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A STUDY ON CLASSIFICATION OF INHIBITORS OF FATTY ACID TRANSPORT PROTEIN-2 IN CELL

Biochemistry

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ABSTRACT

Inhibition of uptake of fatty acids in non-adipose tissues seems an attractive mechanism for treatment of lipotoxicity, dyslipidemia and other elements related to metabolic syndrome and obesity. Fatty acid transport proteins (FATPs) are bifunctional proteins involved in the uptake and activation of fatty acids by esterification with coenzyme A. To date, only inhibitors specific to FATP1 and FATP4 have been identified. Here we characterize a FATP2-specific fatty acid uptake inhibitor, CB5. Identified in a high throughput screening in yeast transformed with humanFATP2, CB5 is effective in inhibiting the uptake of fatty acid at low micro-molar ranges in cell lines that are models for intestines, liver, muscle, pancreas and adipose tissue with varying potencies.

Inhibition was also specific for long and very-long chain fatty acids and not for medium chain fatty acids, which are transported by diffusion. Finally, CB5 was effective in protecting the cell lines that are models for liver and pancreas and primary liver cells from lipotoxic effects of saturated fatty acid, palmitic acid. High throughput screening also identified clozapine and chlorpromazine, atypical antipsychotics drugs, as inhibitors of FATP2-mediated fatty acid uptake in yeast system. However, atypical antipsychotics were ineffective in inhibiting the uptake of FA-analog C1-BODIPY-C12 in HepG2 cells. They were also ineffective in protecting HepG2 cells from the lipotoxic effects generated by saturated fatty acid compared to CB5 that exhibited protection to the cells, demonstrating that they are not effective inhibitors of fatty acid transport compared with CB5.

KEYWORDS

Fatty acid transport proteins, Dyslipidemi, Atypical antipsychotics drugs.

INTRODUCTION

Fatty Acid in diseased states

The rising prevalence of obesity and related disorders is an increasing global threat predicting a decrease in the life expectancy of future generations[1]. According to 2011-2012 data, more than one-third (34.9%) of US adults and 17% of children and adolescents (between 2-19 years) were obese. Since 1980, obesity among children has tripled in United States [2]. A major part of the risk is the increasing prevalence of the pathogenic events that follow obesity; which includes, but is not restricted to, insulin resistance (IR), metabolic syndrome (MS), type II diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD). The etiology of these diseases is still poorly understood as they continue to pose burden on the health management sectors in developed countries.

The role of transport proteins as regulators of lipid metabolism is of great interest specially in understanding the pathophysiology of various diseases such as dyslipidemia, lipotoxicity, cardiomyopathy and so on. However, very few studies have targeted the protein-mediated uptake of fatty acids as therapeutic interventions to reduce the disease conditions. FATP1 and FATP4 inhibitors have been reported, however, these inhibitors were screened on the basis of inhibition of long-chain acyl CoA synthetase activity rather than the transport activity and were not effective in animals [3,4]. In order to understand the linkage and role of FATPs in the excessive accumulation of fat in various diseased states, Sandoval et al., 2010[5] utilized a high-throughput screening assay in yeast transformed with human FATP2 to screen library for inhibitors of fatty acid uptake. This led to the identification of 2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one, also known as CB5 or Grassofermata.

CB5 was shown to be capable of inhibiting the uptake of FA-analog C1-BODIPY-C12 using a live cell based assay in yeast as well as mammalian cell lines that are models for liver, small intestines and adipose tissue (HepG2, Caco-2 cells and 3T3-L1 adipocytes, respectively)[5]. In another HTS of 2000 compounds[6], compounds structurally related to phenothiazine group were identified as inhibitors of FA uptake. This included the atypical antipsychotics, chlorpromazine and clozapine as hits. Both compounds at 80µM concentration when tested in yeast cells caused intermediate levels of inhibition of FA- analog C1-BODIPY-C12 [6]. Thus it was predicted that dyslipidemia caused by these antipsychotics is due the mechanism involving the inhibition of FA uptake using FATPs.

In the current study, we have further characterized CB5 for its activity as an inhibitor in various cell lines that are models for pancreatic islets, muscles and human adipocytes. CB5 inhibition was specific for long and very long chain fatty acid and was protective against lipotoxicity caused by saturated fatty acids. We also compared the inhibition activity of AAP drugs to CB5 and show that their mechanism of action in causing dyslipidemia is different from inhibition of FA uptake and thus do not involve FATPs.

