Novel Effects of Mibefradil, An Anti-Cancer Drug, on White Adipocytes

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NOVEL EFFECTS OF MIBEFRADIL, AN ANTI-CANCER DRUG, ON WHITE ADIPOCYTES

by

SONIA JOSEPH

Under the Direction of Vincent Rehder, PhD

ABSTRACT

The present study was undertaken to investigate the effects of the T-type calcium channel blocker, Mibefradil, on white adipocytes. Unexpected for a T-type channel blocker, Mibefradil was found to increase intracellular calcium levels, cause lipid droplet fusion, and result in cell death. Calcium imaging of white adipocytes showed an increase of calcium concentration by Mibefradil at concentrations ranging from 10-50 µM. The elevation in calcium by Mibefradil was significantly reduced by pretreatment of cells with Thapsigargin, an endoplasmic reticulum (ER) specific Ca ATPase inhibitor. Additionally, lipid droplet fusion and cell death were also attenuated by Thapsigargin pretreatment in white adipocytes. We conclude that Mibefradil elevated intracellular calcium levels, induced lipid droplet fusion and cell death in white
adipocytes via mobilizing intracellular calcium stores from the ER. These results describe novel effects of Mibefradil on white adipocytes and may provide new insight into how this drug might be repurposed in obesity research.
NOVEL EFFECTS OF MIBEFRADIL, AN ANTI-CANCER DRUG, ON WHITE ADIPOCYTES

by

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NOVEL EFFECTS OF MIBEFRADIL, AN ANTI-CANCER DRUG, ON WHITE ADIPOCYTES

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1 METHODS

Cell Culture

All cells were maintained at 37 °C in a 5% CO2 incubator. White preadipocytes were kindly provided by Dr. Bingzhong Xue’s Lab. To differentiate mature adipocytes, we plated preadipocytes with pre-adipocyte medium [Dulbecco’s Modified Eagle Medium (DMEM), 1:1 (v/v) containing 10% fetal calf serum, 15mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and antibiotics]. Cells were grown into 90% confluence in growth medium and were then induced to differentiate with a differentiation medium [DMEM, 1:1 (v/v) containing 10% fetal bovine serum, 15mM HEPES, 33µM biotin, 17 µM pantothenate, 100nM insulin, 1 µM dexamethasone, 0.25 mM isobutyl methylxanthine (IBMX), 1 µM BRL49653, 100 U/ml penicillin, and 100 µg/ml streptomycin]. After two days in differentiation cocktail, cells were changed to and maintained in adipocyte medium [ DMEM/Ham’s F-10, 1:1 (v/v), 0.2% FBS, 15 Mm HEPES, 33 µM biotin, 17 µM pantothenate, 100 U/ml penicillin, and 100 µg/ml streptomycin]. At day 6, all the cells were differentiated.

Image acquisition and analysis

Petri dishes containing white adipocytes were placed on a fixed stage that was centered below the objective of an upright microscope (BS51W1F, Olympus, Japan). The upright microscope was mounted on a manual translation stage (Siskiyou, San Francisco, CA). Phase contrast and fluorescent images of white adipocytes were captured before (-5 and 0 min) and at defined times after Mibefradil application. If pharmacological inhibitors were used in combination with Mibefradil application, the inhibitor was directly applied to the dish for the desired amount of time at the defined 5 min or 5 min 30 sec time marker. In the figures, changes in calcium concentration were expressed as a percent change comparing concentrations just before drug
addition and peak calcium levels after drug addition. In all experiments, the first calcium peak was used for analysis, because this was demonstrated to be the direct result of drug action. For cells in which the calcium concentration did not return to baseline calcium levels and that showed a continued increase in calcium level it was difficult to identify the first peak. In such cases, the time point at which all other cells showed their first peak after treatment was designated as the first peak value for a cell. Statistical analysis was performed with the software ORIGIN DATA ANALYSIS AND GRAPHING software (OriginLab, Northampton, MA) Significance between conditions was determined by using either a Student’s t-test and ANOVA. All data were expressed as mean ± SEM.

Ca2+ imaging

Cells were loaded with 1mM fura-2 AM (Molecular Probes, Eugene, OR, USA) in DMSO and allowed to sit for 30 min to give the dye time to diffuse. Care was taken to load cells just enough to yield sufficient fluorescence and avoid overloading with Fura-2. Cells were washed 3x with extracellular medium (refer to pharmacological agents) then allowed to sit for 20 min for desertification. Acquisition and analysis of calcium images were conducted using Live Acquisition and Offline Analysis software (Till Photonics, FEI, Germany). Images were acquired using 340 nm and 380 nm excitation wavelengths with 100ms exposure time at 5% light intensity every 30 sec. The I cytosolic calcium concentration within an adipocyte was determined by placing a box into the cytosol area of each adipocyte and by calculating the ratio of emission signals at 340 and 380 nm after background subtraction. This ratio was used as a measure of underlying changes in intracellular calcium concentration. No actual calcium concentrations can be reported, because the fluorescence values were not calibrated on the imaging system. Adipocytes were excluded from analysis if their pretreatment ratios exceeded a ratio of 1, which
indicated a calcium concentration above normal resting values, as established in earlier experiments on the system.

**Pharmacological Agents**

Extracellular medium: 152 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 2 mM Calcium Chloride, 10 mM of glucose. Zero calcium extracellular medium: Same ingredients as extracellular medium excluding calcium chloride and addition of 5 mM EGTA. FCCP (Tocris) and Mibefradil (Tocris) were dissolved in DMSO. Final dilutions of DMSO in solutions were less than 1:1000. FCCP was dissolved in 72 µl of DMSO to make a stock solution of 5 mM. Mibefradil was dissolved in 172 µl of DMSO to make a stock solution 100 mM.
2 INTRODUCTION

Obesity refers to a condition where excess fat accumulates on a person’s body posing various health risks to the individual. The word ‘obesity’ is sometimes used synonymously with the word ‘adiposity,’ which refers to morbid or severe overweight. Obesity alters the endocrine and metabolic functions of white adipose tissue and increases the release of proinflammatory molecules, hormones, and fatty acids. In principle, the white adipose tissue functions as an energy reserve or active organ that employs different mechanisms to influence energy homeostasis (Bambace et al., 2013). Adiposity has been described as the part of body mass that comprises neutral lipids stored in the adipose tissue (Weisberg et al., 2003). It correlates closely with vital physiological parameters like leptin concentrations, insulin sensitivity, and blood pressure. Weisberg et al. (2003) reported the existence of positive correlations between adiposity and obesity-related disorders, especially glucose intolerance, dyslipidemia, and hypertension. However, the authors noted that visceral fat mass shows a closer correlation with obesity-related pathology than the overall adiposity.

Adipose tissue plays a vital role in the lives of mammals because it serves as the primary source of free fatty acids (FFA) during postprandial fasting for heat production and energy use (Balistreri et al., 2010). Typically, mammals have two forms of adipose tissue: brown and white tissues (Gesta et al., 2007). The two types of adipose tissue have different functions, as well as different cellular localization and composition. White adipose tissue (WAT) is the major constituent of the adipose tissue and provides the largest proportion of body fat and FFA. In principle, WAT is distributed in various anatomic sites of the body with its primary depots being intra-abdominal areas, peri-renal and intestinal sites, and subcutaneous areas of the abdomen, thighs, and buttocks (Gesta et al., 2007). According to Wozniak et al. (2009), WAT appears to be
associated with two principal functions. First, it helps to control metabolism through insulin sensitivity, adipocyte differentiation, and energy homeostasis. Secondly, it influences inflammation through control mechanisms mediated by various anti-inflammatory molecules, as well as activated immune and anti-inflammatory pathways (Balistreri et al., 2010). Although each WAT subgroup has a specialized role, the excessive accumulation of WAT at any site of the body influences the occurrence of obesity, as well as obesity-related illnesses. The most common WAT excess occurs around the waist resulting in central obesity or ‘android obesity,’ which intensifies the risk of certain inflammatory pathologies (Balistreri et al., 2010). WAT excess may also occur in lower parts of the body causing the development of ‘gynoid obesity,’ which lacks metabolic complications (Gesta et al., 2007).

