

ANTI-OBESITY POTENTIAL OF CALEBIN A

[Para 001] This application is a non-provisional filing of provisional application 61/431157 filed on January 10, 2011.

FIELD OF INVENTION

[Para 002] The invention in general relates to medicaments for obesity management. More specifically, it relates to anti-obesity potential of Calebin A.

DESCRIPTION OF PRIOR ART

[Para 003] Obesity is the most prevalent nutritional disorder in industrialized countries and is a growing problem in developing countries. It is described as a global epidemic and overweight and obese individuals (BMI of 25 and above) are at increased risk for various chronic physical ailments and psychological problems such as depression, eating disorders and low self esteem. It is associated with various diseases like cardiovascular diseases, diabetes mellitus, osteoarthritis, obstructive sleep apnea and cancer. WHO considers obesity to be one of the top 10 causes of preventable death worldwide.

[Para 004] In obesity, there is an increase in the adipose tissue mass due to the production of new fat cells (adipocytes) through the process of adipogenesis and/or the deposition of increased amounts of cytoplasmic triglyceride per cell. A fat cell develops as internally produced lipid droplets coalesce into a single large mass. Eventually, cellulite results due to enhanced adipogenesis and accumulation of chunks of adipocytes under the skin dermis.

[Para 005] Studies of adipogenesis have proceeded with the hope that manipulation of this process in humans might lead to a reduction in the burden of obesity and diabetes. At molecular level, several markers have been targeted in treating obesity such as leptin, adiponectin, TNF- α etc

[Para 006] Though drugs are available for treating the disorder, there is a constant need and search for safe natural approach to help manage obesity and its related socio-economic consequences.

[Para 007] Calebin A is known to protect neuronal cells from β -amyloid insult (Park SY et al, J Nat Prod. 2002 Sep; 65(9):1227-31), induce apoptosis and modulate MAPK family activity in drug resistant human gastric cancer cells (Li Y et al, Eur J Pharmacol. 2008 Sep 4; 591(1-3):252-8). Zeng Y et al.(Chem Pharm Bull (Tokyo) 2007 Jun; 55(6):940-3) discusses two new calebin derivatives, 4''-(4'''-hydroxyphenyl-3'''-methoxy)-2''-oxo-3''-butenyl-3-(4'-hydroxyphenyl)-propenoate and 4''-(4'''-hydroxyphenyl)-2''-oxo-3''-butenyl-3-(4'-hydroxyphenyl-3'-methoxy)-propenoate.

[Para 008] The present invention discloses the potential of Calebin A to prevent fat accumulation during the terminal differentiation of adipocytes (fat cells) and applications thereof in obesity management. The present invention elucidates the potential of Calebin A to favorably modulate biochemical markers associated with obesity. Notable biomodulatory properties of Calebin A include inhibiting leptin production, increasing adiponectin expression and inhibiting local (adipocyte) and systemic inflammation caused by pro-inflammatory cytokines Tumor Necrosis Factor (TNF- α), Interleukin-6 (IL-6) and Interleukin-1 (IL-1 β).

[Para 009] Accordingly, it is the principle objective of the present invention to disclose anti-obesity potential of Calebin A.

[Para 0010] The invention fulfills the aforesaid principle objective and provides further related advantages.

SUMMARY OF THE INVENTION

[Para 0011] The present invention discloses the potential of Calebin A in inhibiting adipogenesis and applications thereof in obesity management. The present invention elucidates the potential of Calebin A to favorably modulate biochemical markers associated with obesity in mammals. Notable biomodulatory properties of Calebin A include inhibiting leptin production, increasing adiponectin expression and inhibiting local (adipocyte) and systemic inflammation caused by pro-inflammatory cytokines Tumor Necrosis Factor (TNF- α), Interleukin-6 (IL-6) and Interleukin-1 (IL-1 β).

[Para 0012] Other features and advantages of the present invention will become apparent from the following more detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, the principle of the invention.

BRIEF DESCRIPTION OF FIGURES

[Para 0013] Fig.1 shows the graphical representation of the percentage adipogenesis inhibition effected by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$ as studied by the Oil-Red-O-Staining method.

[Para 0014] Fig.2 shows the graphical representation of the percentage inhibition of leptin production in human adipocytes effected by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. P value * : < 0.01; ** : <0.001.

[Para 0015] Fig.3 shows the graphical representation of the percentage increase of adiponectin expression in human adipocytes effected by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. P value * : < 0.01.

[Para 0016] Figs.4 and 5 shows the graphical representation of the percentage inhibition of TNF- α expression (P value *: < 0.01; ** : <0.001) and IL-6 expression (P value * : < 0.01) respectively, in human adipocytes effected by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$.

