Mode of Action of RZL-012, a New Fat-Reducing Molecule

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BACKGROUND RZL-012 (5-[3,6-dibromo-9H-carbazol-9-yl]-N,N,N-trimethylpentan-1-aminium chloride) is a novel investigational drug injected subcutaneously into fat tissues in patients with fat-related disorders (Dercum disease) or subjects seeking aesthetic changes.

OBJECTIVE Preclinical studies were undertaken to understand RZL-012's mechanism of action.

MATERIALS AND METHODS The effects of RZL-012 were tested in vitro by measuring adipocyte cell killing, membrane integrity, cytosolic calcium, and mitochondrial membrane potential (MMP). In vivo studies in pigs evaluated RZL-012's adipocyte killing effect and measured pig fat thickness in the injected areas.

RESULTS RZL-012 triggered adipocyte cell killing with IC_{50} values ranging from 25 to 106 μM. RZL-012 demonstrated initial effects on membrane integrity and calcium levels with delayed alterations in MMP. Incubation of RZL-012 with nanoghosts increased membrane permeability, culminating in full membrane destruction. Analysis of injected areas in pigs revealed liponecrosis 24 hours after dosing followed by an inflammatory response and formation of fibrotic tissue. Three months after dosing, an 18% reduction in mean fat thickness was observed in RZL-012 treated pigs.

CONCLUSION RZL-012 destroys adipocytes by directly disrupting cell membrane integrity. Replacement of dead fat tissue by fibrotic tissue enables healing and causes contraction of the injected area. These effects are translated into significant reduction in fat tissue volume.

Patients suffering from fat disorders such as Dercum disease (a very rare disorder characterized by multiple, painful lipomas) and people seeking aesthetic changes often desire reduction of fat volume in specific body areas. Surgery for lipoma removal and liposuction are the most frequently used invasive treatments for fat removal.[1–3] In view of surgery-associated risks, there is a significant demand for nonsurgical alternatives to reduce dimensions of areas of fat accumulation. In the aesthetic field, some novel noninvasive methodologies, such as cryolipolysis,[4] laser irradiation,[5] and injectables, have emerged. Deoxycholic acid (Kybella) is currently the only FDA-approved injectable medication capable of breaking down fat cells by membrane dissolution.[6] The product was approved for the use in people with submental fullness; however, it experienced minimal commercial success partly because of the need for multiple injections.

RZL-012 is a clinical stage product being developed for fat-related disorders, such as Dercum disease, and aesthetic applications. The compound is administered by a single multiinjection session into subcutaneous fat deposits, resulting in reduction of fat tissue volume. We hereby elaborate on RZL-012's mechanism of action on fat tissue by analyzing the compound's in vitro effects on cell viability and membrane permeability. In addition, we demonstrate its efficacy in inducing fat loss in pigs, an accepted animal model for subcutaneous fat removal studies.

Methods

In Vitro Cell Killing

Wi38 Human Lung Fibroblasts Viability Assay

Cryopreserved Wi38 cells were cultured according to the manufacturer's protocol, harvested and seeded into 96-well opaque plates with 95 μL culture media (Eagle's Minimum Essential Medium + 10% Fetal Bovine Serum [FBS]) per well. Cells were incubated overnight at 37°C, 5% CO_2 before the addition of RZL-012 and controls. Immediately before compound addition, culture medium was removed from all plates and replaced with 95 μL fresh medium. RZL-012 was solubilized in dimethyl sulfoxide (DMSO) to form a 500X stock solution, further diluted to a 20X solution and finally added to the cells at 5 μL/well to reach a final volume of 100 μL/well. RZL-012 was tested in triplicate at final assay concentrations of 0, 0.01, 0.03, 0.1, 1, 3, 10, 30, 100, and 300 μM, with a final DMSO vehicle concentration of

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0.2%. Plates were incubated at 37°C, 5% CO_2 for 2, 6, and 24 hours. At the end of the culture period, 50 μL culture media were removed from all wells and replaced with 50 μL Cell Titer Glo 2.0. Plates were placed on an orbital shaker for 2 minutes and incubated at room temperature for 10 minutes before reading in a luminescence plate reader.