Several hypotheses have been developed to shed light on the distribution of WAT and its relationship with inflammatory and metabolic complications. Among the different theories, two principal hypotheses have been given considerable attention. The first theory concentrates on central obesity’s anatomy and its potential to drain inflammatory mediators and FFA into portal circulation. In turn, the drained mediators and FFA act on the liver preferentially and affect metabolic reactions significantly (Gesta et al., 2007). The second hypothesis considers the role of different WAT cell properties in the development of inflammatory and metabolic diseases. Gesta et al. (2006) detected key differences in gene expression between different WAT deposits in both humans and rodents. Interestingly, other researchers have also observed a distinct mediator profile between peripheral and visceral WAT, which seems to elucidate the link that exists between metabolic complications and central obesity (Balistreri et al., 2010). The mediator profile offers additional evidence for the heterogeneity of the cells of WAT (Gesta et al., 2007). Also, WAT comprises fully-developed adipocytes and cell types such as macrophages,
endothelial cells, fibroblasts, and preadipocytes, which are categorized as the stroma vascular fractions (Cancello and Clement, 2006; Subramanian and Ferrante Jr., 2009). The macrophages, preadipocytes, and adipocytes have inflammatory and metabolic functions that allow the WAT to release mediators with varying biological effects (Fantuzzi, 2008; Lago et al., 2009). In particular, macrophages influence the circulating level of different inflammatory molecules and determine low-grade obesity-associated inflammation (Zeyda and Stulnig, 2007).

In the mammalian body, energy is preserved in lipid droplets as triacylglycerols. Recently, researchers have made significant breakthroughs in promoting the understanding of lipid droplets’ biology (Walther and Farese Jr., 2012; Brasaemle and Wolins, 2012). In particular, microscopic observation has been identified as one of the most effective approaches to the visualization of the striking capacity of the white adipocyte lipid droplets (LDs) to store lipids (Konige et al., 2014). The majority of mature adipocytes contain a single lipid droplet within the size range of 25 to about 150 μm in diameter (Konige et al., 2014). The droplet occupies the largest cell volume and, thus, determines the size of the adipocyte. Also, adipose depots have been known to enlarge through the mechanisms of hyperplasia and hypertrophy, which require extensive and coordinated cellular and structural alterations to accommodate the growth and emergence of the LD. Different studies have demonstrated that LDs control the capacity and efficiency of adipocytes to carry out the esterification of fatty acids to form triglyceride and, hence, protect the triglyceride stores inside the cells (Puri et al., 2008). The proteins surrounding LDs comprise the ‘PAT’ proteins perilipin ADRP and TIP47 that target LDs and regulate the biogenesis and size of such organelles (Puri et al., 2008). Investigators have further described Cidec/FSP27 as a unique protein associated with lipid droplets, which negatively controls lipolysis and enhances triglyceride accumulation in adipocytes (Puri et al., 2008). Cidec/FSP27
has been classified as a CIDE-N type of domain-containing proteins in the protein family of CIDE, which includes three protein members found in mice: FSP27; Cideb; and Cide (Puri et al., 2008). The CIDE family also includes four human proteins: CIDEC; CIDEB; and CIDEA. Notably, the proteins have a typical C-terminal containing CIDE-C domain and an N-terminal with a CIDE-N domain. In addition, the proteins have an important homology occurring between CIDE-N domains of the CIDE proteins and regulatory domains associated with apoptotic factors of DNA fragmentation, DFF45 (a DFF40 inhibitor) and DFF40 (nuclease activated by caspase) (Puri et al., 2008). Researchers also found that Cidec/FSP27 acts as an LD-related protein in the adipocytes of mice to control fat deposition. Moreover, Cidea has been known to show high expression in adipose tissues. In mice, the protein is primarily restricted to the brown adipose tissue but highly expressed in the human WAT. Puri et al. (2008) found substantial evidence supporting the view that Cidea localizes to LDs and controls the deposition of triglyceride in various cell types, including adipocytes. Remarkably, the researchers discovered that the expression of both Cidec/FSP27 and Cidea in the WAT of overweight human subjects shows a positive correlation with insulin sensitivity in the body, as well as the LD protein perilipin. Thus, obesity has been identified as one of the primary causes of diabetes.

The magnitude and prevalence of obesity, especially in adolescents, is on the rise. Apart from environmental and genetic factors, the intake of calcium has been described as a key dietary component that shows an inverse relationship with obesity and body mass index (Burbano et al., 2016). Recently, apoptosis modulation in the adipose tissue has emerged as a promising strategy against obesity because the elimination of adipocytes is expected to reduce body fat significantly. Studies have shown that Ca$^{2+}$ signaling induces apoptosis in the adipocytes (Parikh and Yanovski, 2003). Therefore, intracellular calcium has a critical function in the metabolic
derangements linked to obesity. Shi et al. (2000) pointed out that increasing intracellular calcium by stimulating voltage-mediated or receptor calcium channels stimulates the activity and expression of the fatty acid synthase (FAS). FAS is crucial to the commencement of lipogenesis and inhibits both agonist-stimulated lipid breakdown and basal lipolysis in adipocytes. Such effects are often reversed through the antagonism of calcium channels (Shi et al., 2000). Accordingly, increasing intracellular calcium seems to exert a coordinated regulation over lipolysis and lipogenesis, which enhances the accumulation of triglyceride in adipocytes. As such, an increase in intracellular calcium simultaneously stimulates lipogenesis and suppresses lipolysis resulting in adipocyte hypertrophy and lipid filling. Past investigations have further implicated Ca\textsuperscript{2+} in the regulation of adipogenesis, which is thought to promote obesity in humans and rodents (Shi et al., 2000). For instance, increasing intracellular calcium, by inhibiting the CaATPase or stimulating the influx of calcium ions has been known to suppress the initial stages of adipocyte differentiation in murine (Shi et al., 2000). Thus, the effect of calcium signaling on body fat reduction provides a powerful rationale for developing mechanistic approaches to the study of calcium’s anti-obesity properties. Shi et al. (2000) reported that increasing intracellular calcium in the early phases of differentiation resulted in the suppression of adipocyte differentiation in humans, which matched previous observations about adipocyte differentiation in murine. On the other hand, increasing intracellular calcium during the late phases of differentiation enhanced human adipocyte differentiation (Shi et al., 2000). Therefore, considerable evidence suggests that promoting Ca\textsuperscript{2+} signaling has a biphasic regulatory function in the differentiation of human adipocytes. As a result, it inhibits the initial differentiation stages while enhancing the final stages of lipid filling and differentiation (Shi et al., 2000). The role of Ca\textsuperscript{2+} signaling in the differentiation of adipocytes not only elucidates the processes of energy
homeostasis and adipogenesis but also establishes a critical target for the future development of effective therapeutic interventions in obesity. Accordingly, Bagchi and Preuss (2012) recommended the use of dietary calcium as an anti-obesity strategy. The authors demonstrated that an increase in dietary calcium confers protection against obesity by reducing intracellular calcium levels, which in turn decreases lipid storage in the adipocytes.

