Abstract: Disclosed are biomarkers, kits, and methods for diagnosing or treating wounds. In some aspects, the present disclosure provides for a method, comprising: measuring an individual level of an expression or activation of two or more proteins in a sample from the wound. In some aspects, the present disclosure provides for a method of treating a wound in a subject in need thereof, comprising treating the subject with a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof. In some aspects, the present disclosure provides for a kit that comprises reagents for measuring a total amount or a phosphorylation level of two or more proteins.
Methods of Diagnosing and Treating Wounds

Cross-Reference

This application claims the benefit of U.S. Provisional Application No. 62/175,394, filed on June 14, 2015, which is incorporated herein by reference in its entirety.

Incorporation by Reference

All publications, patents, and patent applications disclosed herein are incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term disclosed herein and a term in an incorporated reference, the term herein controls.

Brief Summary

In some aspects, the present disclosure provides for a method, comprising: measuring an individual level of an expression or activation of two or more proteins (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 proteins) in a sample from the wound. In some embodiments, the present disclosure provides for a method, comprising: measuring an individual level of an expression or activation of a first protein and a second protein in a sample from the wound, wherein the first protein and the second protein are individually selected from the group consisting of: IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3p, COX-2, iNOS, NFkB/p65, IxB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, or any combination thereof; and calculating a ratio of the first protein to the second protein. In some embodiments, the method is a method of determining a diagnosis of the wound in a subject. In some embodiments, the measurement is repeated for at least 1, 2, 3, 4, or 5 times, and when the method is repeated more than 1 time, the measurement may be conducted at different time points or at the same time point. In some embodiments, the measurement is repeated during a course of monitoring the wound. In some embodiments, the calculation is implemented at least in part by a computer. In some embodiments, the calculation is implemented at least in part by a computer processor. In some embodiments, the computer process is a computer microprocessor. In some embodiments, the method further comprises determining the diagnosis that a wound will not heal when the ratio is greater than 1, or that the wound will heal when the ratio is 1 or less. In some embodiments, the determination is implemented at least in part by a computer. In some embodiments, the determination is implemented at least in part by a computer processor. In some embodiments, the computer process is a computer microprocessor. In some embodiments, the method further comprises evaluating the wound visually, e.g., by comparing an image (e.g., digital or paper) of the wound with an earlier image of the same wound. In some embodiments, the method further comprises communicating a result with a communication medium. In some embodiments, the
communication medium is a document. In some embodiments, the communication medium is a computer file, an email, a fax, or a paper document. In some embodiments, the ratio is calculated with values generated from a comparison of the measured levels and corresponding reference standards. In some embodiments, the sample is a tissue from the wound. In some embodiments, the sample is from a marginal edge of the wound. In some embodiments, the sample comprises a keratinocyte isolated or purified from the wound. In some embodiments, the first protein and the second protein are individually measured from the keratinocyte or a cellular lysate thereof. In some embodiments, the method comprises measuring the expression of the first protein and the second protein individually. In some embodiments, the method comprises measuring the activation of the first protein and the second protein individually. In some embodiments, the measured level is determined by measuring a total amount of the first protein or the second protein individually. In some embodiments, the measured level is determined by measuring an amount of an activated form of the first protein or the second protein individually. In some embodiments, the activation is phosphorylation. In some embodiments, the measured levels are normalized fluorescence intensity (NFI) per mg protein. In some embodiments, the values are normalized expression values. In some embodiments, the subject has or is suspected of having diabetes, Alzheimer's disease, a cancer, or any combination thereof. In some embodiments, the subject is a human. In some embodiments, the wound will not heal when the ratio is \( \geq 1.2 \), \( \geq 1.3 \), \( \geq 1.4 \), or \( \geq 1.5 \). In some embodiments, the method further comprises treating the subject, e.g., administering to the subject a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein or the second protein are individually selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3p, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof. In some embodiments, a pharmaceutical composition disclosed herein comprises the agonist and the antagonist, which are administered simultaneously, or sequentially. In some embodiments, a pharmaceutical composition disclosed herein further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutically acceptable carrier comprises water. In some embodiments, the second protein is IGF-IR. In some embodiments, the second protein is Akt. In some embodiments, the first protein is PI3 Kinase and the second protein is IGF-IR. In some embodiments, the first protein is PI3 Kinase and the second protein is insulin. In some embodiments, the first protein is PI3 Kinase and the second protein is Akt. In some embodiments, the first protein or the second protein is Akt that is Akt1. In some embodiments, the first protein or the second protein is IGF-IR that is IGF-IRp. In some embodiments, the first protein or the second protein is IGF-IRP that is p-IGF-IRP Y1135-1150 (phospho-tyrosine
1135-1 150). In some embodiments, the first protein or the second protein is HIF that is HIF-la. In some embodiments, the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448). In some embodiments, the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380). In some embodiments, the subject has or is suspected of having diabetes, Alzheimer’s disease, a cancer, or any combination thereof. In some embodiments, the subject is a human. In some embodiments, the subject comprises a complication of trauma, a wound in a cancer patient, a wound in a patient receiving a steroid therapy, a wound from an inflammatory skin disease, a chronically impaired cutaneous wound, or any combination thereof. In some embodiments, the wound is a diabetic foot ulcer.

In some aspects, the present disclosure provides for a method of treating a wound in a subject in need thereof, comprising treating the subject with a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein or the second protein are individually selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3β, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof. In some embodiments, a pharmaceutical composition disclosed herein comprises the agonist and the antagonist, which are administered simultaneously, or sequentially. In some embodiments, a pharmaceutical composition disclosed herein further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutically acceptable carrier comprises water. In some embodiments, the second protein is IGF-IR. In some embodiments, the second protein is Akt. In some embodiments, the first protein is PI3 Kinase and the second protein is IGF-IR. In some embodiments, the first protein is PI3 Kinase and the second protein is insulin. In some embodiments, the first protein is PI3 Kinase and the second protein is Akt. In some embodiments, the first protein or the second protein is Akt that is Akt1. In some embodiments, the first protein or the second protein is IGF-IR that is IGF-IRp. In some embodiments, the first protein or the second protein is IGF-IRp that is p-IGF-IRp Y1135-1150 (phospho-tyrosine 1135-1 150). In some embodiments, the first protein or the second protein is HIF that is HIF-la. In some embodiments, the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448). In some embodiments, the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380). In some embodiments, the subject has or is suspected of having diabetes, Alzheimer’s disease, a cancer, or any combination thereof. In some embodiments, the subject is a human. In some embodiments, the
wound is a diabetic ulcer, a pressure ulcer, a venous stasis ulcer, a radiation ulcer, a skin injury, an unhealed surgical wound, a wound from a surgical procedure, a wound from a peripheral vascular disease, a wound from a complication of trauma, a wound in a cancer patient, a wound in a patient receiving a steroid therapy, a wound from an inflammatory skin disease, a chronically impaired cutaneous wound, or any combination thereof. In some embodiments, the wound is a diabetic foot ulcer.

In some aspects, the present disclosure provides for a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein or the second protein are individually selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3β, COX-2, iNOS, NFκB/p65, IkB-α, p53, p16INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof. In some embodiments, the second protein is IGF-IR. In some embodiments, the second protein is Akt. In some embodiments, the first protein is PI3 Kinase and the second protein is IGF-IR. In some embodiments, the first protein is PI3 Kinase and the second protein is insulin. In some embodiments, the first protein is PI3 Kinase and the second protein is Akt. In some embodiments, the first protein or the second protein is Akt that is Aktl. In some embodiments, the first protein or the second protein is IGF-IR that is IGF-IRp. In some embodiments, the first protein or the second protein is IGF-IRp that is p-IGF-IRP Y1135-1150 (phospho-tyrosine 1135-1150). In some embodiments, the first protein or the second protein is HIF that is HIF-la. In some embodiments, the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448). In some embodiments, the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380). In some embodiments, a pharmaceutical composition disclosed herein further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutically acceptable carrier comprises water.

In some aspects, the present disclosure provides for a method of making a pharmaceutical composition disclosed herein.

In some aspects, the present disclosure provides for a kit that comprises reagents for measuring a total amount or a phosphorylation level of two or more proteins selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3β, COX-2, iNOS, NFκB/p65, IkB-α, p53, p16INK4a, β-Catenin, Bak, Caspase 3, and Caspase 9. In some embodiments, the kit is employed to predict a likelihood of wound healing in a subject. In some embodiments, the kit predicts the prognosis of a wound in a subject. In some embodiments, one of the proteins is PI3 Kinase, IGF-IR, or Akt. In some embodiments, the
proteins are PI3 Kinase and IGF-1R. In some embodiments, the proteins are PI3 Kinase and insulin. In some embodiments, the proteins are PI3 Kinase and Akt. In some embodiments, the first protein or the second protein is Akt that is Aktl. In some embodiments, the first protein or the second protein is IGF-1R that is IGF-1Rβ. In some embodiments, the first protein or the second protein is IGF-1Rβ that is p-IGF-1Rβ Y1135-1150 (phospho-tyrosine 1135-1150). In some embodiments, the first protein or the second protein is HIF that is HIF-1α. In some embodiments, the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448). In some embodiments, the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380). In some embodiments, the subject has or is suspected of having diabetes, Alzheimer's disease, a cancer, or any combination thereof. In some embodiments, the subject is a human. In some embodiments, the wound is a diabetic ulcer, a pressure ulcer, a venous stasis ulcer, a radiation ulcer, a skin injury, an unhealed surgical wound, a wound from a surgical procedure, a wound from a peripheral vascular disease, a wound from a complication of trauma, a wound in a cancer patient, a wound in a patient receiving a steroid therapy, a wound from an inflammatory skin disease, a chronically impaired cutaneous wound, or any combination thereof. In some embodiments, the kit further comprises an instruction manual.

In some instances, a first protein disclosed herein is not identical to a second protein disclosed herein.

**Brief Descriptions of the Drawings**

Figure 1 is a diagram showing DFU (diabetic foot ulcer) proteomic signaling study pathway nodes including HIF-1 and downstream gene expression related to wound repair. Pathway signaling map of the interplay between stimulatory and inhibitory modulation of the various components that have either a defined role or undefined role in the healing of chronic, diabetic ulcers. The ISP Ratio is defined as the ratio between PI3K and IGF-1R.

Figure 2 is a diagram showing a ratio of PI3K to IGF1R in normal, healed diabetic foot ulcers (DFU) and non-healing DFU. In normal and healing DFU the ratio of PI3K to IGF1R ranges from 0.7 to 1.1 while the ratio for non-healing DFU subjects was equal to or greater than 1.1, ranging from 1.1 to 1.7.

Figure 3 is a set of bar charts showing mean, STDev and non-parametric Whitney-Mann U-Test results for 21 proteins levels measured in normal and DFU keratinocytes. Analytes: Receptor Kinases: IGF-IRP - insulin growth factor-1 receptor beta, p-IGF-IRP Y1135-1150 - insulin growth factor-1 receptor beta (phosphor-tyrosine 1135-1150), VEGFR1 - vascular endothelial growth factor receptor-1, and c-MET - hepatocyte growth factor receptor (HGFR); the PI3Kinase/AKT Pathway, p-PTEN S380 - phosphatase tensin homolog (phospho-serine
380), PI3 Kinase - phosphoinosyl-3-kinase (PI3K), Akt - Akt1/protein kinase B, GSK-3β - glycogen synthase kinase-3P; mTOR - mammalian target of rapamycin, and p-mTOR - mammalian target of rapamycin S2448 (phosphor-serine 2448); Pro-inflammatory proteins: COX-2- cyclooxygenase-2, and iNOS - inducible nitric oxide synthase; Survival Pathways: NFKB/p65 - Nuclear Factor Kappa B/p65, 1kB-α - NFKB inhibitor alpha, HIF-la - hypoxia inducible factor-1 alpha, p53 - tumor suppressor transcription factor, pl6INK4a - cyclin-dependent kinase inhibitor/regulator of senescence, and β-Catenin - Catenin beta-1; and Apoptosis Pathways: Bak - BCL2-antagonist/killer 1, Caspase 3 - cysteine-aspartic protease 3, and Caspase 9 - cysteine-aspartic protease 9.

Figure 4 is a set of box-plots showing significantly increased levels of downstream proteins compared to levels of IGF-1R in Healed and Unhealed DFU keratinocytes. Boxplots showing the calculated ratios for the Level of Each Analyte / Level of IGF-1R demonstrate that the Unhealed patients have significantly (p ≤ 0.05) elevated ratios of POKinase, mTOR, Cox2, p53, Bak and Caspase 9 than Healed patients. Not only is the center higher for unhealed patients, but the quantitative independent two-sample t-test analysis suggests that the 2 population means differ beyond random variation.

Figure 5 is a scheme showing the dynamic Akt switch between DFU and Normal tissues and the ratios calculated for significantly modulated analytes (box-plots). Increased levels of PI3 kinase-induced activation of Akt promote angiogenesis and apoptosis through various downstream effectors.

Figure 6 is a diagram showing the ratio of PI3K to IGF-1R can distinguish Normal, Healing, and Nonhealing DFU. This distinction can be used in order to predict healing or not in DFU Subjects.

Figure 7 is a diagram showing ratios of various analytes to Akt in normal and DFU keratinocytes. For example, using the ratios of PI3K/Akt in individual subjects can determine if the subjects DFU would heal or not. Some Subject's ratios are higher in Normal so an antagonist to the analyte would be expected to reduce the ratio and promote healing, and in those Subjects where the ratio is lower, then an agonist would be expected to increase the ratio and promote healing.

Figure 8 shows a computer system 801 that is programmed or configured to implement or regulate a communication with results related to a prognosis of a wound.

**Detailed Description**

In some aspects, the biomarkers disclosed herein can serve as diagnostic markers that predict healing versus nonhealing DFU. In some embodiments, the signaling pathways in which these proteins reside and function are points of therapeutic intervention, wherein combinations of
therapies targeting these interrelated pathway signaling proteins is identified. In some embodiments, the patterns of protein expression and activation are useful in guiding treatment decisions on an individual, per-patient basis. In some aspects, the present disclosure provides a comprehensive assessment of protein signaling cascades related to proliferation, migration, inflammation, and apoptosis/senescence in diabetic wounds.

A protein disclosed herein can mean the entire protein or a fragment thereof.

An agonist disclosed herein can include a partial agonist. An antagonist disclosed herein can include a partial antagonist. An agonist or an antagonist can be purified or isolated.

A ratio (e.g., a ratio of a first protein to a second protein) is calculated with normalized values or quantitated values in weight (e.g., ng, µg, mg), moles (e.g., nmol, µmol, mmol).

The term "subject" as used herein can refer to a mammal (e.g., a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee or baboon). In some cases, the subject is a human subject, male or female. In some cases, the subject is an adult, a senior, or a child. A subject disclosed herein may have one or more of the following conditions: HTN, Lymphedema Rt. Leg, Chronic hyperkalemia and Kidney disease, Hyperlipidemia, PVD-Venous, Chronic Kidney Disease, osteoarthritis, Hyperlipidemia, Degenerative and Rheumatoid Arthritis, CAD, AFIB, PVD-Venous, Sleep Apnea, Hypothyroid, Spinal Sten, Hx Pancreatitis, Gastroparesis, AFIB, PVD-arterial, Prostate Cancer, DJD-bilateral, knee repl, Arterial w Revascularization, Depression, Smoker, Pulmonary HTN, CHF, PVD-Arterial, or Chronic renal disease.

The term "treatment" or 'treating" can refer to cure or ameliorate a condition or disorder including a disease. For example, an effectiveness of a cancer treatment, such as administration of an anti-cancer drug, may be an assessment of the anti-cancer drug to reduce tumor or cancer cell invasiveness, to kill cancer or tumor cells, to eliminate a cancer or tumor in a subject, to reduce a size of a tumor, to weaken or make more susceptible to apoptosis a cancer cell or tumor cell, to reduce or prevent metastasis, or others, or combinations thereof.

The term "effective" as defined herein related to a drug, generally refers to an assessment or determination about whether a drug has achieved the results it is intended to achieve. For example, an effectiveness of an anti-cancer drug or an anti-tumor drug may be an assessment of the anti-cancer drug or the anti-tumor drug to reduce tumor or cancer cell invasiveness, to kill cancer or tumor cells, or to eliminate a cancer or tumor in a subject. An effectiveness of a drug may also include an assessment of the severity and number of side effects or conditions brought on by consuming the drug. The assessment or determination may be performed using methods as described herein.
An "effective amount" when used in connection with a pharmaceutical composition disclosed herein can be an amount sufficient to produce a therapeutic result in a subject in need thereof.

The term "about" can mean the referenced numeric indication plus or minus 15% of that referenced numeric indication.

The term "prognosis," as used herein, generally refers to a prediction of the likely outcome of a subject's current standing or a predication of a future outcome. A prognosis may include a judgment or an opinion of a medical professional of a subject's current standing or future standing. Diagnostic methods or assays may be utilized to make a prognosis. In some cases, a diagnostic method may include performing a tissue biopsy, a cell cytology, an endoscopy, a diagnostic imaging, a blood test, a genetic screening or analysis or any combination thereof.

Wound: a breakdown in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue (i.e. muscle, bone, nerves) following injury to the skin or underlying tissues/ organs caused by surgery, a blow, a cut, chemicals, heat/ cold, friction/ shear force, pressure or as a result of disease, such as leg ulcers or carcinomas.

Nonhealing Wound: a non-healing or chronic wound is defined as a wound that does not improve after four weeks or does not heal in eight weeks. These include: diabetic foot ulcers, venous-related ulcerations, non-healing surgical wounds, pressure ulcers, wounds related to metabolic disease, wounds that repeatedly break down.

Healing Wound: the process of returning to health; the restoration of structure and function of injured or diseased tissues. The healing processes include blood clotting, tissue mending, scarring, and bone healing. Wound healing is a complex and dynamic process of replacing devitalized and missing cellular structures and tissue layers. The human adult wound healing process can be divided into 4-phases: the hemostasis phase, the inflammatory phase, the proliferation phase, and the remodeling phase.

DFU - Diabetic Foot Ulcer is a serious complication of diabetes which aggravates the patient's condition whilst also having significant socioeconomic impact. The aim of the present review is to summarize the causes and pathogenetic mechanisms leading to diabetic foot, and to focus on the management of this important health issue. Increasing physicians' awareness and hence their ability to identify the "foot at risk," along with guidance on how to treat each individual's DFU with proper foot care and therapeutics, may prevent diabetic foot ulceration and thus reduce the risk of amputation.
Wound Healing Diagnostic (WHD)- a diagnostic test that can be administered to a subject with a wound that measures certain biological parameters and can distinguish between wounds that will heal and those that will not heal.

Wound Therapy Diagnostic (WTD)- a diagnostic test that can be administered to a subject with a wound that measures certain biological parameters and can determine the most appropriate course of treatment that modulates biological processes that are preventing wound healing and which results in wounds that will heal.

Phosphorylation- the addition of phosphate to an organic compound, such as a protein, through the action of a phosphotransferase (phosphorylase) or kinase, that results in increased or decreased functions, such as signal transduction, of the protein.

Signal Transduction: occurs when an extracellular signaling molecule, or ligand, activates a specific receptor located on the cell surface, such as a receptor kinase, or inside the cell. Phosphorylation, or other post-translational modifications like methylation or acetylation, receptor triggers a biochemical chain of events inside the cell, eventually eliciting a response. Depending on the cell, the response may alter the cell's metabolism, shape, gene expression, or ability to divide. The signal can be amplified at any step, as signaling involves the interaction of multiple proteins in a step-wise fashion of activation. Thus, one signaling molecule can generate a response involving hundreds to millions of molecules.