Human Adipocyte Viability Assay

Cryopreserved human visceral preadipocytes (Lonza #PT-5005) were cultured according to the manufacturer's protocol. On reaching confluency, cells were harvested and seeded into 96-well opaque plates at 1×10^4 cells/well in 100 μL preadipocyte growth medium (Lonza PGM-2 with 10% FBS, 1% L-glutamine, and 1% gentamycin). Cells were incubated overnight at 37°C and 5% CO_2. Adipogenic differentiation was induced by the addition of 100 μL/well of differentiation medium (PGM-2 plus 2X concentration adipogenic differentiation cocktail, Lonza PT-8002), for a final culture volume of 200 μL/well. Cells were incubated at 37°C and 5% CO_2 for 5 days before the addition of compounds. Adipogenic differentiation was verified before starting the cell viability assay by the presence of lipid vacuoles. Immediately before compound addition, the culture medium in plates was reduced to a volume of 95 μL/well. RZL-012 was added and tested in triplicate at final assay concentrations of 0, 0.01, 0.03, 0.1, 1, 3, 10, 30, 100, and 300 μM, as described above for Wi38 cells.

Effects of RZL-012 on Cell Membrane Integrity, Cytosolic Calcium and Mitochondrial Membrane Potential in Cultured Adipocytes

Preadipocytes were seeded in optical 96-well cell culture plates at 5,000 and 10,000 cells/well in 100 μL cell culture medium. After overnight culture, 100 μL of the 2X adipocytes differentiation medium was added to the cells, which were cultured for 4 to 5 days to induce differentiation, confirmed by visual examination of intracellular lipid vacuoles. After adipocyte differentiation, 100 μL of the medium per well was removed and 25 μL of 5X of the test compound was added to the wells and incubated with the cells for 2, 6, and 24 hours at 37°C, 5% CO_2. At the end of the incubation period, the cells were loaded with a dye cocktail containing tetramethylrhodamine methyl ester perchlorate, 4-(6-acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4′-methyl-2,2′-(ethylenedioxy)dianiline-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl) ester (Fluo-4), and 1,1'-(4,8-bis[dimethyliminio]undecane-1,11-diyl)bis{4-[3-(3-methyl-1,3-benzothiazol-2(3H)-ylidene) prop-1-en-1-yl]quinolinium} (TOTO-3) for 1 hour for the measurements of mitochondrial membrane potential (MMP), intracellular free calcium, and membrane permeability, respectively. Cell plates were scanned by ImageXpress immediately after dye loading. Vehicle control (0.2% DMSO) was tested on the same plate. The test compound was solubilized in DMSO, diluted with medium to a concentration of 5X, and tested at 8 concentrations of 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μM, $n = 3$. The final DMSO concentration was 0.2%.

Effects of RZL-012 on Membrane Permeability in Isolated Cell Membranes (Nanoghosts)