Our research on white adipocytes focused primarily on the presence of voltage gated calcium channels and their functional roles. Several voltage gated calcium channel blockers were utilized in our experiments. However, treatment of one such drug, Mibefradil, had the effect of increasing calcium levels in white adipocytes. Typically, Mibefradil is categorized as a calcium channel blocker because it affects calcium movements into the cells of blood vessels and the heart. Consequently, the agent relaxes the blood vessels while increasing oxygen and blood supply to the heart to minimize its workload. Additionally, Mibefradil functions as a tetraline substituted with benzimidazole. Thus, it selectively inhibits and binds the T-type forms of calcium channels. More specifically, Mibefradil acts as a tetralol-related calcium channel blocker that prevents the movement of calcium across the low-voltage T calcium channels and the high-voltage L calcium channels in the vascular and cardiac smooth muscles (DrugBank, 2016).

However, the drug is relatively more selective for T-type channels. Usually, vascular smooth muscles experience vasodilation, which decreases both blood pressure and peripheral resistance in the vascular vessels. Thus, chronic dosing of the drug often increases cardiac output slightly (DrugBank, 2016). Research has further demonstrated that Mibefradil slows the atrioventricular node and sinus conduction, which leads to an increase in peripheral resistance and decrease in the heart rate. In addition, the drug has been known to prolong the recovery time of the corrected sinus node and lengthen AH interval while raising the Wenckebach point (DrugBank, 2016). The
role of Mibefradil as a calcium channel blocker is essential to the antagonism of calcium channels that reverses the effects of FAS (member of the death receptor family). In our study with adipocytes, the calcium channel blocker Mibefradil had the unusual effect of raising intracellular calcium levels. It also induced lipid droplet fusion and cell death. Since calcium signaling has dual functions in the maintenance of homeostasis and the triggering of adipogenesis and lipid droplets regulate the storage and release of triacylglycerols, investigating the role of Mibefradil in calcium signaling, lipid droplet changes, and adipocyte apoptosis could provide us with possible strategies to control and overcome obesity.
3 RESULTS

*Application of Mibefradil to white adipocytes results in an increase in intracellular calcium.*

The first experiment was to test the effect of Mibefradil on intracellular calcium levels. White adipocytes were loaded with Fura-2 (see Methods for details). We examined the effect of Mibefradil on these Fura-2 loaded adipocytes using a microscopy system specialized for fast calcium imaging (see Methods for details). Baseline values were acquired for 5 minutes prior to the application of Mibefradil, and acquired 30 minutes after the addition of Mibefradil to a dish containing Fura-2 loaded adipocytes. Mibefradil application resulted in an increase in intracellular calcium, which was evident by the increase in the fluorescence intensity ratio following the Mibefradil treatment (Fig. 1A-E). In most cases, after the maximum peak had been reached calcium levels declined close to baseline values seen prior to Mibefradil treatment (Fig. 1). Responses in individual adipocytes were variable though, as shown in Fig. 1D, where one cell upon 40 µM Mibefradil treatment responded with the characteristic rise and subsequent fall of calcium levels, while the other cell showed calcium levels that continued to rise after Mibefradil treatment.

Analyzing experiments of white adipocytes treated with 10 µM Mibefradil showed a significant increase in calcium at P<0.001 (Fig 2). To determine dose-dependency of the drug, Mibefradil concentrations of 10 µM, 25 µM, 30 µM, 40 µM, and 50 µM were each tested. Among the varying concentrations of Mibefradil on average an increase in intracellular calcium levels was seen about 3 min after addition (Fig. 1A-E). A 10 µM Mibefradil treatment resulted in an increase in intracellular calcium levels that reached an average maximum of 0.86± 0.09. 25 µM, 30 µM, 40 µM, and 50 µM Mibefradil treatment resulted in increases in intracellular calcium levels that reached maximum values of 0.96 ± 0.06, 1.13 ± 0.19, 1.07 ± 0.06, and 1.25 ± 0.1,
respectively. Each of these concentrations induced a significant increase in calcium (P< 0.001). A One Way ANOVA test concluded that there was no overall significant difference (P>0.05) between treatments, suggesting that increasing concentrations of Mibefradil did not result in a dose-dependent effect (Fig. 3).
E.

Figure 1 A-E. Representative examples of Mibefradil induced calcium increases in white adipocytes. Fluorescent intensity ratiometric graphs showing the response of different concentrations of Mibefradil. Arrow indicates time of Mibefradil application. Number of cells denoted by N.

Figure 2. Mibefradil (10 µM) induced calcium increase in white adipocytes. Bar graph illustrates percent change of calcium. Mibefradil (MIB) dissolved in DMSO. DMSO treatment showed no significant increase in calcium levels (P>0.05). MIB treatment resulted in a significant increase in calcium peak levels (P<0.001). Number of cells denoted by N.
Figure 3. Different concentrations of Mibefradil induced calcium increases in white adipocytes. Bar graph illustrates average peak of calcium levels. Mibefradil dissolved in DMSO. There was no significant difference among the different concentrations of Mibefradil (ANOVA P>0.05). Number of cells denoted by N.

Application of Mibefradil to white adipocytes in extracellular medium devoid of calcium.

In order to test whether the increase in intracellular calcium upon treatment with Mibefradil was due to calcium influx into the cytoplasm, 10 µM Mibefradil was applied to adipocytes in an extracellular medium with nominally zero calcium and containing the calcium chelator EGTA (5mM). A 30 min treatment of white adipocytes with Mibefradil (10 µM) in a zero calcium medium led to a significant (P<0.001) increase in intracellular calcium. The lack of extracellular calcium did not interfere with Mibefradil’s effect of inducing a calcium increase in white adipocytes (compare to the effect of Mibefradil in normal medium, Figs. 1A, 2), suggesting that Mibefradil’s effect of raising the intracellular calcium concentration in adipocytes was not through calcium influx from the extracellular space, but likely resulted by calcium release from an intracellular source of calcium (Fig. 4). There was an apparent delay in the increase in
calcium levels, as well as a slower rise to peak in zero calcium medium, indicating that extracellular calcium might play an accessory role in producing a normal Mibefradil induced calcium response.

**Figure 4A-B.** Mibefradil induced calcium increase in white adipocytes in a zero calcium environment. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated
with 10 µM Mibefradil (MIB) in a zero calcium medium. B) Bar graph illustrates percent change of calcium levels. Mibefradil dissolved in DMSO. DMSO (5 min) and DMSO peak showed no significant increase in calcium levels (P > 0.05). Adipocytes treated with Mibefradil in a zero calcium environment showed a significant increase in calcium levels (P < 0.001). Number of cells denoted by N.

Mibefradil does not cause a cytosolic calcium increase via release from the mitochondria

Mitochondria are known calcium stores and we next examined the potential role of mitochondria in mediating the effect of Mibefradil on adipocytes. To deplete mitochondria of calcium, white adipocytes were pretreated with the oxidative phosphorylation uncoupler, FCCP. Addition of FCCP (10 µM) resulted in a transient increase in intracellular calcium levels that reached an average maximum value of 0.9 ± 0.05 and returned to pretreatment values within 30 min of application (Fig. 5). The increase in calcium levels following treatment was significantly different from controls (P < 0.001). In order to test whether the effect of a rise in intracellular calcium levels by Mibefradil was attributed to release from the mitochondria, Mibefradil (40 µM) was applied to adipocytes pretreated with FCCP.