IGF1R: The insulin-like growth factor (IGF-1) and its signaling through insulin receptor (IR) or mainly through IGF-1 receptor (IGF-IR) is highly implicated in tumor transformation and survival of malignant cell, but is only partially involved in normal cell growth. This is in part due to the interactions with oncogenes. IGF-IR is mainly involved in regulation of cell proliferation, anti-apoptosis, differentiation and cell motility, whereas IR is mostly of impact for control of glucose uptake and metabolism. Ligand-receptor binding results in phosphorylation of tyrosine residues in the IGF-IR tyrosine kinase domain. In the unstimulated state, the activation loop of IGF-IR activation loop (a-loop) is not phosphorylated and several tyrosine residues (1131, 1135 and 1136) block the active site. Upon ligand binding, the three tyrosines of the activation loop are trans-phosphorylated by IGF-IR's dimeric subunit partner. There are a number of proteins associated with and participate in the functional signaling of IGF-IR, any of which may have altered structure or function and, and have the potential to reduce signaling through IGF-IR. These include ARHGEF12, C-src tyrosine kinase, Cbl gene, EHD1, GRB10, IRS1, Mdm2, NEDD4, PIK3R3, PTPN11, RAS p21 protein activator 1, SHC1, SOCS2, SOCS3, YWHAE. The activation of the tyrosine kinase activity of IGF-IR leads to the phosphorylation of several of the above substrates, including the insulin receptor substrate family of proteins [such as Insulin receptor substrate 1 (IRS-1)].
transforming protein 1] and some others. Once phosphorylated, these docking proteins activate downstream intracellular signaling through the PI3K/Akt pathway, by way of GRB2/Shc activation of the Ras/MAPK pathways that ultimately leads to increased cellular proliferation. IGF-1R is ubiquitously expressed in tissues, playing a role in tissue growth stimulated by insulin, which liberates IGF-1 to activate the IGF-1R. IGF-1 and bFGF regulate transcriptional activity of the IGF-IR gene negatively and positively, respectively. In some embodiments, bFGF increases IGF-IR gene promoter activity by 2-fold and IGF-1 decreases transcriptional activity by 50%. In some embodiments, IGF-IR is not an absolute requirement for normal growth. In some embodiments, IGF-IR is crucial for anchorage independent growth, a property of malignant cells, where anchorage dependency can be acquired when the number of IGF-IRs at the cell surface is increased. In some embodiments, IGF-IR has a critical role in anchorage independent growth in prostate cancer and anchorage independent growth in wounds.

p-IGF-IRP Y1135-1 150 or phospho-tyrosine 1135-1150 Insulin-like growth factor 1-beta) - a protein found on the surface of human cells. It is a transmembrane receptor that is activated by a hormone called insulin-like growth factor 1 (IGF-1) and by a related hormone called IGF-2. It belongs to the large class of tyrosine kinase receptors and it mediates the effects of IGF-1, which is a polypeptide protein hormone similar in molecular structure to insulin. IGF-IR activation by phosphorylation, for example on tyrosines 1135-1150, the earliest major autophosphorylation sites necessary for kinase activation) plays an important role in growth and continues to have anabolic effects in adults - meaning that it can induce hypertrophy of skeletal muscle and other target tissues.

c-MET- a protein that in humans is encoded by the MET gene (MET proto-oncogene, receptor tyrosine kinase), which earlier in the discovery process had also been called MNNG HOS transforming gene. The protein possesses tyrosine kinase activity. The primary single chain precursor protein is post-translationally cleaved to produce the alpha and beta subunits, which are disulfide linked to form the mature receptor. MET is a single pass tyrosine kinase receptor essential for embryonic development, organogenesis and wound healing. Hepatocyte growth factor/Scatter Factor (HGF/SF) and its splicing isoforms (NK1 and NK2) are the only known ligand of the MET receptor. MET is normally expressed by cells of epithelial origin, while expression of HGF/SF is restricted to cells of mesenchymal origin. When HGF/SF binds its cognate receptor MET it induces its dimerization through a not yet completely understood mechanism leading to its activation (phosphorylation).

p-PTEN S380 (phospho-serine 380)- the phosphorylated species of a protein that, in humans, is encoded by the PTEN gene. Mutations of this gene are a step in the development of
many cancers and PTEN orthologs have been identified in most mammals for which complete genome data are available. This gene is considered a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded by this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains atensin-like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating Akt/PKB signaling pathway by inhibiting the signaling activity of the POKinase protein complex. Phosphorylation at serine 380 inactivates PTEN function, thereby releasing the inhibition of the PI3 Kinase complex.

PI3 Kinase-[also known as phosphatidylinositol 3-kinases, phosphatidylinositol-3-kinases, PI 3-kinases, PI(3)Ks, PI-3Ks or by the HUGO official stem symbol for the gene family, PI3K(s)] are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer. PI3Ks are a family of related intracellular signal transducer enzymes capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). The pathway, with oncogene PIK3CA and tumor suppressor PTEN, is implicated in insensitivity of cancer tumors to insulin and IGF1, and in calorie restriction. Activation of the PI3 Kinases results in phosphorylation, or activation, of the AKT/PKB proteins, which is a central signaling hub located in human cells. Examples of PI 3 kinase signaling pathway proteins include PDK1, PKB, AKT, TSC2, Rheb, mTOR, Ribosomal Protein S6, S6, SEBP1, eiF4E, eiF4F, eiF4G, KI67, FOXO, GSK3 A/B, MDM2, TIAM-1, VAV-1, P-Rex-1, ARAP3, Rac, and Rho.

Aktl/protein kinase B- is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. Aktl is involved in cellular survival pathways, by inhibiting apoptotic processes. Aktl is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, and general tissue growth. Complete deletion of Aktl manifests growth retardation and increased spontaneous apoptosis in tissues such as testes and thymus and since it can block apoptosis, Aktl promotes cell survival and has been implicated as a major factor in many types of cancer. Aktl is also known as Akt.

GSK-3P- also known as GSK3B, is an enzyme that in humans is encoded by the GSK3B gene. Abnormal regulation and expression of GSK3β is a proline-directed serine-threonine kinase that was initially identified as a phosphorylating and an inactivating agent of glycogen
synthase. GSK3A is involved in energy metabolism, neuronal cell development, and body
dpattern formation.

mTOR- Mammalian Target of Rapamycin [also known as FK506-binding protein 12-
rapamycin-associated protein 1 (FRAP1)], is a protein that in humans is encoded by the MTOR
gene. The protein complex containing mTOR is a serine/threonine protein kinase that regulates
cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy,
transcription, and fibrosis. Also, mTOR belongs to the phosphatidylinositol 3-kinase-related
kinase protein family. There are two complexes containing mTOR: mTORC1 and mTORC2.
The activity of mTORC1 is stimulated by insulin, growth factors, serum, phosphatidic acid,
amino acids (particularly leucine), and oxidative stress and mTORC2 phosphorylates the
serine/threonine protein kinase Akt/PKB at the serine residue S473, thus affecting metabolism
and survival. Phosphorylation of the serine stimulates Akt phosphorylation at a threonine T308
residue by PDK1 and leads to full Akt activation. MTOR integrates the input from upstream
pathways, including insulin, growth factors (such as IGF-1 and IGF-2), and amino acids. mTOR
also senses cellular nutrient, oxygen, and energy levels. The mTOR pathway is dysregulated in
human diseases, such as diabetes, obesity, depression, and certain cancers. Rapamycin inhibits
mTOR by associating with its intracellular receptor FKBP12. The FKBP12-rapamycin complex
binds directly to the FKBP12-Rapamycin Binding (FRB) domain of mTOR, inhibiting its
activity.

p-mTOR S2448 (phospho-serine 2448)- mTOR phosphorylated at S2448 binds to both,
raptor and rictor. Experiments with chemical inhibitors of the mTOR kinase and of the
phosphatidylinositol-3-kinase revealed that downregulation of mTOR S2448 phosphorylation
correlates with decreased mTORC1 activity.

COX-2 - Cyclooxygenase-2, is an enzyme that acts to speed up the production of certain
chemical messengers, called prostaglandins that play a key role in in promoting inflammation.
When cox-2 activity is blocked, inflammation is reduced. Unlike Cox-1, Cox-2 is active only at
the site of inflammation, and not limited to activity in the stomach.

iNOS - inducible nitric oxide synthase (iNOS), is a small molecule that has various roles
in cellular functions and acts via a cGMP-mediated signal transduction pathway. iNOS is both
tumouricidal and bactericidal, has nitrosylase activity, and mediates cysteine S-nitrosylation of
cytoplasmic target proteins such COX-2. It is regulated by calcium/calmodulin, induced by
endotoxins and cytokines, including IFN-gamma, in synergy with bacterial lipopolysaccharides
(LPS, TNF or IL1B/IL-1 beta and its expression and function are inhibited by aspirin.

NFkB - nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein
complex comprising a subfamily of NFkB proteins, including RelA (NFkB/p65), RelB, and c-
Rel. For example, NfKB/p65 controls transcription of DNA, cytokine production and cell survival. NFKB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NFKB plays a key role in regulating the immune response to infection (light chains are critical components of immunoglobulins). Incorrect regulation of NFKB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development.

IKB-α - the I-kappa-B proteins inhibit the multimeric NFKB complex by trapping it in the cytoplasm. Kinases IκB-α or IκB-β phosphorylates NFKB's serine residues, marking them for destruction via the ubiquination pathway, allowing activation of the NFKB complex which, once activated, translocates to the nucleus, binds DNA and activates transcription.

HIF-1 (hypoxia-inducible factor 1) is a key transcription factor that is responsive to hypoxic conditions and is known to be coordinately acting in hypoxia. The HIF-1 complex, composed of heterodimer pairs containing an alpha and beta subunit, binds to the promoter of hypoxia-responsive genes. The active transcription complex interacts with several transcription factors, such as p300, signal and transducer of transcription 3 (STAT3), and Redox effector factor 1/2/purinic/pyrimidinic endonuclease. There are six different HIFs that can form heterodimers: HIF-la; HIF-1β (ARNT, aryl hydrocarbon receptor nuclear translocator); HIF-2a (EPAS1, endothelial PAS domain protein 1); HIP-2β (ARNT2, aryl-hydrocarbon receptor nuclear translocator 2); HIF-3a; and HIP-3β (ARNTL, aryl-hydrocarbon receptor nuclear translocator 3).

HIF-la hypoxia-inducible factor 1-alpha is a subunit of a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1) that is encoded by the HIF-la gene and is considered as the master transcriptional regulator of cellular and developmental response to hypoxia. The dysregulation and overexpression of HIF-la by either hypoxia or genetic alternations have been heavily implicated in cancer biology, as well as a number of other pathophysologies, specifically in areas of vascularization and angiogenesis, energy metabolism, cell survival, and tumor invasion.

p53 - a specific protein with a mass of 53 kilo Daltons that is produced by a tumor-suppressor gene. Like other tumor-suppressor genes, the p53 gene normally controls cell growth. If p53 is physically lost or functionally inactivated, cells can grow without restraint. Many human tumors have mutations in the gene coding for the p53 protein.

p16INK4a - also known as cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1 and as several other synonyms, is a tumor suppressor protein, that in humans is encoded by the CDKN2A gene. p16 plays an important role in cell cycle regulation by
decelerating cells progression from G1 phase to S phase, and therefore acts as a tumor suppressor that is implicated in the prevention of cancers, notably melanoma, oropharyngeal squamous cell carcinoma, cervical cancer, and esophageal cancer. p16 can be used to improve the histological diagnostic accuracy of CIN3. The CDKN2A gene is frequently mutated or deleted in a wide variety of tumors. p16 is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6. These latter kinases phosphorylate retinoblastoma protein (pRB) which eventually results in progression from G1 phase to S phase. p16 was originally found in an "open reading frame" of 148 amino acids encoding a protein of molecular weight 15,845 comprising four ankyrin repeats. p16Ink4a is named after its molecular weight and its role in inhibiting CDK4.

β-Catenin - a dual function protein, regulating the coordination of cell-cell adhesion and gene transcription. In humans, the CTNNB1 protein is encoded by the CTNNB1 gene, β-catenin is a subunit of the cadherin protein complex and acts as an intracellular signal transducer in the Wnt signaling pathway. It is a member of the catenin protein family and homologous to γ-catenin, also known as plakoglobin. Beta-catenin is widely expressed in many tissues. In cardiac muscle, beta-catenin localizes to adherens junctions in intercalated disc structures, which are critical for electrical and mechanical coupling between adjacent cardiomyocyte. Mutations and overexpression of β-catenin are associated with many cancers, including hepatocellular carcinoma, colorectal carcinoma, lung cancer, malignant breast tumors, ovarian and endometrial cancer. Alterations in the localization and expression levels of beta-catenin have been associated with various forms of heart disease, including dilated cardiomyopathy. β-catenin is regulated and destroyed by the beta-catenin destruction complex, and in particular by the adenomatous polyposis coli (APC) protein, encoded by the tumor-suppressing APC gene.

BAK - this protein functions as a pro-apoptotic regulator involved in a wide variety of cellular activities. In healthy mammalian cells, BAK localizes primarily to the MOM (mitochondrial outer membrane), but remains in an inactive form until stimulated by apoptotic signaling. The inactive form of BAK is maintained by the protein's interactions with VDAC2, Mtx2, and other anti-apoptotic members of the BCL2 protein family. Nonetheless, VDAC2 functions to recruit newly synthesized BAK to the mitochondria to carry out apoptosis. Moreover, BAK is believed to induce the opening of the mitochondrial voltage-dependent anion channel, leading to release of cytochrome c from the mitochondria. Alternatively, BAK itself forms an oligomeric pore, MAC, in the MOM, through which pro-apoptotic factors leak in a process called MOM permeabilization.

Caspase 3 - is a caspase protein that interacts with caspase-8 and caspase-9. It is encoded by the CASP3 gene. CASP3 orthologs have been identified in numerous mammals for which
complete genome data are available. Unique orthologs are also present in birds, lizards, lissamphibians, and teleosts. The CASP3 protein is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6 and 7; and the protein itself is processed and activated by caspases 8, 9, and 10. It is the predominant caspase involved in the cleavage of amyloid-beta 4A precursor protein, which is associated with neuronal death in Alzheimer's disease. Alternative splicing of this gene results in two transcript variants that encode the same protein.

Caspase 9 - is an initiator caspase, encoded by the CASP9 gene. CASP9 orthologs have been identified in all mammals for which complete genome data are available. The aspartic acid specific protease caspase-9 has been linked to the mitochondrial death pathway. It is activated during programmed cell death (apoptosis). Induction of stress signaling pathways JNK/SAPK causes release of cytochrome c from mitochondria and activation of apaf-1 (apoptosome), which in turn cleaves the pro-enzyme of caspase-9 into the active form. Once initiated caspase-9 goes on to cleave procaspase-3 & procaspase-7, which cleave several cellular targets, including poly ADP ribose polymerase.

A "subject," as used herein, includes any animal, e.g., an animal that has diabetes, an Alzheimer's disease, or a cancer. Suitable subjects include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, humans (e.g., human patients), are included.

A "phosphorylation state" of a protein refers to the degree of (total amount of) phosphorylation of the protein. This includes both the number of sites (e.g. suitable Ser, Thr or Tyr amino acid residues) of the protein that are phosphorylated, and the level of phosphorylation at any given acceptor site on the amino acid chain.

A "baseline value" or "threshold level" as used herein, refers to the level of expression or activation (e.g., phosphorylation) of the same protein in a normal subject (e.g., non-diabetic, non-cancerous, or unstimulated subject). For example, a baseline value includes reference standards, where a predetermined threshold value (or range of values) determines whether an expression level of measured protein, or a level of activated state (e.g., phosphorylation state) of the protein, is above the "normal" value. For each protein whose level is determined, the value can be normalized to the total protein in the cell; or to the amount of a constitutively expressed protein (from a housekeeping gene), such as actin; or the amount of a phosphoprotein may be compared to the amount of its non-phosphorylated counterpart.
A "significant" increase, as used herein, means a statistically significant change, using statistical methods that are appropriate and well-known in the art, generally with a probability value of less than five percent chance of the change being due to random variation. As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, "a" member of the tested pathway, as used above, includes 2, 3, 4, 5 or more members of the pathway. Similarly, "an" inhibitor of the pathway includes multiple inhibitors.

Receptor Kinases

Protein kinases are a group of enzymes that possess a catalytic subunit that transfers terminal phosphates from nucleotide triphosphates (often ATP) to one or more amino acid residues in a protein (phosphorylation). Modification of these side-chains results in conformational changes that can alter protein function (i.e., activate its kinase activity). The protein kinase enzymes fall into two broad classes, characterized with respect to substrate specificity: serine/threonine-specific and tyrosine-specific kinases. Phosphorylation of proteins is an important mechanism for communicating signals within a cell (signal transduction) to modulate cellular activity. Tyrosine kinases are divided into two main families: the transmembrane receptor-linked kinases; and those that are cytoplasmic proteins. Receptor tyrosine kinases (RTKs) play pivotal roles in diverse cellular activities including control of cell growth, differentiation, metabolism, adhesion, motility, and apoptosis. (In general, RTKs are composed of: an extracellular domain, some of which may have a specific ligand binding domain; a transmembrane domain that spans the plasma membrane; and an intracellular kinase domain, which binds proteins based on recognition domains on their protein substrates. Ligand binding results in a series of structural rearrangements to the RTK, including activation of the kinase and substrate phosphorylation. These activated substrate proteins, and then initiate a series of subsequent kinase mediated events that transmits a signal from the receptor to multiple downstream effectors. In cancer, many of these signaling pathways result in the alteration in gene expression patterns, as evident by the number of therapies used in cancer that seek to inhibit the RTKs or inhibit the pathways that RTKs modulate downstream.

An increase in kinase-mediated signal transduction may occur in the absence of ligand binding. There are a number of mutations that result in conformational or catalytic changes in RTK signaling. Chromosomal rearrangements, point mutations, deletions, etc. may result in the constitutive activation or hyper-activation in these critical signaling pathways. Additionally the modulation of RTK signaling in the absence of ligand may be modulated by spatial deregulation, controlled by receptor internalization or alterations to the lipid and actin
filament composition of cellular membranes. Such aberrant, ligand-independent, non-regulated RTK signaling in cancer cells results in the hyper-activation of downstream signaling cascades leading to cancerous processes and such processes may occur in the keratinocytes in the epithelia of healing wounds. Ligand-dependent activation is also implicated as an aberrant cancer signaling processes wherein compensatory autocrine- or paracrine-based over expression of ligands can activate RTK signaling, especially as a resistance mechanism. Regardless of the underpinning mechanisms of activation, the common output is increased phosphorylation of the RTK and downstream signaling.

There are a number of therapeutically relevant RTKs (growth factor receptors, for example) that may become aberrantly activated and inappropriately turn on key regulatory processes including cell growth, proliferation, apoptosis, angiogenesis, and cell survival. Receptors become activated, and dysregulation of receptor signaling has been correlated to the development and progression of wound healing and cancers in a wide range of tissues. Many of these receptors become phosphorylated at specific amino acid residues: tyrosine, threonine, and serine. Phosphorylation either activates the receptors intrinsic kinase activity or enhances binding of adaptor proteins that mediate downstream signaling. As tyrosine is a major phosphorylation, many of these receptors are known as Receptor Tyrosine Kinases (RTK).

There are several ways that pharmaceuticals can inhibit RTK signaling, including: blocking ligand binding (the antibody trastuzumab, for example), inhibiting dimer formation where dimer formation is required for activation of signaling (the antibody pertuzumab, for example), or by inhibiting the kinase activity of the RTK with small molecular weight inhibitors (lapatinib, for example) that can cross the cell membrane. Some of these receptors are activated by mutations/translocations/amplifications directly to the receptor itself, such as HER2, EGFR, c-MET, fibroblast growth factor receptor (FGFR), or insulin-like growth factor-1 receptor (IGF-IR). Other mutations in genes coding for proteins that are members of signaling pathways may activate signaling distant and downstream from the receptor, such as in certain mutations/loss of heterozygosity to the tumor suppressor PTEN (phosphatase and tensin homolog) whose loss of function can activate the PTK (phosphoinositide-3 kinase)/Akt (also known as PKB, Protein Kinase B) signaling pathway. Also, signaling may be mediated by the spatial effects of monomer concentration or the sequestration of homo- or heterodimers due to alterations in lipid and actin filaments. Thus, the advent of molecularly targeted drugs has generated a number of therapies that specifically target RTKs or proteins in downstream pathways that the RTKs modulate and these therapies have found clinical utility in treating proliferative diseases like cancer.

**Diabetes-related atherosclerosis**
Overall, people with diabetes mellitus (DM) have an increased incidence of atherosclerosis, thickening of capillary basement membranes, arteriolar hyalinosis, and endothelial proliferation. Calcification and thickening of the arterial media are also noted with higher frequency in the diabetic population, although whether these factors have any impact on the circulatory status is unclear. Diabetics may develop atherosclerotic disease of large-sized and medium-sized arteries, and a common atherosclerotic disease of the infrapopliteal segments is particularly common and significant in the diabetic population. Underlying digital arterial disease, when compounded by an infected ulcer in close proximity, can result in complete loss of digital collaterals and precipitate gangrene. The prevalence of this form of arterial disease in diabetic persons is thought to result from a number of metabolic abnormalities, including high low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) levels, elevated plasma von Willebrand factor, inhibition of prostacyclin synthesis, elevated plasma fibrinogen levels, and increased platelet adhesiveness.

The pathophysiology of peripheral neuropathy in diabetics is multifactorial and some of the contributing factors include: vascular disease occluding the vasa nervorum; endothelial dysfunction; deficiency of myoinositol-altering myelin synthesis; diminishing sodium-potassium adenine triphosphatase (ATPase) activity; chronic hyperosmolarity, causing edema of nerve trunks; and effects of increased sorbitol and fructose. The resultant loss of sensation in the foot is repetitive stress; unnoticed injuries and fractures; structural foot deformity (such as hammertoes, bunions, metatarsal deformities, or Charcot foot). These contributing factors lead to the eventual breakdown of tissue. Blistering and ulceration, the DFU, may develop due to complications resulting from unnoticed excessive heat or cold, pressure from poorly fitting shoes, or physical damage from bumping the tissue sufficiently to produce a bruise or wound. These factors, combined with poor arterial inflow, confer a high risk of developing DFU and can contribute significantly to the loss of limbs in diabetic patients.