Nanoghosts (NGs), nanovesicles derived from the whole membrane of human bone marrow mesenchymal stem cells, were used as a model for cell membranes to detect the influence of RZL-012 on permeability in isolated membranes. This model has significant advantages for studying the interactions of different drugs on and with cell membrane as NGs are generated from whole cells and maintain the normal, living cell membrane structure with transmembrane proteins of mesenchymal stem cells.[7] To detect the loss of cell membrane integrity/increased permeability, the Horseradish Peroxidase (HRP) enzyme was encapsulated into NGs and its release into the medium was measured after 2 hours of incubation with increasing concentrations of RZL-012. NG-encapsulated HRP was produced through an ultrasonication technique described previously.[8,9] The yield of nanoparticles was calculated using a lipid concentration kit (LabAssay Phospholipid, Fujiflm, Japan). RZL-012 was dissolved in DMSO (500X) and diluted with water to its final concentration. RZL-012 was added to HRP-NG at concentrations of 12.5, 25, 50, 100, 200, and 400 μM with final DMSO concentration kept at 0.2% or below. Isosmotic TM-buffer supplemented with 10% sucrose was used to increase NG stability and to prevent precipitation of RZL-012. The resulting solutions, containing HRP-NG and increasing concentrations of RZL-012, were incubated at 37°C for 2 hours at 100 rpm with shaking. After incubation, the number of NGs per mL was counted by Nanoparticle Tracking Analysis (Nanosight NS300; Malvern Panalytical, United Kingdom), and samples were analyzed for the presence of released HRP by the measurement of enzymatic activity (Synergy H1; Biotec). Pyrogallol (50 mg/mL) and hydrogen peroxidase (0.5%) were used as substrates. Cryogenic transmission electron microscopy (Cryo-TEM, FEI Talos 200C; Thermo Fisher) was used for the determination of the morphology of incubated NGs. Results are presented as mean ± SEM as specified for each experiment of at least 4 replicates. Statistical significance in the differences of means was evaluated by a one-way analysis of variance test.

Histopathology and Fat Thickness in RZL-012-Treated Pigs

The Sponsor performed a good laboratory practice toxicology study entitled: *An 84 Day Local and Systemic Toxicity Study of RZL-012 Following Subcutaneous Injection in Farm Pigs*. The study was performed according to US Department of Agriculture's Animal Welfare Act (9 code of federal regulations Parts 1, 2, and 3). The objective of this study was to evaluate the potential local and systemic toxicity as well as efficacy of RZL-012 in pigs.

Histopathological Analysis of Pig Injection Sites

RZL-012 was injected into the subcutaneous abdominal fat layer of domestic Yorkshire crossbred swine (weighting approximately 80 kg) at 500 mg/pig (i.e., as 20 injection sites/pig in which each site was injected with 25 mg/0.5 mL of RZL-012; distance between injections in each site was 2 cm). Three treatment groups were injected with RZL-012, and 3 control groups were injected with saline (20 injection sites/pig, each site injected with 0.5 mL saline). Necropsy was performed at 24 hours and 14 and 84 days postdosing. Male and female animals (initially weighing 73.5–86.5 kg and 74.0–84.5 kg, respectively) were randomly assigned to the study as identified in **Supplemental Digital Content**, Table S1 (http://links.lww.com/DSS/A918).

At each time point, pigs were anesthetized with 6 mg/kg Telazol by intramuscular injection and then euthanized by an intravenous injection of sodium pentobarbital solution followed by exsanguination of the femoral vessels. Injection sites (20 sites/pig) were removed, examined, and fixed in neutral buffered formalin. Study tissues were processed, sectioned, and stained with hematoxylin and eosin (H&E). Slides were graded for liponecrosis, inflammation, and fibrosis using a scale of 1 to 4 as described below:

GRADE 1 = minimal
GRADE 2 = slight
GRADE 3 = moderate
GRADE 4 = marked

Measuring Fat Thickness in Pigs

Subcutaneous fat thickness was measured at necropsy in the saline and RZL-012 groups, 84 days after dosing. Fat thickness was measured between skin and muscle, using a ruler at 9 points along each flank, 4 of which included injection sites and 5 included noninjected areas (considering 2 flanks, 18 points analyzed/pig of which 8 included injection sites and 10 included control noninjection sites). The Wilcoxon scores (rank sums) test was used to test for statistical difference in fat reduction between the RZL-012-treated group versus the saline-treated group.

