Reversal of High Fat Diet-Induced Obesity, Systemic Inflammation, and Astrogliosis by the NLRP3 Inflammasome Inhibitors NT-0249 and NT-0796

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ABSTRACT
Systemic and cerebral inflammatory responses are implicated in the pathogenesis of obesity and associated metabolic impairment. While the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome has been linked to obesity-associated inflammation, whether it contributes to the development or maintenance of obesity is unknown. We provide support for a direct role of saturated fatty acids, such as palmitic acid, as NLRP3 activating stimuli in obese states. To investigate whether NLRP3 activation contributes to the pathogenesis of diet-induced obesity (DIO), we tested two different clinical-stage NLRP3 inflammasome inhibitors. We demonstrate a contributory role of this key inflammasome to established obesity and associated systemic and cerebral inflammation. By comparing their effects to calorie restriction, we aimed to identify specific NLRP3-sensitive mechanisms contributing to obesity-induced inflammation (as opposed to those regulated by weight loss per se). In addition, a direct comparison of an NLRP3 inhibitor to a glucagon like peptide-1 receptor agonist, semaglutide (Wegovy), in the DIO model allowed an appreciation of the relative efficacy of these two therapeutic strategies on obesity, its associated systemic inflammatory response, and cerebral gliosis. We show that two structurally distinct, NLRP3 inhibitors, NT-0249 and NT-0796, reverse obesity in the DIO mouse model and that brain exposure appears necessary for efficacy. In support of this, we show that DIO-driven hypothalamic glial fibrillary acidic protein expression is blocked by dosing with NT-0249/NT-0796. While matching weight loss driven by semaglutide or calorie restriction, remarkably, NLRP3 inhibition provided enhanced improvements in disease-relevant biomarkers of acute phase response, cardiovascular inflammation, and lipid metabolism.

SIGNIFICANCE STATEMENT
Obesity is a global health concern that predisposes individuals to chronic disease such as diabetes and cardiovascular disease at least in part by promoting systemic inflammation. We report that in mice fed a high-fat, obesogenic diet, obesity is reversed by either of two inhibitors of the intracellular inflammatory mediator NLRP3. Furthermore, NLRP3 inhibition reduces both hypothalamic gliosis and circulating biomarkers of cardiovascular disease risk beyond what can be achieved by either the glucagon like peptide-1 agonist semaglutide or calorie restriction alone.

Introduction
The hypothalamus plays a key role in energy homeostasis by balancing food intake and calorie output over long time intervals (Myers et al., 2021). Growing evidence suggests that activation of nonneuronal cells, including microglia and astrocytes, can predispose individuals to excessive weight gain by impairing operation of the hypothalamic energy homeostasis system.

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ABREVIATIONS: ARC, arcuate nucleus; CP-456,773, N-[1,2,3,5,6,7-Hexahydro-s-indacene-4-yl]amino[carbonyl]-4-(1-hydroxy-1-methyl-ethyl)-2-furansulfonamide sodium salt; DIO, diet-induced obesity; DMH, dorsomedial hypothalamic nucleus; GFAP, glial fibrillary acidic protein; GLP-1, glucagon like peptide-1; GLP-1RA, glucagon like peptide-1 receptor agonist; hCES-1, carboxylesterase humanized; HDL, high-density lipoprotein; HFD, high-fat diet; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; ITT, insulin tolerance test; LDL, low-density lipoprotein; LPS, lipopolysaccharide; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; NDT-19795, (2R)-2-[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl]oxy]-3-(pyrimidine-2-yl)propanoic acid sodium salt; NT-0249, sodium [(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl]oxy]-3-(pyrimidine-2-yl)sulfanoyl]azanide; NT-0796, propan-2-yl(2R)-2-[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl]oxy]-3-(pyrimidine-2-yl)propanoate; OD, optical density; OGTT, oral glucose tolerance test; PBMC, peripheral blood mononuclear cell; PCSK9, proprotein convertase subtilisin/kexin type 9; suPAR, soluble urokinase plasminogen activator receptor; sVCAM-1, soluble vascular cell adhesion molecule 1; TG, triglycerides; VMH, ventromedial hypothalamic nucleus.
system (Yoo et al., 2020; Douglass et al., 2023; Sonnefeld et al., 2023). One potential mechanism underlying this “reactive gliosis” response involves the entry of dietary saturated fatty acids into cerebrospinal fluid (Melo et al., 2020) thus promoting inflammatory activation of hypothalamic microglia, potentially via Toll-like receptor 4-dependent mechanisms (Milanski et al., 2009; Valdearcos et al., 2014; Folicik et al., 2022). Regardless of the underlying mechanism, this neuroinflammatory response is implicated in the pathogenesis of diet-induced obesity, since excess weight gain is reduced by interventions that block the gliosis response (Valdearcos et al., 2014; Douglass et al., 2023).

