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(54) Title: ECTO-5'-NUCLEOTIDASE (CD73) USED IN THE DIAGNOSIS AND THE TREATMENT OF PANCREATIC CANCER

(57) Abstract: Using gene expression profiling, the present invention identifies ecto-5'-nucleotidase (5'NT) or CD73 as a diagnostic marker and therapeutic target for pancreatic cancer. The invention therefore provides methods for prediction and detection of CD73 associated cancers, and evaluation of inhibitors of CD73. The invention also provides a method of treating or preventing pancreatic cancer in a subject.



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ECTO-5'-NUCLEOTIDASE (CD73) USED IN THE DIAGNOSIS AND THE TREATMENT OF PANCREATIC CANCER

BACKGROUND OF THE INVENTION

The present application claims the benefit of co-pending U.S. Provisional Patent Application Serial No. 60/486,015, filed July 10, 2003 and U.S. Provisional Patent Application Serial No. 60/451,456, filed March 3, 2003, the entire disclosures of which are specifically incorporated herein by reference.

1. Field of the Invention

The present invention relates generally to the fields of molecular biology and cancer therapy. More particularly, it concerns diagnostic markers and drug targets for pancreatic cancer.

2. Description of Related Art

Pancreatic cancer is the fourth leading cause of cancer death among adults in the United States. In the year 2000 alone, an estimated 28,300 new cases of pancreatic cancer were diagnosed in the United States and nearly 28,200 patients were estimated to have died. Close to 90% of patients diagnosed with pancreatic cancer die within the first year following diagnosis. The deadliness of this disease has encouraged a search for factors that influence incidence and the molecular events that are involved in pancreatic tumor progression. At the molecular level, it is thought that the accumulation of defects in specific genes that contribute to the growth and development of normal tissue are responsible for the progression of cancer. Therefore, understanding the effects of genetic lesions that are common in the development of pancreas cancer will no doubt lead to new and more effective ways to diagnose, treat, and prevent this devastating disease.

The advent of cDNA microarray technology has made possible the identification and validation of new potential targets for drug development and analysis of the secondary effects of agents by monitoring changes in the expression of downstream genes. cDNA expression microarray analysis allows for the rapid identification of potential targets for drug development

by examining the expression of thousands of genes in cancer cells versus normal cells. The changes in gene expression patterns from normal to tumor cells provide a background to determine what pathways are altered in cancer cells on a comprehensive scale.

SUMMARY OF THE INVENTION

The present invention addresses the deficiencies in the art of an efficacious therapy for treating pancreatic cancer by investigating the molecular basis of the disease. In comparing pancreatic cancer cells to that of normal pancreas, by expression profiling, ecto-5'-nucleotidase (CD73) was identified as a diagnostic marker and a therapeutic target in treating this disease. Thus, the present invention provides a method of diagnosing or predicting development of pancreatic cancer in a subject comprising (a) obtaining a pancreatic cell sample from the subject; and (b) assessing ecto-5'-nucleotidase activity or expression in a cell of the sample, wherein increased activity or expression of ecto-5'-nucleotidase in the cell, when compared to a normal cell of the same type, indicates that the subject has or is at risk of developing pancreatic cancer.

A pancreatic cell sample embodied in the present invention may be precancerous pancreatic cell sample, a metastatic pancreatic cell sample, or a malignant pancreatic cell sample. Malignant pancreatic cell samples may further comprise a ductal adenocarcinoma cell sample, an intraductal papillary neoplasm cell sample, a papillary cystic neoplasm cell sample, a mucinous cystadenocarcinoma cell sample, a mucinous cystadenoma cell sample, an acinar carcinoma cell sample, an unclassified large cell carcinoma sample, a small cell carcinoma sample, or a pancreatoblastoma cell sample.

In other embodiments, the cell is a pancreatic tumor cell.

In a particular embodiment, the present invention comprises assessing ecto-5'-nucleotidase expression or activity in a cell or sample, such as a tissue sample, by Northern blotting, quantitative RT-PCR, Western blotting or quantitative immunohistochemistry.

In some embodiments, the subject has previously been diagnosed with cancer or the subject has not previously been diagnosed with cancer and appears cancer free at the time of testing. In another embodiment, the present invention comprises administering a prophylactic cancer treatment, or a cancer therapy to the subject following testing. In other embodiments, the cancer therapy is a chemotherapy, a radiotherapy, an immunotherapy, a gene therapy, a hormonal therapy or surgery.

In still another embodiment, the present invention provides a method of predicting the efficacy of a pancreatic cancer therapy comprising (a) administering a cancer therapy to the subject; (b) obtaining a pancreatic tumor cell sample from the subject; and (c) assessing ecto-5'-nucleotidase activity or expression in the tumor cell of the sample, wherein decreased activity or expression of ecto-5'-nucleotidase in the tumor cell, when compared to a tumor cell of the same type prior to treatment, indicates that the therapy is efficacious.

In further embodiments the present invention comprises assessing ecto-5'-nucleotidase expression comprising measuring ecto-5'-nucleotidase protein levels, or measuring ecto-5'-nucleotidase transcript levels. In other embodiments, the present invention further comprises assessing ecto-5'-nucleotidase activity or expression at multiple time points.

In still yet another embodiment, the present invention comprises a method of screening a candidate compound for anti-cancer activity comprising (a) providing a pancreatic cancer cell; (b) contacting the cell with a candidate compound; and (c) assessing the effect of the candidate compound on ecto-5'-nucleotidase expression or activity, wherein a decrease in the amount of ecto-5'-nucleotidase expression or activity, as compared to the amount of ecto-5'-nucleotidase expression or activity in a similar cell not treated with the candidate compound, indicates that the candidate compound has anti-cancer activity.

The candidate compound of the present invention may be a protein, a nucleic acid or an organo-pharmaceutical.

In some embodiments of the invention the tumor cell is selected from the group consisting of a precancerous pancreatic cell, a metastatic pancreatic cell, or a malignant pancreatic cell. The malignant pancreatic cancer cell may further comprise a ductal adenocarcinoma cell an intraductal papillary neoplasm cell, a papillary cystic neoplasm cell, a mucinous cystadenocarcinoma cell, a mucinous cystadenoma cell, an acinar carcinoma cell, an unclassified large cell carcinoma, a small cell carcinoma, or a pancreatoblastoma cell.

In further embodiments, a method of treating cancer comprises administering to a subject in need thereof a composition that inhibits ecto-5'-nucleotidase activity or expression is provided.

In still further embodiments, the candidate compound may be a protein, a nucleic acid or a organo-pharmaceutical. In yet a further embodiment, the protein may be an antibody that binds immunologically to ecto-5'-nucleotidase. In still yet a further embodiment, the nucleic acid may be a ecto-5'-nucleotidase antisense nucleic acid, a ecto-5'-nucleotidase RNAi nucleic acid, or an antibody encoding a single-chain antibody that binds immunologically to ecto-5'-nucleotidase.

In some embodiments, the invention further comprises administering a second cancer therapy such as a chemotherapy, a radiotherapy, an immunotherapy, a gene therapy, a hormonal therapy or surgery to the subject.

In further embodiments, the composition of the invention may be administered more than once.

In a further embodiment, the present invention provides a method of diagnosing or predicting development of pancreatic cancer in a subject comprising subjecting the subject to whole body scanning for ecto-5'-nucleotidase activity or expression in a cell.

In still a further embodiment, the present invention provides a method of monitoring an anticancer therapy comprising assessing the expression or function of ecto-5'-nucleotidase in a pancreatic cancer cell of a subject following or during provision of the anticancer therapy.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Schematic of gene expression profiling using a microarray.

FIGS. 2A-2B. RT-PCR analysis of ecto-5'-nucleotidase overexpression in pancreatic cancer cells. **FIG. 2A** - mRNA expression levels in pancreatic cell lines as compared to normal pancreas. **FIG. 2B** - Bar graph showing ratio of expression levels to normal pancreas.

FIG. 3. Western blot analysis of ecto-5'-nucleotidase overexpression in pancreatic cancer cells.

FIGS. 4A-4B. Overexpression of ecto-5'-nucleotidase overexpression in pancreatic cell lines by Northern Blot analysis. **FIG. 4A** - Northern blot showing mRNA expression levels as compared to a normal pancreatic cell line. **FIG. 4B.** - Bar graph showing ratio of mRNA expression levels.

FIGS. 5A-5B. Overexpression of ecto-5'-nucleotidase overexpression in pancreatic tumor samples by RT-PCR. **FIG. 5A** - mRNA expression levels in patient samples as compared to normal pancreas. **FIG. 5B** - Bar graph showing ratio of expression levels in patient samples to normal pancreas.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

A. The Present Invention

In order to address the deficiencies of current therapies in treating pancreatic cancer, one of the most deadly of all cancers, the molecular basis for this disease needs to be investigated. Thus, the present inventors examined the expression profiles of pancreatic cancer cells and compared these to normal cells. From these expression profiles, they identified a group of dysregulated genes, the expression of which is greater or less in cancer cells than in a corresponding non-cancerous cell.

One of these genes, ecto-5'-nucleotidase or CD73, was highly overexpressed in all pancreatic cancer cells examined. Overexpression of the CD73 gene in pancreatic cancer cells lines was confirmed using RT-PCR and Western blotting. From these results, CD73 was identified as a diagnostic marker for pancreatic cancer, and a therapeutic target in treating this disease.

In particular aspects, the present invention thus provides methods of assessing the activity or expression of CD73 protein or transcripts levels using a variety of techniques, the goal being the identification of cancers of pancreatic origin. The present invention also provides methods of screening for candidate inhibitors of CD73. Finally, the present invention provides methods of treating a cancer, in particular pancreatic cancer, by providing compositions that inhibit CD73

activity or expression, either as a single agent or in combination with other therapeutic agents. The details of the invention will be provided in the following materials.

B. Ecto-5'-nucleotidase (5'NT) or CD73

CD73 or ecto-5'nucleotidase (5'-NT) is ubiquitously expressed protein in a number of tissues. This protein is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) linkage, and has ecto-enzyme activity and plays a role in signal transduction. The primary function of CD73 is its conversion of extracellular nucleotides (*e.g.*, 5'-AMP), to which cells are generally impermeable, to their corresponding nucleosides (*e.g.*, adenosine), which can readily enter most cells. Thus, ecto-5'-nucleotidase catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleoside. Although CD73 has broad substrate specificity, it prefers purine ribonucleoside. Specifically, CD73 has been reported to dephosphorylate nucleoside monophosphates (AMP) into adenosine in mammalian cells. Hydrolysis of AMP to adenosine is a major signaling pathway in cells.

CD73 is a homodimeric or tetrameric glycoprotein, comprising two or four subunits of 68 to 70 kD (Stochaj *et al.*, 1989, Le Hir *et al.*, 1989 and Loe *et. al.*, 1989). Substrates of CD73 are 5'-adenosine monophosphate (5'-AMP), 5'-uridine monophosphate (5'-UMP), 5'-guanosine monophosphate (5'-GMP), 5'-cytidine monophosphate (5'-CMP) and 5'-inosine monophosphate (5'-IMP). Ecto-5'-NT activity has been shown to be inhibited by the non-hydrolyzable analog of ADP, α,β -methylene ADP (α,β -metADP), and by concanavalin A (Sunderman, 1990).

Rather little is known about the regulation of CD73. As an enzyme that produces nucleosides, particularly adenosine, in the extracellular space, CD73 is thought to modulate neuronal signaling, vascular perfusion, drug metabolism and immune response. It is also thought to have anti-inflammatory and immunosuppressive capabilities. However, like other GPI-anchored proteins, CD73 also has cellular functions that are independent of its enzymatic activity. For instance, CD73 has been reported to bind to the intracellular filament protein actin and the extracellular matrix proteins laminin and fibronectin, suggesting a possible role for 5'-NT in cell adhesion.