[Para 0017] Fig.6 shows the graphical representation of the effect of multiple dose of Calebin A on the expression of TNF- α and IL-1 β in the serum from treated Swiss Albino mice. No. of animals = 6 per group, P-value: * < 0.01; ** < 0.001 students't' test.

[Para 0018] Fig.7 shows the graphical representation of the effect of multiple dose of Calebin A on the expression of IL-6 in the serum from treated Swiss Albino mice. No. of animals = 6 per group, P-value: * < 0.01; ** < 0.001 students't' test.

DETAILED DESCRIPTION OF INVENTION

[Para 019] The present invention discloses the potential of Calebin A to prevent fat accumulation during the terminal differentiation of adipocytes (fat cells) and applications thereof in obesity management. The present invention elucidates the potential of Calebin A to favorably

modulate biochemical markers associated with obesity. Notable biomodulatory properties of Calebin A include inhibiting leptin production, increasing adiponectin expression and inhibiting local (adipocyte) and systemic inflammation caused by pro-inflammatory cytokines Tumor Necrosis Factor (TNF- α), Interleukin-6 (IL-6) and Interleukin-1 (IL-1 β).

[Para 0020] In the most preferred embodiment, the present invention relates to a method of inhibiting adipogenesis, said method comprising step of bringing into contact the adipocytes with an effective amount of Calebin A. In other words, the present invention relates to a method of preventing accumulation of fat during the terminal differentiation of mammalian adipocytes. **(Fig.1).**

[Para 0021] In another preferred embodiment, the present invention relates to a method of inhibiting leptin expression in adipocytes, said method comprising step of bringing into contact the adipocytes with an effective amount of Calebin A **(Fig.2).**

[Para 0022] In another preferred embodiment, the present invention relates to a method of increasing expression of adiponectin in adipocytes, said method comprising step of bringing into contact the adipocytes with an effective amount of Calebin A **(Fig.3).**

[Para 0023] In another preferred embodiment, the present invention relates to a method of inhibiting pro-inflammatory cytokine TNF- α expression in adipocytes, said method comprising step of bringing into contact the adipocytes with an effective amount of Calebin A **(Fig.4).**

[Para 0024] In yet another preferred embodiment, the present invention relates to a method of inhibiting pro-inflammatory cytokine Interleukin-6 expression in adipocytes, said method comprising step of bringing into contact the adipocytes with an effective amount of Calebin A **(Fig.5).**

[Para 0025] In specific embodiment, the adipocytes referred to herein above are human adipocytes.

[Para 0026] In yet another preferred embodiment, the present invention relates to a method of reducing obesity induced systemic expression of pro-inflammatory cytokines in mammals, said method comprising step of administering an effective amount of Calebin A to a subject in need thereof. In specific embodiments, the pro-inflammatory cytokines referred to herein in this

paragraph include Tumor Necrosis Factor- α (TNF- α), Interleukin-6 (IL-6) and Interleukin-1 β (IL-1 β) [Figs 6 and 7].

[Para 0027] In yet another preferred embodiment, the present invention relates to a method of obesity management, said method comprising step of administration of an effective amount of Calebin-A to a subject in need thereof.

[Para 0028] In yet another preferred embodiment, the subject is a mammal.

[Para 0029] In yet another preferred embodiment, the subject is a human.

[Para 0030] The potential therapeutic value of Calebin A as an anti-obesity molecule may be understood through specific examples elucidated herein below.

EXAMPLE I

Acute Oral Toxicity of Calebin A

[Para 0031] Table I lists the parameters studied for Acute Oral Toxicity of Calebin A.

[Para 0032] **Results:** No mortality was observed up to 2000mg/kg p.o. in mice up to two weeks of observation.

Table I: parameters studied for Acute Oral Toxicity of Calebin A

General Behavior	Dermal
Aggression=Nil	Blanching=Nil
Fear=Nil	Hyperaemia=Nil
Passive=Nil	Cyanosis=Nil
General Movement=Normal	
General Locomotor Activity=Normal	
Central Nervous System	General Observations
Excitation=Nil	Muscular Weakness=Nil
Motor Activity=Nil	Salivation=Nil
Tremors=Nil	Pilo-erection=Nil
Clonic Convulsions=Nil	Diarrhea=Nil
Tonic Convulsions=Nil	
Respiratory System	Reflexes
Respiration Rate=Normal	Corneal=No effect

Respiration Depth=Normal	Pinnal=No effect
Autonomic Nervous System	Food and Water (Intake and Excretion)
Motor activity=Normal	Fecal Output=Normal
Atexia=Nil	Urine Output=Normal
Respiration Rate=Normal	
Diarrhea=Nil	

EXAMPLE II

Oil-Red-O-Staining of adipogenic cultures and estimation of leptin, adiponectin, TNF- α and IL-6 by ELISA.