Results

In Vitro Studies: RZL-012's Effect on Cell Necrosis

Cultured Wi38 fibroblasts and adipocytes were exposed to increasing concentrations of RZL-012. Fibroblast viability was determined at 2, 6, and 24 hours after exposure to RZL with IC_{50} concentrations ranging between 30.8 to 25.5 μM RZL-012, respectively, as shown in **Supplemental Digital Content**, Table S2 (http://links.lww.com/DSS/A919). Cultured adipocytes were exposed to increasing concentrations of RZL-012, with IC_{50} concentrations at the same time points ranging between 106.1 and 52.0 μM RZL-012, respectively, as shown in **Supplemental Digital Content**, Table S2 (http://links.lww.com/DSS/A919). In a second in vitro study, cultured adipocytes were examined for viability, intracellular free calcium, membrane permeability, and MMP, respectively, at 2, 6, and 24 hours after exposure. Figure 1 presents the data as mean ± SEM percent of vehicle control at 2 and 24 hours, respectively, after test compound addition (e.g., 100% of vehicle control indicates no change from control values). Two hours after addition of RZL-012, increases in intracellular Ca^{2+} and membrane permeability were apparent at the 100 μM concentration point. These changes imply compromised cell membrane integrity observed before cell death. At this early time point, MMP was still in the normal range. At 24 hours, increases in intracellular Ca^{2+} and membrane permeability, indicating compromised cell membranes, were apparent at the 30 μM concentration. Mitochondrial membrane potential remained in the normal range. An increase in membrane permeability, with low intracellular Ca^{2+} and low MMP, were apparent at the 100 μM concentration, in agreement with cell death evident at this concentration and time point.

RZL-012 Effect on Isolated Cell Membranes (Nanoghosts)

NGs were assessed for size and then incubated with various RZL-012 concentrations for 2 hours, after which HRP release, NG number, and integrity of NG membranes were determined. As depicted in Figure 2, and **Supplemental**

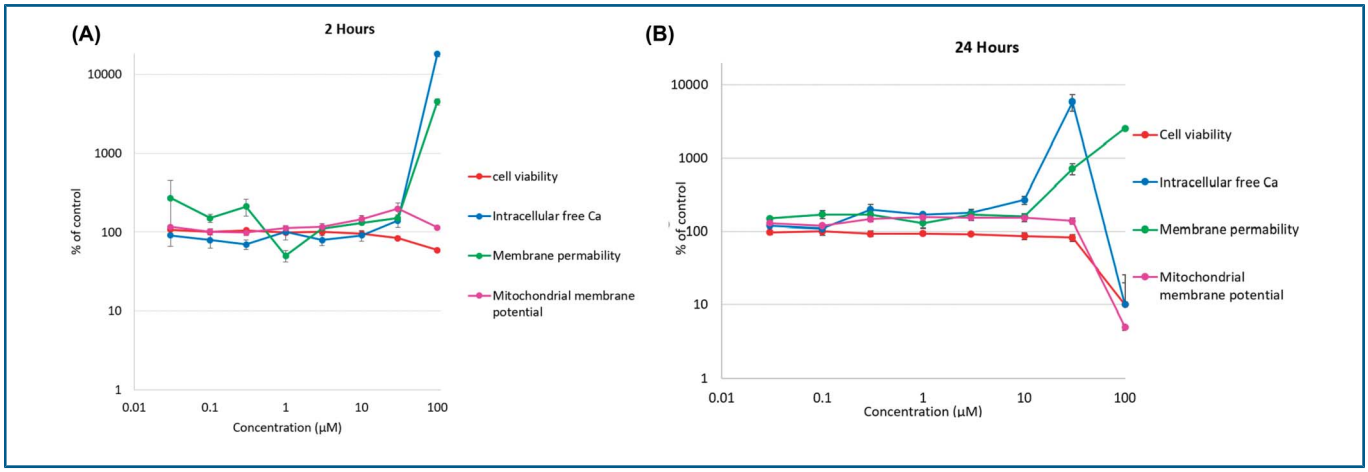

Figure 1. Cellular parameters at (A) 2 hours and (B) 24 hours after RZL-012 exposure.