CD73 expression is tissue and cell type specific, however, it is not clear how such specificity is regulated. Several factors have been reported to regulate the expression of CD73. Protein kinase C (PKC) has been shown to mediate adrenergic stimulation of CD73 activation in the heart, and adenosine release in rat cardiomyocyte (Kitakaze *et al.*, 1995; Kitakaze *et al.*, 1996). Parathyroid hormone has also been shown to stimulate CD73 by a mechanism involving

PKC (Siegfried *et al.*, 1995). Some cytokines were also shown to regulate CD73 activity. In rat, IL-1 β and TNF- α induces CD73 activity (Savic *et al.*, 1990), and in monocytes, interferon- γ and IL-4 reduced its activity (Armstrong *et al.*, 1988).

CD73 production of adenosine by the dephosphorylation of AMP, has been shown to regulate adenosine receptor engagement in many tissues (Resta *et al.*, 1998). This further indicated that adenosine functions in cytoprotection, cell growth, angiogenesis and immunosuppression, and plays a role in tumorigenesis (Spychala, 2000). Spychala *et al.* (2000) have shown adenosine stimulates tumor growth and angiogenesis in cell lines.

To date there are no published data that illustrate the cause versus effect of CD73 in pancreatic cancer. However, elevated CD73 activity has been reported in variety of tumor types, including breast carcinoma (Canbolat *et al.*, 1996), gastric cancer (Durak *et al.*, 1994), glioblastoma (Fenoglio *et al.*, 1997), CML (Gutensohn *et al.*, 1983), and cutaneous T-cell lymphoma (Fukunaga *et al.*, 1989). Flocke *et al.* (1992) have shown that CD73 is expressed in normal pancreatic ductal cells but not the acinar and islet cells. CD73 has also been implicated in cancer cells resistant to nucleoside analog drugs. For example, levels of CD73 activity have been correlated to clinical response to drugs like ara-C in leukemia such as, CLL, HCL and AML (Galmarini *et al.*, 2001).

C. Prognostic and Diagnostic Methods

A variety of methods known to those of ordinary skill in the art are available for assessing the activity or expression of a gene product in a cell, tissue sample or organism. The present invention embodies diagnostic methods and methods for assessing CD73 activity or expression comprising measuring CD73 protein or transcript levels. Methods of assessing for CD73 enzyme activity, or protein expression levels may also be employed. These methods are provided to identify subjects who both may be at risk for developing cancer, and who already have pancreatic cancer. In addition, these same methods may be applied to assess the efficacy of a cancer therapy.

Assays to assess the level of expression of a polypeptide are also well known to those of skill in the art. This can be accomplished also by assaying for CD73 mRNA levels, mRNA stability or turnover, as well as protein expression levels. It is further contemplated that any post-translational processing of CD73 may also be assessed, as well as whether it is being localized or regulated properly. In some cases an antibody that specifically binds CD73 may be used. Assays for CD73 activity also may be used.

1. Northern Blotting Techniques

The present invention employs Northern blotting in assessing the expression of CD73 in a cancer or tumor cell. The techniques involved in Northern blotting are commonly used in molecular biology and is well known to one skilled in the art. These techniques can be found in many standard books on molecular protocols (*e.g.*, Sambrook *et al.*, 2001). This technique allows for the detection of RNA *i.e.*, hybridization with a labeled probe.

Briefly, RNA is separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with, *e.g.*, a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

U.S. Patent 5,279,721, incorporated by reference herein, discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

2. Quantitative RT-PCR

The present invention also employs quantitative RT-PCR in assessing the expression or activity of CD73 in a cancer or tumor cell. Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific mRNA species, such as a CD73 transcript, isolated from a cell. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed.

In PCR, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is not an increase in the amplified target between cycles. If one plots a graph on which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, one observes that a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After some reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target

DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR is directly proportional to the starting concentration of the target before the PCR was begun. By determining the concentration of the PCR products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundances is only true in the linear range portion of the PCR reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR for a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR study to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR study is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample. In such studies, mRNAs for β -actin, asparagine synthetase and lipocortin II may be used as external and internal standards to which the relative abundance of other mRNAs are compared.

Most protocols for competitive PCR utilize internal PCR internal standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant

than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

The discussion above describes the theoretical considerations for an RT-PCR assay for clinically derived materials. The problems inherent in clinical samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of larger size than the target).

Both of the foregoing problems are overcome if the RT-PCR is performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies are available that use a more conventional relative quantitative RT-PCR with an external standard protocol. These assays sample the PCR products in the linear portion of their amplification curves. The number of PCR cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This is very important since this assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR assays can be superior to those derived from the relative quantitative RT-PCR with an internal standard.

One reason for this is that without the internal standard/competitor, all of the reagents can be converted into a single PCR product in the linear range of the amplification curve, increasing the sensitivity of the assay. Another reason is that with only one PCR product, display of the product on an electrophoretic gel or some other display method becomes less complex, has less background and is easier to interpret.

3. Immunohistochemistry

The present invention also employs quantitative immunohistochemistry in assessing the expression of CD73 in a cancer or tumor cell.

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tumor at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections containing an average of about 500 remarkably intact tumor cells.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 h fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

4. Western Blotting

The present invention also employs the use of Western blotting (immunoblotting) analysis to assess CD73 activity or expression in a cell such as a pancreatic cancer cell. This technique is well known to those of skill in the art, see U.S. Patent 4,452,901 incorporated herein by reference and Sambrook *et al.* (2001). In brief, this technique generally comprises separating proteins in a sample such as a cell or tissue sample by SDS-PAGE gel electrophoresis. In SDS-PAGE proteins are separated on the basis of molecular weight, then are transferring to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), followed by incubation of the proteins on the solid support with antibodies that specifically bind to the proteins. For example, in the present invention, anti-CD73 antibodies specifically bind to CD73 proteins on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.* labeled sheep, goat, or mouse antibodies) that specifically bind to the CD73.

5. ELISA

The present invention may also employ the use of immunoassays such as an enzyme linked immunosorbent assay (ELISA) in assessing the activity or expression of CD73 in a cancer or tumor cell. An ELISA generally involves the steps of coating, incubating and binding, washing to remove species that are non-specifically bound, and detecting the bound immune complexes. This technique is well known in the art, for example see U.S. Patent 4,367,110 and Harlow and Lane, 1988.

In an ELISA assay, a CD73 protein sample may be immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of milk powder. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

After binding of the antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from 2 to 4 or more hour to allow effective binding, at temperatures preferably on the order of 25°C to 37°C (or overnight at 4°C). Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting the sample to a second antibody having specificity for the first. To provide a detecting means, the second antibody preferably has an associated enzyme that generates a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) and H₂O₂, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra

spectrophotometer. The use of labels for immunoassays are described in U.S. Patents 5,310,687, 5,238,808 and 5,221,605.

Other immunodetection methods that may be contemplated in the present invention include radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay. These methods are well known to those of ordinary skill and have been described in Doolittle and Ben-Zeev; Gulbis and Galand, 1993; De Jager *et al.*, 1993; and Nakamura *et al.*, 1987, each incorporated herein by reference.

6. Tissue Microarray Immunohistochemistry

This is a recently developed technique that enables the simultaneous examination of multiple tissues sections concurrently as compared to the more conventional technique of one section at a time. This technique is used for high throughput molecular profiling of tumor specimen (Kononen *et al.*, 1998). More specifically, the present invention utilizes a pancreatic tumor tissue microarray containing different adenocarcinoma tissue samples, each of which having two representative 1.5 mm disks from the different areas of the same paraffin-embedded section. These pancreatic tissue microarrays may be used to verify the overexpression of other genes manifested in the cDNA microarray.

7. Determination of Circulating Cancer Cells

With the advent of enrichment techniques, detection of circulating cancer cells can be used for the early detection of cancer recurrence after treatment of a primary tumor, early diagnosis of metastasis, and use in selection and monitoring of treatment strategies for various tumors (Martin *et al.*, 1998; Wang *et al.*, 2000; Hu *et al.*, 2003). Anti-CD73 antibodies of the present invention may be used in conjunction with cancer cell enrichment techniques in the detection of circulating pancreatic cancer cells. One suitable cell enrichment methodology is the magnetic-activated cell separation system as distributed by Miltenyi Biotec Inc. (Auburn, CA). This immunomagnetic method uses magnetically labeled anti-cytokeratin 8 antibodies to separate circulating cancer cells from other circulating cell types (see Martin *et al.*, 1998; Hu *et al.*, 2003). Pancreatic cancer cells express cytokeratin 8 (Rafie *et al.*, 1992; Ditzel *et al.*, 1997; Luttes *et al.*, 1998). Blood samples (20-40 ml) are collected, treated with anticoagulant and stored for up to 23 hr until further processing when they are spun down at 400g for 35 minutes and the leukocyte-rich interphase cells are collected and permeabilized with PBS containing 0.5% BSA and 0.1% saponin and then fixed with 37% formaldehyde. After washing twice with PBS, 0.5% BSA, 0.5% saponin and 0.05% NaN₃, the cells are resuspended in 600 µl PBS, 0.5%

BSA, 0.5% saponin and 0.05% NaN₃, and 200 µl FcR blocking reagent (Miltenyi Biotec) is added and the cancer cells directly magnetically labeled by the addition of 200 µl Cytokeratin Microbeads (Miltenyi Biotec, Auburn, CA) and incubating the cells for 45 min at room temperature. The magnetically labeled cells are passed through a 30 µm filter and applied to a MACS MS enrichment column (Miltenyi Biotec), which is located within a magnetic field. Negative cells are washed off with PBS, 0.5% BSA, and 0.05% NaN₃, and then labeled cells are removed using the same buffer and the plunger supplied with the column after removal of the column from the magnetic field. Pancreatic cancer cells in this fraction can be detected by immunohistochemistry or flow cytometry using suitably labeled anti-CD73 antibodies. Alternatively, magnetically anti-CD73 antibodies may be used to enrich circulating pancreatic cancer cells.

An alternative enrichment technique is Circulating Cancer Cell Test (Cell Works Inc., Baltimore, MD; see Wang *et al.*, 2000). This procedure utilizes a double gradient sedimentation for the removal of most RBC and WBC as well as magnetic cell sorting for the additional removal of WBC before spreading the cancer cells onto a slide utilizing a cytopspin apparatus. The fixed cells on the slide are then stained with a suitably anti-CD73 antibody and positive cells are automatically scanned with an spectroscopic microscope system, first in low magnification, where the fluorescent digital image is captured at a resolution of 0.2 µm using multiple excitation/emission wavelengths, then at higher resolution for further analysis. The system has automatic adjustment of exposure, focus and other parameters required for proper image acquisition and analysis to identify cancer cells and markers on the basis of intensity and blob analysis.

8. Whole Body Imaging

The present invention may further employ the use of whole body imaging techniques to identify subjects who have or may be at risk of developing cancer. Such diagnostic methods may employ positron emission tomography (PET) scanning, electron beam tomography (EBT) scanning, and MRI scanning. Essential to these methods is the use of labeled targeting agents, such as antibodies, that colocalize with ecto-5'-nucleotidase or CD73 in a quantitative fashion.

D. Screening Methods for CD73 Activity or Expression

1. Screening For Inhibitors of CD73

The present invention further comprises methods for identifying inhibitors of CD73 activity or expression. CD73 may be used as a target in screening for compounds that inhibit, decrease or down-regulate its expression or activity in cancer cells, such as pancreatic cancer cells. These assays may comprise random screening of large libraries of candidate substances. Alternatively, the assays may be used to focus on particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to inhibit the function of CD73. By function, it is meant that one may assay for inhibition of expression of CD73 in cancer cells, increase apoptosis, or the inability of the CD73 enzyme to convert extracellular nucleotides, such as 5'-AMP, 5'-CMP, 5'-GMP, 5'-UMP or 5'-IMP, to their corresponding nucleosides.

To identify a CD73 inhibitor, one generally will determine CD73 activity or expression in the presence and absence of the candidate substance, wherein a inhibitor is defined as any substance that down-regulates, reduces, inhibits or decreases CD73 activity or expression. For example, a method may generally comprise:

- a) providing a cell;
- b) contacting the cell with a candidate compound; and
- c) assessing the effect of the candidate compound on CD73 expression or activity,

wherein a decrease in the amount of CD73 expression or activity, as compared to the amount of CD73 expression or activity in a similar cell not treated with the candidate compound, indicates that the candidate compound has anti-cancer activity.