[Para 0033] Terminal differentiation of adipocytes is accompanied by the accumulation of great amounts of lipids in large cytoplasmic vesicles. A common assay to measure adipocyte differentiation in cell culture is with the dye Oil Red-O (ORO). ORO is a lipid-soluble bright red dye which is a reliable indicator of adipocyte differentiation (adipogenesis).

Principle:

[Para 0034] Oil Red O (Solvent Red 27, Sudan Red 5B, C.I. 26125, and C₂₆H₂₄N₄O) is a lysochrome (fat-soluble dye) diazo dye used for staining of neutral triglycerides and lipids on frozen sections and some lipoproteins on paraffin sections. It has the appearance of a red powder with maximum absorption at 518(359) nm. Oil Red O is one of the dyes used for Sudan staining. Similar dyes include Sudan III, Sudan IV, and Sudan Black B. The staining has to be performed on fresh samples, as alcohol fixation removes the lipids. Oil Red O largely replaced Sudan III and Sudan IV, as it provides much deeper red color and the stains are therefore much easier to see.

[Para 0035] Oil red O is an oil soluble dye. Oil soluble dyes exhibit greater solubility of the dye in lipid substances in the tissues/cells, than in the usual hydro alcoholic dye solvents. Hence, it will deeply stain the cells.

Methodology:

[Para 0036] 3T3-L1 cells approximately 60 X 10⁴ cells are seeded for 48-72 hrs to get 70-80% confluence. After 48 hrs 200 µl of AIM (Adipogenesis induction medium) freshly prepared is added. 72 hrs later 200 µl APM (Adipogenesis progression medium) with the test compounds in different concentrations is added to the wells. The cells are incubated for 48 hrs in a humidified atmosphere (37°C) of 5% CO₂ and 95% air. The supernatant is collected and stored for the estimation of leptin, adiponectin, IL-6 and TNF-α by ELISA. Cells are fixed by adding 100 µl of 10% formalin and ORO staining is done. OD is read at 492 nm in microplate reader. The results are expressed as IC₅₀ values using Graphpad prism software.

[Para 0037] The percentage of inhibition of adipogenesis is calculated as follows,

% Inhibition = C-T

..... X 100

T

Where C-absorbance of Oil red O in differentiating/undifferentiated cells

T-absorbance of Oil red O in sample treated differentiating/undifferentiated cells. The estimation of leptin, adiponectin, IL-6 and TNF-α is done according to user's manual from R&D Systems.

[Para 0038] References:

1. Wu Z, Xie Y, Morrison RF, Bucher NLR, Farmer SR 1998. PPAR γ induces the Insulin-dependent Glucose Transporter GLUT4 in the absence of C/EBPβ during the conversion of 3T3 fibroblasts into adipocytes. J Clin Invest. 101:22-32.
2. A pre-adipose 3T3 cell variant highly sensitive to adipogenic factors & to human growth hormone. LA Salazar-Olivo, F Castro-Munozledo & W Kuri-Harcuch. Department of Cell Biology, Centro de Investigation y de Estudios Avanzados del I.P.N., Mexico D.F., Mexico. Journal of Cell Science, Vol 108, Issue 5 2101-2107.
3. A Nuclear Receptor Atlas: 3T3-L1 Adipogenesis. Mingui Fu, Tingwan Sun, Angie L. Bookout, Micheal Downes, Ruth T. Yu, Ronald M. Evans and David J. Mangelsdorf. Molecular Endocrinology 19 (10): 2437-2450.

4. Aimee D, Kohn et al, JBC, Vol 271, No. 49, pp-31372-31378.

Result:

[Para 0039] Fig.1 shows percentage adipogenesis inhibition of 32.43%, 38.59% and 35.8% respectively effected by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$ studied by the Oil-Red-O-Staining method.

[Para 0040] Fig.2 shows percentage inhibition of leptin production (34.92%, 41.04% and 39.48% respectively) in human adipocytes by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. The importance of the effects of Calebin A in inhibiting leptin production in human adipocytes and correlation thereof to obesity management stems from the following facts (Notes on Pathophysiology of the Endocrine System, Colorado State University).

