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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF MARYLAND BALTIMORE** [US/US]; 515 West Lombard Street, 4th Floor, Baltimore, Maryland 21201-1602 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **KEAY, Susan, K.** [US/US]; 3117 Storybook Court, Ellicott City, MD 21042-2371 (US). **ZHANG, Chen-Ou** [US/US]; 5030 Stone Hill Drive, Ellicott City, MD 21043 (US).

(74) Agent: **EVANS, Judith, A.**; Evans & Moliinelli PLLC, 6411 Chapel View Road, Clifton, VA 20124 (US).

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(54) Title: A FACTOR AND METHOD TO INCREASE PERMEABILITY ACROSS A BIOMEMBRANE USING ANTIPROLIFERATIVE FACTOR

(57) Abstract: It has been discovered that antiproliferative factor (APF) increases paracellular permeability by decreasing production of the tight junction proteins zonula occludens-1 and occludin. The present invention is directed to compositions that contain APF for increasing membrane permeability and to methods for improving delivery of a diagnostic or therapeutic agent to a site of action in an animal by pretreating the animal with APF. The compositions and methods are relevant to treating any disease, especially cancer, where local administration of APF increases membrane permeability thereby increasing localized uptake of the anticancer agent into cancer cells.

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ANTIPROLIFERATIVE FACTOR TO INCREASE BIOMEMBRANE PERMEABILITY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of Provisional Application No. 60/663058, filed March 18, 2005, the entire contents of which are hereby incorporated by reference as if fully set forth herein, under 35 U.S.C. §119(e).

STATEMENT OF GOVERNMENT INTEREST

[0002] This work was supported in part by funding from the National Institutes of Health (NIDDK R01 DK52596 and R01 DK68491) and Merit Review Funding from the Veterans Administration. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates to antiproliferative factor (APF), a protein unique to the urine of IC (interstitial cystitis) patients and its use for increasing membrane permeability thereby improving delivery of molecules of interest, such as therapeutic molecules, to or across a biomembrane.

BACKGROUND OF THE INVENTION

[0004] Improved delivery of diagnostic and therapeutic drugs to tumors is important for early detection and prevention of the progression of cancer and other diseases. Methods that enable doctors to increase the preferential delivery of drugs to the tumors are beneficial for imaging and selectively treating tumors while not damaging the surrounding normal tissues. Drugs that target cancer cells must overcome a significant number of structural barriers within the tumor in order to be effective. They must first exit the tumor blood vessels, migrate past the

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support structures that underlie the vessels and eventually make their way to the cancer cells. As a result of these structural barriers, very little drug injected into the blood stream of a patient is able to reach and destroy cancer cells. One potential solution to this problem is to increase the permeability of the blood vessels within the tumor to permit more therapeutic drug to reach and kill substantially more cancer cells, or to increase paracellular permeability of other tissues that impose a barrier between the drug and the cancerous cells.

[0005] U.S. Patent No. 6,737,064, entitled "Method for the diagnosis of neoplastic tissue comprising administering a vasopermeability enhancing peptide of human interleukin-2," covers methods for using a portion of the cytokine interleukin-2 known as a permeability enhancing peptide (PEP) to enhance the diagnosis of cancer. Vasopermeation Enhancement Agents (VEAs) are a class of agents that enhance the efficacy of cancer therapeutics by increasing their uptake into solid tumors. VEAs work by selectively targeting known vasoactive compounds (i.e. molecules that cause tissues to become more permeable) to solid tumors. Once localized at the tumor site, VEAs make blood vessels within a tumor more leaky, allowing administered chemotherapies to better penetrate the tumor mass. In published reports, scientists have seen an almost 400% increase in the normal amount of chemotherapeutic agent taken up by solid tumors with a VEA pre-treatment. The most advanced VEA clinical candidate, NHS76/PEP2, as a pretreatment for six approved chemotherapy drugs to demonstrate the viability of the VEA approach. These studies show that pretreatment with NHS76/PEP2 can markedly increase the clinical efficacy of chemotherapeutic drugs for the treatment of solid tumors, including Doxorubicin, Taxol, Vinblastine, VP-16 and Taxotere in tumor therapy experiments.[25]. This approach may significantly enhance the value of approved drugs.

[0006] Bladder cancer is the second most-common genitourinary cancer in the United States, accounting for approximately two percent of all malignant tumors and approximately seven percent of all urinary tract malignancies in U.S. men. Men are three times more frequently affected than women. The disease usually occurs between 60-70 years of age and the age-adjusted bladder cancer rate in white men is almost twice that of black men. Most bladder

cancers (over 90%) are carcinomas of the transitional epithelium of the bladders mucosal lining (transitional cell carcinoma (TCC)). Although 90 percent of the cases are localized at diagnosis, up to 80 percent recur. More than 13,000 patients died from invasive bladder cancer in 2005 alone. Radical cystectomy is the most commonly prescribed treatment for patients with muscle-invasive bladder cancer, or for those with a nonmuscle-invasive disease that is refractory to intravesical therapy.[26]

[0007] The urinary bladder is lined with an epithelial membrane barrier, i.e., an interior bladder wall that forms an important barrier against noxious substances such as toxins and microbial pathogens. Tight junctions (or zona occludens), established and maintained between epithelial cells in the interior balder wall are crucial to the development and normal functioning of epithelial cells. They create a barrier to the diffusion of solutes through the mucosa and are critical for the generation of chemical and electrical gradients necessary for vectorial transport processes such as absorption and secretion. Tight junctions have both transmembrane components with adhesive functions and cytosolic proteins that provide a direct or indirect link to the cytoskeleton. They are formed by claudin and occludin proteins, joining the cytoskeletons of the adjacent cells. However, while tight junctions are important for normal bladder function, they also present a formidable barrier to agents needed to diagnose and treat bladder cancer.

[0008] A drug product may cost as much as $880 M (million) to develop and obtain FDA approval. However, it has been estimated that about 30-40% of the molecules that reach development are eventually rejected due to problems with absorption, distribution, metabolism, and excretion (ADME). Therefore there is a great need for compounds that enhance drug absorption and delivery in general, and for diagnosing and treating bladder cancer in particular.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] The present invention is illustrated by way of example, and not by way of limitation, in the figures of the accompanying drawings and in which:

[0010] FIG. 1. Paracellular permeability of IC cells vs. normal bladder epithelial cell and APF-treated vs. Mock APF-treated normal cells.

[0011] FIG. 2. Effect of Anti-APF antibody on paracellular permeability of IC cells.

[0012] FIG. 3. Western blot analysis of ZO-1, occludin, E-cadherin, α-1-catenin, vimentin, and α-2-integrin levels in IC cells (IC), normal bladder epithelial cells (NBC), and normal cells treated with 2 ng/flask of HPLC-purified APF (APF) or an equivalent amount of Mock APF (Mock). Actin is an internal control.

[0013] FIG. 4. Immunofluorescence microscopy for ZO-1 in explanted bladder epithelial cells (A, C, E) from IC patients, (B, D, F) from normal controls, (G) from normal cells treated with 0.7 ng/well APF, and (H) from normal cells treated with an equivalent amount of Mock APF.

[0014] FIG. 5. Immunofluorescence microscopy for occludin in explanted bladder epithelial cells (A, C, E) from IC patients, (B, D, F) from normal controls, (G) from normal cells treated with 0.7 ng/well APF, and (H) from normal cells treated with an equivalent amount of Mock APF.

SUMMARY OF THE INVENTION

[0015] Certain embodiments of the present invention are directed to compositions for increasing membrane permeability in an animal containing antiproliferative factor. The forms of APF useful in the compositions and methods of the invention includes isolated and purified animal APF, particularly from humans, recombinant antiproliferative factor, synthetic antiproliferative factor, and fragments of antiproliferative factor. In some embodiments the membrane is epithelium including urinary bladder epithelium, gastric epithelium, lung epithelium, skin epithelium, nasal, kidney, urethra, ureter, and intestinal epithelium. Any membrane having tight junctions can be treated with an APF-containing composition of the present invention to increase paracellular permeability and drug uptake, including endothelium, particularly in the blood brain barrier or in a blood tumor barrier. In

some embodiments the membrane is mucosa. In certain embodiments the amount of antiproliferative factor applied to the membrane is from about 0.001 ng/ml to about 100 ng/ml. In embodiments where APF is administered locally, the amount can be higher.

[0016] Some embodiments are directed to compositions for increasing uptake of a compound in an animal, containing antiproliferative factor, especially where the compound is a diagnostic or therapeutic agent.

[0017] Some other embodiments are directed to methods for improving delivery of a compound to a site of action in an animal by 1) pretreating the animal with antiproliferative factor in an amount sufficient to increase uptake of the compound; and 2) following pretreatment with antiproliferative factor, administering the compound to the animal. The amount of APF used for pretreatment is from about 0.001 ng/ml to about 100 ng/ml in some embodiments, but can be higher if APF is administered locally. APF can be administered by any route known in the art depending on the condition being treated or the type of diagnostic method being used. APF administration after the initial pretreatment period, which is typically from 24-48 hours, also comes within the scope of the present invention. In other words, APF administration does not have to be discontinued once the diagnostic or therapeutic agent is administered. APF can be co-administered with these compounds. The 24 hour minimum pretreatment time is based on the fact that APF was discovered to alter paracellular permeability and open tight junctions by changing expression of tight junction proteins and cellular adhesion molecules. This takes time. 24 hours has so far been required to cause the cells to change their protein expression patterns to affect tight junctions. However if it is discovered that certain cells have an unusually fast metabolism, such as perhaps certain cancer cells or other rapidly dividing cells, the pretreatment time could be shorter than 24 hours.