Assays may be conducted in cell free systems, in isolated cells, or in organisms including transgenic animals. It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

a. Inhibitors

As used herein the term "candidate substance" or "candidate compound" refers to any molecule that may potentially inhibit the expression or activity of CD73. A CD73 inhibitor, may be a compound that overall affects an inhibition of CD73 activity, which may be accomplished by inhibiting CD73 expression, translocation or transport, function, expression, post-translational modification, location, or more directly by preventing its activity, such as by binding CD73. Any compound or molecule described in the methods and compositions herein may be an inhibitor of CD73 activity or expression.

The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to CD73 or other nucleotidases, or that binds CD73. Using lead compounds to help develop improved compounds is known as "rational drug design" and include not only comparisons with known inhibitors, but predictions relating to the structure of target molecules.

Candidate compounds or inhibitors of the present invention will likely function to inhibit, decrease or down-regulate the expression or activity of CD73 in a cancer cell such as a pancreatic cancer cell. Such candidate compounds may be inhibitors or regulators of nucleotidases; may have the ability to inhibit the dephosphorylation of purine or pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding nucleosides; or may likely be involved in controlling cellular proliferation in a cancer or tumor cell, such as pancreatic cancer cells. These candidate compounds may be antisense molecules, ribozymes, interfering RNAs, antibodies (including single chain antibodies), or organopharmaceuticals, but are not limited to such.

b. Rational Drug Design

The present invention also provides methods for developing drugs that inhibit CD73 activity or expression that may be used to treat a cancer, such as pancreatic cancer. One such method involves the prediction of the three dimensional structure of a validated 5'-nucleotidase target using molecular modeling and computer stimulations. The resulting structure may then be used in docking studies to identify potential small molecule inhibitors that bind in the enzyme's active site with favorable binding energies. Inhibitors identified may then be tested in biochemical assays to further identify CD73 drug target for pancreatic cancer treatment.

Rational drug design is therefore used to produce structural analogs of substrates for CD73. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for the CD73 targets of the invention or a fragment thereof. This could be accomplished by X-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-

idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

Candidate compounds may include fragments or parts of naturally-occurring compounds, or may be found as active combinations of known compounds, which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators.

Other suitable compounds include antisense molecules, ribozymes, and antibodies (including single chain antibodies), each of which would be specific for the target molecule. Such compounds are described in greater detail elsewhere in this document. For example, an antisense molecule that bound to a translational or transcriptional start site, or splice junctions, would be ideal candidate inhibitors.

In addition to the inhibiting compounds initially identified, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the structure of the inhibitors. Such compounds, which may include peptidomimetics of peptide inhibitors, may be used in the same manner as the initial inhibitors.

An inhibitor according to the present invention may be one which exerts its inhibitory or activating effect upstream, downstream or directly on or other related nucleotidases. Regardless of the type of inhibitor identified by the present screening methods, the effect of the inhibition by such a compound results in the regulation in CD73 activity or expression as compared to that observed in the absence of the added candidate substance.

The term "drug" is intended to refer to a chemical entity, whether in the solid, liquid, or gaseous phase which is capable of providing a desired therapeutic effect when administered to a subject. The term "drug" should be read to include synthetic compounds, natural products and macromolecular entities such as polypeptides, polynucleotides, or lipids and also small entities such as neurotransmitters, ligands, hormones or elemental compounds. The term "drug" is meant to refer to that compound whether it is in a crude mixture or purified and isolated.

2. 5'-Nucleotidase Assays

Assays that measure 5'-nucleotidase activity may also be employed in the present invention. One method of screening for drug targets would involve measuring inhibition of CD73 to dephosphorylate purine and pyrimidine ribo- and dexoyribonucleoside monophosphates to their corresponding nucleosides. This procedure detects the amount of inorganic phosphate that is released from 5'-nucleotide, such as AMP, using a molybdate color reaction. Ecto-5'-nucleotidase activity may also be measured using the Berthelot indophenol reaction. In this procedure CD73 hydrolyzes 5'-AMP to produce adenosine, which is deaminated by adenosine deaminase (ADA) to yield inosine and ammonia. The ammonia that is produced is then measured colorimetrically by the Berthelot indophenol reaction. Such assays or systems are commercially available from suppliers such as Molecular Probes Inc., (Eugene, OR; EnzChek™ phosphate assay kit).

3. *In vitro* Assays

A quick, inexpensive and easy assay to run is an *in vitro* assay. Such assays generally use isolated molecules, and can be run quickly and in large numbers, thereby increasing the amount of information obtainable in a short period of time. A variety of vessels may be used to run the assays, including test tubes, plates, dishes and other surfaces such as dipsticks or beads.

One example of a cell-free assay is a binding assay. While not directly addressing function, the ability of a compound to bind to a target molecule such as CD73 in a specific fashion is strong evidence of a related biological effect, which can be assessed. For example,

binding of a molecule to CD73 may, in and of itself, be inhibitory, due to steric, allosteric or charge-charge interactions. The CD73 may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the CD73 or the compound may be labeled, thereby permitting measuring of the binding. Competitive binding formats can be performed in which one of the agents is labeled, and one may measure the amount of free label versus bound label to determine the effect on binding.

A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. Bound polypeptide is detected by various methods.

4. *In cyto* Assays

The present invention also contemplates the screening of compounds for their ability to inhibit CD73 in cells. Various cell lines can be utilized for such screening assays, including cells specifically engineered for this purpose. The present invention particularly contemplates the use of pancreatic cancer cells, which express a higher level of CD73 activity, and thus may provide an easier baseline for measurement. Depending on the assay, culture may be required. The cell is examined using any of a number of different physiologic assays. Alternatively, molecular analysis may be performed, for example, looking at protein expression, mRNA expression (including differential display of whole cell or polyA RNA) and others by methods as described herein and that are well known to those of skill in the art.

5. *In vivo* Assays

In vivo assays involve the use of various animal models, including transgenic animals that have been engineered to have specific defects such as CD73 overexpression, or that carry markers that can be used to measure the ability of a candidate substance to reach and affect different cells within the organism. Due to their size, ease of handling, and information on their physiology and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses and monkeys (including chimps, gibbons and baboons). Assays for inhibitors may be conducted using an animal model derived from any of these species.

In such assays, one or more candidate substances are administered to an animal, and the ability of the candidate substance(s) to alter one or more characteristics, as compared to a similar animal not treated with the candidate substance(s), identifies an inhibitor. The characteristics

may be any of those discussed above with regard to CD73 expression or function, or it may be broader in the sense of "treating" the condition present in the animal.

Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated routes are systemic intravenous injection, regional administration via blood or lymph supply, or directly to an affected site.

Determining the effectiveness of a compound *in vivo* may involve measuring toxicity and dose response can be performed in animals in a more meaningful fashion than in *in vitro* or *in cyto* assays.

E. Cancer Treatment

The present invention embodies a method of treating cancer such as pancreatic cancer, by the delivery of a CD73 inhibitor to a subject having a cancer. Examples of cancers contemplated for treatment include leukemia, ovarian cancer, breast cancer, lung cancer, colon cancer, liver cancer, prostate cancer, testicular cancer, stomach cancer, brain cancer, bladder cancer, head and neck cancer, melanoma, and any other cancer that may be treated by inhibiting or decreasing the activity of CD73 activity.

1. Ecto-5'-Nucleotidase or CD73 Inhibitors

a. Antisense

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription,

RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs may include regions complementary to intron/exon splice junctions. Thus, antisense constructs with complementarity to regions within 50-200 bases of an intron-exon splice junction may be used. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

b. Ribozymes

The present invention also contemplates the use of CD73-specific ribozymes to down-regulate or inhibit or CD73 expression. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess

endonuclease activity (Kim and Cech, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992) is particularly suited to therapeutic applications of the present invention. It has been reported that ribozymes elicited genetic changes in some cell lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. In light of the information included herein and the knowledge of one of ordinary skill in the art, the preparation and use of additional ribozymes that are specifically targeted to a given gene will now be straightforward.

Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down-regulation or inhibition of Ecto-5'-nucleotidase or CD73 include sequences from the Group I self splicing introns including tobacco ringspot virus (Prody *et al.*, 1986), avocado sunblotch viroid (Palukaitis *et al.*, 1979), and Lucerne transient streak virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozymes based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992; Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and hepatitis virus based ribozymes (Perrotta and Been, 1992). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988; Symons, 1992; Chowrira, *et al.*, 1994; and Thompson, *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozymes, the cleavage site is a dinucleotide sequence on the target RNA, uracil (U) followed by either an adenine, cytosine or uracil (A,C or U; Perriman, *et al.*, 1992; Thompson, *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target mRNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible.

Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.* (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in CD73-targeted ribozymes is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

c. RNA Interference (RNAi)

RNA interference (also referred to as "RNA-mediated interference" or RNAi) is a mechanism by which gene expression can be reduced or eliminated. Double stranded RNA (dsRNA) has been observed to mediate the reduction, which is a multi-step process. dsRNA activates post-transcriptional gene expression surveillance mechanisms that appear to function to defend cells from virus infection and transposon activity (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin and Avery, 1999; Montgomery *et al.*, 1998; Sharp and Zamore, 2000; Tabara *et al.*, 1999). Activation of these mechanisms targets mature, dsRNA-complementary mRNA for destruction. RNAi offers major experimental advantages for study of gene function. These advantages include a very high specificity, ease of movement across cell membranes, and prolonged down-regulation of the targeted gene (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin *et al.*, 1999; Montgomery *et al.*, 1998; Sharp, 1999; Sharp *et al.*, 2000; Tabara *et al.*, 1999). Moreover, dsRNA has been shown to silence genes in a wide range of systems, including plants, protozoans, fungi, *C. elegans*, *Trypanosoma*, *Drosophila*, and mammals (Grishok *et al.*, 2000; Sharp, 1999; Sharp *et al.*, 2000; Elbashir *et al.*, 2001). It is generally

accepted that RNAi acts post-transcriptionally, targeting RNA transcripts for degradation. It appears that both nuclear and cytoplasmic RNA can be targeted. (Bosher *et al.*, 2000).

siRNAs must be designed so that they are specific and effective in suppressing the expression of the genes of interest. Methods of selecting the target sequences, *i.e.* those sequences present in the gene or genes of interest to which the siRNAs will guide the degradative machinery, are directed to avoiding sequences that may interfere with the siRNA's guide function while including sequences that are specific to the gene or genes. Typically, siRNA target sequences of about 21 to 23 nucleotides in length are most effective. This length reflects the lengths of digestion products resulting from the processing of much longer RNAs as described above. (Montgomery *et al.*, 1998).

The making of siRNAs has been mainly through direct chemical synthesis; through processing of longer, double stranded RNAs through exposure to *Drosophila* embryo lysates; or through an *in vitro* system derived from S2 cells. Use of cell lysates or *in vitro* processing may further involve the subsequent isolation of the short, 21-23 nucleotide siRNAs from the lysate, *etc.*, making the process somewhat cumbersome and expensive. Chemical synthesis proceeds by making two single stranded RNA-oligomers followed by the annealing of the two single stranded oligomers into a double stranded RNA. Methods of chemical synthesis are diverse. Non-limiting examples are provided in U.S. Patents 5,889,136; 4,415,732; 4,458,066, expressly incorporated herein by reference, and in Wincott *et al.* (1995).

Several further modifications to siRNA sequences have been suggested in order to alter their stability or improve their effectiveness. It is suggested that synthetic complementary 21-mer RNAs having di-nucleotide overhangs (*i.e.*, 19 complementary nucleotides + 3' non-complementary dimers) may provide the greatest level of suppression. These protocols primarily use a sequence of two (2'-deoxy) thymidine nucleotides as the di-nucleotide overhangs. These dinucleotide overhangs are often written as dTdT to distinguish them from the typical nucleotides incorporated into RNA. The literature has indicated that the use of dT overhangs is primarily motivated by the need to reduce the cost of the chemically synthesized RNAs. It is also suggested that the dTdT overhangs might be more stable than UU overhangs, though the data available shows only a slight (< 20%) improvement of the dTdT overhang compared to an siRNA with a UU overhang.