Experiments with white adipocytes pretreated with FCCP followed by a treatment with Mibefradil (40 µM) did not inhibit Mibefradil from inducing an increase in intracellular calcium levels (Fig. 6A). Interestingly, calcium levels continued to rise in pretreated cells and did not recover (Fig. 6A). This appeared to indicate that the uncoupling of mitochondria and the depletion of mitochondrial stores made cells increasingly susceptible to the Mibefradil induced calcium increase.

Decreasing the concentration of Mibefradil to 30 µM produced similar results (Fig 7). Preincubation of 10 µM FCCP for 30 min did not block the effect of increasing intracellular calcium levels normally seen when Mibefradil was applied onto white adipocytes. When
comparing calcium levels before and after addition of Mibefradil in pretreated adipocytes with FCCP there was a significant difference at P<0.001 (Fig. 6B & 7B). Calcium levels after addition of Mibefradil did not return to pretreatment values and this result indicated that the source of calcium mobilized by Mibefradil was not the mitochondria. Given the strong and irreversible effect of 30µM Mibefradil on FCCP pre-treated adipocytes, future experiments should evaluate the effects of lower concentrations of Mibefradil.

Figure 5. FCCP induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated with 10 µM FCCP. Number of cells denoted by N.
Figure 6A-B. Pretreatment of FCCP (10 µM) did not block Mibebradil (40 µM MIB) induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over time in pretreated white adipocytes with 10 µM FCCP followed by 40 µM Mibebradil (MIB). B) Bar graph illustrates percent change of calcium levels. FCCP and Mibebradil dissolved in DMSO. DMSO (5 min) and DMSO (10min) showed no significant increase in calcium levels (P>0.05). Pre-FCCP (5 min)
and FCCP peak (10 min) showed a significant increase in calcium levels (P < 0.001). DMSO (35 min 30 sec) and DMSO (44 min) showed no significant increase in calcium levels (P>0.05). Post-FCCP (35min 30 sec) and Post-MIB peak (44 min) showed a significant increase in calcium levels (P < 0.001). The mitochondrial calcium store was depleted by treating cells for 30 min with a mitochondrial oxidative phosphorylation uncoupler, 10 µM FCCP. Number of cell denoted by N.
Pretreatment of FCCP (10 µM) did not block Mibefradil (30 µM MIB) induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over time in pretreated white adipocytes with 10 µM FCCP followed by 30 µM Mibefradil (MIB). B) Bar graph illustrating percent change of calcium levels. FCCP and Mibefradil dissolved in DMSO. DMSO (5 min) and DMSO (8 min) showed no significant increase in calcium levels (P > 0.05). Pre-FCCP (5 min) and FCCP peak (8 min) showed a significant increase in calcium levels (P < 0.001). DMSO (35 min) and post-MIB peak (1 hr-5 min) showed a significant increase in calcium levels (P < 0.001).
Post-FCCP (35 min 30 sec) and Post-MIB peak (1 hr 5 min) showed a significant increase in calcium levels (P < 0.001). The mitochondrial calcium store was depleted by treating cells for 30 min with a mitochondrial oxidative phosphorylation uncoupler, 10 µM FCCP. Number of cells denoted by N.

Mibefradil causes a cytosolic calcium increase via release from the endoplasmic reticulum

To test whether the endoplasmic reticulum was involved in the mechanism mediating the effects of Mibefradil on calcium levels in white adipocytes, we employed an inhibitor of the calcium ATPase pump in the ER, Thapsigargin. Thapsigargin (10 µM) application by itself induced a calcium transient in adipocytes (Fig. 8). Intracellular calcium levels reached a maximum of 1.07 ± 0.06 following Thapsigargin application and returned to pretreatment values within 30 minute of application. The increase in calcium levels following treatment was significant (P<0.001). We tested next the effects of application of Mibefradil to adipocytes pretreated with Thapsigargin. Following a 30 min treatment of Thapsigargin, Mibefradil was applied for a period of 30 min. Interestingly, the Mibefradil induced calcium increase was blocked in pretreated cells (Fig. 9). Preincubation of 10 µM Thapsigargin for 30 min blocked the effect of increasing intracellular calcium levels normally seen when Mibefradil was applied onto white adipocytes (P<0.001 compared with 10µM MIB alone; Fig 9c). Higher concentrations of Mibefradil (25 µM and 50 µM) were also tested (Figs. 10-11). At each of these concentrations, Thapsigargin abolished the Mibefradil induced calcium increase (Fig. 9-11). Taken together, these results suggested that Mibefradil induced calcium increase in white adipocytes was likely caused by calcium release from the endoplasmic reticulum.
Figure 8. Thapsigargin (THAP) induced a calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated with 10 µM THAP.
Figure 9A-C. Pretreatment of Thapsigargin (10 μM THAP) blocked Mibefradil (10 μM MIB) induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated with 10 μM Mibefradil (MIB). B) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over time in pretreated white adipocytes with 10 μM Thapsigargin (THAP) followed by 10 μM Mibefradil (MIB). C) Bar graph illustrates percent change of calcium levels. Thapsigargin and
Mibefradil dissolved in DMSO. DMSO (5 min) and DMSO (8 min) showed no significant increase in calcium levels (P>0.05). Pre-THAP (5 min) and THAP peak (8 min) showed a significant increase in calcium levels (P < 0.001). DMSO (36 min) and DMSO (46 min) showed no significant increase in calcium levels (P >0.05). Post-THAP (36 min) and Post-MIB peak (46 min) showed no significant increase in calcium levels (P > 0.05). Intracellular Ca stores were depleted by treating cells for 30 min with a Ca/ATPase inhibitor, 10 µM THAP. Pretreatment with 10 µM THAP abolished the MIB-induced increase in calcium levels (P <0.001 when comparing pre-MIB calcium levels with Post MIB peak calcium levels.) Number of cells denoted by N.
C.

Figure 10A-C. Pretreatment of Thapsigargin (10 µM THAP) blocked Mibefradil (25 µM MIB) induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated with 25 µM Mibefradil (MIB). B) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over time in pretreated white adipocytes with 10 µM Thapsigargin (THAP) followed by 25 µM Mibefradil (MIB). C) Bar graph illustrates percent change of calcium levels. Thapsigargin and Mibefradil dissolved in DMSO. DMSO (5 min) and DMSO (9 min) showed no significant increase in calcium levels (P>0.05). Pre-THAP (5 min) and THAP peak (9 min) showed a significant increase in calcium levels (P < 0.001). DMSO (35 min 30 sec) and DMSO (44 min 30 sec) showed no significant difference (P>0.05). Post-THAP (35 min 30 sec) and Post-MIB peak (44 min 30 sec) showed no significant increase in calcium levels (P>0.05). Intracellular Ca stores were depleted by treating cells for 30 min with a Ca/ATPase inhibitor, 10µM THAP. Pretreatment with 10 µM THAP abolished the MIB-induced increase in calcium levels (P<0.001 when comparing pre-MIB calcium levels with Post MIB peak calcium levels.) Number of cells denoted by N.
C. Figure 11A-C. Pretreatment of Thapsigargin (10 μM THAP) blocked Mibefradil (50 μM MIB) induced calcium increase in white adipocytes. A) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over a period of 30 min in white adipocytes treated with 50 μM Mibefradil (MIB). B) Representative graph of fluorescence excitation ratio (340nm/380nm) corresponding to intracellular calcium levels over time in pretreated white adipocytes with 10 µM Thapsigargin (THAP) followed by 50μM Mibefradil (MIB). C) Bar graph illustrates percent change of calcium levels. Thapsigargin and Mibefradil dissolved in DMSO. DMSO (5 min) and DMSO (8 min 30 sec) showed no significant increase in calcium levels (P>0.05). Pre-THAP (5 min) and THAP peak (8 min 30 sec) showed a significant increase in calcium levels (P < 0.001). DMSO (17 min) and DMSO (34 min 30 sec) showed no significant increase in calcium levels (P>0.05). Post-THAP (17 min) and Post-MIB peak (34min 30 sec) showed no significant increase in calcium levels (P>0.05). Intracellular Ca stores were depleted by treating cells for 30 min with a Ca/ATPase inhibitor, 10μM THAP. Pretreatment with 10 μM THAP abolished the MIB-induced increase in calcium levels (P<0.001 when comparing pre-MIB calcium levels with Post MIB peak calcium levels.). Number of cells denoted by N.
Application of Mibebradil to white adipocytes results in lipid droplet fusion.