[0018] In some other embodiments APF is administered multiple times before administering the diagnostic, imaging, or therapeutic agent. In those embodiments where an anticancer agent is administered after pretreatment with APF, the site of action can be a tumor. In some embodiments the tumor is cancer and the cancer is a member selected from the group

comprising bladder cancer, stomach cancer, lung cancer, skin cancer, nasal cancer, brain cancer, vaginal cancer, kidney cancer, esophageal cancer, urethra cancer, ureter cancer, colon cancer and intestinal cancer. In one embodiment APF is administered intravesicaly prior to administering an anticancer drug to treat bladder cancer.

[0019] APF used in the embodiments of the methods of the invention can be isolated and purified animal APF (especially human), or recombinant antiproliferative factor, synthetic antiproliferative factor, and fragments of antiproliferative factor. In certain embodiments APF is administered 24-48 hours before administering the diagnostic or therapeutic compound to increase uptake and efficacy of these compounds by first increasing paracellular permeability.

DEFINITIONS

[0020] A tight junction is an intercellular junction between cells in a membrane, including epithelial cells and endothelial cells lining blood vessels, in which the outer layers of the cell membranes fuse, reducing the ability of larger molecules and water to pass between the cells. Tight junctions reduce paracellular permeability which reduces the ability of larger molecules and water to pass between the cells.

[0021] Paracellular permeability means the permeability (or leakiness) between cells in a tissue membrane (as opposed to through the cell membrane itself). When tight junctions become leaky, paracellular permeability increases.

[0022] Antiproliferative Factor (APF) means a 1.485 kDa (or 1,485 Da) sialoglycopeptide that is secreted specifically by bladder epithelial cells from patients with interstitial cystitis (IC), a chronic bladder disorder commonly associated with denudation or thinning of the bladder epithelium. The peptide sequence of APF is identical to amino acids 541–549 in the sixth transmembrane region of frizzled 8, a Wnt ligand receptor. The glycosyl moiety of APF is sialic acid α-2,3 linked to galactose β1–3 N-acetyl-galactosamine, which is O-linked to the

N-terminal threonyl residue of the nonapeptide. For the purpose of this document, APF also includes APF with amino acid substitutions that do not change its ability to increase paracellular permeability as described herein, and includes recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments of antiproliferative factor. APF also includes functional derivatives and agonists as described in U.S. Patent No. 5,962,645.

[0023] Increased permeability means having a property of allowing an increased mass of a drug or compound to travel through a cellular (tissue) barrier relative to a cellular barrier that has not been exposed to a permeabilizing reagent. An effective amount of the permeabilizing reagent will increase the permeability of the vessel wall and/or tissue membrane such that sufficient quantities of a drug may pass through a membrane having tight junctions, including epithelium and endothelium, and into the adjacent target tissue, where it can exert a therapeutic or diagnostic effect. (APF does not affect the cell membrane itself).

[0024] APF-induced uptake of diagnostic agents or therapeutic agents in an animal, means uptake via increased paracellular permeability to diagnostic and therapeutic agents through one or more membranes. It does not refer to active transport of these compounds into a cell. APF-treated membranes become leaky thereby increasing paracellular permeability of diagnostic and therapeutic agents that are too large to pass through the intercellular spaces of untreated membranes.

DETAILED DESCRIPTION

[0025] Some embodiments of the present invention relate to compositions and methods for increasing the permeability of a membrane in an animal by administering the drug antiproliferative factor (APF). APF includes recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments of antiproliferative factor. In some embodiments the membrane is epithelium, including urinary bladder epithelium, gastric epithelium, lung epithelium, skin epithelium, nasal, kidney, urethra, ureter, and intestinal epithelium. In other embodiments the membrane is mucosa

including urinary bladder mucosa, intestinal mucosa, gastric mucosa and lung mucosa. In still other embodiments the membrane is vascular endothelium, including the blood brain barrier.

[0026] Certain embodiments are directed to methods for improving delivery of a diagnostic or therapeutic compound to a site of action in an animal by pretreating the animal with antiproliferative factor in an amount sufficient to facilitate transport of the diagnostic or therapeutic compound across one or more membranes in the animal to the site of action. Pretreatment with APF is typically done about 24 to 48 hours before administering the compound. In certain other embodiments uptake of a diagnostic or therapeutic agent is increased by administering APF locally to the site of action. For example APF can be administered to an area of the body having cancer, or even into a solid tumor itself, prior to administration of the diagnostic or therapeutic agent. In a preferred embodiment APF is administered intravesicaly or locally prior to administering an agent to diagnose or treat bladder cancer. The present methods and compositions are effective on any membrane having tight junctions. An effective amount of APF for increasing membrane permeability is typically an amount from about 0.001 ng/ml to about 100 ng/ml, which is about one hundred times the level of APF found in patients having intersticial cystitis. Where APF is applied locally, for example to a cancerous tumor, the concentration of APF can be increased above 100 ng/ml. Some embodiments of the invention are directed to pharmaceutical compositions that include antiproliferative factor (APF) in an amount from about 0.001 ng/ml to about 100 ng/ml to increases membrane permeability.

[0027] Epithelial cells line the interior bladder wall and form an important barrier against noxious substances. While the bilipid membrane of these cells, uroplakins, and a mucus layer may contribute to the barrier against water, urea, protons, and ammonia[1,2], the barrier against the flux of larger substances such as inulin and mannitol in other epithelia is thought to result from the formation of intercellular junctions (including tight junctions and adherens junctions between cells)[3-9]. Tight junctions impede paracellular permeability of compounds between cells in a membrane (as opposed to through the cell membrane itself). When we discuss APF-

induced uptake of diagnostic agents or therapeutic agents in an animal, we are not referring to active transport of these compounds into a cell. By uptake we refer to the paracellular permeability of diagnostic and therapeutic agents through one or more membranes. APF-treated membranes become leaky thereby increasing paracellular permeability of diagnostic and therapeutic agents that are too large to pass through the intercellular spaces of untreated membranes. In this way a tissue such as urinary bladder, the brain or a tumor, for example, takes up the agents.

[0028] Tight junctions have both transmembrane components with adhesive functions and cytosolic proteins that provide a direct or indirect link to the cytoskeleton, and they have been demonstrated in terminally differentiated epithelial cells as well as underlying cell layers of mammalian tissue. Tight junctions are the most apical junctional complexes in urinary bladder epithelium and were recently shown to include zonula occludens -1 (ZO-1) and occludin proteins in the mammalian urinary bladder[8].

[0029] Previous reports indicate that the bladder epithelial barrier is abnormal in patients having interstitial cystitis (IC), a debilitating chronic painful bladder disorder characterized by thinning or denudation of the bladder epithelium. IC patients have increased absorption of urea given intravesically[10], and increased pain following intravesical potassium chloride infusion[11], indicating the possibility that bladder epithelial permeability may be increased in IC patients as compared to controls. Bladder biopsy specimens from IC patients also have decreased levels of ZO-1[12], indicating a possible decrease in zonula occludens tight junction formation in IC. The entire contents of references 10-12 are hereby incorporated by reference as if fully set forth herein.

[0030] We previously reported the discovery of an antiproliferative factor (APF) peptide that is unique to bladder epithelial cells in patients with IC[13, 37] APF is a frizzled 8 protein-related sialoglycopeptide[14] that profoundly inhibits normal bladder epithelial cell growth and production of heparin-binding epidermal growth factor-like growth factor (HB-EGF)[15, 16]. Microarray analysis indicated that APF applied to normal bladder cells induces changes in the pattern of epithelial cell gene expression with increased mRNA for E-cadherin and

decreased mRNA for vimentin, alpha 2 catenin and alpha 1 integrin[17]. Because some of these proteins function directly or indirectly in bladder epithelial cell adhesion, we evaluated the role of APF on the integrity of the bladder epithelial barrier. We discovered that APF inhibits expression of the tight junction proteins ZO-1 and occlude in normal bladder epithelium, and microscopic examination revealed that tight junctions were decreased after a 48 hour exposure to APF. Moreover, the decrease in tight junctions resulted in increased epithelial paracellular (intercellular) permeability to the otherwise impermeable tracers mannitol and inulin. The entire contents of references 13-17, and 37 are hereby incorporated by reference as if fully set forth herein.

[0031] The bladder itself is made up of four layer: the epithelium, the lamina propria, the muscularis propria, and the perivesical soft tissue. These layers are important landmarks in determining how deeply a bladder tumor has invaded and the ultimate stage of the cancer. The epithelium, which lines the bladder and is in contact with the urine, is referred as transitional epithelium or urothelium. Most bladder cancers originate from the cells of this transitional epithelium. The urethra, ureters and the pelvis of the kidney are also lined by this transitional epithelium, therefore, the same types of cancers seen in the bladder can also occur in these sites. Under the epithelium is the lamina propria, a layer of connective tissue and blood vessels. The endothelium of blood vessels also has tight junctions. Within the lamina propria, there is a thin and often discontinuous layer of smooth muscle called the muscularis mucosae. This superficial layer of smooth muscle is not to be confused with the true muscular layer of the bladder called the muscularis propria or detrusor muscle. Muscularis propria or detrusor muscle is a deep muscle layer consists of thick smooth muscle bundles that form the wall of the bladder. For purposes of staging bladder cancer, the muscularis propria has been divided into a superficial (inner) half and a deep (outer) half. The outermost layer consists of fat, fibrous tissue and blood vessels called perivesical soft tissue. When the tumor reaches this outer layer, it is considered out of the bladder. Once bladder cancer has penetrated the outer layer of the muscularis propria, it metastasizes readily to other organs.

