 2) containing a colorimetric enzyme immunoassay for the quantitative determination of secreted hGH utilizing the sandwich ELISA principle; the key for this plate is shown in Table 18.

Figure 35 is a photograph of immunostained Fa2N-4 cells stained for CD81 expression; CD81 was visualized by indirect immunofluorescence with a fluorescein conjugated secondary antibody.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Terms

In accordance with the present invention and as used herein, the following terms and abbreviations are defined with the following meanings, unless explicitly stated otherwise. These explanations are intended to be exemplary only. They are not intended to limit the terms as they are described or referred to throughout the specification. Rather, these explanations are meant to include any additional aspects and/or examples of the terms as described and claimed herein.

The following abbreviations are used herein:

MCT = MultiCell Technologies

MFE = Multi-functional Enhancing media

TPP = therapeutic plasma proteins

IαIp = inter-alpha-inhibitor proteins

SV40 = simian virus 40 T antigen and t antigen

AAT = α-1-antitrypsin

The term "cell line" refers to a population or mixture of cells of common origin growing together after several passages *in vitro*. By growing together in the same medium and culture conditions, the cells of the cell line share the characteristics of generally similar growth rates, temperature, gas phase, nutritional and surface requirements. The presence of cells in the cell line expressing certain substances, for example albumin, can be ascertained, provided a sufficient proportion, if not all, of the cells in the line produce a measurable quantity of the substance. An enriched cell line is one in which cells having a certain trait, e.g. expressing albumin, are present in greater proportion after one or more subculture steps, than the original cell line.

The term "clonal cells" are those, which are descended from a single cell. As a practical matter, it is difficult to obtain pure cloned cell cultures of mammalian cells. A high degree of cell purity can be obtained by successive rounds of cell enrichment. As used herein, a cell culture in which at least 80% of the cells possess a defined set of traits is termed a cloned cell culture. Preferably, a cell culture in which at least 90% of the cells possess a defined set of traits is termed a cloned cell culture. More preferably, a cell culture in which at least 98% of the cells possess a defined set of traits is termed a cloned

cell culture. The Fa2N-4 and Ea1C-35 cell lines claimed in this invention are clonal cell lines.

The term "immortalization" is defined as the acquisition of an infinite life span. Immortalization may be induced in finite cell lines by tranfection with telomerase, oncogenes, or the large T antigen of the SV40, or by infection with SV40. Immortalization is not necessarily a malignant transformation, though it may be a component of malignant transformation.

The term "immortalized" refers to the cell line that grows continually without senescence when cultured *in vitro* in a suitable growth medium.

The term "virally-immortalized" refers to hepatocytes being transfected or infected with all or part of the viral genome of a wild type or mutant virus. Preferably, the virus is a DNA virus. More preferably, the virus is SV40, which binds to p53 and Rb tumor suppressor proteins, leading to inactivation of their tumor suppressor pathways.

The term "substantially pure" refers to a DNA which has been purified from the sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome in which it naturally occurs, and which has been substantially purified from other components which naturally accompany the DNA, e.g., DNA which has been purified from the proteins which naturally accompany it in the cell.

The term "hepatocytes" refers to liver cells that are capable of considerable regeneration in response to loss of liver mass (e.g., through hepatotoxic processes, disease, or surgery), and constitute about 80% of the cell population of the liver. They are large polygonal cells measuring between 20-30 μm. Hepatocytes have as many as 200-300 peroxisomes per cells, which are involved in the breakdown of hydrogen peroxide, produced in many of the general cytoplasmic metabolic activities. In addition, peroxisomes have specific oxidative functions in gluconeogenesis, metabolism of purines, alcohol and lipids. The smooth endoplasmic reticulum (sER) in hepatocytes contain enzymes involved in degradation and conjugation of toxins and drugs. Under conditions of hepatocyte challenge by drugs, toxins or metabolic stimulants, the sER may become the predominant

organelle in the cells. Hepatocytes perform multiple finely-tuned functions which are critical to homeostasis. Of the variety of cell types in the mammalian body, only hepatocytes combine pathways for synthesis and breakdown of carbohydrates, lipids, amino acids, protein, nucleic acids and co-enzymes simultaneously to accomplish a unique biological task.

The term "isolated hepatocyte" refers to a hepatocyte that has been physically separated from other cells to which it is attached in its natural environment.

The term "primary hepatocyte" refers to a hepatocyte that has been recently isolated from intact liver tissue.

The term "normal primary human hepatocyte" refers to a hepatocyte derived from a nondiseased human liver and maintained *in vitro* for a finite period when cultured in a suitable medium.

The term "cryopreserved human hepatocyte" refers to a normal primary human hepatocyte that was cryopreserved prior to being cultured in a suitable medium.

The term "metabolic activity" refers to the sum total of the chemical reactions that proceed in a cell, including catabolism (breaking down) and anabolism (building up). The metabolic activity in a hepatocyte includes, but is not limited to, the ability to process potentially toxic compounds, e.g., a drug or endogenous metabolite, into a less toxic or non-toxic compound.

The term "cytochrome P450 enzyme" or "CYP" refers to a family of heme-based oxidase enzymes found predominantly in the liver. These enzymes form the first line of defense against toxins and they are involved in the metabolism of hydrophobic drugs, carcinogens, and other potentially toxic compounds and metabolites circulating in the blood. They are found tethered to the surface of the endoplasmic reticulum, where they attach a chemical handle onto carbon-rich toxins. Then other enzymes attach large soluble groups to these handles, making the entire molecule more water soluble. This allows the toxins to be eliminated by the urinary and digestive systems. The CYP family is divided into subfamilies, which include, but are not limited to, CYP1A, CYP2A, CYP2C, CYP2D, CYP2E, and CYP3A. Within these subfamilies there are numerous human CYP enzymes, often termed "isozymes" or "isoforms." The human CYP3A, CYP2D6, CYP2C, and

CYP1A isoforms are known to be important in drug metabolism. See, e.g., Murray, M., 23 Clin. Pharmacokinetics 132-46 (1992). CYP3A4 is by far the major isoform in human liver and the small intestines, comprising 30% and 70% respectively of the total CYP450 protein in those tissues. Based primarily on in vitro studies, it has been estimated that the metabolism of 40% to 50% of all drugs used in humans involve CYP3A4 catalyzed oxidations. See Thummel, K. E. & Wilkinson, G. R., In Vitro and In Vivo Drug Interactions Involving Human CYP 3A, 38 Ann. Rev. Pharmacol. Toxicol., 389-430 (1998).

The term "hepatic function" refers to liver specific biological functions, which include, but are not limited to, (1) gluconeogenesis; (2) glycogen synthesis, storage, and breakdown; (3) synthesis of serum proteins including, but not limited to, albumin, hemopexin, ceruloplasmin, the blood clotting factors (including, but not limited to, Factors V, VII, VIII, IX, X, prothrombin, and fibrinogen), α-l-antitrypsin, transferrin, and anti-thrombin III; (4) conjugation of bile acids; (5) conversion of heme to bile pigments; (6) lipoprotein synthesis; (7) vitamin storage and metabolism; (8) cholesterol synthesis; (9) ammonia metabolism, including urea synthesis and glutamine synthesis; (10) amino acid metabolism, including metabolic conversion and re-utilization of aromatic amino acids; and (11) detoxification and drug metabolism.

Hepatocyte-derived proteins provide a safer, more reproducible approach for producing native plasma proteins for therapeutic applications. These plasma proteins include Factor VIII, Factor IX, human growth hormone (hGH), and α-1-antitrypsin, but can also include other plasma proteins, such as growth factors. This finding is based upon MCT's data that demonstrates its proprietary, immortalized human hepatocyte cell lines, continue to produce inter-alpha-inhibitor proteins, a complex family of plasma proteins made by four different polypeptides that are produced from four different genes. Salier, J.-P., et al., *The inter-a-inhibitor family: from structure to regulation.* Biochem J, 1996. **351**: p. 1-9.

Methods of Use of Immortalized Cell to Produce Proteins

One aspect of the present invention is a method of use of immortalized human hepatocyte cells to produce a protein, preferably, a therapeutic protein, and more preferably a therapeutic plasma protein.

In general, such a method comprises the steps of:

(1) providing an immortalized human hepatocyte cell that includes DNA that encodes and can express a protein;

(2) culturing the immortalized hepatocyte cell under conditions in which a gene or genes encoding the protein are expressed so that the protein is produced and processed in the immortalized hepatocyte cell; and

(3) isolating the processed protein from the immortalized hepatocyte cell;

wherein the protein is expressed such that the protein is processed and glycosylated, if necessary, so that its *in vivo* function is substantially preserved after its isolation.

The protein to be produced can be a plasma protein, such as a therapeutic plasma protein. Examples of therapeutic plasma proteins include, but are not limited, to, proteins such as Factor VIII, Factor IX, human growth hormone (hGH), α-1-antitrypsin, or a growth factor. Alternatively, the protein to be produced can be an IαIp protein complex, such as either IαI or PαI, or both of these protein complexes. In still another alternative, the protein can be a protein useful for diagnostic uses or other uses such as albumin, transcobalamin II, C-reactive protein, fibronectin, or ceruloplasmin, as well as other proteins having structural, enzymatic, or transport activities. In yet another alternative, the protein to be produced can be a mutein of a protein such as growth factors, blood clotting factors such as Factor VIII or Factor IX, human growth hormone, antitrypsins such as α-1-antitrypsin, or another protein whose primary structure is modified by standard techniques of genetic engineering, such as site-specific mutagenesis. Similarly, the protein can be a mutein of a protein useful for diagnostic uses or other uses such as albumin, transcobalamin II, C-reactive protein, fibronectin, or ceruloplasmin, as well as other proteins having structural, enzymatic, or transport activities.

The protein to be produced can be a protein that is naturally produced by hepatocytes, either constitutively or in response to one or more outside stimuli, such as

hormonal signals. Alternatively, the protein to be produced can be a protein that is not naturally produced by hepatocytes. In the latter case, the protein can be a mutein of a protein that is naturally produced by normal hepatocyte cells.

Many biologically-active proteins comprise at least two different subunits. When such a protein is expressed, the immortalized hepatocyte cell can be transformed or transfected with at least two vectors, each vector including: (1) DNA including at least one gene that encodes at least one subunit of the protein; and at least one control element operably linked to the DNA encoding at least one gene that encodes the subunit of the protein.

The immortalized liver cells can be the immortalized liver cells disclosed in Provisional Patent Application No. 10/510,509, by Liu et al., filed October 10, 2003, and incorporated herein by this reference. In contrast to heterologous proteins produced by genetic recombination in mammalian cells, such as Chinese Hamster Ovary cells, proteins derived from immortalized liver cells as used in methods according to the present invention behave more normally since the secondary post-translational modifications required for complete function are carried out by the hepatocytes directly. A significant advantage of using immortalized liver cells to produce proteins, preferably therapeutic proteins, and more preferably therapeutic plasma proteins, is that the producer cell line is of human origin and therefore leads to a more natural protein. Therefore, since a number of therapeutic plasma proteins (TPP) are synthesized by human hepatocytes, human hepatocyte-based expression systems of the cell lines of the present invention are used to produce TPP in their "native" form. For one thing, this eliminates a possible immune response if a non-human protein were given to a human subject, as either a humoral or a cellular antibody response could develop if a protein recognized as non-self were administered.

A large number of proprietary immortalized human hepatocyte cell lines are disclosed in Provisional Patent Application No. 60/510,509, by Liu et al., filed October 10, 2003, and incorporated herein by this reference. The majority of these cell lines were created using simian virus 40 (SV40) T antigen as the immortalization gene. This strategy was chosen because transfection of human cells with T antigen results in cell lifespan

extension and frequently in nontumorigenic immortalization since the cells are semipermissive to viral infection. T antigen is a nuclear protein of 90,000 daltons. Cascio, S., *Novel strategies for immortalization of human hepatocytes.* Artificial Orgs, 2001. 25: p. 529-538.

The multiple mechanisms of T antigen action are still under investigation, but many studies demonstrate that this viral protein binds to and inactivates Rb and p53, two key tumor suppressor genes of the host cell. Ludlow, J., *Interactions between SV40 large-tumor antigen and the growth suppressor proteins pRB and p53.* FASEB J, 1993. 7: p. 866-871.

While inactivation of Rb and p53 extends the lifespan of the cell, immortalization requires a secondary genetic event in order for the cell to escape senescence and proliferate indefinitely. The nature of this event is poorly understood, but occurs when the cells proceed through crisis. Most SV40 T antigen immortalized cell lines retain varying levels of the differentiated characteristics associated with the primary cell type and do not display tumorigenicity prior to extensive passage *in vitro*. Kuroki, T. and N. Huh, *Why are human cells resistant to malignant cell transformation in vitro?* Jpn J Cancer Res, 1993. **84**: p. 1091-1100.

The normal human liver primary cells can be made to grow continuously by transfecting the cells with the T antigen gene of SV40 virus. Transfection or infection can be accomplished by use of a virus or a plasmid containing the T antigen gene of the SV40 virus. Either transfection or infection may lead to transformation of the cell line. Other transformation vectors may also be useful, such as papilloma virus or Epstein Barr virus. The techniques for making continuous human cell lines are described in the following references: Grahm. F. L., Smiley J., Russell, W. C. and Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol., 36:59-72 (1977); Zur Hausen, H. Oncogenic herpes viruses In: J. Tooze (ed.), DNA tumor viruses, Rev. Ed. 2, pp 747–798. Cold Spring Harbor, N.Y., Cold Spring Harbor Press (1981); Popovic, M., Lange-Wantein, G., Sarin, P. S., Mann, D. and Gallo, R. C. Transformation of a human umbilical cord blood T-cells by human T-cell leukemia/lymphoma virus (HTLV), Proc. Natl. Acad. Sci. USA,80:5402-5406 (1983);

DiPaolo, J. A. Pirisi, I., Popeseu, N. C., Yasumoto, S., Poniger, J. Progressive changes induced in human and mouse cells by human Papillomavirus Type-16 DNA, Cancer Cells 5:253-257 (1987).

Preferably, immortalized human hepatocytes useful in methods according to the present invention are derived from primary cryopreserved human hepatocytes. Preferably, immortalized human hepatocytes useful in methods according to the present invention are immortalized by introduction of a substantially pure SV40 DNA. Preferably, the substantially pure SV40 DNA includes wild type SV40 large T and small t antigens (TAg). Typically, the hepatocytes include DNA from which substantially pure DNA encoding a tumor suppressor gene can be isolated. Preferably, the tumor suppressor gene encoded by the DNA is human Rb. Preferably, the hepatocytes also include DNA from which substantially pure DNA encoding human p53 can be isolated.

Typically, the virally-immortalized human hepatocyte has the ability to be maintained and grow in serum-free media. Preferably, the serum-free media is MCT serum-free media.

Suitable hepatocyte cell lines include the virally-immortalized human primary hepatocyte cell line Fa2N-4, and the virally-immortalized human hepatocyte cell line Ea1C-35. Other human hepatocyte cell lines can also be used.

Creation of Immortalized Human Hepatocyte Cell Lines

Primary Cell Isolation

Digestion of donor, human liver was performed *in vitro* with pre-perfusion of oxygen-saturated, calcium-free buffer at 37°C. Pre-perfusion continued until the liver was blanched and followed by perfusion with oxygen-saturated, collagenase buffer until the liver was thoroughly digested (approximately 45 minutes).

To harvest cells, the liver was minced into 1 cm^2 pieces with the resulting suspension filtered through a #10 wire screen, then filtered again through a 253um nylon mesh. The suspension was centrifuged at 20xg for five minutes at 4°C to sediment intact parenchymal cells. The pellet was resuspended at 4°C and washed with washing buffer

(3X) to remove all collegenase. The cell pellet was resuspended in 150ml tissue culture media to yield a final volume of 400-500ml with a density of 3-4x10^7 cells/ml. Trypan blue and lactate dehydrogenase viability assessment was performed on aliquots of this suspension.

Cryopreservation of Primary Human Hepatocytes

The freshly isolated human hepatocytes isolated from donor liver as described above were washed with washing buffer three times by centrifuging at 50 x g for 5 minutes. The cell pellet was resuspended in chilled freezing medium (serum-free MFE medium: FBS:DMSO (8:1:1)) at a final cell density of 5x 10^6/mL. Aliquots of the cell suspension were transferred to Nunc Cryovials (1.0 mL/1.5ml cryovial, 4.5mL/5 ml cryovial). The cells in cryovials were equilibrated at 4°C for 15-30 minutes, the vials were then placed in a styrofoam container at -80°C for at least 3 hours. The vials were then plunged in LN_2.

Creation of Cell Lines

Cryopreserved human hepatocytes were rapidly thawed in a 42 degree Celsius water bath, washed and plated in MFE culture medium. Two days later the immortalizing gene was introduced by lipofection-mediated transfection. The Ea1C-35 cell line was derived from transfection with an immortalization vector containing the 2.5kb early region of the SV40 genome, which encompasses both the large-T and small-t antigens, and whose expression is driven by the SV40 early promoter. This early region was inserted into the Stratagene pBluescript SK vector backbone and was named pBlueTag. Neomycin resistance was conferred on the transfected cells as a selectable marker by co-transfection of a neo plasmid. Clones were initially selected based on their ability to grow in G418 containing media. The Ea1C-35 cell line was established and maintained in CSM medium.

The Fa2N-4 cell line was immortalized via lipofection-mediated transfection with a single immortalization vector. The early region of the SV40 genome, contained in the pBlueTag vector, was inserted into a backbone based upon the InvivoGen pGT60mcs plasmid and was named pTag-1. The T-antigen coding region is under the influence of a

hybrid hEF1-HTLV promoter. The vector also encodes a hygromycin resistance gene as a drug selectable marker. Clones were selected based on their ability to grow in hygro containing media. The Fa2N-4 cell line was established and maintained in MFE.

The Fa2N-4 cell line was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md., on October 6, 2003. The Ea1C-35 cell line was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md., on October 6, 2003.

Suitable immortalized hepatocyte cell lines, such as those described above, can be used for the production of proteins as described above. The proteins can be therapeutic plasma proteins such as Factor VIII, Factor IX, α-1-antitrypsin, human growth hormone, growth factors, or other proteins. The proteins can also be muteins of proteins such as growth factors, blood clotting factors, antitrypsins such as α-1-antitrypsin, and other proteins whose primary structure is modified by standard techniques of genetic engineering, such as site-specific mutagenesis. The proteins can further also include other proteins of therapeutic or diagnostic interest including albumin, transcobalamin II, C-reactive protein, fibronectin, or ceruloplasmin, as well as other proteins having structural, enzymatic, or transport activities.

