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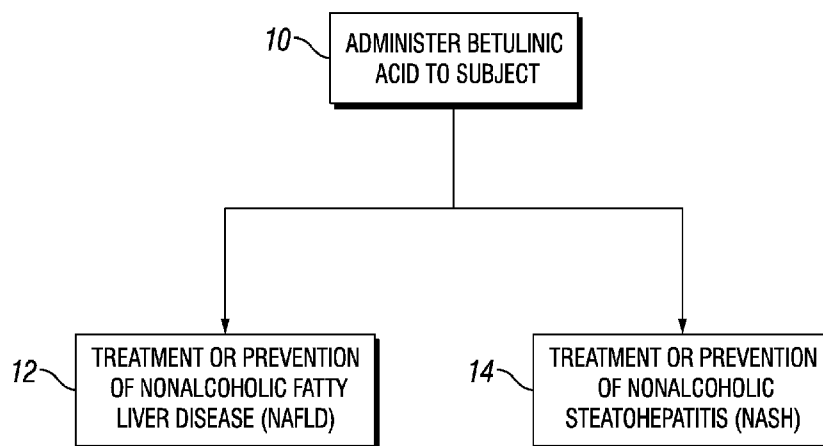


FIG. 1

(57) Abstract: Embodiments of the present disclosure pertain to methods of treating or preventing nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) in a subject by administering a therapeutic composition that includes betulinic acid to the subject. The betulinic acid in the therapeutic composition may be in crystalline or encased form. The subject to be treated may be a human being, such as a human being suffering from at least one of NASH or NAFLD. Additional embodiments of the present disclosure pertain to the aforementioned therapeutic compositions.

TITLE

USE OF BETULINIC ACID FOR THE TREATMENT OR PREVENTION OF NONALCOHOLIC STEATOHEPATITIS AND NONALCOHOLIC FATTY LIVER DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/365,229, filed on July 21, 2016. The entirety of the aforementioned application is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. 3092-51000-059-01S, awarded by the U.S. Department of Agriculture. The government has certain rights in the invention.

BACKGROUND

[0003] Current methods of treating nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD) through controlling body weight have numerous limitations. Notably no drugs are currently approved by the FDA for the treatment of NASH. Various embodiments of the present disclosure address the aforementioned limitations.

SUMMARY

[0004] In some embodiments, the present disclosure pertains to therapeutic compositions for treating or preventing nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) in a subject. In some embodiments, the therapeutic composition includes betulinic acid. In some embodiments, the betulinic acid is in crystalline or encased (e.g., nanoparticle-encased) form.

[0005] In some embodiments, the present disclosure pertains to methods of treating or preventing NAFLD or NASH in a subject by administering the therapeutic compositions of the present disclosure to the subject. In some embodiments, the administration occurs by intestine-specific (or restricted) pharmacological delivery approaches. In some embodiments, the subject

to be treated is a human being. In some embodiments, the subject is suffering from at least one of NASH or NAFLD.

[0006] The administration of the therapeutic compositions of the present disclosure to a subject can treat or prevent NAFLD or NASH through various mechanisms. For instance, in some embodiments, the administration of the therapeutic compositions induces a physiological or pharmacological effect in the subject. In some embodiments, the physiological or pharmacological effect includes, without limitation, anti-diabetic effects, improvement of insulin resistance, increase in insulin sensitivity, improvement of glycemic control, increase in glucose tolerance, reduction of blood glucose levels, reduction of endogenous glucose production (EGP), promotion of body weight loss, promotion of energy homeostasis, suppression of food intake, reduction of nutrient absorption by the gastrointestinal tract, enhancement of basal energy metabolism, increase in energy expenditure (heat production), reduction of respiratory exchange ratio, increase in utilization (β -oxidation) of body fats, reduction of body fat mass, reduction of liver fat accumulation, reduction of hepatic inflammation, and combinations thereof.

FIGURES

[0007] **FIGURE 1** illustrates a method of treating or preventing nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) in a subject.

[0008] **FIGURE 2** provides data and images relating to a new platform to identify potent secretagogues on enteroendocrine L cells in the mouse mini-guts.

[0009] **FIGURE 3** provides data indicating that identified secretagogues stimulate glucagon-like peptide 1 (GLP-1) release from the mouse mini-guts.

[0010] **FIGURE 4** provides data indicating that an oral form of crystalline betulinic acid (B5022 or BA5022) acutely stimulates secretion of gut hormones in the high-fat diet (HFD)-induced obese mouse model.

[0011] **FIGURE 5** provides data indicating that oral BA5022 showed anti-diabetic effects in a HFD-induced obese mouse model.

[0012] **FIGURE 6** provides data indicating that BA5022 showed anti-obesity effects in a HFD-induced obese mouse model.

[0013] **FIGURE 7** provides data indicating that BA5022 promotes energy homeostasis in the HFD-induced obese mouse model.

[0014] **FIGURE 8** provides images and data indicating that BA5022 ameliorates hepatic steatosis and inflammation in a manner that depends on the receptors for GLP-1 (GLP-1R) and GLP-2 (GLP-2R).

[0015] **FIGURE 9** provides data indicating that BA5022 improves glucose homeostasis in a GLP-1R/GLP-2R-dependent manner.

[0016] **FIGURE 10** provides data indicating that BA5022 reduces body weight and fat mass in a GLP-1R/GLP-2R-dependent manner.

[0017] **FIGURE 11** provides images and data indicating that BA5022 inhibits cell proliferation in the mouse mini-guts and HFD-induced obese mouse model.

[0018] **FIGURE 12** provides data indicating that BA5022 mimics vertical sleeve gastrectomy (VSG)-induced glucose tolerance in the HFD-induced obese mouse model.

DETAILED DESCRIPTION

[0019] It is to be understood that both the foregoing general description and the following detailed description are illustrative and explanatory, and are not restrictive of the subject matter, as claimed. In this application, the use of the singular includes the plural, the word “a” or “an” means “at least one”, and the use of “or” means “and/or”, unless specifically stated otherwise. Furthermore, the use of the term “including”, as well as other forms, such as “includes” and “included”, is not limiting. Also, terms such as “element” or “component” encompass both elements or components comprising one unit and elements or components that comprise more than one unit unless specifically stated otherwise.

[0020] The section headings used herein are for organizational purposes and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated herein by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar materials defines

a term in a manner that contradicts the definition of that term in this application, this application controls.

[0021] Non-alcoholic fatty liver disease (NAFLD) is recognized as the hepatic manifestation of metabolic syndrome and present in 50–60% patients of type 2 diabetes (T2D). NAFLD affects approximately 20% of Americans. In addition, NAFLD is associated with fat accumulation in the liver, but with no inflammation or liver damage.

[0022] Approximately 20% of patients with NAFLD develop and progress to hepatic inflammation and fibrosis (namely nonalcoholic steatohepatitis or NASH). Such progression provides for an increased risk for the development of cirrhosis, liver cancer, and liver failure-related death.

[0023] NASH affects approximately 2-5% of Americans. As the prevalence of NASH continues to rise, it threatens to become a global epidemic.

[0024] While the underlying reasons for NASH are not known, a multi-factorial process is accounted for the development and progression of NASH. Such factors include insulin resistance, T2D, metabolic syndrome, metabolic inflammation, oxidative stress, impaired glucose and lipid metabolism, visceral adiposity, dyslipidemia, and gut microbial dysbiosis.

[0025] Both NASH and NAFLD are becoming more common, possibly because of the prevalence of obesity. However, treatment options for NAFLD and NASH remain limited. In fact, there are no specific therapies currently available for NASH. Thus, a need exists for improved methods of treating NASH and NAFLD.

[0026] In some embodiments, the present disclosure pertains to therapeutic compositions for treating or preventing NAFLD or NASH in a subject. In some embodiments, the therapeutic composition includes betulinic acid. In additional embodiments illustrated in **FIG. 1**, the present disclosure pertains to methods of treating or preventing NAFLD or NASH in a subject by administering the aforementioned therapeutic composition to the subject (step 10). In some embodiments, the administering results in the treatment or prevention of NAFLD (step 12). In some embodiments, the administering results in the treatment or prevention of NASH (step 14).

[0027] As set forth in more detail herein, the methods and therapeutic compositions of the present disclosure can have various embodiments. For instance, various methods may be utilized to administer the therapeutic compositions of the present disclosure to various subjects in order to treat or prevent NAFLD or NASH.

[0028] Therapeutic compositions

[0029] Therapeutic compositions generally refer to compositions that include betulinic acid. Betulinic acid may be in various forms in the therapeutic compositions of the present disclosure. For instance, in some embodiments, betulinic acid can be in a crystalline form. In some embodiments, betulinic acid can be in an encased form (e.g., a nanoparticle-encased form).

[0030] The therapeutic compositions of the present disclosure may also be in various states. For instance, in some embodiments, the therapeutic compositions of the present disclosure may be in the form of a liquid, a solid, a gas, and combinations thereof. In some embodiments, the therapeutic compositions of the present disclosure may be in the form of a liquid, such as a syrup. In some embodiments, the therapeutic compositions of the present disclosure may be in the form of a solid, such as a pill.

[0031] The therapeutic compositions of the present disclosure may also include components in addition to betulinic acid. For instance, in some embodiments, the therapeutic compositions of the present disclosure can also include other active agents. In some embodiments, the other active agents can include, without limitation, peptides, hormones, betulinic acid derivatives, bile acids, bile acid derivatives, activators of bile acid membrane receptor (TGR5), agonists against glucagon-like peptide 1 (GLP-1) receptor (GLP-1R), agonists against glucagon-like peptide 2 (GLP-2) receptor (GLP-2R), chemicals, and combinations thereof.

[0032] In some embodiments, the therapeutic compositions of the present disclosure also include a carrier. In some embodiments, the carrier includes, without limitation, carbon-based nanomaterials, liposomes, polymers, micelles, microspheres, nanostructures, dendrimers, and combinations thereof.

[0033] In some embodiments, the therapeutic compositions of the present disclosure also include formulation materials for modifying, maintaining, or preserving various parameters (e.g., pH,

osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution, rate of release, rate of adsorption, rate of penetration, and combinations thereof). In some embodiments, suitable formulation materials can include, without limitation, amino acids (e.g., glycine), antimicrobials, antioxidants (e.g., ascorbic acid), buffers (e.g., Tris-HCl), bulking agents (e.g., mannitol and glycine), chelating agents (e.g., EDTA), complexing agents (e.g., hydroxypropyl-beta-cyclodextrin), and combinations thereof.

[0034] Administration of therapeutic compositions

[0035] Various methods may be utilized to administer the therapeutic compositions of the present disclosure to subjects. For instance, in some embodiments, the administration occurs by methods that include, without limitation, oral administration, inhalation, subcutaneous administration, intravenous administration, intraperitoneal administration, intramuscular administration, intrathecal injection, topical administration, central administration, peripheral administration, intestine-specific administration, intestine-restricted administration, and combinations thereof. In some embodiments, the administration occurs by intravenous administration. In some embodiments, the administration occurs by central administration. In some embodiments, the administration occurs by peripheral administration. In some embodiments, the administration occurs by oral administration.

[0036] In some embodiments, the administration of betulinic acid (e.g., in encased form, such as in nanoparticle-encased form) is specifically targeted to the intestine. In some embodiments, the administration of betulinic acid is realized through intestine-specific delivery approaches.

[0037] Subjects

[0038] The methods and therapeutic compositions of the present disclosure may be utilized in various subjects. For instance, in some embodiments, the subject is a human being. In some embodiments, the subject has been selected for or undergone bariatric surgery, such as roux-en-Y gastric bypass (RYGB) or vertical sleeve gastrectomy (VSG). In some embodiments, the subject is a non-human animal. In some embodiments, the non-human animal includes, without limitation, mice, rats, rodents, mammals, cats, dogs, monkeys, pigs, cattle and horses.

[0039] In some embodiments, the subject to be treated is suffering from NAFLD. In some embodiments, the subject to be treated is suffering from NASH. In some embodiments, the subject to be treated is suffering from NAFLD and NASH.

[0040] Treatment or prevention of NAFLD and NASH

[0041] The methods and therapeutic compositions of the present disclosure may be utilized to treat or prevent NASH, NAFLD, and combinations thereof. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat at least one of NASH or NAFLD in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to prevent at least one of NASH or NAFLD in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat and prevent at least one of NASH or NAFLD in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat or prevent NASH in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat or prevent NAFLD in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat and prevent NASH in a subject. In some embodiments, the methods and therapeutic compositions of the present disclosure may be utilized to treat and prevent NAFLD in a subject.