[0032] In order for diagnostic or chemotherapeutic agents to penetrate deep enough to reach tumors or cancer cells in the outer muscularis propria, they must penetrate these four layers of the bladder. If the tight junctions in the bladder epithelium can be opened, these agents can travel between cells in the paracellular spaces instead of being endocytosed by the cell. They could penetrate deeper into the tissue, and they would not be at risk of being degraded by cellular enzymes. Studies indicating that bladder epithelial permeability is increased in IC patients as compared to controls, led us to study whether APF is involved in the regulation of bladder epithelial paracellular barrier function. We tested this hypothesis by looking at the effect of APF on the transport or flux of radiolabeled inulin and mannitol through intercellular junctions in normal bladder epithelial cell monolayers that express ZO-1 and occludin in a peripheral distribution *in vitro*. We also determined the effects of APF on expression of several additional proteins involved directly or indirectly in the formation of tight junctions and adherens junctions in bladder epithelial cells.

[0033] It has now been discovered that APF increases paracellular permeability in normal epithelium, i.e. permeability between and around cells, in normal bladder epithelium. The data presented below show that treating normal bladder epithelial monolayers with APF in amounts of 1 ng/ml for 48 hours increased paracellular permeability to the otherwise impermeable tracers mannitol and inulin. This increased permeability was associated with a significant decrease in expression of the 220 kilo Dalton tight junction protein Zona Occludens-1 and the 60 kDa protein occludin. Increased paracellular permeability induced by APF is also accompanied by a decrease in expression of intracellular proteins α-1-catenin, vimentin, and α-2-integrin, and an increase in E-cadherin. Further, microscopic observation of APF-treated epithelium showed a decrease in tight junctions. Thus, various embodiments of the present invention are directed to compositions and methods for increasing membrane permeability by applying APF. Other methods are directed to methods to treat cancer by pretreating a patient with APF to facilitate uptake of an anticancer agent, prior to administering anticancer agents. The cancer can be any cancer, but especially cancer that is a member selected from the group comprising bladder cancer, stomach cancer, lung cancer,

skin cancer, nasal cancer, kidney cancer, esophageal cancer, urethra cancer, ureter cancer, colon cancer and intestinal cancer, where the anticancer agent has to pass through a membrane barrier to reach the cancer cells.

[0034] Cadherins constitute a superfamily that shares a basic structure. They include E-cadherin [epithelial or uvomorulin], P-cadherin [placental], N-cadherin [neural or A-CAM], and L-CAM [liver cell adhesion molecule]. At least two major subfamilies such as E- and P-cadherin are expressed by tumor cells. E-cadherin is the prime mediator of epithelial cell-cell adhesion via calcium dependent, homotypic interactions.[36] It is required for the induction and maintenance of normal epithelial integrity. It is complexed in the cytoplasm with a-, ß- and y catenin to form a functional unit called the E-cadherin-catenin unit (ECCU). This unit is then thought to bind to the actin cytoskeleton. Disruption of any of the components of the ECCU results in loss of E-cadherin-mediated adhesion. [27-32]

[0035] Integrins are a family of transmembrane glycoproteins consisting of noncovalent heterodimers. They interact with a wide variety of ligands including extracellular matrix glycoproteins, complement, and other cells; their intracellular domains interact with the cytoskeleton. Vimentin is a polypeptide that copolymerizes with other subunits to form the intermediate filament cytoskeleton of mesenchymal cells.

[0036] The formation of tight junctions is thought to occur through the recruitment and assembly of the highly related PDZ-containing proteins [PDZ stands for conserved sequence elements within e proteins: PSD95/SAP90, dlgA, and ZO-1], ZO-1,[1] ZO-2, and ZO-3, tethered to the integral membrane protein, occludin . The tight junction is also associated with the actin cytoskeleton through direct interaction with ZO-1. Loss of E-cadherin expression is associated with high grade and advanced stage breast, prostatic and bladder tumors. [33-34] It has been reported that reduced cadherin expression is correlated with invasiveness rather than metastasis.35 Importantly, APF increased E-cadherin expression in epithelium.

[0037] Without being bound by theory, the decrease in ZO-1 and occludin in epithelium caused by APF also contributes to increased paracellular permeability. Tight junctions in any

tissue of the body have ZO-1 and occluding. Further, adherins, integrins and vimentin are also ubiquitous cell adhesion proteins. Therefore the compositions and methods of the present invention for increasing membrane permeability and increasing uptake of diagnostic agents and therapeutic drugs with APF can be used on any membrane having tight junctions, including endothelium that lines blood vessels. As is discussed below, this has implications for improving the delivery of agents across the blood brain barrier.

[0038] To test the effects of APF on normal bladder epithelium grown in confluent monolayers normal bladder epithelial cell monolayers were grown on Transwell™ membrane inserts, serum-starved, and treated with APF or Mock APF for 48 hrs prior to performance of the permeability assay. Mock APF means a like preparation prepared using supernatant of bladder epithelial cells from a normal control and the same purification procedure, described in U.S. Patent No. 5,962,645, the entire contents of which are hereby incorporated by reference as if fully set forth herein. For these experiments 1 ng/ml of purified APF was applied, a concentration that is within the range of approximate APF concentrations found in human urine samples from IC patients in previous studies (0.1 – 2 ng/ml).

[0039] To determine whether altered permeability of bladder epithelial cells from IC patients could be demonstrated *in vitro*, paracellular flux of membrane impermeable [^{14}C]-mannitol or [^{3}H]-inulin tracers across a confluent monolayer of cells explanted from three IC patients and three normal controls was assessed (2 females, 1 male in each group) by measuring the amount of applied tracer found in the basal medium at various times. FIG. 1 shows the paracellular permeability of IC cells vs. normal bladder epithelial cell and APF-treated vs. Mock APF-treated normal cells. Paracellular flux of [^{3}H]-inulin (molecular weight: 5,200) or [^{14}C]-mannitol (molecular weight: 184) was measured in the basal medium of cells cultured on Transwell™ membranes at 0.5 hr, 1 hr, 2 hrs, 4 hrs, 6 hrs. The vertical axes plot the % of total inulin or mannitol flux versus time measured in hours on the horizontal axis. FIG. 1A shows the paracellular permeability of [^{3}H]-inulin in normal bladder epithelial cells (open bars), IC cells (upward slashes), normal cells treated with 1 ng/well purified APF(black

bars), and normal cells treated with an equivalent amount of Mock APF (downward slashes). FIG. 1B shows the paracellular permeability of [^{3}H]-inulin in normal bladder epithelial cells exposed to Ca^{2+}-free medium (black bars) (positive control) vs. normal medium (open bars). FIG. 1C shows the paracellular permeability of [^{14}C]-mannitol in normal bladder epithelial cells (open bars), IC cells (upward slashes), normal cells treated with 1 ng/ml HPLC-purified APF (black bars) and normal cells treated with an equivalent amount of Mock APF (downward slashes). FIG. 1D shows paracellular permeability of [^{14}C]-mannitol in normal bladder epithelial cells exposed to Ca^{2+}-free medium (black bars) (positive control) vs. normal medium (open). Values are means ± SD of data from triplicate experiments. + $p < 0.05$, * $p < 0.001$.

[0040] IC cells (upward slashes) had significantly increased paracellular permeability of both tracers by 1 hour. The levels of [^{14}C] - mannitol flux increased in the IC cell monolayers by 345.3 ± 159.3%, 210.1 ± 6.1%, 481.6 ± 34.4%, and 651.1 ±29.4% over normal bladder cell monolayers. FIG. IC The levels of [^{3}H]-inulin flux also increased in IC cells by 260.3 ± 11.1%, 478.3 ± 30.8%, 592.3 ± 27.8%, and 667.2 ± 138.7%, at 1, 2, 4, and 6 hours, respectively, compared to controls. FIG. 1A These results compared favorably with those obtained following culture in Ca^{2+}-free medium, which served as a positive control (FIG.s 1B and 1D).

[0041] Experiments were also conducted to determine whether APF affects bladder epithelial paracellular barrier function by increasing inter-cellular permeability in normal cells. To do this, normal bladder epithelial cell monolayers were grown on Transwell™ membrane inserts, serum-starved, and treated with APF or Mock APF for 48 hrs prior to performance of the permeability assay. Mock APF means a like preparation prepared using supernatant of bladder epithelial cells from a normal control and the same purification procedure. For these experiments 1 ng/ml of purified APF; a concentration that is within the range of approximate APF concentrations found in human urine samples in previous studies (0.1 – 2 ng/ml).[15]

[0042] APF pre-treatment of normal epithelium (black bars) significantly increased paracellular permeability of both tracers by 1 hour following their introduction into the cell

medium (FIG.s 1A and 1C). The levels of [^{14}C]- mannitol flux in APF-treated monolayers increased by 252.3 ± 88.6%, 372.9 ± 118.3%, 471.5 ± 161.2%, and 607.1 ± 69.1% at 1, 2, 4, and 6 hours, respectively, as compared to cell controls. The levels of [^{3}H]-inulin flux through the intercellular space increased by 138.1 ± 24.7%, 221.0 ± 44.5%, 406.8 ± 89.8%, and 480.9 ± 115.1% at 1, 2, 4, and 6 hours, respectively, as compared to cell controls. By contrast, the level of paracellular permeability for Mock APF-treated cells (downward slashes) did not differ significantly from cell controls at any time point.

[0043] To provide additional evidence that APF is the factor responsible for increasing paracellular permeability in IC cell monolayers, polyclonal rabbit antibodies against synthetic APF peptide were made. It was determined whether these antibodies could normalize permeability in IC cell monolayers in a dose-dependent manner. These antibodies bind specifically to both synthetic and HPLC-purified native APF in a dot-blot format, and they inhibit the antiproliferative effects of native APF in a thymidine incorporation assay (whereas preimmune antibodies from the same animals had no effect in either assay - data not shown). Paracellular flux of [^{3}H]-inulin or [^{14}C]-mannitol was measured in the basal medium of cells cultured on Transwell™ membranes following a 4 hour incubation immunohistochemically with labeled tracers. The results show that treatment of IC cells with these antibodies for 48 hours prior to performance of the permeability assay resulted in a dose-dependent decrease in the flux of both labeled tracers. The vertical axis of FIG. 2 shows the % decrease in 3H-inulin flux at four hours, and the horizontal axis shows the amount of Anti-APF antibody in micrograms/ml. FIG. 2A shows the paracellular permeability of [^{3}H]-inulin in IC cells treated with anti-APF antibody (black bars) or control normal cells treated with anti-APF antibody (white bars). FIG. 2B shows the paracellular permeability of [^{14}C]-mannitol in IC cells treated with anti-APF antibody (black bars) or control normal cells treated with anti-APF antibody (white bars), Values are mean ± SD of data from duplicate experiment. * $p < 0.05$.