When the hepatocyte cell line is used for the production of a naturally-occurring plasma protein, such as, but not limited to, Factor VIII, Factor IX, human growth hormone (hGH), or α-1-antitrypsin, several strategies can be used to maximize expression of the plasma protein.

In one strategy, expression of the naturally-occurring gene encoding the protein is enhanced by introduction of a high-level promoter. Such promoters are known in the art, and are described, for example, in S.B. Primrose et al., "Principles of Gene Manipulation" (6th ed., 2001, Blackwell, Oxford, England), p. 199, incorporated herein by this reference. Such promoters can also be accompanied by additional control elements, such as enhancers. Such promoters or promoter-enhancer combinations include the SV40 early promoter and enhancer, the Rous sarcoma virus long-terminal-repeat promoter and enhancer, and the human cytomegalovirus immediate early promoter.

However, it is generally preferred to enhance expression by introducing multiple copies of the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed via the use of one or more recombinant vectors that include: (1) the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed; and (2) at least one control element affecting the transcription of the gene, the control element being operably linked to the gene. The control element is typically a promoter or a promoter-enhancer combination. The characteristics of a suitable vector also include: (1) an origin of replication; (2) restriction endonuclease cleavage sites allowing the insertion of DNA encoding the desired genes; and (3) a selection marker, typically one that confers antibiotic resistance. In one particularly preferred embodiment, the control elements comprise at least one promoter and at least one enhancer.

Suitable recombinant vectors include, but are not limited to, SV40-derived vectors, murine polyoma-derived vectors, BK virus-derived vectors, Epstein-Barr virus-derived vectors, adenovirus-derived vectors, adeno-associated virus-derived vectors, baculovirus-derived vectors, herpesvirus-derived vectors, lentiviral-derived vectors, retrovirus-derived vectors, alphavirus-derived vectors, vaccinia virus-derived vectors, and others. Such vectors typically include a strong and constitutive promoter, at least one intron in the DNA to be expressed, and a polyadenylation signal at the 3′-terminus of the sequence to be transcribed. The addition of a signal peptide to ensure appropriate post-translational modification, such as glycosylation, can be desirable. These vectors and characteristics of vectors are described in S.B. Primrose et al., "Principles of Gene Manipulation" (6th ed., 2001, Blackwell, Oxford, England), pp. 174-201, and in T.A. Brown, "Gene Cloning and DNA Analysis: An Introduction" (4th ed., 2001, Blackwell, Oxford, England), both of which are incorporated herein by this reference.

Methods for isolating DNA encoding proteins to be expressed and for inserting such DNA into these vectors are also well known in the art. These methods are described, for example, in S.B. Primrose, "Principles of Gene Manipulation" (6th ed., Blackwell, Oxford, 2001), incorporated herein by this reference. In general, suitable DNA for cloning can be obtained from reverse transcription of specific mRNAs, which can be followed by

application of the polymerase chain reaction (PCR) to amplify the DNA; such DNAs are generally known as cDNA. DNA can be inserted into the vectors by techniques that generally involve cleavage of the vectors with specific restriction endonucleases and insertion of the DNA at the cleavage sites.

Methods for transforming or transfecting the virally-immortalized human hepatocytes are well-known in the art and need not be described further in detail here. In general, such methods include, but are not limited to, lipofection, calcium-phosphate-mediated transfection, transfection mediated by DEAE-dextran, transfection by electroporation, transfection by biolistics, and transfection using polybrene. These transfection methods are described in J. Sambrook & D.W. Russell, "Molecular Cloning: A Laboratory Manual (3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001), vol. 3, ch. 16, incorporated herein by this reference.

In many cases, it is desirable to incorporate one or more reporter genes into the vector to assess the efficiency of transfection. The gene of choice is under the control of strong ubiquitous promoter-enhancer combinations. These include those from the immediate early genes of human cytomegalovirus, the Rous sarcoma virus long terminal repeat, or the human β-actin gene. An example of a suitable reporter gene is the chloramphenicol acetyltransferase (CAT) gene found in the *Escherichia coli* transposon. Detection of expression of the reporter gene can be done by a variety of techniques, such as detection of fluorescence or detection of radioactive products. Reporter genes and their assay are further described in M.A. Aitken et al., "Gene Transfer and Expression in Tissue Culture Cells of Higher Eukaryotes," in Molecular Biomethods Handbook (R. Rapley & J.M. Walker, ed., Humana Press, Totowa, New Jersey, 1998), pp. 235-250, incorporated herein by this reference.

Once the protein has been expressed, it is then necessary to isolate the expressed protein. This is typically performed by standard methods for protein purification. These methods include, but are not limited to, precipitation with salts such as ammonium sulfate, ion-exchange chromatography, gel filtration chromatography, reverse phase high pressure liquid chromatography, electrofocusing, chromatofocusing, and/or immunoaffinity chromatography, using any readily ascertainable property, such as protease activity, to

detect the protein. Other purification methods are also known in the art. Protein purification methods are described, for example, in R.K. Scopes, "Protein Purification: Principles and Practice" (3d ed., Springer-Verlag, New York, 1994), incorporated herein by this reference.

In some cases, the expressed protein can be secreted from the cell into the surrounding culture medium. The efficiency of this process depends on the pattern of post-transcriptional modification, such as glycosylation, that the protein undergoes. This pattern affects the processing of the protein within the rough endoplasmic reticulum and the Golgi apparatus and its subsequent secretion. This is described in A.J. Dorner & R.J. Kaufman, "Analysis of Synthesis, Processing, and Secretion of Proteins Expressed in Mammalian Cells" in Gene Expression Technology (D.V. Goeddel, ed., Academic Press, San Diego, 1991), pp. 577-598, incorporated herein by this reference. The cloning vector can also be chosen so that the protein being expressed is fused to another protein, called a tag, which can be used to facilitate protein purification. Examples of tags include glutathione *S*-transferase, the MalE maltose-binding protein, and a polyhistidine sequence. The resulting fusion protein can then be cleaved with specific proteolysis to remove the tag and result in purified plasma protein. This technique can be applied to both therapeutic and non-therapeutic proteins, including plasma proteins.

In one example of the application of the present invention to the production of proteins, such virally-immortalized primary human hepatocytes can be transformed or transfected with vectors that include the genes for the various precursors for the IαIp proteins, such as IαI and PαI. This is done to increase the expression of the IαIp proteins. In one version of this example, two vectors are used: (1) a first vector that includes the genes *ITH3* and *AMBP*; and (2) a second vector that includes the genes *ITH2* and *ITH1*.

In one example of the production of therapeutic plasma proteins, the vector that includes DNA encoding the *ITH1* and *ITH2* genes is a variant of a commercially-available vector sold by InvivoGen. The vector sold by InvivoGen is designated pVITRO2 and contains a hygromycin resistance gene. The variant vector is designated pVITRO2-Blasti. The vector is modified by replacing the hygromycin resistance gene with a blasticidin resistance gene. The vector uses two human ferritin composite promoters. The 5′-UTR of

FerH and FerL are replaced by the 5′-UTR of the mouse and chimpanzee EF1a genes. The activity of both promoters is increased by the addition of the SV40 and CMV enhancers to yield activity similar to that of the CMV promoter. This promoter backbone was chosen in order that similar levels of expression for the two cDNAs that have been incorporated are driven from each vector. This is particularly desirable because the IαIp proteins are built in a 1:1:1:1 ratio using the polypeptides encoded by the cDNAs. In the plasmid pVITRO2-Blasti, *ITH1* is cloned into MCS1 and *ITH2* into MCS2. The cDNAs have unique restriction sites added to their ends to facilitate possible downstream subcloning. The linear schema for the cDNA encoding *ITH1* is 5′-BamHI-AgeI-EcoRV-cDNA-MluI-AvrII-BamHI-3′. The linear schema for the cDNA encoding *ITH2* is 5′-FspI-SgrA1-cDNA-XhoI-FspI-3′.

Similarly, in one example of the production of therapeutic plasma proteins, the vector that includes DNA encoding the *AMBP* and *ITH3* genes is another variant of the commercially-available pVITRO2 vector. This variant is designated pVITRO2-Neo and contains a neomycin resistance gene in place of the hygromycin resistance gene. This vector uses the same promoter backbone and 5′-UTRs as pVITRO2-Blasti. This vector contains the DNA encoding *AMBP* in MCS1 and the DNA encoding *ITH3* in MCS2. The linear schema for the cDNA encoding *AMBP* is 5′-BamHI-AgeI-EcoRV-cDNA-MluI-AvrII-BamHI-3′. The linear schema for the cDNA encoding *ITH3* is 5′-XhoI-NheI-cDNA-BglII-XhoI-3′. Other vectors can be used, but it is generally preferred that the vectors express each of the encoded proteins at approximately equal rates because of the 1:1 ratios between protein subunits that exist in the protein complexes.

Accordingly, another example of the application of the present invention in producing plasma proteins is a vector that includes DNA encoding the genes *ITH3* and *AMBP* operably linked to at least one control sequence compatible with a suitable host cell. The control sequence can be a promoter, enhancer, or other control sequence.

Yet another example of the application of the present invention in producing plasma proteins of the present invention is a vector that includes DNA encoding the genes *ITH2* and *ITH1* operably linked to at least one control sequence compatible with a suitable host cell. The control sequence can be a promoter, enhancer, or other control sequence.

Yet another example of the application of the present invention in producing therapeutic plasma proteins is an isolated and purified nucleic acid sequence that includes nucleic acid encoding the genes *ITH3* and *AMBP* operably linked to at least one control sequence compatible with a suitable host cell. The control sequence can be a promoter, enhancer, or other control sequence. The nucleic acid sequence is typically DNA, but can be RNA or an RNA-DNA hybrid.

Similarly, yet another example of the application of the present invention in producing therapeutic plasma proteins is an isolated and purified nucleic acid sequence that includes nucleic acid encoding the genes *ITH2* and *ITH1* operably linked to at least one control sequence compatible with a suitable host cell. The control sequence can be a promoter, enhancer, or other control sequence. The nucleic acid sequence is typically DNA, but can be RNA or an RNA-DNA hybrid.

Therefore, as a result of the existence of these vectors, another aspect of the invention is a virally immortalized human hepatocyte that is transformed or transfected with at least one vector that includes: (1) DNA including at least one gene encoding a plasma protein; and (2) at least one control element operably linked to the DNA encoding the plasma protein in order to enhance expression of the plasma protein. This virally immortalized human hepatocyte therefore encodes DNA that encodes and can express a plasma protein. An example of this is a virally immortalized human hepatocyte that is transformed or transfected with at least one vector that includes: (1) DNA including at least one gene for a precursor of a protein that is part of an IαIp protein complex; and (2) at least one control element operably linked to the DNA encoding at least one precursor gene in order to enhance expression of the precursor gene.

Yet another aspect of the invention is transformed or transfected eukaryotic cells, other than human hepatocyte cells, that produce active IαIp, either IαI or PαI. In general, these cells are transformed or transfected with at least one vector that includes: (1) DNA including at least one gene for a precursor of a protein that is part of an IαIp protein complex; and (2) at least one control element operably linked to the DNA encoding at least one precursor gene in order to enhance expression of the precursor gene. These cells can be CHO cells, COS cells, yeast cells, or other eukaryotic cells that can be transformed or

transfected, preferably with the vectors described above. These cells produce either IαI or PαI, or both; they also may produce other proteins. These cells, therefore, are transformed or transfected with either: (1) DNA encoding the genes *ITH3* and *AMBP*; (2) DNA encoding the genes *ITH2* and *ITH1*; or (3) both DNA encoding the genes *ITH3* and *AMBP* and DNA encoding the genes *ITH2* and *ITH1*.

Yet another example of the production of therapeutic plasma proteins according to the present invention is a method of using these transformed or transfected eukaryotic cells, other than human hepatocyte cells, to produce IαIp.

In general, such a method comprises the steps of:

(1) providing a transformed or transfected eukaryotic cell, other than a human hepatocyte cell, that includes DNA that encodes and can express proteins forming an IαIp protein complex;

(2) culturing the immortalized eukaryotic cell under conditions in which genes encoding proteins forming an IαIp protein complex are expressed so that an IαIp complex is produced; and

(3) isolating the expressed IαIp protein complex from the immortalized eukaryotic cell.

The protein produced can be either IαI or PαI, or both of these protein complexes.

Isolation of such protein complexes from cells expressing them, either virally immortalized human hepatocytes or cells other than human hepatocytes, is performed by standard methods for protein purification as described above.

Accordingly, another aspect of the present invention is the use of a protein produced by the immortalized hepatocyte cells to treat a disease or condition. The disease or condition can be a disease or condition affecting the liver, such as sepsis, liver cancer, hepatitis, or liver failure, or can be a disease or condition affecting an organ other than the liver, such as cancer at sites other than the liver, joint inflammation, or arthritis. An example of such a protein is an IαIp protein complex, but the method is applicable to proteins in general.

An example of the use of therapeutic plasma proteins according to the invention is the use of an IαIp protein complex.

In general, such a method comprises the steps of:

(1) providing an active IαIp protein complex; and

(2) administering the active IαIp protein complex to a patient suffering from a disease or condition in a therapeutically active quantity to treat the disease or condition.

As stated above, the disease or condition can affect the liver, such as sepsis, cancer, hepatitis, or liver failure. Alternatively, the disease or condition can affect an organ other than the liver, such as cancer at sites other than the liver, joint inflammation, or arthritis. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See, e.g. A.S. Nies & S.P. Spielberg, "Principles of Therapeutics" in J.G. Hardman & L.E. Limbird, eds., "Goodman & Gilman's The Pharmacological Basis of Therapeutics" (9th ed., McGraw-Hill, New York, 1996), ch. 3., pp. 43-62.) It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose of an IαIp protein complex in the management of a liver disease or condition will vary with the severity of the disease or condition and with the route of administration. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient, as well as other conditions affecting pharmacodynamic parameters such as liver and kidney function. In general, however, for therapeutic protein compositions, routes other than oral are typically preferred in order to avoid the proteolytic activity of the digestive tract. These routes can be intravenous, intramuscular, intraperitoneal, intralymphatic, subcutaneous, or other routes.

As described above, the active IαIp protein complex can be produced in immortalized hepatocytes, or can be produced in other eukaryotic cells.

Yet another aspect of the present invention is pharmaceutical compositions for treating a disease or condition comprising an active protein produced by eukaryotic cells, either virally immortalized human cells or other eukaryotic cells, in a quantity that is therapeutically effective to treat the disease and a pharmaceutically acceptable carrier. The

pharmaceutical compositions of the invention comprising the active protein can be in a variety of dosage forms which include, but are not limited to, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the particular therapeutic application.

Conventional pharmaceutically acceptable carriers for the compositions may include those known in the art such as serum proteins including human serum albumin, buffer substances such as phosphates, water or salts or electrolytes.

Yet another aspect of the present invention is antibodies that specifically bind protein produced according to the methods of the present invention. The protein can be used to prepare antibodies, including polyclonal antibodies that bind to purified protein or peptides isolated from the protein. These antibodies can be used to purify the protein in larger quantities. For example, the protein can be purified by fixing the antibody to a solid support and reacting the antibody fixed to the solid support with a sample containing the protein to bind the protein to the antibody. Alternatively, the antibody can be labeled with a detectable label, reacting the antibody labeled with the detectable label with a sample containing protein to bind the protein to the antibody, thereby forming an antigen-antibody complex, and separating the antigen-antibody complex from other proteins present in the sample. In either case, the protein can then be dissociated from the antibody by standard techniques, such as high salt, change of pH, or low concentrations of chaotropic agents.

The purified protein complex can then be used, for therapeutic or screening uses.

Monoclonal antibodies reactive with vesicle membrane transport protein can be produced by hybridomas prepared using known procedures, such as those introduced by Kohler and Milstein (see Kohler and Milstein, Nature, 256:495-97 (1975)), and modifications thereof, to regulate cellular interactions.

These techniques involve the use of an animal which is primed to produce a particular antibody. The animal can be primed by injection of an immunogen (e.g. the IαIp protein complex) to elicit the desired immune response, i.e. production of antibodies from the primed animal. Lymphocytes derived from the lymph nodes, spleens or peripheral blood

of primed (immunized) animals can be used to search for a particular antibody. The lymphocyte chromosomes encoding desired immunoglobulins are immortalized by fusing the lymphocytes with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines. These myeloma lines are available from the ATCC, Rockville, Md. Other myeloma lines can be used.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of the desired specificity, e.g. by immunoassay techniques using the protein that have been used for immunization. Positive clones can then be subcloned under limiting dilution conditions, and the monoclonal antibody produced can be isolated.

Both polyclonal and monoclonal antibodies can be obtained that are specific for the protein of interest.

Various conventional methods can be used for isolation and purification of the monoclonal antibodies so as to obtain them free from other proteins and contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see Zola et al., in Monoclonal Hybridoma Antibodies: Techniques and Applications, Hurell (ed.) pp. 51-52 (CRC Press, 1982)). Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art (see generally Fink et al., Prog. Clin. Pathol., 9:121-33 (1984), Fig. 6-1 at p. 123).

Generally, the individual cell line can be propagated *in vitro*, for example, in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration, or centrifugation.

In addition, fragments of these antibodies containing the active binding region reactive with the protein, such as Fab, $F(ab')_2$ and F_v fragments can be produced. Such fragments can be produced using techniques well established in the art (see e.g. Rousseaux et al., in Methods Enzymol., 121:663-69, Academic Press (1986)).

Yet another aspect of the present invention is a screening method for the detection of a protein for the detection of disease. As disease develops, there is a drop in the expression of active protein. In general, one embodiment of a screening method according to the present invention comprises:

(1) providing a plasma sample from a patient;

(2) determining the concentration of an active protein in the plasma sample; and

(3) correlating the concentration of the active protein in the plasma sample with the presence or absence of disease in the patient to determine the presence or absence of disease in the patient.

Typically, the concentration of active protein in the plasma sample is determined by an immunoassay using the antibodies described above. A number of immunoassay formats can be used, such as ELISA, radioimmunoassay, and other formats well known in the art. Either competitive or non-competitive immunoassays can be used. Typically, the antibody is labeled, but it is also possible to label the antigen, such as for a competitive immunoassay. Various types of labels can be used, including radioactive labels, fluorescent labels, chemiluminescent labels, bioluminescent labels, and enzyme labels. Immunoassays are described in E. Harlow & D. Lane, "Antibodies: A Laboratory Manual" (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988), ch. 14, pp. 553-612, incorporated herein by this reference.