Our first experiment of treating adipocytes with Mibebradil did not only result in increased intracellular calcium levels, but also induced smaller lipid droplets within cells to fuse to form larger droplets. Lipid droplet fusion was evident by comparing differential interference contrast (DIC) images of adipocytes before and after treatment (Fig. 12A). Also, shown are images of Fura-2 loaded adipocytes (380nm excitation) before and after lipid droplet fusion (Fig. 12B). To investigate any dose-dependent effect of Mibebradil on lipid droplet fusion, the following concentrations were used: 10 µM, 25 µM, 30 µM, 40 µM, and 50 µM. Each of these Mibebradil concentrations induced fusion (Tables 2a-6a). 10 µM, 25 µM, 30 µM, 40 µM, and 50 µM Mibebradil treatment resulted in lipid droplet fusion percentage values of 7.2 ± 2.9% (4 experiments), 34.5% (1 experiment), 45.5% (1 experiment), 16.4±8.1% (2 experiments), and 39.4±4.8% (9 experiments), respectively. Both 10 µM and 50 µM Mibebradil concentrations based on average values showed dose dependency whereby increasing concentrations produced an increased amount of lipid droplet fusion. Intermediate concentrations (25 µM, 30 µM, 40 µM) did not based on average values did show dose dependency, which could be due to the low number experiments performed with these concentrations. Only one experiment was completed for each of the 25 µM and 30 µM Mibebradil treatment and two experiments were completed for the 40 µM Mibebradil treatment. Therefore, dose dependency for these intermediate concentrations cannot be determined and additional experiments utilizing these Mibebradil concentrations need to be performed.
Figure 12A-B. A) Differential interference contrast image of unstained white adipocytes before (left) and after treatment with Mibefradil (right). Mibefradil (25 µM) treatment induced lipid droplets to fuse (above). B) Ratiometric image of fura-2 loaded white adipocytes before (left) and after treatment with Mibefradil (right). Mibefradil (25 µM MIB) treatment induced lipid droplets to fuse. Some fusion events are shown by arrows. Regions of interest (ROI) indicate areas of the cells used to measure Fura-2 fluorescence. White bar: 10 µm.

Application of Mibefradil to white adipocytes in an extracellular medium devoid of calcium induced lipid droplet fusion.

Lipid droplet fusion was a common occurrence after treating white adipocytes with Mibefradil. We investigated next whether the Mibefradil induced lipid droplet fusion depended on the presence of extracellular calcium. When adipocytes were treated with Mibefradil in a zero-calcium environment lipid droplet fusion was present in adipocytes (Table 1). In a medium containing calcium, Mibefradil (40µM) treatment resulted in 16.4±8.1% of cells (n= 2 experiments) showing lipid droplet fusion, while 49.4±12.0% of cells (n=3 experiments) showed fusion with the same treatment in a medium without calcium. These results suggested that
Mibefradil induced lipid droplet fusion was not inhibited by the lack of extracellular calcium. Fusion events in zero calcium were actually numerically higher, but given the low number of experiments and variability in results, additional experiments would have to be performed to test whether this trend was significant.

Table 1. Lipid droplet fusion in white adipocytes treated with 40 µM Mibefradil in normal and in zero calcium containing extracellular medium. Note that lipid droplet fusion ranged widely between experiments. Number cells denoted by N.

<table>
<thead>
<tr>
<th>40 µM Mibefradil in calcium containing medium.</th>
<th>40 µM Mibefradil in zero calcium medium.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment</strong></td>
<td><strong>Fusion</strong></td>
</tr>
<tr>
<td>E1</td>
<td>24.4%</td>
</tr>
<tr>
<td>E2</td>
<td>8.3%</td>
</tr>
<tr>
<td>Average</td>
<td>16.4±8.1%</td>
</tr>
</tbody>
</table>

Application of Mibefradil to white adipocytes pretreated with FCCP induced lipid droplet fusion.

Previous experiments had demonstrated that Mibefradil treatment of adipocytes pretreated with an oxidative phosphorylation uncoupler, FCCP, still resulted in an increase in intracellular calcium levels attributed to Mibefradil. To test whether FCCP pretreatment had an effect on Mibefradil induced lipid droplet fusion, cells were pretreated with FCCP (10µM) for 30min. 30 min treatment with FCCP (10 µM) alone induced 0% lipid droplet fusion (2 experiments). The application of Mibefradil to FCCP pretreated adipocytes induced lipid droplet fusion (Table 2). A 40 µM Mibefradil application to FCCP pretreated cells induced fusion in 10.7±6.4% of cells (n=2 experiments), compared to 16.4±8.1% in 40µM Mibefradil alone (n=2 experiments). At 30
μM, Mibefradil application induced fusion in 39.1±29.3% of FCCP pretreated cells (n=2 experiments), compared to 45.5% of cells for Mibefradil alone (n=1 experiment). Clearly, FCCP pretreatment of adipocytes did not inhibit Mibefradil induced lipid droplet fusion, but due to the low numbers of experiments and variability in results between experiments, no statistical analysis was warranted. A 40 μM Mibefradil treatment in pretreated adipocytes did not show a significant difference (P<0.001) in lipid droplet fusion when compared to a Mibefradil (30 μM) treatment alone. It would be important to increase the number of experiments in future studies to test possible dose dependency of the effect of Mibefradil and any effect of FCCP pretreatment on lipid droplet fusion.

Table 2. Lipid droplet fusion in white adipocytes pretreated with FCCP followed by different Mibefradil concentrations. R1: 30 μM Mibefradil, R2: 40 μM Mibefradil. Note that lipid droplet fusion ranged widely between experiments. Number of cells denoted by N.

<table>
<thead>
<tr>
<th>R1. 30 μM Mibefradil treatment only.</th>
<th>R1. 10 μM FCCP followed by a 30 μM Mibefradil treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Experiment</td>
<td>Fusion</td>
</tr>
<tr>
<td>E1</td>
<td>45.5%</td>
</tr>
<tr>
<td>E2</td>
<td>68.4%</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>39.1±29.3%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R2. 40 μM Mibefradil treatment only.</th>
<th>R2. 10 μM FCCP followed by a 40 μM Mibefradil treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Experiment</td>
<td>Fusion</td>
</tr>
<tr>
<td>E1</td>
<td>24.4%</td>
</tr>
<tr>
<td>E2</td>
<td>8.3%</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>16.4±8.1%</td>
</tr>
</tbody>
</table>

| E2         | 17.0%   | 53     |
| **Average** | 10.7±6.4% | 2 exp. |
Application of Mibefradil to white adipocytes pretreated with Thapsigargin prevented lipid droplet fusion.