[0042] Without being bound by theory, the methods and therapeutic compositions of the present disclosure may treat or prevent NAFLD or NASH by various mechanisms. For instance, in some embodiments, the administration of the therapeutic compositions of the present disclosure can treat or prevent NAFLD or NASH by inducing various physiological or pharmacological effects in subjects. Such physiological or pharmacological effects can include, without limitation, anti-diabetic effects, improvement of insulin resistance, increase in insulin sensitivity, improvement of glycemic control, increase in glucose tolerance, reduction of blood glucose levels, reduction of endogenous glucose production (EGP), promotion of body weight loss, promotion of energy homeostasis, suppression of food intake, reduction of nutrient absorption by the gastrointestinal tract, enhancement of basal energy metabolism, increase in energy expenditure (heat production), reduction of respiratory exchange ratio, increase in utilization (β -oxidation) of body fats,

reduction of body fat mass, reduction of liver fat accumulation, reduction of hepatic inflammation, and combinations thereof.

[0043] In some embodiments, the administration of the therapeutic compositions of the present disclosure can treat or prevent NAFLD or NASH by inducing various cellular effects in subjects. Such cellular effects can include, without limitation, an increase in intracellular calcium (Ca^{2+}) on enteroendocrine cells, stimulation of gut hormone (e.g., GLP-1/2) secretion, reduction of cell proliferation in the intestine, and combinations thereof.

[0044] In some embodiments, the administration of the therapeutic compositions of the present disclosure can treat or prevent NAFLD or NASH by inducing gut hormone secretion in subjects. In some embodiments, the secreted gut hormones can include, without limitation, glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), peptide YY (PYY), gastric inhibitory polypeptide (GIP), and combinations thereof.

[0045] In some embodiments, the therapeutic compositions of the present disclosure treat or prevent NAFLD or NASH by activating the receptors for GLP-1 (GLP-1R) and GLP-2 (GLP-2R). As such, in some embodiments, the therapeutic compositions of the present disclosure treat or prevent NAFLD or NASH in a GLP-1R/GLP-2R-dependent manner.

[0046] In more specific embodiments, the therapeutic compositions of the present disclosure treat or prevent NAFLD or NASH by activating bile acid membrane receptors (e.g., TGR5, a G-protein coupled receptor) on enteroendocrine L cells. Thereafter, TGR5 activation stimulates the secretion of GLP-1 and GLP-2, which in turn activates GLP-1R and GLP-2R.

[0047] **Additional Embodiments**

[0048] Reference will now be made to more specific embodiments of the present disclosure and experimental results that provide support for such embodiments. However, Applicants note that the disclosure herein is for illustrative purposes only and is not intended to limit the scope of the claimed subject matter in any way.

[0049] **Example 1. Metabolic Effects of Betulinic Acid in Mice**

[0050] In this Example, Applicants have demonstrated that an optimal crystalline form of betulinic acid (i.e., one particular polymorph referred to herein as BA5022 or B5022) ameliorates

hepatic steatosis and inflammation of high fat diet (HFD)-induced obese mice in a GLP-1R/GLP-2R-dependent manner; improves obesity-associated metabolic syndrome, insulin resistance, and glucose intolerance; and promotes body weight loss. Applicants also demonstrate in this Example that betulinic acid improves insulin resistance and glycemic control; promotes body weight loss by suppressing food intake; reduces body fat accumulation by enhancing basal energy metabolism; and resolves liver inflammation completely.

[0051] Example 1.1. Design of a new platform to identify secretagogues on enteroendocrine L cells in mouse mini-guts

[0052] As shown in **FIG. 2A**, Enteroendocrine L cells were labeled in glucagon-Cre-mediated green fluorescence in the Cre reporter Rosa26^{mT/mG} mouse. The Rosa26^{mT/mG} is a cell membrane-targeted, two-color fluorescent Cre reporter allele, where membrane-localized red fluorescence is expressed in cells prior to Cre recombinase exposure, and membrane-localized green fluorescence is expressed in Cre recombinase expressing cells and derived cell lineages.

[0053] **FIG. 2B** shows a mouse mini-guts model (of crypt-villus-like, polarized-monolayer intestinal epithelium) after H&E staining. **FIG. 2C** shows enteroendocrine L cells (in green fluorescence) that are *in situ* scattered in polarized epithelial monolayer of cultured mouse mini-guts.

[0054] Applicants also assessed L cell calcium transients in response to potential activators by high throughput Ca²⁺ imaging on L cells scattered in the mouse mini-guts. In brief, Applicants isolated the mouse jejunal crypts and cultured them in a 3D Matrigel support with defined growth factors. After about 4 days of culture, newly formed villous-crypt enteroids (named mini-guts) were harvested, passaged, and seeded onto glass coverslips for 24-48 hours. L cells (identified by glucagon-Cre-mediated GFP) on the coverslips were monitored for imaging intracellular [Ca²⁺] transients in response to bioactive compounds after loading with Fura-2 (a fluorescent Ca²⁺ indicator).

[0055] **FIG. 2D** shows a Ca²⁺ imaging on single L cells after loading Ca²⁺ indicator Fura-2 in cultured mouse mini-guts consisting of crypt-villus like epithelial cells. **FIG. 2E** shows selective

cellular Ca^{2+} influx in endocrine L cells in the mini-guts, as measured by ratiometric Ca^{2+} excitability.

[0056] FIG. 2F shows that intracellular $[\text{Ca}^{2+}]$ in L cells niched in the mini-guts increased in response to a bioactive compound. In particular, BA5022 showed a potent stimulation (at 2.5 μM) to increase intracellular $[\text{Ca}^{2+}]$ in L cells in the intestinal epithelium under physiologically-relevant conditions. ** indicate $P < 0.01$.

[0057] Example 1.2. Identified secretagogues stimulate GLP-1 release from the mouse mini-guts

[0058] To identify selective and potent secretagogues on primary L cells, the mouse mini-guts were used for screening potent secretagogues. One group of pentacyclic triterpenoids with the same molecular weight and composition (putative TGR5 agonists) were identified to stimulate GLP-1 secretion. Peptide concentrations in the medium and cells were determined with multiplex immunoassays. Potent secretagogues on L-cell-specific peptides were identified using cultured mouse enteroids (or mini-guts) under physiologically relevant settings. The medium and cells were collected in the mini-guts treated with pentacyclic triterpenoids for 30 minutes. BA5022 was discovered as the most potent compound. * and ** indicate $P < 0.05$ and 0.01, respectively.

[0059] FIG. 3A shows the GLP-1 concentrations in the medium. **FIG. 3B** shows the GLP-1 release defined as the percentage of the GLP-1 in medium over the GLP-1 in both medium and cells.

[0060] Example 1.3. Oral BA5022 acutely stimulates secretion of gut hormones in mice

[0061] Mice were pair-fed for 4 weeks with high-fat diet (HFD) and administered orally with BA5022 (also referred to as B5022) or oil. As summarized in **FIG. 4**, circulating concentrations of gut satiety hormones (GLP-1, PYY, and GIP) were increased after 30 minutes of administration (**FIGS. 4A-B and D**). In contrast, blood insulin concentration was not increased (**FIG. 4C**). The decreased basal level of insulin suggest increased insulin sensitivity after a 4-week treatment of BA5022. The decreased basal level of insulin also suggests little incretin effect without oral intake of glucose at that moment. * and ** indicate $P < 0.05$ and 0.01, respectively.

[0062] Example 1.4. Oral BA5022 showed anti-diabetic effects in the HFD-induced obese mouse model

[0063] As summarized in **FIG. 5**, oral BA5022 showed anti-diabetic effects in the HFD-induced obese mouse model. As shown in **FIG. 5A**, fasting glucose levels after 4-hour and 18 -hour fasts are reduced in HFD-induced obese mice after dietary supplementation of BA5022, respectively, for 8 and 12 weeks. However, the other compounds in the same group of pentacyclic triterpenoids with the same molecular weight and chemical composition showed no effects on basal glucose.

[0064] As shown in **FIG. 5B**, oral administration of BA5022 for one week acutely increases glucose tolerance in HFD-induced obese mice after ip glucose tolerance challenge. However, the other compounds in the same group of pentacyclic triterpenoids with the same molecular weight and chemical composition showed no effects on glucose tolerance.

[0065] As shown in **FIG. 5C**, basal endogenous glucose production (EGP) is reduced in mice pair-fed for 4 weeks with the HFD supplemented with BA5022, which indicates hepatic insulin sensitivity is increased. As shown in **FIG. 5D**, insulin sensitivity is increased in mice pair-fed for 4 weeks with the HFD supplemented with BA5022. Insulin sensitivity was assessed using stable isotopic glucose tracers coupled with hyperinsulinemic euglycemic clamp. Note that whole-body insulin sensitivity (indicated by glucose infusion rate, GIR) and peripheral glucose utilization (indicated by insulin-stimulated disappearance of glucose, Rd) are increased. * and ** indicate $P < 0.05$ and 0.01 , respectively.

[0066] Example 1.5. BA5022 showed anti-obesity effects in the HFD-induced obese mouse model

[0067] As summarized in **FIG. 6**, BA5022 showed anti-obesity effects in the HFD-induced obese mouse model. **FIG. 6A** shows the general structure of BA5022 (betulinic acid). **FIG. 6B** shows that the body weight is reduced after 8- and 12-week supplementation of BA5022 in the HFD. ** indicates $P < 0.01$. **FIG. 6C** shows that body fat mass is reduced after 5, 8, and 12 week supplementation of BA5022 in the HFD. * and ** indicate $P < 0.05$ and 0.01 , respectively. **FIG. 6D** shows that, in contrast to the aforementioned results, body lean mass is increased after

5 and 8 week supplementation of BA5022 in the HFD. * and ** indicate $P < 0.05$ and 0.01 , respectively.

[0068] Body composition was assessed by quantitative magnetic resonance imaging (qMRI). Other compounds in the same group of pentacyclic triterpenoids with the same molecular weight and chemical composition showed no anti-obesity effects. Instead, dietary supplementation of UA1062 increased body weight and body fat mass.

[0069] Example 1.6. BA5022 promotes energy homeostasis in the HFD-induced obese mouse model

[0070] As summarized in **FIG. 7**, BA5022 promotes energy homeostasis in the HFD-induced obese mouse model. **FIG. 7A** shows that heat production (i.e., energy expenditure) during the light period (basal state) is enhanced in HFD-pair fed mice after dietary supplementation of BA5022. **FIG. 7B** shows that respiratory exchange ratio is reduced in HFD-pair fed mice after dietary supplementation of BA5022, indicating increased utilization (β -oxidation) of body fats. **FIG. 7C** shows that basal metabolic rate (i.e., basal energy expenditure) during the light period is enhanced in HFD-pair fed mice after dietary supplementation of BA5022. **FIG. 7D** shows that food intake is reduced in HFD-pair fed mice after dietary supplementation of BA5022.

[0071] Note that mice were pair-fed for 4 weeks with HFD supplemented with BA5022. * and ** indicate $P < 0.05$ and 0.01 , respectively. Indirect calorimetry was performed with a computer-controlled open-circuit calorimetry system (the *Oxymax-CLAMS* system).

[0072] Example 1.7. BA5022 ameliorates hepatic steatosis and inflammation in a GLP-1R/GLP-2R-dependent manner

[0073] As shown in **FIG. 8**, BA5022 ameliorates hepatic steatosis and inflammation in a GLP-1R/GLP-2R-dependent manner. Mice were fed for 12 weeks with HFD supplemented with BA5022. Hepatic steatosis and inflammation were histologically evaluated in the liver samples. Paraffin-embedded liver samples were sectioned for H&E staining.

[0074] **FIG. 8A** shows liver histology from the wild-type mouse fed with the HFD. **FIG. 8B** shows the liver histology from the wild-type mouse fed with the HFD supplemented with BA5022. **FIG. 8C** shows the liver histology from a double-knockout (DKO) mouse (with the

double knockout of GLP-1R and GLP-2R) fed with the HFD. **FIG. 8D** shows liver histology from the DKO mouse fed with the HFD supplemented with BA5022.

[0075] In particular, images were amplified by 20x, showing that dietary supplementation of BA5022 reduced hepatic steatosis, ballooning, and inflammation in the wild-type mice (**FIG. 8B**). This histological resolution of NASH was negated in the double knockout of GLP-1R and GLP-2R (**FIG. 8D**).

[0076] The amount of macrophages in the liver (quantified by cell sorting, an indication of inflammation) was reduced in the wild-type mouse fed with the HFD supplemented with BA5022, compared to that in the wild-type mouse fed with the HFD. However, the amount of macrophages in the liver was not altered in the DKO mouse fed with the HFD supplemented with BA5022, compared to that in the DKO mouse fed with the HFD (Data not shown).

[0077] Therefore, hepatic inflammation is reduced by dietary supplementation of BA5022 in a GLP-1R/GLP-2R-dependent manner. These results suggest that oral BA5022 resolves NASH through the GLP-1R/GLP-2R-dependent pathway.