Combined with evidence that this response is mounted early after initiating a high-fat diet, prior to excessive weight gain, available data suggest a contributory role for hypothalamic gliosis in obesity pathogenesis (Sonnefeld et al., 2023). Proper function of the energy homeostasis system hinges on responsiveness of key hypothalamic neurons to afferent input from humoral “adiposity negative feedback” signals such as insulin and leptin. These signals are generated in proportion to body fat mass, and the obesogenic effect of hypothalamic gliosis likely results from impaired neuronal responsiveness to these cues (Myers et al., 2021; Sonnefeld et al., 2023).

Inflammammasomes are intracellular sensors of the innate immune system that control caspase-1 activation and subsequent release of the key inflammatory cytokine interleukins (IL-1β) and IL-18. Saturated fatty acids, present during obesity, are potential activators of the NOD-, LRR-and pyrin domain-containing protein 3 (NLRP3) inflammasome (Karasaki et al., 2018; Gianfrancesco et al., 2019). Previous research has highlighted that NLRP3−/− mice are protected from diet-induced obesity (Skiens et al., 2011). In addition, prophylactic dosing of MCC950 ([N-[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl]-4-(1-hydroxy-1-methylylethyl)-2-furansulfonyamide sodium salt (CP-456773); CRID3], a potent, selective, but non-central nervous system penetrant, NLRP3 inhibitor has previously been shown to limit weight gain in response to high-fat diet feeding in mice (Pavillard et al., 2017). However, it is unclear whether therapeutic dosing of an NLRP3 inhibitor can reverse preestablished obesity and associated metabolic sequelae, such as systemic acute phase responses, dyslipidemia, and vascular inflammation. It is also unclear if brain exposure is required for full efficacy of NLRP3 inhibitors in preestablished obesity. Here, using two structurally distinct, clinical-stage NLRP3 inhibitors, we demonstrate that NLRP3 inhibition reverses established diet-induced obesity (DIO) in mice, and this effect is associated with greater resolution of systemic inflammatory markers than either semaglutide or simple calorie restriction. This finding suggests that in mice consuming an obesogenic high-fat diet, NLRP3 inhibition confers metabolic benefit that extends well beyond reversal of obesity per se.

Methods

Animals. For sodium [(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl](1-methyl-1H-pyrazol-4-yl)([(2S)-oxan-2-yl]methyl)sulfamoyl]azanide (NT-0249) studies, 5-week-old male C57BL/6J mice were purchased from Nanjing GemPharmatech LLC. Studies with esterol pro-drug propan-2-yl[(2R)-2-[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyloxy]-3-pyrinimid-2-yl]propanoate (NT-0796) were performed in humanized carboxylesterase (hCES1) mice, a novel mouse line engineered to possess more human-like traits with respect to carboxylesterase biology (Smolak et al., 2024), specifically, a lack of murine plasma Ces1c and targeted expression of human CES1 (hCES1) to myeloid cells. This strain originated on and was maintained on a B6.129 background as a homozygous colony. hCES1 mice were provided by NodThera and maintained at HD Biosciences Co., Ltd. (China). Experimental procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at HDB. Animals were acclimatized for at least 1 week prior to experiments. All euthanasia was performed using carbon dioxide inhalation, and all efforts were made to minimize animal suffering. Animals were group-housed (five animals per cage) with bedding under controlled temperature (20–25°C), noise, humidity (40%–70%), and lighting (12-hour light and 12-hour dark) conditions. All animals had free access to purified water and standard certified rodent chow (Beijing Keao Xieli Feed Co., Ltd., Beijing, China, #2112/2151) ad libitum. The general condition (appearance and activity) of all animals was carefully monitored daily by a veterinarian and was normal throughout the study.