MATERIALS AND METHODS:

Materials:

CB5 (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)- one) and its analogs were purchased from ChemBridge Corporation (San Diego, CA, USA). Fluorescent fatty acid analog C1-BODIPY-C12 (4,4-Difluoro-5-Methyl-4-Bora- 3a,4a-Diaza-s-Indacene-3-Dodecanoic Acid) (Catalogue no. D-3283), BODIPY-FL-C5 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoic Acid) and BODIPY-FL-C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3- Hexadecanoic Acid) were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA). First-generation antipsychotic drug Haloperidol (H1512), second-generation drugs Clozapine (C6305) and Risperidone (R3030); 13C labeled oleic acid (catalog no. 490431) and tyloxapol (catalog no. T0307) were purchased from Sigma Aldrich. Olanzapine (O253750) was purchased from Toronto Research Chemicals (Ontario, Canada) and Quetiapine purchased from Key Organics/BIONET (External ID: KS-1099). Pancreatic lipase inhibitor, Orlistat, was a gift from GlaxoSmithKline (60mg per capsule). Palmitic- acid (PA) (P-0500) was purchased from Sigma and 50mM stock prepared in 100% ethanol. Fatty acid- free and nuclease, protease-free Bovine Serum Albumin (BSA) was purchased from CalBiochem (Cat. No. 126609). Verapamil, 0.1M PBS (pH 7.4), 10mM NADPH (freshly prepared in PBS), acetonitrile, human and mouse (Balb-C) microsomes (as used by Kansas University).

METHODS

For each cell type, fatty acid transport kinetics were evaluated according to the method described in Arias-Barrau, et al. [7]. A range of

C1-BODIPY-C12 concentrations, as specified in Figure 2.1, were used in a 5 min assay conducted in kinetic mode on a BioTek Synergy plate reader using Gen5.2 software. Uptake was measured every 5 sec. The substrate was presented to the cells as a complex with fatty acid-free BSA to give BODIPY-FA to BSA ratios of 0.5:1 to 20:1 (2.5-100 µM C1-BODIPY-C12 and 5µM BSA). Non-cell associated fluorescence was quenched with trypan blue as described. Uptake was measured at 485 nm excitation and 528 nm emission. The level of C1- BODIPY-C12 transported into the cell was calculated by conversion of the relative fluorescence units (RFU) to concentration of C1-BODIPY-C12 calculated from a standard curve generated for each lot of ligand. These experiments allowed the apparent KT and maximal rate (Vmax) of C1-BODIPY-C12 transport to be defined. The experiments were repeated three times in triplicate for each cell type and then the data was analyzed using Prism® 5.0 software.

STATISTICALANALYSIS

A minimum of 3 experiments, each assayed in triplicate, were used for statistical comparison as stated within the legends of the figures. Data were compared using JMP v11 analysis software (SAS Inst., Inc., Cary, N.C., USA). Significance of difference was determined using ANOVA, Student's paired t distribution, or bivariate fit Y by X. Values were considered statistically significant at $p < 0.05$.

RESULTS AND ANALYSIS

Kinetics of C1-BODIPY-C12 in different cell lines

The Kinetics of transport of C1-BODIPY-C12 was determined in HepG2, Caco-2, INS-1E, C2C12 and human adipocytes. This was done to monitor the transport of fatty acids in mentioned cell lines in real time using an assay previously described by our lab [7]. As shown in Figure 2.1, the transport of FA-analog C1-BODIPY-C12 follows a typical Michaelis-Menton kinetics. The accumulation of FA-analog was least efficient in INS-1E cells. These cells had a Vmax of 17.1±1.5 µmol/min/106 cells which was 2.4-fold lower than muscle cell line C2C12, 4-fold lower than Caco-2 and HepG2 cells, and 32-fold lower than human adipocytes. The KT values were more similar in all the cell lines tested which suggests that affinity for fatty acids may be equivalent between the cell lines but the uptake and storage capacity is variable.