In some embodiments, the management of diabetic foot ulcers requires offloading the wound by using appropriate therapeutic footwear, daily saline or similar dressings to provide a moist wound environment, debridement when necessary, antibiotic therapy if osteomyelitis or cellulitis is present, optimal control of blood glucose, or evaluation and correction of peripheral arterial insufficiency. In some embodiments, wound coverage by cultured human cells or heterogeneic dressings/grafts, application of recombinant growth factors and hyperbaric oxygen treatments may also be beneficial. In some embodiments, topical wound management is to provide a moist, but not wet, wound bed. After debridement, application of moist sodium chloride dressings or isotonic sodium chloride gels (e.g., Normlgel®, IntraSite® gel) or a hydroactive paste (e.g., DuoDERM®) is typically utilized to manage the ulcer. Optimal wound
coverage requires wet-to-damp dressings, to support autolytic debridement, absorb exudate, and to protect the surrounding healthy tissues. A polyvinyl film dressing (e.g., OpSite, Tegaderm) that is semipermeable to oxygen and moisture and impermeable to bacteria is also used by physicians to treat wounds that are neither very dry nor highly exudative.

Wound coverage may also be based upon other approaches:

Dry wounds: Hydrocolloid dressings, such as DuoDERM® or IntraSite® Hydrocolloid, are impermeable to oxygen, moisture, and bacteria; maintain a moist environment; and support autolytic debridement. They are a good choice for relatively desiccated wounds;

Exudative wounds: Absorptive dressings, such as calcium alginates (e.g., Kaltostat®, Curasorb®), are highly absorptive and are appropriate for exudative wounds. Alginates are available in a rope form, which is useful for packing deep wounds;

Very exudative wounds: Impregnated gauze dressings (e.g., Mesalt®) or hydrofiber dressings (e.g., Aquacel®, Aquacel-Ag®) are useful for extremely exudative wounds. In these cases, twice-daily dressing changes may be needed;

Infected wounds: For infected superficial wounds, use Silvadene® (silver sulfadiazine) if the patient is not allergic to sulfa drugs; if a sulfa allergy exists, either bacitracin-zinc or Neosporin ointment is a good alternative. Where heavy bacterial contamination of deeper wounds exists, irrigation using one-fourth strength Dakin solution and 0.25% acetic acid may be useful for a brief period of time; a hydrofiber-silver dressing (Aquacel-Ag®) can help control wounds that are both exudative and potentially colonized;

Wounds covered by dry eschar: In this case, simply protecting the wound until the eschar dries and separates may be the best management. Occasionally, painting the eschar with povidone iodine (Betadine) is beneficial to maintain sterility while eschar separation occurs; an uninfected dry heel ulcer in a well-perfused foot is perhaps best managed in this fashion;

Areas that are difficult to bandage: Bandaging a challenging anatomical area, such as around a heel ulcer, requires a highly conformable dressing, such as an extra thin hydrocolloid; securing a dressing in a highly moist challenging site, such as around a sacrococcygeal ulcer, requires a conformable and highly adherent dressing, such as a wafer hydrocolloid;

Fragile periwound skin: Hydrogel sheets and nonadhesive forms are useful for securing a wound dressing when the surrounding skin is fragile.

In some embodiments, topical preparations useful in the management of diabetic foot ulcers are as follows:

Platelet-derived growth factors (PDGF): Topically applied PDGF has a modestly beneficial effect in promoting wound healing. Becaplermin gel 0.01% (Regranex®), a recombinant human PDGF that is produced through genetic engineering is approved by the US Food and Drug
Administration (FDA) to promote healing of diabetic foot ulcers. Regranex® is meant for a healthy, granulating wound, not one with a necrotic wound base, and is contraindicated with known skin cancers at the site of application;

Enzymatic debridement: Collagen comprises a significant fraction of the necrotic soft tissues in chronic wounds; the enzyme collagenase, derived from fermentation of C. histolyticum, helps remove nonviable tissue from the surface of wounds. In some cases, it is not a substitute for an initial surgical excision of a grossly necrotic wound;

Miscellaneous topical agents: Various other topical agents that have been used for wound management include sugar, antacids, and vitamin A and D ointment;

Cytotoxic agents, such as hydrogen peroxide, povidone iodine, acetic acid, and Dakin solution (sodium hypochlorite), should be avoided, except as noted above under infected wounds.

In some embodiments, mechanical methods are employed to effect healing, including:

Vacuum-assisted closure: Clean but nonhealing deep cavity wounds may respond to repeated treatments by application of negative pressure under an occlusive wound dressing (vacuum-assisted closure [VAC]);

Hydrotherapy: Intractable, infected, cavity wounds sometimes improve with hydrotherapy using saline pulse lavage under pressure (PulsEvac®);

Debridement: Surgical management is indicated for debridement of nonviable and infected tissue from the ulceration, removal of excess callus, curettage of underlying osteomyelitic bone, skin grafting, and revascularization. The wound usually requires an initial surgical debridement and probing to determine the depth and involvement of bone or joint structures. Visible or palpable bone implies an 85% chance of osteomyelitis.

In some aspects, once a wound has reached a steady clean state, a decision has to be made about allowing healing by natural processes or expediting healing by a surgical procedure. In some embodiments, surgical options include skin grafting, application of bioengineered skin substitutes, and flap closures. Methods used may include skin grafts, including autologous skin grafts or cadaveric skin to cover relatively deep wounds following surgical excision when the wound bed does not appear appropriate for application of an autologous skin graft.

In some embodiments, appropriate skin substitutes include:

Dermagraft® is a cryopreserved human fibroblast-derived dermal substitute produced by seeding neonatal foreskin fibroblasts onto a bioabsorbable polyglactin mesh scaffold;

Dermagraft® is useful for managing full-thickness chronic diabetic foot ulcers. It is not appropriate for infected ulcers, those that involve bone or tendon, or those that have sinus tracts. A multicenter study of 314 patients demonstrated significantly better 12-week healing rates with
Dermagraft® (30%) versus controls (17%). Allergic reactions to its bovine protein component have been reported;

Apligraf® is a living, bilayered human skin substitute. It is not appropriate for infected ulcers, those that involve tendon or bone, or those that have sinus tracts. Allergic reactions to the agarose shipping medium or its bovine collagen component have been reported;

Xenograft: Oasis® is a xenogeneic, acellular collagen matrix derived from porcine small intestinal submucosa in a way that allows an extracellular matrix and natural growth factors to remain intact. This provides a scaffold for inducing wound healing. The use of Oasis® is not recommended for patients with allergies to porcine materials.

In some embodiments, Hyperbaric Oxygen Therapy can also be used in promoting DFU healing. In the presence of an intractable wound and associated non-correctible ischemic arterial disease, hyperbaric oxygen therapy may be beneficial (in selected cases), e.g., 40 hyperbaric oxygen treatments (85 min daily, 5 d/wk for 8 wk)

**Therapeutic Approaches**

Diabetes herein include Type 1, Type 2, or gestational diabetes. Many medications may have a role in the treatment of diabetes, the complications of diabetes, and the etiologies of diabetic ulcer. For example, hemorheologic agents and antiplatelet agents are used in the management of underlying atherosclerotic disease.

Hemorheologic agents such as pentoxifylline (Trental®) improve intermittent claudication in approximately 60% of patients after 3 months. Cilostazol (Pletal®) is an alternative hemorheologic agent for patients who cannot tolerate pentoxifylline. Cilostazol is contraindicated in patients with congestive heart failure.

Antiplatelet therapy with aspirin or clopidogrel (Plavix®) may be warranted in some cases for the prevention of the complications of atherosclerosis. Antiplatelet agents inhibit platelet function by blocking cyclooxygenase and subsequent platelet aggregation.

Therapies used with varying levels of success in treating DFU include:

1) Clopidogrel (Plavix) selectively inhibits ADP binding to platelet receptor and subsequent ADP-mediated activation of glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation. It is indicated as antiplatelet therapy in some patients with atherosclerotic disease.

2) Aspirin inhibits prostaglandin synthesis, preventing formation of platelet-aggregating thromboxane A2 and may be used in low dose to inhibit platelet aggregation and to improve complications of venous stases and thrombosis. The recommended dose varies with indication, and, often, the literature is unclear on the optimal dosing.
3) Topically applied platelet-derived growth factors (PDGF) such as becaplermin gel (Regranex®) have a modestly beneficial effect in promoting wound healing.

4) Becaplermin (Regranex®), a recombinant human PDGF that is produced through genetic engineering, is approved by the US Food and Drug Administration (FDA) to promote healing of diabetic foot ulcers. Regranex® is meant for a healthy, granulating wound, not one with a necrotic wound base, and it is contraindicated with known skin cancers at the site of application.

In some aspects, the present disclosure provides for a method of predicting a diabetic foot ulcer subject's wound healing by a wound healing diagnostic (WHD) provided by the invention.

In some aspects, the present disclosure provides for a method for providing a specific, or individualized, wound therapy diagnostic (WTD) through the development of signaling protein-pathway guided pharmaceutical strategies.

The present disclosure describes the WHD as a panel of ratios of protein expression between IGF-1R and PI3K (PI3K/IGF-1R) and/or mTOR (mTOR/IGF-1R) and/or COX-2 (COX-2/IGF-1R) and/or p53 (p53/IGF-1R) and/or Bak (Bak/IGF-1R) and/or Caspase-9 (Caspase-9/IGF-1R) obtained from tissue analysis of DFU patients. Each WHD ratio determination value would be used to associate the subject's cellular signaling protein expression ratios to those for healed or unhealed DFUs obtained from the clinical studies that established the likely outcome for wound healing. In some embodiments, a WTD as a panel of signaling protein expression ratios that characterize the mediating processes of normal or impaired wound healing that would be used to identify pharmaceutical therapies to optimize signaling protein expression relationships associated with DFU wound closure or normal tissue signaling.

In some aspects, the present disclosure provides several important cellular processes associated with normal healing for the DFU subjects which include angiogenesis (HIF-1α, iNOS and VEGFR) and intrinsic cell death/apoptosis (Bak and Caspase-9) signaling proteins. Consequently, the cellular processes associated with impaired DFU subject healing include survival (NFkB, GSK-3P and β-Catenin) and senescence (p53 and p56INK4a) signaling proteins. WTD values are obtained from the ratio of Akt protein expression and the protein expression of Bak (Bak/Akt) and/or Caspase-9 (Caspase-9/Akt) and/or VEGFR1 (VEGFR1/Akt) and/or iNOS (iNOS/Akt) and/or HIF-1α (HIF-1α/Akt) and/or GSK-3P (GSK-3P/Akt) and/or β-Catenin (β-Catenin/Akt) and/or NFkB (NFkB/Akt) and/or p53 (p53/Akt) and/or p56INK4a (p56INK4a/Akt).

Signaling protein ratio signatures of tested subjects would be profiled against these four categories of values obtained from the clinical study to provide corrective pharmaceutical strategies for wound treatment that would promote wound healing. These therapies would be designed for, but not limited to, topical applications of appropriately compounded...
pharmaceutical agents selected using signaling protein-pathway guided strategies that would alter the WTD protein expression ratio signature values of nonhealing DFU subjects to those values associated with normal or healing DFU subjects. In some embodiments, the use of signaling protein specific pathway therapies would promote the use of specific nodal targeting for corrective wound treatment.

In some aspects, the present disclosure provides information for the development of pharmaceutical wound healing therapies based on the abnormal signaling protein ratios obtained from the unhealed subject's wound being evaluated. The example is given of a non-healing DFU subject demonstrating WTD ratio values below normal subject values for iNOS/Akt, HIF-1α/Akt and VEGFR/Akt (angiogenesis panel) and with WTD ratio values abnormally elevated for NFkB/Akt (survival panel). Therapeutic evaluations and considerations for these groupings of abnormal values would prioritize the signaling protein pathway relationships for therapy to achieve the broadest impact for protein expression modulation through supplementation, or inhibition, of pharmaceuticals selected for topical therapy. In this case, a candidate topical pharmaceutical for wound healing therapy would include the combination of an iNOS donor pharmaceutical with a pharmaceutical inhibitor of NFkB expression. Clinical testing of these compounds would be performed from additional RPPA tissue analysis and observations of outcomes for validation and/or assessment of additional cellular processes that may contribute to impaired wound healing. The following factors would be used from the information obtained from the WTD panel analysis to support the selection of the pharmaceuticals suggested for the treatment of this unhealed wound.

For the correction of deficient iNOS protein ratio expression nitric oxide (NO) pharmaceutical wound supplementation by the use of an NO donor compound for wound healing would provide direct enhancement of wound NO bioactivity - and iNOS protein expression - that enhances wound epithelialization (keratinocyte migration), angiogenesis (keratinocyte VEGF activation), collagen deposition and wound remodeling. NO plays a primary role in the control of VEGF-synthesis and in cell adaptations with upregulated activity. HIF-1α accumulation is achieved by attenuated prolyl hydroxylase (PHD) activity with NO production and PHD activity is subject to regulation by NO. NO not only modulates the HIF-1 response but also functions as a HIF-1 inducer during sustained formation. Levels of wound fluid NO metabolites (wound fluid nitrates/NOx) have also been determined to accurately predict the wound healing trajectory of chronic wounds and may individually provide diagnostic information for wound healing to support the need for iNOS supplementation. In some embodiments, iNOS supplementation of the example wound would not only improve the protein
expression of iNOS but also the protein expression of VEGF and HIF-1α, possibly promoting the necessary corrective influence of the angiogenesis signaling proteins to optimize wound recovery.

Altered regulation of inflammation in wound repair with diabetes is suggested to be a byproduct of dysfunction of the transcription factor nuclear factor-kappa B (NF-kB). During normal wound repair, local inflammation and immune responses are facilitated by tumor necrosis factor-a (TNF-a) regulated activation and upregulation of NF-kB-dependent gene expression for host defense proteins, cytokines, growth factors, proteases and inflammatory enzymes. Several important moderators of impaired wound healing are identified as target genes or byproducts of NFkB activation in the diabetic foot model. These include early phase NF-kB-dependent genes activated by TNF-a for metalloproteases MMP9 and MMP10, that are observed in higher concentrations in chronic diabetic wounds and the anti-apoptosis factor c-FLIP that has been demonstrated to significantly inhibit cellular apoptosis through TNF-a regulated NFkB activation. Additional NFkB-mediated processes associated with suppressive factors associated with DFU wound healing include the inhibition of insulin signaling through enhanced serine phosphorylation of IRS1 and the upregulation of cytokine, chemokine and inflammatory factors from the senescence associated secretory phenotype (SASP) during the establishment of cellular senescence. In some embodiments, NFkB is a mediator of induced p53-directed programmed cell death/apoptosis by a RAF/MAPK pathway, bypassing TNF-a regulated NFkB activation, signaling the repression of p53-mediated cell death following NFkB inhibition. In some embodiments, the reduction of NFkB protein expression promotes improved recovery of wound healing, reduces dysregulated inflammatory cytokine and chemokine production (SASP), facilitates improved insulin pathway signaling, and/or modulate excessive p53-mediated cell death.

In some embodiments, the topical application (or other use) of a pharmaceutical combination of an acceptable NO (iNOS) donor with an acceptable inhibitor of NFkB protein expression can provide appropriate therapy for the correction of impaired healing of the example wound based on protein expression profiles provided from RPPA tissue analysis. In some embodiments, the WTD may provide clinical information useful in the selection of currently available therapies to improve wound healing as well as for the development of unique or novel pharmaceutical therapies for impaired DFU wound subjects who are evaluated by the use of RPPA tissue analysis.

**Diabetic Wound Healing Research**

The paradigm for wound healing is divided into four overlapping stages: hemostasis, inflammation, proliferation and remodeling. This process requires a complex coordination of key molecular, cellular and physiologic events by facilitative signaling between hematopoietic,
immunologic, and resident skin cells. Depending upon which diabetic wound healing models are evaluated, increased expression of the tumor suppressor transcription factor p53 and ischemia-induced apoptosis may result in senescence and the inhibition of signaling pathways driving inflammation or cell survival. Other proteins identified in healing models, like the hypoxia-inducible factor-1 (HIF-1) and HIF-1 α proteins (which modulate angiogenesis, cell proliferation, and wound healing, as well as cancer invasion/metastasis) may be altered in the diabetic environment, contributing to impaired wound healing.

RPPA

Understanding the pathogenesis of disease states has traditionally focused on the application of genomic techniques to assess alterations at the DNA level that ultimately results in altered gene expression, aberrations in intercellular location, or the production of proteins that harbor mutations affecting function. The regulation of protein expression and function can occur at many levels but ultimately the modulation of activity can be assessed by measuring the levels of activating or inhibiting posttranslational modifications across multiple proteins in the disease state. While the functional consequences of DNA alterations cannot be ascertained by measuring genomic events directly, an assessment of each protein’s posttranslational modifications can provide valuable data that allows for the identification of activated signaling cascades that are aberrantly regulated and contributing to the disease state.

Gene expression arrays, which measure the cellular levels of mRNA are routine and informative for some of these alterations but from such arrays it is difficult to determine the actual level of protein expression, and are unable to detect the posttranslational modifications of proteins. The development of reliable proteomic characterizations that allow for multiple proteins and their posttranslational modifications can provide the necessary global understanding of a disease state, allowing for the identification of therapeutic points of intervention or the development of diagnostic tests that can identify the best therapy for each molecularly unique sub-classification of a disease.

Proteomics is defined as the large-scale study of proteins, including their structure, function, and, for those in signal transduction pathways, their activation status. Comparative analyses are made difficult because the proteome differs from cell to cell, the patterns change dynamically over time, and there are many proteins that contain polymorphisms, splice variants, and different levels and types of posttranslational modifications. Attempts at the proteomic characterization of diseased tissues have for the most part relied on technologies that assess a single protein (immunohistochemistry, ELISA, Western Blot analysis, for example) and the newer technologies related to Mass Spectroscopy following an initial parsing based upon two-dimensional gel electrophoresis.
The Reverse Phase Protein micro-Array (RPPA) is a highly sensitive, high-throughput, functional proteomic technology that offers many of the advantages needed. It extends beyond the power of immunoblotting by providing a quantitative measure on the differential expression of total proteins and their activation, which is usually the phosphorylated species. Total proteins and their corresponding phosphoproteins, across multiple proteins that are involved in signaling cascades, can be assessed across multiple patient samples, simultaneously, providing a 'snap-shot' of the activation state/functionality of entire pathways. With RPPA, all samples are spotted at the same time, making this method ideally suited for the retrospective analysis of a large number of specimens, in a manner that is analogous to genomic-dot blots. Compared with a conventional Western blot, RPPA analysis uses 20,000 cells to generate quantitative data across multiple. From a lysate of 20,000 cells, multiple, identical nitrocellulose slides are printed and each slide is interrogated with a single highly specific antibody that recognizes either the total protein or a modified protein in the multiplexed samples (over 300 individual samples per array). Thus, samples prepared from 20,000 cells are sufficient to analyze many different protein targets. This enables the simultaneous analysis of a much larger number of proteins from each sample and makes this technique suitable for the analysis of cell populations present in low numbers. Although newer Western blot, signal detection reagents, such as SuperSignal West Femto (Pierce, Rockford, IL), have signal sensitivities comparable to RPPA, they cannot compete with the high-throughput nature or miniscule cell numbers and volume requirements achievable with RPPA. This broad assessment of protein modifications and the determination of a disease tissue's pathway signaling architecture have the potential to recognize meaningful protein and pathway interactions of known proteins, which can lead to the discovery of new protein interactions and the formation of novel signaling pathways.

In some aspects, the present disclosure is related to the area of wound healing in diabetes. In some embodiments, it is related to the novel use of RPPA proteomic analysis of chronic wound tissues from subjects with diabetes for the documentation of the prediction and mechanisms of healing using the ratios of selected sites of proteomic phosphorylation and cellular signaling from prepared chronic wound specimens.

In some aspects, impaired neuropathic-diabetic foot ulcer (DFU) wound healing may be predicted by determination of keratinocyte PI3K (phosphatidyl inositol-3-kinase)/IGF-IR (insulin-like growth factor-1-receptor) proteomic ratios. In Example 1., comparative, quantitative proteomic assessments of selected signaling pathways of marginal DFU keratinocytes (n=19) and non-diabetic (control), plantar foot skin keratinocytes (n=3), were performed using reverse phase microarray (RPPA) and laser-capture microdissection (LCM)
technology. When compared with non-diabetic control subjects, DFU wound keratinocyte signaling activity was significantly (p<0.05) elevated for: 1) the pro-inflammatory transcription factor, NF-kB; 2) the tumor suppressor transcription factor, P53; 3) the senescence biomarker, P16INK4a; 4) the cellular survival and growth transduction pathway proteins: PI3K, AKT (protein kinase B), β-catenin, glycogen synthase kinase-3p (GSK-3p), and C-Met; and 5) the P53-directed apoptosis cleavage protein Caspase-3. During a 12-week post-debridement observation period all DFU subjects received standard of care wound treatment and wound off-loading. During this time three (n=3) of the nineteen DFU subjects (16 percent) demonstrated complete healing of the study ulcer. Following study completion DFU study subject wound status (healed vs. un-healed) correlated with proteomic activity ratios for PI3K/IGF-1R (“P/I-ratio”). PI3K- AKT/mTOR (mammalian target of rapamycin) is the major signaling pathway activated by insulin and insulin-like growth factors-1 and 2 (IGF-1 and IGF-2) through IGF-IR. IGF-IR stimulation activates AKT signaling transduction pathways leading to hypoxia-inducible factor-1 (HIF-1) expression. Increased HIF-1 expression promotes cell proliferation/survival and is documented as a critical factor for diabetic wound healing. Conversely, phosphatase tensil homolog (PTEN), a p53-directed PI3K antagonist documented with diabetes, inhibits AKT/mTOR signal pathway activation and induction of HIF-1 mediated gene expression. Our data documents P/I-ratio values (±SEM) of: 0.7 (±0.01) for control study subjects, 1.0 (±0.06) for healed-DFU study subjects, and 1.3 (±0.03) for unhealed-DFU study subjects. Unpaired t-test analysis for healed and unhealed DFU group P/I-ratios and ANOVA analysis of all study group P/I-ratios documented significant mean differences. In some embodiments, a P/I-ratio provides a novel quantitative gauge of IGF-IR initiated AKT/mTOR signal transduction activity predictive for DFU healing and/or HIF-1-mediated cellular processes. In some embodiments, increasing P/I-ratio values is inversely proportional to decreasing AKT/mTOR signal transduction activity associated with impaired DFU wound repair. In some embodiments, P53-directed dysfunctional regulation of IGF-IR, PI3K, and AKT/mTOR are important factors associated with impaired DFU wound healing.