DIO Model and Compound Dosing. Male C57BL/6J (for studies with NT-0249) or hCES1 mice (for studies with NT-0796) were fed with standard chow diet until week 5, then switched to high-fat diet (HFD). Research Diets, New Brunswick, NJ, USA, #D12492, Rodent Diet with 60 kcal% Fat, Research diet) for an additional 15 weeks. Prior to compound treatment, animals were randomized based on their body weight and total plasma high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TG) values. NT-0249 and NT-0796 were prepared in vehicle (0.5% methylcellulose + 0.2% Tween80) at 2 to 10 mg/ml with a maximum dosing volume of 10 ml/kg (by mouth, three times a day). Three times a day dosing was conducted to compensate for the short half-life of these compounds in mouse and to ensure ≥ 50% brain target cover (Supplemental Fig. 1). For NT-0249 and NT-0796, the three times a day regimen was given at 8 AM, 3 PM, and 10 PM. Semaglutide was prepared in vehicle containing 0.05% Tween80 in PBS [Thermo Fisher (China), Shanghai, China, #20012-043] at a final concentration of 0.001 ng/ml with a dosing volume of 10 ml/kg (subcutaneously, once a day).

Food Intake and Body Weight. Food intake was measured by group (per cage) daily until day 24. At 8:00 AM every day (the beginning of the light cycle), food pellet was weighed and provided to animals ad libitum. At 8:00 AM on the following day (the beginning of the light cycle), the remaining food pellet was weighed again, and the difference was recorded as the total food consumption by the group of animals over 24 hours. The results were expressed as average food consumption per mouse (g; total food consumption/number of animals in the cage). The body weight of each animal was measured daily after food intake measurement. Overnight fasting into day 25 was performed during DIO studies to facilitate the analysis of further exploratory endpoints (e.g., diabetic readouts, Supplemental Fig. 5).

Weight Matching by Calorie Restriction. For the calorie restriction group of animals, food intake was measured daily for 1 week prior to the initiation of compound dosing studies. Throughout the study, the body weight of mice in the calorie-restricted group was matched as closely as possible to that of mice receiving NT-0249 or NT-0796 by daily adjustment of the allocated food. In the NT-0249 study, calorie-restricted animals received 50% calorie restriction from day 0 to day 4, 40% calorie restriction from day 5 to day 8, 30% calorie restriction from day 9 to day 10, 20% calorie restriction from day 11 to day 15, 10% calorie restriction from day 16 to day 18, 0% calorie restriction from day 19 to day 20, and 10% calorie restriction from day 21 to day 28. In the NT-0796 hCES1 mouse study, calorie-restricted animals received 20% calorie restriction from day 0 to day 11 and 40% calorie restriction from day 12 to study end.

Compound Exposure Analysis. For studies using NT-0796, 160 mg/ml sodium fluoride/potassium oxalate (NaF/KOx) 1/3, NaF: Shanghai Aladdin Biochemical Technology Co., Ltd, Shanghai, China, #S111586; KO: Shanghai Aladdin Biochemical Technology Co., Ltd, Shanghai, China, # P111576 with 5% ABEFS (100 mM in DMSO, MCE, Shanghai, China, #HY12821) as stabilizer was added to the terminal blood/EDTA-K3 mixture. Acetonitrile (Innochem, Beijing, China, #20012-043) at a final concentration of 0.001 ng/ml with a dosing volume of 10 ml/kg (subcutaneously, once a day).
China, #A6400) containing internal standard for protein precipitation was added to each sample, then vortexed and centrifuged at 12000 g for 10 minutes. Resulting supernatants were mixed with H2O and the final solutions were injected for liquid chromatography and tandem mass spectrometry analysis. For studies using NT-0796, chromatographic separation was achieved on the Waters UPLC BEH C18 column 50×2.1 mm ID, 1.7 μm. The column temperature was maintained at room temperature. The flow rate was maintained at 0.6 ml/min using mobile phases of H2O (0.1% formic acid, Sigma-Aldrich, Shanghai, China, #F50507-500ML) and acetonitrile (0.1% formic acid). Both (2R)-2-[1,2,3,5,6,7-hexahydro-s-indacen-4-yl]carbonyl[ox]-3-(pyrimidin-2-yl) propanoic acid sodium salt (NDT-19795) and NT-0796 show positive ion mode ionization with mass transitions of 368.00/169.00 and 410.00/211.00, respectively. For studies using NT-0249, chromatographic separation was achieved on XBridge BEH C8 (Waters, Shanghai, China, #M6600) for compound exposure analysis. Blood samples were collected via retroorbital vein on day 0 for plasma lipids analysis (HDL, LDL, TG), at the end of the study terminal blood samples were collected via retroorbital vein on day 1 for plasma lipids analysis (HDL, LDL, TG) (FUJIFILM Wako Pure Chemical Corporation, L-Type Triglyceride Kit, #991-09001/997-09010), LDL (FUJIFILM Wako Pure Chemical Corporation, L-Type LDL-C Kit, #991-39891/997-39991), and TG (FUJIFILM Wako Pure Chemical Corporation, L-Type Triglyceride M Kit, #999-32991/999-33091) were sourced from FUJIFILM Wako Pure Chemical Corporation and measured using Hitachi 7180 Clinical Analyzer (Hitachi, Tokyo, Japan, #7180) according to manufacturers’ instructions. A Meso Scale Discovery U-PLEX assay was used to measure plasma levels of IL-1β, IL-6, and interleukin-1 receptor antagonist (IL-1RA). Plasma was diluted 1:1 in PBS.

