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Obesity-Causes, Treatment and *in vitro* Antiobesity Studies - A Review

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Abstract : Obesity is a present global issue due to drastic changes in life style, food habits, intake of too many drugs and stressful or sedentary life. Obesity is associated with substantial increases in morbidity, premature mortality. Athough several antiobesity drugs are introduced into the market, many of them have been withdrawn due to fatal side effects. Exploration of new and effective antiobesity drugs with less or negligible side effects is highly essential especially patients with morbid obesity. The present paper summerizes the causes, various treatments and in vitro evaluation techniques for new antiobesity drugs.

Obesity has increased at an alarming rate and has become a worldwide health problem. It is gradually being regarded as a specific, distinct medical problem rather than just a consequence of overeating. Obesity is associated with many chronic diseases including type 2 diabetes mellitus, cardiovascular disease (stroke, hypertension and dyslipidemia), certain types of cancer (endometrial, breast, colon), depression, sleep disorders, musculoskeletal disorders[1] and formation of gallstones.

Obesity, most often defined as a body mass index (BMI) of ≥30 kg/m^2 and morbid obesity is defined as BMI of ≥40 kg/m^2. Prevalence of the disorder in adults has more than tripled in the past decade globally.Obesity in children and adolescents is of particular concern. The current prevalence of 7–10% in these populations is predicted to at least double by 2025[2] and there is strong evidence of persistence into adulthood[3,4.]

Obesity is associated with substantial increases in morbidity, premature mortality and impaired quality of life[5,6,7]. Although prevention and changes to the obesogenic environment are long-term goals, treatment is required obese. However, however, treatment options remain quite limited. Lifestyle changes in the form of dieting and/or exercise do not generally produce marked and sustainable weight loss[8,9], whereas effective psychological therapies, such as cognitive behavioural therapy, cannot easily be delivered on a mass scale[10] and long-term results are disappointing. Bariatric surgery, such as Roux-en-Y bypass or gastric banding, is much more effective in terms of weight loss, co-morbidity reduction and enhanced survival[11,12]. However, owing to concerns about mortality, surgical complications and the frequent need for reoperation, these procedures tend to be reserved for the morbidly obese[13,14.]

Keywords: Antiobesity Studies.

Causes of Obesity:

Although obesity is mainly caused due to increased calories intake and decreased utilisation of energy, a number of factors contribute to obesity. These include lack of physical activity, lack of sleep, sedentary or stressful life style, genetic disorders and certain medications like corticosteroids, antidepressants or antiepileptic agents that slow down calorie burn, increase appetite or cause water retention.

Following health conditions also can lead to weight gain:

- Hypothyroidism, an underactive thyroid gland that slows metabolism and causes fatigue and weakness.
- PCOS, or polycystic ovarian syndrome, which affects up to 10 percent of women of childbearing age and can also lead to excess body hair and reproductive problems.
- Cushing's syndrome, which stems from an overproduction of the hormone cortisol by the adrenal glands and is characterized by weight gain in the upper body, face and neck.
- Prader-Willi syndrome, a rare condition in which people never feel full, are tempted to eat constantly.

Treatment-Antiobesity Agents

Although diet and exercise is recommended for loss of weight, anti-obesity drugs are prescribed after diagnosis of the cause of obesity in patients who do not respond to diet and exercise.

Antiobesity drugs exert their effect through various mechanisms which assist with weight loss.

- A**ppetite suppression** with medications acting through alteration of monoamine neurotransmitters or cannabinoid receptor systems in the central nervous system (brain).
- **To reduce nutrient absorption** from the gastrointestinal (GI) tract. It is mainly the absorption of fats that are targeted.
- **Increasing metabolism** also promotes weight loss.

Medications exerting actions through these mechanisms form the basis for all currently used anti-obesity agents. However, most of the currently available drugs are associated with significant health risks. Hence anti-obesity drugs are to be prescribed only when the advantages are more than the risks associated with the use of the medication.

Appetite suppressing drugs

Appetite-suppressing drugs, also called anorexiants, reduce appetite by causing early satiety (feeling of satisfaction or fullness after food intake) and subsequently reducing hunger. This helps patients to reduce calorie intake without a sense of deprivation. These drugs mainly exert the effect on appetite by modulating the neurotransmission of three monoamines namely norepinephrine, serotonin (5-hydroxytryptamine or 5-HT) and dopamine. However, the disadvantages of appetite suppressants are that the effects of the drugs are short-lived and wear off after discontinuation. The individuals are likely to regain weight if the drug is discontinued at the same time use of most appetite suppressants beyond 3 months is associated with significant toxicities.