[0078] Example 1.8. BA5022 improves glucose homeostasis in a GLP-1R/GLP-2R-dependent manner

[0079] As shown in **FIG. 9**, BA5022 improves glucose homeostasis in a GLP-1R/GLP-2R-dependent manner. **FIG. 9A** shows basal glucose levels after an 18-hour fast are reduced in HFD-induced obese mice after dietary supplementation of BA5022, respectively, for 8 and 12 weeks. In contrast, the BA5022-induced hypoglycemic effect was negated in mice with the double knockout of GLP-1R and GLP-2R (DKO).

[0080] **FIG. 9B** shows that oral administration of BA5022 for 8 weeks acutely increases glucose tolerance after an ip glucose tolerance challenge in HFD-induced obese mice. However, this was not the case in HFD-fed DKO mice.

[0081] In particular, basal glucose levels were elevated in HFD-fed DKO mice. ^{abc} indicates $P < 0.05$ and 0.01 between genotypes within the same dietary intervention. * and ** indicate $P < 0.05$ and 0.01 between dietary interventions within the same genotype. These results indicate that

BA5022 improves glycemic control (by decreasing fasting glucose levels and increasing glucose tolerance). ipGTT (ip glucose tolerance test) was performed in mice after a 6 hour fast.

[0082] Example 1.9. BA5022 reduces body weight and fat mass in a GLP-1R/GLP-2R-dependent manner

[0083] As shown in **FIG. 10**, BA5022 reduces body weight and fat mass in a GLP-1R/GLP-2R-dependent manner. **FIG. 10A** shows an 8-week gain of fat mass, lean mass and body weight in HFD-induced obese mice after dietary supplementation of BA5022. Both fat accumulation and body weight are reduced while lean accumulation tended to increase in the wild-type mice. In contrast, BA5022-induced anti-obesity effects were negated in mice with the double knockout of GLP-1R and GLP-2R (DKO). ^{ac} indicates $P < 0.01$ between genotypes within the same dietary intervention. * and ** indicate $P < 0.05$ and 0.01 between dietary interventions within the same genotype.

[0084] **FIG. 10B** shows a 12-week gain of fat mass, lean mass and body weight in HFD-induced obese mice after dietary supplementation of BA5022. Both fat accumulation and body weight are reduced while lean accumulation tended to increase in the wild-type mice. In contrast, BA5022-induced anti-obesity effects were negated in mice with the double knockout of GLP-1R and GLP-2R (DKO). ^{ac} indicates $P < 0.01$ between genotypes within the same dietary intervention. * and ** indicate $P < 0.05$ and 0.01 between dietary interventions within the same genotype.

[0085] Example 1.10. BA5022 inhibits cell proliferation in the mouse mini-guts model

[0086] As shown in **FIG. 11**, BA5022 inhibits cell proliferation in the mouse mini-guts model. **FIG. 11A** shows an image where the mouse mini-guts were treated with ethanol (Control). Cell proliferation was assessed by *in vivo* BrdU labelling. **FIG. 11B** shows an image where the mouse mini-guts were treated with BA5022. Cell proliferation was assessed by *in vivo* BrdU labelling.

[0087] The mouse mini-guts were cultured from isolated ileum crypts of glucagon-Cre-mediated dual florescence Rosa26^{mT/mG} reporter mouse. The mini-guts were cultured in the Matrigel for about 4 days and passaged for 48 hours when treated with vehicle (ethanol) or pentacyclic triterpenoids (at 25 μ M for 48 hours) and labeled *in vivo* with BrdU (for 4 hours). The mini-guts

were fixed in 4% paraformaldehyde solution, embedded in paraffin, and then sectioned for immunostaining. Images were captured and BrdU-positive cells (i.e., proliferative cells) were counted. Each treatment was repeated three times with 3 wells each. These results show that BA5022 reduce cell proliferation in the mouse mini-guts..

[0088] Example 1.11. BA5022 inhibits cell proliferation in HFD-induced obese mouse model

[0089] Dietary supplementation of BA5022 inhibits crypt cell proliferation in the jejunums of mice fed with HFD for 12 weeks. Cell proliferation was assessed by *in vivo* BrdU labelling. Mice were ip injected with BrdU two hours prior to euthanization. The jejunum samples were harvested, fixed in 4% paraformaldehyde solution, embedded in paraffin, and then sectioned for immunostaining. BrdU-positive cells (i.e., proliferative cells) in the crypt were accounted. There were 10 mice per treatment. These results show that BA5022 reduce *in vivo* cell proliferation mice (Data not shown). BA-mediated suppression of cell proliferation in the gut would lead to a reduction of absorption surface, decreasing nutrient uptake by the intestine.

[0090] Example 1.12. BA5022 mimics VSG-induced glucose tolerance in the HFD-induced obese mouse model

[0091] As shown in **FIG. 12**, BA5022 mimics VSG-induced glucose tolerance in the HFD-induced obese mouse model. In particular, glucose tolerance was increased in mice after the VSG procedure. Importantly, VSG-induced glycemic improvement was reproduced in the sham mice after oral administration of BA5022. These results suggest that BA5022 may augment secretion of multiple L hormones (namely GLP-1/2 and PYY), key contributors to metabolic benefits after VSG.

[0092] Without further elaboration, it is believed that one skilled in the art can, using the description herein, utilize the present disclosure to its fullest extent. The embodiments described herein are to be construed as illustrative and not as constraining the remainder of the disclosure in any way whatsoever. While the embodiments have been shown and described, many variations and modifications thereof can be made by one skilled in the art without departing from the spirit and teachings of the invention. Accordingly, the scope of protection is not limited by the description set out above, but is only limited by the claims, including all equivalents of the

subject matter of the claims. The disclosures of all patents, patent applications and publications cited herein are hereby incorporated herein by reference, to the extent that they provide procedural or other details consistent with and supplementary to those set forth herein.

WHAT IS CLAIMED IS:

1. A method of treating or preventing nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) in a subject, said method comprising:

5

administering a therapeutic composition to the subject,
wherein the therapeutic composition comprises betulinic acid.

2. The method of claim 1, wherein the administration occurs by a method selected from the
10 group consisting of oral administration, inhalation, subcutaneous administration, intravenous administration, intraperitoneal administration, intramuscular administration, intrathecal injection, topical administration, central administration, peripheral administration, intestine-specific administration, intestine-restricted administration, and combinations thereof.

15 3. The method of claim 1, wherein the administration occurs by oral administration.

4. The method of claim 1, wherein the administration occurs by intestine-specific or intestine-restricted delivery approaches.

20 5. The method of claim 1, wherein the betulinic acid is in a crystalline form.

6. The method of claim 1, wherein the betulinic acid is in an encased form.

7. The method of claim 6, wherein the betulinic acid is encased in nanoparticles.

25

8. The method of claim 1, wherein the therapeutic composition is in the form of a liquid, a solid, a gas, and combinations thereof.

9. The method of claim 1, wherein the therapeutic composition further comprises a carrier.

10. The method of claim 9, wherein the carrier is selected from the group consisting of carbon-based nanomaterials, liposomes, polymers, micelles, microspheres, nanostructures, dendrimers, and combinations thereof.

5

11. The method of claim 1, wherein the method treats at least one of NASH or NAFLD in the subject.

12. The method of claim 1, wherein the method prevents at least one of NASH or NAFLD in the
10 subject.

13. The method of claim 1, wherein the method treats and prevents at least one of NASH or NAFLD in the subject.

15 14. The method of claim 1, wherein the method is utilized to treat or prevent NASH.

15. The method of claim 1, wherein the method is utilized to treat and prevent NASH.

16. The method of claim 1, wherein the method is utilized to treat or prevent NAFLD.

20

17. The method of claim 1, wherein the method is utilized to treat and prevent NAFLD.

18. The method of claim 1, wherein the subject is a human being.

25 19. The method of claim 1, wherein the subject has undergone bariatric surgery.

20. The method of claim 1, wherein the subject is suffering from at least one of NASH or NAFLD.

21. The method of claim 1, wherein the administering induces a physiological or pharmacological effect in the subject, wherein the physiological or pharmacological effect is selected from the group consisting of anti-diabetic effects, improvement of insulin resistance, increase in insulin sensitivity, improvement of glycemic control, increase in glucose tolerance, reduction of blood glucose levels, reduction of endogenous glucose production (EGP), promotion of body weight loss, promotion of energy homeostasis, suppression of food intake, reduction of nutrient absorption by the gastrointestinal tract, enhancement of basal energy metabolism, increase in energy expenditure (heat production), reduction of respiratory exchange ratio, increase in utilization (β -oxidation) of body fats, reduction of body fat mass, reduction of liver fat accumulation, reduction of hepatic inflammation, and combinations thereof.

22. A therapeutic composition for treating or preventing nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) in a subject, wherein the therapeutic composition comprises betulinic acid.

23. The therapeutic composition of claim 22, wherein the betulinic acid is in a crystalline form.

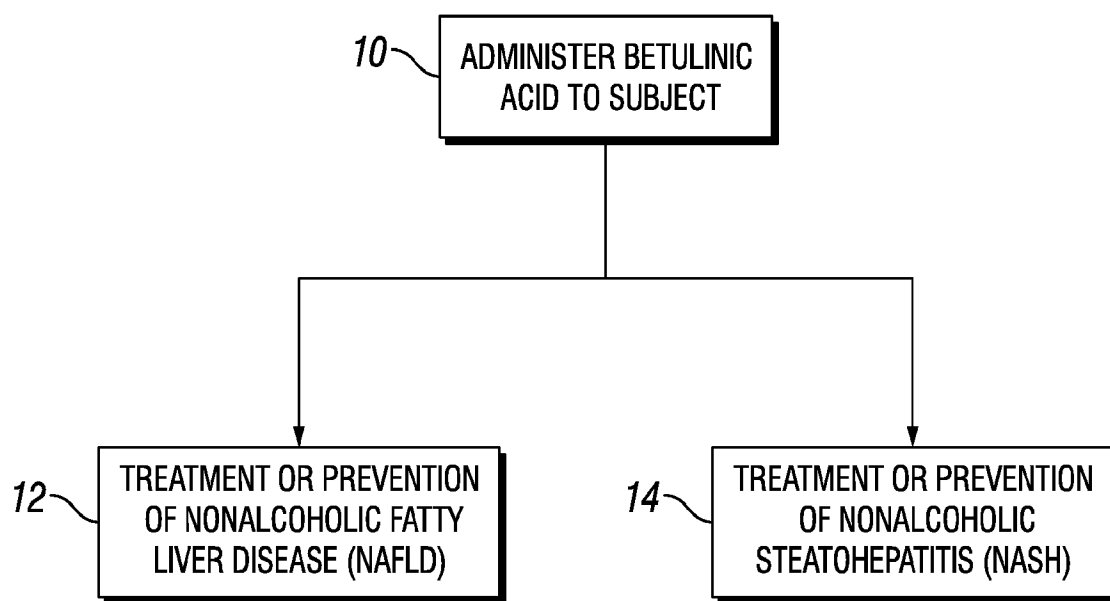
24. The therapeutic composition of claim 22, wherein the betulinic acid is in an encased form.

25. The therapeutic composition of claim 24, wherein the betulinic acid is encased in nanoparticles.

26. The therapeutic composition of claim 22, wherein the therapeutic composition is in the form of a liquid, a solid, a gas, and combinations thereof.

27. The therapeutic composition of claim 22, wherein the therapeutic composition further comprises a carrier.

28. The therapeutic composition of claim 27, wherein the carrier is selected from the group consisting of carbon-based nanomaterials, liposomes, polymers, micelles, microspheres, nanostructures, dendrimers, and combinations thereof.