In an alternative screening method, the quantity of mRNA encoding the protein is detected, as this is related directly to expression. In one embodiment of this alternative, the screening method comprises:

(1) taking a sample of cells from a patient;

(2) isolating the mRNA from the sample;

(3) determining the quantity or concentration of mRNA in the sample encoding the protein; and

(4) correlating the quantity or concentration of mRNA in the sample encoding the protein with the presence or absence of disease in the patient to determine the presence or absence of disease in the patient.

Methods for isolation of mRNA from cells and tissues are well known in the art and need not be described further in detail here. Such methods are described in detail in J. Sambrook & D.W. Russell, "Molecular Cloning: A Laboratory Manual" (3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001), vol. 1, ch. 7, incorporated herein by this reference. One method for performing the step of determining the quantity or concentration of mRNA in the sample encoding the IαIp protein complex is to perform the polymerase chain reaction (PCR) using an oligo-dT primer, with reverse transcriptase, to make a DNA copy of the polyadenylated mRNA in the sample. The DNA of the copy is then amplified in a second PCR reaction using primers specific for the IαIp protein complex genes. This approach is described in C.R. Cantor & C.L. Smith, "Genomics: The Science and Technology Behind the Human Genome Project" (John Wiley & Sons, 1999), pp. 119-120, incorporated herein by this reference. Another method for performing the step of determining the quantity or concentration of mRNA in the sample encoding the IαIp protein complex is the use of Northern blotting to detect specific mRNAs by hybridization. This requires that a suitable DNA labeled probe be available; typically, the labeled probe is radioactively labeled. This method is well known in the art and is described, for example, in P.A. Sabelli, "Northern Blotting" in Molecular Biomethods Handbook (R. Rapley & J.M. Walker, eds., Humana Press, Totowa, New Jersey, 1998), ch. 9, pp. 89-94, incorporated herein by this reference.

Post-translational modifications of therapeutic proteins may affect bioactivity, clearance rate *in vivo*, immunogenicity and/or stability. Proteins secreted by our hepatocyte-based expression systems of the present invention behave more naturally than recombinant counterparts. For example, the inventors have demonstrated that its immortalized human hepatocyte cell lines produce biologically active IαIp and therefore is a strong commercial source for this protein that cannot be produced by recombinant technology. Therefore, the inventors' production of IαIp in its "native" form leads to a

more effective, safe, and cost effective solution to treating life threatening diseases such as sepsis.

The use of cell lines suitable for the production of protein also allows the use of a sequential protein purification scheme that generates multiple products similar to plasma-derived proteins without the reoccurring risk of viral contamination. These hepatocyte-derived plasma proteins provide a safe, effective, and cost efficient strategy to commercially produce native plasma proteins, which overcomes the shortcomings of the prior art.

UTILITY

Examples of Uses of Virally Immortalized Liver Cell Lines That Produce Plasma Protein Complexes

The following are uses of virally immortalized liver cell lines that produce plasma proteins, more particularly, IαIp protein complexes. These uses are related to their production of IαIp protein complexes, as well as to their possible production of other proteins.

(1) Identification of potential chemotherapeutic drugs: These cells are useful for screening chemicals suitable for the treatment of cancer and related diseases, by growing them *in vitro* in medium containing the chemical to be tested and then, after a suitable period of exposure, determining whether and to what extent cytotoxicity has occurred, e.g. by trypan blue exclusion assay or related assays (Paterson, Methods Enzymol, 58:141 (1979)), or by growth assays such as colony forming efficiency (MacDonald et al, Exp. Cell. Res., 50:417 (1968)), all of which are standard techniques well known in the art.

(2) Investigation of the controls of gene expression by biological agents that induce or inhibit gene expression. Chemical and biological substances are screened for their ability to induce or inhibit gene expression or metabolic pathways by adding them to the growth medium of these liver cells and then after a suitable period of time, determine whether a complex of changes, including cessation of DNA synthesis, induction or inhibition of gene expression (as measured by RT-PCR analysis) and production of liver

specific proteins (as determined by immunochemical techniques) occurs. Identification of the effects of chemical and biological substances on the induction or inhibition of gene expression and metabolic pathways is a way to identify new drug targets for treating diseases such as cancer.

(3) Studies of metabolism of carcinogens and other xenobiotics: Carcinogens and other xenobiotics may be added to the growth medium of these cells and the appearance of metabolic products of these compounds may be monitored by techniques such as thin layer chromatography or high performance liquid chromatography and the like, and the interaction of the compounds and/or their metabolites with DNA is determined.

(4) Studies of DNA mutagenesis: Substances known or suspected to be mutagens may be added to the growth medium of the cells and then mutations may be assayed, e.g., by detection of the appearance of drug resistant mutant cell colonies (Thompson, Methods Enzymol, 58:308, 1979).

(5) Studies of malignant transformation by chemical, physical and viral agents, and transferred genes including oncogenes and high molecular weight genomic DNA from tumors, using standard assays such as anchorage independent growth or tumor formation in athymic nude mice. For example, a cloned viral oncogene N-ras (an oncogene present in many liver cell cancers) can be introduced into the hepatocyte cells using strontium phosphate transfection. The subsequent ability of the newly transfected cells to form tumors in mice as well as grow in an anchorage-independent fashion can be assessed.

(6) Use of cells altered by transfer of oncogenes as in paragraph (5) above to screen for potential chemotherapeutic agents (by the techniques described in paragraph (1) above) especially those which may be specific for cells transformed by the activation of particular oncogenes or combination of oncogenes.

(7) Studies of cellular biochemistry, including changes in intracellular pH and calcium levels, as correlated with cell growth and action of exogenous agents including but not limited to those described in paragraphs (1) through (6) above. To study intracellular pH and calcium levels, cells in suitable culture vessels are exposed to fluorescent indicator

dyes and then fluorescence emissions are detected with a fluorescence spectrophotometer (Grynkiewicz et al, J. Biol. Chem., 260:3440-3450 (1985)).

(8) Studies of cellular responses to growth factors and production of growth factors: Identification and purification of growth factors important for growth and differentiation of human liver hepatocyte cells. These cells are particularly useful for such an application since they grow in serum-free media. Therefore, responses to growth factors can be studied in precisely defined growth medium and any factors produced by the cells may be identified and purified without the complication of the presence of serum.

(9) Use of recombinant DNA expression vectors to produce proteins of interest. This is described above for IαIp protein complexes; similar techniques can be used for other proteins. For example, the gene encoding a protein of therapeutic value may be recombined with controlling DNA segments (i.e. containing a promoter with or without an enhancer sequence), transferred into the cell (e.g., by strontium phosphate transfection) and then the protein produced may be harvested from the culture supernatant or a cellular extract by routine procedures well known in the art.

(10) Studies of intracellular communication e.g., by dye scrape loading assays, to determine whether the cells growing *in vitro* have the ability to communicate via gap junctions. The cultures may be scraped, e.g., with a scalpel, in the presence of a fluorescent dye in the growth medium. Cells at the edge of the wound are mechanically disrupted and therefore take up dye; whether intercellular communication has occurred may be ascertained by determining whether cells distant from the wound also contain dye.

(11) Characterization of cell surface antigens: The cells are incubated with an antibody against the cell surface antigen of interest, and then reacted with a second antibody, which is conjugated to a fluorescent dye. The cells are then evaluated using a fluorescence activated cell sorter to determine whether they are fluorescent and therefore posses the cell surface antigen.

(12) Cell-cell hybrid studies for identification of tumor suppressor activity (Stranbridge et al, Science, 215:252-259 (1982)). To determine whether these cell lines contain tumor suppressor genes, they are fused to malignant tumor cells. The presence of

tumor suppressor genes is indicated by loss of malignancy e.g., as detected by loss of ability to form tumors in athymic nude mice, in the hybrid cells.

(13) Identification of novel genes, including transforming genes in the naturally occurring cancer described in paragraph (5) above, growth factor genes as described in paragraph (8) above, tumor suppressor genes as described in paragraph (12) above, using standard molecular biological techniques (Davis et al, Methods in Molecular Biology, New York: Elsevier (1986)) and techniques such as cDNA subtraction cloning and similar processes.

(14) Growth of replicating hepatitis virus (as e.g., HBV, non-A non-B, HAV and other livertropic virus, e.g., CMV). Establishment of a clonal cell line of human liver hepatocyte cells containing replicating hepatitis virus using methods of transfection established for human liver cancer cell lines (Sells, M. A. et al, Proc. Natl. Acad. Sci., 84:444-448). Using human liver hepatocyte lines, which contain HBV, the ability of HBV, alone as well as in conjunction with chemical liver carcinogens such as aflatoxin B, can be evaluated for malignant transformation using anchorage independent growth assays as well as growth in athymic nude mice. Cell--cell hybrid techniques similar to those in paragraph (13) can be used to evaluate possible inactivation of tumor suppressor genes by fusion with malignant cells before and after HBV transfection.

The screening kits are easily assembled as other screening kits containing cell lines with other conventional components and labeling instructions for performing the test.

(15) The immortalized cells may be used as a way of expanding cells for liver transplant and liver function assist devices, both implanted and extracorporeal. Also, these cells can have additional genes transfected/infected into them for organ transplant for therapy of inherited metabolic disorders, especially those diseases associated with hepatic degradation (i.e., certain diseases are due to a deletion or abnormality of a particular gene). This gene could then be transfected into cells useful for production of IαIp protein complexes, and the cells then expanded for organ transplant.

(16) Studies of cytotoxicity of drugs, carcinogens, xenobiotics: Drugs, carcinogens, xenobiotics may be added to the growth medium of the cells and the viability

of the cells as a function of time of exposure may be ascertained using gene expression profiling, dye exclusion, enzyme leakage, colony forming efficiency, etc. assays.

(17) Studies of gene expression: Drugs, chemicals, new chemical entities, etc., may be added to the culture medium of the cells and changes in gene expression as a function of exposure may be monitored using RNA and protein expression as biological endpoints. Changes may reflect either induction or inhibition of specific genes. For example, cells may be cultured with drugs, chemicals, new chemical entities, etc to identify those agents that modulate the expression of drug metabolism enzymes including but not limited to cytochrome P450s designated CYP3A4 or CYP1A2, the multi drug resistance gene, biliary transporters, glucuronyl transferases, glutathione transferases, sulfatases, etc.

(18) Studies of liver parasites: The cultured cells could prove efficacious for studying the life cycle of parasites that invade hepatocytes.

(19) Production of hepatocyte–derived proteins. This is described extensively above with respect to the expression and production of plasma proteins, particularly therapeutic plasma proteins, by these cells. Cells maintained in suitable medium will naturally express proteins such as blood clotting factors (e.g. Factor VIII and Factor IX), α-1-antitrypsin, human growth hormone, growth factors, etc., that may be purified and used. These proteins can be any proteins that are expressible by differentiated human hepatocytes. This category of proteins includes proteins that are naturally encoded and expressed by those cells, either constitutively or in response to one or more outside stimuli, such as hormonal signals. This category of proteins also includes proteins that can be expressed by those cells in such a way that they are processed and glycosylated so that their *in vivo* function is substantially preserved when genes for those proteins are introduced into those cells. This can include muteins of proteins such as growth factors, blood clotting factors, antitrypsins such as α-1-antitrypsin, and other proteins whose primary structure is modified by standard techniques of genetic engineering, such as site-specific mutagenesis. This can also include other proteins of therapeutic or diagnostic interest including albumin, transcobalamin II, C-reactive protein, fibronectin, or

ceruloplasmin, as well as other proteins having structural, enzymatic, or transport activities.

Proteins produced by methods according to the present invention can be used to treat a large variety of conditions, both conditions affecting the liver and conditions affecting organs other than the liver. The latter can include cancer at sites other than the liver, joint inflammation, and arthritis. These proteins can include IαIp complexes and other proteins produced by these methods, as described above.

The properties of the cell lines provide the ability to perform additional screening assays of value.

For example, the invention includes a method of screening a compound for chemotherapeutic activity comprising the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to the compound to be screened; and

(3) determining the extent of cytotoxicity induced by the compound to be screened in the cell line to determine whether the compound has chemotherapeutic activity.

The invention also includes a method of screening a compound for mutagenic activity comprising the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to the compound to be screened; and

(3) determining the extent of mutagenesis induced by the compound to be screened in the cell line to determine whether the compound has mutagenic activity.

Furthermore, the invention also includes a method of screening a compound for the activity of inducing or inhibiting gene expression comprising the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to the compound to be screened; and

(3) determining the inhibititory or inductive effect of by the compound to be screened in the cell line to determine whether the compound has the activity of inducing or inhibiting gene expression.

Similarly, the invention also includes a method of screening a compound for cytotoxicity comprising the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to the compound to be screened; and

(3) determining the extent of cytotoxicity induced by the compound to be screened in the cell line by determining the viability of the cell line as a function of either or both of concentration and time of exposure in order to determine whether the compound has cytotoxic activity.

One of the most significant screening methods according to the present invention is a method of screening for drug-drug interactions. These can be mediated by either a cytochrome P450 (CYP) enzyme or a multi-drug transporter (MDR) protein, such as MDR1. These drug-drug interactions, where administration of one drug interferes with the metabolism of a second drug, are of considerable clinical importance. They can occur with prescription drugs or over-the-counter drugs.

One method for screening for the existence of a drug-drug interaction according to the present invention comprises the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to a first drug;

(3) determining whether the first drug induces the production of a cytochrome P450 enzyme that can metabolize the second drug by measuring the production of the cytochrome P450 enzyme induced by the first drug to screen for the existence of an interaction between the first drug and the second drug.

Another method for screening for the existence of a drug-drug interaction comprises the steps of:

(1) providing a hepatocyte cell line selected from the group consisting of Fa2N-4 and Ea1C-35;

(2) exposing the cell line to a first drug;

(3) determining whether the first drug induces the production of a MDR protein by measuring the production of the MDR protein induced by the first drug to screen for the existence of an interaction between the first drug and the second drug.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

The following examples are provided by way of describing specific embodiments of the present invention without intending to limit the scope of the invention in any way.

Example 1

Characterization of Immortalized Human Hepatocytes

Over 100 human hepatocyte clonal cell lines were established by transfecting human hepatocytes with the simian virus 40 large T and small t antigen genes under control of the SV40 early promoter. Two cell lines designated Ea1C-35 and Fa2N-4 are described.

Both cell lines were created by lipofection-mediated transfection of primary cryopreserved human hepatocytes with vectors containing the SV40 largeT and small t antigens. The Ea1C-35 cell line was derived from transfection of cryopreserved human hepatocytes with the immortalization vector pBlue Tag, a recombinant plasmid containing the early region of wild-type SV40. The pBlue Tag vector was constructed as follows: pBR/SV (ATCC) was digested with restriction enzymes KpnI and BamHI to release a 2995 bp fragment (239-2468 bp, numbering according to Fiers, W et al Science, 273:113-120) containing the SV40 early promoter and the coding regions from small t and large T antigens. This KpnI/BamHI fragment was inserted into the pBluescript SK vector (Strategene) to produce pBlueTag; a Bluescript based vector that uses the SV40 promoter to drive T antigen expression. Neomycin resistance was conferred on the transfected cells as a selectable marker by co-transfection of a neo plasmid. Clones were initially selected based on their ability to grow in G418 containing media. The Ea1C-35 cell line was established and maintained in MCT's proprietary serum containing media, CSM.

The Fa2N-4 cell line was immortalized via lipofection-mediated transfection with a single immortalization vector. The early region of the SV40 genome, contained in the pBlueTag vector, was inserted into a backbone based upon the InvivoGen pGT60mcs plasmid and was named pTag-1. The T-antigen coding region is under the influence of a hybrid hEF1-HTLV promoter. The vector also encodes a hygromycin resistance gene as a

drug selectable marker. Clones were selected based on their ability to grow in hygromycin containing media. The Fa2N-4 cell line was established and maintained in MFE.

Example 2

Expression of Liver Specific Transcription Factors

Since retention of liver specific transcription factors is a prerequisite for expression of hepatic functions, clonal cell lines were initially screened by RT-PCR using primers for human HNF1, HNF3, HNF4α, HNF4γ and C/EBP and albumin. Briefly, total RNA was prepared from 10^6 cells of each clonal cell line using the micro-isolation method of Brenner et al. (55). Where is the information for the previous reference? 50 µg of *E. coli* rRNA (Sigma) was used as a carrier to facilitate the isolation of RNA from a small number of cells. RT-PCR reactions were carried out using the Perkin Elmer Cetus, GeneAmp RNA PCR Kit. One µg of total RNA was reverse transcribed using random hexamers and M-MLV reverse transcriptase according to the supplier's instructions. The PCR reaction was carried out using oligonucleotide primers that defined nucleotide fragments unique for each transcription factor. The primers were commercially synthesized and purified by Cruachem (Fisher Scientific). The PCR reaction was carried out for 30 cycles using an annealing temperature of 58°C for 1 min. The PCR products were visualized in a 1% agarose gel after staining with ethidium bromide. Positive control samples included RT-PCR analysis of total RNA of freshly isolated human hepatocytes (not shown). Both cell lines expressed all five hepatocyte associated transcription factors, as shown below in Table 1. Albumin production was measured as an indicator of hepatocyte specific gene expression. As shown below in Table 1, both cell lines secrete albumin into the serum free conditioned medium as detected by ELISA assay using an antibody that recognizes human albumin.

Table 1

Clones	HNF - 1	HNF – 3α	HNF – 4α	HNF – 4γ	hC/EBP	Albumin (μg/mg protein)
Fa2N-4	+	+	+	+	+	2.79
Ea1C-35	+	+	+	+	+	0.3

Example 3

SV40 Mediated Proliferative Activity

Primary human hepatocytes have limited proliferative activity when cultured. In order to overcome this characteristic, SV40 large T and small t antigens were introduced into the genome. The resulting clonal cell lines, Fa2N-4 and Ea1C-35 have subsequently been maintained in culture for up to 18 months. Both immortalized lines grow and function when maintained in MFE medium and can be cryopreserved and banked. Indirect immunofluorescent staining using polyvalent antibodies against large T antigen and albumin demonstrated that the cell lines continue to express the nuclear localized immortalizing gene (Figure 1a) as well as express a hepatocyte specific gene characteristic of differentiated function (Figure 1b). The morphology of the Ea1C-35 cell line is shown below (Figure 1c).