[0044] To determine whether proteins involved in cell adhesion or tight junctions are altered in IC bladder epithelial cells, cells from IC patients (IC, column1) and normal controls

(NBC, column 2) were grown to confluence and total protein extracted for Western blot analysis. Experiments were also conducted to determine whether APF (column 3) could affect expression of tight junction proteins in normal cells. Confluent normal bladder epithelial cells from control patients were treated with APF or Mock APF (Mock APF, column 4) for 48 hours prior to determining the levels of ZO-1 (220 kDa), occludin (65 kDa), E-cadherin (120 kDa), α-1-catenin (102 kDa), vimentin (57 kDa), and α-2-integrin (150 kDa) proteins using Western blot. β-actin (42 kDa) was also identified as an internal control for equal loading.

[0045] FIG. 3A shows that the levels of ZO-1, occludin, vimentin and α-2-integrin are significantly decreased by 82.8 ± 0.04%, 93.5 ± 0.01%, 84.9 ± 0.04% and 66.4 ± 0.1%, respectively, after a 48 hour pre-treatment with APF compared to Mock APF. By contrast, the level of E-cadherin protein significantly increased by 78.5 ± 0.1% in normal bladder epithelial cells treated with APF compared to Mock APF. Although there was also a trend toward decreased α-1-catenin production as a result of APF treatment, the amount in APF-treated cells was not significantly different from the amount in cells treated with Mock APF. The amounts of ZO-1, occludin, E-cadherin, α-1-catenin, vimentin, and α-2-integrin proteins in cells treated with Mock APF did not differ significantly from levels of the same proteins in untreated normal bladder epithelial cells. These results show that a 48 hour exposure to APF decreased expression of certain tight junction proteins and increased E-cadherin.

[0046] FIG.s 3 B-G show the quantitative densitometric analysis of autoradiograms from the cells described in 3A: IC is indicated by bar "1"; NBC by bar "2", APF-treated NBC by bar "3", and Mock APF-treated NBC by bar "4". Values are means ± SD of data from replicate experiments. The relative levels of ZO-1, occludin, E-cadherin, α-1-catenin, vimentin, and α-2-integrin proteins were corrected for loading as measured by densitometry of β-actin control. * $p < 0.02$; # $p = 0.01$; + $p < 0.01$. The results show that the levels of ZO-1, occludin, α-1-catenin, vimentin, and α-2-integrin proteins were decreased by 79.6 ± 0.1%, 84.1 ± 0.14%, 85.8 ± 0.1%, 97.5 ± 0.01% and 58.7 ± 0.04%, respectively, in the IC cells as

compared to normal bladder cells. By contrast, the levels of E-cadherin were significantly increased by 55.2 ± 0.1%.

[0047] Decreased expression of ZO-1 protein in epithelial cells from IC patients was confirmed by immunohistochemistry using FITC-labeled antibodies against human ZO-1. FIG. 4 shows immunofluorescence micrographs of ZO-1 in explanted bladder epithelial cells (A, C, E) from IC patients, (B, D, F) from normal controls, (G) from normal cells treated with 0.7 ng/well APF, and (H) from normal cells treated with an equivalent amount of Mock APF. Cells were fixed with acetone/ethanol and incubated with FITC-labeled anti-ZO-1 antibody. FIG. 4 is representative of results from three separate experiments.

[0048] As shown in FIG. 4, IC patients cells (FIG. 4 A, C, and E) expressed decreased ZO-1 compared with normal bladder epithelial cells (FIG. 4 B, D, and F). APF-treatment of normal bladder epithelial cells resulted in decreased ZO-1 expression (FIG. 4G) compared to Mock APF treatment (FIG. 4H) and microscopic examination showed evidence for decreased paracellular tight junction formation. The observed fluorescence was determined to result from specific binding of the antibodies rather than cellular autofluorescence; control cells incubated with PBS alone did not have any fluorescent signal (data not shown).

[0049] Decreased expression of occludin protein was observed in bladder epithelium from IC patients with immunohistochemistry using primary antibodies against human occludin and FITC-labeled secondary antibodies. FIG. 5 shows immunofluorescence micrographs for occludin in explanted bladder epithelial cells (A, C, E) from IC patients, (B, D, F) from normal controls, (G) from normal cells treated with 0.7 ng/well APF, and (H) from normal cells treated with an equivalent amount of Mock APF. Cells were fixed with acetone/ethanol and incubated with anti-occludin primary antibody followed by FITC-labeled goat anti-rabbit secondary antibody. FIG. 5 is representative of results from three separate experiments. It can be seen that cells from IC patients (FIG. 5 A, C, and E) expressed decreased levels of occludin compared with normal epithelial cells (FIG. 5 B, D, and F). APF-treated normal bladder epithelial cells also expressed decreased amounts of occludin (FIG. 5G) compared to Mock APF-treated normal bladder epithelial cells (FIG. 5H). The observed fluorescence was

determined to result from specific binding of the primary antibodies [FITC-labeled antibodies against human ZO-1] rather than cellular autofluorescence or nonspecific binding of the secondary antibodies; control cells incubated with PBS alone, or secondary antibody diluted in PBS alone, did not have any fluorescent signal (data not shown).

[0050] The experiments described above demonstrate that APF causes changes in paracellular permeability in normal bladder epithelial cells and in expression of specific proteins involved in tight junction formation that mirror those seen in untreated epithelial cells from IC patients *in vitro*. Importantly, APF treatment of normal bladder epithelial cells *in vitro* significantly increased paracellular permeability; i.e. permeability between cells. APF also caused a coordinated decrease in expression of tight junction proteins ZO-1 and occludin as well as intracellular proteins α-1-catenin, vimentin, and α-2-integrin, while causing an increase in the expression of the adhesion protein E-cadherin.

[0051] The ability of APF to increase in paracellular permeability makes it a powerful new tool for increasing uptake of diagnostic and therapeutic agents through any membrane that has tight junctions, such as endothelium. Endothelial cells line blood vessels including those in tumors and those in blood vessels in the brain that form the blood brain barrier. Some embodiments of the present invention are directed to compositions containing APF that increase membrane permeability generally. The amount of APF in the compositions and methods of the present invention ranges from about 0.001 ng/ml to about 100 ng/ml. Where APF is applied locally the amount can be increased substantially above 100 ng/ml without causing toxic side effects. Recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments or variants of antiproliferative factor come within the scope of the present invention. In certain embodiments the membrane is epithelium including urinary bladder epithelium, gastric epithelium, lung epithelium, skin epithelium, nasal, kidney, urethra, ureter, and intestinal epithelium. Certain other embodiments are directed to APF-containing compositions for increasing uptake of a compound in an animal, including diagnostic and therapeutic agents. Yet other embodiments of the invention include methods for improving delivery of a

compound to a site of action in an animal by pretreating with antiproliferative factor in an amount sufficient to increase uptake of the compound.

[0052] Because our results show that APF alters membrane permeability by altering expression of tight junction proteins and other cell adhesion proteins, pretreatment with APF needs to allow time for the cells to change their protein expression patterns. A recommended time for pretreating with APF is between 24 and 48 hours before administering the diagnostic or therapeutic compound. However, routine experimentation will determine the optimum time for exposing a cell to APF to increase paracellular permeability. Administration of APF can be in any of the conventional ways known, with some routes of administration preferred depending on the condition being diagnosed or treated. Administration can be systemic or localized, by injection, inhalation, oral, transdermal, topical application, or other known routes. If stomach diseases are treated, the preferred route is oral administration. If nasal diseases are treated, inhalation is preferred. If skin diseases are treated topical or transdermal application is preferred. If a tumor is treated, APF may be injected locally to the tumor. When administered locally, APF may be applied in higher doses that if it is given systemically. The amount of antiproliferative factor that can be used in the compositions of the present invention ranges from about 0.001 ng/ml to about 100 ng/ml. Routine experimentation monitoring, for example, uptake of radioactively labeled compounds can help physicians determine the amount of APF to apply for the various diagnostic and therapeutic compounds as well as for the various diseases being treated. Where APF is applied locally, for example to a cancerous tumor, the concentration of APF can be increased above 100 ng/ml.

[0053] The blood brain barrier (BBB, also called the blood tumor barrier) poses a significant obstacle for the treatment of metastatic brain tumors. The BBB represents a specialized structure that lines the central nervous system (CNS) vasculature. The CNS vasculature is lined by specialized endothelial cells attached to each other by tight junctions, encased by a basement membrane and lined by astrocytic foot processes. This structure tightly regulates the entrance of molecules into the CNS. Most chemotherapeutic agents are too large to readily pass through the BBB. Although metastatic brain lesion(s) "enhance" on post-contrast

images indicating alteration in the permeability of the cerebral vasculature and the BBB, alterations in BBB permeability are very limited. There are several methods that have been evaluated to improve chemotherapy delivery for the treatment of brain tumors. Pharmacologic disruption of the BBB in particular has several important advantages over other methods of blood-brain barrier disruption. The identification and characterization of an important pathway that results in transient blood-brain tumor permeability has been identified. Activation of the bradykinin-2 receptor system by bradykinin or a bradykinin analog, labradimil, has been shown to transiently alter the permeability of the BBB. Localized pharmacologic disruption of the BBB is a noninvasive method of improving chemotherapy delivery to brain tumor, and chemotherapy is delivered only to the tumor, thereby limiting the exposure of normal brain. The compositions and methods of the present invention for increasing paracellular permeability across membranes having tight junctions like the BBB endothelium by administering APF is another embodiment of the present invention.