Inhibition of C1-BODIPY-C12 uptake in C2C12, INS-1E and human adipocytes CB5 was identified as a FATP2-mediated FA uptake inhibitor in a high throughput screening (HTS) of chemical compounds in humanized yeast. Further testing of CB5 in HepG2 and Caco-2 cells, models for liver and small intestines, confirmed the role of CB5 as an inhibitor of fatty acid uptake [5]. However, the process of fatty acid metabolism is also interlinked with muscle and fat metabolism. Additionally, pancreatic control of glucose homeostasis is coordinated with regulation of FA metabolism and pancreatic β- cells are particularly sensitive to FA-mediated lipotoxicity [8]. The action or effect of changes on one tissue/organ, affects the nearby tissues in one way or another. To test the sensitivity of different model cell lines to CB5, we compared the inhibition activity of CB5 in C2C12, INS-1E and human adipocyte cell lines, models for muscle, pancreatic β- cells and adipose tissues. CB5 was effective in inhibiting the uptake of FA analog C1- BODIPY-C12 in C2C12 and INS-1E (FATP2 expressing cell lines [9]) cells with IC50s of 10.6 and 8.3µM, respectively. On the other hand, the half-maximal inhibition value (IC50) was much higher in human adipocytes (58.2µM). This is almost 6- fold higher than HepG2 cells and 9-fold higher than Caco-2 cells that express FATP2 at high concentrations [9]. Furthermore, CB5 inability to inhibit uptake of FA analog BODIPY in human adipocytes is at advantage because it is a store house of fat and we expect it to store maximum fat which is re-routed from other non-adipose tissues such as liver, muscle and pancreas.

Examination of CB5 activity in inhibiting uptake of fatty acids with different chain lengths

To assess the inhibitory effect of CB5 against transport of FA with different chain length, we employed BODIPY FL-C5, a medium chain FA- analog and BODIPY FL-C16, a very long chain FA-analog in studies employing HepG2 and Caco-2 cell lines. Medium chain length fatty acids (C6-C10) are transported into the cells via diffusion whereas protein- mediated processes are required for transport of long chain and very long chain fatty acids. Our data demonstrated that the uptake of medium chain FA-analog was not saturable and exhibited a protein-independent mechanism of uptake [9]. However, the uptake of very long chain FA-analog was saturable and displayed a typical Michaelis- Menton kinetics [9]. CB5 was effective in inhibiting the uptake of BODIPY FL-C16 in HepG2 and Caco-2 cells with IC50 values of 17.0±2.5µM and 13.5±1.1µM, respectively. As expected, CB5 did not affect the transport of BODIPY FL- C5 in either of the cell lines since the uptake of small and medium chain fatty acids occur via simple diffusion [10].

CB5 protects against lipotoxicity induced by saturated fatty acids

Saturated free fatty acids, particularly, palmitic acid are known to induce lipotoxicity and apoptosis in various cell lines. We predicted CB5 would be protective against palmitate-mediated lipotoxicity. Therefore, HepG2, INS-1E cells (model for liver and pancreatic β-cells) and freshly isolated primary hepatocytes from 129S1/SvImJ mice were treated with varying concentrations of palmitic acid (0-500µM) without or with CB5 (5-50µM). As the concentration of PA increases, lipid accumulation increases in a dose dependent manner. However, when CB5 is added along with PA, 50µM of CB5 significantly reduces lipid accumulation in HepG2 cells and primary hepatocytes. We also see a reduction in the lipid accumulation without palmitate addition and at 5 and 10µM CB5 conc., which could be the effect of CB5 in reducing the entry of fatty acids present in the media, and thus decreasing lipid accumulation. In INS-1E cells, 5µM CB5 significantly ($p=0.001$) reduced lipid accumulation at ≤250µM PA. The effectiveness of CB5 in reducing lipid accumulation at much lower concentrations in INS-1E cells is advantageous since these cells do not generally store fat. This would help in further reducing or preventing the state of lipotoxicity and thus associated cellular dysfunction in diseased states of obesity, T2DM and hyperlipidemia [11].

High concentrations of saturated fatty acid, particularly palmitic acid also affect the nuclear integrity of the cells and thus leads to apoptosis. This was assessed by using DAPI staining of live cells treated with increasing concentrations of PA. Increasing concentrations of DAPI staining is indicative of apoptosis. As the concentration of palmitate increases, nuclear integrity of the cells is compromised thus leading to an increase in DAPI staining. Ten µM of CB5 was effective in maintaining the nuclear integrity and thus protection from apoptosis in HepG2 and INS-1E cells treated with 500µM PA. A trend was evident at lower concentrations of CB5 (5- 10µM) in primary hepatocytes and 50µM of CB5 significantly reduced DAPI staining and thus, protected the nuclear integrity of cells.