In some aspects, the present disclosure provides a method for predicting whether or not a wound in a diabetic patient will heal, comprising the steps of:

A. measuring the levels of protein expression and/or activation in tissue excised from the wound (e.g., a leading edge), wherein one or more of the measured proteins are selected from the insulin signaling pathway or in the POKinase signaling pathway,
B. comparing the levels of the proteins in (A) to positive and/or negative reference standards to determine the level of expression and/or the level of activation of the one or more proteins,

C. determining the ratio of POKinase signaling activity to insulin signaling activity, wherein, a ratio level determined for PBKinase/Insulin signaling that is greater than 1 signifies that the wound will not heal and a ratio of 1 or less signifies that the wound will heal, and

D. providing a report of expression and/or activation levels of the proteins in (A), in which the levels are compared to the levels in a control population and a non-healing wound population, to physicians that characterizes whether a wound will heal or not.

In some aspects, the present disclosure also provides a method of treating a wound in a diabetic patient identified by the method described above as having a wound that will not heal, comprising the steps of:

A. administering to the patient a therapeutic agent that inhibits the PI3 Kinase signaling pathway, and/or

B. administering to the patient a therapeutic agent that increases signaling in the insulin signaling pathway.

In some embodiments, the antagonist is an antagonist of PBKinase complex, Akt, mTOR, 4E.BP1, Ribosomal Protein S6 Kinase, HIF-1α, PTEN, POKinase, IGFRPB3, Src, GSK3β, β-catenin, or any protein that is a member of the insulin signaling or PBKinase signaling pathways of proteins, or any combination thereof.

In some embodiments, the agonist is an agonist of insulin, insulin-like growth factor 1 (IGF-1), IGF-2, PTEN, PBKinase, mTOR, 4E.BP1, Ribosomal Protein S6 Kinase, HIF-la, PTEN, IGFRPB3, Src, GSK3β, β-catenin, any protein that is a member of the insulin signaling or PBKinase signaling pathways of proteins, or any combination thereof.

In some aspects, disclosed herein are novel biomarkers of diabetic foot ulcer (DFU) wound healing identified by proteomic RPPA methodology used to quantify the level of signaling proteins and the activation states (e.g., phosphorylation sites) in the insulin signaling pathway (ISP), which is exemplified in a comparative clinical study of chronic DFU keratinocytes and non-diabetic (control) plantar skin specimens (See Figure 1). Patterns of proteomic pathway and nodal analysis for this study were selected to investigate the following as factors correlating to wound outcomes in DFU study subjects: 1) Senescence-related pl6INK4a antagonism of NF-kB expression and apoptosis (Bak, Caspase-3, and Caspase-9); 2) Expression of insulin signaling pathway (ISP) nodes: IGF-1R, PI3K, GSK-3P, β-Catenin, AKT and mTOR; 3) NF-kB-regulated angiogenesis factors - TNF-a, NF-kB,
VEGF, NOS2 and COX2; and 3) Correlations of P53, NF-kB, P16INK4a and ISP nodal expression and signal transduction to DFU subject wound study outcomes (Figure 1). During the data analysis of the clinical study it was determined that the clinical status of DFU subject wound healing demonstrated a significant (p<0.05) correlation with the ratio of phosphatidylinositol 3-kinase (PI3K) and insulin-like growth factor-1 receptor (IGF-1R) expression from wound keratinocytes (PI3K/IGF-1R). Using linear regression analysis a significant (p<0.05) correlation between P/I ratio values and mTOR expression was also demonstrated for the control and DFU study subject population. This suggests that P/I ratio values may contribute to the expression of mTOR, a downstream insulin-signaling pathway, influencing HIF-1 expression and, perhaps, patterns of wound repair signaling in DFU subjects.

This proteomic expression ratio determination may function as a quantitative measure of ISP signal transduction for AKT/mTOR pathways predictive for DFU wound healing and, perhaps, increased production of the hypoxia-inducible factor-1 (HIF-1) protein as part of the cellular injury response for wound keratinocytes (Figure 1). These expression ratio determinations obtained from wound keratinocytes have not previously been identified as candidate biomarkers for DFU wound-healing nor have they provided valuable personalized clinical information useful for the clinical evaluation and management of DFU subjects.

Briefly, following a cellular injury response within non-diabetic tissue IGF-1R mediates a cellular signaling response to PI3K where, in turn, phosphatidylinositol (3,4)- bisphosphate (PIP2) is converted to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). At this point, once PIP3 activity is sufficient, activation of the AKT/mTOR pathways is initiated to downstream pathways and transcription factors. To engage the cellular injury response, critical levels of IGF-1/PI3K mediate signaling of the AKT/mTOR pathways for the increased expression of the hypoxia-inducible factor-1 (HIF-1) protein. The production of the HIF-1 protein activates the transcription of numerous genes for proteins required for normal wound healing processes such as angiogenesis, glucose metabolism, cell proliferation and survival (Figure 1). Alternatively, in the cancer cell increased HIF-1 protein production stimulates tumor cell invasion and metastasis. It is suggested that the effect of growth factor (IGF-1) signaling initiation and increased ISP signal transduction (via AKT/mTOR) is an increase in the rate at which the HIF-1 protein is produced. This would suggest that below a threshold of ISP signal transduction, required by AKT/mTOR pathways, that HIF-1 protein production would be insufficient to elevate HIF-1 stimulated gene expression to the levels required for unimpaired wound healing to proceed or for effective promotion of the cellular injury response.

Impaired diabetes wound healing is associated with attenuation of HIF-1 production. An important assumption is that attenuation of ISP signal transduction, decreased HIF-1 protein
production and diabetes-related impaired wound healing, is in great part related to the presence of P53-regulated genes inhibiting ISP signal propagation. Two of these genes, insulin-like growth factor binding protein-3 (IGFBP-3) and phosphatase and tensil homolog (PTEN) function to decrease IGF-1R signal initiation and inhibit activation of AKT/mTOR pathways by decreasing pathway signal transduction, respectively. This is achieved by: 1) IGFBP-3 binding to the IGF-1 ligand blocking its receptor initiation, and 2) by PTEN inhibition of PI3K-produced PIP required for signal transduction and activation of AKT/mTOR and downstream signaling pathways. With increased P53-directed inhibition of the cellular injury response in diabetes AKT/mTOR cellular signal transduction following PTEN-mediated inhibition of PI3K is variably affected. This results in wound recovery and healing in a small fraction of DFU subjects (those with sufficient ISP signal transduction) and irreversible inhibition of recovery processes and complete healing failure in the remaining chronic wound subjects (those with insufficient ISP signal transduction).

In some aspects, in the non-diabetic environment, PI3K signaling routinely directs phosphorylation of PIP2→PIP3 where PIP3 is required for binding and signal initiation for the AKT/mTOR pathways. In some embodiments, increased PI3K expression that is documented by RPPA reflects the transmission of signal propagation from the growth factor receptor in transit to the AKT/mTOR pathway.

In some embodiments, with diabetes, and the presence of the P53-directed PI3K inhibitor PTEN, PIP3 produced at PI3K is diverted back to PIP2 preventing signal pathway transduction and adding this "recycled" PIP2 into position for re-phosphorylation by PI3K. In some embodiments, with diabetes, an overall increased level of PIP2 phosphorylation and increased site activation for PI3K now reflects the presence of "inhibited" PIP3 redirected by PTEN. Increased PI3K expression in diabetes, as documented in our clinical DFU study, now provided a quantitative gauge of the impact of PTEN inhibition on insulin signaling pathway transduction. In the DFU environment, increasing PTEN inhibition as related to the diversion of PIP3 signal initiation at PI3K was determined by comparison of DFU study subject PI3K/IGF-1 ratio values with control samples. In the DFU study data PI3K/IGF-1 ratio values (±S.E.M.) were lowest for the study control subjects (n=3/0.7 ±0.01). [Control subject ratio values: 0.71-0.73] DFU subjects experiencing wound healing within 12-weeks were documented with ratio values that were statistically elevated as compared to control subjects (n=3/1.0 ±0.06). [Healed DFU subject ratio values: 0.9-1.1] The remaining DFU study subjects that did not experience wound healing were documented with ratio levels that were significantly higher than control subjects or the healed DFU subjects (n=16/1.3 ±0.03). [Unhealed DFU subject ratio values: 1.1-1.6]. See Figure 2.
By quantifying cellular signaling relationships describing a correlation between DFU subjects that experience complete wound healing (healed DFU subjects) as compared to DFU subjects not experiencing complete wound healing (unhealed DFU subjects), the PI3K/IGF-1 ratio defines a "threshold" value for DFU healing based on ISP signal transduction. As illustrated in Figure 2, this threshold is defined by the change in correlation of ratio values to clinical wound status and is observed at the ratio value of 1.1. Control subjects and healed DFU subjects are identified with ratio values ≤ 1.1 (range 0.7-1.1) while unhealed DFU subject are identified with ratio values ≥ 1.1 (range 1.1-1.6). As the ratio determinations have been designed, subjects with values below the threshold should experience higher levels of ISP signal transduction at the AKT/mTOR pathway than those subjects with ratio values above the threshold. The identification of a "threshold" for healing for DFU subjects based on proteomic measures of ISP signal transduction is a significant clinical tool for the management of the DFU subject. This information may be clinically applied immediately and will provide for the design of novel platforms for research of the relationships between the ISP cellular injury responses, reparative processes of wound biology and HIF-1-coordinated gene expressions supporting tissue repair.

The value afforded by the invention of the PI3K/IGF-1 ratio determination as a clinical tool for the evaluation and management of DFU subjects is meaningful and reflects a unique combination of significance and novelty that is underscored by the priority of its potential impact on the treatment of wound healing complications of diabetes.

Active Agents

In some aspects, the present disclosure provides for a pharmaceutical composition that comprises an antagonist and/or an antagonist of one or more proteins (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 proteins). In some embodiments, the pharmaceutical composition comprises an antagonist a first protein, an agonist of a second protein, or a combination thereof. For example, the first protein or the second protein are individually selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3p, COX-2, iNOS, NFKB/p65, 1xB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof.

In some embodiments, antagonists (e.g., of GSK-3beta) include Beryllium, Lithium, Anilino maleimides (SB216763, SB415286), Arylpyrazolopyridazines (e.g., 6-aryl pyrazole [3,4-b] pyridine 4), Bisindole maleimides (e.g., Ro 31-8220, GF 109203x), Indirubins (6-bromoindirubin-3’-oxime, aka BIO), Paullones (alsterpaullone), Psuedosubstrate peptide, Dibromocantherelline, Meridianine A, CHIR908014, CHIR908023, CHIR908021, SB216763, AR-A014418, Kenpaullone, Alsterpaullone, Cazpaullone, SB415286, TWS119, Aloisine A,
TDZD-8, NP00111, HMK-32, Manzamine A, Palinurin, Tricantin, L803-mts. Additional examples of antagonists (e.g., of PI3K/AKT/mTOR) include Everolimus (Novartis), Temsirolimus (Pfizer), BEZ235 (Novartis), GDC-0980 (Genentech), PF-05212384 (Pfizer), SAR245409 (XL-765; Sanofi/Exelixis), BAY80-6946 (Bayer), Buparlisib (BKM120; Novartis), Pictilisib (GDC-0941; Genentech), PX-866 (Oncothyreon), SAR245408 (XL-147; Sanofi/Exelixis), ZSTK474 (Zenaku Kogyo), BYL719 (Novartis), GDC-0032 (Genentech), MLN01117 (INK1117; Intellikine), GSK2636771 (GSK), SAR260301 (Sanofi), Ikelalisib (CAL-101; GS-1101; Gilead/Calistoga), AMG319 (Amgen), Perifosine (KRX-0401; Keryx), MK2206 (Merck), GDC-0068 (Genentech), GSK2110183 (GSK), GSK2141795 (GSK), ARQ 092 (ArQule/Daiichi Sankyo), AZD5363 (AstraZeneca), AZD2014 (AstraZeneca), MLN0128 (INK128; Intellikine), or CC-223 (Celgene). Additional antagonists (e.g., of β-catenin signaling) include PKF118-310, CGP049090, PKF115-584, PKF222-815, PKF118-744, ICG001, CCT036477, XAV939, Acyl hydrazones, HQBA, Molecules with 2,3,6-trisubstituted pyrido[2,3-b] pyrazine core skeletons, Carnosic acid, CCT031374, iCRT-3,5,14, NC043, Ibuprofen, or Aspirin. Additional antagonists (e.g., of p53) include Nutlins RG7112 (RO5045337), Benzodiazepinediones (TDP665759), Spiro-oxindoles (MI-219), RITA, JNJ-26854165 (Sermedetan), Tenovin 1 and 6, SJ-172550, RO-2443/RO-5693, XI-Oil, Re-activate mutant p53, CP-31398, PRIMA-1 (APR-246), MIRA-1, Phikan083, or NSC319726. Examples of agonists (e.g., of GSK-3beta) include Celecoxib, Stauoropine, Trichostatin A, Curcumin, AKT/protein kinase B signaling inhibitor-2(API-2), Wortmannin, LY294002, Rapamycin, Differentiation-inducing factors (DIFs), and Retinoids. Advexin may inhibit p53; a number of flavonoids are being evaluated for inhibiting GSK3B along with Tidoglusib and indirubin; there are several HDAC inhibitors (PCI-24781) which would inhibit NFkbp65 expression, and CX4945 which is an inhibitor of CK2 which would reduce NFkbp65.

Another approach may be to modulate the PTEN/Akt signaling by shifting p-PTEN to non-phosphorylated version by inhibiting the kinase or activating the phosphatase activity on PTEN. One embodiment increases p-AKT levels (at one or more sites of phosphorylation, i.e. SC79: a brain-penetrable Akt phosphorylation activator and an inhibitor of Akt-PH domain translocation) or seek to increase signaling downstream of Akt, such as through the mTOR pathway (MHY1485: a potent, and cell-permeable mTOR activator, and also potently inhibits autophagy) which is related to cell survival and may control senescence through p53.

**Excipients/Carriers/Additives**

In some instances, a pharmaceutical composition disclosed herein comprises one or more excipients, carriers, or additives. Exemplary excipients, carriers, or additives can include
antioxidant agents, binders, coating materials, colorant agents, diluents, disintegrants, dispersants, emulsifying agents, flavoring agents, glidants, lubricants, pH modifying agents (e.g., buffering agents), plasticizers, preservative agents, solubilizing agents, stabilizers or stabilizing agents, surfactants, sweetening agents, thickening agents, or pharmaceutically inert materials. In some instances, excipients can comprise nontoxic auxiliary substances.

Exemplary antioxidants can include flavonoids, anthocyanidins, anthocyanins, proanthocyanidins, or combinations thereof. In some instances, one or more antioxidants can be included in the liquid dosage form. In some instances, antioxidants help provide long term stability to liquid compositions, e.g., at ambient conditions for at least about one month, at least about 3 months, at least about 24 months, or longer, depending on the type and concentration of antioxidant used and depending on other components of the storage microenvironment, such as pH, buffering agent, etc.

Exemplary binders include celluloses such as hydroxypropylcellulose, methylcellulose, and hydroxypropyl methylcellulose; starches such as com starch, pregelatinized starch, and hydroxypropyl starch; sugars such as glucose, dextrose, sucrose, lactose and sorbitol; alcohols such as polyvinyl alcohol and polyethylene glycol; waxes and natural and synthetic gums such as acacia, tragacanth, sodium alginate; synthetic polymers such as polymethacrylates and polyvinylpyrrrolidone; and povidone, dextrin, pullulan, agar, gelatin, tragacanth, macrogol, or combinations thereof. Binders can impact cohesive qualities to a tablet formulation, or a particle formulation in a capsule. Tablets can remain intact after compression by including a binder in the pharmaceutical composition.

Exemplary coating materials include hydroxypropyl methyl cellulose 2910, aminoalkyl methacrylate copolymer E, polyvinylacetel diethylaminoacetate, macrogol 6000, titanium oxide, or combinations thereof. Exemplary plasticizers include triethyl citrate, triacetin, macrogol 6000, or combinations thereof.

Exemplary colorant agents include one or more synthetic organic food additives (e.g., food dyes such as food red dye Nos. 2 and 3, food yellow dye Nos. 4 and 5 and food blue dye Nos. 1 and 2), water-insoluble lake dyes (e.g., aluminum salts of the above synthetic organic food additives, etc.), natural pigments (e.g., beta-carotene, chlorophyll, iron oxide red, etc.), or combinations thereof. Other suitable colorant agents can include D&C Red No. 33, FD&C Red No. 3, FD&C Red No. 40, D&C Yellow No. 10, and C Yellow No. 6, or any combination of these or the above colorants. A colorant agent, when included in the liquid dosage form, can be provided in an amount sufficient to provide the pharmaceutical compositions with a more aesthetic and/or distinctive appearance.
Exemplary diluents include cellulose and cellulose derivatives such as microcrystalline cellulose; starches such as dry starch, hydrolyzed starch, and starch derivatives such as corn starch; cyclodextrin; sugars such as powdered sugar and sugar alcohols such as lactose; D-mannitol; inorganic diluents such as aluminum hydroxide gel, precipitated calcium carbonate, carbonate, magnesium alumino-metasilicate, dibasic calcium phosphate; and sodium chloride, silicon dioxide, titanium dioxide, titanium oxide, dicalcium phosphate dihydrate, calcium sulfate, alumina, kaolin, talc, or combinations thereof. Diluents, also terms "fillers", can increase the bulk of a tablet so that a practical size is provided for compression.

Exemplary disintegrants include starches, alginic acid, crosslinked polymers such as, e.g., crosslinked polyvinylpyrrolidone, croscarmellose sodium, potassium or sodium starch glycolate, clays, celluloses, starches, gums, or combinations thereof. Disintegrants can facilitate tablet disintegration after administration, or following contact with dissolution fluid, or as measured in an in vitro dissolution study.

Exemplary emulsifying agents include gelatin, egg yolk, casein, cholesterol, acacia, tragacanth, chondrus, pectin, methyl cellulose, carbomer, cetostearyl alcohol, cetyl alcohol, or combinations thereof. Emulsifying agents can be included in the liquid dosage form in an amount sufficient to facilitate more uniform dispersion of one or more active ingredients or other pharmaceutically acceptable excipient that is not generally soluble in the liquid.

Exemplary glidants include silicon dioxide, talc, dried aluminum hydroxide gel, magnesium silicate, or combinations thereof. Exemplary lubricants include magnesium stearate, calcium stearate, stearic acid, glyceryl behenate, polyethylene glycol, talc, or combinations thereof. Lubricants can also facilitate tablet manufacture.

Exemplary buffering agents include gluconate, lactate, citrate, acetate, phosphate, benzoate, carbonate salts, or combinations thereof. The buffering agent can be present in an amount sufficient to buffer the pH of the solution and minimize degradation of the active ingredients. Some buffering agents can also modulate active ingredient solubility in the liquid dosage form. The pH can be adjusted with a combination of two or more of these buffering agents, e.g. citric acid and sodium benzoate. The buffering agent can be present as a buffer solution. In some instances, the buffering agent can include a phosphate, such as a potassium phosphate or sodium phosphate, or any combination thereof.

Exemplary preservative agents include sodium benzoate, paraoxybenzoic acid esters, methyl, ethyl, butyl, and propyl parabens, chlorobutanol, benzyl alcohol, phenylethylalcohol, dehydroacetic acid, sorbic acid, benzalkonium chloride (BKC), benzethonium chloride, phenol, phenylmercuric nitrate, thimerosal, or combinations thereof. Preservative agents can
be included in the liquid dosage form. The preservative agents can be in an amount sufficient to extend the shelf-life or storage stability, or both, of the liquid dosage form.