Chemically synthesized siRNAs are found to work optimally when they are in cell culture at concentrations of 25-100 nM. This had been demonstrated by Elbashir *et al.* wherein concentrations of about 100 nM achieved effective suppression of expression in mammalian

cells. siRNAs have been most effective in mammalian cell culture at about 100 nM. In several instances, however, lower concentrations of chemically synthesized siRNA have been used (Caplen *et. al.*, 2000; Elbashir *et. al.*, 2001).

WO 99/32619 and WO 01/68836 suggest that RNA for use in siRNA may be chemically or enzymatically synthesized. Both of these texts are incorporated herein in their entirety by reference. The enzymatic synthesis contemplated in these references is by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, SP6) via the use and production of an expression construct as is known in the art. For example, see U.S. Patent 5,795,715. The contemplated constructs provide templates that produce RNAs that contain nucleotide sequences identical to a portion of the target gene. The length of identical sequences provided by these references is at least 25 bases, and may be as many as 400 or more bases in length. An important aspect of this reference is that the authors contemplate digesting longer dsRNAs to 21-25mer lengths with the endogenous nuclease complex that converts long dsRNAs to siRNAs *in vivo*. They do not describe or present data for synthesizing and using *in vitro* transcribed 21-25mer dsRNAs. No distinction is made between the expected properties of chemical or enzymatically synthesized dsRNA in its use in RNA interference.

Similarly, WO 00/44914, incorporated herein by reference, suggests that single strands of RNA can be produced enzymatically or by partial/total organic synthesis. Preferably, single stranded RNA is enzymatically synthesized from the PCR products of a DNA template, preferably a cloned cDNA template and the RNA product is a complete transcript of the cDNA, which may comprise hundreds of nucleotides. WO 01/36646, incorporated herein by reference, places no limitation upon the manner in which the siRNA is synthesized, providing that the RNA may be synthesized *in vitro* or *in vivo*, using manual and/or automated procedures. This reference also provides that *in vitro* synthesis may be chemical or enzymatic, for example using cloned RNA polymerase (*e.g.*, T3, T7, SP6) for transcription of the endogenous DNA (or cDNA) template, or a mixture of both. Again, no distinction in the desirable properties for use in RNA interference is made between chemically or enzymatically synthesized siRNA.

U.S. Patent 5,795,715 reports the simultaneous transcription of two complementary DNA sequence strands in a single reaction mixture, wherein the two transcripts are immediately hybridized. The templates used are preferably of between 40 and 100 base pairs, and which is equipped at each end with a promoter sequence. The templates are preferably attached to a solid surface. After transcription with RNA polymerase, the resulting dsRNA fragments may be used for detecting and/or assaying nucleic acid target sequences.

2. Pharmaceutical Compositions and Routes of Administration

Pharmaceutical compositions of the present invention comprise administering an effective amount of one or more inhibitors that inhibit or down-regulate the CD73 activity (and/or an additional agent) dissolved or dispersed in a pharmaceutically acceptable carrier to a subject. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one CD73 inhibitor or additional active ingredient will be known to those of skill in the art in light of the present disclosure, and as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

A pharmaceutical composition of the present invention may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it needs to be sterile for such routes of administration as injection. A pharmaceutical composition of the present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctival, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, inhalation (*e.g.*, aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the foregoing as would

be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

The actual dosage amount of a composition of the present invention administered to an subject can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The number of doses and the period of time over which the dose may be given may vary. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s), as well as the length of time for administration for the individual subject.

In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

A CD73 inhibitor(s) of the present invention may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts,

e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine.

In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, *etc.*), lipids (*e.g.*, triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof.

In certain aspects of the invention, the CD73 inhibitors are prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (*e.g.*, hard or soft shelled gelatin capsules), sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with the food of the diet. Preferred carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention, the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

In certain preferred embodiments an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent,

such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, *etc.*; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both.

Additional formulations which are suitable for other modes of administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein.

In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

F. Combination Therapies with CD73 Inhibitor(s)

In order to increase the effectiveness of a cancer treatment with the compositions of the present invention, such as a CD73 inhibitor, it may be desirable to combine these compositions with other cancer therapy agents. For example, the treatment of a cancer may be implemented with therapeutic agents of the present invention in conjunction with other anti-cancer therapies. Thus, in the present invention, it is contemplated that a CD73 inhibitor(s) may be used in conjunction with a chemotherapeutic, a radiotherapeutic, an immunotherapeutic or other biological intervention, in addition to pro-apoptotic or cell cycle regulating agents or nucleotidase regulators.

This process may involve contacting the cell(s) with a CD73 inhibitor and a therapeutic agent at the same time or within a period of time wherein separate administration of the inhibitor and an agent to a cell, tissue or organism produces a desired therapeutic benefit. The terms "contacted" and "exposed," when applied to a cell, tissue or organism, are used herein to describe the process by which a CD73 inhibitor and/or therapeutic agent are delivered to a target cell, tissue or organism or are placed in direct juxtaposition with the target cell, tissue or organism. The cell, tissue or organism may be contacted (*e.g.*, by administration) with a single composition or pharmacological formulation that includes both a CD73 inhibitor and one or more agents, or by contacting the cell with two or more distinct compositions or formulations, wherein one composition includes a CD73 inhibitor and the other includes one or more agents.

1. Regimens

The CD73 inhibitor may precede, be concurrent with and/or follow the other agent(s) by intervals ranging from minutes to weeks. In embodiments where the CD73 inhibitor and other agent(s) are applied separately to a cell, tissue or organism, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the inhibitor and agent(s) would still be able to exert an advantageously combined effect on the cell, tissue or organism. For example, in such instances, it is contemplated that one may contact the cell, tissue or organism with two, three, four or more modalities substantially simultaneously (*i.e.*, within less than about a minute) as the inhibitor. In other aspects, one or more agents may be administered within of from substantially simultaneously, about 1 minute, about 5 minutes, about 10 minutes, about 20 minutes about 30 minutes, about 45 minutes, about 60 minutes, about 2 hours, or more hours, or about 1 day or more days, or about 4 weeks or more weeks, or about 3 months or more months, or about one or more years, and any range derivable therein, prior to and/or after administering the CD73 inhibitor.

Various combinations of a CD73 inhibitor(s) and a cancer therapeutic may be employed in the present invention, where a ecto-5'-nucleotidase or CD73 inhibitor is "A" and the secondary agent, such as a chemotherapeutic or radiotherapeutic agent, or any other cancer therapeutic agent is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	B/B/B/A	B/B/A/B
A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B	A/A/A/B	
B/A/A/A	A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B			

Administration of a CD73 inhibitor of the present invention to a patient will follow general protocols for the administration of that particular secondary therapy, taking into account the toxicity, if any, of the CD73 inhibitor treatment. It is expected that the treatment cycles would be repeated as necessary. The compositions employed in the present invention may be administered once or more than once to a subject. It also is contemplated that various cancer therapies, such as chemotherapy, radiotherapy, as well as surgical intervention, may be applied in combination with the described pancreatic cancer therapy.

2. Anti-Cancer Therapies

An "anti-cancer" agent as contemplated for use with the present invention would be capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. Anti-cancer agents include biological agents (biotherapy), chemotherapy agents, and radiotherapy agents. The combination of chemotherapy with biological therapy is known as biochemotherapy.

In the present invention a composition that inhibits CD73 activity and an anti-cancer agent would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the CD73 inhibitor and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both the CD73 inhibitor and the other

agent, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the CD73 inhibitor and the other includes the second agent(s).

a. Chemotherapy

It is also contemplated in the present invention a CD73 inhibitor(s) may be used in combination with chemotherapeutic agents. Such chemotherapeutic agents may include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatinum, vincristine, vinblastine and methotrexate, Temazolomide (an aqueous form of DTIC), or any analog or derivative thereof. One example of a chemotherapeutic agent currently used to treat pancreatic cancer is gemcitabine.

1. Pyrimidine and Purine Analogs

Other chemotherapeutic agents that may be employed with the CD73 inhibitors of the present invention include pyrimidine analogs and purine analogs. These agents are categorized as antimetabolites that disrupt DNA and RNA synthesis, particularly influencing the cell cycle during S phase.

Pyrimidine analogs include such compounds as cytarabine (cytosine arabinoside), 5-fluorouracil (fluorouracil; 5-FU) and floxuridine (fluorode-oxyuridine; FudR). Cytarabine has been used in the treatment of cancers such as acute granulocytic leukemia and acute lymphocytic leukemias. Floxuridine and 5-fluorouracil have been used in the treatment of cancers such as breast, colon, stomach, pancreas, ovary, head and neck, urinary bladder and topical premalignant skin lesions.

5-Fluorouracil (5-FU), also known as 5-fluoro-2,4(1H,3H)-pyrimidinedione, is thought act by blocking the methylation reaction of deoxyuridylic acid to thymidylic acid. Thus, 5-FU interferes with the synthesis of deoxyribonucleic acid (DNA) and to a lesser extent inhibits the formation of ribonucleic acid (RNA). Since DNA and RNA are essential for cell division and proliferation, it is thought that the effect of 5-FU is to create a thymidine deficiency leading to cell death. Thus, the effect of 5-FU is found in cells that rapidly divide, a characteristic of metastatic cancers.

Purine analogs and related compounds include, but are not limited to, mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2-deoxycoformycin).

Mercaptopurine has been used in acute lymphocytic, acute granulocytic and chronic granulocytic leukemias. Thioguanine has been used in the treatment of such cancers as acute granulocytic leukemia, acute lymphocytic leukemia and chronic lymphocytic leukemia. Pentostatin has been used in such cancers as hairy cell leukemias, mycosis fungoides and chronic lymphocytic leukemia.

b. Radiotherapy

Another therapy that may be used in conjunction with a CD73 inhibitor(s) of the present invention to treat a cancer is radiotherapy. It is contemplated that radiotherapeutic factors that may be employed in the present invention are factors that cause DNA damage and have been used extensively, such as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the cancer or tumor cells.

c. Immunotherapy

The present invention also contemplates the use of immunotherapy in conjunction with a CD73 inhibitor(s). Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, *i.e.*, inhibition or reduction of CD73 expression or activity would provide therapeutic benefit in the treatment of cancer, such as pancreatic cancer.

Immunotherapy could also be used as part of a combined therapy. The general approach for combined therapy is discussed herein. In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells.

Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers which have been found to be upregulated in pancreatic cancer include, but are not limited to carcinoembryonic antigen, CA 27-29 antigen, neuron-specific enolase (NSE), CA 125 antigen, and human chorionic gonadotropin (HCG).

Other types of immunotherapy that may be employed with a CD73 inhibitor(s) of the present invention are passive and active immunotherapy.

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotypic antibodies; and finally, purging of tumor cells in bone marrow. It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune enhancers as described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is well known to those of skill in the art (see Harlow and Lane, 1988).

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993).

d. Gene Therapy

The present invention also contemplates gene therapy in conjunction with CD73 inhibitor therapy. As with the majority of human cancers, numerous genetic alterations have been identified that play a role in adenocarcinoma of the pancreas. These include mutations in the tumor suppressor genes p53, Rb, p16, BRCA2 and DPC4. Several activated oncogenes have also been identified as contributing to pancreas cancer including K-*ras*, HER-2/*neu*, NFkappaB and AKT2. There are, no doubt, many other genetic defects that contribute to the onset and progression of pancreatic cancer and identifying these mutants and the specific consequences of the defects will lead to a better understanding of how to treat this disease. Gene therapy make also be combined with chemo- and radiotherapy to further improve the efficacy of the inhibitor of the present invention. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver *et al.*, 1992).

Inhibitors of cell proliferation, such as tumor suppressor genes, may be employed with the CD73 inhibitor(s) of the present invention. The tumor suppressor oncogenes function to

inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16, Rb, and MMAC1/PTEN may be employed with a CD73 inhibitor(s) of the present invention in treating a cancer, such as pancreatic cancer. Other genes that may be employed with a CD73 inhibitor of the present invention include APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, *zac1*, p73, VHL, DBCCR-1, FCC, *rsk-3*, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (*e.g.*, COX-1, TFPI), PGS, Dp, E2F, *ras*, *myc*, *neu*, *raf*, *erb*, *fms*, *trk*, *ret*, *gsp*, *hst*, *abl*, E1A, p300, genes involved in angiogenesis (*e.g.*, VEGF, FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC. These genes are provided herein as examples and are not meant to be limiting.