As shown above, pretreatment of adipocytes with Thapsigargin inhibited Mibefradil induced calcium increase. Next, we investigated whether Thapsigargin pretreatment would, in addition to its effect on abolishing Mibefradil induced calcium increase, also have an effect on lipid droplet fusion attributed to Mibefradil. White adipocytes treated only with Thapsigargin (10 μM) did not show any lipid droplet fusion. Pretreatment with Thapsigargin followed by the application of 10μM and 25μM Mibefradil fully blocked the Mibefradil induced effects of fusion (Table 3), whereas fusion was observed in 3 out of 7 experiments at 50μM Mibefradil, suggesting that higher doses of Mibefradil might have overcome the effect of emptying the ER stores of calcium. Thus, in most cases, fusion was inhibited when endoplasmic reticulum (ER) calcium stores were emptied by Thapsigargin, suggesting that calcium release from the ER is an important factor in causing Mibefradil induced lipid droplet fusion.
Calcium levels in white adipocytes undergoing lipid droplet fusion induced by Mibefradil vary considerably.

The finding that Thapsigargin inhibited both Mibefradil induced calcium increase and lipid droplet fusion suggested a possible link between calcium and lipid droplet fusion. To further investigate the relationship between the intracellular calcium concentration and lipid droplet
fusion, we analyzed the fusion events and correlated them to the calcium levels. Fusion of lipid droplets was associated with a wide range of calcium levels (Fig. 13). A more detailed analysis of correlating the intracellular calcium concentration of the adipocyte as lipid droplets fused is shown in Figure 14. Fig. 14A, shows adipocytes that fused after the concentration of calcium had been reduced from its initial peak. In fig. 14B, fusion also did not coincide with the initial calcium peak, but occurred at a later time point at which the calcium concentration was still elevated close to the level of the initial calcium peak. Fig. 14C shows examples of cells in which the calcium concentration continued to increase and fusion occurred at a time when the concentration of calcium was higher than the calcium concentration of the 1st peak. Taken together, adipocytes underwent lipid droplet fusion at varying intracellular calcium concentrations. Additionally, lipid droplet fusion occurred at varying times after the first calcium peak had been reached, suggesting that the initial rise in calcium may have triggered signaling events that resulted in lipid fusion further downstream. The majority of lipid droplet fusion events in white adipocytes treated with Mibefradil took place between 13-19 min (Fig. 15). Thus, lipid droplet fusion occurred 8-14 min after Mibefradil addition.
Figure 13. Calcium levels corresponding to lipid droplet fusion in white adipocytes treated with 50 µM Mibebradil. This plot illustrates lipid droplet fusion occurred at varying levels of calcium, ranging from ratios as high as 2.6 to as low as 0.5. Each dot represents a single cell.
Figure 14. Calcium levels of white adipocytes treated with 50 µM Mibefradil (MIB) at three major stages: Pre-Mibefradil levels, 1st peak (Post-Mibefradil), and Fusion. A-C) Graphs show fusion of lipid droplets within adipocytes occurred at varying calcium levels. Pre-Mibefradil represent calcium concentration of adipocytes prior to Mibefradil treatment. The maximum calcium levels post Mibefradil treatment is represented as 1st peak. The calcium levels at which lipid droplets fused within adipocytes is represented by fusion. Number of cells denoted by N.
Mibefradil induced lipid droplet fusion preceded cell death in white adipocytes.

Previous experiments demonstrated that Mibefradil, in addition to causing an increase in intracellular calcium levels and subsequent lipid droplet fusion, also resulted in the death of some white adipocytes at various times throughout the 30-minute treatment period. Death was accompanied by the disintegration of the plasma membrane of white adipocytes and the release of the cells’ contents into the extracellular medium (Fig. 16). The effect of the different concentrations of Mibefradil on cell death appeared to show dose dependency between 10 µM and 50 µM, whereby increasing concentrations produced an increased amount of cell death (Table 6). A 10 µM Mibefradil treatment induced on average 1.6±1.0% cell death in white adipocytes (n=4 experiments) (Table 6). A 50 µM Mibefradil treatment induced on average 36.8±9.7% cell death (n=9 experiments). Intermediate concentrations (25 µM, 30 µM, 40 µM) based on average values did not show dose dependency. A 25 µM Mibefradil treatment induced on average 3.4% cell death (n= 1 experiment) (Table 6). A 30 µM Mibefradil treatment induced on average 30.3% cell death (n=1 experiment) (Table 5). A 40 µM Mibefradil treatment induced on average 29.9±25.7% cell death in adipocytes (2 experiment) (Table 4). However, the low
number of experiments could be the reason for the wide range in cell deaths seen at the intermediate concentrations. Only one experiment was completed for each of the 25 µM and 30 µM Mibefradil treatment and two experiments were completed for the 40 µM Mibefradil treatment. While cell death was clearly higher at 50µM compared to 10µM of Mibefradil, additional experiments utilizing intermediate Mibefradil concentrations needed to be performed to establish a statistically significant dose dependency for the effect of Mibefradil on cell death.

Taken together, these experiments suggested that Mibefradil had a detrimental effect on the long-term viability of adipocytes. Next, we investigated whether the source of the Mibefradil induced calcium increase would have an effect on Mibefradil induced cell death. Mibefradil induced cell death was still present in adipocytes either deprived of extracellular calcium (Table 4) or depleted of calcium from the mitochondrial store (Table 5). However, cell death was nearly abolished in adipocytes deprived of intracellular calcium from the endoplasmic reticulum (Table 6). Adipocytes pretreated with Thapsigargin followed by a 10 µM Mibefradil treatment showed 0% cell death. Zero percent cell death was also seen in 25 µM Mibefradil treatment in pretreated cells. Only 0.3±0.3% cell death was seen in pretreated adipocytes following a 50 µM Mibefradil treatment. These results suggested that Mibefradil induced cell death could be nearly completely blocked when the endoplasmic reticulum calcium store had been previously emptied by Thapsigargin. The important role of the endoplasmic reticulum in enabling Mibefradil to induce cell death merits further research to better understand the mode of Mibefradil’s action in white adipocytes.
Figure 16. Differential interference contrast image of unstained white adipocytes treated with Mibefradil. Mibefradil (25 µM) treatment induced cell death in white adipocytes. Right image shows cell membrane disintegration and diffusion of cell contents into the extracellular medium. White arrow shows intact plasma membrane before (left) and after disintegration of adipocytes treated with Mibefradil (right).

Table 4. Cell death in white adipocytes treated with 40 µM Mibefradil concentrations in zero calcium and normal calcium containing extracellular medium. Note that cell death ranges widely between experiments. Number of cells denoted by N.