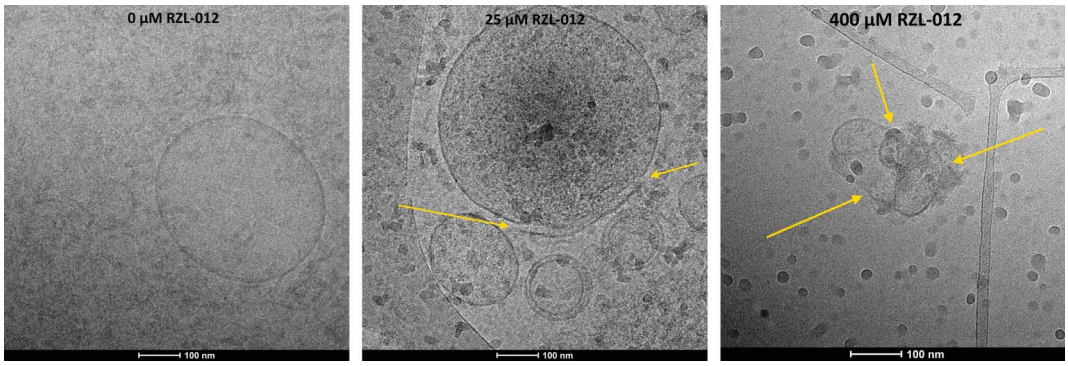

Figure 2. Representative cryo-TEM images; arrows show broken membranes and aggregates.

Digital Content Fig S1a,b,c (http://links.lww.com/DSS/A917), HRP-loaded NGs had a mode size of 188 nm (SD = 42 nm) and a narrow size distribution. In Fig. S1b, http://links.lww.com/DSS/A917, HRP release from HRP-loaded NGs showed a statistically significant dose-related elevation in RZL-012–treated versus Vehicle-treated NGs, although basal leakage of HRP was noted in vehicle-treated NGs. In Fig. S1c, http://links.lww.com/DSS/A917, NG counts showed a statistically significant reduction at low (25 μM) RZL-012 concentration versus vehicle and almost complete elimination of NGs at the high (400 μM) RZL-012 concentration. Finally, in Figure 2, representative cryo-TEM images were taken from vehicle, low (25 μM), and high (400 μM) concentration RZL-012–treated NGs.

In Vivo Pig Studies

Histopathological Analysis of Pig Injection Sites

Pig injection sites were analyzed at 24 hours ($n = 6$), 14 days ($n = 4$), and 84 days ($n = 4$) after RZL-012 dosing. Twenty injection sites per pig were stained with H&E and assessed semiquantitatively for liponecrosis, inflammation, and fibrosis on a scale of 1 (minimal) to 4 (marked). Figure 3 shows the mean scores per group of pigs injected with RZL-012.

Effect of RZL-012 Injection on Fat Thickness in Pigs

RZL-012–treated ($n = 4$; 2 M and 2 F) and saline-treated ($n = 4$; 2 M and 2 F) pigs were analyzed for fat thickness at 84 days after dosing. Predefined sections were taken through injected (8 sections/pig) and noninjected (10 sections/pig) areas. The change in fat thickness (injected vs noninjected areas per each pig) in the control group was +0.06 cm ± 0.13 (+2.8%) versus −0.45 ± 0.08* (−18%) in the RZL-012–treated group (*$p < .05$), as summarized in **Supplemental Digital Content,** Table S3 (http://links.lww.com/DSS/A920).