IL-1β Release Assays in Human Peripheral Blood Mononuclear Cells. To assay NLRP3 activity in vitro, cytokine output was measured from human peripheral blood mononuclear cells (PBMCs). Heparin-stabilized blood from healthy volunteers was obtained from Bloodworks Northwest (Seattle, WA, USA). PBMCs were prepared using Ficoll-Paque Plus centrifugation (Cytivia, Marlborough, MA, USA, #171140003). Total cell numbers were determined using a hemacytometer and adjusted to 2.6×10^6 cells/ml in RPMI Glutamax medium (Thermo Fisher Scientific, Waltham, MA, USA, #61870-036) containing 1% FBS (Thermo Fisher Scientific, #10082-147), 1% penicillin/streptomycin (Thermo Fisher Scientific, #15140-122), and 20 mM HEPES (Thermo Fisher Scientific, #15630-080), pH 7.3. To assess IL-1β output, 0.1 ml (2.6×10^6 cells) of this cell suspension was added to each well of 96-well plates. After 2-hour incubation at 37°C in a 5% CO2 incubator to allow adherence of monocytes, media and nonadherent cells were aspirated; 0.1 ml of RPMI Glutamax medium containing 5% FBS, 1% penicillin/streptomycin, 20 mM HEPES, pH 7.3 (Base Medium) was added to all wells and the plates were incubated overnight at 37°C in a 5% CO2 incubator. To wells designated to receive lipopolysaccharide (LPS), 0.04 ml of Base Medium containing 350 ng/ml LPS (Sigma-Aldrich, St. Louis, MO, USA, #L3391) was introduced; wells not receiving LPS received 0.04 ml of Base Medium. Plates were incubated at 37°C for 2 hours to allow transcription/translation of proIL-1β. At this point, media were removed by aspiration and 0.143 ml of fresh RPMI Glutamax medium containing 1% FBS and 1% penicillin/streptomycin and test compound at indicated concentrations [or 0.2% DMSO (VWR, Radnor, PA, USA, #BDH1115-LP)] were added; the presence of LPS was maintained in wells previously exposed to this stimulus. Plates were placed at 37°C in a 5% CO2 incubator for 60 minutes. To wells designated to receive ATP, 0.0075 ml of a 100 mM ATP solution (final concentration = 5 mM, Sigma-Aldrich, #A6419) was then introduced to promote NLRP3 activation and the plates returned to a 37°C/5% CO2 incubator for 60 minutes after which the plates were subjected to centrifugation and media supernatants were harvested. A 5 mM palmitate (TCI Chemicals, Portland, OR, USA, #P0007/0.8 mM fatty acid free bovine serum albumin solution (BSA) (MP Biomedicals, Irvine, CA, USA, #152401) was prepared fresh on the day of assay. Fatty acid free BSA was dissolved in prewarmed PBS (Cytivia, Logan, UT, USA, #SH90256.01) to yield a 0.8 M solution to which sodium palmitate was added to a final concentration of 5 mM. This solution was shaken vigorously for 60 minutes at 30°C. To wells designated to receive palmitate, 0.0075 ml of the 5 mM palmitate/0.8 M BSA solution was introduced (final concentration = 250 μM palmitate/40 μM BSA) and the plates returned to a 37°C/5% CO2 incubator overnight. Plates were subjected to centrifugation after which the media supernatants were harvested. Media supernatants were assessed for IL-1β levels by ELISA (Thermo Fisher Scientific, #88-7281-88). Birb-796 was obtained from Tocris Bioscience (Minneapolis, MN, USA, #5898). Semaglutide (#29969) and liraglutide (#24727) were obtained from Cayman Chemical (Ann Arbor, MI, USA).