Example 4

Drug Metabolism Data

Both cell lines continue to catalyze Phase I (cytochrome P450) and Phase II conjugative reactions in monolayer cultures based on the metabolism of model substrates. One of the most important Phase I enzymes is CYP3A4, which is responsible for the metabolism of approximately 50% of all drugs. The expression of CYP3A4 can be modulated by many factors including multiple drug intake that may induce or inhibit the overall expression of this P450. Therefore the effective therapeutic dose of a drug is determined in part by CYP3A4 expression.

CYP3A4 modulators can be identified by monitoring the transcriptional responsiveness of the gene and by measuring enzymatic activity towards model substrates (i.e. testosterone). For example, transcriptional responsiveness to prototypical pharmacological CYP3A4 inducers (i.e. rifampin) can be assayed by the reverse transcription polymerase chain reaction (RT-PCR) using specific primers to detect CYP3A4 mRNA. Rifampin-induced CYP3A4 enzymatic activity can also be measured by the production of the 6β-OH-testosterone metabolite when cells are incubated with testosterone. As shown below in Table 2, the Fa2N-4 cell line is more sensitive to CYP inducers than the Ea1C-35 cell line.

In order to demonstrate that the cell lines continue to express Phase II conjugating enzymes, cells were exposed to acetaminophen for 24 hours and conditioned culture medium was collected and analyzed for the production of acetaminophen glucuronide or sulfate conjugates. The production of the acetaminophen glucuronide and acetaminophen sulfate conjugates was measured by HPLC analysis. The results are shown in Table 2. To determine the effect of passage number, the production of acetaminophen glucuronide and acetaminophen sulfate was measured for Fa2N-4 cells after 11, 14, 27, 32, 40, and 41 passages. For passage 41, ammonia clearance was also measured as an indicator of nitrogen metabolism. The results are shown in Table 3. These results indicate that both pathways are intact.

Table 2
Characteristics of the Fa2N-4 and Ea1C-35 cell lines

Cell line	Rifampin treated CYP3A4 (mRNA fold induction)[1]	Control (μg 6β-OH testosterone/ mg protein)[2]	Rifampin (μg 6β-OH testosterone/ mg protein)[2]	Acetaminophen glucuronide (μg/mg protein)	Acetaminophen sulfate (μg/mg protein)
Fa2N-4 (p13)	15.4	5.44	15.28	20.9	16.1
Ea1C-35 (p29)	2.2	4.53	9.25	15	21.5

[1]Cells were exposed to vehicle or rifampin for 72 hours. Data is expressed relative to vehicle treated controls.

[2]Cells were exposed to vehicle or rifampin for 72 hours and then incubated with testosterone for 24 hours. Production of the 6β-OH-testosterone metabolite was quantitated by HPLC analysis and data is expressed per mg total cell protein.

Table 3

Effect of Passage Number for Fa2N-4 Cells on Metabolism of Acetaminophen

Cells	Passage	Acetaminophen Glucuronide (μg/mg protein)	Acetaminophen Sulfate (μg/mg protein)	Ammonia Clearance (mg NH_3/mg protein/24hr)
Fa2N-4	11	15.18 ± 0.74	30.26 ± 0.31	
	14	16.43 ± 1.26	29.87 ± 1.83	
	27	7.93 ± 2.37	27.48 ± 2.2	
	32	10.42 ± 1.45	25.37 ± 0.84	
	40	12.68 ± 2.76	25.25 ± 1.99	
	41	21.4 ± 4.5	36.6 ± 1.2	246 ± 5.87

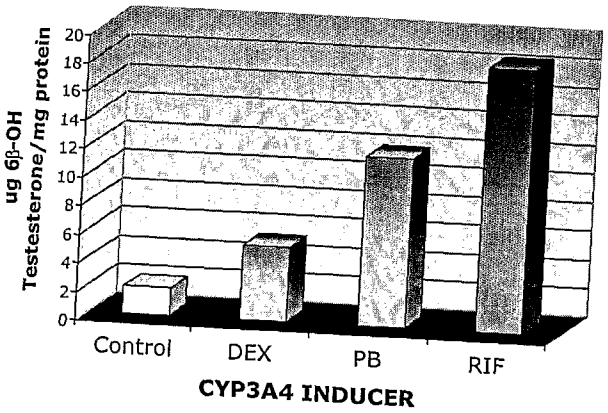
Example 5

Use of Immortalized Hepatocytes to Identify and Rank CYP Inducers

Two lines of evidence indicate that immortalized human hepatocytes can be employed to identify and rank CYP3A4 inducers based on 'induction potency". First, exposing Fa2N-4 cells to rifampin (10 μM) results in a greater production of the 6-β–OH–testosterone metabolite than treating cells with weaker CYP3A4 inducers such as dexamethasone (50 μM) or phenobarbital (1 mM), as shown below in Table 4. Secondly, immunoblot analysis demonstrated that exposure of each of the cell lines to rifampin or phenobarbital for 48-72 hours increased expression of CYP3A4 protein in comparison to vehicle-treated controls; however, exposure to rifampin resulted in a greater increase expression of CYP3A4 protein. This data is shown in Figure 2.

Table 4

Inducibility of testosterone metabolism after treating Fa2N-4 cells with different CYP3A4 inducers



Example 6

Expression of Plasma Proteins by Fa2N-4 and Ea1C-35 Cell Lines

The utilization of immortalized human hepatocytes as protein biofactories requires that the cell lines continue to proliferate and secrete plasma proteins when maintained in mass culture. In order to address this question, the expression of plasma proteins by these cell lines was analyzed. The well-differentiated nature of these cell lines is further supported by their continued secretion of adult hepatocyte function specific plasma proteins (Figure 3). Culture medium was harvested from Fa2N-4 and Ea1C-35 cells seeded into either 60 mm plates or roller bottles and analyzed by Western blot analysis. Medium was concentrated 50X by ultrafiltration and 40 μg of total protein was loaded per lane except for albumin (10 μg total protein/lane). Blots were incubated with either monoclonal or affinity purified polyclonal antibodies against albumin, α–1-antitrypsin, Factor VIII and Factor IX and visualized using secondary antibodies conjugated to horseradish peroxidase followed by incubation with DAB substrate. As shown below in Figure 3, both cell lines continue to express albumin, α-1-antitrypsin, and Factor IX. The expression of Factor VIII was variable and highly dependent on cell line and culture conditions. There was

heterogeneity in the processing of Factor IX, an observation also seen in the human plasma-derived protein.

As a confirmation, Fa2N-4 cells were grown to confluence in T-150 flasks in serum free medium and albumin production was measured by an ELISA assay. The results are shown in Table 4. The results indicate that production of albumin persists through at least 41 passages of the cells.

Table 4

Production of Albumin by Fa2N-4 Cells at Different Passage Numbers

Cells	Passage	Albumin in Media (μg/ml)
Fa2N-4	13	3.73 ± 0.64
	16	3.06 ± 0.11
	33	9.5 ± 0.5
	41	6.17 ± 0.29

Inter-α-inhibitor proteins (IαIp), natural serine protease inhibitors found in relatively high concentration in plasma have been shown to play roles in inflammation, wound healing and cancer metastasis reviewed by Bost et al.[19]. IαIp is a family of plasma proteins made and secreted by hepatocytes. The major forms of IαIp are inter-α-inhibitor (IαI, containing one light chain peptide called bikunin and two heavy chains) and pre-a-inhibitor (PαI, containing one light and one heavy chain). Recently, a monoclonal antibody that recognizes the light chain of human IαIp (MAb 69.31) was developed by scientists at Prothera Biologics. Using MAb 69.31 in a competitive ELISA, these investigators demonstrated that plasma IαIp levels were significantly decreased in severe septic patients compared to healthy controls (Lim YP, Bendelja K, Opal SM, Siryaporn E, Hixson DC, Palardy JE. *Correlation Between Mortality and the Levels of Inter-alpha Inhibitors in Plasma of Severely Septic Patients.* Journal of Infectious Disease, 188:919-926, 2003).

Western blot analysis, using MAb 69.31 revealed that both the Fa2N-4 and Ea1C-35 cell lines continue to synthesize immunoreactive IαIp (data not shown). Subsequently,

the amount of IαIp secreted into the condition medium was quantitated using an ELISA assay (see example below).

Example 7

Quantitative ELISA and Trypsin Inhibition Assay

The total trypsin inhibitory activity of the conditioned media includes the activity from the major serine protease inhibitors, α-1-antitrypsin and IαIp. IαIp can be functionally and quantitatively measured using 1) an *in vitro* assay that measures inhibition of proteases such as trypsin and 2) a competitive ELISA using MAb 69.31, a monoclonal antibody from Prothera Biologics (providence, Rhode Island) that specifically recognizes human IαIp), respectively. The trypsin inhibition assay is used to examine the biological activity of serine protease inhibitors by using the chromogenic trypsin substrate L-BAPA (N(alpha)-Benzoyl-L-arginine-4-nitroanilide hydrochloride, Fluka Chemicals). The assay is based on the ability of serine protease inhibitors to inhibit the hydrolysis of L-BAPA. Inhibition can be monitored by a decrease in the rate of Δ absorbance/minute at 410 nm. The specific activity was calculated based on the biological activity per ug protein. Conditioned media of both hepatocyte cell lines Fa2N4 and Ea1C35 were collected and concentrated 50x by using Amicon Ultra ultrafiltration device with 30 kD cut off (Millipore). The total trypsin inhibitory activity of the conditioned media includes the activity from both major serine protease inhibitors, α-1-antitrypsin and IαIp which were present in the hepatocyte conditioned media as detected by western blot (see above) and ELISA assay (see below). The amount of IαIp in the media was also measured in the competitive ELISA. The ELISA were performed as follows: 96 well Immunolon-4 plates (Dynex, USA) were coated with purified IαIp (300 ng) in 50 mM carbonate buffer pH 9.6 and incubated overnight at 4°C. A serial dilution of purified human plasma derived IαIp in PBS containing 1% rat serum was used to establish a standard curve. For the quantitative analysis of IαIp levels in culture media, 50 μL of media or serially diluted IαIp were added to individual wells of a 96 well plate. After the addition of 50 μL of MAb 69.31 to each well, plates were incubated for 1 hr at 37°C and subsequently washed using an automated plate washer (Labsystem). The bound MAb 69.31 was detected by adding HRP-conjugated

goat anti-mouse IgG (human absorbed) (Biosource, Camarillo, CA, USA) for 1 hr at 37°C. After washing, 100 µL 1-Step ABTS (Pierce, Rockford, IL, USA) was added to the wells and the absorbance at 405 nm was measured on ELISA plate reader (BioTek). Each sample was tested in triplicate. Unconditioned culture media was used as baseline control. The results are shown in Table 5:

Table 5

Cultured media	Protein Conc. after 50x ultrafiltration (UF) [mg/mL]	Trypsin Inhibition Activity after UF [TIU/mg]	IαIp conc. after UF [µg/mL]
Ea1C35	4.50	115.0	20.08
Fa2N4	9.02	45.10	4.03

Example 8

Enzyme Induction in Fa2N-4 and Ea1C-35 Cell Lines

Induction of cytochrome P450 (CYP) and related drug metabolizing enzymes (including transporters) is a well recognized cause of clinically significant drug interactions, as well as a cause of pharmacokinetic tolerance or auto-induction (the process whereby a drug induces its own hepatic metabolism) (1,2). Recent evidence implicates enzyme induction as an important determinant of certain types of drug-induced hepatotoxicity (3). Guidelines for assessing enzyme induction *in vitro* have been outlined by Tucker *et al.* (4) and Bjorsson *et al.* (5). These two "consensus reports" identify primary cultures of human hepatocytes as the method of choice – the gold standard –for assessing the enyzyme-inducing potential of new chemical entities (NCE's) and drug candidates. This *in vitro* approach, based on a human-derived test system, is superior to an *in vivo* approach based on tests in laboratory animals because drugs are known to cause enzyme induction in a species-specific manner (1). In fact, the two prototypical inducers used in the studies described later in this Example, namely omeprazole and rifampin,are efficacious inducers of human CYP1A2 and CYP3A4 and yet they do not induce the corresponding enzymes in rats or mice. The basic procedures for conducting enzyme induction studies in

primary cultures of human hepatocytes and Fa2N-4 cells are shown in the flowchart of Figure 4.

Following their attachment to collagen, hepatocytes are cultured for two days, in accordance with the recommendations of the consensus reports (4,5). During this so-called adaptation period, the hepatocytes restore their normal hepatocellular morphology and function. Prior to this redifferentiation, the hepatocytes are refractory to the enzyme-inducing effects of drugs. Hepatocytes are treated with test articles and negative and positive controls (*i.e.,* solvent control and prototypical inducers of human CYP enzymes) once daily for three consecutive days. Enzyme induction is assessed 24 hours after the last treatment by a variety of techniques, including mRNA analysis, western immunoblotting and/or measurement of enzyme activity either in the hepatocytes themselves or, preferably, in microsomes prepared from the hepatocytes. The latter approach permits an *in vitro-ex vivo* comparison between microsomes isolated from hepatocytes (*in vitro*) and microsomes isolated directly from human liver (*ex vivo*). Measurement of enzyme activity is the end-point advocated in both consensus reports (4,5).

The procedure for assessing enzyme induction in Fa2N-4 cells is remarkably similar to that described for human hepatocytes, as illustrated in Fig. 4. The Fa2N-4 cells are propagated on a collagen substratum in a proprietary medium developed by MCT. This medium, called **MFE Support Medium F** (formerly known as Multi-Function Enhancing (MFE) medium) is available from XenoTech. The cells are detached by trypsinization, isolated by centrifugation, and re-attached to collagen in the desired format (*e.g.,* 6-, 12-, 24- or 96-well plates). After a two-day adaptation period, the cells are treated once daily for three consecutive days with test article or the appropriate negative and positive controls (*i.e.,* solvent controls and prototypical inducers of human CYP enzymes). Enzyme induction is assessed 24 hours after the last treatment.

Morphologically, Fa2N-4 cells closely resemble human hepatocytes, as shown in Fig. 5. This is significant because normal hepatocellular morphology is intimately linked with normal hepatocellular function; both reflect the expression of highly differentiated properties of hepatocytes (7-9).

The consensus reports (4,5) recommend that assessment of enzyme induction utilize measurements of enzyme activity, rather than measurments of mRNA or immunoreactive protein levels, although these latter end-points often provide valuable information about the mechanism of induction. For example, they can also reveal induction by compounds that inhibit CYP activity so strongly that their inhibitory effect masks their inductive effect, as recently reported for ritonavir, which is both a CYP3A4 inhibitor and inducer (13). This issue will be discussed later in the section on *Chemical specificity of enzyme induction in Fa2N-4 cells.*

When conducting enzyme induction studies in human hepatocytes, XenoTech measures enzyme activity in microsomes. CYP activity measured in microsomes prepared from the cultured hepatocytes (*in vitro* activity) can be compared with that measured in microsomes prepared directly from human livers (*ex vivo* activity). Such comparisons provide compelling evidence that XenoTech's technique for culturing human hepatocytes support CYP enzyme activities that are comparable to those present in hepatocytes *in vivo*.

In order to prepare microsomes for such analysis, XenoTech cultures human hepatocytes in large (60 mm) dishes, which generally restricts such studies to an analysis of drug candidates that are fairly well advanced in the drug development process. Fa2N-4 cells provide the capability of analyzing the enzyme-inducing properties of larger numbers of new chemical entities (NCEs), allowing an assessment of enzyme-inducing potential in preclinical drug development or discovery. With this goal in mind, XenoTech has focused on measuring enzyme induction in Fa2N-4 cells in a variety of higher throughput formats, including 6-, 12-, 24- and 96-well plates. When cells are cultured under such conditions, it is impractical to prepare microsomes, hence, an assessment of induction based on measurements of enzyme activity must involve adding marker substrates to the Fa2N-4 cells.

XenoTech assesses enzyme induction in Fa2N-4 cells by incubating the cells with phenacetin (to measure CYP1A2), bupropion (to measure CYP2B6), diclofenac (to measure CYP2C9) or midazolam (to measure CYP3A4). In each case, the final concentration of substrate is 100 μM. Metabolite formation is determined by assaying aliquots of the cell culture medium at various times (up to 8 hours) by LC/MS/MS. To

facilitate a comparison of different CYP activities under a variety of conditions, the results are expressed relative to control activity determined at the 8-hour time point.

Fa2N-4 cells respond appropriately to enzyme inducers. As in the case of human hepatocytes, CYP1A2 is highly inducible by those agents that activate the Ah receptor, whereas those agents that activate PXR and/or CAR cause induction of CYP3A4 and, to a lesser extent, CYP2B6 and CYP2C9. As shown in Fig. 6, treatment of Fa2N-4 cells with 100 μM omeprazole causes marked induction of CYP1A2 activity, whereas treatment with 20 μM rifampin induces CYP3A4 and, to a lesser extent, CYP2B6 and CYP2C9 activity. For the experiment depicted in Fig. 6, the Fa2N-4 cells were cultured in 6-well plates.

Enzyme induction in Fa2N-4 cells is reproducible from one experiment to the next, and across different sized multi-well plates. Fig. 7 depicts the results of a comparison of the reproducibility of induction of CYP2B6 (bupropion hydroxylase) activity by rifampin, across three different plate formats. In addition, reproducibility of CYP1A2 and CYP3A4 induction across multiple cell passages was assessed, and those results are shown in Fig. 8. The reproducibility in magnitude of induction across passages 32-47 is excellent for both CYP enzymes, and is superior to the reproducibility of induction typically seen with individual preparations of human hepatocytes. Preparations of human hepatocyte from different individuals can demonstrate enormous variability in magnitude of induction, under identical conditions. Thus, reproducibility of induction of multiple passages of the Fa2N-4 cells is markedly superior to reproducibility with the same, or different, fresh human hepatocyte preparations.

It is not widely known, but enzyme induction in human hepatocytes is affected by the format of the cell culture system, such that the magnitude of induction tends to decline and become less reproducible as well size decreases. This feature (together with the limited and erratic supply of human liver) complicates the use of human hepatocytes for higher throughput screening in a 96-well format. This complication does not occur with Fa2N-4 cells.

Enzyme induction in Fa2N-4 cells was assessed in 6-, 12-, 24-and 96-well plates. Induction of CYP2B6 by rifampin is the same in 6-, 12- and 24-well plates, as shown in Fig. 9 (studies in 96-well plates are in progress). Identical results were obtained with

CYP2C9 (results not shown). Fig. 10 shows the effect of cell culture format on the induction of CYP1A2 by omeprazole and the induction of CYP3A4 by rifampin. Cell culture format appears to influence CYP1A2 induction, in that the magnitude of induction was greater in a 6-well than in a 12-, 24- or 96-well format. However, in all cases, omeprazole induced CYP1A2 activity at least 9 fold over control. (Note: CYP1A2 induction in the 96-well plate was probably greater than 9.3 fold because, in this case, phenacetin *O*-dealkylation was measured after one hour, which is not optimal for measuring CYP1A2 activity, as shown in Fig. 6.) In the case of CYP3A4, induction by rifampin is similar in the 6-, 12-, 24- and 96-well format (Fig. 10).