Genes that regulators of apoptosis, or programmed cell death, may also be employed with CD73 inhibitor(s) of the present invention in treating pancreatic cancer. Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins have been demonstrated, in the art, to be important regulators and effectors of apoptosis in numerous systems. Some members of this family *e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri, are known to promote cell death and thus may be employed with the CD73 inhibitor(s) of the present invention.

e. Hormonal Therapy

Hormonal therapy may also be used in conjunction with a CD73 inhibitor(s) of the present invention or in combination with any other cancer therapy described herein. The use of hormones may be employed to lower the level or block the effects of certain hormones that may play a role in the tumor cell proliferation. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases in cancers which include but are not limited to breast, prostate, ovarian, or cervical cancer.

f. Surgery

The present invention may also be used in conjunction with surgery. Surgery may also be used in combination with any of the other cancer therapies described herein such as radiation therapy and chemotherapy.

Surgery may be used to remove all or part of the pancreas. The extent of surgery depends on the location and size of the tumor, the stage of the disease, and the patient's general health. Surgery may employ various procedures. One type of surgical procedure that may be use to treat

pancreatic cancer is the Whipple procedure. In this procedure, if the tumor is in the head (the widest part) of the pancreas, the surgeon removes the head of the pancreas and part of the small intestine, bile duct, and stomach. The surgeon may also remove other nearby tissues. Another surgical procedure is a distal pancreatectomy in which the surgeon removes the body and tail of the pancreas if the tumor is in either of these parts. A total pancreatectomy may also be performed in which the surgeon removes the entire pancreas, part of the small intestine, a portion of the stomach, the common bile duct, the gallbladder, the spleen, and nearby lymph nodes.

G. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Material and Methods

Microarray Sample Preparation and Hybridization. cDNA microarray slides used in this study were fabricated in the microarray core facilities at the Arizona Cancer Center (Calaluce *et al.*, 2001). Briefly, each slide has 5760 spots divided into four blocks, with each containing eight identical ice plant genes from *Mesembryanthemum crystallinum* and 23 different housekeeping genes as references for data normalization. Each slide had 5289 unique human cDNA sequences. Poly(A)⁺ RNA was directly isolated from cell pellets using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA), following the instruction manual provided by the manufacturer. Normal pancreas Poly(A)⁺ RNA was isolated from total RNA, which was purchased from Clontech Laboratories (Palo Alto, CA) using the Oligotex Direct mRNA kit (Qiagen, Inc., Valencia, CA). This "normal pancreata" consisted of a pool of two tissue specimens donated by two male Caucasians 18 and 40 years of age. Labeling and purification of cDNA probes were carried out using the MICROMAX direct cDNA microarray system (NEN Life Science Products, Boston, MA). Two to 4 µg of the Poly(A)⁺ RNA samples were used for each labeling. Probes for each pancreatic cell line were labeled with cyanine 5 (Cy5), and probes

for HeLa cells were labeled with cyanine 3 (Cy3). For HeLa cell *versus* normal pancreas hybridization, a normal pancreas sample was labeled with Cy3, and a HeLa cell sample was labeled with Cy5.

Purified cDNA probes were dried and dissolved in 15 μ l of hybridization buffer (included in the MICROMAX direct cDNA microarray system kit). The probes were then denatured by heating at 95°C for 2 min and applied to the array area of a predenatured microarray slide. The microarray slide was covered with a 22 $\times$ 22-cm slide coverslip and incubated in a HybChamber (GeneMachines, San Carlos, CA) at 62°C for overnight. On the second day, the slide was washed in 0.5 $\times$ SSC, 0.01% SDS for 5 min; 0.06 $\times$ SSC, 0.01% SDS for 5 min; and 0.06 $\times$ SSC for 2 min. Finally, the slide was dried by spinning at 500 $\times$ g for 1 min and scanned in a dual-laser (635 nm for red fluorescent Cy5 and 532 nm for green fluorescent Cy3) microarray scanner (GenePix 4000; Axon Instruments, Foster City, CA).

RT-PCR. Two μ g of total RNA isolated from pancreatic cancer cell pellets or frozen pancreatic tumor tissues were used for reverse transcriptase reactions (20 μ l in total volume), which were carried out using the Omniscript RT kit (Qiagen, Inc.), following the manufacturer's protocol. The PCRs were then carried out by mixing 2 μ l of reverse transcriptase reaction mixture, 5 μ l of 10 $\times$ PCR buffer containing 15 mM Mg²⁺, 1 μ l of 10 mM deoxynucleotide triphosphate mixture, 2.5 μ l of 5 μ M PCR primer pair for specific gene, 1 μ l of β -actin primer pair, 1 μ l of β -actin competimers (Ambion, Inc., Austin, TX), 37 μ l of H₂O, and 0.5 μ l of 5 units/ μ l Taq polymerase (Promega Corp., Madison, WI). The amplification cycle (94°C for 30 s; 56°C for 45 s; and 72°C for 1 min) was repeated 29 times. PCR primers for individual genes were designed to generate a DNA fragment ~600 bp in length (if the mRNA itself is less than 600 bases, PCR products were generated in maximal length) using the Primer3 program (Rozen *et al.*, 1998).

Northern Blot. RNA electrophoresis and transferring to Zeta-Probe GT membranes (Bio-Rad, Hercules, CA) were performed as described previously (Calaluce *et al.*, 2001). ³²P-labeled probes were made from the agarose gel-purified RT-PCR products of each gene using the RadPrime DNA Labeling System (Invitrogen). The probe hybridization and stripping buffers and conditions were as provided by the membrane manufacturer. Hybridized membranes were exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and signals were quantified using the ImageQuant software.

Pancreatic Tumor Tissue Array Construction and Immunohistochemistry.

Morphologically representative areas of 42 archival cases of pancreatic tumors, 35 of which are documented ductal adenocarcinomas, from the University of Arizona Health Sciences Center and the Tucson Veterans Administration Medical Center, are selected from formalin-fixed tissue samples embedded in paraffin blocks. Two 1.5-mm-diameter cores/case are reembedded in a tissue microarray using a tissue arrayer (Beecher Instruments, Silver Spring, MD) according to a method described previously (Kononen *et al.*, 1998). Serial sections of the paraffin-embedded pancreatic tissue array are deparaffinized and reacted with primary antibodies specific for c-*Myc* (clone 9E10.3; Neo-Markers, Fremont, CA) or *Rad51* (Oncogene, Boston, MA). Before antibody incubation, the slides are processed for antigen retrieval. This consist of microwaving the slides in citrate buffer (0.1 M, pH 6.0) in a pressure cooker for 25 min and then leaving them to cool. The slides will be incubated with the antibody for 1 h. Biotinylated anti-mouse/anti-rabbit secondary antibodies are applied, followed by streptavidin-peroxidase complex (DAKO, Carpinteria, CA). Colored products are produced using the diaminobenzidine substrate. Staining reactions are scored as diffuse or focal and graded (from 0, negative to 4+, intensely positive) for both neoplasm and background stroma.

Antisense Experiments. To perform antisense experiments cells (5×10^5) are incubated in triplicate in 6-well plates in 1 ml of culture medium supplemented with 10% heat-inactivated FCS for 30 min at 65°C to destroy nuclease activity. These cells are then cultured (24 h) in the presence or absence of antisense, sense, or randomly scrambled phosphorothioate oligonucleotides (ODNs). The ODN sequences are tested against sequences in the GenBankTM database. Two distinct antisense ODNs complementary to sequences that encompass the translation initiation site of the specific target are used. The ability of the antisense to inhibit expression of the target is verified by RT-PCR and with an antibody for Western blotting. Once it is confirmed that expression of the target is downregulated, the phenotypic consequences of inhibition of the target can be determined using the assays describe below.

Cell Proliferation Assays. Cell proliferation assays with these cell lines are performed to determine the effect of target inhibition on cell growth. Cells are seeded at $2.0\text{-}5.0 \times 10^5$ cells in 100-mm culture dishes and allow to attach overnight at 37°C. Adherent cells are washed and incubated with serum-free RPMI 1640 or RPMI containing 10% FBS for 48 h, after which they are trypsinized and counted using a hemocytometer. In addition, parallel experiments are perform and instead of cell counts, the proliferative status of the cell lines is determined using flow cytometric analysis of DNA content. For flow analysis, cells are stained with propidium

iodide using a modified Krishan technique (Krishan, 1975). All samples are analyzed with an FACSCAN flow cytometer (Becton Dickinson) using a 15 mWatt argon ion laser operated at 6 mWatts of power at 488 nm. Photomultiplier tube voltage is adjusted for each control sample to position the G0/G1 to channel 240 on a 1024 channel presentation. Histograms are analyzed for cell cycle compartments using CELLQUEST (Becton Dickinson) analysis software. Histograms having 50K events are collected to maximize the statistical validity of the compartmental analysis. The results of this flow analysis allow the examination of the cell cycle distribution of the pancreatic cancer cell lines.

Alternative analysis of proliferative rate can be estimated by a number of other techniques, including BrdU incorporation or PCNA or Ki67 immunostaining, however, flow analysis is preferred, since it provides an estimate of the fraction of cells in the G1 and G2/M stages of the cell cycle as well as in S-phase. By measuring the proliferative status of the cell lines a better understanding of whether or not the target plays a role in regulating the growth of pancreatic cancer cells is achieved.

Apoptosis Assays. For the measurement of spontaneous and serum starvation induced apoptosis before and after target inhibition, the cells are seeded at $2.0\text{-}5.0 \times 10^5$ cells in 100-mm culture dishes and allow to attach overnight at 37°C. Adherent cells are washed and incubated with serum-free RPMI 1640 media or RPMI 1640 media containing 10% FBS for 48 hr, at which time they are harvested by trypsinization. Any floating cells in the media will be saved and pooled with the harvested cells for the apoptosis analysis. An annexin V based assay is used to quantitate apoptosis. After initiating apoptosis, cells translocate phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be detected using a GFP or FITC conjugate of annexin V (Clontech), a protein that has a strong, natural affinity for PS. This simple assay is a one-step staining procedure of live cells that takes 10 minutes. After incubation with the conjugated annexin V the cells are analyzed by flow cytometry and the percentage of labeled cells determined.

Anchorage Dependent Cell Growth. To assess anchorage-independent growth, the target inhibited cells are suspended in reduced-serum (2%) medium containing 0.3% agar, and overlaid onto a 0.6% agar base at a density of 2×10^4 cells/60-mm dish. Colony formation is monitored for up to 1 month. The number of colonies formed by the target inhibited and uninhibited cells is counted and compared for statistical differences. The ability of target inhibition to alter anchorage-independency of the pancreatic cancer cell lines is a good indication of whether the target is involved in promoting tumorigenicity in pancreatic cancer cell lines.

Cell Migration. In addition to anchorage-independent cell growth, the role of target inhibition in suppressing cell migration can be assessed. Multiple signaling pathways are believed to play a role in directed cell migration. Cell migration is assessed by quantitating the number of cells that directionally migrate through membranes to a collagen undercoating. Briefly, $1 \times$ target inhibited and uninhibited cells are loaded into modified Boyden chambers (tissue culture- treated, 6.5-mm diameter, 10- μ m thickness, 8- μ m pores, Transwell®; Costar Corp) containing collagen type I-undercoated membranes. Cells are allowed to migrate through membranes by incubating them at 37°C for various time points. Nonmigratory cells on the upper membrane surface are removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane are stained with 0.1% crystal violet in 0.1 M borate, pH 9.0, and 2% ethanol for 20 min at room temperature. The number of migratory cells per membrane is either counted with an inverted microscope using a 40 $\times$ objective, or the stain is eluted with 10% acetic acid and the absorbance at 600 nm determined and migration is enumerated from a standard curve. Differences in the migration capacity of cells between target inhibited and uninhibited cells is evaluated by comparing the percentages. A decrease in the migration capacity indicates that the target plays a role in regulating cell invasiveness.