GFAP Immunohistochemistry in Hypothalamus. Coronal brain sections were embedded in paraffin (Arcadia H, Leica, Deer Park, IL, USA) and cut to a thickness of 4 μm with a microtome (Leica, #RM2235). After heat-induced citrate antigen (pH = 6.0, MXB, Fuzhou, China, #MVS-0066) unmasking, sections were immersed in 3% hydrogen peroxide solution for 5 minutes. To avoid nonspecific staining, the sections were then incubated in blocking serum for 15 minutes at room temperature, followed by anti-GFAP antibody (Abcam, Waltham, MA, USA, #68428) staining in dilution of 1:1000 for 1 hour. Secondary antibody (Dako, Aglient, Beijing, China, #4003) in dilution solution (Dako, Aglient, #S2022) were then added and nuclei stained with hematoxylin stain (Baso, Zhuhai, Fujian, China, #BA0411). Slides were
scanned using Aperio Scanner (Leica, #GT450) at 400X magnification. Images were processed using HALO Image Analysis Platform (Indica Laboratories, Albuquerque, NM, USA). The areas of the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMH), and ventromedial hypothalamic nucleus (VMH) were assigned within the HALO software (Indica Laboratories) and regions of interest annotated for each picture individually using a mouse brain atlas (bregma, −1.70 mm). The bilateral ARC area was measured to be 230498.7 μm² and the bilateral VMH area was 469961.6 μm², while the bilateral DMH area was 354096.6 μm². Percent areas of the ARC, DMH, and VMH displaying weak, moderate, and strong GFAP immunoreactivity were quantified using optical density (OD) threshold values of 0.15, 0.25, and 0.35, respectively, in the Multiplex HIC v3.2.3 module of the HALO Image Analysis Platform (Indica Laboratories). The strongest staining assessed visually was set to 0.35 OD, the weakest staining assessed visually was set to 0.15 OD, and the moderate staining value was the mean of strong and weak values.

**Statistical Analysis.** Statistical analyses were performed in GraphPad Prism 9.2.0. (GraphPad Software, USA) using either one-way or two-way ANOVA followed by Tukey or Dunnett post hoc statistical analysis. A P value of < 0.05 was considered to be statistically significant.

**Results**

One potential mechanism linking obesity to chronic low-grade inflammation involves activation of Toll-like receptor 4 by saturated fatty acids such as palmitate (Korbecki and Bajdak-Rusinek, 2019). To determine if the response to palmitate may also be associated with NLRP3 activation, as suggested by previous studies (Wen et al., 2011; Karasawa et al., 2018), we first primed PBMCs with LPS. Subsequent overnight incubation with palmitate induced significant IL-1β release (Fig. 1A), indicative of NLRP3 activation, whereas treatment with either LPS or palmitate alone failed to drive IL-1β release (Fig. 1A). The prototypical NLRP3 inhibitor, CP-456,773, but not p38 kinase inhibitor BIRB-796, blocked palmitate-driven IL-1β release by 75% (Fig. 1A), indicating that a large proportion of the palmitate response is NLRP3-dependent. In addition, our novel NLRP3 inhibitor, NT-0249 (structure shown in Fig. 1B and synthesis described in the Supplemental Material) potently inhibited palmitate-driven IL-1β release from human PBMCs with an IC50 value of 0.011 μM (Fig. 1B).