Exemplary solubilizing agents include an alcohol, e.g., 95% ethyl alcohol, a glycol, glycerin, D-mannitol, trehalose, benzyl benzoate, trisaminomethane, cholesterol, triethanolamine, sodium carbonate, sodium citrate, sodium salicylate, sodium acetate, or combinations thereof. Exemplary alcohols can include ethanol, isopropanol, t-butanol, phenol, cresol, a benzyl alcohol, or any combination thereof. Exemplary glycols include C2-20 alkenes functionalized with a glycol, including propylene glycol, polypropylene glycol, polyethylene glycol, etc., or any combination thereof. Solubilizing agents can be included in the liquid dosage form, e.g., in an amount sufficient to facilitate greater or more rapid dissolution of one or more active ingredients or other excipients. A solubilizing agent can be included in an amount of about 1 volume percent to 20 volume percent (v/v), or about 4 volume percent to 15 volume percent (v/v), based on the total volume of the solution. Exemplary amounts of solubilizing agent include about 7 volume percent to 12 volume percent (v/v) based on the total volume of the solution.

Exemplary stabilizing agents include one or more liquid excipients such as ethanol or glycerin; one or more glycols, such as polyethylene glycol, e.g., PEG-400, propylene glycol, or polypropylene glycol; a cellulose-based component, such as hydroxypropyl methylcellulose (HPMC) or hydroxymethylcellulose (HMC); or combinations thereof. Stabilizers can inhibit or retard drug decompositions reactions including oxidative reactions. A stabilizing agent can include any suitable monohydroxy phenol component or polyhydroxy phenol component, or any combination thereof. Such stabilizing agents can also function as antioxidant agents, or antimicrobial agents. Stabilizing agent(s) can be included in the liquid dosage form. Thus, it should be understood that certain solubilizing agents can function effectively as a stabilizing agent. For example, propylene glycol can function as both a solubilizing agent and as a stabilizing agent.

Exemplary surfactants include sucrose esters of fatty acids, polyoxyl stearate, polyoxyethylene hydrogenated castor oil, polyoxyethylene polyoxypropylene glycol, sorbitan sesquioleate, sorbitan trioleate, sorbitan monostearate, sorbitan monopalmitate, sorbitan monolaurate, polyorbate, glyceryl monostearate, sodium lauryl sulfate, lauromacrogol, or combinations thereof. Surfactants can also be anionic, cationic, amphoteric, or nonionic.

Exemplary sweetening agents include sorbitol, saccharin, acesulfame, e.g., acesulfame potassium, sucralose, xylitol, maltitol, sucrose, aspartame, fructose, neotame, glycerin, sodium saccharate, glycyrrhizin dipotassium, acesulfame K, mannitol, invert sugar, or combinations thereof. In some instances, a sweetening agent, such as one or more sucralose-containing
components or saccharin-containing components, can be added to the pharmaceutical composition to modify the taste of the pharmaceutical composition. In some instances, a viscous sweetener such as one or more of a sorbitol solution, a syrup (sucrose solution), or high-fructose corn syrup can increase viscosity and retard sedimentation. In some instances, the sweetening agent can include an acesulfame-containing, sucralose-containing, or saccharin-containing component. The sweetening agent can include glycerin, saccharin, liquid sugar (sucrose solution), or any combination thereof. In some instances, a sweetening agent can be present in an amount sufficient to minimize or mask any off-flavors in the taste of the active agents (e.g., opioid analgesic, non-opioid analgesic, antiemetic, laxative, barbiturate, etc), and also to minimize or mask any other off-flavor components included in the pharmaceutical composition.

In some instances, a sweetening agent is present in an amount of about 0.1 volume percent to 85 volume percent (v/v), based on the total volume of the solution. In one example, the sweetening agent is present in an amount of about 5 volume percent to 70 volume percent (v/v), based on the total volume of the solution. Exemplary amounts of glycerin can include about 2 volume percent to 18 volume percent (v/v), or about 5 volume percent to 10 volume percent (v/v). Exemplary amounts of liquid sugar can include about 40 volume percent to 75 volume percent (v/v), or about 60 volume percent to 70 volume percent (v/v), based on the total volume of the solution. Certain types of thickening agent or sweetening agent can also act as a solubilizing agent or a stabilizing agent, or both, or have other properties, when included as a component of a pharmaceutically acceptable carrier. For example, a sweetening agent such as glycerin can also act as a thickening agent. An oral liquid dosage form can also contain, in addition to a sweetening agent, a flavoring agent, for example, one or more of natural and artificial fruit, artificial banana, strawberry, and pineapple.

Exemplary thickening agents include acacia, alginic acid bentonite, carboxymethylcellulose calcium or sodium, cetostearyl alcohol, methyl cellulose, ethylcellulose, glycerin, gelatin guar gum, hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose ("HPMC"), any other suitable cellulose-based component, maltodextrin, polyvinyl alcohol, povidone, propylene carbonate, propylene glycol alginate, sodium alginate, sodium starch glycolate, starch tragacanth, and xanthan gum, or combinations thereof. A thickening agent or viscosity-enhancing agent can improve the mouth-feel of the liquid oral dosage form and/or to help coat the lining of the gastrointestinal tract.

In some instances, a thickening agent is present in an amount of about 0.1 volume percent to 20 volume percent (v/v), based on the total volume of the solution. In one example, glycerin can be present in an amount of about 1 volume percent to 10 volume percent (v/v), based on the
total volume of the solution. Exemplary amounts of thickening agent can include from about 1 volume percent to 12 volume percent (v/v), or at an amount of about 4 volume percent to 10 volume percent (v/v), based on the total volume of the solution. An exemplary amount can include about 6 to 10 volume percent (v/v).

In some instances, an excipient includes cellulose ethers such as hydroxypropyl methylcellulose (e.g., Methocel K4M) or silicified microcrystalline cellulose; polyvinylacetate-based excipients such as, e.g., Kollidon SR, and polymers and copolymers based on methacrylates and methacrylic acid such as, e.g., Eudragit NE 30D; microcrystalline cellulose, sodium carboxymethyl cellulose, sodium starch glycolate, corn starch, colloidal silica, sodium laurel sulphate, magnesium stearate, Prosolve SMCC (HD90), croscarmellose sodium, Crospovidone NF, Avicel PH200 or combinations thereof.

In some instances, an excipient includes acesulfame potassium, glacial acetic acid, acetone, acetyltributyl citrate, acetyltriethyl citrate, adipic acid, albumin, aliphatic polyester, alitame, almond oil, alpha tocopherol, aluminum monostearate, aluminum oxide, aluminum phosphate adjuvant, ammonia, ammonium alginate, ammonium chloride, anthocyanidin, anthocyanin, ascorbic acid, ascorbyl palmitate, aspartame, attapulgite, bentonite, benzalkonium chloride, benzethonium chloride, benzoic acid, benzyl alcohol, benzyl benzoate, boric acid, bronopol, butylated hydroxyanisole, butylated hydroxytoluene, butylene glycol, butylparaben, calcium acetate, calcium alginate, calcium chloride, calcium hydroxide, calcium lactate, calcium phosphate (tribasic), calcium silicate, canola oil, carbomer, carbon dioxide, carboxymethylcellulose calcium, carboxymethylcellulose sodium, carrageenan, casein, castor oil, podere cellulose, cellulose acetate, cellulose acetate phthalate, ceratonia, cerasin, cetostearyl alcohol, cetrimide, cetyl alcohol, cetylpyridinium chloride, chitosan, chlorhexidine, chlorobutanol, chlorocresol, chlorodifluoroethane, chlorofluorocarbon, chloroxylenol, cholesterol, chondrus, citric acid monohydrate, coconut oil, copovidone, com oil, cottonseed oil, cresol, cyclomethicone, denatonium benzoate, dextrose, dibutyl phthalate, dibutyl sebacate, diethanolamine, diethyl phthalate, difluoroethane, dimethicone, dimethyl ether, dimethyl phthalate, dimethyl sulfoxide, dimethylacetamide, disodium edetate, docusate sodium, edetic acid, egg yolk, erythorobic acid, erythritol, ethyl acetate, ethyl lactate, ethyl maltol, ethyl oleate, ethyl vanillin, ethylene glycol stearate, ethylene vinyl acetate, ethylparaben, flavonoid, fructose, fumaric acid, glycerin, glycercyl monooleate, glycercyl palmitostearate, glycine, glycofurol, guar gum, hectorite, heptafluoropropane, hexetidine, hydrocarbon, hydrochloric acid, hydrophobic colloidal silica, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl betadex, hydroxypoyl starch, hypromellose acetate succinate, imidurea, inulin, iron oxide, isomalt, isopropyl alcohol, isopropyl myristate, isopropyl palmitate, lactic acid, lactitol, lanolin, lanolin
(hydrous), lanolin alcohol, lauric acid, lecithin, leucine, linoleic acid, magnesium aluminum silicate, magnesium carbonate, magnesium oxide, magnesium trisilicate, maleic acid, malic acid, maltitol, maltitol solution, maltodextrin, maltol, maltose, medium-chain triglyceride, meglumine, menthol, methionine, methylparaben, mineral oil, lanolin alcohol, monoethanolamine, monosodium glutamate, monothioglycerol, myristic acid, myristyl alcohol, neohesperidin dihydrochalcone, neotame, nitrogen, nitrous oxide, octyldodecanol, oleic acid, oleyl alcohol, olive oil, palmitic acid, paraffin, peanut oil, pectin, pentetic acid, petrolatum, petrolatum alcohol, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric acetate, phenylmercuric borate, phenylmercuric nitrate, phospholipid, phosphoric acid, polacrilin potassium, poloxamer, polycarbophil, polydextrose, poly (dl-lactic acid), polyethylene oxide, poly(methyl vinyllether/maleic anhydride), polyoxyethylene alkyl ether, polyoxyethylene sorbitan fatty acid ester, polyoxyethylene stearate, polyoxyethylglyceride, polyvinyl acetate phthalate, potassium alginate, potassium alum, potassium benzoate, potassium bicarbonate, potassium chloride, potassium citrate, potassium hydroxide, potassium metabisulfite, potassium sorbate, proanthocyanidin, propionic acid, propyl gallate, propylene carbonante, propylene glycol, propylene glycol alginate, propylparaben, propylparaben sodium, pyrrolidone, raffinose, saccharin, saccharin sodium, safflower oil, saponite, sesame oil, shellac, simethicone, sodium acetate, sodium ascorbate, sodium benzoate, sodium bicarbonate, sodium borate, sodium carbonate, sodium citrate dihydrate, sodium cyclamate, sodium formaldehyde sulfoxylate, sodium hyaluronate, sodium lactate, sodium metabisulfite, sodium phosphate (dibasic), sodium phosphate (monobasic), sodium propionate, sodium sulfate, sodium thiosulfate, sorbic acid, sorbitan fatty acid ester, soybean oil, stearyl alcohol, sucralose, sucrose octaacetate, sulfobutylether b-cyclodextrin, sulfur dioxide, sulfuric acid, sunflower oil, suppository bases (hard fat), tagatose, tartaric acid, tetrafluoroethane, thaumatin, thimerosal, thymol, trehalose, tributyl citrate, tricaprylin, triethanolamine, triolein, vanillin, hydrogenated vegetable oil, vitamin e polyethylene glycol succinate, water, wax (anionic emulsifying), wax (cetyl esters), wax (microcristalline), wax (nonionic emulsifying), wax (white), wax (yellow), xanthan gum, xylitol, zein, zinc acetate, zinc stearate, food red dye No. 2, food red dye No. 3, food yellow dye No. 4, food yellow dye No. 5, food blue dye No. 1, food blue dye No. 2, beta-carotene, chlorophyll, iron oxide red, titanium dioxide, gluconate, lactate, paraoxybenzoic acid ester, phenylethylalcohol, dehydroacetic acid, ethyl alcohol, trisaminomethane, sodium salicylate, ethanol, isopropanol, t-butanol, polypropylene glycol, hydroxyethylcellulose, acesulfame, sodium saccharate, glycyrhrizin dipotassium, acesulfame K, or ethylcellulose.

Examples of excipients include acacia, acesulfame potassium, acetic acid (glacial), acetone, acetyltributyl citrate, acetyltributyl citrate, adipic acid, agar, albumin, alcohol, alginic
acid, aliphatic polyesters, alitame, almond oil, alpha tocopherol, aluminum hydroxide adjuvant, aluminum monostearate, aluminum oxide, aluminum phosphate adjuvant, ammonia solution, ammonium alginate, ammonium chloride, ascorbic acid, ascorbyl palmitate, aspartame, attapulgite, bentonite, benzalkonium chloride, benzethonium chloride, benzoic acid, benzyl alcohol, benzyl benzoate, boric acid, bronopol, butylated hydroxyanisole, butylated hydroxytoluene, butylene glycol, butylparaben, calcium acetate, calcium alginate, calcium carbonate, calcium hydroxide, calcium lactate, calcium phosphate (dibasic anhydrors), calcium phosphate (dibasic dihydrate), calcium phosphate (tribasic), calcium silicate, calcium stearate, calcium sulfate, canola oil, carbomer, carbon dioxide, carboxymethylcellulose calcium, carboxymethylcellulose sodium, carrageenan, castor oil, castor oil (hydrogenated), cellulose (microcristalline), cellulose (microcrystalline and carboxymethylcellulose sodium), cellulose (podere), cellulose (silificed microcristalline), cellulose acetate, cellulose acetate phthalate, ceratonia, cerasin, cetostearyl alcohol, cetrimide, cetyl alcohol, cetylpyridinium chloride, chitosan, chlorhexidine, chlorobutanol, chlorocresol, chlorodifluoroethane (HCFC), chlorofluorocarbons (CFC), chloroxylenol, cholesterol, citric acid monohydrate, coconut oil, colloidal silicon dioxide, colorant agents, copovidone, corn oil, corn starch and pregelatinized starch, cottonseed oil, cresol, croscarmellose sodium (AC-Di-Sol), crospovidone, cyclodextrins, cyclomethicone, denatonium benzoate, dextrates, dextrin, dextrose, dibutyl phthalate, dibutyl sebacate, diethanolamine, diethyl phthalate, diffuroethane (HFC), dimethicone, dimethyl ether, dimethyl phthalate, dimethyl sulfoxide, diethylacetamide, disodium edetate, docusate sodium, edetic acid, erythorbic acid, erythritol, ethyl acetate, ethyl lactate, ethyl maltol, ethyl oleate, ethyl vanillin, ethylcellulose, ethylene glycol stearetes, ethylene vinyl acetate, ethylparaben, fructose, fumaric acid, gelatin, glucose (liquid), glycerin, glyceryl behenate, glyceryl monooleate, glyceryl monostearate, glyceryl palmitostearate, glycine, glycofurol, guar gum, hectorite, heptafluoropropane (HFC), hexetidine, hydrocarbons (HC), hydrochloric acid, hydrophobic colloidal silica, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl betadex, hydroxypropyl cellulose, hydroxypropyl cellulose (low-substituted), hydroxypropyl starch, hypromellose, hypromellose acetate succinate, hypromellose phthalate, imidurea, inulin, iron oxides, isomalt, isopropyl alcohol, isopropyl myristate, isopropyl palmitate, kaolin, lactic acid, lactitol, lactose (anhydrors), lactose (inhalation), lactose (monohydrate), lactose (monohydrate and corn starch), lactose (monohydrate and microcrystalline cellulose), lactose (monohydrate and povidone), lactose (monohydrate and powdered cellulose), lactose (spray-dried), lanolin, lanolin (hydror), lanolin alcohols, lauric acid, lecithin, leucine, linoleic acid, macrocol 15 hydroxystearate, magnesium aluminum silicate, magnesium carbonate, magnesium oxide, magnesium silicate, magnesium stearate, magnesium trisilicate, maleic acid, malic acid, maltitol,
maltoitol solution, maltodextrin, maltol, maltose, mannitol, medium-chain triglycerides, meglumine, menthol, methionine, methylcellulose, methylparaben, mineral oil, mineral oil (light), mineral oil and lanolin alcohols, monoethanolamine, monosodium glutamate, monothioglycerol, myristic acid, myristyl alcohol, neohesperidin dihydrochalcone, neotame, nitrogen, nitrous oxide, octyldodecanol, oleic acid, oleyl alcohol, olive oil, palmitic acid, paraffin, peanut oil, pectin, pentetic acid, petrolatum, petrolatum and lanolin alcohols, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric acetate, phenylmercuric borate, phenylmercuric nitrate, phospholipids, phosphoric acid, polacrilin potassium, poloxamer, polycarbophil, polydextrose, poly (dl-lactic acid), polyethylene glycol, polyethylene oxide, polymethacrylates, poly(methyl vinylether/maleic anhydride), polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene stearates, polyoxylglycerides, polyvinyl acetate phthalate, polyvinyl alcohol, potassium alginate, potassium alum, potassium benzoate, potassium bicarbonate, potassium chloride, potassium citrate, potassium hydroxide, potassium metabisulfite, potassium sorbate, povidone, propionic acid, propyl gallate, propylene carbonate, propylene glycol, propylene glycol alginate, propylparaben, propylparaben sodium, pyrrolidone, raffinose, saccharin, saccharin sodium, safflower oil, saponite, sesame oil, shellac, simethicone, sodium acetate, sodium alginate, sodium ascorbate, sodium benzoate, sodium bicarbonate, sodium borate, sodium carbonate, sodium chloride, sodium citrate dihydrate, sodium cyclamate, sodium formaldehyde sulfoxylate, sodium hyaluronate, sodium hydroxide, sodium lactate, sodium lauryl sulfate, sodium metabisulfite, sodium phosphate - dibasic, sodium phosphate (monobasic), sodium propionate, sodium starch glycolate, sodium stearyl fumarate, sodium sulfite, sodium thiosulfate, sorbic acid, sorbitan esters (sorbitan fatty acid esters), sorbitol, soybean oil, starch, starch (pregelatinized), starch (sterilizable maize), stearic acid, stearyl alcohol, sucralose, sucrose, sucrose octaacetate, sugar (compressible), sugar (confectioner’s), sugar spheres, sulfobutylether b-cyclodextrin, sulfur dioxide, sulfuoric acid, sunflower oil, suppository bases (hard fat), tagatose, talc, tartaric acid, tetrafluoroethane (HFC), thaumatin, thimerosal, thymol, titanium dioxide, tragacanth, trehalose, triacetin, tributyl citrate, tricaprylin, triethanolamine, triethyl citrate, triolein, vanillin, vegetable oil (hydrogenated), vitamin e polyethylene glycol succinate, water, wax (anionic emulsifying), wax (camauba), wax (cetyl esters), wax (microcristalline), wax (nonionic emulsifying), wax (white), wax (yellow), xanthan gum, xylitol, zein, zinc acetate, zinc stearate, or combinations thereof. Furthermore, dosage forms herein can comprise acceptable carriers or salts known in the art, such as those described in the Handbook of Pharmaceutical Excipients, American Pharmaceutical Association (1986), incorporated by reference herein in its entirety.
Salts

In some instances, a pharmaceutical composition disclosed herein comprises a pharmaceutically active agent that can be in the form of its free base, its pharmaceutically acceptable salt, prodrug, analog, or complex. Exemplary pharmaceutically acceptable salts include metal salts, such as sodium salts, potassium salts, lithium salts; alkaline earth metals, such as calcium salts, magnesium salts; organic amine salts, such as triethylamine salts, pyridine salts, picoline salts, ethanolamine salts, triethanolamine salts, dicyclohexylamine salts, N,N’-dibenzylethlenediamine salts; inorganic acid salts such as hydrochloride salts, hydrobromide salts, sulfate salts, phosphate salts; organic acid salts such as formate salts, acetate salts, trifluoroacetate salts, maleate salts, tartrate salts; sulfonate salts such as methanesulfonate salts, benzenesulfonate salts, p-toluenesulfonate salts; and amino acid salts, such as arginate salts, asparginate salts, glutamate salts, or combinations thereof.

In some instances, a pharmaceutically acceptable salt includes bitartrate, bitartrate hydrate, hydrochloride, p-toluenesulfonate, phosphate, sulfate, trifluoroacetate, bitartrato hemipentahydrate, pentafluoropropionate, hydrobromide, mucate, olate, phosphate dibasic, phosphate monobasic, acetate trihydrate, bis(heptfluorobutyrate), bis(pentafluoroacrylate), bis(pyridine carboxylate), bis(trifluoroacetate), chloride, sulfate pentahydrate, or combinations thereof. In some instances, exemplary pharmaceutically acceptable salts include, e.g., water-soluble and water-insoluble salts, such as the acetate, amsonate(4,4-diaminostilbene-2,2-disulfonate), benzenesulfonate, benzonate, bicarbonate, bisulfate, bitartrate, borate, butyrate, calcium edetate, camphorsulfonate, camsylate, carbonate, citrate, clavulinate, dihydrochloride, edetate, edisylate, estolate, esylate, flunorate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexafluorophosphate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isothionate, lactate, lactobionate, laurate, malate, maleate, mandelate, mesylate, methylboronate, methylnitrate, methylsulfate, mucate, napsylate, nitrate, N-methylglucamine ammonium salt, 3-hydroxy-2-naphthoate, oleate, oxalate, palmitate, pamoate (l,l-methene-bis-2-hydroxy-3-naphthoate, einbonate), pantothenate, phosphate/di phosphate, picrate, polygalacturonate, propionate, p-toluenesulfonate, salicylate, stearate, subacetate, succinate, sulfate, or combinations thereof. In some instances, a pharmaceutically acceptable salt includes bitartrate, bitartrate hydrate, hydrochloride, p-toluenesulfonate, phosphate, sulfate, trifluoroacetate or bitartrato hemipentahydrate.