Analysis of Gene Expression Patterns. From frozen pancreatic cancer specimens, frozen tissue sections can be made and examined independently of the original pathological report. Total RNA is extracted, using the standard Triazol RNA isolation protocol (Life Technologies, Gaithersburg, MD), from tissue blocks that contained over 75% of neoplastic cells. The amount and the quality of RNA is checked by electrophoresis on a 1% formamide agarose gel. Normal tissue RNA samples can be obtained from Clontech (Palo Alto, CA). The RNA is labeled by reverse transcription and array hybridizations to the new 10,000-gene chip is performed as described above. After analysis, gene expression patterns from the frozen tissue are compared to those from the cell lines to look for significant differences and for potential new targets.

EXAMPLE 2

Microarray Analysis

The gene expression patterns of genes from pancreatic cancer cell lines were analyzed and compared to gene expression in normal pancreas cells. The strategy employed is shown in FIG. 1. Instead of performing straight comparisons of gene expression in the pancreatic cancer cell lines to normal pancreas, a universal reference RNA (Hela cell RNA) was used to hybridize

to both the cancer cell lines and normal pancreas. The gene expression ratios were then calculated by dividing out the ratio data from the reference as shown in FIG. 1. The reference was used in this analysis because it allows for a comparison of multiple hybridizations when the control RNA (normal pancreas) is limiting.

EXAMPLE 3

Overexpression of Ecto-5'-Nucleotidase or CD73 in Pancreatic Tumors

Ecto-5'-nucleotidase (5'-NT) or CD73 was identified as one of the most highly upregulated genes in pancreatic cancer cells by a genome-wide gene expression profiling using cDNA microarray. The microarray slide contained 4,000 unique sequences, each of which represented a gene that has at least some known function. As shown in Table 1, all pancreatic cancer cell lines for which gene expression was compared to that of normal pancreas cells showed more than 10-fold upregulation of 5'-nucleotidase. Ecto-5'-nucleotidase or CD73 was overexpressed in 11 cell lines with an expression ratio ranging from 12.6 to 74.4.

TABLE 1: Expression Ratios of Pancreatic Cells Expressing Ecto-5'-Nucleotidase

Cell Line	Expression ratio	Cell Line	Expression Ratio
	(Cell Line/Normal)		(Cell Line/Normal)
HPDE6E7	1.2	HPAF II	15.7
AsPC-1	40.9	Hs766T	24.9
BxPC-3	19.4	Mia Paca-2	32.7
Capan-1	16.2	Mutj	22.2
Capan-2	56.4	Panc-1	31.2
CFPAC	12.6	Su86.86	74.4

EXAMPLE 4

Expression of CD73 in Pancreatic Cancer Cell Lines

To verify the overexpression of CD73, semi-quantitative RT-PCR, Western blot and Northern blot experiments were conducted. FIGS. 2A and 2B show RT-PCR detection of CD73 at the mRNA level. It is apparent from analysis of the results that CD73 is highly expressed in all pancreatic cell lines but not in normal pancreas. Western blot results shown in FIG. 3, confirmed the overexpression of CD73 at the protein level. Similarly, Northern blot results

confirmed the overexpression of CD73 at the mRNA level (FIGS. 4A-4B). In all experiments conducted, CD73 protein was found to be present in all pancreatic cell lines but not in the normal pancreatic ductal cell line HPDE6E7. Interestingly, the CD73 protein levels at the cell surface of these cell lines shown by Western blot correlated well with its mRNA levels inside the cell shown by microarray.

CD73 expression in pancreatic cancer patient samples was also investigated using RT-PCR (FIGS. 5A-5B). Compared to normal pancreatic tissue, 6 of the 7 patient samples (86%) showed CD73 overexpression. Three of the samples demonstrated a high degree of upregulation (>15 fold), and the other three samples (43%) showed moderate upregulation (between 3- and 7-fold).

EXAMPLE 5

Pancreatic Cancer Tissue Array

In addition to confirming overexpression of the target genes in the pancreatic cancer cell lines, a tissue array can be developed that allows the determination of the expression of specific gene products in tumors taken from pancreatic cancer patients. The sampling of the original pancreatic cancer tissues for arraying is performed from morphologically representative regions of formalin-fixed paraffin-embedded tumor and normal tissue blocks. Core tissue biopsies (diameter 0.6 mm, height 3-4 mm) are taken from individual "donor" blocks and arrayed into a new "recipient" paraffin block (45 x 20 mm) using a tissue microarraying instrument (Beecher Instruments). Once constructed, the tissue microarray slide is stained using immunohistochemistry with antibodies directed against the proteins of interest and evaluated either manually or utilizing a high-throughput digital imaging system. This tissue array system greatly enhances the ability to quickly validate the expression of potential target genes and analyze the frequency of expression across a number of patient tumors.

EXAMPLE 6

Effect of siRNA Inhibition of CD73 on Pancreatic Cancer Cell Growth

Short interfering RNA (siRNA) can be used to suppress CD73 expression in pancreatic cancer cells. Double stranded siRNA complexes may be designed using the following guidelines: (1) a double stranded RNA complex may be composed of a 21-nucleotide sense and 21-nucleotide anti-sense strand, both with a 2-nucleotide 3' overhang, *i.e.*, a 19 nucleotide

complementary region; (2) a 21 nucleotide sequence may be chosen in the coding region of the mRNA with a G:C ratio as close to 50% as possible, preferably within about 60% to about 40%, or alternatively within about 70% to about 30%; (3) preferably regions within about 75 nucleotides of the AUG start codon or within about 75 nucleotides of the termination codon are avoided; (4) preferably more than three guanosines in a row are avoided as poly G sequences can hyperstack and agglomerate; (5) preferably choose a sequence that starts with AA as this results in siRNA's with dTdT overhangs that are potentially more resistant to nucleases; (6) preferably the sequence is not homologous to other genes to prevent silencing of unwanted genes with a similar sequence. A negative control may be included, such a negative control suitably being the same nucleotide sequence as the test siRNA but scrambled such that it lacks homology to any other gene.

The above guidelines are solely an aid to designing suitable RNA oligonucleotides and are not a limitation of the interfering RNA oligonucleotides and related methods of use in the present invention.

EXAMPLE 7

Screening of Compound Libraries for Ecto-5'-Nucleotidase or CD73 Inhibitors Using an Enzymatic Assay

Two different but complementary approaches can be applied to identify novel small molecular weight inhibitors of CD73. One is the high throughput screen of small molecule libraries using an *in vitro* enzymatic assay of CD73. The other is the CD73 homolog model, based on virtual screening of chemical structure libraries and optimization of lead compounds.

To produce CD73 protein for *in vitro* nucleotidase assays, the CD73 gene (Accession No: NM002526) can be cloned into an expression vector with a tag and the protein expressed *in vitro* using the TNT coupled transcription and translation kit (Promega). This assay can then be optimized and used to screen various compound libraries for CD73 inhibitors.

All of the compositions and/or methods and/or apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the

compositions and/or methods and/or apparatus and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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U.S. Patent 4,452,901
U.S. Patent 4,458,066
U.S. Patent 5,221,605
U.S. Patent 5,238,808
U.S. Patent 5,279,721
U.S. Patent 5,310,687
U.S. Patent 5,354,855
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CLAIMS

1. A method of diagnosing or predicting development of pancreatic cancer in a subject comprising:
 - (a) obtaining a pancreatic cell sample from said subject; and
 - (b) assessing ecto-5'-nucleotidase activity or expression in said cell of said sample,wherein increased activity or expression of ecto-5'-nucleotidase in said cell, when compared to a normal cell of the same type, indicates that said subject has or is at risk of developing pancreatic cancer.
2. The method of claim 1, wherein said cell is a pancreatic tumor cell.
3. The method of claim 1, wherein assessing comprises assessing ecto-5'-nucleotidase expression.
4. The method of claim 3, wherein assessing ecto-5'-nucleotidase expression comprises Northern blotting.
5. The method of claim 3, wherein assessing ecto-5'-nucleotidase expression comprises quantitative RT-PCR.
6. The method of claim 3, wherein assessing ecto-5'-nucleotidase expression comprises Western blotting.
7. The method of claim 3, wherein assessing ecto-5'-nucleotidase expression comprises quantitative immunohistochemistry.
8. The method of claim 1, wherein assessing comprises assessing ecto-5'-nucleotidase activity.
9. The method of claim 1, wherein said subject has previously been diagnosed with cancer.

10. The method of claim 1, wherein said subject has not previously been diagnosed with cancer and appears cancer free at the time of testing.
11. The method of claim 1, further comprising administering a prophylactic cancer treatment to said subject following testing.
12. The method of claim 1, further comprising administering a cancer therapy to said subject following testing.
13. The method of claim 12, wherein said cancer therapy is a chemotherapy, a radiotherapy, an immunotherapy, a gene therapy, a hormonal therapy or surgery.
14. A method of predicting the efficacy of a pancreatic cancer therapy comprising:
 - (a) administering a cancer therapy to said subject;
 - (b) obtaining a pancreatic tumor cell sample from said subject; and
 - (c) assessing ecto-5'-nucleotidase activity or expression in said tumor cell of said sample,wherein decreased activity or expression of ecto-5'-nucleotidase in said tumor cell, when compared to a tumor cell of the same type prior to treatment, indicates that said therapy is efficacious.
15. The method of claim 14, wherein assessing ecto-5'-nucleotidase expression comprises measuring ecto-5'-nucleotidase protein levels.
16. The method of claim 14, wherein assessing ecto-5'-nucleotidase expression comprises measuring ecto-5'-nucleotidase transcript levels.
17. The method of claim 14, further comprising assessing ecto-5'-nucleotidase activity or expression at multiple time points.
18. A method of screening a candidate compound for anti-cancer activity comprising:
 - (a) providing a pancreatic cancer cell;
 - (b) contacting said cell with a candidate compound; and

(c) assessing the effect of said candidate compound on ecto-5'-nucleotidase expression or activity,

wherein a decrease in the amount of ecto-5'-nucleotidase expression or activity, as compared to the amount of ecto-5'-nucleotidase expression or activity in a similar cell not treated with said candidate compound, indicates that said candidate compound has anti-cancer activity.

19. The method of claim 18, wherein said candidate compound is a protein, a nucleic acid or an organo-pharmaceutical.
20. The method of claim 18, wherein said cell is a pancreatic tumor cell.
21. The method of claim 18, wherein assessing comprises assessing ecto-5'-nucleotidase expression.
22. The method of claim 21, wherein assessing ecto-5'-nucleotidase expression comprises Northern blotting.
23. The method of claim 21, wherein assessing ecto-5'-nucleotidase expression comprises quantitative RT-PCR.
24. The method of claim 21, wherein assessing ecto-5'-nucleotidase expression comprises Western blotting.
25. The method of claim 21, wherein assessing ecto-5'-nucleotidase expression comprises quantitative immunohistochemistry.
26. The method of claim 18, wherein assessing comprises assessing ecto-5'-nucleotidase activity.
27. A method of treating cancer comprising administering to a subject in need thereof a composition that inhibits ecto-5'-nucleotidase activity.

28. The method of claim 27, wherein said composition inhibits ecto-5'-nucleotidase expression.
29. The method of claim 27, wherein said composition is a protein, a nucleic acid or an organo-pharmaceutical.
30. The method of claim 29, wherein said protein is an antibody that binds immunologically to ecto-5'-nucleotidase.
31. The method of claim 29, wherein said nucleic acid is a ecto-5'-nucleotidase antisense nucleic acid, a ecto-5'-nucleotidase RNAi nucleic acid, or an antibody encoding a single-chain antibody that binds immunologically to ecto-5'-nucleotidase.
32. The method of claim 27, further comprising administering a second cancer therapy to said subject.
33. The method of claim 32, wherein said second cancer therapy is a chemotherapy, a radiotherapy, an immunotherapy, a gene therapy, a hormonal therapy or surgery.
34. The method of claim 27, wherein said composition is administered more than once.
35. A method of diagnosing or predicting development of pancreatic cancer in a subject comprising subjecting said subject to whole body scanning for ecto-5'-nucleotidase activity or expression in a cell.
36. A method of monitoring an anticancer therapy comprising assessing the expression or function of ecto-5'-nucleotidase in a pancreatic cancer cell of a subject following or during provision of said anticancer therapy.