Having established a critical role for NLRP3 as a trigger of inflammatory responses to fatty acids of obesity, we wondered whether NLRP3 inhibition contributed to the anti-inflammatory mechanism(s) of glucagon like peptide-1 receptor agonist (GLP-1RA), as previously suggested (Chen et al., 2021; Zhang et al., 2022). However, the “incretin-mimetic” drug liraglutide (GLP-1RA) failed to inhibit NLRP3 (following canonical activation with LPS + ATP) at concentrations up to 10 μM in the PBMC assay (Fig. 1C), despite widely recognized anti-inflammatory effects in multiple tissues (Mehdi et al., 2023). In contrast, our novel, specific NLRP3 inhibitor, NT-0249, potently blocked (LPS + ATP)-induced IL-1β release with an IC50 value of 0.012 μM (Fig. 1D). Furthermore, both liraglutide (Fig. 1E) and semaglutide (Fig. 1F) failed to block palmitate-induced IL-1β release from PBMCs. These data suggest that the GLP-1RAs anti-inflammatory mechanisms(s) are independent of NLRP3.

To investigate the role of NLRP3 inflammasome activation in obesity pathogenesis, mice were fed a HFD for 15 weeks prior to compound treatment. At 15 weeks of HFD, when DIO mice weighed considerably more than chow-fed control mice (Fig. 2A), the effect of dosing NLRP3 inhibitor NT-0249 was compared with that of semaglutide or calorie restriction for a further 28 days. The peripheral pharmacokinetics of NT-0249 (even at 100 mg/kg) highlights its short plasma half-life in mouse (Supplemental Fig. 1A) and the requirement for three times a day dosing to maintain target cover (Supplemental Fig. 1B) in mouse models (see Supplemental Methods). When orally gavaged three times daily at 100 mg/kg, NT-0249 caused significant weight loss within 8 days compared with vehicle-treated mice (Fig. 2A). Whereas the vehicle-treated DIO mice increased their body weight by 7.4% over the study period, mice receiving NT-0249 lost 6.8% of their starting weight while consuming the same HFD (Fig. 2B). Although we observed a slight trend toward weight loss in nonobese, normal chow fed mice receiving NT-0249 (100 mg/kg, three times a day), the effect did not achieve statistical significance (Fig. 2C). Thus, NT-0249 does not cause nonspecific weight loss in normal, lean mice, suggesting that the mechanism of action is specific to obese animals. Relative to vehicle-treated DIO mice, the weight loss driven by NT-0249 was approximately one third below that induced by semaglutide (0.01 mg/kg), which induced a 14.3% reduction in starting body weight (Fig. 2B). To identify the effects of NLRP3 inhibition on systemic inflammatory biomarkers that are not explained by weight loss, we included a weight-matched control group that was fed the same but calorie-restricted HFD. By the end of the study (day 28), this calorie restricted group had lost similar weight (7.4% below starting weight) to the NT-0249-treated group (Fig. 2B).

As expected for a preestablished obese state, average calorie intake between vehicle-treated DIO control mice and chow-fed control mice was comparable across the study dosing period (Fig. 3, A–C). DIO mice treated with semaglutide or NT-0249 or DIO mice on calorie restriction all consumed similar calories over the study period, but their calorie consumption was significantly reduced relative to the vehicle-treated DIO mice (Fig. 3, B and C). Relative to chow-fed mice, DIO mice displayed significant elevations of fat mass, and this effect was markedly reduced by each of the three interventions. Semaglutide, NT-0249, or calorie restriction reduced body fat in DIO mice by 39.1%, 23.2%, or 30.8%, respectively (Fig. 4A).

Specifically, NT-0249 induced significant reductions in perirenal and inguinal fat mass, and semaglutide and calorie restriction drove reductions in perirenal, inguinal, and epididymal fat mass (Fig. 4, B–D). Plasma leptin levels were elevated in DIO mice; treatment with NT-0249, semaglutide or calorie restriction markedly reduced leptin levels (Fig. 4E). Adiponectin levels were also increased in DIO mice, yet therapeutic interventions had no effect on circulating adiponectin (Fig. 4F).