The prominent appetite suppressing agents are :

- Sympathomimetic agents
- Sibutramine
- Cannabinoid receptor antagonists

Sympathomimetic agents

Sympathomimetic agents include amphetamine derivatives like: benzphetamine, phendimetrazine, diethylpropion, mazindol, phenylpropanolamine, fenfluramine, dexfenfluramine and phentermine.These drugs function by stimulating norepinephrine release or by blocking its reuptake. Phenylpropanolamine, which was commonly used as appetite suppressant preparations, was associated with hemorrhagic strokes in young women and increase in blood pressure.

Fenfluramine and dexfenfluramine primarily exerts action by increasing serotonin levels in brain. These drugs were commonly usedbecause of its ability to suppress appetite. However, the effectiveness of these drugs gradually declines if it is used continuously beyond a period of 3 months. These drugs have very less brain stimulating property but can produce a sense of relaxed state of mind (tranquillizing role). Use of these drugs is associated with certain cardiac valvular abnormalities especially in patients with carcinoid syndrome (conditions associated with increased serum level of serotonin).

Sympathomimetic drugs are associated with stimulant effect on central nervous system and can potentially cause disturbances in sleep. These drugs are also associated with significant potential for abuse, mood disturbances, and cardiovascular toxicity. Amphetamine derivatives are thus no longer recommended for routine treatment of obesity.

Sibutramine

Sibutramine acts by inhibiting reuptake of serotonin and norepinephrine. It is effective in suppressing appetite and also promotes fat breakdown by increasing metabolism and heat generation in the body. These effects of sibutramine are responsible for reducing the body weight. Sibutramine can produce a loss of about 5 to 10 kgs over a period of 12 months and it can maintain the weight loss for about 2 years. Unlike other appetite suppressants which were popular previously, sibutramine is not pharmacologically related to amphetamine and has no addictive potential.

The undesirable effects of sibutramine are constipation, sleep disturbance, alterations in the heart rate and blood pressure, acute cardiovascular complications, mood disturbances, and dry mouth. Use of sibutramine is contraindicated in patients with uncontrolled hypertension (high blood pressure) and ischemic heart disease. Sibutramine has been withdrawn from markets in several countries including the United States, due to a high incidence of the cardiovascular complications like myocardial infarction (heart attack) and stroke.

Cannabinoid receptor antagonist

Rimonabant (Acomplia) is a recently developed anti-obesity medication. It is a cannabinoid (CB1) receptor antagonist that acts centrally on the brain thus decreasing appetite.It may also act peripherally by increasing thermogenesis and therefore increasing energy expenditure.[17]

Weight loss with Rimonabant however has not been shown to be greater than other available weight-loss medication. The most common side effects include depression, anxiety and significant nausea. The drug was never approved in the United States and Canada even though it was available in the Europe. The manufacturer has discontinued the drug worldwide due to serious psychiatric side effects.

Peripherally acting weight loss drugs

These drugs affect sites other than the brain and its hormones. One of the more common of these agents is a lipase inhibitor. Other peripherally acting weight loss drugs include fiber supplements and diabetes medication.

Lipase inhibitors

Orlistat is the only approved weight loss medication which is widely used at present. It is a synthetic derivative of a lipase inhibitor called lipostatin which is produced naturally by the fungi *Streptomyces toxytricini*. Orlistat inhibits absorption of fat by blocking the action of lipase enzymes (fat dissolving enzymes) produced in the body. In this way, orlistat reduces the absorption of certain fats from the gut. Significant weight-loss can be obtained with continuous use of orlistat. Orlistat can reduce a 10% weight loss over a period of 12 months.

Undesirable effects of orlistat are mainly due to the presence of undigested fat in the stools. The fecal fat increases considerably after starting the therapy leading to significant gastrointestinal side effects in about 10% of the patients. The adverse effects of orlistat mostly includes flatulence, fatty stool, pain in the abdomen, fecal urgency, deficiency of fat soluble vitamins and foul smelling stools. The intensity of side effects generally diminishes with control of dietary fat intake. The GI side effects may be minimized with concomitant intake of *Psylliummucilloid* along with orlistat

Biguanides and other antidiabetics

The biguanides, like metformin, is associated with reduction of absorption of glucose from intestine. At the same time it helps increase utilization of glucose in the peripheral tissues mainly in muscles and fat tissue. Metformin is a popular antidiabetic drug and it may be of some use in reducing weight, especially in obese diabetic patients.