**FIG. 1**

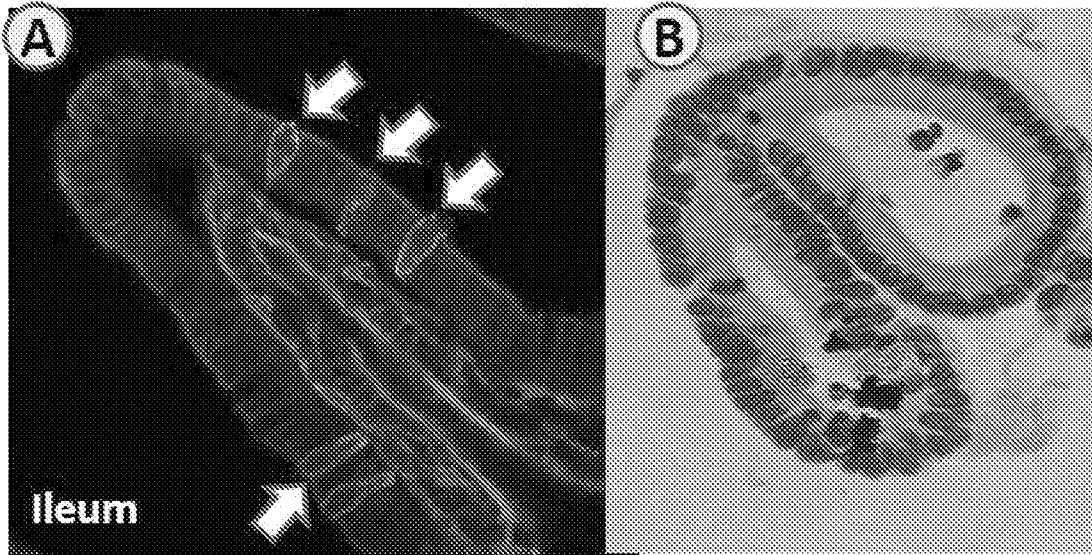


FIG. 2A

FIG. 2B

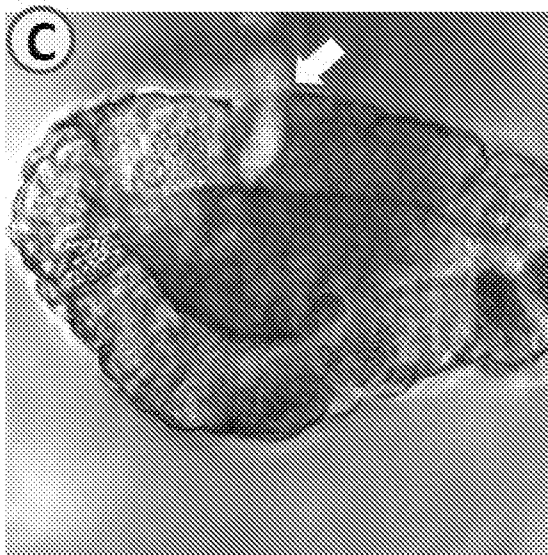


FIG. 2C

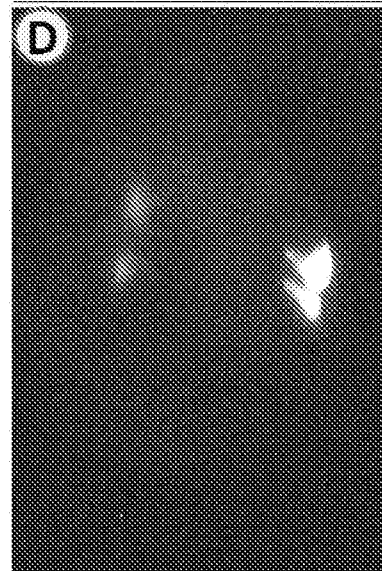


FIG. 2D

FIG. 2

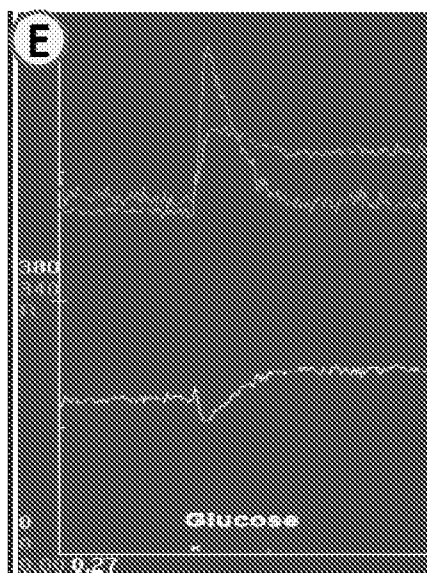


FIG. 2E

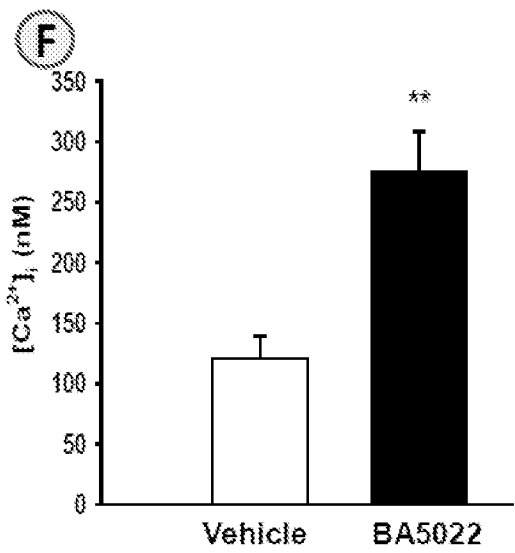
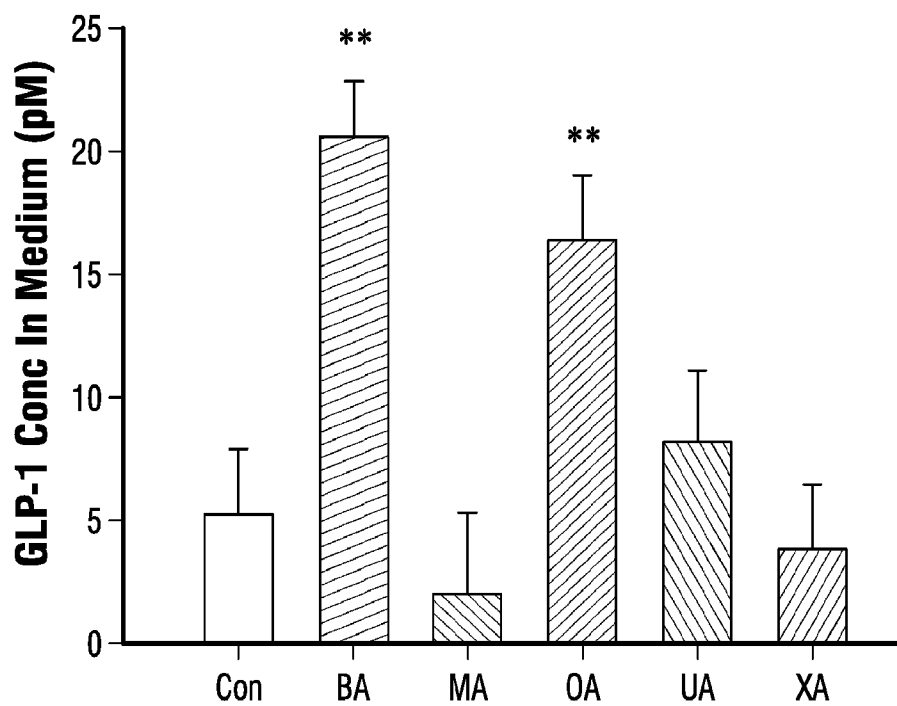
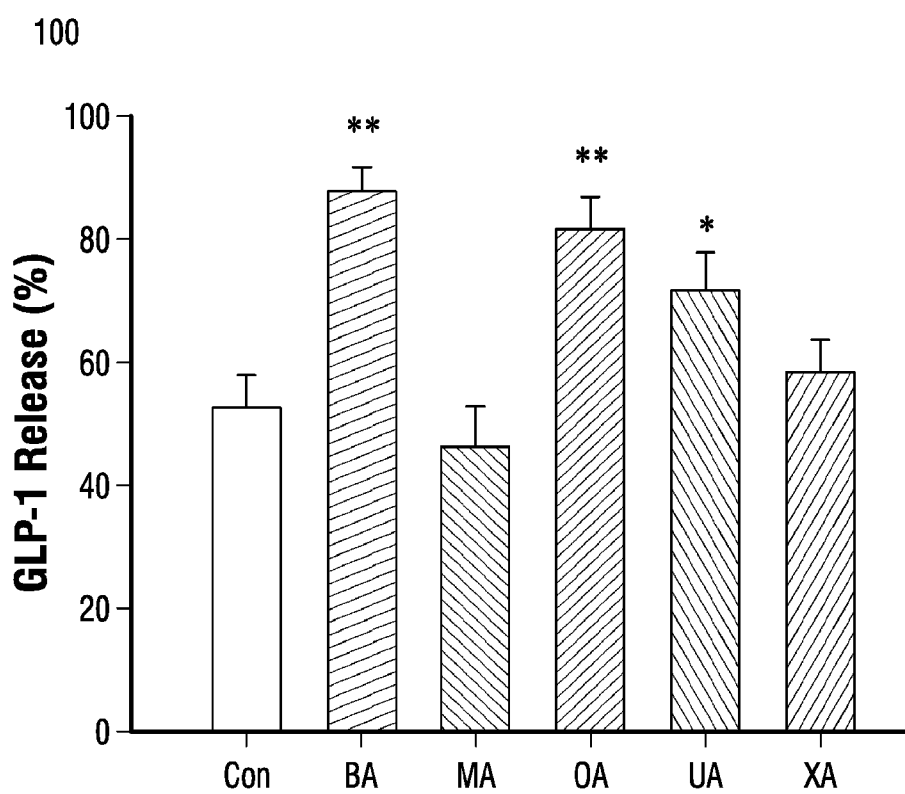
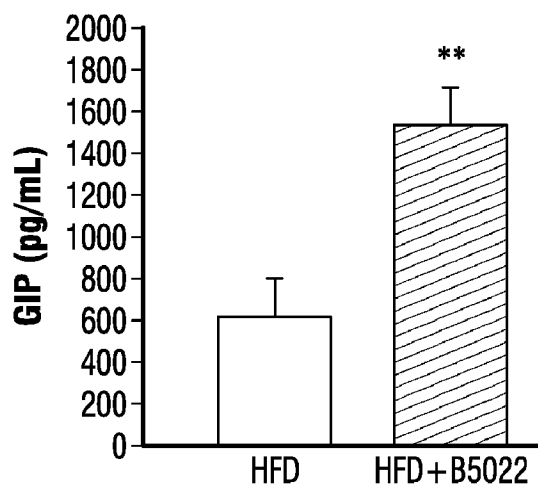
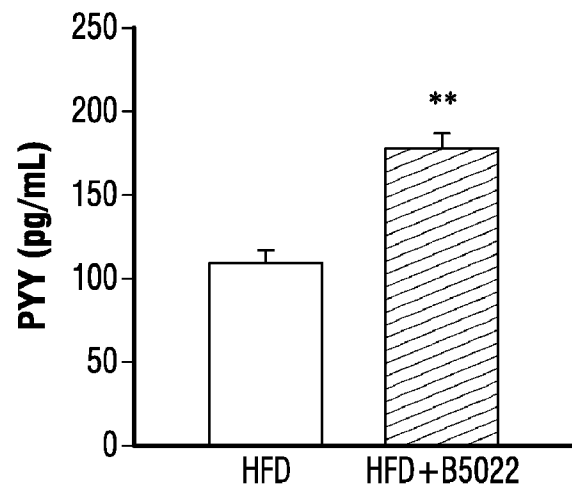
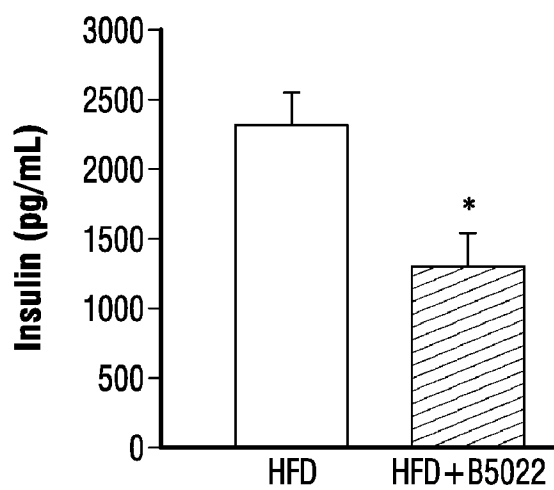
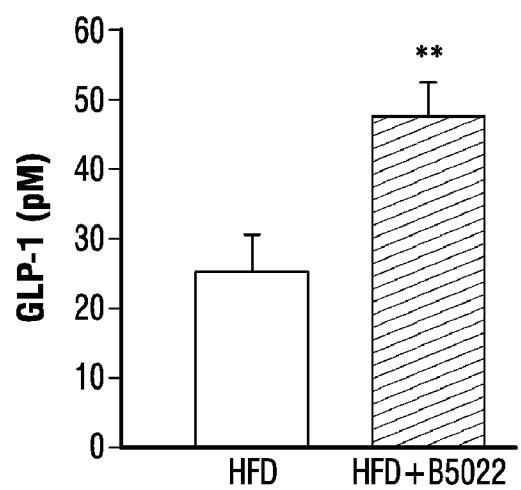
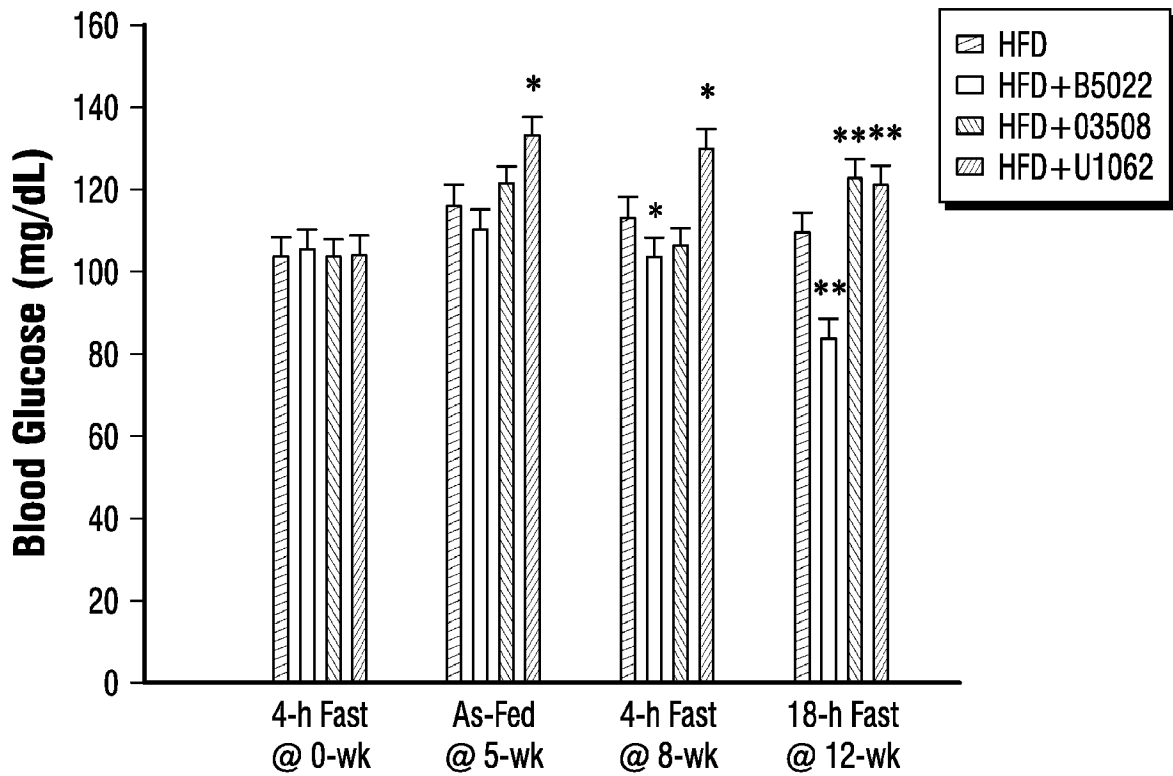
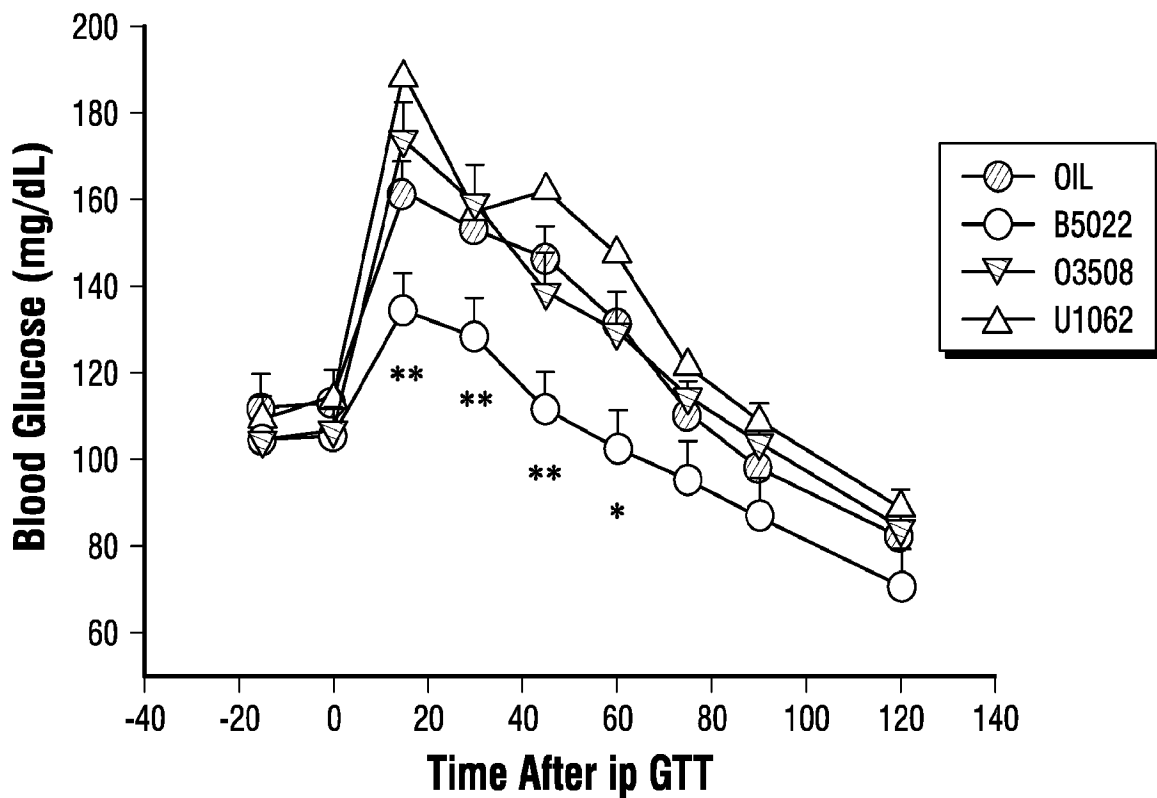