Overall, the results in Figs. 9 and 10 indicate that enzyme induction in Fa2N-4 cells can be assessed in a variety of cell culture formats, including 96-well plates, which bodes well for higher throughput screening of enzyme inducers.

The induction of CYP enzyme activity in Fa2N-4 follows an appropriate and anticipated time course. To achieve maximum induction of CYP enzymes, human hepatocytes are treated for three to five consecutive days with test articles and prototypical inducers (positive controls), as recommended in the consensus reports (4,5). The time course of CYP1A2 and CYP3A4 induction in Fa2N-4 cells is shown in Fig. 11. The results are similar to those observed in primary cultures of human hepatocytes (12).

Enzyme induction in Fa2N-4 cells occurs over an appropriate range of inducer concentrations. The concentration-response curves for CYP1A2 induction by omeprazole and for CYP3A4 induction by rifampin in Fa2N-4 are shown in Fig. 12. Similar results are observed in human hepatocytes (12,13). For studies with human hepatocytes, 100 μM omeprazole and 20 μM rifampin can be routinely used to achieve maximum induction of CYP1A2 and CY3A4, respectively. These same concentrations are recommended for induction studies in Fa2N-4 cells. In the case of rifampin, concentrations above 20 μM caused less than maximum induction in Fa2N-4 cells. A similar phenomenon has been observed in some preparations of human hepatocytes (13) but not others (12).

Fa2N-4 cells respond appropriately to those compounds that do and that do not induce CYP enzymes in human hepatocytes. For example, compounds shown previously to activate PXR and induce CYP3A4 in human hepatocytes (13) induce CYP3A4 activity in

Fa2N-4 cells, whereas Ah receptor agonists do not, as shown in Fig. 13. An exception is clotrimazole, which is both a CYP3A4 inducer of enzyme biosynthesis and inhibitor of enzyme activity. In this case, induction of CYP3A4 was masked by the inhibitory effect of clotrimazole (which is consistent with clinical observation). The results obtained with clotrimazole in Fa2N-4 cells are reminiscent of those observed in human hepatocytes treated with ritonavir, which is also a CYP3A4 inhibitor and inducer (13). When compounds function as both inhibitor and inducer, it is helpful to assess enzyme induction by measuring both enzyme activity and either mRNA or immunoreactive protein levels. This lesson applies to human hepatocytes as well as Fa2N-4 cells.

Investigators at Pfizer (Mills *et al.* [14]) and Hoffmann-La Roche (Morris *et al.* [15]) have examined enzyme induction in Fa2N-4 cells based largely on measurements of mRNA levels, which were determined by the Invader© cleavase assay (14) or by TaqMan RT-PCR (15). In both studies, induction of mRNA encoding CYP1A2, CYP2C9, CYP3A4 and P-glycoprotein (MDR-1) was measured in Fa2N-4 cells treated with a variety of inducers (rifampin, phenobarbital, dexamethasone, clotrimazole, β-naphthoflavone and chrysin in the case of Mills *et al.* and rifampin, phenobarbital and omeprazole in the case of Morris *et al.*) in 6-and 24-well plates (14) or in 6- and 96-well plates (15). Mills *et al.* (14) reported that β-naphthoflavone (10 μM) induces CYP1A2 mRNA up to 6 fold; rifampin (20 μM) induces CYP3A4 and CYP2C9 mRNA by up to 15 and 3 fold, respectively, and phenobarbital (1 mM) induces 3A4 and CYP2C9 mRNA by 12 and 2.5-fold, respectively. Slightly less induction was observed in 24-well plates compared with 6-well plates. For example, rifampin induced CYP3A4 mRNA 15 fold in 6-well plates *versus* 9 fold in 24-well plates. In general, the results described in this report resemble those reported by Mills *et al.* (14), although a comparison of the two studies suggests that, in the case of CYP3A4, induction at the mRNA level is greater than induction at the level of enzyme activity, whereas the converse appears to be true in the case of CYP1A2. However, comparing the results of studies conducted in different laboratories is not straightforward, especially when different end-points are measured. In the study by Morris *et al.* (15), induction of CYP2C9 and CYP3A4 was measured at both the activity and mRNA levels in a 96-well format. In the case of CYP3A4, mRNA levels increased more

than activity (*e.g.*, 10 μM rifampin increased mRNA levels 11 fold *versus* a 7.7-fold increase in CYP activity) whereas the opposite was observed in the case of CYP2C9 (mRNA increased 1.4 fold *versus* a 2.6 fold increase in CYP2C9 activity). Morris *et al.* (15) also demonstrated that induction of CYP3A was due to induction of CYP3A4, not CYP3A5 (a predominantly kidney form) or CYP3A7 (a predominantly fetal form), which supports the contention that Fa2N-4 cells behave like well differentiated adult hepatocytes. Like Mills *et al.* (14), Morris *et al.* (15) demonstrated that treatment of Fa2N-4 cells with rifampin (or high concentrations of phenobarbital) causes a ~2-fold increase in the mRNA encoding the transporters P-glycoprotein (MDR-1) and MRP2. Overall, there is excellent agreement between the studies conducted at XenoTech and those conducted by Mills *et al.* at Pfizer (14) and Morris *et al.* at Hoffmann-La Roche (15).

Table 6 summarizes the magnitude of induction of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 in Fa2N-4 cells and primary cultures of human hepatocytes. The latter data are from XenoTech's recent publication (Madan *et al.*, 2003). In the case of CYP1A2, the magnitude of induction in Fa2N-4 cells was greater than the average fold induction in human hepatocytes. In the case of CYP2B6, CYP2C9 and CYP3A4, the magnitude of induction in Fa2N-4 cells was comparable to the median fold induction in human hepatocytes, but less than the average fold induction. Median induction differs considerably from mean induction in human hepatocytes because the latter is markedly affected by the occasional samples with extremely high values of fold induction. This is illustrated in Fig. 14 for CYP3A4 induction, which ranges from zero (less than 1.5 fold) to 145 fold.

Table 6

Comparison of CYP Enzyme Induction in Fa2N-4 Cells and Human Hepatocytes

Enzyme (Inducer)	**Fa2N-4** Average induction (range)	**Human hepatocytes *** Average induction (range)	**Human hepatocytes *** Median induction
CYP1A2 (Omeprazole or BNF) **	20 fold (9.3 – 29)	13 fold (2 – 56)	8.4 fold
CYP2B6 (Rifampin)	2.5 fold (2.0 – 3.9)	4.1 or 13 fold *** (up to 14 or 71)	2.9 or 8.5 fold
CYP2C9	2.0 fold	3.5 fold	3.1 fold

(Rifampin)	(1.6 – 2.8)	(1.5 – 10)	
CYP3A4 (Rifampin)	5.1 fold (4.0 – 6.9)	10 fold (0 – 145)	3.8 fold

* Data from Maden et al., *Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes*, Drug Metab. Dispos. 31: 421-431, 2003.

** BNF (β-naphthoflavone) was the inducer for human hepatocytes, whereas omeprazole was the inducer for Fa2N-4 cells.

*** CYP2B6 activity based on 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation (4 fold) or S-mephenytoin *N*-demethylation (13 fold).

Figure 15 also shows the effect of enzyme inducers on CYP1A2 and CYP3A4 activity in Fa2N-4 cells. The left panel shows the fold induction for CYP1A2, which catalyzes phenacetin *O*-dealkylation. The compounds tested were omiprazole, 3-methylcholanthrene, lanoprazole, 1,2-benzanthracene, β-naphthoflavone, resveratrol, probenecid, benzo[a]pyrene, oltipraz, phenytoin, and benzo[e]pyrene. The right panel shows the fold induction for CYP3A4, which catalyzes midazolam 1′-hydroxylation. The compounds tested were rifampin, dexamethasone, hyperforin, phenobarbital, sulfinpyrazone, ciglitazone, phenytoin, efavirenz, troleandomycin, simvastatin, vitamin D3, probenecid, troglitazone, carbemazepine, tamoxifen, omeprazole, fexofenadine, 3-methylcholanthrene, and clotrimazole.

Although the mean fold induction of CYP3A4 in human hepatocytes is 10 fold, the median induction, which is a more meaningful comparator, is about 4 fold.

Fa2N-4 cells in culture are morphologically and functionally similar to primary cultures of human hepatocytes. The response of this cell line to enzyme inducers closely resembles that observed in human hepatocytes, which are considered the *in vitro* system of choice — the gold standard — for assessing the enzyme-inducing potential of drug candidates. Fa2N-4 cells offer a number of advantages over human hepatocytes; some of which make Fa2N-4 cells a promising *in vitro* test system for higher throughput screening of new chemical entities. In contrast to human liver, the supply of which is limited and erratic, Fa2N-4 cells are available in unlimited supply. Induction of CYP enzyme activity in Fa2N-4 cells is more reproducible than that in human hepatocytes. Furthermore, CYP induction in Fa2N-4 cells can be measured in a variety of cell culture formats, including 96-well plates, whereas this is not always possible with human hepatocytes. Primary

cultures of human hepatocytes are currently acknowledged by regulatory agencies as being an appropriate *in vitro* test system for assessing the enzyme-inducing potential of drug candidates, provided the studies are conducted in accordance with recommendations outlined in the consensus reports (4,5). The Fa2N-4 cell line is a new cell line with unique properties. As such, it is not approved by regulatory agencies, but the similarity between Fa2N-4 cells and primary cultures of human hepatocytes suggests that such approval is a future possibility.

This report focuses on enzyme induction in the Fa2N-4 cell line. Studies at Pfizer by Mills *et al.* (14) suggest that the Ea1C-35 cell line can also be used for enzyme induction studies, although the Ea1C-35 have higher basal CYP enzyme activity, which may blunt the magnitude of induction. The recent study by Morris *et al.* at Hoffmann-La Roche supports this observation (15). Both cell lines can be used to examine compounds for their ability to cause cellular toxicity. In fact, it is desirable to include one or two tests of cellular toxicity (*e.g.*, enzyme leakage to assess membrane integrity and Alamar blue reduction to assess mitochondrial respiration) so that a true lack of enzyme induction can be distinguished from a failure of enzyme induction to occur due to cellular toxicity.

Drug-induced liver toxicity is an important clinical problem, and several drugs have been withdrawn from the market because of their ability to cause rare but severe (even lethal) cases of hepatotoxicity. XenoTech scientists have completed a preliminary analysis of the response of the Fa2N-4 cells to different concentrations of known toxicants and non-toxicants, using the endpoints of compromised membrane integrity, determined by release of intercellular proteins (αGST or LDH), and perturbation of mitochondrial respiration.

Results on the use of the immortalized hepatocytes in toxicity studies are shown in Figure 16. Treatment of cells with toxic concentrations (up to 100 μM) of several agents, namely 3-methylcholanthrene, methotrexate, menadione, rotenone, and troglitazone, caused a loss of membrane integrity, resulting in the release into the medium of an intracellular enzyme, namely α-glutathione S-transferase (α-GST), which was measured with Biotrin High Sensitivity Alpha GST EIA (Biotrin International, Dublin, Ireland). In contrast, little or no α-GST was released from Fa2N-4 cells treated with non-toxic

concentrations of omeprazole, acetaminophen, probenecid, felbamate, or rifampin. It should be noted that some of these agents, such as acetaminophen, can cause clinically significant liver toxicity, but only at high doses (and hence at much higher concentrations than those used in the study depicted in Figure 16.) The toxicity data is also summarized in Table 7.

Table 7
Comparison of the Toxicity of 22 Compounds in Fa2N-4 Cells and Primary Human Hepatocytes

Cellular Response	Non-toxic Compound	Toxic Compound
Same	Rifampin Phenobarbital Phenytoin Carbamazepine Troleandomycin Lansoprazole Omeprazole Probenicid Felbamate Acetaminophen Ciglitazone Sulfinpyrazone Simvastatin Fexofenadine	3-Methylcholanthrene Methotrexate Rotenone Efavirenz
Different		Troglitazone * Benzo[a]pyrene * Hyperforin * Menadione **

* Fa2N-4 cells more sensitive than human hepatocytes
** Fa2N-4 cells less sensitive than human hepatocytes

The Fa2N-4 cells may offer a superior alternative to other systems for identifying compounds with a high potential to cause clinically significant hepatotoxicity.

As of this writing, XenoTech has detected CYP1A2, 2B6, 2C9and 3A4 activity in Fa2N-4 cells, and even greater activity is present in Ea1C-35 cells. The cell lines have been shown by MCT to conjugate acetaminophen with glucuronic acid. These findings suggest that one or both cell lines may be useful in assessing the metabolic stability of drug candidates. However, the basal metabolic rate of both lines is sufficiently low that we are

unable to currently recommend their use in studies of metabolic stability. We are researching different approaches with theFa2N-4 or Ea1C-35 cells, which may result in availability of immortalized hepatocytes capable of properly supporting metabolic stability studies.

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Example 9

Induction of Drug Metabolism Enzymes and MDR1 Using the Cell Line Fa2N-4

Drug metabolizing enzymes, including cytochrome P450s (CYPs), and transporters are involved in the clearance of drugs. The CYPs carry out various drug metabolism reactions, including oxidations and hydroxylations. Drug-drug interactions involving drug metabolizing enzymes and transporters are of increasing interest due to reports of adverse reactions and loss of efficacy (Baciewicz et al., 1987; Spina et al., 1996). During drug development, in vitro assays can be used to avoid inducers, and characterize drug-drug interaction potential due to increased drug clearance by the liver. CYPs are involved in the metabolism of drugs, primarily in the liver. Induction of CYP3A gene expression is caused by a variety of marketed drugs including rifampin, phenobarbital, clotrimazole, and dexamethasone (Meunier et al., 2000; Sahi et. al., 2000, Luo et al., 2002; Madan et al., 2003) and represents the basis for a number of common drug-drug interactions. CYP1A2 is inducible by 3-methylcholanthrene, beta-naphthoflavone, and tetrachlorodibenzodioxin (Li et al., 1998; Breinholt et al., 1999; Meunier et al., 2000; Madan et al., 2003). CYP2C9 can be induced by rifampin and phenobarbital, however, the magnitude of induction is less than that for CYP3A4 (Li et al., 1997; Madan et al., 2003). Inducers of the UGT1A family include rifampin, chrysin, and betanaphthoflavone (Li et al., 1997, Abid et al., 1997; Breinholt et al., 1999). The MDR1 gene product P-glycoprotein (P-gp) is an important drug efflux transporter. Inducers of P-gp include rifampin, phenobarbital, clotrimazole, and dexamethasone (Schuetz et al., 1996; Geick et al., 2001; Sahi et. al., 2003).

The pregnane X receptor (PXR) is the major determinant of CYP3A gene regulation by drugs and other xenobiotics (Lehmann et al., 1998; Bertilsson et al., 1998, Pascussi et al., 2003). In addition, PXR mediates induction of CYPs 2B6, 2C8/9, and 3A7, as well as the drug transporters MDR1, OATP-C, BSEP and MRP2 (Pascussi et al., 2003, Tirona et al., 2003). Other nuclear hormone receptors involved in induction of ADMET endpoints include glucocorticoid receptor (GR) (CYP2B6, CYP2C8/9, CYP3A4/5), constitutive androstane receptor (CAR) (UGT1A, CYP2B6, CYP3A4, and CYP2C9) and peroxisome proliferator-activated receptor (PPAR) (CYP4A) (Ferguson et al., 2002;

Pascussi et al., 2003). A cytosolic receptor, the aryl hydrocarbon (Ah) receptor, is involved in the induction of the CYP1A subfamily (Whitlock et al., 1996).

The ability to evaluate CYP induction in human hepatocytes is highly desirable because several drugs are known to induce CYP enzymes in humans but not rats, and *vice versa* (Bertilsson et al., 1998; Moore and Kliewer, 2000). For example, pregnenolone 16-alpha-carbonitrile induces CYP3A in rats but not humans, whereas rifampin is a known inducer of CYP3A in humans but not rats. Primary cultures of human hepatocytes have the distinct advantage of exhibiting species-specific induction of CYP isoforms, but are dependent on the availability of fresh cells and donor-to-donor variability. Cell lines such as HepG2, LS180, and LS174T, have been useful in studying induction of a limited subset of CYPs and drug transporters (Schuetz et al., 1996; Li et al., 1998; Geick et al., 2001), but lack adequate response for other inducible targets (Silva and Nicoll-Griffith, 2002).

Induction of drug metabolizing enzymes and drug transporters can be detected at the mRNA level (Schuetz et al., 1996; Abid et al., 1997; Li et al., 1998; Ferguson et al., 2002). The Invader® assay (Kwiatkowski et al., 1999; Eis et al, 2001) quantifies transcript expression from total RNA extracted from cultured cells. It is an isothermal detection of RNA and does not require a PCR amplification step. An overlap between oligonucleotides consisting of an upstream invasive deoxyoligonucleotide and a downstream deoxynucleotide probe are both annealed to the RNA target, followed by cleavage by a 5′ nuclease of the downstream probes. A second cleavage reaction utilizes a fluorescence resonance energy transfer (FRET) oligonucleotide that further amplifies the signal. This assay can differentiate between closely related RNA transcripts, such as in CYP subfamilies (Eis et al, 2001). For the current studies, we have used the immortalized human hepatocyte cell line Fa2N-4. We have characterized these cells by studying their drug metabolizing enzymes, both at the level of the transcript and enzyme activity. We have also studied the induction potential of the Fa2N-4 cells by treating them with a few prototypical inducers of the major drug metabolizing enzymes and monitoring changes in mRNA and enzyme activities. This Example describes the utilization of the immortalized human hepatocytes Fa2N-4 in combination with the mRNA detection Invader assay as a

potential method to predict clinical drug-drug interactions due to increase in the transcription of genes encoding drug metabolizing enzymes or transporters.