Exenatide

Exenatide is a long-acting analogue of the hormone GLP-1, which the intestines secrete in response to the presence of food. GLP-1 delays gastric emptying and promotes a feeling of satiety. Some obese people are deficient in GLP-1, and dieting reduces GLP-1 further.[30] Byetta is currently available as a treatment for diabetes type 2. Some, but not all, patients find that they lose substantial weight when taking Byetta. Drawbacks of Byetta include that it must be injected subcutaneously twice daily, and that it causes severe nausea in some patients, especially when therapy is initiated. Byetta is recommended only for patients with Type 2 Diabetes. A somewhat similar drug, Symlin, is currently available for treating diabetes and is in testing for treating obesity in non-diabetics.

***In Vitro* Evaluation of Antiobesity Activity:**

Many in-vitro tests are done to confirm the anti-obesity action of compounds. Some of the in-vitro tests using plant extracts have been summerized below:

Canine in vitro MTP assay:

(A) Canine hepatic microsome isolation:canine microsomes are first isolated from canine liver by thawing frozen liver on ice and rinsing several times with 0.25 M sucrose. A 50% liver homogenate (w/v) is made in 0.25 M sucrose. The homogenate is diluted 1:1 with 0.25 M sucrose, and centrifuged at 10,000g at 4 C for 20 min. The supernatant is saved. The pellet is re-suspended in a minimal volume of 0.25 M sucrose and re-centrifuged at 10,000g for 20 min at 4 C. The supernatants are combined and centrifuged at 105,000g for 75 min at 4 C. The supernatant is discarded and the resulting microsomal pellet is saved. The microsomal pellet is re-suspended in a minimum volume of 0.25 M sucrose and diluted to 3 ml/g liver weight in 0.15 M Tris–HCl, pH 8.0. The resulting suspension is divided into 12 tubes and centrifuged at 105,000g for 75 min. The resulting microsomal pellets are stored at 80 C until needed. MTP is isolated by thawing the microsomal pellet tube and suspending in 12 ml/tube of cold 50 mMTris–HCl, 50 mMKCl, 2 mMMgCl, pH 7.4, and slowly adding 1.2 ml of a 0.54% deoxycholate, pH 7.4 solution. After 30 min incubation on ice with gentle mixing, the solution is centrifuged at 105,000g for 75 min at 4 C. The supernatant, containing soluble MTP, is dialyzed for 2– 3 days with 5 changes of assay buffer (15.0 mMTris–HCl, 40 mMNaCl, 1 mM EDTA, 0.02% NaN3, pH 7.4).

(B) MTP activity assay reagents: Donor liposomes are created by adding 447 mM egg phosphatidylcholine (68/20 ml), 83 mM bovine heart cardiolipin (169/20 ml) and 0.91 mM [14C] triolein (110 Ci/mol) (20/20 ml). The lipids are available in chloroform and are first dried under nitrogen and then hydrated in assay buffer to the volume needed. To create liposomes, lipids are sonicated for 7 min. Lipids are centrifuged at 105,000g for 2 h and liposomes are harvested by removing the top 80% of supernatant into separate tube. Acceptor liposomes are created by adding 1.33 mM egg phosphatidylcholine (404/40 ml), 2.6 mMtriolein (100/ 40 ml) and 0.5 nM [3 H]egg phosphatidylcholine (50 Ci/mol) (10/40 ml). The lipids are available in chloroform and are first dried under nitrogen and then hydrated in assay buffer to the volume needed. To create liposomes, lipids are sonicated for 20 min. Lipids are centrifuged at 105,000g for 2 h and are harvested by removing the top 80% of supernatant into separate tube.

(C) MTP in vitro lipid transfer inhibition assay: Appropriately diluted drug or control samples in 100 ml assay buffer containing 5% BSA are added to reaction tubes containing assay buffer, 50 ml donor liposomes, 100 ml acceptor liposomes, and partially purified liver MTP. The tubes are vortexed and incubated on a tube shaker for 1 h at 37 C to allow lipid transfer reaction to occur. Donor liposomes are precipitated by adding 300 ml of a 50% (w/v) DEAE cellulose suspension in assay buffer to each tube, followed by gentle/repeated inversion for5 min at room temperature. Tubes are then centrifuged at 1000 rpm to pellet resin. Four hundred milliliters of supernatant is transferred into a scintillation vial with scintillation fluid and DPM counts for both [3 H] and [14C] are determined. Triolein transfer is calculated by comparing the amount of [14C] and [3 H] remaining in the supernatant to [14C] and [3 H] in the original donor and acceptor liposomes, respectively. % Triolein transfer = ([14C]supernatant/[14C]donor) · ([3 H]acceptor/[3 H]supernatant) · 100 IC50 values are obtained using standard methods and first order kinetic calculations.