[0054] The compositions of the present invention containing APF can also be delivered through the skin ("transdermal drug delivery" or "TDD"). TDD provides many advantages; primarily, such a means of delivery is a comfortable, convenient and non-invasive way of administering drugs. The variable rates of absorption and metabolism encountered in oral treatment are avoided, and other inherent inconveniences--e.g., gastrointestinal irritation and the like--are eliminated as well. Transdermal drug delivery also makes possible a high degree of control over blood concentrations of any particular drug.

[0055] Skin is a structurally complex, relatively impermeable membrane. Molecules moving from the environment into and through intact skin must first penetrate the stratum corneum and any material on its surface. They must then penetrate the viable epidermis, the papillary dermis, and the capillary walls into the blood stream or lymph channels. To be so absorbed, molecules must overcome a different resistance to penetration in each type of tissue. Transport across the skin membrane is thus a complex phenomenon. "Transdermal" (or "percutaneous") shall mean passage of a material into and through the skin to achieve effective therapeutic blood levels or deep tissue therapeutic levels. The compositions and

methods of the present invention for increasing paracellular permeability across epithelial membranes having tight junctions, such as the epidermis of the skin, are also be beneficial in the transdermal administration of pharmacological agents, including chemotherapeutic agents to treat various skin cancers, including oral cancers.

[0056] The presently disclosed and claimed compounds and methods also encompasses the "transmucosal" and "topical" administration of drugs. With respect to skin location, virtually any area of the body surface may be selected so long as it is intact, however, the thickness and permeability of skin at the site of exposure will affect the treatment conditions, i.e., intensity, frequency, contact time, exposure time, and the like.

[0057] The present invention will employ, unless otherwise indicated, conventional pharmaceutical methodology. For example, the present compositions can include APF, a formulation vehicle, and a bulking agent. In one exemplary embodiment, lactose can be utilized as the bulking agent. As those skilled in the art will appreciate, it is contemplated that other bulking agents known or developed in the art may be utilized. According to another exemplary embodiment, the composition of the present invention can be buffered. The composition can be buffered with any known or developed buffering agents. The compositions of the present invention can either be compounded for intravesical delivery or lyophilized. As those skilled in the art will appreciate, the compositions of the present invention can be lyophilized by those methods known or developed in the art. The lyophilized compositions can be reconstituted by a reconstitution vehicle. According to one exemplary embodiment, the reconstitution vehicle comprises 2% sodium bicarbonate, 0.02% disodium edetate and propylene glycol: water (60:40 V/V).

[0058] In another aspect of the present invention, the compositions of the present invention also comprise coating agents. The coating agents of the present invention provide better adhesion of the composition to the bladder wall thereby increasing contact of the APF with bladder epithelium. In one embodiment of the present invention, the coating agent is propylene glycol. In other exemplary embodiments of the present invention, the coating agent can be selected from the group consisting of hydroxypropylcellulose, carboxymethylcellulose, chitosan hydrochloride, lectin, or polycarbophil.

[0059] In yet another embodiment of the present invention, the compositions of the present invention can be delivered, for example to the bladder wall, by a liposome. According to one embodiment of the present invention, the liposomes used are unilamellar or multilamellar and contain at least one cationic phospholipid such as stearylamine, 1,2-diacyl-3-trimethylammonium-propane (TAP) or 1,2-triacyl-3-dimethylammonium-propane (DAP). In another embodiment of the present invention, the surface liposomes may be coated with polyethylene glycol to prolong the circulating half-life of the liposomes. In yet another embodiment of the present invention, neutrally charged liposomes such as, but not limited to, phosphatidylcholine and cholesterol can also be used for liposomal entrapment of the compositions of the present invention. In another embodiment, the compositions of the present invention can be delivered to the bladder wall by a microsphere such as those known or developed in the art.

[0060] The pharmaceutical compositions can comprise one or more excipients (e.g., buffer, protein, detergent, lipid, a water-soluble polymer, preservative, etc.) and may comprise one or more addition active pharmaceutical ingredients, such as one or more additional bioactive agents. In one embodiment of the invention, the pharmaceutical compositions of the present invention will be administered using a drug delivery system. Such drug delivery systems may include formulations that provide site-specific release, or that enhance protection for the intestinal mucosa, etc. Suitable formulations include: dry powder formulations, delivery via particles, liposome encapsulation, transdermal patches, electrically aided transport (electroporation therapy), etc. The drug delivery system may comprise agents to facilitate the controlled release of APF-containing compositions of the invention. Such agents may include: poly(urethanes), poly(siloxanes), poly(methyl methacrylate), poly(vinyl alcohol) for hydrophilicity and strength, poly(ethylene), poly(vinyl pyrrolidone), poly(2-hydroxy ethyl methacrylate), poly(n-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol), poly(acrylic acid), polyacrylamide, poly(ethyleneco-vinyl acetate), poly(ethylene glycol), poly(methacrylic acid), etc. In a further preferred embodiment, biodegradeable polymers may be employed to facilitate drug delivery. Such biodegradeable polymers incude polylactides (PLA), polyglycolides (PGA), poly(lactide-co-glycolides) (PLGA), polyanhydrides, and

polyorthoesters. Drug delivery devices, and methods for their use are described in U.S. patents; U.S. Pat. Nos. 6,072,041; 6,041,253; 6,018,678; 6,017,318; 6,002,961; 5,879,712; 5,849,331; 5,792,451; 5,783,212; 5,766,633; 5,759,566; 5,690,954; 5,681,811; 5,654,000; 5,641,511; 5,438,040; 4,810,499; and 4,659,558.

[0061] Administration can be via any accepted systemic or local route, for example, via parenteral, intramuscular, subcutaneous, oral or sublingual (particularly for infant and animal formulations), intravenous, intravaginal, intraural, intraocular, nasal, bronchial inhalation (i.e., aerosol formulation), transdermal or topical routes, in the form of solid, semi-solid or liquid or aerosol dosage forms, such as, for example, tablets, pills, gel caps, capsules, powders, liquids, solutions, emulsion, injectables, suspensions, suppositories, aerosols or the like. The compositions of the invention can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, pills, transdermal (including electrotransport) patches, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages. The compositions can include a conventional pharmaceutical carrier or excipient and in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, etc. Carriers can be selected from the various oils, including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water, saline, aqueous dextrose, and glycols are preferred liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. Other suitable pharmaceutical carriers and their formulations are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

[0062] Oral formulations are particularly suited for treatment of gastrointestinal disorders. Oral bioavailability for general systemic purposes can be adjusted by utilizing excipients that improve uptake to systemic circulation, such as formulation comprising acetylated amino acids. See, e.g., U.S. Pat. Nos. 5,935,601 and 5,629,020.

[0063] Oral compositions may take the form of a lozenge, capsule, pill or tablet and thus the composition will contain, along with the herbal composition, a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant such as croscarmellose sodium, starch or derivatives thereof; a lubricant such as magnesium stearate and the like; and a binder such as a starch, polyvinylpyrrolidone, gum acacia, gelatin, cellulose and derivatives thereof, and the like.

[0064] Liquid pharmaceutically administrable compositions (such as a syrup, elixir, etc.) can, for example, be prepared by dissolving, dispersing, etc. the herbal composition of the invention and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, glycols, ethanol, preservatives and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting agents, suspending agents, emulsifying agents, or solubilizing agents, pH buffering agents and the like, for example, sodium acetate, sodium citrate, cyclodextrine derivatives, polyoxyethylene, sorbitan monolaurate or stearate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa. The composition or formulation to be administered will, in any event, contain a quantity of APF with or without a diagnostic agent or therapeutic drug, in an amount effective to prevent or alleviate the symptoms of the subject being treated. For oral administration to infants, a liquid formulation (such as a syrup or suspension) is preferred.

[0065] The compositions of the present invention may be administered parenterally. Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously, and can include intradermal or intraperitoneal injections as well as intrasternal injection or infusion techniques. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, as emulsions or in biocompatible polymer-based microspheres (e.g., liposomes, polyethylene glycol derivatives, poly(D,C)lactide and the like). Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the

like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, solubility enhancers, protein carriers and the like, such as for example, sodium acetate, polyoxyethylene, sorbitan monolaurate, triethanolamine oleate, cyclodextrins, serum albumin etc. The composition may be administered as a bolus injection or as a continuous infusion.

[0066] The specific dose of APF administered is determined by the particular circumstances surrounding each situation. These circumstances include the route of administration whether it is local or systemic. However, it will be understood that the effective dosage of APF administered to increase paracellular permeability will be determined by the physician in the light of the relevant circumstances.

EXAMPLES

Example 1: Materials and Methods

[0067] Patients IC patients had previously undergone cystoscopy and fulfilled modified NIDDK diagnostic criteria for IC (without measurement of bladder capacity)[18]; age- and gender-matched controls were asymptomatic for urinary tract disease. All participants were at least 18 years old and enrolled in accordance with guidelines of the Institutional Review Board of the University Of Maryland School Of Medicine.

[0068] Cell Culture Cystoscopy was performed under general anesthesia, and 4-mm^2 pieces of transitional epithelium with submucosal bladder tissue were obtained from IC patients and controls for the growth of primary bladder epithelial cells, as previously described[15-17]. Epithelial cells were propagated in DMEM-F12 (Media-Tech, Herndon VA) with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, 1% L-glutamine, 0.25 units/ml insulin (all from Sigma, St. Louis, MO), and 5 ng/ml hEGF (R & D Systems, Minneapolis, MN) at 37°C in a 5% CO_2 atmosphere and characterized by binding of AE-1/AE-3 pancytokeratin antibodies (Signet, Dedham, MA), as previously described[15-17].