Conclusions

The clinical effects of RZL-012 are currently being evaluated for lipomas of Dercum disease and the aesthetic reduction of submental fat. In vitro studies testing the effects of RZL-012 on cultured adipocytes and fibroblasts did not hint toward selectivity to adipocytes, hence indicating a nonspecific cell-killing mechanism with IC_{50} values in the range of 25 to 106 μM. To further clarify the cellular target for RZL-012 activity, several parameters connected to very early events in the cell necrosis process were examined. At the 100 μM concentration, initial signs of adipocyte injury were already evident at 2 hours. Enhanced membrane permeability and high intracellular Ca^{2+} levels indicated irreversible cellular deterioration. Yet, at this 2-hour time point, cell viability was about 60% and MMP was still preserved. At the 24-hour time point, the 100 μM concentration resulted in almost complete cell death with very high membrane permeability and low Ca^{2+} and MMP, indicating full-blown necrosis. At the 24-hour time point, adipocytes exposed to 30 μM RZL-012 demonstrated similar parameters to those seen at 2 hours for the 100 μM concentration.

To test for a direct effect of RZL-012 on cell membrane integrity, HRP release from HRP-encapsulated NGs was determined. Spontaneous release of protein from NGs has been previously shown,[7] yet although HRP leakage existed in vehicle-treated samples, addition of RZL-012 for 2 hours enhanced HRP release in a dose-dependent manner. This implied that RZL-012's effect on enhancing membrane permeability was a direct effect, not requiring any energy-dependent cellular mechanisms. Counting of NG numbers/mL demonstrated a significant reduction in NG counts versus vehicle, at 25 μM, whereas at 400 μM, full destruction of NG membranes was evident. Hence, RZL-012 seemed to exert a dose-dependent cell killing effects by directly damaging the cell membrane.

Histopathological analysis of the injected regions in pigs allowed for semiquantitative analysis of processes taking place at various time points after RZL-012 injection into subcutaneous fat. The presence of significant liponecrosis at the injected areas, as early as 24 hours after dosing, indicated that rapid fat cell death occurs very early on in the process. This was in line with in vitro data demonstrating an immediate increase in membrane permeability, seen

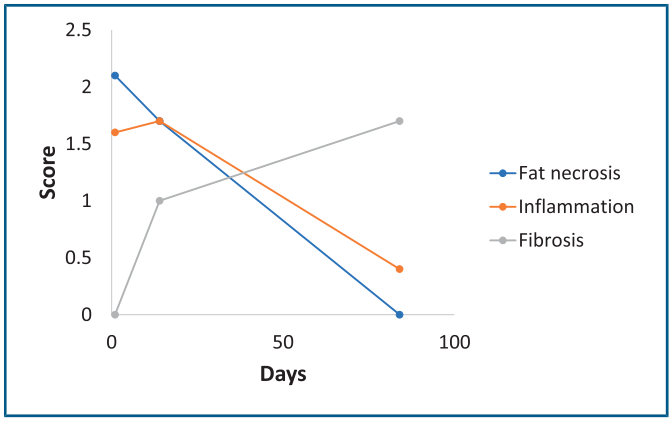

Figure 3. Histopathological analysis of pig injection sites at various time points after RZL-012 dosing. Data presented as mean ± SEM. No abnormalities were found in the saline-treated groups (data not shown). As the tissues heal, note the reduction in liponecrosis and inflammation versus the increase in fibrosis on Day 84.

at 2 hours of exposure to 100 μM RZL-012. Liponecrosis was still evident at 14 days after dosing but scored 0 on Day 84, indicating complete clearance of dead fat tissue. An inflammatory reaction was involved in fat clearance and presence of macrophages at the injection sites was detected as early as 24 hours after dosing.[10] Although inflammation was noted at 14 days after dosing, it was almost absent on Day 84 after injection, indicating a healing process. In contrast to liponecrosis and inflammation, fibrosis was not seen at 24 hours, yet rose steadily and peaked at 84 days after RZL-012 injection. Formation of fibrotic tissue is also considered a part of the healing process.[11] Analysis of pig fat at injected versus noninjected sites revealed an 18% reduction in fat thickness in RZL-012–injected pigs, whereas about a 3% increase in fat thickness was noted in vehicle-injected pigs.

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