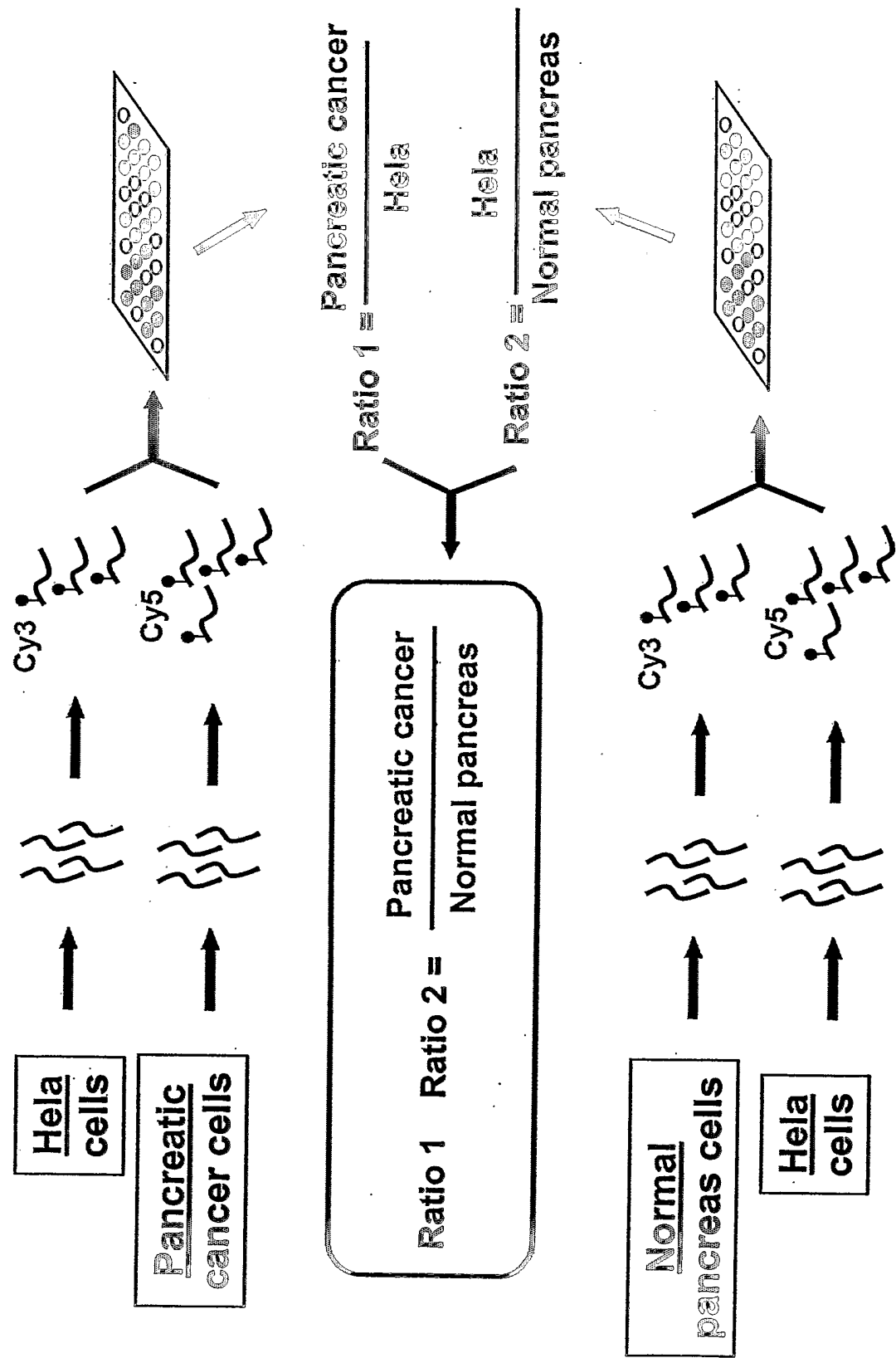


FIG. 1

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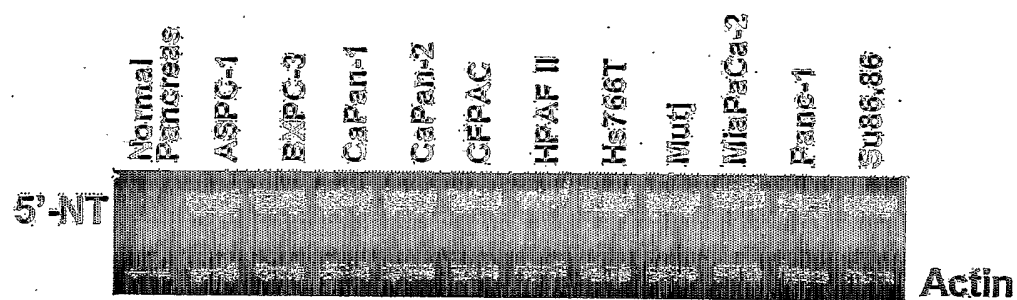


FIG. 2A

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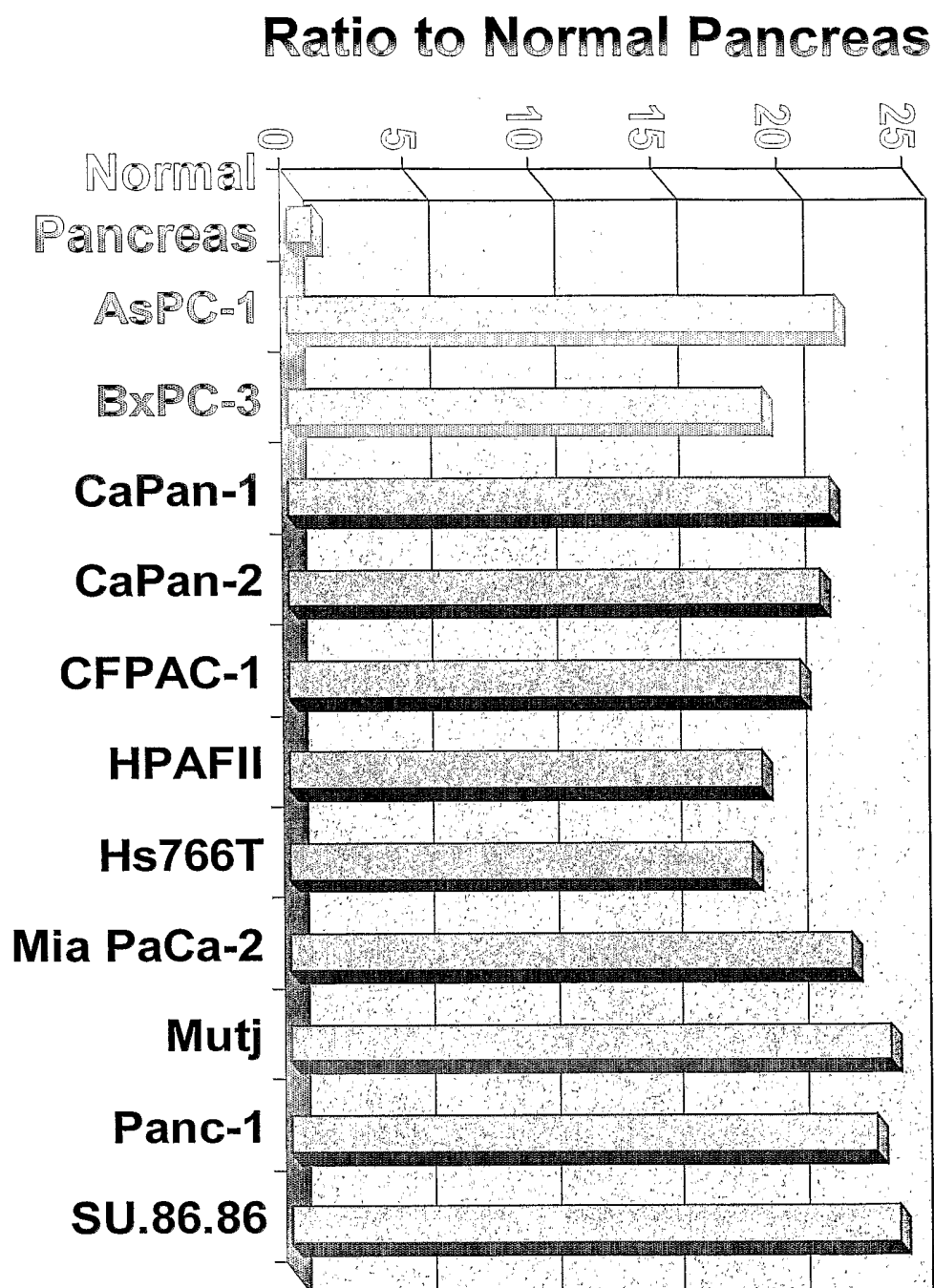


FIG. 2B

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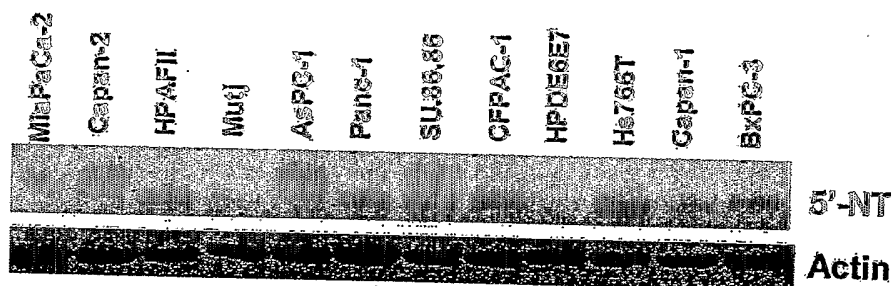


FIG. 3

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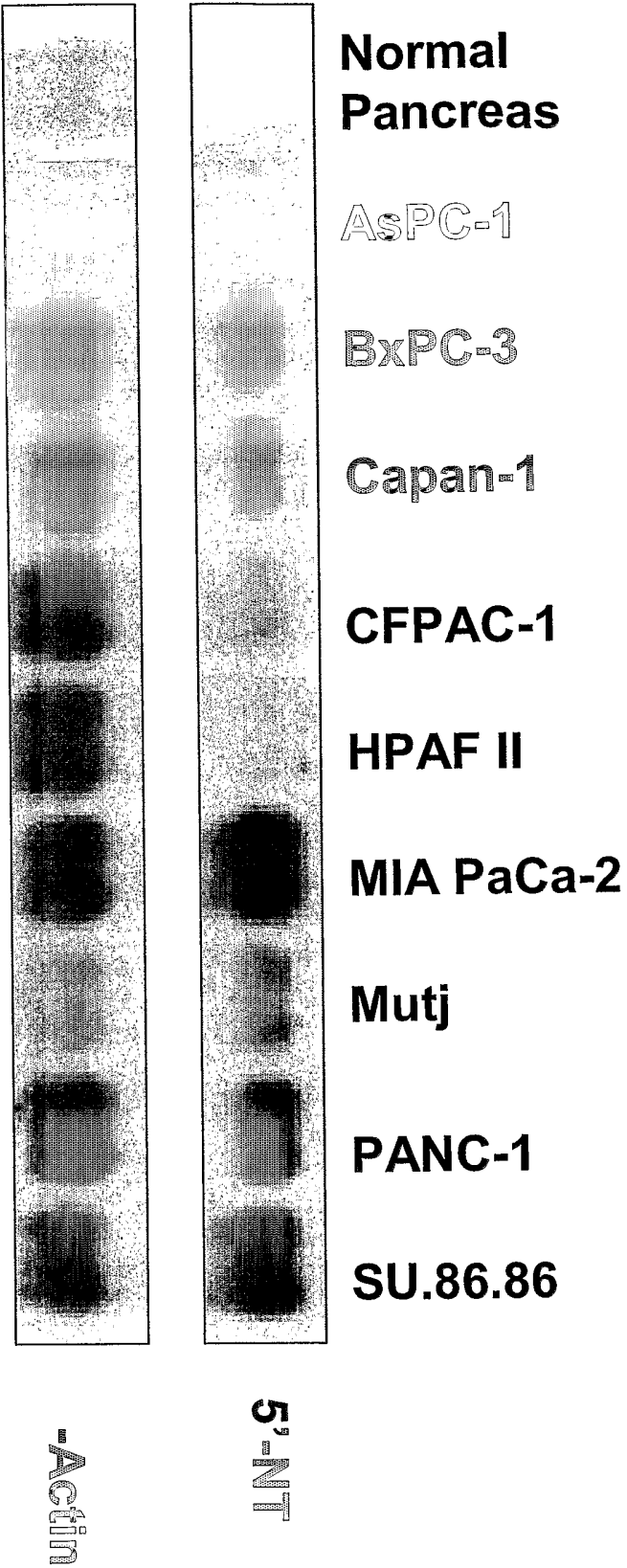