<table>
<thead>
<tr>
<th></th>
<th>R1. 40 µM Mibefradil treatment in an extracellular calcium medium.</th>
<th>R1. 40 µM Mibefradil treatment in an extracellular medium excluding calcium.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (%)</td>
<td>Experiment</td>
</tr>
<tr>
<td>E1</td>
<td>55.6%</td>
<td>E1</td>
</tr>
<tr>
<td>E2</td>
<td>4.2%</td>
<td>E2</td>
</tr>
<tr>
<td>Average</td>
<td>29.9±25.7%</td>
<td>Average</td>
</tr>
</tbody>
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Table 5. Cell death in white adipocytes pretreated with FCCP followed by different Mibefradil concentrations. R1: 30 µM Mibefradil, R2: 40 µM Mibefradil. Note that cell death ranges widely between experiments. Number of cells denoted by N.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mibefradil Treatment</th>
<th>Mean (%)</th>
<th>Cell Death</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1. 30 µM Mibefradil treatment only.</td>
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<td></td>
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<tr>
<td>E1</td>
<td></td>
<td>30.3%</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>R1. 10 µM FCCP followed by a 30 µM Mibefradil treatment.</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>E1</td>
<td></td>
<td>34.1%</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>5.3%</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>19.7±14.4%</td>
<td>2 exp.</td>
<td></td>
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<tr>
<td>R2. 40 µM Mibefradil treatment only.</td>
<td></td>
<td></td>
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<tr>
<td>E1</td>
<td></td>
<td>55.6%</td>
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<tr>
<td>Average</td>
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<td>29.9±25.7%</td>
<td>2 exp.</td>
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<tr>
<td>R2. 10 µM FCCP followed by a 40 µM Mibefradil treatment.</td>
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<tr>
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<td></td>
<td>40.4%</td>
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<tr>
<td>Average</td>
<td></td>
<td>58.9±18.5%</td>
<td>2 exp.</td>
<td></td>
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Table 6. Cell death in white adipocytes pretreated with Thapsigargin followed by different Mibebradil concentrations. R1: 10 µM Mibebradil, R2: 25 µM Mibebradil, R3: 50 µM Mibebradil. Note that cell death ranges widely between experiments. Number of cells denoted by N.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell Death</th>
<th>N</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1. 10 µM Mibebradil treatment only</td>
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<tr>
<td>E1</td>
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<td>E2</td>
<td>2.8%</td>
<td>36</td>
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</tr>
<tr>
<td>E3</td>
<td>0%</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>0%</td>
<td>33</td>
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</tr>
<tr>
<td>Average</td>
<td>1.6±1.0%</td>
<td>4 exp.</td>
<td></td>
</tr>
<tr>
<td>R2. 25 µM Mibebradil treatment only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>3.4%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>R3. 50 µM Mibebradil treatment only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>100%</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>30%</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>6.3%</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>15%</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>37.8%</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>22.2%</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>62.2%</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>15.6%</td>
<td>32</td>
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<tr>
<td>E9</td>
<td>42.1%</td>
<td>38</td>
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</tr>
<tr>
<td>Average</td>
<td>36.8±9.7%</td>
<td>9 exp.</td>
<td></td>
</tr>
<tr>
<td>R1. 10 µM Thapsigargin followed by a 10 µM Mibebradil treatment.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0%</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>0%</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>R2. 10 µM Thapsigargin followed by a 25 µM Mibebradil treatment.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>0%</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>R3. 10 µM Thapsigargin followed by a 50 µM Mibebradil treatment.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0%</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>0%</td>
<td>56</td>
<td></td>
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<tr>
<td>E3</td>
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</tr>
<tr>
<td>E4</td>
<td>0%</td>
<td>44</td>
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<td>0%</td>
<td>35</td>
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<tr>
<td>E6</td>
<td>2.2%</td>
<td>45</td>
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<tr>
<td>E7</td>
<td>0%</td>
<td>44</td>
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<tr>
<td>Average</td>
<td>0.3±0.3%</td>
<td>7 exp.</td>
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4 CONCLUSIONS

The role of white adipose tissue in our bodies is of central interest to many researchers interested in combating the epidemic of obesity among developed nations around the world. White adipose tissue (WAT) is mainly located in subcutaneous regions of the abdomen and wraps around internal organs often referred to as visceral fat. White adipose tissue functions as the storage site for fat and in obese individuals there is an excessive amount of dietary fat (Bjorndal 2011). Normally in WAT, fat in the form of triglycerides is broken down into fuel, or free fatty acids, for instances when energy expenditure exceeds food intake. However, in obese individuals, there is an abundance of free fatty acids in the blood being released by an abnormally enlarged adipose tissue mass (Boden 2008). The excess of triglycerides, composed of free fatty acids, is stored in lipid droplets. The study of lipid droplets in the scientific community now known as lipid droplet biology has, through genetic techniques, focused primarily on the various proteins located on the membrane of lipid droplets (Konige 2014). Lipid droplets are dynamic organelles that function to store energy mainly in the form of triacylglycerol. The mobilization of these lipids is dependent on whether the mammal is under fed or fasting conditions. Fed conditions typically accompany an excess of calories which promote the generation and storage of triacylglycerols into the large, unilocular lipid droplets found within white adipocytes. While at a time of fasting conditions associated with caloric deficit the mobilization of fatty acids via regulated lipolysis of stored triacylglycerols ensues (Brasaemle 2009; Paar 2012).

Mibefradil is a calcium channel antagonist that binds to transient low voltage activated T-type calcium channels mainly located in vascular smooth muscle, neurosecretory cells in the brain, kidneys, adrenal gland and pacemaker cells (Ernst and Kelly 1998). Once bound Mibefradil blocks the influx of extracellular calcium through T-type channels and has numerous effects on these
different tissues. Some recent studies have shown that Mibefradil is potentially capable of preventing cancer cell proliferation and induce tumor suppression activity (Huang, Lu et al. 2015, Gomez-Lagunas, Carrillo et al. 2016). An interesting phenomenon occurred in the process of using Mibefradil in preliminary experiments on white adipocytes meriting future investigation. Mibefradil induced an increase in intracellular calcium levels, unexpected for a T-type calcium channel blocker. Additionally, it also induced lipid droplet fusion in white adipocytes.

**Intracellular calcium increase in murine white adipocytes due to Mibefradil**

Calcium participates in a variety of complex cellular processes, as it is a vital intracellular secondary messenger. A critical function of this messenger is to produce calcium signals (Berridge 1997). This messenger depends on intracellular calcium stores and influx to produce these signals. Consequently, we predicted that the mechanism by which Mibefradil caused a rise in intracellular calcium was due either to calcium influx or calcium release from internal stores. Accordingly, the present experiments tested whether an external medium deficient in calcium would attenuate Mibefradil induced calcium increase. Subsequently we tested whether Thapsigargin-induced depletion of intracellular endoplasmic reticulum calcium store as well as FCCP-induced depletion of the mitochondrial store would prevent Mibefradil induced calcium increase.

Our first experiment aimed at confirming that Mibefradil increased cytoplasmic calcium within white adipocytes. Mibefradil application (10-50 µM) repeatedly increased the intracellular calcium concentration in Fura-2-loaded 3L1L cells. The next question was to determine whether Mibefradil’s mechanism to induce calcium increase was due to calcium influx. Cells were tested in a calcium-free medium and treated with Mibefradil. Mibefradil produced an increase in cytosolic calcium on white adipocytes of the same magnitude than Mibefradil in regular
extracellular medium that contained normal levels of calcium. The finding that Mibefradil still induced an increase in calcium levels under conditions of calcium depleted extracellular environment suggested that intracellular stores likely served as the main source of calcium.