Administration
In some instances, a pharmaceutical composition disclosed herein is administered to a subject in need thereof at about every 4 to about 8 hours. In some instances, a pharmaceutical composition disclosed herein is administered to a subject in need thereof at about every 4 to about 6 hours as needed. In some instances, a pharmaceutical composition disclosed herein is administered to a subject in need thereof at about every 4 to about 8 hours. In some instances, a pharmaceutical composition disclosed herein is administered to a subject in need thereof at about every 6 to about 8 hours as needed. In some instances, a pharmaceutical composition disclosed herein is administered to a subject in need thereof at about every 4 hours, about every 5 hours, about every 6 hours, about every 7 hours, or about every 8 hours. In some instances, a pharmaceutical composition disclosed herein is administered once daily. In some instances, a pharmaceutical composition disclosed herein is administered not more than 2-6 times daily. In another instance, a pharmaceutical composition disclosed herein is administered not more than 4 times daily. In another instance, a pharmaceutical composition disclosed herein is administered 1, 2, 3, 4, 5, 6, 7, or 8 times daily.

In some instances, active agents disclosed herein are formulated to be administered through oral dosage forms (e.g., tablets, capsules, gels, lollipops), inhalations, nasal sprays, patches, absorbing gels, liquids, liquid tannates, suppositories, injections, I.V. drips, other delivery methods, or any combination thereof to treat subjects in need thereof. Administration can be performed in a variety of ways, including, but not limited to orally, subcutaneously, intravenously, intranasally, intraoptically, transdermally, topically (e.g., gels, salves, lotions, creams, etc.), intraperitoneally, intramuscularly, intrapulmonary (e.g., AERx.RTM. inhalable technology commercially available from Aradigm, or Inhance, pulmonary delivery system commercially available from Inhale Therapeutics), vaginally, parenterally, rectally, or intraocularly.

To prepare a pharmaceutical composition disclosed herein, an effective amount of active agents can be mixed with a suitable pharmaceutically acceptable carrier. Upon mixing of the compounds, the resulting pharmaceutical composition can be a solid, a half-solid, a semi-solid, a solution, suspension, or an emulsion. Such pharmaceutical compositions can be prepared according to methods known to those skilled in the art. The forms of the resulting pharmaceutical compositions can depend upon a variety of factors, including the intended mode of administration and the solubility of the compounds in the selected carrier or vehicle. The effective concentration of analgesics is sufficient for lessening or alleviating pain. In some instances, the components of the present pharmaceutical compositions are at least one opioid analgesic agent (e.g., hydrocodone/oxycodone), one non-opioid analgesic agent (e.g., acetaminophen), and one antiemetic agent (e.g., promethazine). In other instances,
administration comprises administration of an antiemetic (e.g., promethazine) separately, prior to, or during administration of the analgesic formulations described herein (e.g., which comprises hydrocodone and acetaminophen). In another instance, the components of the present pharmaceutical compositions are at least one opioid analgesic agent, a non-opioid analgesic agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, the components of the present pharmaceutical compositions are at least one opioid analgesic agent, a non-opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, the components of the present pharmaceutical compositions are at least one opioid analgesic agent, a non-opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent. In another instance, the components of the present pharmaceutical compositions are at least one opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, the components of the present pharmaceutical compositions are at least one opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, the components of the present pharmaceutical compositions are at least one non-opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, the components of the present pharmaceutical compositions are at least one non-opioid analgesic agent, a barbiturate agent, an agent that reduces side effects of the opioid analgesic agent. In another instance, components of the present pharmaceutical compositions are at least one opioid analgesic agent, an agent that reduces side effects of the opioid analgesic agent and a stimulant agent. In another instance, components of the present pharmaceutical compositions are at least one opioid analgesic agent, an agent that reduces side effects of the opioid analgesic agent.

In some instances, an agent described herein is administered by the nasal inhalation route using conventional nebulizers or by oxygen aerosolization to provide convenient pain relief with reduced adverse effects. The agents can be suspended or dissolved in a pharmacologically acceptable inhalation carrier. Examples of such carriers are distilled water, water/ethanol mixtures, and physiological saline solution. Conventional additives including sodium chloride, glucose, citric acid and the like can be employed in these dosage forms to stabilize or to provide isotonic media. In some instances, the pharmaceutical compositions suitable for nasal inhalation by oxygen aerosolization administration comprise hydrocodone or oxycodone, acetaminophen, and promethazine. In another instance, the pharmaceutical compositions suitable for nasal inhalation by oxygen aerosolization administration comprise hydrocodone or oxycodone, and
promethazine. In other instances, an antiemetic (e.g., promethazine) can be administered separately, prior to, or during administration of the pharmaceutical compositions described herein (e.g., those comprising hydrocodone and acetaminophen).

In some instances, an agent described herein can also be administered as a self-propelled dosage unit in an aerosol form suitable for inhalation therapy. Suitable means for employing the aerosol inhalation therapy technique are described, for example, in U.S. Pat. No. 6,913,768 to Couch et al., a reference which is incorporated herein by reference in its entirety. The agent can be suspended in an inert propellant such as a mixture of dichlorodifluoromethane and dichlorotetrafluoroethane, together with a co-solvent such as ethanol, together with flavoring materials and stabilizers. In some instances, the agents useful for a self-propelled dosage unit in aerosol form administration are hydrocodone or oxycodone, acetaminophen, and promethazine. In another instance, the agents useful for a self-propelled dosage unit in aerosol form administration are hydrocodone or oxycodone, and promethazine. In a further instance, the dosage unit can further comprise an agent such as a bronchodilator (e.g., albuterol).

In some instances, an agent described herein can also be administered as nasal spray/drop pharmaceutical compositions, which can conveniently and safely be applied to subjects in need thereof to effectively treat pain with reduced adverse effects. The pharmaceutical compositions can further comprise a water soluble polymer such as polyvinylpyrrolidone, together with other medications and together with bioadhesive material. In some instances, the components of a pharmaceutical composition for nasal spray or drop administration are hydrocodone or oxycodone agent, acetaminophen, and promethazine, or a pharmaceutically acceptable salt of any one of the foregoing, or any combination thereof. In another instance, the components of a pharmaceutical composition for nasal spray or drop administration are hydrocodone or oxycodone agent, and promethazine, or a pharmaceutically acceptable salt of any one of the foregoing, or any combination thereof.

In some instances, a pharmaceutical composition described herein can also be administered topically to the skin of a subject in need thereof. The agents can be mixed with a pharmaceutically acceptable carrier or a base which is suitable for topical application to skin to form a dermatological pharmaceutical composition. Suitable examples of carriers or bases include but not limited to: water, glycols, alcohols, lotions, creams, gels, emulsions, and sprays. A dermatological pharmaceutical composition comprising an analgesic agent can be integrated into a topical dressing, medicated tape, dermal patch absorbing gel and cleansing tissues. In some instances, the dermatological pharmaceutical composition comprises hydrocodone or oxycodone, acetaminophen, and promethazine. In another instance, the
dermatological pharmaceutical composition comprises hydrocodone or oxycodone, and promethazine.

The pharmaceutical compositions described herein can also be in liquid or liquid tannate form. The liquid formulations can comprise, for example, an agent in water-insoluble and/or suspension form; and a vehicle comprising polyethoxylated castor oil, alcohol and/or a polyoxyethylated sorbitan mono-oleate with or without flavoring. Each dosage form comprises an effective amount of an active agent and can comprise pharmaceutically inert agents, such as conventional excipients, vehicles, fillers, binders, disintegrants, pH adjusting substances, buffer, solvents, solubilizing agents, sweeteners, colorant, color, color agents and any other inactive agents that can be included in pharmaceutical dosage forms for oral administration. Examples of such vehicles and additives can be found in Remington's Pharmaceutical Sciences, 17th edition (1985).

In some cases, the pharmaceutical compositions described herein are administered in a suppository form, comprising an outer layer containing the pharmaceutical composition in a suppository base. The suppository base can, for example, be any conventional suppository base material such as glycocholate, polyethylene glycol, fractionated palm kernel oil, or one or more natural, synthetic or semi synthetic hard fats such as cocoa butter. In some cases, the pharmaceutical compositions described herein are administered in injection-ready stable liquids for injection or I.V. drip.

**Dosage**

In some instances, concentrations and dosages (e.g., a single unit dosage or multiple unit dosages) of active agents in a pharmaceutical composition can be varied as desired, as further described herein. Depending on the subject and/or condition being treated and on the administration route, the active agent in a pharmaceutical composition can generally be administered in dosages of 0.01 mg to 500 mg per kg body weight per day, e.g. about 20 mg/day for an average person. The dosage can be adjusted based on the mode of administration. A typical dosage can be one administration daily or multiple administrations daily. In some instances, for a controlled-release dosage form the unit dose can be designed for administration over a defined period of time. In some instances, dosage for one or a combination of agents can be from about 0.01 to 5mg, 1 to 10 mg, 5 to 20 mg, 10 to 50 mg, 20 to 100 mg, 50 to 150mg, 100 to 250mg, 150 to 300mg, 250 to 500mg, 300 to 600mg or 500 to 1000mg V/kg body weight. Dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to adverse effects. In another instance, a pharmaceutical composition comprises multiple active agents at the same or different dosages, where the pharmaceutical composition comprises an effective
amount of: an opioid analgesic; an antiemetic; and a stimulant. In some instances, the pharmaceutical composition can further comprise a barbiturate or a non-opioid active agent, or both. The dosage can be adjusted according to the particular actives selected.

In some instances, a pharmaceutical composition comprises an effective amount of one or more active agents that are present at about 0.5 mg to about 60 mg, including but not limited to a dose of about: 0.5 mg, 1.0 mg, 1.5 mg, 2.0 mg, 2.5 mg, 3.0 mg, 3.5 mg, 4.0 mg, 4.5 mg, 5.0 mg, 5.5 mg, 6.0 mg, 6.5 mg, 7.0 mg, 7.5 mg, 8.0 mg, 8.5 mg, 9.0 mg, 9.5 mg, 10 mg, 10.5 mg, 11.0 mg, 11.5 mg, 12.0 mg, 12.5 mg, 13 mg, 13.5 mg, 14 mg, 14.5 mg, 15 mg, 15.5 mg, 16 mg, 16.5 mg, 17 mg, 17.5 mg, 18 mg, 18.5 mg, 19 mg, 19.5 mg, 20 mg, 20.5 mg, 21 mg, 21.5 mg, 22 mg, 22.5 mg, 23 mg, 23.5 mg, 24 mg, 24.5 mg, 25 mg, 25.5 mg, 26 mg, 26.5 mg, 27 mg, 27.5 mg, 28 mg, 28.5 mg, 29 mg, 29.5 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, 36 mg, 37 mg, 38 mg, 39 mg, 40 mg, 4.1 mg, 12 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg, 50 mg, 55 mg, or 60 mg, individually or in a total amount.

**Communication Media**

The present disclosure provides communication media (e.g., computer control systems) that are programmed to implement methods of the disclosure. Figure 8 shows a computer system 801 that is programmed or otherwise configured to implement or regulate a communication with determining or recording a prognosis of a wound. The computer system 801 can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device. The computer system 801 includes a central processing unit (CPU, also "processor" and "computer processor" herein) 805, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system 801 also includes memory or memory location 810 (e.g., random-access memory, read-only memory, flash memory), electronic storage unit 815 (e.g., hard disk), communication interface 820 (e.g., network adapter) for communicating with one or more other systems, and peripheral devices 825, such as cache, other memory, data storage and/or electronic display adapters. The memory 810, storage unit 815, interface 820 and peripheral devices 825 are in communication with the CPU 805 through a communication bus (solid lines), such as a motherboard. The storage unit 815 can be a data storage unit (or data repository) for storing data. The computer system 801 can be operatively coupled to a computer network ("network") 830 with the aid of the communication interface 820. The network 830 can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network 830 in some cases is a telecommunication and/or data network. The network 830 can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network 830, in
some cases with the aid of the computer system 801, can implement a peer-to-peer network, which may enable devices coupled to the computer system 801 to behave as a client or a server. The CPU 805 can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory 810. The instructions can be directed to the CPU 805, which can subsequently program or otherwise configure the CPU 805 to implement methods of the present disclosure. Examples of operations performed by the CPU 805 can include fetch, decode, execute, and writeback. The CPU 805 can be part of a circuit, such as an integrated circuit. One or more other components of the system 801 can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC). The storage unit 815 can store files, such as drivers, libraries and saved programs. The storage unit 815 can store user data, e.g., user preferences and user programs. The computer system 801 in some cases can include one or more additional data storage units that are external to the computer system 801, such as located on a remote server that is in communication with the computer system 801 through an intranet or the Internet. The computer system 801 can communicate with one or more remote computer systems through the network 830. For instance, the computer system 801 can communicate with a remote computer system of a user. Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system 801 via the network 830. Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system 801, such as, for example, on the memory 810 or electronic storage unit 815. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor 805. In some cases, the code can be retrieved from the storage unit 815 and stored on the memory 810 for ready access by the processor 805. In some situations, the electronic storage unit 815 can be precluded, and machine-executable instructions are stored on memory 810. The code can be pre-compiled and configured for use with a machine having a processor adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion. The computer system 801 can include or be in communication with an electronic display 835 that comprises a user interface (UI) 840. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface. Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An
algorithm can be implemented by way of software upon execution by the central processing unit 805.

Aspects of the systems and methods provided herein, such as the computer system 801, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

A machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a
PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

**Kits**

The present disclosure provides a kit that comprises reagents for measuring a total amount or a phosphorylation level of two or more proteins disclosed herein, e.g., selected from the group consisting of IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3β, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, and Caspase 9.

In some embodiments, the reagents may include H2O2, Amp, PB, Avidin, Biotin, TPER reagent. Use of a kit may involve one or more of the following procedures:

Cells of interest are isolated from a FFPE biopsy using LCM or macro-dissection.

Cells are disrupted by Boiling in a strong denaturing (SDS) buffer to release denatured proteins.

Denatured proteins in the lysate are then arrayed on a nitrocellulose slide using a touch pin spotter (we use Aushon).

Slides are stained with a non-specific total protein dye (Ruby Sypro) to determine the amount of protein in each spot.

Slides are reacted with a primary Ab (i.e., specific for GSK-3β) that is specific for the protein or p-protein (i.e., p-PTEN S380) of interest.

The slide is then reacted with a biotin-labeled secondary antibody that recognizes the primary Ab (can bind to multiple primaries, so each slide is a specific primary Ab).

The Biotin is then reacted with a SA-labeling system (tyramide) to decorate the spot with fluorophores.

The slide is scanned and the fluorescence in each spot is normalized to the amount of protein.

On each array are + and - controls and a series of known dilutions of the target antigen so that you can extrapolate the unknown to the known and generate a score based on semi-quantitative assessment (or quantitative if the linear part of the Std. curve is tight).

Then scores (ratio in his application) are calculated, compared to the score for healers/nonhealers, and a determination is made as to which signals are up and which are down, and does the pattern match nonhealers or healers. Depending on what analytes are up in the inhibition of survival or activation of senescence signaling pathway are then considered targets for a drug.
In some aspects, the present disclosure provides a method for using a protein microarray to identify a combination of therapeutic agents for individualized treatment of a disease in a patient caused by a peculiar, abnormal network of interconnected cell signaling proteins that leads to an aberrant cellular response in the patient, comprising the steps of: a) measuring with the protein microarray activity states for a plurality of different signaling proteins extracted from diseased cells obtained from the patient; b) providing activity states of signaling proteins from reference cells corresponding to the signaling proteins extracted from the diseased cells obtained from the patient; c) detecting differences between the activity states of the individual signaling proteins from the diseased cells and the activity states of the corresponding individual signaling proteins from the reference cells, thereby providing a snapshot of the signaling proteins in the diseased cells that form an abnormal network of interconnected cell signaling proteins that leads to the aberrant cellular response in the patient; and/or d) reporting a combination of at least two different therapeutic agents for administration to the patient for the purpose of correcting the abnormal network by reducing the difference that was detected in the activity states of two or more of the signaling proteins in the abnormal network of interconnected cell signaling proteins identified by steps (a)-(c) in the diseased cells from the activity states of the corresponding signaling proteins in the reference cells.

**Microarray and Data Analysis**

In some cases, each array is scanned, spot intensity analyzed, data normalized, and a standardized, single data value is generated for each sample on the array (Image Quant v5.2, GE Healthcare, Piscataway, N.J.). Spot intensity is integrated over a fixed area. Local area background intensity is calculated for each spot with the imprinted adjacent slide background. This results in a single data point for each sample, for comparison to every other spot on the array. The Ward method for two-way hierarchical clustering is performed using JMP v5.0 (SAS Institute, Cary N.C.). Wilcoxon two-sample rank sum test is used to compare values between two groups. P values less than 0.05 are considered significant. Kaplan-Meier (log-rank) survival estimates are used for univariate survival analysis.

In some instances, every array has on-board calibrators (with known amounts of each specific analyte) that allow knowing how much of each protein is detected. Each protein signal on the assay is normalized to the amount of protein in each individual spot on the array. A value of Normalized Fluorescence Intensity (NFI) per mg protein is generated and compared to the linear range of the calibrators to calculate the amount of each protein per mg total protein in the test samples. This gives a semi-quantitative value used to calculate a ratio. For example, a value for an analyte HIF-1 alpha is divided by one that does not change (IGF-IR or Akt for example) to
get the ratio. If the ratio is high, e.g., >1, then there is an increased level of the HIF-la that correlates to healing or nonhealing. In some embodiments, total proteins are measured to gauge the effect of transcription factors on gene expression. In some embodiments, phosphoproteins are measured to see if actively signaling or not.

In some cases, expression or activation levels of proteins are first measured and then compared to their reference standards. Each comparison gives a value, which is used in the next step of determining a ratio to another protein. In some embodiments, calibrators are generated, and a large population of patients is run to get a distribution, which allows to bin the patients on a per analyte basis from low to high. Then the unknown is compared to the distribution to place the patient on a per analyte basis somewhere in the population. Multiple analytes are evaluated, for example those in a signaling pathway (HER2 \( \rightarrow \) Akt \( \rightarrow \) mTOR \( \rightarrow \) 4EBP1), to see if a pathway is on or not.

**Examples**

**Example 1: A comparative clinical study of chronic DFU keratinocytes and non-diabetic (control) plantar skin specimens**

A panel of biomarkers comprised of proteins or the activated-state (phosphorylation) of proteins was examined in isolated keratinocytes from the margins of healing and nonhealing diabetic foot ulcers (DFU) and compared to the levels or activation of the same proteins in normal plantar tissues. Alterations in the intracellular levels these proteins and/or in the level of activation were observed that identified the dysregulation of multiple signal transduction pathways and from an analysis of these biomarkers, a correlation was found between sets of biomarkers was identified that can predict healing.

Patient information for fourteen patients who presented with Diabetic Foot Ulcers (DFU). Clinical data includes sex, age, whether they were Type 1 or 2 diabetics, their use of insulin, presence or absence of neuropathy or co-morbidities, the age and size of their ulcer, recent infection histories, whether they had recent surgery and if any amputations had been performed is listed. The information on the patients used to establish the P/I ratio are shown in Table 1.

RPPA measurements on 19 analytes were performed on biopsies (formalin preserved) taken from a patient population that included 18 patients who presented with chronic ulcers (DFU) and 3 biopsies from DFU patients whose ulcer healed, and 3 biopsies taken from normal patients (NOR) and the information on these patients are shown in Table 1. For the two cohorts, the sex (DFU 14 male, 10 female; NOR 3 male, 1 female) and age (DFU Mean 59.96 years, Range36-81 years of age; NOR Mean 29.75, Range21-51) as shown in Table 1.
The 19 analytes measured by RPPA included a number of important signaling markers that have been associated with various processes that may be involved in the healing or non-healing of diabetic ulcers are:

1. Akt
2. iNOS
3. mTOR
4. NF kappa B p65
5. P16INK4a
6. P53
7. POKinase
8. VEGF Receptor 1
9. Bak
10. Catenin-beta
11. Caspase.3
12. Caspase.9
13. GSK.3.beta
14. IGF.1R beta
15. IkB alpha
16. Met
17. Cox.2
18. p-mTOR S2448
19. IGF IR Y1135, 1150

From the 30 patient biopsies that were analyzed, shown in Tables 1 and 2 (cont), only 22 of these had sufficient cellularity to allow for RPPA analysis. Those that were suitable for RPPA analysis included 3 normal patient biopsies, 3 diabetic patients whose ulcers healed, and 16 diabetic patients whose ulcers did not heal following standard treatment modalities.

RPPA analysis was performed on lysates made from the tissues obtained for each patient, and the results of this analysis for 19 analytes are shown in Tables 2 and 2 (cont.). Values represent normalized results for the analytes measured in 18 DFU patients and 4 Normal Controls.

When compared with non-diabetic control subjects, DFU wound keratinocyte signaling protein levels were significantly (p<0.05) elevated for:

1) the pro-inflammatory transcription factor, NF-kB;
2) the tumor suppressor transcription factor, P53;
3) the senescence biomarker, P16INK4a;
4) the cellular survival and growth transduction pathway proteins: PI3K, AKT (protein kinase B), β-catenin, glycogen synthase kinase-3p (GSK-3P), and C-Met; and
5) the P53-directed apoptosis cleavage protein Caspase-3.