[0069] For Western blots, cells were plated in Corning T75 tissue culture flasks (VWR Scientific Products, Bridgeport, NJ) at a density of 1 x 10^4 cells/ml. When confluent, cells were cultured in serum-free Eagle's minimal essential medium (MEM, GIBCO-BRL; Life Technologies, Grand Island, NY) containing 1% antibiotic/antimycotic solution, and 1% L-glutamine at 37°C in a 5% CO_2 atmosphere overnight. For experiments involving HPLC-purified APF or Mock APF these were added to the medium the next day, and cells further incubated at 37°C in a 5% CO_2 atmosphere. Two days later cells were rinsed with phosphate-buffered saline (PBS), harvested, and protein extracted as described below.

[0070] Flux assays were performed using 12-mm Transwell™ culture plates (Corning Incorporated, Corning, NY), as previously described for studying paracellular flux in kidney, lung, and intestinal epithelial cells[3-6,19,20]. Cells were plated at 4 x 10^5 cells/cm^2 on the insert and grown in DMEM-F12 medium containing 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution, 1% L-glutamine, 0.25 units/ml insulin (all from Sigma, St. Louis, MO), and 5 ng/ml hEGF (R & D Systems, Minneapolis, MN) to establish tight monolayers. On day 2, the medium was changed to MEM medium (GIBCO/InVitrogen) containing 1% antibiotic/antimycotic solution and 1% L-glutamine (Sigma).

[0071] For experiments involving APF treatment of normal bladder cells, on day 3 all cells had the same MEM medium reapplied, and APF- or Mock APF-treated cells also received the appropriate addition; cells were then cultured for an additional 48 hours. For anti-APF neutralization experiments, on days 3 and 4, IC and normal bladder epithelial cells were also treated with anti-APF antibodies in varying concentrations, and cells were cultured for an additional 24 hours before application of radiolabeled tracers.

[0072] APF Purification: APF was harvested from the supernatant of bladder epithelial cells explanted from one IC patient, and purified using molecular weight fractionation, ion-exchange chromatography, hydrophobic interaction chromatography, and reversed-phase HPLC, as previously described[14,16]. Mock APF was prepared using supernatant of bladder epithelial cells from a normal control and the same purification procedure.

[0073] Immunofluorescence Assay: Cells (2 x 10^4 cells/well) were plated on 8-well LabTek chamber slides (Nalge Nunc International, Naperville, IL), grown to confluence in

DMEM-F12 containing supplements as listed above, fixed using ethanol/acetone (1:1) for 15 min at room temperature, washed three times with PBS, and incubated with FITC-labeled mouse anti- ZO-1 1:200 (Zymed) or anti-occludin 1:200 (Zymed) antibodies diluted in PBS (Sigma)] for 2 hrs at 37°C. For the anti-occludin antibodies, cells were then washed three times with PBS and further incubated with FITC-labeled secondary antibody diluted in PBS [goat anti-rabbit Ig 1:100 (Southern Biotechnology Associates, Birmingham, AL)], for 2 hrs at 37°C. Following five washes with PBS, cells were examined using a Zeiss LSM510 confocal laser-scanning microscope. Negative controls for the method included cells incubated without primary and/or secondary antibodies, as well as cells incubated with secondary antibody alone.

[0074] Western blot analysis: Cells were scraped into ice-cold RIPA-buffer (50mM Tris/HCL, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM sodium orthovanadate), sonicated and centrifuged for 15 min at 4°C. Protein concentration of the supernatant was measured using a Folin reagent-based protein assay kit (Bio-Rad). The supernatant was boiled for 5 minutes in reducing buffer, each lane was loaded with 20 μg protein, and proteins separated by electrophoresis using 7.5% NuPAGE Novex Bis-Tris polyacrylamide (InVitrogen) according to the manufacturer's instructions, and transferred to NitroBind nitrocellulose membranes (Osmonics) according to the NuPAGE gel manufacturer's protocol for Western transfer (at 30 V constant current for 1 hour). Following protein transfer, the nitrocellulose membranes were blocked with 5% nonfat dry milk in TBS-T buffer (Tris-buffered saline, pH 7.4, with 0.1% Tween 20), and incubated overnight at 4°C in 5% blocking buffer containing specific antibodies against E-cadherin (1:5000) (BD Bioscience), α-1-catenin (1:500) (BD Bioscience), ZO-1 (1:1000) (Zymed), occludin (1:500) (Zymed), vimentin (1:500) (BD Bioscience), α-2-integrin (1:250) (BD Bioscience), or β-actin (1:8000) (BD Bioscience). The membranes were subsequently washed with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature, and developed for with ECL Chemiluminiscence Reagent (Amersham Biosciences).

[0075] Permeability assay: Two different membrane impermeable molecules, [^{14}C]-mannitol (molecular weight: 184 Daltons) and [^{3}H]-inulin (molecular weight: 5,200 Daltons), served as paracellular tracers. At the beginning of the flux assay, both sides of the bathing wells of Transwell^{TM} filters were replaced with fresh medium containing either 5 mM unlabeled mannitol or 0.5 mM unlabeled inulin. Each tracer was added at a final concentration of 3.6 nM for [^{14}C]-mannitol and 0.36 nM for [^{3}H]-inulin to the apical bathing wells. The basal bathing well contained the same medium as the apical compartment but without tracers. lux assays were performed at 37°C; basal medium was collected at 0.5 - 6 hrs after addition of [^{14}C]-mannitol or [^{3}H]-inulin, and the amount of radioactivity determined using a Beckman LS 5000 scintillation counter. Results were expressed as percentage of total counts for each tracer.

[0076] Anti-APF Antibodies: Synthetic APF peptide was generated with a terminal cysteine residue^{14}, coupled to KLH, and injected into two New Zealand White rabbits for antibody production. Following two monthly booster injections, serum was harvested and the immunoglobulin fraction purified using protein A sepharose (antibodies generated by Lampire Laboratories).

[0077] Statistics: The percentage of total counts in the basal medium was determined in quadruplicate experiments, and. expressed as mean $\pm$ standard deviation. The significance of the difference between mean values was determined by an analysis of variance.

[0078] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

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WHAT IS CLAIMED IS:

1. A composition for increasing membrane permeability in an animal comprising antiproliferative factor.

2. The composition of claim 1, wherein the membrane is epithelium.

3. The composition of claim 2, wherein the epithelium is selected from the group comprising urinary bladder epithelium, gastric epithelium, lung epithelium, skin epithelium, nasal, kidney, urethra, ureter, and intestinal epithelium.

4. The composition of claim 1, wherein the membrane is mucosa.

5. The composition of claim 4, wherein the mucosa is selected from the group comprising urinary bladder, intestinal mucosa, gastric mucosa, nasal mucosa, vaginal mucosa, and lung mucosa.

6. The composition of claim 1, wherein antiproliferative factor is a member selected from the group comprising recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments and variants of antiproliferative factor.

7. The composition of claim 1, wherein the antiproliferative factor is human antiproliferative factor.

8. The composition of claim 1, wherein the amount of antiproliferative factor is in an amount of from about 0.001 ng/ml to about 100 ng/ml.

9. A composition for increasing uptake of a compound in an animal, comprising human antiproliferative factor.

10. The composition of claim 9, wherein the compound is a diagnostic or therapeutic agent.

11. The composition of claim 9, wherein antiproliferative factor is a member selected from the group comprising recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments and variants of antiproliferative factor.

12. The composition of claim 9, wherein the amount of antiproliferative factor is from about 0.001 ng/ml to about 100 ng/ml.

13. A method for improving delivery of a compound to a site of action in an animal comprising:

1) pretreating the animal with antiproliferative factor in an amount sufficient to increase uptake of the compound; and

2) following pretreatment with antiproliferative factor, administering the compound to the animal.

14. The method of claim 13, wherein antiproliferative factor is administered in an amount of from about 0.001 ng/ml to about 100 ng/ml.

15. The method of claim 13, wherein antiproliferative factor is administered locally to the site of action.

16. The method of claim 13, wherein antiproliferative factor is administered systemically.

17. The method of claim 13, wherein antiproliferative factor is administered multiple times before administering the compound.

18. The method of claim 13, wherein the compound is a diagnostic agent or an imaging agent.

19. The method of claim 13, wherein the compound is a therapeutic agent.

20. The method of claim 13, wherein the site of action is an area of the body having cancer and the compound is an anticancer agent.

21. The method of claim 13, wherein the site of action is a tumor and the compound is a diagnostic agent.

22. The method of claim 24, wherein the tumor is cancerous and the cancer is a member selected from the group comprising bladder cancer, stomach cancer, lung cancer, skin cancer, nasal cancer, brain cancer, vaginal cancer, kidney cancer, esophageal cancer, urethra cancer, ureter cancer, colon cancer and intestinal cancer.

23. The method of claim 13, wherein antiproliferative factor is administered locally to the site of action.

24. The method of claim 13, wherein antiproliferative factor is administered systemically.

25. The method of claim 13, wherein the antiproliferative factor is a member selected from the group comprising recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments and variants of antiproliferative factor.

26. The method of claim 13, wherein antiproliferative factor is administered 24-48 hours before administering the compound.

27. A method for treating cancer in an animal, comprising:

1) pretreating the animal with antiproliferative factor in an amou facilitate uptake an anticancer agent; and

2) following pretreatment with antiproliferative factor, administering a therapeutic amount of the anticancer agent to the animal.

28. The method of claim 27, wherein antiproliferative factor is administered in an amount of from about 0.001 ng/ml to about 100 ng/ml.

29. The method of claim 27, wherein antiproliferative factor is administered systemically.

30. The method of claim 27, wherein antiproliferative factor is administered locally to a cancerous tumor.

31. The method of claim 27, wherein antiproliferative factor is administered multiple times before administering the anticancer agent.