We next investigated the source of Mibefradil induced calcium increase in cells treated with 10µM FCCP to deplete mitochondria calcium stores. The effects of the pretreatment of white adipocytes with FCCP did not prevent the Mibefradil induced calcium increase. Mibefradil still caused a rise in calcium levels in white adipocytes that had been pretreated with the mitochondrial uncoupler FCCP, indicating that this organelle is unlikely the source of the Mibefradil induced increase in calcium in white adipocytes.

We next investigated the role of endoplasmic reticulum stores of calcium as the source of the Mibefradil induced calcium increase. Thapsigargin irreversibly blocks the endoplasmic reticulum Ca-ATPase pump and thereby prevents the refilling of the ER stores, resulting in calcium store depletion. Thapsigargin (10µM) application to white adipocytes resulted in a transient increase in intracellular calcium levels. Depletion of Thapsigargin-sensitive calcium stores did prevent the Mibefradil induced calcium increase in white adipocytes, suggesting that the calcium source mobilized by Mibefradil was the endoplasmic reticulum. Therefore, we propose that the direct source of calcium after Mibefradil treatment in mouse white adipocytes is through endoplasmic reticulum channels. Identifying the mechanism in which the blocking of T-type calcium channels by Mibefradil resulted in calcium release from the endoplasmic reticulum requires further research. Alternatively, Mibefradil might have acted by a mechanism other than the known inhibition of T-type calcium channels.

L Lipid droplet fusion in murine white adipocytes due to Mibefradil
Adipose tissue is highly specialized to be the main lipid storage tissue in the body. These cells are responsible for the management and packaging of lipid stores. Unilocular lipid droplets present in white adipocytes are well adapted to store large amounts of lipids, and the size of its lipid droplet takes up a large portion of the cytoplasm. The increasing size of lipid droplets is frequently seen because of overnutrition in obese individuals. These dynamic organelles are devoted to providing the most efficient recycling and storing of excessive and gross amounts of fatty acids. Disruptions in these processes lead to the disabling of lipid droplets to protect against lipotoxicity (Krahmer 2013; Walther 2012). In white adipocytes, the chief event in the progression of obesity is the evolution of lipid droplets transforming from a small lipid droplet to a large lipid droplet (WU 2014). In our study, time lapse fluorescence microscopy showed that Mibefradil treatment also induced lipid droplet fusion in white adipocytes. Live cell imaging of murine adipocytes revealed that pretreatment with the endoplasmic reticulum CaATPase pump inhibitor, Thapsigargin, prevented lipid droplet fusion in addition to blocking Mibefradil induced calcium increase. These data indicate that the release of calcium from intracellular stores is required for lipid droplet fusion because in the presence of Thapsigargin both fusion events and calcium increase induced by Mibefradil were blocked. To test whether the release of calcium from stores is sufficient for lipid droplet fusion, other experiments such as the release of calcium from intracellular stores using caged IP3 should be performed.

*Cell death in murine white adipocytes due to Mibefradil*

Many proteins are located on the surface of lipid droplets. These proteins are devoted towards regulating the synthesis of lipids, enacting fusion of lipid droplets and mediating the hydrolysis of lipids. Lipid droplet fusion has been directly linked to FSP27/CIDE protein complexes. It has been
demonstrated that the overexpression of these proteins generates larger and fewer lipid droplets via homotypic fusion (Urritia 2015). The deviation of a controlled amount of triacylglycerol in storage is regulated by specialized proteins on lipid droplets. However, inhibition of these adipocyte-specific lipid droplet-associated proteins can induce lipotoxicity. An essential role of lipid droplets is to quarantine overaccumulated lipids from entering the cytosol of adipocytes and thereby safeguarding the adipocyte from the damaging effect of amassing excessive free fatty acids (FFA). An elevated release of FFA from enlarged adipocytes initiates inflammation and lipolysis in adipose tissue (Matsuda 2013). In our study, Mibefradil caused the lipid droplets to fuse into large unilocular lipid droplets and subsequently caused the disintegration of the plasma membrane of the adipocyte. Mibefradil induced adipocyte apoptosis and while its mode of action is presently unclear, it is possible that the drug could be targeting surface proteins on lipid droplets and interfere with their purpose of managing and providing the most efficient packaging of lipids.

**Mibefradil as a prospective drug treatment**

The work on Mibefradil’s effects on adipocytes is noteworthy because currently Mibefradil is in the spotlight in the disciplines of cancer research and drug development. It has been found that T-type calcium channels are highly expressed in numerous cancer cells. This includes a wide range of cells such as ovarian, glioma, breast, leukemic, and many others (Huang 2015). The drug Mibefradil is highly specific for T-type calcium channels and is a “potent” inhibitor of these channels, making Mibefradil a very promising therapeutic for fighting cancer (Huang 2015). Recently the National Cancer Institute (NCI) Adult Brain Tumor Consortium has placed Mibefradil in a phase 1b clinical trial as an anticancer agent that can be used for tumor treatment. The drug company Tau Therapeutics LLC has been developing Mibefradil to be a suitable drug
for the treatment of cancer. They are introducing Mibefradil as a sequential combination treatment with other antitumor applications to provide the maximal efficiency of targeted cancer treatment (Krouse 2015). The unexpected effects of Mibefradil in white adipocytes described in this study need to be considered as Mibefradil is advanced as a drug in human subjects. Changes in calcium dynamics, such as increases in calcium levels resulting in lipid droplet fusion in response to Mibefradil in white adipocytes require attention as they could signal previously unreported side effects of the drug.

Adipocyte function can be altered by abnormal deviations in calcium levels. There is mounting evidence that dysfunction of calcium homeostasis in adipose tissue is coupled to metabolic diseases. It was reported that isolated adipocytes from obese individuals in the senescent population have increased levels of cytosolic calcium (Byyny 1992). Elevated cytosolic calcium levels are implicated to be a part of obesity-related diseases due to the calcium deposits surrounding lipid droplets within hypertrophic adipocytes (Giordano 2013). In addition, it has been found that an escalation of cytosolic calcium led to the modulation of de novo lipogenesis via regulation of FAS expression and in turn stimulates triglyceride build-up and expands lipid storage. FAS is part of the death-inducing signaling complex and functions to signal apoptosis (Zemel 2000). In this study, Mibefradil’s effect of accumulating excess calcium in the cytoplasm of white adipocytes led to compromised cells and cell death. Coupled with Mibefradil emerging as a potential therapeutic application for the treatment of cancer, this drug can also be designed to target hypertrophic adipocytes. Today medicines in design and development are focused on having multi-purpose pharmacological and interventional approaches that treat more than a single underlying disease. One such development the compound 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), an active metabolite of vitamin D, which is being used as a preventive and
therapeutic agent against both breast cancer and obesity. 1,25-dihydroxyvitamin D3 also acts as a hormone that activates an apoptotic calcium signal that induces an increase in intracellular calcium levels in breast cancer cells and adipocytes causing apoptosis. The study of this compound has not only shed light on the link between obesity and breast cancer, but also discovered that the modulation of apoptosis is a viable and effective means to attenuate the pathways in obesity and breast cancer (Sergeey 2013).

In the world of obesity research, understanding the way Mibefradil exerts effects such as calcium release, lipid droplet fusion, and cell death on adipocytes is crucial in unearthing its potential as a therapeutic. Manmade drugs that in addition to their known function also indirectly activate other unknown processes within a cell are an opportunity for scientists to design novel drugs that exploit the cells and their natural functions in our bodies for other purposes. Mibefradil changes adipocytes in interesting ways and further research into engineering pharmacological changes and repurposing its function in adipocytes should be pursued.
REFERENCES