[Para 0041] Leptin is a protein hormone expressed predominantly in adipocytes. It has important effects in regulating body weight, metabolism and reproductive function. Encoded by the obese (ob) gene, the protein is approximately ~16 kDa in mass. At normal concentrations, Leptin's biological function is predominantly vested in its effects on hypothalamic centers of the brain that control hunger, appetite, regulation of body temperature and energy metabolism. Thus leptin, in a non-obese individual could result in weight loss by two important mechanisms. (i) Decrease in hunger and food consumption most probably through the inhibition of neuropeptide Y that controls feeding behavior and (ii) increase in energy expenditure through increased body temperature, oxygen consumption and loss of adipose tissue mass. However, excessive secretion of leptin as in case of obesity or experimental models of induced obesity leads to disrupted functions of hypothalamic centers that an obese subject fails to attain satiations and tends to go on a over feeding mode. Hence it becomes imperative to bring about effective reduction of the over excessive levels of leptin in obesity and Calebin A shows promise in this area as indicated in Fig.2.

[Para 0042] Fig.3 shows percentage enhancement of adiponectin expression (27.12%, 34.06% and 32.8% respectively) in human adipocytes by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. Adiponectin is a cytokine produced almost exclusively by adipocytes and is expressed in very high levels by lean and healthy individuals. Obese individuals on the other hand express

reduced levels of this adipokine and are prone to coronary heart disease (CAD), diabetes mellitus and hypertension.

[Para 0043] References

1. Tamar.R.Aprahamian and Flora Sam, "Adiponectin in Cardiovascular Inflammation and Obesity, *Int J Inflam.* 2011; 2011: 376909;
2. Hotta K, Funahashi T, Arita Y, et al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arteriosclerosis, Thrombosis and Vascular Biology.* 2000; 20(6):1595–1599;
3. Iwashima Y, Katsuya T, Ishikawa K, et al. Hypoadiponectinemia is an independent risk factor for hypertension. *Hypertension.* 2004; 43(6):1318–1323;
4. Kumada M, Kihara S, Sumitsuji S, et al. Association of hypoadiponectinemia with coronary artery disease in men. *Arteriosclerosis, Thrombosis and Vascular Biology.* 2003; 23(1):85–89 and
5. Lindsay RS, Funahashi T, Hanson RL, et al. Adiponectin and development of type 2 diabetes in the Pima Indian population. *The Lancet.* 2002; 360(9326):57–58.

[Para 0044] Calebin A is shown (**Fig.3**) to effectively increase levels of adiponectin in human adipocytes and thus show promise in the area of obesity management.

[Para 0045] **Figs. 4 and 5** show the percentage inhibition of TNF- α (36.03%, 40.81% and 45.47% respectively) and IL-6 (21.31%, 32.37% and 31.7% respectively) by Calebin A at concentrations of 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. Bastard JP et al, "Recent Advances in the relationship between obesity, inflammation and insulin resistance", *Eur Cytokine Netw.* 2006 Mar;17(1):4-12 cite that obesity is associated with low-grade inflammation of the white adipose tissue (WAT). The authors also remark that in obesity, WAT is characterized by increased expression of pro-inflammatory molecules like TNF- α and IL-6 which not only exert effects on WAT but also on other systemic organs of the body. **Figs.4 and 5** demonstrate that Calebin A is effective in reducing TNF- α and IL-6 expression in adipocytes and would be a useful agent to modulate effects of local and systemic inflammation in obesity.

EXAMPLE III**Modulation of systemic inflammation by Calebin A**

[Para 0046] The present inventors also adduce extra evidence to support the ability of Calebin A to suppress intracellular TNF and extracellular IL-1 β in murine neutrophil systems (**Table II**, **Table III**). Neutrophils are isolated by histopaque gradient method tested for their ability to produce in vitro TNF- α following stimulation with Lipopolysaccharide (LPS). The cells were incubated with phycoerythrin (PE)-labeled anti-mouse TNF- α . in the dark, and after being washed with sterile PBS, samples were resuspended in PBS (pH 7.4) and acquired directly on the flow cytometer (BDLSR; Becton Dickinson). A fluorescence trigger was set on the PE (FL1) parameter of the gated neutrophil populations (10,000 events). Rolipram at 100 μ g/ml was used as standard inhibitor of TNF- α in this study. Fluorescence compensation, data analysis, and data presentation were performed using Cell Quest Pro software (Becton Dickinson).

[Para 0047] References

1. Clara, B., R. C. Arancha, G. M. Andre's, P. Atanasio, A. Julia, and O. Alberto. 2003. A new method for detecting TNF- α -secreting cells using direct immunofluorescence surface membrane stainings. J. Immuno. Methods 264:77–87.
2. Khurshid A. Bhat, Bhahwal A. Shah, Kuldeep K. Gupta, Anjali Pandey, Sarang Bani, Subhash C. Taneja. Semi-synthetic analogs of pinitol as potential inhibitors of TNF- α cytokine expression in human neutrophils. Bioorganic & Medicinal Chemistry Letters 19 2009, 1939–1943.