Pancreatic lipase activity:

(A)Measurement of pancreatic lipase activity:

The lipase activity was measure on emulsions of TAG using a pH-stat at 37^0C .For PPL the reaction medium containe 10ml of olive oil emulsion(10ml olive oil and 90 ml of arabic um in 20 ml distilled water, 4mM sodium taurodeoxycholate (NaTDC). Pure colipase was added in the assay medium at a molar excess of 100 to PPL. The lipase activity was determined by titration of the fatty acids released using NaOH (0.1N). One lipase unit (IU) was defined as 1 µmole of free fatty acid per minute.

(B) Measurement of inhibitory effects

Three methods were used to determine the inhibitory effect of various exracts depending upon the order of addition of lipase, substrate and inhibitor.

Method 1: Lipase inhibitor pre-incubation. This method was used to test the possible reaction between lipase and inhibitor in the absence of substrate. The reaction medium contained 20 µl of enzyme and 100µl of pomegranate peel extract in the absence of 4 mM of NaTDC.

Method 2: In this case, the inhibitor was added during lipolysis. This method was designed to test the possible inactivation reaction in the presence of substrate.

Method 3:The experimental conditions were run as in method A with the addition of NaTDC at 4mM final concentration to create a miceller solution containing the inhibitory molecules.

The lipase activity was measured titrimetrically at pH 12 and 60°C with a pH-stat under standard conditions using tributyrin (0.25 mL) in 30 ml of 2.5 mMTris-HCl pH 12, 2 mM $CaCl_2$, 1 mMNaDC or olive oil emulsion (10 mL in 20 mL of 9‰ NaCl pH 12, 2 mM $CaCl_2$, 2 mMNaDC) as substrate. Lipase activity was also measured at pH 7 and 37°C using TC_3 as substrate (0.25 mL TC_3) in 30 mL of 2.5 mM phosphate buffer pH 7, 2 mM $CaCl_2$. The olive oil emulsion was obtained by mixing (3 × 30 s in a Waring blender) 10 mL of olive oil in 90 ml of 10% GA. When measuring SL1 lipase activity in the absence of $CaCl_2$, EDTA or EGTA was added to the lipolytic system. Lipolytic activity was expressed as units. One unit corresponds to 1 µmol of fatty acid released per minute.

Mammalian lipase (Sigma Porcine pancreatic lipase) inhibitionassay:

Mammalian lipase (Sigma Porcine pancreatic lipase) inhibition assay was carried out using triolein as a substrate. The hydrolysis was carried out at pH 8.0 and 37°C over the period of 10min using a pH stat. The substrate emulsion (1.5ml/assay) was prepared by ultrasonication of triolein (30mg/ml) in a solution containing 1mMtaurochenodeoxycholate, 9mM-taurocholate, 0.1mMcholesterol, 2mM-tris/ hydrochloric acid, 1Mm phosphatidylcholine, 15mg/ml bovine serum albumin (BSA), 100mM-NaCl and 1mM-calcium chloride. After the addition of the test compound, which was dissolved in 150µl of DMSO, the pH was adjusted to 8.0 and the reaction was started within 1min by the addition of 15-20µl of lipase (dissolved in saline/ 4% BSA at a concentration of 70µg/ml). A drop in pH was monitored over the period of 10mins. A blank DMSO without the test compound was a solvent control for substrate inhibition [6]. The amount of lipolytic activity was adjusted to result in the generation of 0.2 to 0.3µmol fatty acid/ml per min.

Reagents 1. Tris-HCl buffer (13 mM, pH 8.0, with 150 mMNaCl and 3 mM CaCl2) 2. Porcine pancreatic lipase (Sigma, L3126) 3. Glyceryltrioleate (Sigma, T7140-1G) 4. L-α-Phosphatidylcholine (Sigma, P4279, purity>99%) 5. Taurocholic Acid Sodium Salt (Nacalaitesque, 32729-74) 6. Orlistat (Lipase inhibitor, sold from Roche as Xenical®) 7. LabAssay NEFA (Wako Chem., 294-63601) 8. Several solvents . Preparation of reagent solution A. Porcine pancreatic lipase solution Dissolve 4.5 mg of Porcine pancreatic lipase in 30 mL of Tris-HCl buffer. Prepare fresh solution before the assay B. Glyceryltrioleate solution Dissolve 1.0 g of Glyceryltrioleate in 6.25 mL of chloroform. Store under nitrogen atmosphere at -20 ºC. C. L-α-Phosphatidylcholine solution Prepare 100 mg/mL ethanol solution. Store at -20 ºC. D. Stop reagent Prepare 10 µg/mL solution in 50% DMSO aq. Prepare fresh solution before the assay. (Prepare 1 mg/mL DMSO solution and then dilute with 50% DMSO aq. to the desired concentration) E. Positive control Prepare 1 µg/mL solution in 50% DMSO aq. (dilute stop reagent ten times) Preparation of glyceryltrioleate micelle 1. Add 100 µL of