Mechanism of action of atypical antipsychotics differs from CB5

CB5 and atypical antipsychotics, chlorpromazine and clozapine and related compounds, were identified as inhibitors of FA uptake in humanized yeast during the HTS of two different libraries[5,6]. In those screens, 80µM of chlorpromazine and clozapine showed intermediate levels of inhibition activity in yeast cells. In Caco-2 cells, the percent inhibition by chlorpromazine was only 50% at concentrations of 100µM and 10% at 10µM [6]. In order to compare the inhibitory effects of atypical antipsychotics with CB5, a set of second generation antipsychotics (SGA) known to cause hyperlipidemia in schizophrenic patients were subjected to live cell assay in HepG2 cells. The set of compounds tested included the SGAs Clozapine, Olanzapine, Quetiapine and Risperidone; along with a first generation antipsychotic (FGA) Haloperidol and a pancreatic lipase inhibitor, Orlistat. Orlistat was used as a control because it blocks the activity of pancreatic lipase in the gut and thus prevents the breakdown of triglycerides from the diet. Unhydrolyzed TAGs are not converted into absorbable FA and are excreted undigested. However, it is associated with various adverse effects such as diarrhea, hypertension, depression, diabetic ketoacidosis and oedema. These side effects makes it unsuitable for use as a drug.

All of the tested antipsychotic drugs were ineffective in inhibiting C1-BODIPY-C12 uptake in HepG2 cells (concentration ranging from 0-640µM) (data not shown). As expected, Orlistat also had no effect on C1-BODIPY-C12 uptake since it's a pancreatic lipase inhibitor and does not affect fatty acid uptake directly. CB5, on the other hand, could inhibit the uptake of C1-BODIPY-C12 with an IC50 of 9.6µM. We also determined the extent of lipid accumulation in the presence of varying concentrations of PA (500-100µM) and 50 or 100µM AAP drugs, orlistat or CB5. All the compounds exhibited only modest decreases in lipid accumulation at concentrations of 50 and 100µM. Cells were also not protected against nuclear fragmentation, except modest protection by clozapine and quetiapine as evidenced by DAPI staining.

Orlistat, which inhibits FA absorption by inhibiting pancreatic lipase, did not show any effect either, as expected. CB5, on the other hand, was protective against palmitate induced lipid accumulation and apoptosis.

DISCUSSION

In a previous study from our lab, CB5 was identified as an inhibitor of

FATP2-mediated fatty acid uptake in a HTS screening in yeast expressing humanFATP2 and was subsequently characterized further in cell lines that are models for liver, enterocytes and adipocytes[5]. In the present study, we have further assessed the activity of CB5 in cell lines that are models for pancreas, muscle and human adipocytes. CB5 was effective in inhibiting the uptake of fatty acid analog in INS-1E and C2C12 cells but the uptake inhibition was least efficient in human adipocytes, the store-house of fat. This is advantageous to us since we expect to re-route the excessive fat to adipose tissue and thus, reduce the propensity to develop lipotoxic diseases. Importantly, we have demonstrated that CB5 was able to attenuate lipid accumulation and apoptosis in HepG2 cells, INS-1E cells and primary hepatocytes in a dose dependent manner thus confirming its role as a protectant against lipotoxic diseases.

CB5 inhibits the uptake of long chain fatty acids as determined using FA analog C1- BODIPY-C12 [5]. Long chain and very-long chain fatty acids move across the cell membrane using proteins in a carrier-mediated manner. However, short and medium chain fatty acid move across the cell membrane via diffusion. CB5 was ineffective in inhibiting the uptake of the medium chain FA analog BODIPY FL-C5 suggesting its role as a long chain FA uptake inhibitor. Further, it was the effective in inhibiting the uptake of very-long chain FA analog BODIPY FL-C16 in low micro-molar ranges. Thus, CB5 is specifically an inhibitor for long and very long chain fatty acids. This is in accordance with our earlier data that show FATP2 has a preference for the uptake of long chain fatty acid and activation of very long chain fatty acids [12].