Elevated acute phase reactants and cardiovascular inflammatory biomarkers are key aspects of obesity-related metabolic impairment. Treatment with NT-0249 (100 mg/kg by mouth, three times a day) for 28 days led to significant reductions in DIO-driven acute phase protein fibrinogen (Fig. 5A) and cardiovascular inflammatory biomarkers sVCAM-1 (Fig. 5B) and suPAR (Fig. 5C). By comparison, semaglutide treatment failed to reduce these peripheral markers of inflammation, whereas calorie restriction did reduce plasma suPAR levels (Fig. 5, A–C). We attempted to measure elevations in circulating cytokines, but there was no significant induction of plasma IL-1β, or IL-6 in response to DIO (Fig. 5D). However, plasma IL-1RA was significantly elevated in DIO mice, and treatment with NT-0249 (100 mg/kg by mouth, three times a day)
for 28 days inhibited its induction (Fig. 5E). In contrast, treatment with semaglutide or calorie restriction failed to significantly lower DIO-driven plasma IL-1RA (Fig. 5E).

We suspected that NLRP3 inhibition in peripheral tissues was a key driving factor for the reduction in obesity-related inflammatory biomarkers in the DIO mice. With this in mind, we explored whether lower doses of NT-0249 (sufficient for significant peripheral but not brain target cover) could also lower systemic inflammatory biomarkers, such as fibrinogen. We show that a lower dose of NT-0249 (20 mg/kg by mouth, three times a day) also significantly reduced DIO-driven elevations in fibrinogen (Fig. 5F). The robust blockade of peripheral inflammation by both 20 and 100 mg/kg aligns with the predicted target cover as determined by the $E_{\text{max}}$ model (Daryaee and Tonge, 2019).
Indeed, NT-0249 at 100 mg/kg or 20 mg/kg by mouth three times a day are projected to achieve average peripheral target covers of 70% or 63%, respectively (Supplemental Fig. 1, B and D).