Methods

Chemicals

Phenobarbital (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine), dexamethasone (9-alpha-fluoro-16-alpha-methylprednisolone), β-naphthoflavone (5,6-benzoflavone), rifampin (3-[4-methylpiperazinyliminomethyl]rifamycin SV), clotrimazole (1-[*o*-chloro-α-,α-diphenylbenzyl]-imidazole), and 1-cyclohexyl-3-(morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate, testosterone, 6-hydroxytestosterone, 7-ethoxyresorufin, resorufin, hydrocortisone, and diclofenac were purchased from Sigma (St. Louis, MO). 4'-hydroxydiclofenac was purchased from Gentest (Bedford, MA). [$^{13}C_6$]-4'-OH-diclofenac was produced internally at Pfizer.

Induction of Fa2N-4 cells

This cell line originated from human hepatocytes isolated from a 12-year old female donor and were immortalized via transfection with the Simian virus 40 large T antigen as described above. Fa2N-4 cells (Figure 17) were obtained from MultiCell Technologies (Warwick, RI) and cultured as follows. For RNA analysis, multiwell plates were pre-coated with a rigid collagen complex composed of 2.75 mM 1-cyclohexyl-3-(morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate and 4% (v/v) Vitrogen 100 purified collagen (Cohesion, Palo Alto, CA) in sterile saline (0.9% NaCl). Excess collagen was removed prior to cell plating. For enzyme activity analysis, Biocoat type I collagen plates were used (Becton-Dickinson, Bedford, MA). Fa2N-4 cells were plated at confluency in MFE media (MultiCell Technologies, Warwick, RI) supplemented with 100 units/ml penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). Media was replaced with serum-free MFE media supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin after cell attachment (approximately 3 hours). Cells were kept in an incubator set at 37°C, 5% carbon dioxide, and 95% relative humidity. Media was replaced with fresh serum-free MFE media supplemented with 100

units/mL penicillin and 100 µg/mL streptomycin every 24 hours. Treatment of cells with drug was initiated 48 hours after plating. For RNA quantification, cells were exposed to drug for 48 hours. For enzyme activity studies, cells were exposed to drug for 72 hours. Fa2N4 cells are depicted in Figure 17. Phase contrast image of confluent Fa2N-4 cells plated in 96-well Biocoat Type I collagen plates (Becton Dickinson, Bedford, MA) in MFE media (MultiCell Technologies, Warwick, RI) at 200X magnification are shown in Figure 17.

RNA Analysis

Total RNA was extracted from cells using the mini RNeasy kit according to instructions provided by the manufacturer (Qiagen, Valencia, CA). RNA (100 ng) was analyzed using the Invader® RNA assay reagent kits according to instructions provided by the manufacturer (Third Wave Technologies, Madison, WI). Statistical analysis for increased levels of RNA in samples as compared to vehicle-treatment was conducted using the 2-sample, unpaired Student's t-Test, $p < 0.05$ indicating significant differences. Statistical analysis for increased levels of RNA to compare multiple treatments (> 2 samples) was conducted using ANOVA analysis, $p < 0.05$ indicating significant differences, for the purpose of rank ordering multiple inducers.

Enzyme Activity

CYP3A4. Activity was determined by measuring the extent of 6β-hydroxytestosterone formation from testosterone by mass spectrometry, essentially as described by Wood *et al* (1983) and Sonderfan *et al* (1987; 1988), with the following modifications: Test drugs were washed from cells by removing dosing media, replacing with fresh media, and incubating cells for 1 hour. After removing wash media, reactions were started with the addition of 250 µL MFE media containing 200 µM testosterone to the tissue culture well. At 30 minutes, aliquots were removed for analysis via HPLC or LC/MS/MS. For LC/MS/MS analysis, the aliquot was mixed with 1 volume of acetonitrile spiked with 250 ng/ml hydrocortisone. Mass spectrometry was carried out with a Perkin Elmer 200 HPLC system and a Micromass Quattro II detector. Samples were injected and

were ionized utilizing the electrospray positive ion mode in a mobile phase of 70:30 methanol: trifluoroacetic acid 0.02% (v/v) at 0.20 ml/min (isocratic) and a Keystone Aquasil C18, 100 · 2.1 mm, 5 μm particle size column.Some of the studies used an HPLC –UV assay for testosterone metabolism, as follows: 200 μl of medium was mixed with 5 μl of internal standard (IS) solution (20 μg/ml prednisolone in acetonitrile) and evaporated to approximately 50 μl. Samples (20 μl) were then injected on an Agilent 1100 HPLC system utilizing an Agilent Zorbax Eclipse XDB-C8 column (4.6 X 150 mm) with UV detection at 254 nm. Mobile phase A consisted of 10 mM ammonium phosphate in water, and mobile phase B consisted of 100 % acetonitrile. Initial conditions were 35% B for 3 min, increasing to 65% B over 2 min, then at 10 min, returning to 35% B, for a total run time of 15 min. Retention times were 2.8 min, 3.0 min, and 7.0 min for 6-β-hydroxy-testosterone, prednisolone, and testosterone, respectively. The standard curve for 6-β-hydroxytestosterone was linear from 25 ng/ml to at least 1000 ng/ml. Peak area for 6-β-hydroxy-testosterone was normalized to IS, and reported as fold-change from DMSO-treated cells.

CYP2C9. Activity was determined by measuring the extent of 4′-hydroxydiclofenac formation using the method of Leemann *et al.* (1993), modified as follows. Test drugs were washed from cells by removing dosing media, replacing with fresh media, and incubating cells for 15 minutes. After removing wash media, reactions were started with the addition of 250 μl MFE media containing 7.5 μM diclofenac to each well. Aliquots were removed at 60 minutes for LC/MS/MS analysis. Mass spectrometry was carried out with a Perkin Elmer 200 HPLC system and a Micromass Quattro II detector. Samples were injected and were ionized utilizing the electrospray positive ion mode in a mobile phase of 50:50 acetonitrile: 0.1% formic acid in water (v/v) at 0.27 ml/min (isocratic) and a Phenomenex, Synergi Max RP, 50 · 2.0 mm, 4 μm particle column.

CYP1A2. Activity was determined by measuring the extent of O-dealkylation of 7-ethoxyresorufin using the fluorometric method of Burke *et al.*(1985), with minor modifications (Rodrigues and Prough, 1991). Test drugs were washed from cells by removing dosing media, replacing with fresh media, and incubating cells for 15 minutes. After removing wash media, reactions were started with the addition of 250 μL MFE

media containing 7-ethoxyresorufin (20 μM) to each well. Aliquots were removed at 15 minutes for fluorometric analysis. Metabolites were quantified by comparing measurements to standard curves. The concentration of protein for each cell treatment was determined with Biorad DC reagents (Hercules, CA) according to instructions provided by the manufacturer, using bovine serum albumin as standard. Values were used to calculate enzyme activities as picomoles of metabolite per milligram protein per minute of incubation.

Results

Inductive response of Fa2N-4 cells to known inducers of drug metabolizing enzymes and drug transporters

As illustrated in Figure 18, Fa2N-4 cells are useful for monitoring several endpoints including CYP1A2, CYP2C9, CYP3A4, UGT1A, and MDR1 using the known inducers rifampin (induces CYP2C9, CYP3A4, UGT1A, and MDR1), phenobarbital (induces CYP2C9, CYP3A4, and MDR1), dexamethasone (induces CYP3A4 and MDR1), and β-naphthoflavone (induces CYP1A2 and UGT1A). In Figure 18, induction of CYP1A2, CYP2C9, CYP3A4, UGT1A, and MDR1 transcripts in Fa2N-4 cells is shown. Fa2N-4 cells were plated in 24-well plates and exposed to 0.1% DMSO vehicle (open bars), 10 μM rifampin (red bars), 1000 μM phenobarbital (blue bars), 50 μM dexamethasone (green bars), and 10 μM beta-naphthoflavone (black bars) for 48 hours is shown. The levels of transcripts were quantified from total RNA isolated from the treated cells. Plot represents the mean ± SD from the data of quadruplicate samples. Asterisk denotes statistically significant increase in transcript versus vehicle control treatment (Student's t-Test, $p<0.05$). CYP3A4 inducers were significantly different from each other using ANOVA analysis ($p < 0.05$). Increases in transcripts can be observed for all positive controls. In comparison to the vehicle control, CYP1A2 transcript was increased 15–fold after treatment with 10 μM β-naphthoflavone, but not significantly increased with other inducers. CYP2C9 transcript was increased 3.8–fold with 10 μM rifampin, 2.6–fold with 1 mM phenobarbital, and not induced by treatment with 50 μM dexamethasone, nor 10 μM beta-naphthoflavone. CYP3A4 transcript was increased 17–fold with 10 μM rifampin, 9.2–

fold with 1 mM phenobarbital, and 1.3–fold with 50 μM dexamethasone. UGT1A transcript was increased 2.1–fold with 10 μM beta-naphthoflavone, and not induced by treatment with 1 mM phenobarbital, nor 50 μM dexamethasone. Rifampin induction of UGT1A was not statistically significant ($p = 0.08$). MDR1 transcript was increased 3.1–fold with 10 μM rifampin, 2.3–fold induction with 1 mM phenobarbital, 1.3–fold induction with 50 μM dexamethasone, and there was no MDR1 induction by 10 μM β-naphthoflavone. Table 8 summarizes the induction data in Fa2N-4 cells for three CYPs expressed as fold-increase in mRNA compared to published data in primary hepatocytes.

Table 8

Summary of Reported Inductive Response in Fa2N-4 Cells as Compared to Response of Primary Human Hepatocytes

Parameter	Inducer	Fa2N-4 cells Fold-increase	Primary cells Fold-increase
CYP1A2	B-Naphthoflavone	1.5	13
CYP2C9	Rifampin	3.8	3.5
	Phenobarbital	2.6	1.8
CYP3A4	Rifampin	17	10
	Phenobarbitol	9.3	3.3

CYP enzyme activity in Fa2N-4 cells

CYP3A4 activity increased 8.9-fold and 2.1–fold, as assessed by increases in formation of the 6-β-hydroxytestosterone with 10 μM rifampin and 50 μM dexamethasone, respectively, as compared to vehicle-treated control (Figure 19A). In Figure 19, measurement of induction by cytochrome-450 enzyme activity is shown. Induction of CYP3A4, CYP2C9, and CYP1A2 enzyme activity in Fa2N-4 cells after 72 hour exposure to 0.1% DMSO vehicle (VEH) or inducer is shown. Study was conducted using a 12-well plate format. Data represents enzyme activity in terms of metabolite formed per milligram of total Fa2N-4 protein per minute of incubation with parent compound. Data is from duplicate assays denoted by open and closed bars. **(A)** Measurement of CYP3A4 activity by formation of the testosterone metabolite 6-β-hydroxytestosterone in cells induced with

vehicle, 10 μM rifampin (RIF), and 50 μM dexamethasone (DEX). **(B)** Measurement of CYP2C9 activity by formation of the diclofenac metabolite 4'-hydroxydiclofenac in cells induced with vehicle, 10 μM rifampin (RIF), and 1000 μM phenobarbital (PB). **(C)** Measurement of CYP1A2 activity by O-dealkylation of 7-ethoxyresorufin in cells induced with vehicle or 50 μM beta-naphthoflavone (BNF). Formation of 4'-hydroxydiclofenac for assessment of CYP2C9 activity was increased approximately 2-fold for treatments with 10 μM rifampin and 1 mM phenobarbital (Figure 19B). Fold changes in the EROD assay for CYP1A2 were 27-fold with 10 μM β-naphthoflavone (Figure 19C).

In addition to examining the inductive effect of a single concentration of drug, the Fa2N-4 cells can also be used to look at dose-response relationships. For example, EC50 values were calculated based on the response of Fa2N-4 cells dosed with multiple concentrations of rifampin ranging from 100 nM to 50 μM. Figure 20 contains EC50 plots for Fa2N-4 cells using increased CYP3A4 transcript values (Figure 20A), as well as increased CYP3A4 enzyme activity (Figure 20B). In Figure 20, dose-response dependence of CYP3A4 induction by rifampin in Fa2N-4 cells is shown. Measurement of induction of CYP3A4 was performed in Fa2N-4 cells treated with 100 nM to 50 μM rifampin. Data was fitted using SigmaPlot (version 8) using a 3-parameter sigmoidal curve. **(A)** Total RNA was analyzed to determine level of CYP3A4 transcript and then compared to vehicle control to determine fold-induction. Data represents mean ± SD from the data of triplicate samples. **(B)** CYP3A4 activity was measured by formation of the testosterone metabolite 6-β-hydroxytestosterone and then compared to vehicle control to determine fold-induction. Data represents mean ± SD from the data of triplicate samples. The calculated EC50s were 0.43 μM ($r^2 = 92$) and 0.77 μM ($r^2 = 94$), for the transcript and enzyme activity, respectively. In addition, the calculated maximum induction (Imax) values were 13–fold for the transcript endpoint and 9.7–fold for the enzyme activity endpoint.

Fa2N-4 inductive response over multiple passages

Multiple passages of the Fa2N-4 cells have been tested for CYP3A4 induction. Figure 21 shows response of multiple passages of Fa2N-4 cells to a CYP3A4 inducer with a weak response (50 μM dexamethasone) and a CYP3A4 inducer that exhibits a strong

response (10 μM rifampin). In Figure 21, various passages of Fa2N-4 cells were plated in 24-well plates and exposed to 0.1% DMSO vehicle (open bars), 50 μM dexamethasone (striped bars), and 10 μM rifampin (black bars). **(A)** The levels of CYP3A4 transcripts were quantified from isolated total RNA. Plot represents the mean ± SD from the data of quadruplicate samples. **(B)** CYP3A4 activity was measured by formation of the testosterone metabolite 6-β-hydroxytestosterone. Plot represents the mean of duplicate samples. All compounds showed statistically significant increase in transcript versus vehicle control treatment (Student's t-Test, $p<0.05$). Treatment with dexamethasone increased CYP3A4 transcripts, 1.6-fold and 1.5-fold at passages 21 and 36, respectively. Treatment with 10 μM rifampin increased CYP3A4 transcripts, 17-fold and 16-fold at passages 21 and 36, respectively (Figure 21A). CYP3A4 enzyme activity was increased 2.1-fold and 2.0-fold for dexamethasone and 8.9-fold and 4.9-fold for 10 μM rifampin at passages 28 and 36, respectively (Figure 21B).

Capacity for HTS with Fa2N-4 cells and Invader® assay

Figure 22 compares various multiwell plate formats. In Figure 22, induction of CYP3A4 transcript in Fa2N-4 cells after 48 hour exposure to 10 μM rifampin (closed bars) is shown in comparison with vehicle (open bars). Data is from studies conducted in each multiwell plate format as indicated. Plot represents the mean ± SD from the data of quadruplicate samples. All compounds showed statistically significant increase in transcript versus vehicle control treatment (Student's t-Test, $p<0.05$). Regardless of the plate format, Fa2N-4 cells exhibit substantial CYP3A4 inductive response to rifampin. Fold changes in CYP3A4 transcript were 17.1-fold when using a 24-well plate, 6.6-fold when using a 24-well plate, and 5.7-fold for when using a 96-well plate.

Discussion

The Fa2N-4 cells have the ability to induce CYP1A2, CYP2C9, CYP3A4, UGT1A, and MDR1 mRNA in response to known inducers. Using CYP3A4 transcript as an endpoint, we have demonstrated the ability of the assay to rank inducers according to potency and demonstrate dose-response for rifampin as previously observed in primary

human hepatocytes (Li et al., 1997; Sahi et al., 2000). In addition to distinguishing inducers from noninducers, this assay has a wide dynamic range for some endpoints such as CYP3A4 and CYP1A2, enabling rank ordering for induction potency. The same decreasing potency for CYP3A4 inducers (rifampin > phenobarbital >dexamethasone) has been previously reported in the literature for studies in primary human hepatocytes using both mRNA and enzyme activity endpoints (Luo et al., 2002). Our results using mRNA induction in Fa2N-4 cells are in good agreement with the publication by Madan et al. (2003), who reported the effects of prototypical inducers for CYP1A2, CYP2C9, and CYP3A4 in cultured primary human hepatocytes by measuring enzyme activity in microsome preparations from treated cells. Our induction results using the immortal cell line Fa2N-4 and mRNA measurements are in good agreement with the publication by Madan et al. (2003) using primary human hepatocytes. Madan et al. used livers from several different donors and measured enzyme activity towards several CYPs, after treatment with the same prototypical inducers as in our study. The rank order for CYP3A4 induction potency for rifampin and phenobarbital expressed as fold induction over vehicle controls was the same in both studies. CYP2C9 was also induced, albeit to a lesser extent than CYP3A4, and CYP1A2 had the highest response in both systems. Another study (Sahi et al. 2000) using primary human hepatocytes reported EC50 for rifampin in CYP3A4 induction, using an activity assay. Those results are also in good agreement with our own EC50 results using the Fa2N-4 cells and mRNA measurements.

This immortal hepatocyte clone was identified in a screen of several of clones where the best response to rifampin induction of CYP3A4 was the selection criteria. Hence, the most appropriate utilization of this assay is for CYP3A4 induction. Further characterization of this clone indicated that it had high response to a CYP1A2 inducer, enabling also the detection of this endpoint in a screening format. The dynamic range of the responses to CYP2C9 and MDR1 were smaller, but they were in the same proportion as the inductive response found in fresh hepatocytes (Li et al., 1997; Madan et al. 2003; Sahi et al. 2003).

Although the average UGT1A transcript was higher in rifampin-treated Fa2N-4 cells than in vehicle-treated cells, the level of induction was not statistically significant.

Previous induction studies in primary human hepatocyte cite inter-individual variation in the effects of rifampin, using 1-naphthol glucuronidation as an endpoint. Abid *et al.* (1997) reported that the variability may be attributed to differential induction of two UGT1A isoforms . The Invader® UGT1A oligonucleotides used here span a common region in the RNA among all isoforms, and measurement of mRNA is a sum of all UGT1A isoforms. Thus, the inductive effect on rifampin could have been minimized by the non-induced UGT1A isoforms. It is likely that probes designed for individual UGT1A isoforms would be able to detect significant increases in their mRNA.

Induction in the Fa2N-4 cells is not limited to mRNA and can also be assessed at the enzyme activity level. The extent of induction using mRNA quantified with Invader® correlated well with enzyme activity data as indicated by similar rank order for several prototypical inducers. The ability to induce CYP enzyme activity provides further evidence on the expression of a comprehensive array of CYPs in the Fa2N-4 cells. In addition, it shows the potential of these cells for alternative applications, such as CYP inhibition or metabolite generation.