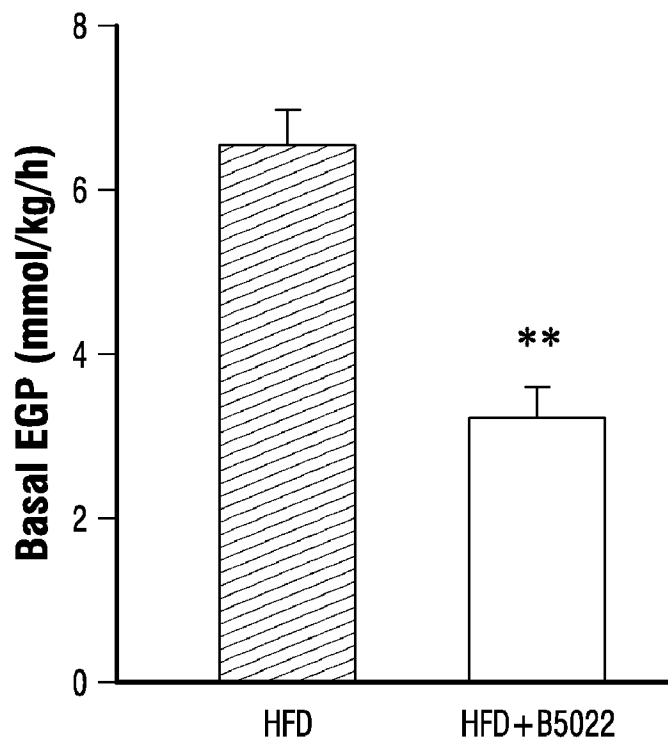
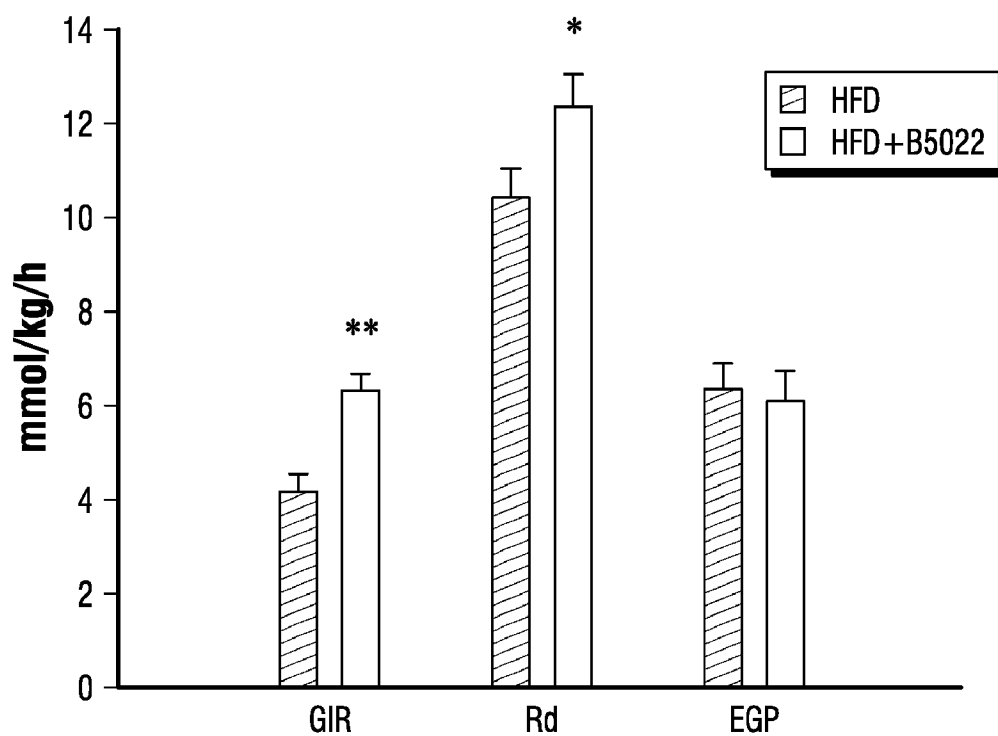
FIG. 2F

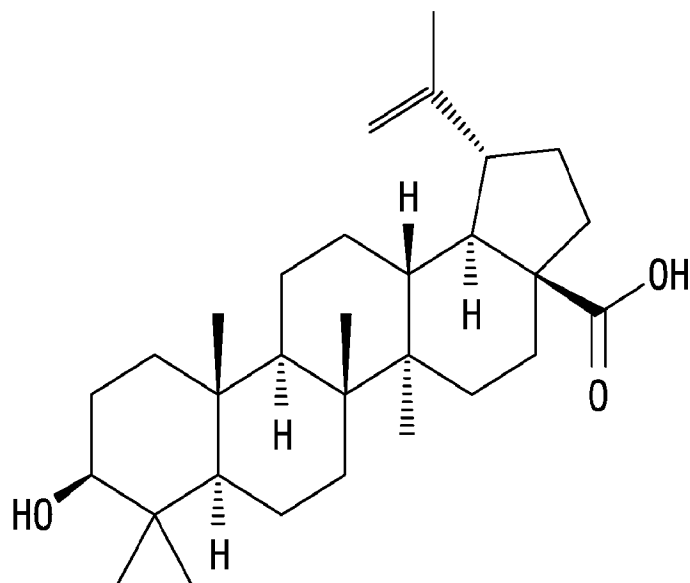
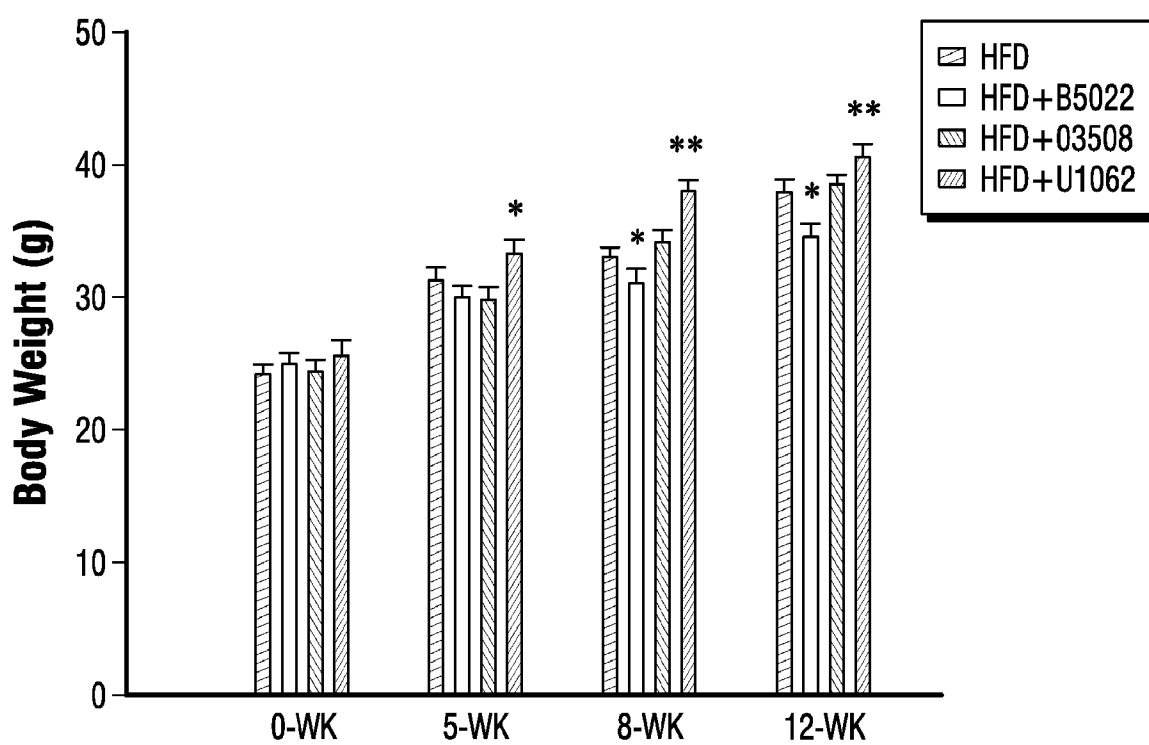
FIG. 2