During a 12-week post-debridement observation period all DFU subjects received standard of care wound treatment and wound off-loading. During this time three (n=3) of the
nineteen DFU subjects (16 percent) demonstrated complete healing of the study ulcer. Following study completion, DFU study subject wound status (healed vs. un-healed) correlated with proteomic activity ratios for PI3K/IGF-1R (P/I-ratio). PI3K-AKT/mTOR (mammalian target of rapamycin) is the major signaling pathway activated by the hormone insulin and insulin-like growth factor-1 (IGF-1). IGF-1 cellular stimulation of AKT signaling has been demonstrated to induce the expression of hypoxia-inducible factor-1 (HIF-1) that may be critical to the initiation of angiogenesis and the healing of diabetic wounds. Conversely, phosphatase tension homolog (PTEN), a p53-directed PI3K antagonist documented with diabetes, inhibits AKT/mTOR activation and suppresses induction of hypoxia-inducible factor-1 (HIF-1) mediated gene up-regulation. The data documents P/I-ratio values (±SD) of: 0.7 (±0.02) for control study subjects, 1.0 (±0.1) for healed-DFU study subjects, and 1.3 (±0.1) for unhealed-DFU study subjects. Unpaired t-test analysis for healed and unhealed DFU group P/I-ratios and ANOVA analysis of all study group P/I-ratios documented significant mean differences.

The P/I-ratio may provide a novel quantitative gauge of IGF-1 initiated AKT/mTOR signal transduction. In this case, increasing P/I-ratio values would be inversely proportional to decreasing AKT/mTOR signal transduction associated with impaired DFU wound repair. Proteomic assessments from this pilot study document the neuropathic DFU as a complex, senescent wound and suggest P53-directed dysfunctional regulation of IGF-1, PI3K, and AKT/mTOR signaling as critical factors responsible for impaired DFU wound healing.

The results indicate that:

1) The unexpected finding that, when the ratio of PKBKinase to IGF-1R is greater than about 1, a diabetic ulcer will not heal and alternate therapies should be employed to promote healing.

2) The unexpected finding of novel specific protein drug targets in non-healing diabetic ulcers that should be inhibited to promote healing.

3) The unexpected finding that a therapeutic strategy for treating non-healing wounds would be to inhibit PKB signaling, opening up the use of such inhibitors as potential therapeutic agents for treating non-healing diabetic ulcers.

4) A cut point for DFU management can be discerned from the data, wherein the ratio of PI3K to IGF-1R is greater than 1.5 can predict non-healing and a therapeutic approach of inhibiting PKB signaling in non-healing diabetic ulcers discerned from the data and that this cut point can be used for prospective patient selection.

In summary, the results of the DFU wound healing study report the correlation of keratinocyte insulin pathway PI3K/IGF-1R ratio values to wound healing outcomes in DFU.
subjects. These findings provide the basis for the invention described with this report. These novel signal pathway relationships have not been previously reported and describe quantifiable thresholds of ISP signal transduction and P53-directed processes that may be responsible for promoting impaired diabetic wound healing. This invention will have immediate application and impact on the clinical management of wound healing complications with diabetes and will provide a novel platform for future research and development of combined clinical and pharmacological treatment pathways for the devastating morbidity associated with this problem. Study findings reported with this invention also provide irrefutable evidence of the value provided by proteomic analysis technology (RPPA and LCM) in support of investigations of mechanisms of wound healing and impaired tissue repair. The role of P53-mediated dysfunctional insulin signaling pathway transduction and supporting data suggesting a downstream link with HIF-1 expression in diabetes impaired wound healing would not have been achieved without RPPA and LCM technology. Additionally, this study has appropriately underscored the importance of understanding the critical roles played by P53-directed processes as they impact the cellular injury response and the pro-inflammatory cellular dynamics required for wound repair. Wound repair is a productive extrinsic stress response generally unaffected by the intrinsic stresses that threaten unchecked cell division and proliferation. As isolated phenomena P53-directed processes may appear to promote dysfunctional cellular responses following cellular injury. P53 can limit the consequences of extrinsic stress from the cellular injury response and wound repair by inhibiting pro-inflammatory processes, initiating cell death, senescence, or cell cycle arrest and promoting oxidative cellular metabolism. Ultimately, it is the balance (or imbalance) achieved following the regulation of these intrinsic and extrinsic processes that provides the environment and outcomes that are achieved in wound repair. The active antagonism demonstrated by P53-directed processes during wound healing can be a constant factor.

Example 2: Comparative, quantitative proteomic assessment of DFUs.

DFU subjects, adults with chronic, full-thickness, neuropathic foot ulcers selected from a hospital patient population, and control study subjects, non-diabetic adults who volunteered to submit plantar foot tissue specimens for control tissue analysis were evaluated using a comparative, quantitative proteomic assessment of twenty-one (n=21) signaling pathway proteins, spanning multiple pathways. The assessment of signaling proteins of DFU (n=18) and non-diabetic (normal) plantar skin (n=3), was performed on keratinocytes isolated from full thickness plantar foot skin samples obtained using a three-millimeter punch biopsy technique. Punch specimens were obtained from the viable ulcer margin immediately following clinical debridement of the wound and from control subject's plantar foot areas. For tissue biopsies all
study subjects were allowed to receive a suitable volume of injectable local anesthetic for tissue retrieval, if needed. The punch biopsy sites were closed by use of non-absorbable sutures and dressed with occlusive foam or silicon bandages. All subject tissue specimens were immediately placed in formalin to preserve morphology and phosphoprotein levels in tissues.

DFU study participants continued with normally scheduled wound evaluations as routinely coordinated by the medical and nursing staff. A twelve-week post-debridement observation period was scheduled for all DFU study patients to assess wound healing, study subject compliance and for evaluation of off-loading methods assigned for patient care. During a 12-week post-debridement observation period three (n=3) DFU subjects demonstrated complete healing of their study ulcers. RPPA quantitatively measured the expression levels and activation (phosphorylation) status of proteins in signaling pathways relevant to wound healing. RPPA allowed for the simultaneous assessment from all biopsies, providing a high-dimensional 'snap-shot' of signaling activity in DFU keratinocytes.

In the DFU study group there were seventeen subjects with Type II diabetes, one with Type I diabetes, fifteen male and three female subjects. The average age (mean value) of the DFU subjects was 60.3 years (ranging from 48-75; median age = 60). The average age of the DFU study ulcer was nineteen (19) months, ranging from 2-120 months (median ulcer age = 11 months). Comparing the healed and unhealed DFUs wound age in months (±STDev): Mean ulcer age - healed DFUs = 18.0 months (±16.1) vs. unhealed DFUs=19.2 months (±19.2). The mean DFU wound sizes were measured by planometric methods and were: 7.5cm² (±13.2cm) for unhealed DFUs vs. 1.6cm (±1.0cm) for healed DFUs. Healed and unhealed DFU wound sizes were not significantly different. The control study group (n=3) was comprised of three healthy adult males with an average age of 23 years.

DFU subject wound care consisted of moist to dry dressings, foams and alginates with and without nanocrystalline silver, collagenase, antibiotic ointments or hydrogels with or without nanocrystalline silver. No study patients received allograft, xenograft, living, single or bi-layered human cell based dressings (e.g., Dermagraft™ or Apligraf™) or adjunctive hyperbaric oxygen therapy (HBOT) during or within six months of this study.

DFU study subjects had been taking medications for glycemic control prior to tissue specimen retrieval. These medications were not changed during the study observation period and are listed in Table 3- Diabetic Medications Taken During the Study. DFU study subjects listed by number with their pre-study medications for glycemic control. HEALED- indicates subjects with healed study DFU wounds at the completion of the twelve-week observation period. TIDM-indicates study subject with Type I DM; all other study subjects diagnosed with Type II DM.
Significant differences were observed in multiple signaling proteins following non-parametric Whitney-Mann U-Test comparison of normal and DFU keratinocytes (Figure 3). Unexpectedly, many of these elevated proteins that are abnormally expressed in DFUs are known to be dysregulated to some degree in most cancer types, including breast, colon, lung, pancreatic, ovarian, and hepatic tumors. The proteins exhibiting significantly (p < 0.01) increased levels in nonhealing DFU keratinocytes compared to normal tissue included: members of the PI3K/AKT signaling pathway (p-PTEN S380, PI3 Kinase, AKT, GSK-3P and p-mTOR S2448); the NFKB pathway (NFKB/p65 and p53); the β-catenin pathway (GSK-3P, pl6INK4a, and Caspase 3). Total mTOR and COX-2 were also elevated (p < 0.05) in nonhealing DFUs.

When the data cross all analytes was subjected to hierarchical clustering to see if there were patterns of significant dysregulated in the analytes of the signaling pathways between normal and DFU patients. Normal (Subjects 19, 20, and 21) and DFU patients were separated. Based upon the signaling architecture of the two populations, DFU patients significantly exhibit higher levels of multiple proteins across the PI3 Kinase/Akt pathway, the survival pathways (NFkB/p65 and β-catenin), and the cell surface receptor, c-MET a target of the TCF/p-catenin transcription complex. Thus, RPPA analysis identified an activity-relationship that corresponds to major components that have been observed individually in wound healing found in the literature, but the analysis of multiple pathways allows for correlative and combinatorial relationships to be determine, critical to determining the salient biomarker groups that can predict healing versus nonhealing DFU and to identify combinatorial therapeutic approaches to treating DFU.

The level of expression (and activation) of IGF-1R, a cell surface receptor involved in insulin and IGF-1 signaling, was not significantly different between normal and DFUs (healed or unhealed). To assess correlative alterations in downstream signaling, a ratio of the downstream proteins to IGF-1R was calculated in an attempt to identify perturbations to signaling that may distinguish healing from nonhealing DFUs (Figure 6). There were several proteins whose ratios of expression levels were significantly elevated in nonhealing DFU, including: PI3 Kinase and mTOR, members of the Akt signaling pathway that facilitates cell cycle progression and inhibits apoptotic events; p53, a regulator of cell cycle arrest, apoptosis, and senescence; Caspase 9, indicative of intrinsic apoptosis; Bak, the pro-apoptotic protein that is increased in response to p53 and which is activated by Caspase 9; and COX-2, whose transcription and stabilization may be modulated by Akt and/or NfKB/p65 signaling, indicative of a pro-inflammatory state.

In our analysis, there were no significant differences between normal, healing DFU, or nonhealing DFU levels for total or phosphorylated IGF-1R, suggesting that increased IGF-1R
signaling in DFUs was not inhibiting wound healing. However, there was a significant increase in PI3 Kinase/mTOR signaling in DFUs.

Expression of c-Met was significantly increased in DFUs compared to normal. The observed increased levels of c-Met may be responsible for the observed increased PI3 Kinase/Akt signaling in DFU. The IGF-1R and IR receptors, are known to form heterodimer pairs with c-Met, mediating transactivation of c-Met in the absence of its ligand, HGF (hepatocyte growth factor), thereby potentiating un-regulated signaling in multiple pathways (DFU levels compared to normal, Figure 4). Our observed signaling architecture in DFUs suggests patterns of cross-talk between pathways involved in apoptosis (p53, caspases), proliferation and migration (PI3Kinase/Akt/mTOR), inflammation (Cox-2, NFKB), and senescence (p53, pl6INK4a).

RPPA identified active signaling pathways in DFUs which may help identify points of therapeutic intervention to treat nonhealing DFUs (Figure 5). Observed differences between healing and nonhealing DFUs suggest an increased level of pro-apoptotic proteins and a potential role for c-Met/IGF-1R heterodimer signaling through the PI3Kinase/Akt pathway as an important driver in the nonhealing wound. In the absence of altered levels of IGF-1R or its activation by phosphorylation, a significant modulation of pathways related to increased PI3Kinase/AKT signaling coupled with increased inflammatory and apoptotic signaling occurs, perhaps in response to c-Met activation.

The nonhealing DFU is a disease of dysregulated signal transduction potentially influenced by somatic mutations to many of the same genes as those involved in cancer. Altered signaling due to somatic mutations can interfere with mRNA transcription or protein function. These mutations change signaling efficiency, altering the control of transcription factors that modulate the intercellular levels of key proteins or the regulated patterns of post-translational modifications (PTM). An interesting observation in DFUs is the inactivating phosphorylation of PTEN (p-PTEN S380), resulting in increased PI3Kinase/Akt signaling which can trigger senescence in addition to survival (Figure 7).

A PI3Kinase/Akt imbalance can contribute to the prevention of healing in DFUs and can potentiate certain cancers. Many of the active signaling pathways observed in DFUs are evident in cancers, suggesting that drugs developed for one disease may have utility in treating the other. For example, the diabetes drug metformin, an activator of AMPK-directed inhibition of Akt/mTOR-mediated proliferation and Cyclin D1 synthesis, reduces the incidence and progression of some cancers. In diabetics, metformin may be associated with larger ulcerations and inhibition of keratinocyte proliferation, but these DFUs do not progress to amputation. Activation of survival and senescence signaling pathways, like NFKB, p53 and β-catenin, can
contribute to nonhealing DFUs. In some embodiments, nonhealing DFUs can be treated using topical applications of anti-cancer therapies targeting proteins in these pathways or for the development of diagnostic tests to guide therapeutic decisions when treating DFUs.

**Example 3**

This example illustrates an assessment of the wound healing potential of DFU wound subjects A and B from a comparative analysis of the IGF-1R protein expression ratio of the subject ulcer following RPPA analysis in the Wound Healing Diagnostic (WHD). For their evaluation each subject has completed the steps of having a small (<3 mm diameter) punch biopsy of the wound marginal epidermis performed after wound debridement. This is followed by the step of immediately placing the epidermal wound margin biopsy tissue in formalin fixative solution. Following tissue fixation the specimen is dissected using macro-dissection or laser capture microdissection methods to adequately isolate the basal keratinocyte cells from the specimen for RPPA analysis. Protocols are then followed for the completion of RPPA cell lysate analysis against a panel of protein analytes that would include those used in the study performed with the invention. The WHD panel for healing assessment would include values of IGF-1R protein expression ratios with the following signaling proteins: PI3K (PI3K/IGF-1R) and/or mTOR (mTOR/IGF-1R) and/or COX-2(COX-2/IGF-1R) and/or Bak (Bak/IGF-1R) and/or p53 (p53/IGF-1R).

Following the completion of the RPPA WHD ratio analysis on two subjects presenting with DFU at the time of diagnosis, Subject A’s tissue specimen PI3K/IGF-1R ratio value was 0.9, while the Subject B tissue specimen PI3K/IGF-1R ratio value was 1.4. After comparing these values with the DFU study WHD ratios for this invention (see Figure 7) it was determined that the values obtained from Subject A’s DFU wound demonstrated that the PI3K/IGF-1R value was associated with wounds that experienced healing, while the PI3K/IGF-1R value determine for Subject B’s DFU wound value was associated with wounds that did not heal. Subsequent clinical observation was continued with both subjects while optimal wound dressings and off-loading methods were continued with Subject A’s wound demonstrating complete healing in 12 weeks, or less, while Subject B’s wound remained unhealed at the end of a twelve week period of observation. During this time the Subject B DFU wound was prepared for evaluation using the wound therapy diagnostic (WTD) panel assessment to determine topical corrective pharmaceutical therapies for the promotion of wound healing as presented in the following example.

As can be seen from Figure 6, using the ratio of PI3K to IGF-1R as a guide, the PI3K/IGF-1R, analysis of Subject A’s signaling generated a ratio of 0.9 which is within the Box for Healing wounds while the ratio of PI3K to IGF-1R for Subject B was 1.3, which is within the
Box for Healing wounds. Thus based on the ratio of 0.9 for Subject A's DFU, this ulceration was expected to heal, while that of Subject B, ratio of 1.3, was not expected to heal.

As Subject B was expected to have a nonhealing DFU, a subsequent analysis was performed to identify potential points of therapeutic intervention based upon the ratio of a number of analytes to Akt. The analytes included: VEGFR1, iNOS, HIF-la, Bak, GSK-3b, Catenin b, NFkb p65, pl6INK4a, and p53 (Figure 7). As the levels of these protein analytes increased there is a decrease in the ratio, and those with significant differences would be considered points of therapeutic intervention. For example in Subject B the ratio of pl6INK4a / Akt was found to be 1.4 and this ratio in Normal keratinocytes is 0.5. By inhibiting the level of pl6INK4a the ratio would be expected to decrease and by shifting the level of pl6INK4a lower, there would be less contribution to the activation of senescence, which is a terminal event that causes irreversible cell death. So by treating with a pINK3A inhibitor the over activation of senescence would be lessened, keratinocytes would live longer and the wound would heal.

**Example 4. Treatment.**

One or more subjects are treated with a pharmaceutical composition disclosed herein.

The pharmaceutical composition comprises an antagonist and/or an agonist as described herein. For example, antagonists (e.g., of GSK-3beta) include Beryllium, Lithium, Anilino maleimides (SB216763, SB415286), Arlypyrazolopyridazines (e.g., 6-aryl pyrazole [3,4-b] pyridine 4), Bisindole maleimides (e.g., Ro 31-8220, GF 109203x), Indirubins (6-bromoindirubin-3'-oxime, aka BIO), Paullones (alsterpaullone), Psuedosubstrate peptide, Dibromocantherelline, Meridian 4e A, CHIR908014, CHIR908023, CHIR908021, SB216763, AR-A014418, Kenpaullone, Alsterpaullone, Cazpaullone, SB415286, TWS119, Aloisine A, TDZD-8, NP00111, HMK-32, Manzamine A, Palinurin, Tricantin, L803-mts. Additional examples of antagonists (e.g., of PI3K/AKT/mTOR) include Everolimus (Novartis), Temsirolimus (Pfizer), BEZ235 (Novartis), GDC-0980 (Genentech), PF-05212384 (Pfizer), SAR245409 (XL-765; Sanofi/Exelixis), BAY80-6946 (Bayer), Bupariisib (BKM120; Novartis), Pictlisib (GDC-0941; Genentech), PX-866 (Oncothyreon), SAR245408 (XL-147; Sanofi/Exelixis), ZSTK474 (Zenyaku Kogyo), BYL719 (Novartis), GDC-0032 (Genentech), MLN01117 (INK1117; Intellikine), GSK2636771 (GSK), SAR260301 (Sanofi), Idelalisib (CAL-101; GS-1101; Gilead/Calistoga), AMG319 (Amgen), Perifosine (KRX-0401; Keryx), MK2206 (Merck), GDC-0068 (Genentech), GSK2110183 (GSK), GSK2141795 (GSK), ARQ 092 (ArQule/Daiichi Sankyo), AZD5363 (AstraZeneca), AZD2014 (AstraZeneca), MLN0128 (INK128; Intellikine), or CC-223 (Celgene). Additional antagonists (e.g., of β-catenin signaling) include PKF118-310, CGP049090, PKF115-584, PKF222-815, PKF118-744, ICGO01, CCT036477, XAV939, Acyl hydrazones, HQBA, Molecules with 2,3,6-trisubstituted
pyrido[2,3-b] pyrazine core skeletons, Carnosic acid, CCT031374, iCRT-3,5,14, NC043, Ibuprofin, or Aspirin. Additional antagonists (e.g., of p53) include Nutlins RG7112 (RO5045337), Benzodiazepinediones (TDP665759), Spiro-oxindoles (MI-219), RITA, JNJ-26854165 (Serdemetan), Tenovin 1 and 6, SJ-172550, RO-2443/RO-5693, XI-Oil, Re-activate mutant p53, CP-31398, PRIMA-1 (APR-246), MIRA-1, Phikan083, orNSC319726. Examples of agonists (e.g., of GSK-3beta) include Celecoxib, Staurosporine, Trichostatin A, Curcumin, AKt/protein kinase B signaling inhibitor-2(API-2), Wortmannin, LY294002, Rapamycin, Differentiation-inducing factors (DIFs), and Retinoids.