The Invader® assay can adequately quantify induction based on mRNA level increases. Advantages of the mRNA endpoint include increased throughput and target specificity. For enzyme activity, a separate well in a multiwell plate must be used for each enzyme activity endpoint, whereas a single well can be used to assess multiple mRNA targets. The recovery of RNA from each sample (24-well plate) is high enough to run up to 50 separate mRNA endpoints. The Invader assay is able to discriminate among closely related CYPs, whereas enzyme activity assays are not always specific. For example, *O*-dealkylation of 7-ethoxyresorufin characterizes the combination of CYP1A1 and CYP1A2, and 6-β-hydroxytestosterone can also be formed by both CYP3A4 and CYP3A5 (Williams et al., 2002).

In contrast to fresh human hepatocytes, Fa2N-4 cells are readily available. Since accessibility to fresh human hepatocytes is reliant on availability of a suitable liver tissue donor, it can take a long time to conduct experiments using hepatocytes isolated from three different livers to verify that a certain compound is an inducer. In addition, plating efficiency of fresh hepatocytes is unpredictable, so it is not uncommon to have a suitable

donor, but find that the cells are not usable due to poor plating efficiency or substandard cell health. Fa2N-4 cells can be passaged and used over several passages while retaining activity of the major drug metabolizing enzymes. With fresh human hepatocytes, cells can only be used one time, making it difficult to compare data between studies. Plateable cryopreserved primary human hepatocytes are an improvement by theoretically allowing multiple experiments at different times from a single donor, or potentially the use of multiple donors at one time. However, plateable cryopreserved primary human hepatocytes are in limited supply. Both fresh primary human hepatocytes and plateable cryopreserved primary human hepatocytes have donor-to-donor variability, based on the influence of genetics, the environment, and co-medications. There are vast differences seen in the drug metabolizing enzyme profile of donors, leading to the current recommendation of obtaining data from three donors before reaching a conclusion for induction potential of a chemical. In addition, some authors cite the necessity for potency indexes in order to compare data between donors (Silva and Nicoll-Griffith, 2002). The potency index standardizes data between donors by reporting the ratio of induction response (i.e. fold-induction) of the test compound to that of a prototypical inducer.

Thus, our preliminary data using a few prototypical inducers demonstrates that Fa2N-4 cells can be a suitable substitute for fresh human hepatocytes in induction studies, and provide the additional attribute of being amenable for higher throughput studies. Fa2N-4 cells are superior to previously published immortal cell lines, as they show induction of a varied number of genes. These cells can be used to determine the induction potential of a drug, with findings consistent with monitoring increased enzyme activity in primary human hepatocytes. Higher throughput cell culturing and analysis via mRNA endpoint enables more compounds to be tested and reduces the cost per compound; two favorable traits for drug discovery assays. This induction assay has the potential of becoming a useful tool for pharmaceutical companies to eliminate compounds with drug-drug interaction potential and to understand the likelihood and extent of DDI for compounds in development.

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Example 10

Reverse Transcription-Polymerase Chain Reaction Analysis for Expression of mRNA Transcripts After Exposure of Immortalized Hepatocyte Cell Lines to Inducers of Cytochrome P450

RT-PCR analysis was performed on the two immortalized human hepatocyte cell lines designated Ea1C-35 and Fa2N-4. Cells were plated on type I collagen coated dishes and maintained in MultiCell's proprietary media. Cultured cells were treated with rifampin (10μM) for 3 days or an equal volume of DMSO (e.g. Control).

This was done to screen for expression of hepatocyte specific transcription factors (e.g. HNF-1α, HNF-3, HNF-4 α, HNF4γ, cEBP), liver specific genes (e.g. albumin and asialoglycoprotein receptor), transcription factors controlling drug metabolizing genes (e.g. SXR, RXR α, RXRβ, CAR) and phase I and phase II drug metabolizing enzymes (e.g. CYP1A2, CYP2A6, CYP2C9, CYP3A4, CYP2D6, CYP2E1, and UGT 1A1, UGT 2B4, respectively).

Analysis was performed with and without exposure to rifampin, a known pharmacological inducer of CYP3A4 expression. RT-PCR analysis revealed that all transcripts examined were expressed by both cell lines but to various levels. Rifampin induction increased the expression of CYP3A4 transcripts.

The following primers were used for the RT-PCR analysis: Albumin, Asialoglycoprotein II receptor, HNF-1α, HNF-3, HNF-4 α, HNF4γ, c/EBP, UGT 1A1, UGT 2B4, SXR, RXR α, RXRβ, CAR, CYP1A2, CYP2A6, CYP2C9, CYP3A4, CYP2D6, CYP2E1, Cytochrome c, and NADPH. The figure legends for gels 1-4 (Figures 23-26) are given in Table 9. The figure legends for gels 5-8 (Figures 27-30) are given in Table 10.

Table 9

#1	PCR Product	Gel #1	Gel #2	Gel #3	Gel #4
#2	Ea1C-35 p17, DMSO Ctrl	UGT 1A1	SXR	HNF-1α	Albumin
#3	Ea1C-35 p17, Rifampin	UGT 1A1	SXR	HNF-1α	Albumin
#4	Fa2N-4 p34, DMSO Ctrl	UGT 1A1	SXR	HNF-1α	Albumin
#5	Fa2N-4 p34, Rifampin	UGT 1A1	SXR	HNF-1α	Albumin
#6					
#7	Ea1C-35 p17, DMSO Ctrl	UGT 2B4	RXRα	HNF-3	ASGPR II
#8	Ea1C-35 p17, Rifampin	UGT 2B4	RXRα	HNF-3	ASGPR II
#9	Fa2N-4 p34, DMSO Ctrl	UGT 2B4	RXRα	HNF-3	ASGPR II
#10	Fa2N-4 p34, Rifampin	UGT 2B4	RXRα	HNF-3	ASGPR II
#11					
#12	Ea1C-35 p17, DMSO Ctrl	CAR	RXRβ	HNF-4α	GAPDH
#13	Ea1C-35 p17, Rifampin	CAR	RXRβ	HNF-4α	GAPDH
#14	Fa2N-4 p34, DMSO Ctrl	CAR	RXRβ	HNF-4α	GAPDH
#15	Fa2N-4 p34, Rifampin	CAR	RXRβ	HNF-4α	GAPDH
#16					
#17	Ea1C-35 p17, DMSO Ctrl	c/EBP	GAPDH	HNF-4γ	
#18	Ea1C-35 p17, Rifampin	c/EBP	GAPDH	HNF-4γ	
#19	Fa2N-4 p34, DMSO Ctrl	c/EBP	GAPDH	HNF-4γ	
#20	Fa2N-4 p34, Rifampin	c/EBP	GAPDH	HNF-4γ	kit Ctrl

Table 10

	PCR Product	Gel #5	Gel #6	Gel #7	Gel #8
#1	100bp marker	5ul	5ul	5ul	5ul
#2	Ea1C-35 p17, DMSO Ctrl	CYP 3A4	CYP 2D6	GAPDH,RT(+),60°C	Kit Ctrl,61°C
#3	Ea1C-35 p17, Rifampin	CYP 3A4	CYP 2D6	GAPDH,RT(+),60°C	Kit Ctrl,60°C
#4	Fa2N-4 p34, DMSO Ctrl	CYP 3A4	CYP 2D6	GAPDH,RT(+),60°C	Kit Ctrl,59°C
#5	Fa2N-4 p34, Rifampin	CYP 3A4	CYP 2D6	GAPDH,RT(+),60°C	Kit Ctrl,60°C
#6					
#7	Ea1C-35 p17, DMSO Ctrl	CYP 2C9	CYP 2E1	GAPDH,RT(+),61°C	
#8	Ea1C-35 p17, Rifampin	CYP 2C9	CYP 2E1	GAPDH,RT(+),61°C	
#9	Fa2N-4 p34, DMSO Ctrl	CYP 2C9	CYP 2E1	GAPDH,RT(+),61°C	
#10	Fa2N-4 p34, Rifampin	CYP 2C9	CYP 2E1	GAPDH,RT(+),61°C	
#11					
#12	Ea1C-35 p17, DMSO Ctrl	CYP 1A2	Cyto c	GAPDH,RT(+),59°C	
#13	Ea1C-35 p17, Rifampin	CYP 1A2	Cyto c	GAPDH,RT(+),59°C	
#14	Fa2N-4 p34, DMSO Ctrl	CYP 1A2	Cyto c	GAPDH,RT(+),59°C	
#15	Fa2N-4 p34, Rifampin	CYP 1A2	Cyto c	GAPDH,RT(+),59°C	
#16					
#17	Ea1C-35 p17, DMSO Ctrl	CYP 2A6	NADPH	GAPDH,RT(-),60°C	
#18	Ea1C-35 p17, Rifampin	CYP 2A6	NADPH	GAPDH,RT(-),60°C	
#19	Fa2N-4 p34, DMSO Ctrl	CYP 2A6	NADPH	GAPDH,RT(-),60°C	
#20	Fa2N-4 p34, Rifampin	CYP 2A6	NADPH	GAPDH,RT(-),60°C	

Example 11

Conditions for the Expression of Plasma Proteins by Fa2N4 and Ea1C35 Cell Lines

Two-dimensional gel electrophoretic analysis was used to separate the secreted proteins of the Fa2N4 and Ea1C35 cell lines. Using Invitrogen's ZOOM IPGRunner system, the first IEF separation of the proteins was carried out using fixed pH gradient strip (pH range of 3-10) followed by the second dimension separation using 4-12% Tris-Gycine SDS-PAGE. In both cell lines multiple spots of proteins could be identified as possible candidates for therapeutic proteins. (Fig. 31A. Fa2N4; 31B, Ea1C35). After the 2-dimensional gel separation the secreted proteins of the Ea1C35 cell line were transferred

onto nitrocellulose and Western blot analysis using anti-Factor IX antibody was performed. Reactive protein with MW of 70 kD and pI 6.5-7.0 was detected (Fig. 31C).

Immunostaining of Fa2N-4 cells for albumin expression was also carried out. Cells were plated on type I collagen and cultured in serum free medium for 72hr. Albumin was visualized by indirect immunofluorescence with a fluorescein conjugated secondary antibody. As shown in Figure 1b, virtually all of the cells express albumin (e.g. green color).

In order to determine if the Ea1C-35 and Fa2N-4 cells from various passages made and secreted transferrin, the cells were cultured in serum free medium without transferring for 7 days . Conditioned culture medium was collected after 7 days and immunoblot analysis was performed using a commercially available antibody against transferrin. Human plasma was used as the positive control. Immunoblots revealed that the cells from all passages continue to express this plasma protein. The results are shown in Figure 32. The lanes for Figure 32 are shown in Table 11.

Table 11

Lane #	Samples
1	**Marker**
2	Ea1C-35 p15
3	Ea1C-35 p24
4	Ea1C-35 p29
5	Ea1C-35 p43
6	Fa2N-4 p10
7	Fa2N-4 p23
8	Fa2N-4 p31
9	Fa2N-4 p39
10	Human plasma

The economical production of therapeutic plasma proteins using cultured immortalized human hepatocytes as producer cells can only be accomplished if the cells continue to make and secrete these plasma proteins when expanded in mass culture. In order to initially evaluate this question, Fa2N-4 cells were grown to confluence in T25,

T75 and T150 culture flasks and selected plasma proteins were quantitated using ELISA assays in combination with capture antibodies that recognized albumin, AAT or IαIp. An equivalent number of cells were initially plated per square cm, 5, 15 and 30 million cells, respectively. Conditioned medium was collected for 3 days, pooled, concentrated 10x by ultrafiltration and assayed. As shown in Tables 12-14, the total expression of each plasma protein was approximately proportional to cell number. Values represent the mean ± SD for triplicate samples. Over the 3-day period cells cultured in T150 flasks produced approximately 200 ug albumin, 500 ng IαIp and 150 ng AAT.

Table 12

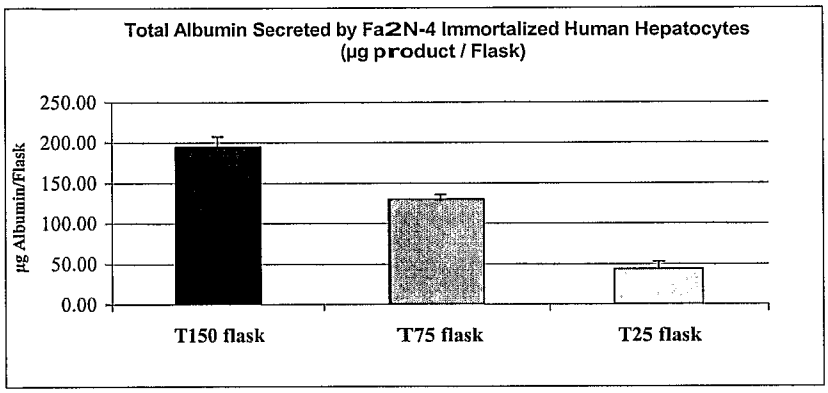


Table 13

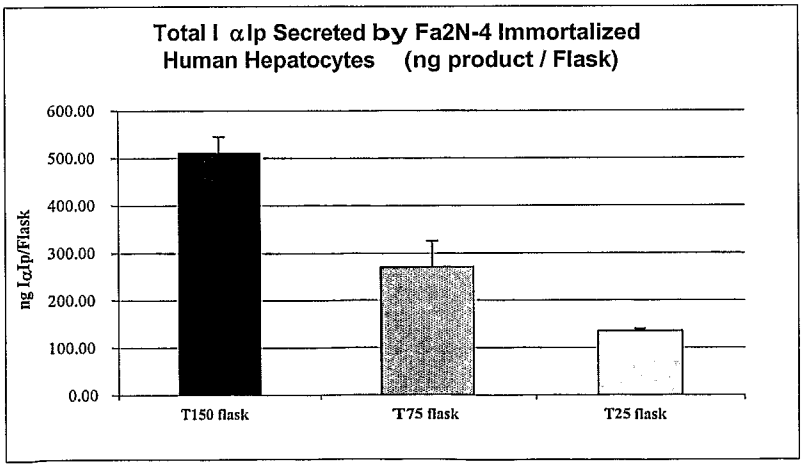
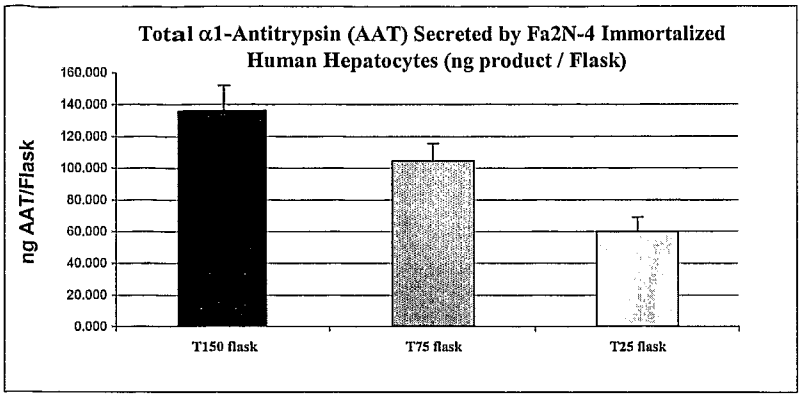
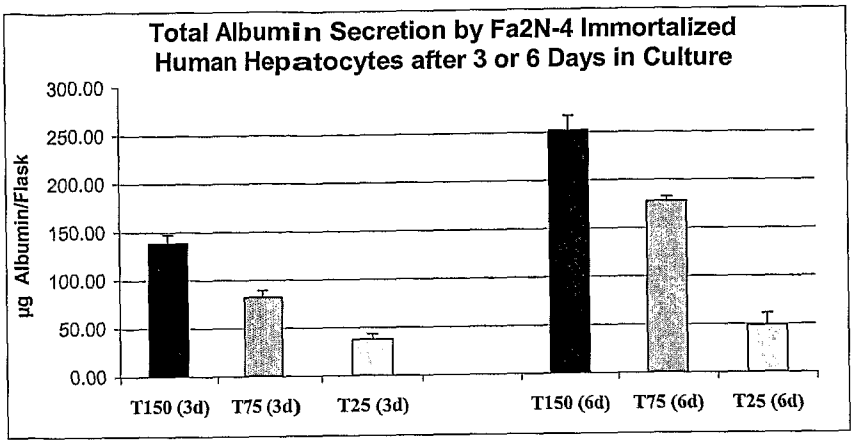


Table 14



We plan to use immortalized human hepatocytes as biofactories for the commercial production of therapeutic proteins. Therefore, it is essential that plasma protein secretion must not be significantly decreased in long-term culture. We recently initiated a study in order to evaluate this question, Fa2N-4 cells were grown in T25, T75 and T150 culture flasks as described above and albumin production was measured as an indicator of overall protein secretion. Conditioned medium was collected on Day 3, cells were refed and resampled on Day 6. Albumin secretion was analyzed by an ELISA assay. The results indicate that albumin secretion continues to increase over the 6 day collection period irrespective of the plating format (see Table 15). Of particular note, there is a dramatic increase in albumin when cells were cultured in the T75 and T150 flasks. Since total cellular protein does not significantly increase with time in culture (data not shown), it seems likely that these results are due to enhanced production as a result of adaptation to culture conditions and not the result of a dramatic increase in cell number per flask.

Table 15



Since the production of some plasma proteins can be modulated by acute phase proteins such as TNFα *in vivo* we reasoned that this cytokine might enhance the secretion of plasma proteins by immortalized human hepatocytes. In the present study, we examined the effects of TNFα on the secretion of AAT. Fa2N-4 cells were maintained in serum free proprietary MFE media containing TNFα (0, 1, 5, or 10 ng/ml) for 3 days. The results are shown in Table 16. Values are the average of duplicate samples. As shown in Table 16, the secretion of AAT was most notably increased by the inclusion of 5 ng/ml TNFα in the serum-free culture medium. Therefore, it might be possible to increase AAT production using this cytokine.

Table 16

Sample	**Concentration of Tumor Necrosis Factor Alpha**	Antitrypsin (ng)/µg Protein	Antitrypsin (ng)/ well
#1	TNF 0 ng/ml	0.21	14.00
#2	TNF 1 ng/ml	0.34	21.00
#3	TNF 5 ng/ml	0.43	44.73
#4	TNF 10 ng/ml	0.48	32.93

Albumin expression is regulated in part by a dexamethasone inducible promoter. In order to examine the effects of dexamethasone on the production and secretion of albumin by immortalized human hepatocytes, Fa2N-4 cells (passage 32) were cultured on

type I collagen dishes with or without dexamethasone in the culture medium for 48hrs and albumin expression was measured by an ELISA assay. Values represent the average of duplicate samples. As summarized in the table below (Table 17), the secretion of albumin was significantly decreased in the absence of dexamethasone.