FIG. 4A

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Ratio to Normal Pancreas

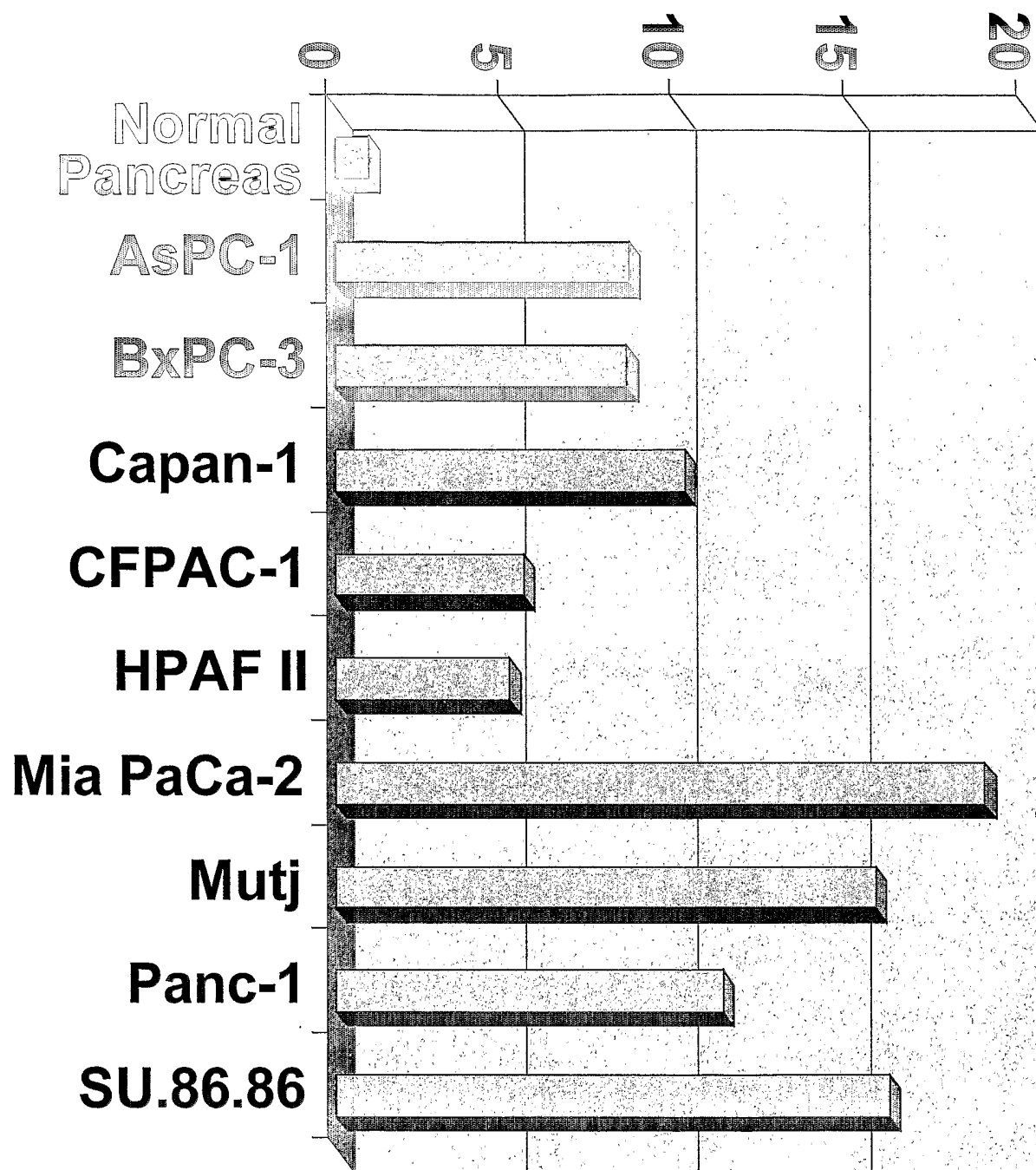


FIG. 4B

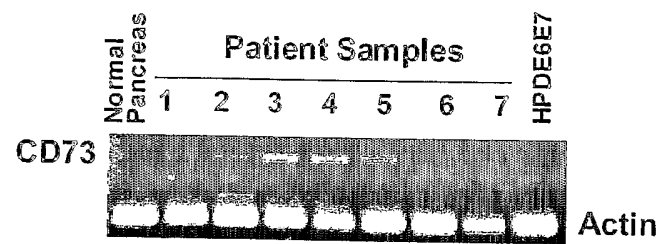


FIG. 5A

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Ratio to Normal Pancreas

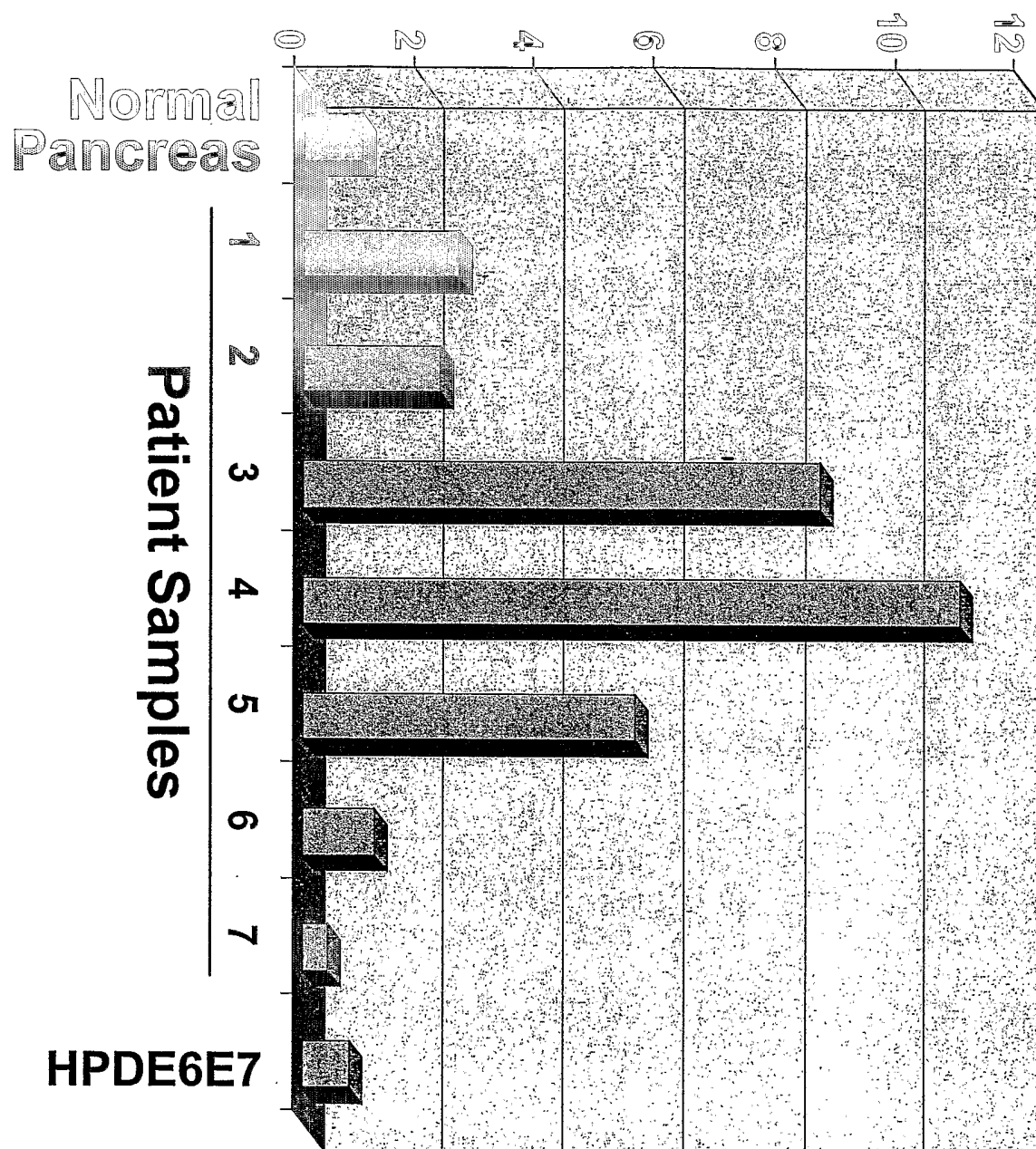


FIG. 5B

INTERNATIONAL SEARCH REPORT

ational Application No

/US2004/006404

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 C12N15/11 A61K39/395

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q C12N A61K

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FLOCKE K ET AL: "Monoclonal antibodies against 5'-nucleotidase from a human pancreatic tumor cell line: their characterization and inhibitory capacity on tumor cell adhesion to fibronectin substratum." EUROPEAN JOURNAL OF CELL BIOLOGY. JUN 1992, vol. 58, no. 1, June 1992 (1992-06), pages 62-70, XP009033209 ISSN: 0171-9335	1-17
Y	page 68, right-hand column, lines 24-26 ----- -/--	18-26

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

13 July 2004

Date of mailing of the international search report

27/07/2004

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Knudsen, H

INTERNATIONAL SEARCH REPORT

ational Application No

/US2004/006404

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FLOCKE K ET AL: "Isolation and characterization of 5'-nucleotidase of a human pancreatic tumor cell line." BIOCHIMICA ET BIOPHYSICA ACTA. 29 JAN 1991, vol. 1076, no. 2, 29 January 1991 (1991-01-29), pages 273-281, XP002288216 ISSN: 0006-3002	1-17
Y	page 279; table 1	18-26
X	MARTIN J M ET AL: "Activities of gamma-glutamyl transferase, 5'-nucleotidase and alkaline phosphatase in human duodenal aspirate." ENZYME. 1986, vol. 35, no. 4, 1986, pages 189-196, XP009033208 ISSN: 0013-9432	1-17
Y	page 196, left-hand column, last paragraph; table 3	18-26
X	WO 01/80884 A (IDEC PHARMA CORP) 1 November 2001 (2001-11-01) claims 36,37,43,44	27-34
Y	WO 01/94629 A (AVALON PHARMACEUTICALS) 13 December 2001 (2001-12-13) SEQ ID NO:299 & claim 1	18-26
A	KOZIOLKIEWICZ MARIA ET AL: "The mononucleotide-dependent, nonantisense mechanism of action of phosphodiester and phosphorothioate oligonucleotides depends upon the activity of an ecto-5'-nucleotidase" BLOOD, vol. 98, no. 4, 15 August 2001 (2001-08-15), pages 995-1002, XP002230446 ISSN: 0006-4971 page 999, left-hand column, lines 12,13	1-36
A	HAN HAIYONG ET AL: "Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray." CANCER RESEARCH. 15 MAY 2002, vol. 62, no. 10, 15 May 2002 (2002-05-15), pages 2890-2896, XP002267297 ISSN: 0008-5472 abstract	1-36
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INTERNATIONAL SEARCH REPORT

ational Application No

PCT/US2004/006404

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	HAN HAIYONG ET AL: "Overexpression of ecto-5'-nucleotidase in pancreatic cancer." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 44, July 2003 (2003-07), page 1001, XP001182183 & 94TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH; WASHINGTON, DC, USA; JULY 11-14, 2003 ISSN: 0197-016X the whole document -----	1-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/006404

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-36
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-13, 18-26 and 35-36 are directed to a method involving surgery practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 14-17 and 27-34 are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International Application No

/US2004/006404

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