The examples and embodiments described herein are for illustrative purposes only and various modifications or changes suggested to persons skilled in the art are to be included within the spirit and purview of this application and scope of the appended claims.
<p>| Study # | Type 1 or Type 2 | Sex | Age | On or Off Insulin | Y/N | Age Of Ulcer | Co-Morbidities | Wound Size | Amputations | Recent Surgery | Recent Infection | Recent Weight Loss | Recent Amputations |
|---------|----------------|-----|-----|------------------|-----|--------------|----------------|------------|-------------|---------------|----------------|------------------|------------------|-------------------|
| 1       | DFU            | Male| 51  | Y                | N   | 10 years     | HTN, Lymphedema and hyperkalemia and Kidney disease | L 3.6 x W 2.3 x D | 0.6 cm      | No           | No            | No              | No               | No               |
| 2       | DFU            | Female| 81  | Y                | Y   | 1 year       | HTN, Hypertension, Hyperlipidemia, PVD-Varicose, Chronic Kidney Disease | L 0.5 x W 0.3 x D | 0.2 cm      | No           | No            | Yes             | Yes              | Yes               |
| 3       | DFU            | Male| 60  | Y                | Y   | 1.5 years    | HTN, Hypertension, Hyperlipidemia, PVD-Varicose, Chronic Kidney Disease | L 2.2 x W 1.3 x D | 0.1 cm      | No           | No            | Yes             | Yes              | Yes               |
| 4       | DFU            | Male| 58  | Y                | N   | 5 months     | CAD, HTN, APLB, Degenerative and Rheumatoid Arthritis &amp; Scleroderma, Stenosis | L 1.2 x W 0.8 x D | 0.1 cm      | Yes          | Yes           | Yes             | Yes              | Yes               |
| 5       | DFU            | Male| 36  | Y                | Y   | 8 months     | HTN, Hypertension, Hyperlipidemia, Chonic Kidney Disease, Sleep Apnea | L 3.3 x W 2.0 x D | 0.3 cm      | Yes          | Yes           | Yes             | Yes              | Yes               |
| 6       | DFU            | Male| 69  | Y                | Y   | 4 months     | HTN, Hyperlipidemia, Chronic Kidney Disease, Diabetes, Pancreatitis, Gastroparesis | L 2.1 x W 0.9 x D | 0.1 cm      | Yes          | Yes           | Yes             | Yes              | Yes               |</p>
<table>
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<tr>
<th>Study #</th>
<th>DFU or Control</th>
<th>Sex</th>
<th>Age</th>
<th>Type 1 or Type 2</th>
<th>On Insulin Y/N</th>
<th>Neuropathy Y/N</th>
<th>Co-Morbidities</th>
<th>Age Of Ulcer</th>
<th>Recent Infection Y/N</th>
<th>Recent Surgery Y/N</th>
<th>Any Toe or Foot Amputations?</th>
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<td>Y</td>
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<td>14 months</td>
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<td>N</td>
<td>Y Aortogram</td>
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<td>N</td>
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<td>Y</td>
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<td>23 months</td>
<td>L 8.6 X W 3.2 X D 0.5 CM</td>
<td>Y abcess 4 months prior</td>
<td>Y debridement</td>
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<td>Y</td>
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<td>Y 11/29/12 EXO-</td>
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<td>Co-Morbidities (Wound Size)</td>
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<td>Recent Surgery Y/N</td>
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<td>Y</td>
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<td>Y</td>
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<td>Y</td>
<td>COPD, PAD, Angioplasty lt. leg, HTN, Hyperlipidemia, osteomyelitis, spinal stenosis</td>
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<td>15 months</td>
<td>Y osteomyelitis</td>
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<td>Type 2</td>
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<td>Y</td>
<td>Smoker, HTN, Degenerative disc disease, arthritis, depression</td>
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<td>Y</td>
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<td>3 months</td>
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Table 2. Normalized expression values from DFU patients that healed, did not heal, and normal controls and from whose biopsy RPPA data was possible.

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<th>mTOR</th>
<th>NT kappa p65</th>
<th>P16INK4a</th>
<th>P53</th>
<th>1PI3. Kinase</th>
<th>VEGF Receptor 1</th>
<th>Bak</th>
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Table 2 (Cont.).

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<th>Met</th>
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Table 3. Diabetic Medications Taken During the Study.

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<tr>
<th>Patient Number</th>
<th>Prior Therapy</th>
<th>DFU Status</th>
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<tbody>
<tr>
<td>1</td>
<td>Tradjenta (Linagliptin)</td>
<td>Non-Healing</td>
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<tr>
<td>2</td>
<td>Lantus / Humalog Insulin</td>
<td>Non-healing</td>
</tr>
<tr>
<td>3</td>
<td>Glimeperide</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>4</td>
<td>Glipizide; Metformin</td>
<td>Healed</td>
</tr>
<tr>
<td>5</td>
<td>Lantus / Humalog Insulin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>6</td>
<td>Humalog / Humulin N Insulin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>7</td>
<td>Humalog / Humulin N Insulin (TIDM)</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>8</td>
<td>Humulin 70 / 30 Insulin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>9</td>
<td>Glipizide / Novolog and Lantus Insulin</td>
<td>Healed</td>
</tr>
<tr>
<td>10</td>
<td>Glimeperide / Victoza and Invokana Insulin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>11</td>
<td>Glyburide / Lantus and Humalog Insulin; Metformin</td>
<td>Healed</td>
</tr>
<tr>
<td>12</td>
<td>Glimeperide; Metformin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>13</td>
<td>Humulin 70 / 30 Insulin</td>
<td>Non-Healing</td>
</tr>
<tr>
<td>14</td>
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<td>Non-Healing</td>
</tr>
<tr>
<td>16</td>
<td>Lantus and Novolog Insulin; Metformin</td>
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<tr>
<td>17</td>
<td>Novolon N and Novolin R Insulin; Actos</td>
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</tr>
<tr>
<td>18</td>
<td>Glipizide; Metformin, Januvia</td>
<td>Non-Healing</td>
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</table>
Claims

What is claimed is:

1. A method, comprising:
   A. measuring an individual level of an expression or activation of a first protein and a second protein in a sample from a wound, wherein the first protein and the second protein are individually selected from the group consisting of: IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3P, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, or any combination thereof, and
   B. calculating a ratio of the first protein to the second protein.

2. The method of claim 1, wherein the method is a method of determining a diagnosis of the wound in a subject.

3. The method of claim 1 or 2, wherein the measurement is repeated for at least 1, 2, 3, 4, or 5 times, and wherein when the method is repeated more than 1 time, the measurement may be conducted at different time points or at the same time point.

4. The method of any preceding claim, wherein the measurement is repeated during a course of monitoring the wound.

5. The method of any preceding claim, wherein the calculation is implemented at least in part by a computer.

6. The method of claim 5, wherein the calculation is implemented at least in part by a computer processor.

7. The method of claim 6, wherein the computer processor is a computer microprocessor.

8. The method of any preceding claim, further comprising determining a likelihood that a wound will not heal when the ratio is greater than 1, or that the wound will heal when the ratio is 1 or less.

9. The method of claim 8, wherein the determination is implemented at least in part by a computer.

10. The method of claim 9 wherein the determination is implemented at least in part by a computer processor.

11. The method of claim 10, wherein the computer processor is a computer microprocessor.

12. The method of any preceding claim, further comprising evaluating the wound visually.

13. The method of any preceding claim, further comprising communicating a result via a communication medium.

14. The method of claim 13, wherein the communication medium is a document.
15. The method of claim 14, wherein the communication medium is a computer file, an email, a fax, or a paper document.

16. The method of any preceding claim, wherein the ratio is calculated with values generated from a comparison of the measured levels and corresponding reference standards.

17. The method of any preceding claim, wherein the sample is a tissue from the wound.

18. The method of any preceding claim, wherein the sample is from a marginal edge of the wound.

19. The method of any preceding claim, wherein the sample comprises a keratinocyte isolated or purified from the wound.

20. The method of claim 19, wherein the first protein and the second protein are individually measured from the keratinocyte or a cellular lysate thereof.

21. The method of any preceding claim, comprising measuring the expression of the first protein and the second protein individually.

22. The method of any preceding claim, comprising measuring the activation of the first protein and the second protein individually.

23. The method of any preceding claim, wherein the measured level is determined by measuring a total amount of the first protein and/or the second protein individually.

24. The method of any preceding claim, wherein the measured level is determined by measuring an amount of an activated form of the first protein and/or the second protein individually.

25. The method of any preceding claim, wherein the activation is phosphorylation.

26. The method of any preceding claim, wherein the measured levels are normalized fluorescence intensity (NFI) per mg protein.

27. The method of any preceding claim, wherein the values are normalized expression values.

28. The method of any preceding claim, wherein the wound will not heal when the ratio is \( \geq 1.2, \geq 1.3, \geq 1.4, \) or \( \geq 1.5. \)

29. The method of any preceding claim, further comprising treating the subject.

30. The method of claim 29, wherein the treating comprises administering to the subject a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein, the second protein, or the first protein and the second protein are individually selected from the group consisting of: IGF-IR, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET,
GSK-3P, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof.

31. The method of claim 29, wherein the treating comprises administering to the subject a pharmaceutical composition that comprises an inhibitor of POKinase signaling, an activator of insulin signaling through IGF-1R, or any combination thereof.

32. The method of claim 31, wherein the inhibitor of POKinase signaling is an inhibitor of POKinase complex, Akt, mTOR, 4E.BP1, Ribosomal Protein S6 Kinase, HIF-1α, PTEN, POKinase, IGFRBP3, Src, GSK3β, β-catenin, any protein that is a member of the insulin signaling or POKinase signaling pathways of proteins, or any combination thereof.

33. The method of claim 31 or 32, wherein the activator of insulin signaling through IGF-1R is selected from the group consisting of insulin, insulin-like growth factor 1 (IGF-1), IGF-2, PTEN, POKinase, mTOR, 4E.BP1, Ribosomal Protein S6 Kinase, HIF-1α, PTEN, IGFRBP3, Src, GSK3β, β-catenin, any protein that is a member of the insulin signaling or POKinase signaling pathways of proteins, and any combination thereof.

34. A method of treating a wound in a subject in need thereof, comprising treating the subject with a pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein, the second protein, or the first protein and the second protein are individually selected from the group consisting of: IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3P, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof.

35. The method of any one of claims 30-34, wherein the pharmaceutical composition comprises the agonist and the antagonist, which are administered simultaneously.

36. The method of any one of claims 30-34, wherein the pharmaceutical composition comprises the agonist and the antagonist, which are administered sequentially.

37. The method of any one of claims 30-36, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

38. The method of claim 37, wherein the pharmaceutically acceptable carrier comprises water.

39. The method of any one of claims 1-38, wherein the second protein is IGF-1R.

40. The method of any one of claims 1-38, wherein the second protein is Akt.

41. The method of any one of claims 1-38, wherein the first protein is PI3 Kinase and the second protein is IGF-1R.

42. The method of any one of claims 1-38, wherein the first protein is PI3 Kinase and the second protein is insulin.
43. The method of any one of claims 1-38, wherein the first protein is PI3 Kinase and the second protein is Akt.
44. The method of any preceding claim, wherein the first protein or the second protein is Akt that is Akt1.
45. The method of any preceding claim, wherein the first protein or the second protein is IGF-1R that is IGF-1Rp.
46. The method of any preceding claim, wherein the first protein or the second protein is IGF-1Rβ that is p-IGF-1Rε Y1135-1150 (phospho-tyrosine 1135-1150).
47. The method of any preceding claim, wherein the first protein or the second protein is HIF that is HIF-la.
48. The method of any preceding claim, wherein the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448).
49. The method of any preceding claim, wherein the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380).
50. The method of any preceding claim, wherein the subject has or is suspected of having diabetes, Alzheimer's disease, a cancer, or any combination thereof.
51. The method of any preceding claim, wherein the subject is a human.
52. The method of any preceding claim, wherein the wound is a diabetic ulcer, a pressure ulcer, a venous stasis ulcer, a radiation ulcer, a skin injury, an unhealed surgical wound, a wound from a surgical procedure, a wound from a peripheral vascular disease, a wound from a complication of trauma, a wound in a cancer patient, a wound in a patient receiving a steroid therapy, a wound from an inflammatory skin disease, a chronically impaired cutaneous wound, or any combination thereof.
53. The method of any preceding claim, wherein the wound is a diabetic foot ulcer.
54. A pharmaceutical composition that comprises an antagonist of a first protein, an agonist of a second protein, or a combination thereof, wherein the first protein or the second protein are individually selected from the group consisting of: IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGFRBP-3, c-MET, GSK-3p, COX-2, iNOS, NFkB/p65, p1B-α, p53, p6INK4a, β-Catenin, Bak, Caspase 3, Caspase 9, and any combination thereof.
55. The pharmaceutical composition of claim 54, wherein the second protein is IGF-1R.
56. The pharmaceutical composition of claim 54, wherein the second protein is Akt.
57. The pharmaceutical composition of claim 54, wherein the first protein is PI3 Kinase and the second protein is IGF-1R.
58. The pharmaceutical composition of claim 54, wherein the first protein is PI3 Kinase and the second protein is insulin.

59. The pharmaceutical composition of claim 54, wherein the first protein is PI3 Kinase and the second protein is Akt.

60. The pharmaceutical composition of any one of claims 54-59, wherein the first protein or the second protein is Akt that is Akt1.

61. The pharmaceutical composition of any one of claims 54-60, wherein the first protein or the second protein is IGF-1R that is IGF-1Pβ.

62. The pharmaceutical composition of any one of claims 54-61, wherein the first protein or the second protein is IGF-1Pβ that is p-IGF-1Pβ Y1135-1150 (phospho-tyrosine 1135-1150).

63. The pharmaceutical composition of any one of claims 54-62, wherein the first protein or the second protein is HIF that is HIF-1a.

64. The pharmaceutical composition of any one of claims 54-63, wherein the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448).

65. The pharmaceutical composition of any one of claims 54-64, wherein the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380).

66. The pharmaceutical composition of any one of claims 54-65, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

67. The pharmaceutical composition of claim 66, wherein the pharmaceutically acceptable carrier comprises water.

68. A method of making a pharmaceutical composition of any one of claim 54-67.

69. A kit that comprises a reagent for measuring a total amount or a phosphorylation level of two or more proteins selected from the group consisting of: IGF-1R, PI3 Kinase, Akt, insulin, mTOR, HIF, PTEN, IGF-RBP-3, c-MET, GSK-3p, COX-2, iNOS, NFKB/p65, IκB-α, p53, pl6INK4a, β-Catenin, Bak, Caspase 3, and Caspase 9, wherein the kit is employed to predict a likelihood of wound healing in a subject.

70. The kit of claim 69, wherein the kit predicts a prognosis of a wound in a subject.

71. The kit of claim 69 or 70, wherein one of the proteins is PI3 Kinase, IGF-1R, or Akt.

72. The kit of claim 69 or 70, wherein the proteins are PI3 Kinase and IGF-1R.

73. The kit of claim 69 or 70, wherein the proteins are PI3 Kinase and insulin.

74. The kit of claim 69 or 70, wherein the proteins are PI3 Kinase and Akt.

75. The kit of any one of claims 69-74, wherein the first protein or the second protein is Akt that is Akt1.
76. The kit of any one of claims 69-75, wherein the first protein or the second protein is IGF-1R that is IGF-IRp.

77. The kit of any one of claims 69-76, wherein the first protein or the second protein is IGF-1Rβ that is p-IGF-IRβ Y1135-1150 (phospho-tyrosine 1135-1150).

78. The kit of any one of claims 69-77, wherein the first protein or the second protein is HIF that is HIF-1α.

79. The kit of any one of claims 69-78, wherein the first protein or the second protein is mTOR that is p-mTOR S2448 (phospho-serine 2448).

80. The kit of any one of claims 69-79, wherein the first protein or the second protein is PTEN that is p-PTEN S380 (phosphor-serine 380).

81. The kit of any one of claims 69-80, wherein the subject has or is suspected of having diabetes, Alzheimer's disease, a cancer, or any combination thereof.

82. The kit of any one of claims 69-81, wherein the subject is a human.

83. The kit of any one of claims 69-82, wherein the wound is a diabetic ulcer, a pressure ulcer, a venous stasis ulcer, a radiation ulcer, a skin injury, an unhealed surgical wound, a wound from a surgical procedure, a wound from a peripheral vascular disease, a wound from a complication of trauma, a wound in a cancer patient, a wound in a patient receiving a steroid therapy, a wound from an inflammatory skin disease, a chronically impaired cutaneous wound, or any combination thereof.

84. The kit of any one of claims 69-83, wherein the kit further complies an instruction manual.
FIG. 1

PROTEOMIC ASSESSMENT OF NEUROPATHIC DIABETIC FOOT ULCER (DFU) WOUND HEALING

\[ \text{p16INK4a} \]
Regulator of Senescence

\[ \text{AKT} \]
\[ \text{MAPK} \]
\[ \text{PTEN}^* \]
\[ \text{ Chronic Wound Factors } \]
\[ \text{GSK-3β/β-Catenin} \]

\[ \text{NF-kB} \]
\[ \text{ P65/p52} \]

\[ \text{TNF-α} \]

\[ \text{AKT/mTOR} \]
\[ \text{PIP3} \]
\[ \text{PIP2} \]
\[ \text{PI3K} \]
\[ \text{IGF-1R} \]

Insulin Signaling Pathway/ISP

\[ \text{HIF-1} \]

\[ \text{NF-kB} \]

Angiogenesis and Vascular Tone
\[ \text{-VEGF} \]
\[ \text{-NOS2} \]
\[ \text{-COX2} \]

\[ \text{ISP Ratio} = \text{PI3K/IGF-1R} \]

Positive Effect
Inhibitory Effect

Apoptosis
\[ \text{-Bak} \]
\[ \text{-Caspase-3} \]
\[ \text{-Caspase-9} \]

Cellular Growth, Metabolism and Survival
\[ \text{-bFGF} \]
\[ \text{-EGFR} \]
\[ \text{-PDGF} \]
\[ \text{-TGFα} \]
\[ \text{-TGFβ3} \]
\[ \text{-c-MET} \]
\[ \text{-EPO} \]
\[ \text{-KRTs} \]
\[ \text{-Hmox1} \]
\[ \text{-UPAR} \]
\[ \text{-SDF1} \]
\[ \text{-MMPs} \]
\[ \text{-ADM} \]
\[ \text{-FN1} \]
FIG. 2

Clinical Signaling Response
PI3K/IGF-1R Ratios vs. DFU Wound Status

DFU Wound Status
1 = Healed
5 = Not Healed

N = 3

Controls

N = 16

DFU Subjects

PI3K/IGF-1R Ratio
FIG. 3

Fluorescence units / mg protein

IGF-1Rβ T1135-1135 P13K 5380 Akt 6Sk38 mTOR P-mTOR

Fluorescence units / mg protein

COX-2 INOS NfκB p65 HIF-1α P53 p16INK4a B-Catenin Bak Caspase 3 Caspase 9

Normal DFU * P ≤ 0.01 # P ≤ 0.05
FIG. 4

- PI3 Kinase: p=0.033
- mTOR: p=0.015
- Cox-2: p=0.018
- p53: p=0.020
- Bak: p=0.049
- Caspase 9: p=0.049

Ratio Measured Level of Analyte / Level of IGF-1R

Healed | Unhealed
--- | ---
Healed | Unhealed
Healed | Unhealed
Healed | Unhealed
Healed | Unhealed
Healed | Unhealed
FIG. 5

Ratio PI3K / IGF-1R

Normal  Healing  Nonhealing
FIG. 7

Ratio: Analyte/Akt

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<thead>
<tr>
<th>Analyte</th>
<th>Value</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>VEGFR1</td>
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<td>0.002</td>
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<tr>
<td>iNOS</td>
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<td>0.0002</td>
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<td>HIF-1α</td>
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<td>0.010</td>
</tr>
<tr>
<td>Bak</td>
<td>0.5</td>
<td>0.0001</td>
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</tbody>
</table>

DFU Normal DFU Normal DFU Normal DFU Normal

<table>
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<tr>
<th>Analyte</th>
<th>Value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK-3β</td>
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<td>0.001</td>
</tr>
<tr>
<td>Catenin β</td>
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</tr>
<tr>
<td>NFκβ p65</td>
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</tr>
<tr>
<td>P16 INK4a p53</td>
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<td>0.0004</td>
</tr>
<tr>
<td>p53</td>
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<td>0.018</td>
</tr>
</tbody>
</table>

DFU Normal DFU Normal DFU Normal DFU Normal
Figure 8

830

801

820

825

810

815

835

840

805
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/037455

A. CLASSIFICATION OF SUBJECT MATTER

IPC (8) - A61K 38/30; A61K 45/06; A61P 17/02; C12N 9/12 (2016.01)
CPC - A61K 38/30; A61K 45/06; A61L 26/0028; C12N 9/12; C12N 9/1229 (2016.08)

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 - A61K 38/30; A61K 45/06; A61P 17/02; C12N 9/12
CPC - A61K 38/30; A61K 45/06; A61L 26/0028; C12N 9/12; C12N 9/1229

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

USPC - 433/3T3, 3 14/8.3, 0 14/β.β, 3 14/9.4 (key words delimited)

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

Orbit, Google Patents, Google Scholar

Search terms used: wound, healing, PI3K, Akt, Akt1, PKB, IGF1R, insulin, antagonist, inhibitor, decrease, block, tissue, ulcer, injury, ulceration

C. DOCUMENTS CONSIDERED TO BE CITED

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 2013/0040953 A1 (PALLER) 14 February 2013 (14.02.2013) entire document</td>
<td>55, 57, 58, 60, 72, 73</td>
</tr>
<tr>
<td>Y</td>
<td>SOMANATH et al. &quot;Akt1 is necessary for the vascular maturation and angiogenesis during cutaneous wound healing.&quot; Angiogenesis, 16 April 2008 (16.04.2008), Vol. 11, No. 3, Pgs. 277-88. entire document</td>
<td>55, 57, 58, 72, 73</td>
</tr>
</tbody>
</table>

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 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
  "&" document member of the same patent family

Date of the actual completion of the international search
24 August 2016

Date of mailing of the international search report
19 SEP 2016

Name and mailing address of the ISA/
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Authorized officer
Blaine R. Copenheaver
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)
## INTERNATIONAL SEARCH REPORT

### Box No. 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.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

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.: 4-33, 3G-G3, 61-68, 75-84  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box No. 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 additional fees, this Authority did not invite payment of additional fees.

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 and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)