32. The method of claim 27, wherein antiproliferative factor is administered 24-48 hours before administering the anticancer agent.

33. The method of claim 27, wherein antiproliferative factor is a member selected from the group comprising recombinant antiproliferative factor, isolated and purified antiproliferative factor, synthetic antiproliferative factor, and fragments and variants of antiproliferative factor.

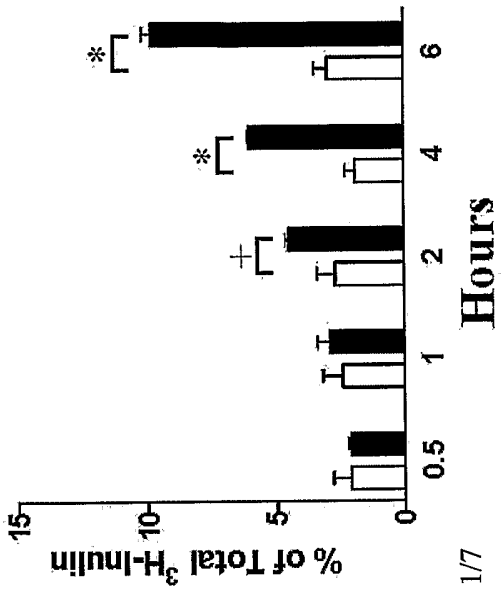
34. The method of claim 27, wherein the cancer is a member selected from the group comprising bladder cancer, vaginal cancer, stomach cancer, lung cancer, skin cancer, nasal cancer, kidney cancer, esophageal cancer, urethra cancer, ureter cancer, colon cancer, brain cancer and intestinal cancer.

35. The method of claim 27, wherein the cancer is bladder cancer and the anticancer agent is administered intravesicaly.

36. The method of claim 27, wherein the step b further comprises continuing administration of antiproliferative factor.

37. A method for increasing membrane permeability in an animal or organism, comprising contacting the membrane with antiproliferative factor in an amount that increases membrane permeability.

FIG. 1B



1/7

FIG. 1A

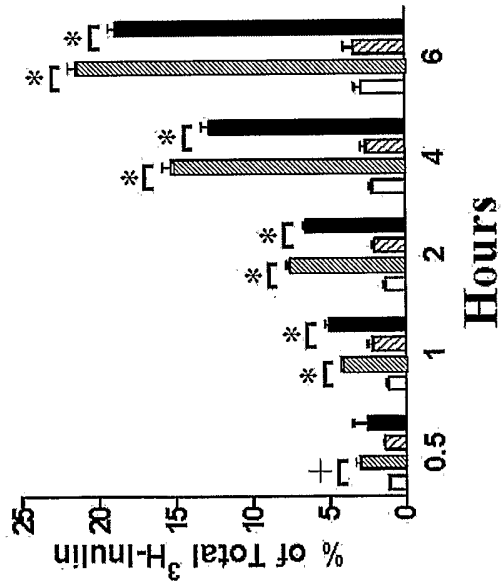


FIG. 1D

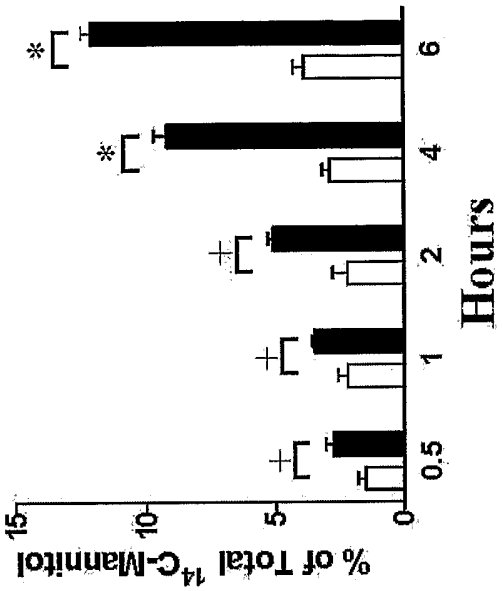

% of Total ^{14}C-Mannitol
0
5
10
15
0.5
1
2
4
6
Hours

FIG. 1C

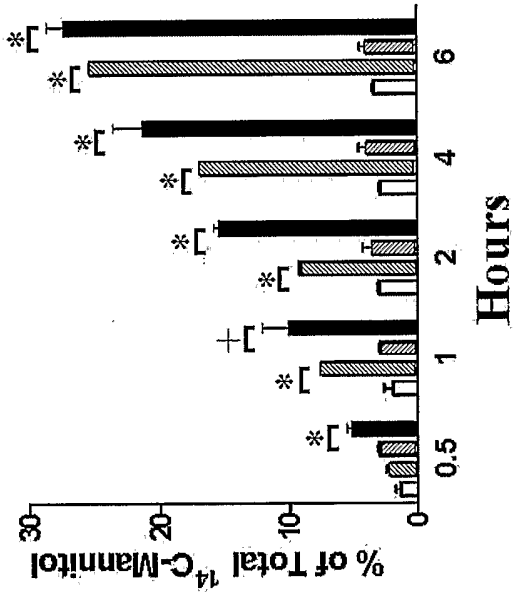

% of Total ^{14}C-Mannitol
0
10
20
30
0.5
1
2
4
6
Hours

FIG. 2A

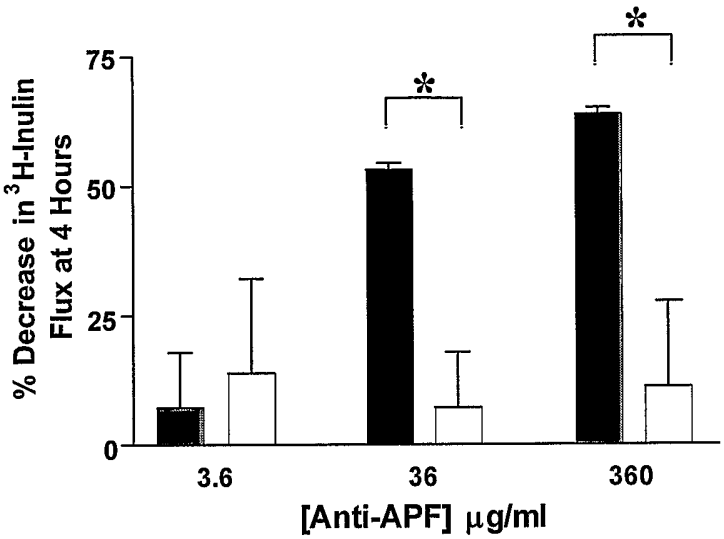

% Decrease in ^{3}H-Inulin Flux at 4 Hours
75
50
25
0
*
*
3.6
36
360
[Anti-APF] µg/ml

FIG. 2B

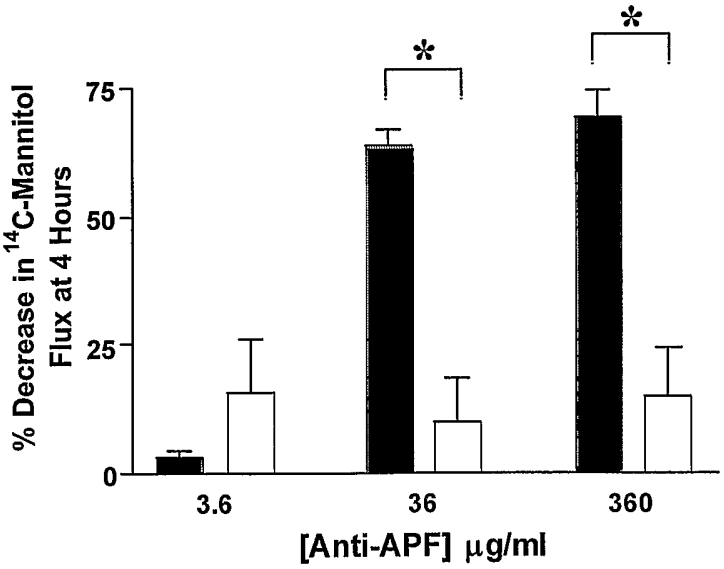

% Decrease in ^{14}C-Mannitol Flux at 4 Hours
75
50
25
0
*
*
3.6
36
360
[Anti-APF] µg/ml