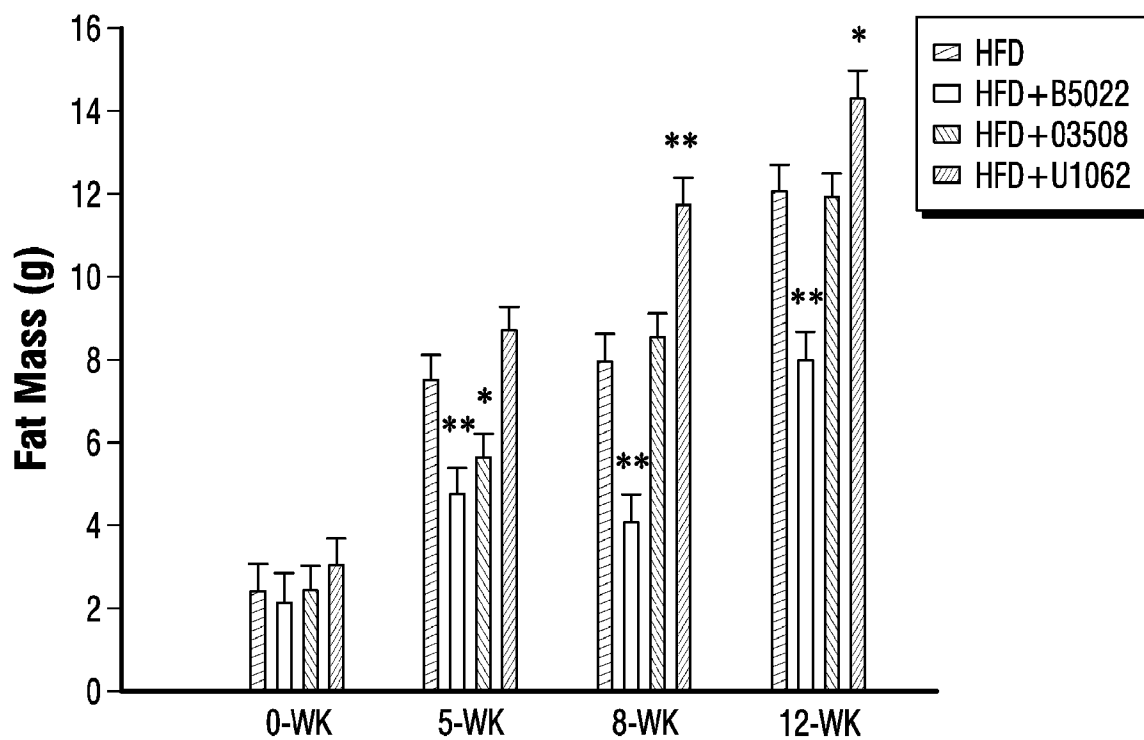
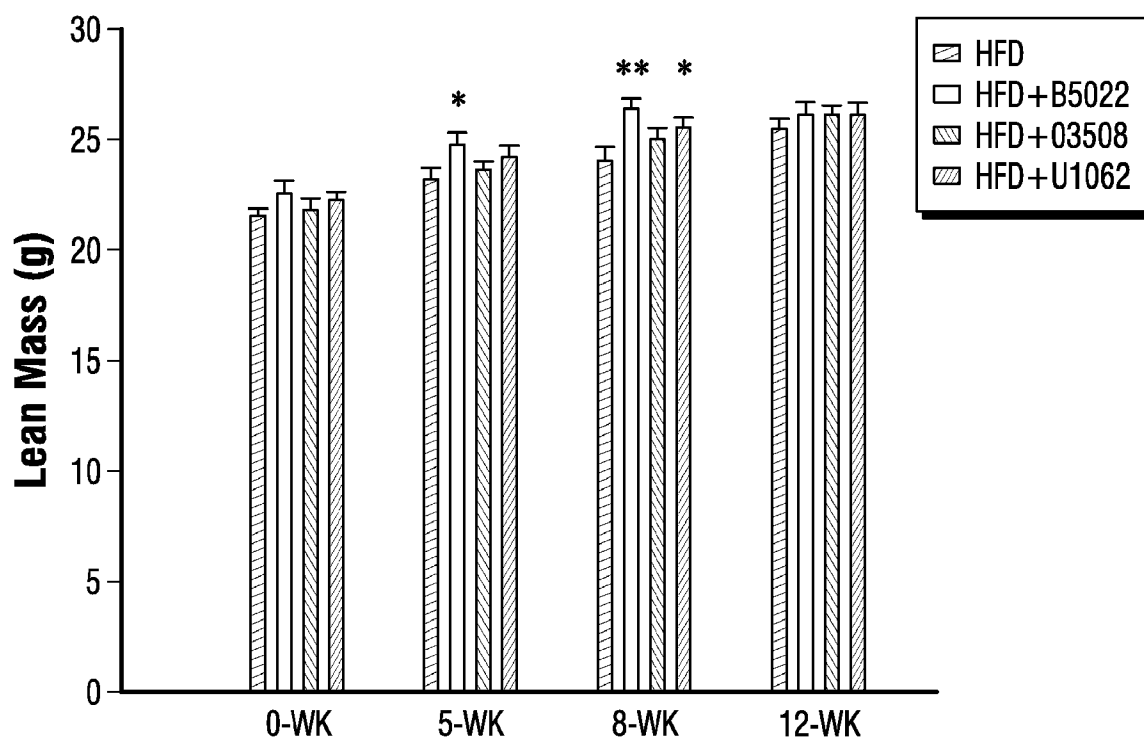
**FIG. 3A****FIG. 3B**

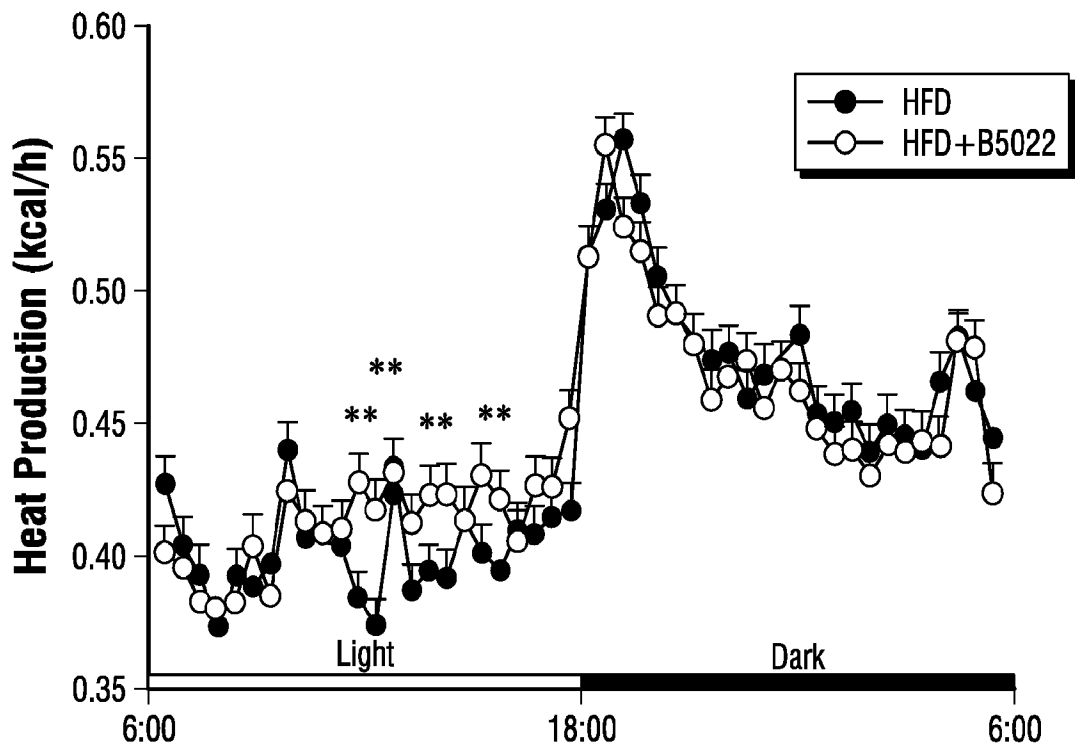
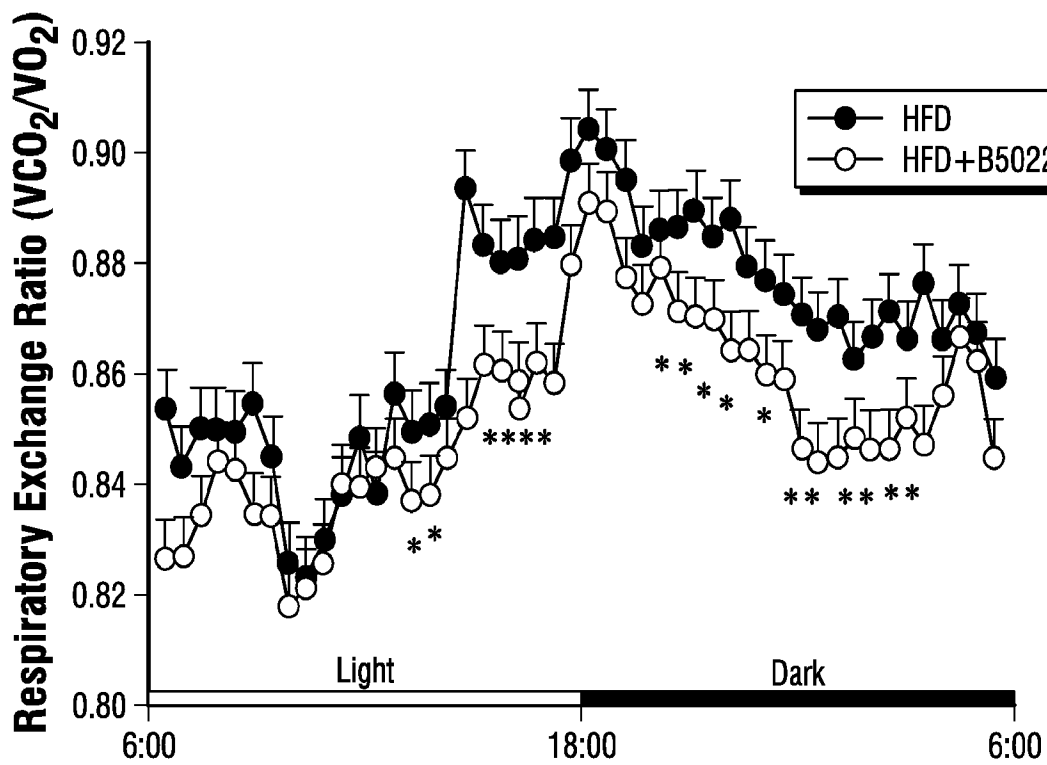
**FIG. 4A****FIG. 4B****FIG. 4C****FIG. 4D**