Glyceryltrioleate solution and 100 µL of L-α-Phosphatidylcholine solution in a glass tube and evaporate the solvent. 2. Add 5 mg of Taurocholic Acid Sodium Salt and 9 mL of Tris-HCl buffer in the tube 3. Vortex the tube and make a suspension 4. Sonicate the suspension using ultrasonic homogenizer for 5 min and create a micelle solution Assay procedure 1. Add 100 µL micelle solution and 50 µL sample solution (in 50% DMSO aq.), mix and pre-incubate for 5 min at 37 ºC 2. Add 50 µL of lipase solution, mix and start the reaction 3. After reacting for 30 min at 37 ºC, add 50 µL of stop reagent 4. Add 20 µL of 1 M hydrochloric acid and 400 µL of hexane 5. Mix rigorously for 40 sec. and leave settled until phase separation 6. Take 200 µL from hexane layer to the new tube 7. Evaporate the hexane and dissolve the residue in 100 µL of DMSO. 8. Measure the oleic acid in the DMSO solution by LabAssay NEFA kit.

Determination of Pancreatic Lipase Activity:

The pancreatic lipase activity was measured by using 4-MU oleate as a substrate, as reported by Nakai and his co-workers.5 An aliquot of 50 µL of the pancreatic lipase solution (2 U/mL) in a 50 mMTris-HCl (pH 8.0) buffer solution was added into 100 µL of the diluted sample solution and mixed with 50 µL of a 0.5 mM 4-MU solution dissolved in the above buffer in the well of a 96-well microplate to start the enzyme reaction. The plate was immediately placed in the 37 _C preheating FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc.) to measure the amount of 4-methylumbelliferone released by the lipase every minute for 30 min at an excitation wavelength of 360 nm with a tolerance of (40 nm and an emission wavelength of 455 nm with a tolerance of (20 nm. The enzymatic reaction rate for each sample was measured as that as mentioned above. All of the samples were investigated for the pancreatic lipase inhibition, for which 20 mg/mL of FME, 10 mg/mL of SME, 0.04 mg/mL of cyanidin, 0.2 mg/mL of cyanidin- 3,5-diglucoside, and the control were studied against the substrate 4-MU oleate at different concentrations to explore the enzymatic kinetic constants and inhibitive mode.

Calorimetric Assay:

The extra-cellular lipase activity was assayed in crude or purified lipase obtained following DEAE anion exchange chromatography. The stock solution (20mM) pNPP was prepared in HPLC grade iso-propanol. The reaction mixture contained 75µl of pNPP stock solution, 5-50µl of test sample (CL, PL or commercial lipase) and Tris buffer (0.05M, pH-8.5) to make final volume 3ml. the reaction mixture was incubated at 45° C for 20 mins in a water bath. The reaction was stopped by adding 1 ml of chilled acetone:ethanol mixture (1:1 kept at-20° C overnight). Control containing heat inactivated (5 mins in boiling water bath) enzyme (in duplicate was also incubated with each assay.

The absorbance of heat-inactivated lipase was substracted from the absorbance of the corresponding test sample. The absorbance (A_{410}) of p-nitrophenol released was measured. The unknown concentration of p-nitrophenol released was determined from a previously prepared reference curve of p-nitrophenol(2-20µg/ml in 0.05 M Tris buffer, pH 8.5). each of the assays was performed in triplicate, unless otherwise stated and mean values were recorded. One unit (IU) of lipase activity was defined as micromoles of p-nitrophenol released by hydrolysis of pNPP by one ml of enzyme at 45° C under assay conditions.

Conclusion:

Several safe antiobesity agents are under clinical trials. Although a lot of in vitro antiobesity evaluation techniques are available, it is essential to ascertain the results by in vivo evaluation. A successful discovery of safe antiobesity drugs are anticipated to replace the invasive antiobesity treatments in future.

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