FIG. 3A

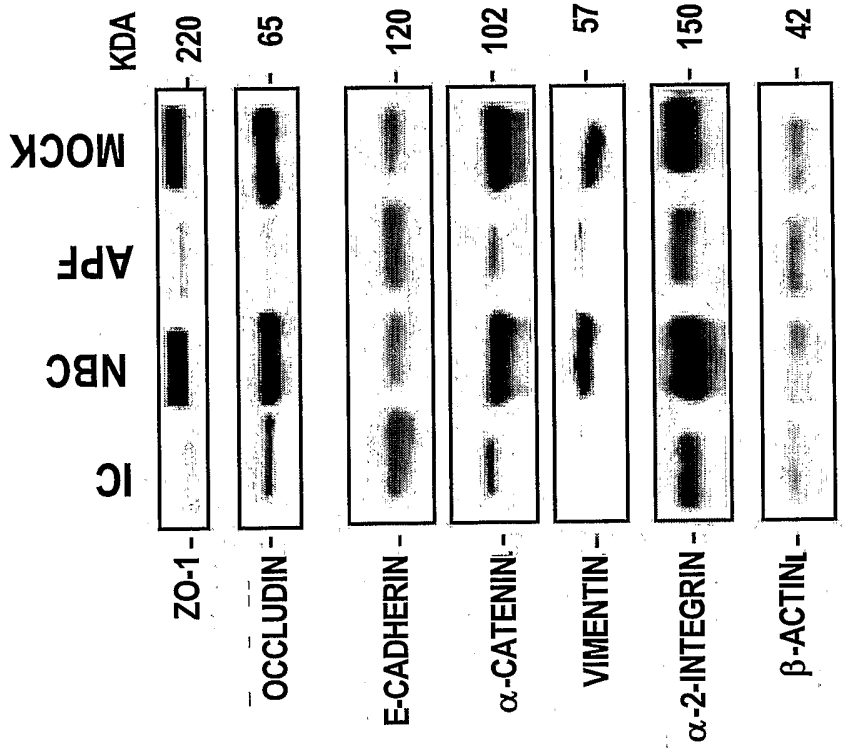

KDA
220
65
120
102
57
150
42
MOCK
APF
NBC
IC
ZO-1
OCCLUDIN
E-CADHERIN
α-CATENIN
VIMENTIN
α-2-INTEGRIN
β-ACTIN

FIG. 3B

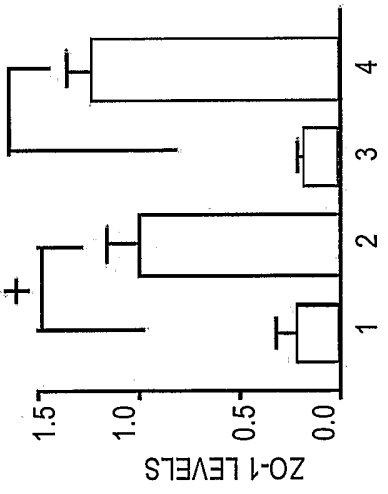


FIG. 3C

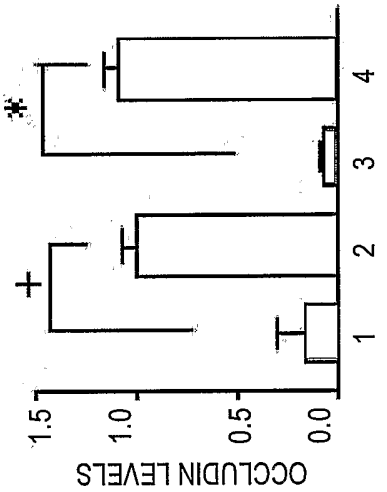


FIG. 3D

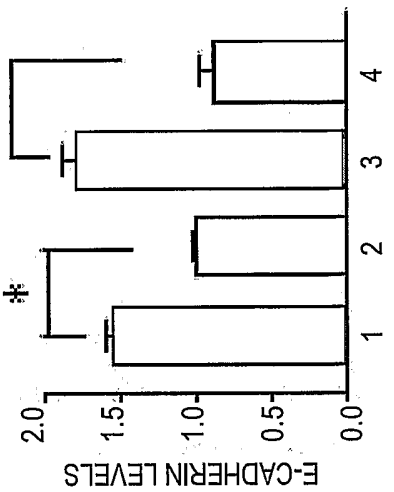


FIG. 3E

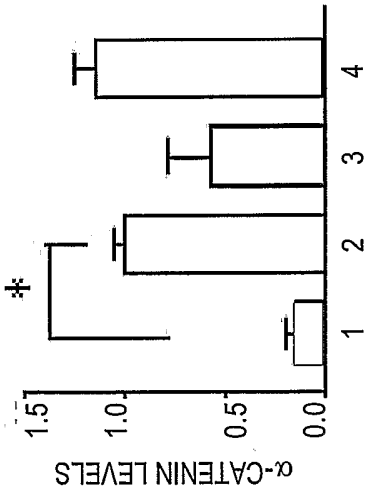


FIG. 3F

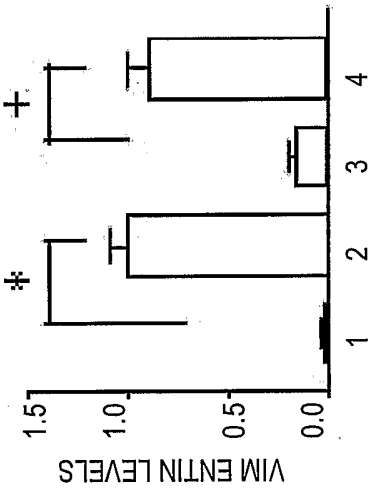


FIG. 3G

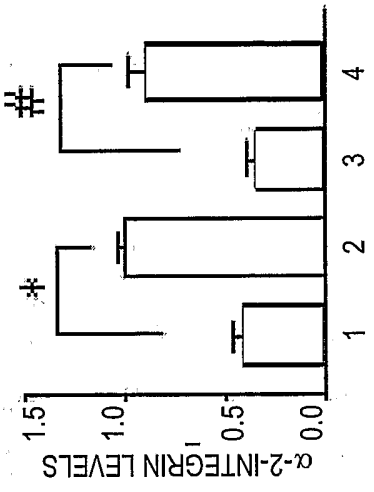
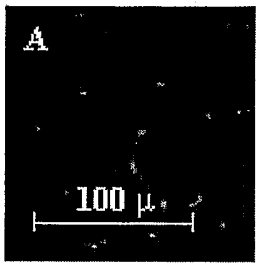


FIG. 4A



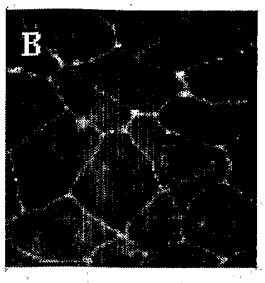
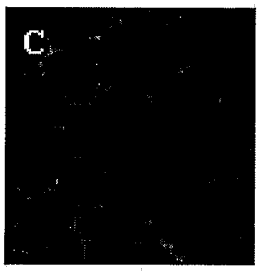


FIG. 4B

FIG. 4C



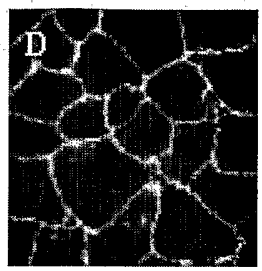
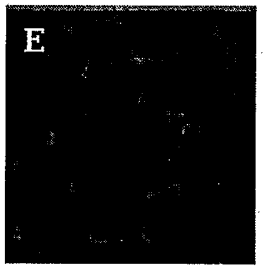


FIG. 4D

FIG. 4E



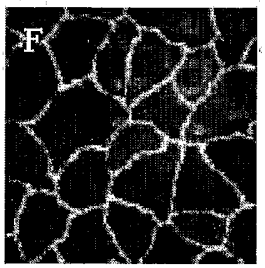
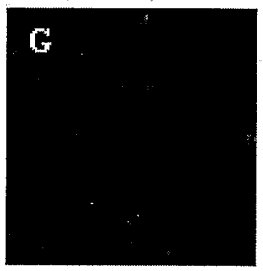


FIG. 4F

FIG. 4G



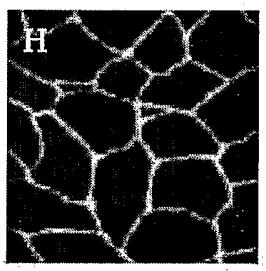
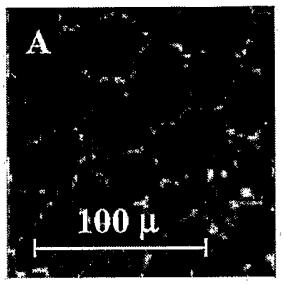


FIG. 4H

FIG. 5A



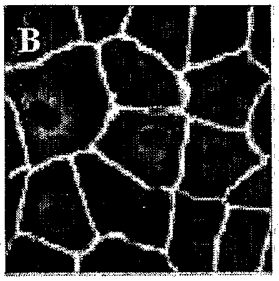
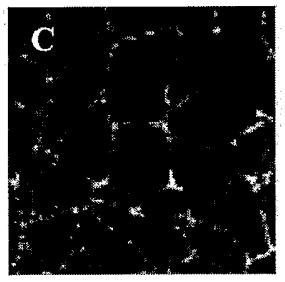


FIG. 5B

FIG. 5C



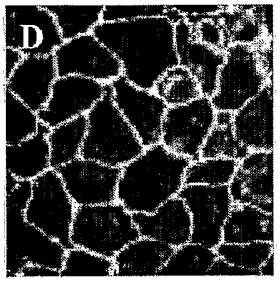
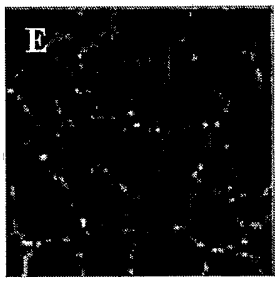


FIG. 5D

FIG. 5E



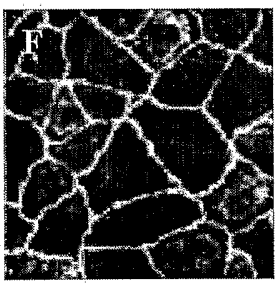


FIG. 5F

FIG. 5G



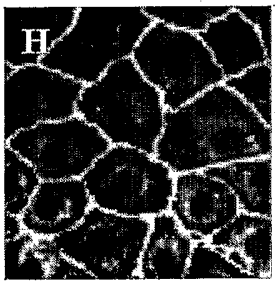


FIG. 5H

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/09936

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **A61K 38/00**(2006.01)

USPC: 514/2,576
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)
U.S. : 514/2, 576

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 practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEMOY et al. Time- and Concentration-dependent apoptosis and necrosis induced by free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells. Journal of Controlled Release. 2000, Vol. 69, pagaes 185-196 especialy abstract.	1-7, 9-11
X	AU et al. Clinical aspects of drug delivery to tumors. Journal of Controlled Release. 2002, Vol. 78, pages 81-95, especially Table 2, pages 84, Figs 5-6, page 91.	1-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family 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 application or patent 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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2006 (26.06.2006)	28 JUL 2006
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer MISOOK YU, Ph.D. Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/09936

Continuation of B. FIELDS SEARCHED Item 3:
Dialog (155)
search terms: membrane permeability, antiproliferative factor