On the basis of aforementioned properties of CB5, we predicted that CB5 could be useful in attenuating lipotoxicity caused by saturated fatty acids in the diet. Western diet is constituted mainly of saturated fatty acids which is correlated with various life style diseases including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM) and insulin resistance [13]. Also, palmitic acid is known to induce lipid accumulation and apoptosis in hepatocytes as well as pancreatic β-cells. Thus, CB5 was used to assess its protective effects against the saturated fatty acid, palmitic acid. After 24hrs of treatment with palmitic acid and CB5, CB5 was able to attenuate the lipid accumulation and protect from apoptosis in primary hepatocytes as well as cell lines that are model for liver and pancreatic β-cells in a dose dependent manner.

Other potential hits identified during HTS involved the structurally related phenothiazine group of compounds. Chlorpromazine and clozapine, the atypical antipsychotic drugs, were also identified as hits [6]. Typical (or first generation antipsychotics-FGA) and atypical (or second generation antipsychotics-SGA) antipsychotics are used for the treatment of various mental disorders ranging from psychosis, bipolar disorder, depression and schizophrenia. The ability of FGA to cause extrapyramidal effects (EPS) such as acute dyskinesia's and dystonic reactions, tardive dyskinesia, Parkinsonism etc. led to the discovery of SGA, which had a greater ability to treat mental disorders without EPS. Unfortunately, atypical antipsychotics are known to cause other metabolic side effects including weight gain, dyslipidemia, insulin resistance, glucose intolerance, overt diabetes and in rare cases diabetic ketoacidosis, thus increasing the risk of cardiovascular disease events [14].

Since clozapine and chlorpromazine were identified as inhibitors of fatty acid uptake in the yeast based assay system [6], we tested and compared a set of antipsychotic drugs in HepG2 cells with CB5 to provide insight into the mechanism of dyslipidemia caused by them. All the antipsychotics tested were unable to inhibit the uptake of FA-analog C1-BODIPY-C12 in HepG2 cells. Also, they were unable to prevent lipid accumulation in HepG2 cells that were treated with palmitic acid along with compounds (antipsychotics and Orlistat). Olanzapine and clozapine have the greatest propensity for inducing weight gain followed by risperidone and quetiapine and least for FGA haloperidol [15].

However, olanzapine exhibited modest reductions in accumulation of lipid in cells at high concentrations of 50 or 100µM. CB5 was still the best compound in protecting the cells against the lipid accumulation and apoptosis caused by saturated fatty acid, palmitate.

Various studies have recently associated the alterations in lipid metabolism caused by AAP to transcriptional dysregulation caused by sterol regulatory element-binding protein, SREBP1 and 2 in liver.

Inability of the SREBPs to control AMPK signaling and triglyceride metabolism [16] leads to an increase in lipid synthesis and thus dyslipidemia. This suggests that antipsychotics cause weight gain from mechanisms that do not involve inhibition of fatty acid uptake using FATPs.

Table: Kinetic parameters of C1-BODIPY-C12 transport in different cell lines. The values are represented as ± standard error.

Cell Line	V_{max} (µmol/min/10^6 cells)	K_T (µM)
HepG2	67.3±6.6	40.1±8.7
Caco-2	63.5±5.8	32.8±7.1
C2C12	40.1±1.9	20.9±2.6
INS-1E	17.1±1.5	18.1±4.3
Human Adipocytes	559.2±57.5	43.7±9.7

CONCLUSION

This study confirms the role of CB5 as a FATP2-mediated fatty acid uptake inhibitor. The exact mechanism by which CB5 inhibits fatty acid uptake is still not clearly understood. However, CB5 is effective in inhibiting specific class of fatty acid that are transported in a protein-mediated manner and does not affect fatty acids that are transported via diffusion. It is also effective in inhibiting the transport of fat into the cell lines that do not normally store excess fat and is least effective in inhibiting the transport in the adipose tissue that is considered the storehouse of fat. CB5 was also protective against the accumulation of lipids and apoptosis generated by saturated fatty acid, palmitic acid, in cell lines that are models for liver and pancreatic β-cells. However, inability of atypical antipsychotics to inhibit the uptake of FA-analog in HepG2 cells or to protect them against palmitate-induced lipid accumulation and nuclear fragmentation confirms that a different mechanism is causing weight gain and dyslipidemia in schizophrenic patients and it does not involve FATPs. Thus, CB5 blocks fatty acid transport without affecting other cellular functions and helps to partition toxic fatty acids away from cells thus attenuating weight gain and dyslipidemia in lipotoxicity related diseases.

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