Table 17

Concentration of Dexamethasone	Albumin (μg/ml)
0	40.0
1.0 μM	100.0

Example 12

Ability to Produce and Express Therapeutic Plasma Proteins

The ability of our Fa2N-4 cell line to correctly produce an immunologically reactive therapeutic plasma protein was illustrated with the production of immuno-reactive human growth hormone (hGH). On the day prior to transient transfection, Fa2N-4 cells were plated at a density of 0.5-0.8x10^6 cells per well in six-well Nunc plates using 10% NBCS-MFE medium. On the day of transfection the cells were washed one time to remove serum and a CMV-based plasmid, containing the complete cDNA for hGH, was transiently transfected into the Fa2N-4 cells using either an Invitrogen Lipofectamine Plus or a Qiagen Effectene transfection reagent kit. The transfections were performed as per the manufacturers' protocols.

Conditioned media was withdrawn from each well after 24 and/or 48 hours and was subsequently used for an ELISA-based immunodetection assay. The ELISA assay is a colorimetric enzyme immunoassay for the quantitative determination of secreted hGH utilizing the sandwich ELISA principle. Microtiter plate pre-bound antibodies to hGH

bind to secreted hGH contained in the conditioned media. Subsequently, a digoxigenin labeled hGH antibody binds to a second epitope of the hGH peptide contained in the conditioned media and retained on the microtiter plate. An antibody to digoxigenin, which is conjugated to peroxidase is then added and followed by the peroxidase substrate ABTS. The peroxidase-catalyzed cleavage of the substrate yields a colored reaction product that can be easily detected using a microtiter plate reader.

Our results confirm that using either transfection kit and harvesting the conditioned media at either 24 or 48 hours post-transfection, the Fa2N-4 cells produce extraordinarily large quantities of double immunodetected hGH while transfection with *LacZ* or no plasmid negative controls produced no detectable levels of hGH. A photograph of the ELISA plates 1 and 2 are shown in Figure 33 and Figure 34, respectfully. The key for Figures 33 and 34 is shown in Table 18.

Table 18

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 0	Std 80	1Q*LacZ* 2	1Q(1:10)3	1Q(1:30)1	1Neg2'	1Q(1:10)3'	1Q(1:30)1'	2Q*LacZ* 2	2Q(1:10)3	2Q(1:30)1
B	Blank	Std 0	Std 80	1Q*LacZ* 2	1Q(1:10)3	1Q(1:30)1	1Neg2'	1Q(1:10)3'	1Q(1:30)1'	2Q*LacZ* 2	2Q(1:10)3	2Q(1:30)1
C	Blank	Std 10	Std 160	1Q*LacZ* 3	1Q(1:20)1	1Q(1:30)2	1Neg3'	1Q(1:20)1'	1Q(1:30)2'	2Q*LacZ* 3	2Q(1:20)1	2Q(1:30)2
D	Blank	Std 10	Std 160	1Q*LacZ* 3	1Q(1:20)1	1Q(1:30)2	1Neg3'	1Q(1:20)1'	1Q(1:30)2'	2Q*LacZ* 3	2Q(1:20)1	2Q(1:30)2
E	Blank	Std 20	Std 320	1Q(1:10)1	1Q(1:20)2	1Q(1:30)3	1Q(1:10)1'	1Q(1:20)2'	1Q(1:30)3'	2Q(1:10)1	2Q(1:20)2	2Q(1:30)3
F	Blank	Std 20	Std 320	1Q(1:10)1	1Q(1:20)2	1Q(1:30)3	1Q(1:10)1'	1Q(1:20)2'	1Q(1:30)3'	2Q(1:10)1	2Q(1:20)2	2Q(1:30)3
G	Blank	Std 40	1Q*LacZ* 1	1Q(1:10)2	1Q(1:20)3	1Neg1'	1Q(1:10)2'	1Q(1:20)3'	2Q*LacZ* 1	2Q(1:10)2	2Q(1:20)3	2Neg1'
H	Blank	Std 40	1Q*LacZ* 1	1Q(1:10)2	1Q(1:20)3	1Neg1'	1Q(1:10)2'	1Q(1:20)3'	2Q*LacZ* 1	2Q(1:10)2	2Q(1:20)3	2Neg1'

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			2Neg3'	2Q(1:20)1'	2Q(1:30)2'	I*LacZ* 3	I(1.0)1	I(2.0)2			Blank'
B	Blank			2Neg3'	2Q(1:20)1'	2Q(1:30)2'	I*LacZ* 3	I(1.0)1	I(2.0)2			Blank'
C	Blank			2Q(1:10)1'	2Q(1:20)2'	2Q(1:30)3'	I(0.5)1	I(1.0)2	I(2.0)3			Blank'
D	Blank			2Q(1:10)1'	2Q(1:20)2'	2Q(1:30)3'	I(0.5)1	I(1.0)2	I(2.0)3			Blank'
E	Blank			2Q(1:10)2'	2Q(1:20)3'	I*LacZ* 1	I(0.5)2	I(1.0)3				Blank'
F	Blank			2Q(1:10)2'	2Q(1:20)3'	I*LacZ* 1	I(0.5)2	I(1.0)3				Blank'
G	Blank		2Neg2'	2Q(1:10)3'	2Q(1:30)1'	I*LacZ* 2	I(0.5)3	I(2.0)1				Blank'
H	Blank		2Neg2'	2Q(1:10)3'	2Q(1:30)1'	I*LacZ* 2	I(0.5)3	I(2.0)1				Blank'

Key-

Blank=Substrate
Std X=Standard of X ng/ml hGH
XQ*LacZ* Y=Sample Y obtained X days after transfection of a *LacZ* control plasmid into 0.5x10(6) cells using the Qiagen kit
XQ(1:Y)Z=Sample Z obtained X days after transfection of a 1:Y ratio of DNA:Effectene reagent into 0.5x10(6) cells using the Qiagen kit
XNegY'=Sample Y obtained X days after transfection of no DNA into 0.8x10(6) cells using the Qiagen kit
XQ(1:Y)Z'=Sample Z obtained X days after transfection of a 1:Y ratio of DNA:Effectene reagent into 0.8x10(6) cells using the Qiagen kit
I*LacZ* X=Sample X obtained one day after transfection of a *LacZ* control plasmid into 0.7x10(6) cells using the Invitrogen kit
I(X)Y=Sample Y obtained one day after transfection of Xug DNA into 0.7x10(6) cells using the Invitrogen kit
Blank'=Buffer

Example 13

Immunophehenotypic characterization of the EA1C-35 and Fa2N-4 cell lines

Both the Ea1C-35 (passage 26) and Fa2N-4 (passage 30) cell lines were phenotyped by indirect immunofluorescence analysis using a panel of antibodies against different hepatocyte or bile duct markers as well as against the SV40 immortalizing gene. The results from this analysis are summarized in the Table 19 below:

Table 19

Marker	Ea1C-35 (% positive Cells)	Fa2N-4 (% positive Cells)
Albumin	90	100
Alpha Fetoprotein	0	0
Connexin 32	50	80
CD 81	100	100
CD49f (integrin alpha 6 chain)	0	0
SV40 T-antigen	100	100

The expression of connexin 32 was density dependent. At low plating density expression of these two proteins was undetectable. However when cells grew to confluent monolayers, a subpopulation of Ea1C-35 and fa2N-4 cells express this gap junctional protein which is only expressed by hepatocytes in adult liver tissue.

All cells expressed SV40 T-antigen, the immortalizing gene in their nucleus. The well-differentiated nature of the immortalized liver cells is indicated by the strong expression of the adult hepatocyte specific lineage markers, albumin and connexin 32 and

the lack of the fetal hepatocyte marker, alpha fetoprotein. The cells do not express CD49f, a bile duct marker. The cells express CD81, the putative receptor for hepatitis C virus glycoprotein-mediated viral infection. A photomicrograph of Fa2N-4 cells immunstained for CD81 is shown in Figure 35. Note that expression of CD81 is localized to the plasma membrane.

Taken together, all the above examples strongly indicate that the two immortalized human hepatocyte cell lines maintain many functional attributes characteristic of hepatocytes *in vivo* and are an invaluable *in vitro* system to produce plasma proteins, including therapeutic plasma proteins.

The inventions illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the future shown and described or any portion thereof, and it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions herein disclosed can be resorted by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the scope of the generic disclosure also form part of these inventions. This includes the generic description of each invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised materials specifically resided therein.

In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. It is also to be understood that the above description is intended to be illustrative

and not restrictive. Many embodiments will be apparent to those of in the art upon reviewing the above description. The scope of the invention should therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent publications, are incorporated herein by reference.

We claim:

1. A method of using immortalized human hepatocyte cells to produce a protein comprising the steps of:

(a) providing an immortalized human hepatocyte cell that includes DNA that encodes and can express a protein;

(b) culturing the immortalized hepatocyte cell under conditions in which a gene or genes encoding the protein are expressed so that the protein is produced and processed in the immortalized hepatocyte cell; and

(c) isolating the processed protein from the immortalized hepatocyte cell;

wherein the protein is expressed such that the protein is processed and glycosylated, if necessary, so that its *in vivo* function is substantially preserved after its isolation.

2. The method of claim 1 wherein the protein is a plasma protein that is naturally produced by human hepatocytes.

3. The method of claim 1 wherein the protein is a protein that is not naturally produced by human hepatocytes.

4. The method of claim 3 wherein the protein is a mutein of a protein that is normally produced by human hepatocytes.

5. The method of claim 1 wherein the protein is a therapeutic protein.

6. The method of claim 5 wherein the therapeutic protein is a therapeutic plasma protein.

7. The method of claim 6 wherein the protein is selected from the group consisting of Factor VIII, Factor IX, human growth hormone (hGH), α-1-antitrypsin, and a growth factor.

8. The method of claim 6 wherein the protein is selected from the group consisting of muteins of Factor VIII, muteins of Factor IX, muteins of human growth hormone, muteins of α-1-antitrypsin, and muteins of a growth factor.

9. The method of claim 1 wherein the protein is an IαIp protein complex.

10. The method of claim 1 wherein the protein is a protein selected from the group consisting of albumin, transcobalamin II, C-reactive protein, fibronectin, ceruloplasmin, and other proteins having structural, enzymatic, or transport activities.

11. The method of claim 1 wherein the protein is a mutein of a protein selected from the group consisting of albumin, transcobalamin II, C-reactive protein, fibronectin, ceruloplasmin, and other proteins having structural, enzymatic, or transport activities.

12. The method of claim 1 wherein the protein is expressed by a gene that occurs naturally in the hepatocytes, and expression of the naturally-occurring gene encoding the protein is enhanced by introduction of a high-level promoter into the hepatocytes.

13. The method of claim 1 wherein expression is enhanced by introducing multiple copies of the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed via the use of one or more recombinant vectors that include: (1) the gene encoding the protein to be expressed, a subunit of the protein to be expressed, or a precursor of the protein to be expressed; and (2) at least one control element affecting the transcription of the gene, the control element being operably linked to the gene.

14. The method of claim 13 wherein the recombinant vector is selected from the group consisting of SV40-derived vectors, murine polyoma-derived vectors, BK virus-

derived vectors, Epstein-Barr virus-derived vectors, adenovirus-derived vectors, adeno-associated virus-derived vectors, baculovirus-derived vectors, herpesvirus-derived vectors, lentiviral-derived vectors, retrovirus-derived vectors, alphavirus-derived vectors, and vaccinia virus-derived vectors.

15. The method of claim 14 wherein the vector incorporates one or more reporter genes.

16. The method of claim 1 wherein the expressed protein is secreted from the cell into the surrounding culture medium.

17. The method of claim 1 wherein the protein is glycosylated.

18. The method of claim 1 wherein the protein is processed post-translationally.

19. The method of claim 1 wherein the protein is expressed in a form wherein it is fused to a cleavable tag.

20. The method of claim 19 wherein the cleavable tag is selected from the group consisting of glutathione *S*-transferase, the MalE maltose-binding protein, and a polyhistidine sequence.

21. The method of claim 1 wherein the protein comprises at least two different subunits, and wherein the immortalized hepatocyte cell is transformed or transfected with at least two vectors, each vector including: (1) DNA including at least one gene that encodes at least one subunit of the protein; and at least one control element operably linked to the DNA encoding at least one gene that encodes the subunit of the protein.

22. The method of claim 1 wherein the immortalized human hepatocyte cell is virally immortalized.

23. The method of claim 22 wherein the hepatocyte is immortalized by transformation or transfection with substantially pure simian virus (SV40) DNA.

24. The method of claim 23 wherein the substantially pure SV40 DNA encodes large T and small t antigens (Tag).

25. The method of claim 1 wherein the immortalized human hepatocyte cell is derived from primary cryopreserved human hepatocytes.

26. The method of claim 1 wherein the hepatocyte includes tumor-suppressor-encoding DNA such that substantially pure DNA encoding tumor suppressor can be isolated and purified from the hepatocyte.

27. The method of claim 1 wherein the hepatocyte includes DNA encoding Rb such that substantially pure DNA encoding Rb can be isolated and purified from the hepatocyte.

28. The method of claim 1 wherein the hepatocyte includes DNA encoding p53 such that substantially pure DNA encoding p53 can be isolated and purified from the hepatocyte.

29. The method of claim 1 wherein the hepatocyte is nontumorigenic, has the ability to be maintained in a serum-free medium, and produces plasma proteins.

30. The method of claim 1 wherein the hepatocyte is a hepatocyte of the Fa2N-4 cell line.

31. The method of claim 1 wherein the hepatocyte is a hepatocyte of the Ea1C-35 cell line.

32. A method of using eukaryotic cells, other than human hepatocytes, to produce an IαIp protein complex comprising the steps of:

(a) providing a eukaryotic cell, other than a human hepatocyte, that includes DNA that encodes and can express proteins forming an IαIp protein complex, the eukaryotic cell having been transformed or transfected with at least one vector that includes: (1) DNA including at least one gene for a precursor of a protein that is part of an IαIp protein complex; and (2) at least one control element operably linked to the DNA encoding at least one precursor gene in order to enhance expression of the precursor gene;

(b) culturing the transformed or transfected eukaryotic cell under conditions in which genes encoding proteins forming an IαIp complex are expressed so that an IαIp complex is produced; and

(c) isolating the expressed IαIp protein complex from the transformed or transfected eukaryotic cell.

33. The method of claim 32 wherein the eukaryotic cell is selected from the group consisting of CHO cells, COS cells, and yeast cells.

34. The method of claim 32 wherein the hepatocyte is transformed or transfected with two vectors: (1) a first vector that includes the genes *H3* and *AMBP*; and (2) a second vector that includes the genes *H2* and *H1*.

35. An immortalized human hepatocyte cell that includes DNA that encodes and can express a protein, the immortalized human hepatocyte cell having been transformed or transfected with at least one vector that includes: (1) DNA including at least one gene encoding a protein; and (2) at least one control element operably linked to the DNA encoding the protein in order to enhance expression of the protein.

36. The cell of claim 35 wherein the protein is a protein that is naturally produced by human hepatocytes.

37. The cell of claim 35 wherein the protein is a protein that is not naturally produced by human hepatocytes.

38. The cell of claim 37 wherein the protein is a mutein of a protein that is normally produced by human hepatocytes.

39. The cell of claim 35 wherein the protein is a therapeutic protein.

40. The cell of claim 39 wherein the therapeutic protein is a therapeutic plasma protein.

41. The cell of claim 40 wherein the protein is a therapeutic plasma protein selected from the group consisting of Factor VIII, Factor IX, human growth hormone (hGH), α-1-antitrypsin, and a growth factor.

42. The cell of claim 40 wherein the protein is a plasma protein is selected from the group consisting of muteins of Factor VIII, muteins of Factor IX, muteins of human growth hormone, muteins of α-1-antitrypsin, and muteins of a growth factor.

43. The cell of claim 35 wherein the plasma protein is an IαIp protein complex.

44. The cell of claim 35 wherein the protein is a protein selected from the group consisting of albumin, transcobalamin II, C-reactive protein, fibronectin, ceruloplasmin, and other proteins having structural, enzymatic, or transport activities.

45. The cell of claim 45 wherein the protein is a mutein of a protein selected from the group consisting of albumin, transcobalamin II, C-reactive protein, fibronectin, ceruloplasmin, and other proteins having structural, enzymatic, or transport activities.

46. A method of treating a disease or condition comprising the steps of:

(a) providing an active protein produced according to the method of claim 1; and

(b) administering the active protein to a patient suffering from the disease or condition in a therapeutically active quantity to treat the disease or condition.

47. The method of claim 46 wherein the disease or condition is a disease or condition affecting the liver.

48. The method of claim 47 wherein the disease or condition affecting the liver is selected from the group consisting of sepsis, cancer, hepatitis, and liver failure.

49. The method of claim 46 wherein the disease or condition is a disease or condition affecting an organ other than the liver.

50. The method of claim 49 wherein the disease or condition is selected from the group consisting of cancer, joint inflammation, and arthritis.

51. A pharmaceutical composition for treating a disease or condition comprising:

(a) an IαIp protein complex produced by eukaryotic cells in a quantity terapeutically effective to treat a disease or condition; and

(b) a pharmaceutically acceptable carrier.

52. The pharmaceutical composition of claim 51 wherein the disease or condition is a disease or condition affecting the liver.

53. The pharmaceutical composition of claim 52 wherein the disease or condition affecting the liver is selected from the group consisting of sepsis, cancer, hepatitis, and liver failure.

54. The pharmaceutical composition of claim 51 wherein the disease or condition is a disease or condition affecting an organ other than the liver.

55. The pharmaceutical composition of claim 54 wherein the disease or condition is selected from the group consisting of cancer, joint inflammation, and arthritis.

56. A method of treating a disease or condition comprising the steps of:

(a) providing an active plasma protein produced by the method of claim 1; and

(b) administering the active plasma protein to a patient suffering from the disease or condition in a therapeutically effective quantity to treat the disease or condition.

57. The method of claim 56 wherein the disease or condition is a disease or condition affecting the liver.

58. The method of claim 57 wherein the disease or condition affecting the liver is selected from the group consisting of sepsis, cancer, hepatitis, and liver failure.

59. The method of claim 56 wherein the disease or condition is a disease or condition affecting an organ other than the liver.

60. The method of claim 59 wherein the disease or condition is selected from the group consisting of cancer, joint inflammation, and arthritis.

61. The method of claim 56 wherein the active plasma protein is selected from the group consisting of Factor VIII, Factor IX, human growth hormone (hGH), α-1-antitrypsin, and a growth factor.