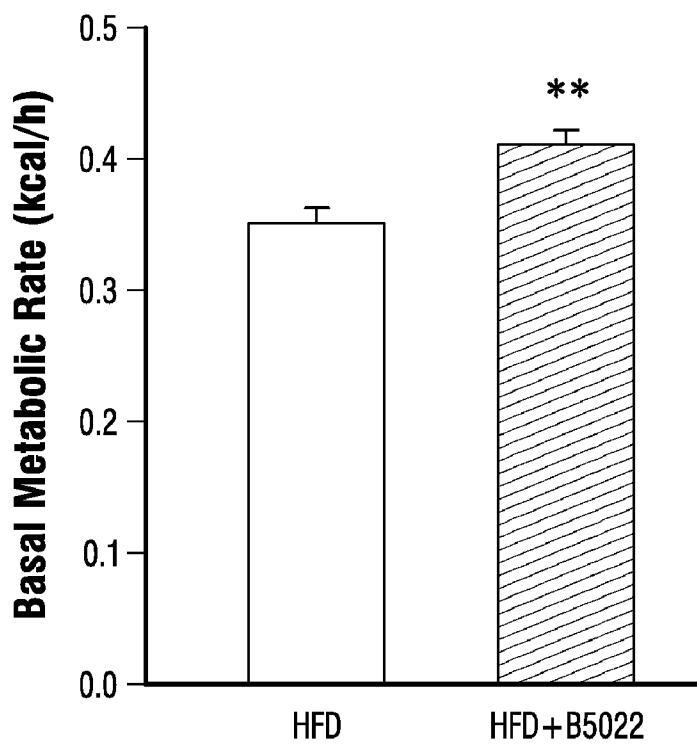
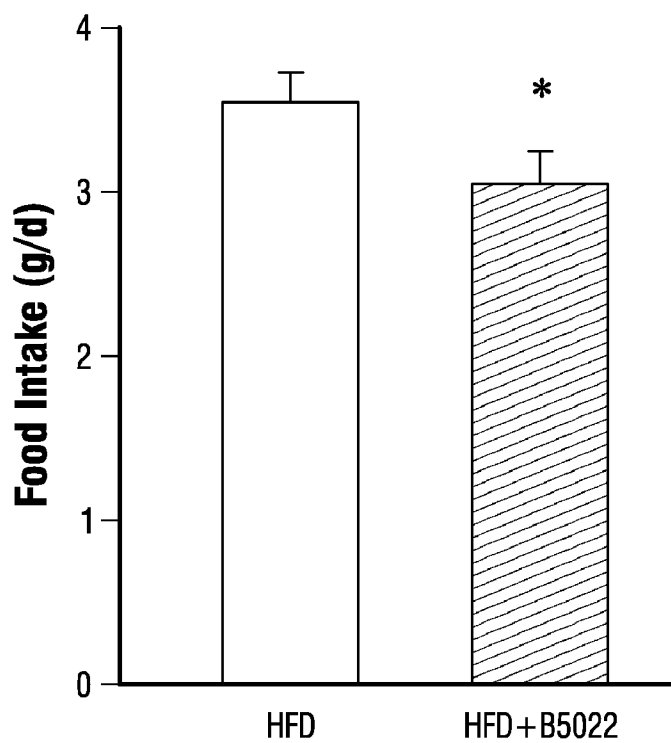
**FIG. 5A****FIG. 5B**

**FIG. 5C****FIG. 5D**

**FIG. 6A****FIG. 6B**

**FIG. 6C****FIG. 6D**

**FIG. 7A****FIG. 7B**

**FIG. 7C****FIG. 7D**

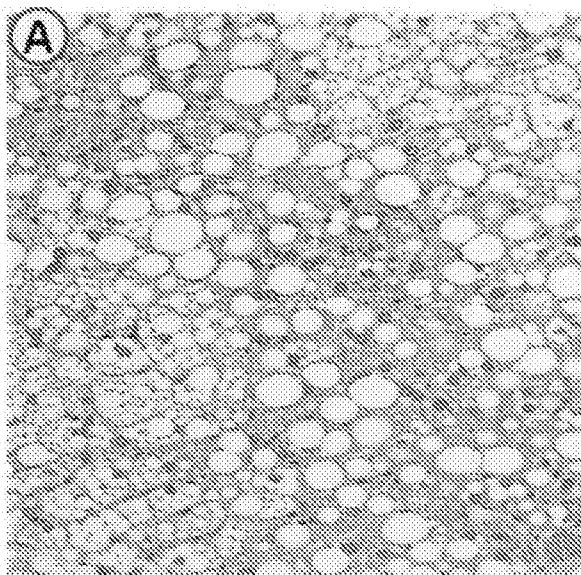


FIG. 8A

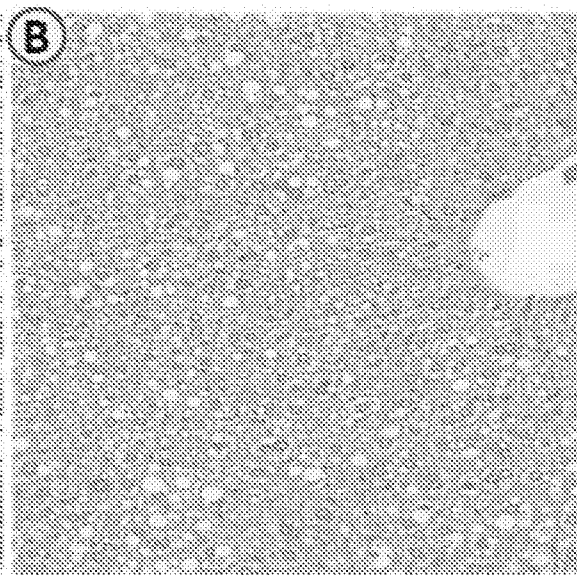


FIG. 8B

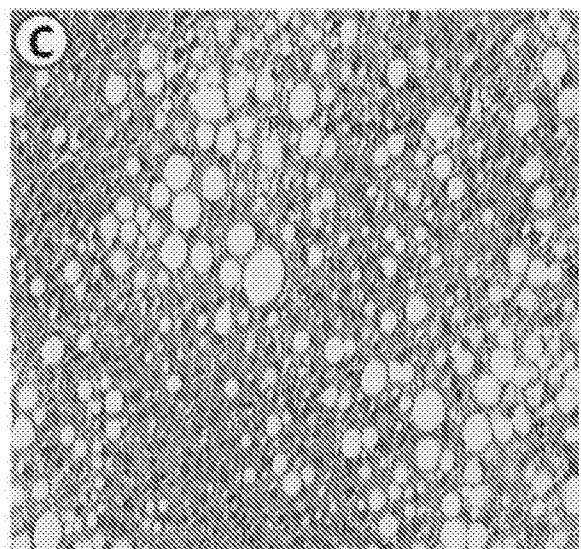


FIG. 8C

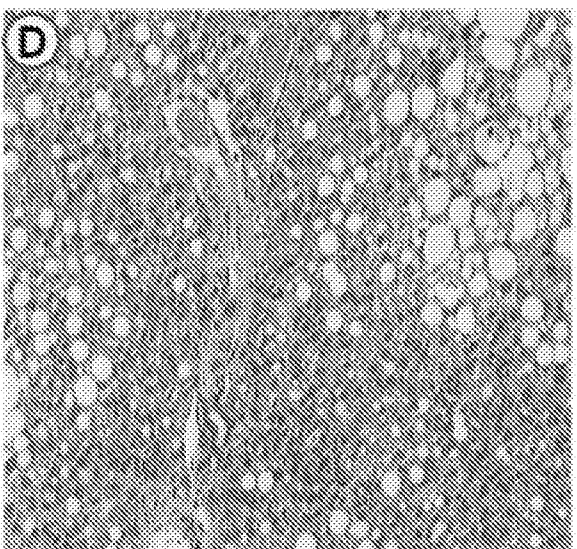
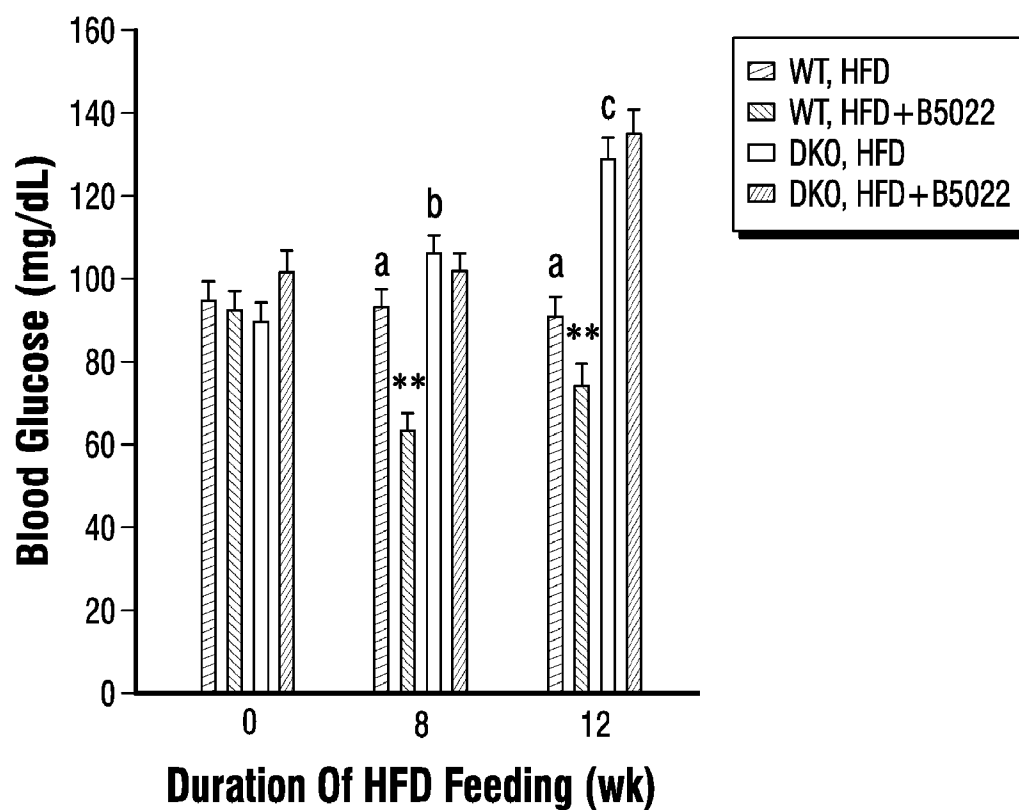
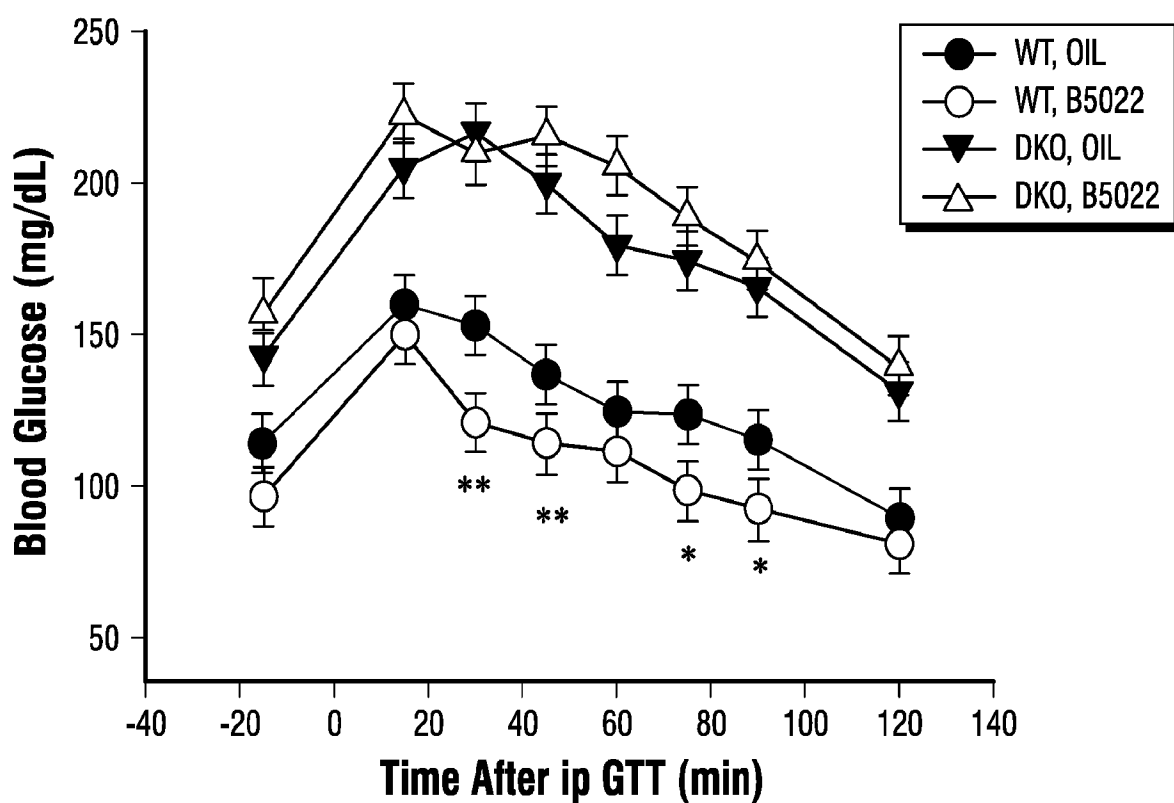
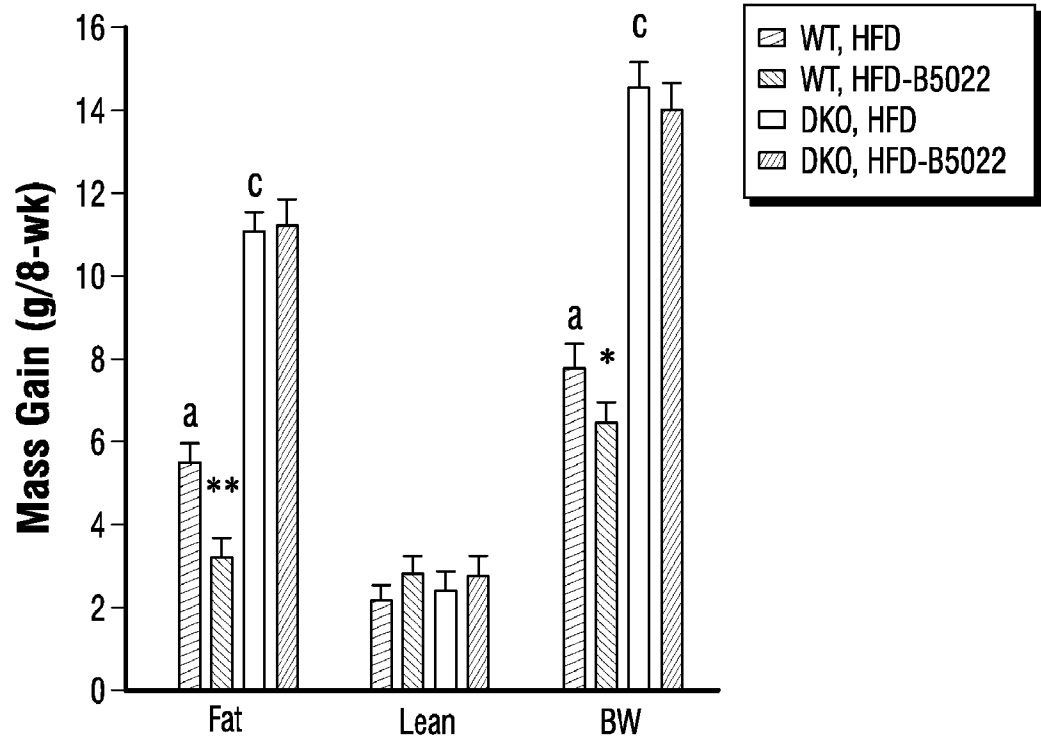
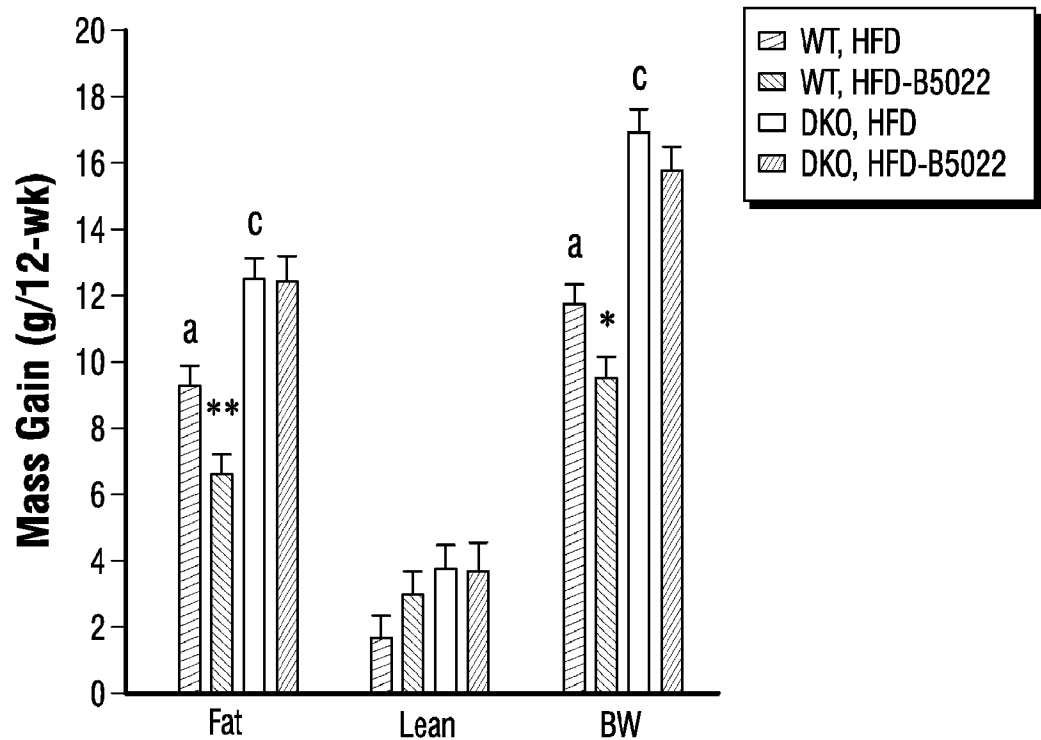