TABLE II

Serial No	Sample	Concentration ($\mu\text{g/ml}$)	Expression of TNF- α Mean $\pm$ S.E	% Activity
1	LPS Control	-	2.62 $\pm$ 0.01	-
2	Calebin A	0.5	1.87 $\pm$ 0.04*	28.62% $\downarrow$
3	Calebin A	1.0	1.70 $\pm$ 0.02**	35.11% $\downarrow$
4	Calebin A	2.0	1.59 $\pm$ 0.05**	39.31% $\downarrow$
5	Rolipram	100	0.73 $\pm$ 0.09**	72.13 % $\downarrow$

% $\downarrow$: indicates suppression of TNF- α expression

No. of observations = 3

P-value: * < 0.01; ** < 0.001 students't' test

TABLE III

Samples Treatment	Concentration (pg/ml)	% Activity
LPS Control	51.80 $\pm$ 2.18	-
Calebin-A		
0.5 $\mu\text{g/ml}$	41.24 $\pm$ 1.16*	20.38% $\downarrow$
1.0 $\mu\text{g/ml}$	39.26 $\pm$ 2.52*	24.20% $\downarrow$
2.0 $\mu\text{g/ml}$	37.16 $\pm$ 2.11**	28.26% $\downarrow$
Rolipram (Standard)	22.52 $\pm$ 1.60**	56.52% $\downarrow$
100 $\mu\text{g/ml}$		

% $\downarrow$: indicates suppression of IL-1 β expression

No. of observations = 3

P-value: * < 0.01; ** < 0.001 students 't' test

[Para 0048] The present inventors also adduce study data on the ability of Calebin-A to reduce expression of Extracellular TNF- α , IL-1 beta **[Fig.6]** and IL-6 **[Fig.7]** in serum from treated mice (in-vivo models). Swiss albino male mice aged 6–8 weeks were maintained at 22 $\pm$ 2 $^{\circ}\text{C}$

under 12/12 h light dark cycle. Mice received oral treatment of test drugs at graded doses (w/v) for 6 days, followed by intravenous injection of 1 mg/kg of LPS according to the method described by Brieva A, Guerrero A, Alonso-Lebrero J L and Pivel JP. 2001. Immuferon, a glycoconjugate of natural origin, inhibits LPS-induced TNF- α production and inflammatory responses. *International Immunopharmacology* 1, 1979–1987. Six mice were employed in each group and experiments were performed in triplicates. TNF- α , IL-1 beta and IL-6 production was evaluated by a commercial ELISA kits (R&D Systems) in serum from treated mice, 90 min after LPS injection. Rolipram at 30 mg/kg was used as standard drug.

[Para 0049] Figs.6 and 7 demonstrate that Calebin A is effective in reducing TNF- α , IL-1 beta and IL-6 thus indicating that the compound is a useful agent to modulate effects of local and systemic inflammation in obesity.

[Para 0050] While the invention has been described with reference to a preferred embodiment, it is to be clearly understood by those skilled in the art that the invention is not limited thereto. Rather, the scope of the invention is to be interpreted only in conjunction with the appended claims.

CLAIMS:

1. Use of an effective amount of Calebin A for the manufacture of a medicament for inhibiting adipogenesis.
2. Use of an effective amount of Calebin A for the manufacture of a medicament for inhibiting leptin expression in adipocytes.
3. Use of an effective amount of Calebin A for the manufacture of a medicament for increasing expression of adiponectin in adipocytes.
4. Use of an effective amount of Calebin A for the manufacture of a medicament for inhibiting obesity induced pro-inflammatory cytokines in adipocytes.
5. The use according to claim 4, wherein the pro-inflammatory cytokine is Tumor Necrosis Factor- α (TNF- α).
6. The use according to claim 4, wherein the pro-inflammatory cytokine is Interleukin-6 (IL-6).
7. Use of an effective amount of Calebin A for the manufacture of a medicament for reducing obesity induced systemic expression of pro-inflammatory cytokines.
8. The use according to claim 7, wherein the pro-inflammatory cytokine is Tumor Necrosis Factor- α (TNF- α).
9. The use according to claim 7, wherein the pro-inflammatory cytokine is Interleukin-6 (IL-6).
10. The use according to claim 7, wherein the pro-inflammatory cytokine is Interleukin-1 β (IL-1 β).

11. Use of an effective amount of Calebin-A for the manufacture of a medicament for obesity management.