FIG. 8D

FIG. 8

**FIG. 9A****FIG. 9B**

**FIG. 10A****FIG. 10B**

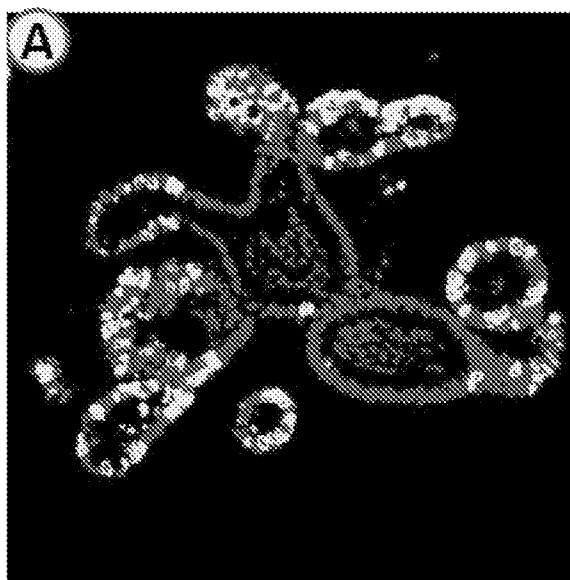


FIG. 11A

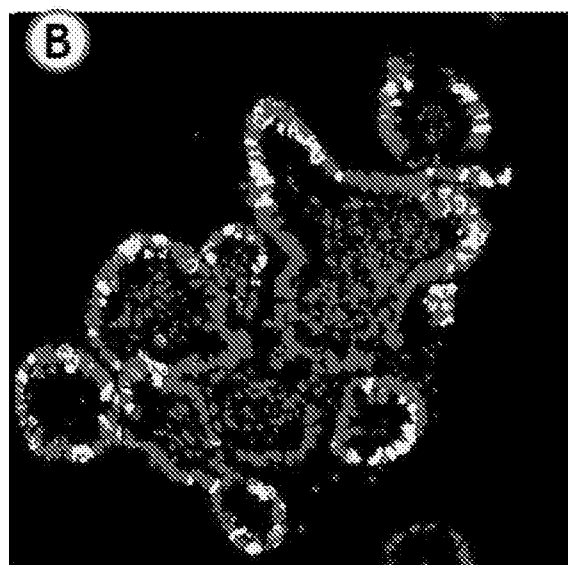


FIG. 11B

FIG. 11

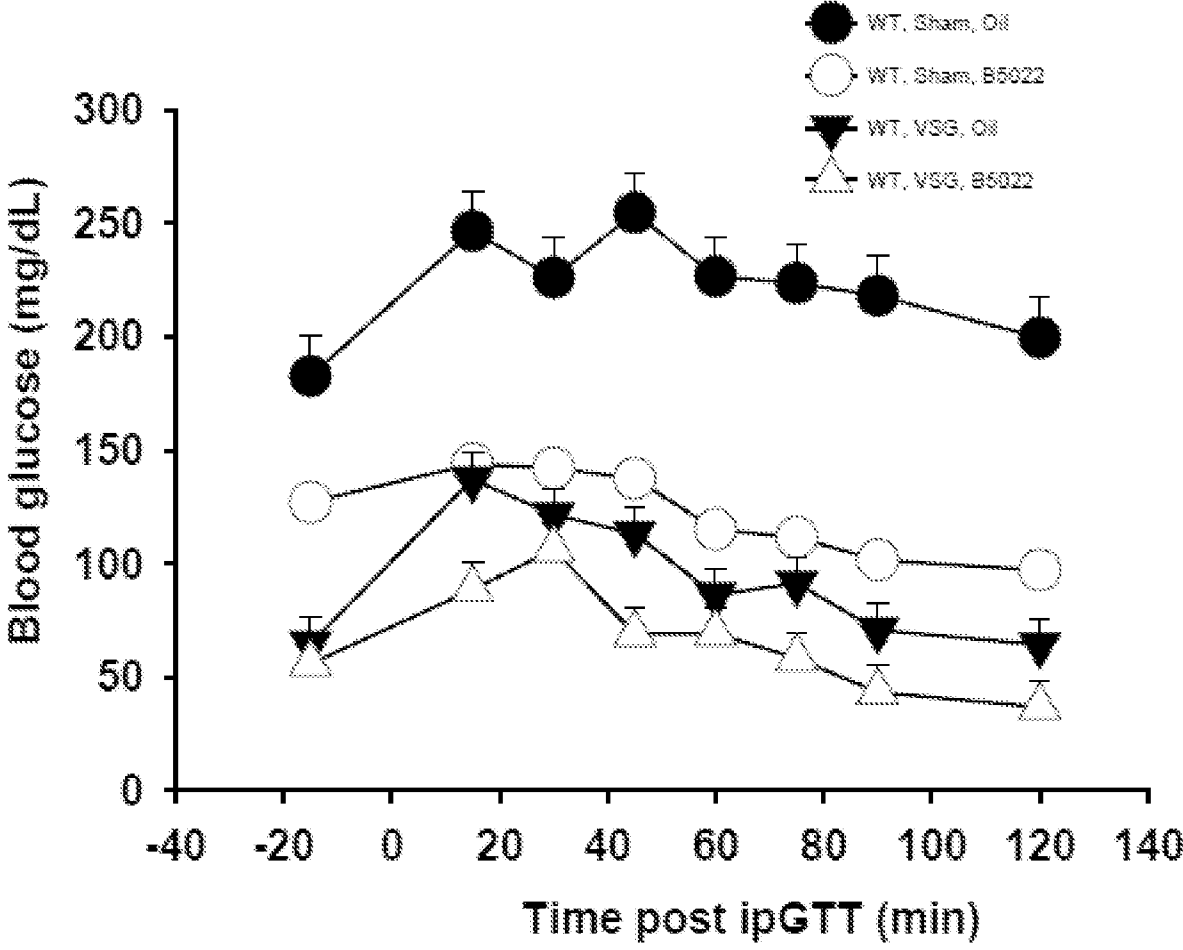


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/43259

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/4184, A61K 31/4164 (2017.01)

CPC - C07D 233/84, A61K 31/4184, A61K 31/4164

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Quan et al. "Betulinic acid alleviates non-alcoholic fatty liver by inhibiting SREBP1 activity via the AMPK-mTOR-SREBP signaling pathway" Biochemical Pharmacology. 19 February 2013 (19.02.2013) vol 85, pg. 1330-1340; pg. 1330, right col, para 2, pg. 1331, left col, para 1, pg. 1333, right col, para 1, pg. 1339, left col, para 3	1-3, 8, 11-18, 20-22, 26
Y		4-7, 9-10, 19, 23-25, 27-28
Y	Xu et al. "Polymeric Micelles, a Promising Drug Delivery System to Enhance Bioavailability of Poorly Water-Soluble Drugs" Journal of Drug Delivery. 11 June 2013 (11.06.2013); vol. 2013; pg. 1-15; pg. 1, right col, para 1-2, pg. 4, left col, para 2	4
Y	US 2003/0149286 A1 (Sauter) 07 August 2003 (07.08.2003); para [0003], [0008], [0036]	5, 23
Y	Das et al. "Nanopharmaceutical Approach for Enhanced Anti-cancer Activity of Betulinic Acid in Lung-cancer Treatment via Activation of PARP: Interaction with DNA as a Target" Journal of Pharmacopuncture. 01 March 2016 (01.03.2016) vol 19, pg. 37-44; pg. 037, left col, para 1, right col, para 1	6-7, 24-25
Y	Zhou et al. "Preparation and release kinetics of betulinic acid/ CS drug-loaded microspheres" MATEC Web of Conferences. 12 July 2016 (12.07.2016) < https://www.matec-conferences.org/articles/mateconf/pdf/2016/26/mateconf_mmmme2016_03008.pdf >; pg. 1, left col, para 1, right col, para 6	9-10, 27-28
Y	Sasaki et al. "Bariatric surgery and non-alcoholic fatty liver disease: current and potential future treatments" Frontiers in Endocrinology. 27 October 2014 (27.10.2014) vol 5, pg. 1-6; pg. 1, right col, para 2	19

☒ 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

15 September 2017 (15.09.2017)

Date of mailing of the international search report

16 OCT 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/43259

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Reiner et al. "Betulinic Acid Selectively Increases Protein Degradation and Enhances Prostate Cancer-Specific Apoptosis: Possible Role for Inhibition of Deubiquitinase Activity" PLOS ONE. 12 February 2013 (12.02.2013) pg. 1-11; entire document	1-28
A	US 8,129,429 B2 (Sporn et al.) 06 March 2012 (06.03.2012); entire document	1-28