Despite robust peripheral target cover achieved by NT-0249 at 20 mg/kg by mouth three times a day, this dose (unlike 100 mg/kg) failed to affect body weight of DIO mice (Fig. 5G). We speculated that the higher average brain target cover of 50% achieved by NT-0249 at 100 mg/kg by mouth three times a day may contribute to its improved efficacy in DIO mice (over the 20 mg/kg dose, which would achieve an 18% target cover in brain; Supplemental Fig. 1, B and D). If brain NLRP3 inhibition is required for full efficacy in preestablished obesity, we...
reasoned that our recently described (Harrison et al., 2023; Smolak et al., 2024), fully brain-penetrant NLRP3 inhibitor NT-0796 should also drive weight loss in DIO mice. As described previously, the pharmacology of NT-0796, itself a pro-drug of the active metabolite NDT-19795, is enhanced in hCES1 mice, which mimics human expression of carboxylesterase-1 in myeloid cells (Smolak et al., 2024). We fed mice that transgenically overexpress myeloid hCES1 a high-fat diet for 15 weeks prior to compound treatment. Similarly to NT-0249, the pharmacokinetics of NT-0796 highlights its short plasma half-life in mouse (Supplemental Fig. 1E) and the requirement for three times a day dosing to maintain target cover (Supplemental Fig. 1F) in mouse models. When oral gavaged three times daily at 100 mg/kg, NT-0796 led to a significant weight loss within 14 days compared with vehicle-treated mice (Fig. 6A). Using the E_{max} model (Supplemental Methods) and an in vivo IC_{50} (Supplemental Fig. 2C), this dose of NT-0796 delivers active metabolite NDT-19795 both peripherally and centrally with projected target covers of 61% and 66% in the periphery and brain, respectively (Supplemental Fig. 1F). By 28 days, NT-0796-dosed mice had lost 19.0% of their starting body weight (Fig. 6B). Importantly, we observed no effect of NT-0796 (100 mg/kg, three times a day) on body weight (Supplemental Fig. 3A) or average daily food intake (Supplemental Fig. 3B).
Fig. 5. Exploration of NT-0249 efficacious dose on the regulation of cardiovascular risk biomarkers and weight loss in DIO mice. Plasma fibrinogen (A), sVCAM-1 (B), or suPAR (C), IL-1β, IL-6 (D), or IL-1RA (E) in control or DIO mice following 28 days treatment with NT-0249 (100 mg/kg by mouth, three times a day), semaglutide (0.01 mg/kg s.c., once a day), or calorie restriction. Plasma fibrinogen (F) in control or DIO mice following 28 days treatment with NT-0249 (20 or 100 mg/kg by mouth, three times a day). (G) Body weight of normal chow fed mice or DIO mice treated therapeutically with vehicle (by mouth, three times a day), NT-0249 (20 mg/kg or 100 mg/kg by mouth, three times a day) for 28 days. Data are expressed as mean ± S.E.M. (A–C: n = 20 per group; D–G: n = 10 per group) and analyzed using a one-way ANOVA (A–C) or two-way ANOVA (G) with Dunnett’s multiple comparisons test using GraphPad Prism v10.0.2. Significance is calculated relative to vehicle/DIO group. ****P < 0.0001 (A); **P = 0.0044 (B); ***P = 0.0003, ****P < 0.0001 (C); *P = 0.0295 (E); **P = 0.0099, ***P = 0.0003 (F); days 12–19 *P < 0.05, days 20–23 **P < 0.01, day 24 ***P = 0.0004, day 25 **P = 0.0011, days 26–27 ***P < 0.0005, day 28 ****P < 0.0001 versus NT-0249 (100 mg/kg by mouth, three times a day); ns, nonsignificant (G).
Fig. 6. Effects of NT-0796, semaglutide, or calorie restriction on body weights, food intake, and lipid metabolism biomarkers in control or DIO hCES1 mice. (A) Body weight of normal chow fed or DIO hCES1 mice treated therapeutically with vehicle (by mouth, three times a day), NT-0796 (100 mg/kg by mouth, three times a day), or semaglutide (0.01 mg/kg s.c., once a day) for 28 days. Body weights of calorie restricted subjects were maintained as close to NT-0796-treated subjects as possible by controlling the degree of calorie restriction throughout the experiment. An overnight fast into day 25 (dashed line) was performed. (B) Body weights of hCES1 mice from (A) expressed as percent change from day 0. (C) Average food consumption (over 28 days) of control or DIO hCES1 mice treated with vehicle (by mouth, three times a day), NT-0796 (100 mg/kg by mouth, three times a day), or semaglutide (0.01 mg/kg s.c., once a day). (D) Average calorie intake per treatment group was calculated for the 28-day experiment. (E) Total fat mass of control or DIO hCES1 mice following 28 days treatment with NT-0796 (100 mg/kg by mouth, three times a day), semaglutide (0.01 mg/kg s.c., once a day), or calorie restriction. Plasma TG (F), TC (G), HDL (H), LDL (I), or PCSK9 (J), in control or DIO hCES1 mice following 28 days treatment with NT-0796 (100 mg/kg by mouth, three times a day), semaglutide (0.01 mg/kg s.c., once a day), or calorie restriction. Data are expressed as mean ± S.E.M. (n = 10 per group) and analyzed by two-way ANOVA (A and B) or one-way ANOVA (C–J) with Dunnett’s multiple comparisons test using GraphPad Prism v10.0.2. Significance is calculated relative to vehicle/
in nonobese, normal chow fed hCES1 mice (Supplemental Methods), demonstrating that, similar to NT-0249, NT-0796 reverses body weight specifically in obese animals. The effect of NT-0796 on weight loss appeared more pronounced than that of NT-0249 and comparable to semaglutide (0.01 mg/kg), which itself induced a 21.5% weight loss over the 28-day study period (Fig. 6B). A calorie-restricted group of hCES1 DIO mice was also included, and by 28 days, the calorie-restricted regimen provided 16.9% body weight loss in hCES1 DIO mice (Fig. 6B).

Similar to C57BL/6j mice, hCES1 HFD-fed mice consumed less food mass compared with normal chow fed animals (Fig. 6C), yet average calorie intake was similar between both groups over the study period (Fig. 6D). Treatment of DIO hCES1 mice with semaglutide or NT-0796 or calorie-restricted hCES1 DIO mice consumed less food and calories over the study period relative to the vehicle-treated hCES1 DIO mice (Fig. 6, C and D). Relative to chow-fed mice, hCES1 DIO mice displayed enhanced total body fat (Fig. 6E). Treatment with semaglutide or NT-0796 reduced body fat in DIO mice by 37.8% or 31.8%, respectively, over 28 days (Fig. 6E). Specifically, NT-0796 and semaglutide drove significant reductions in epididymal, perirenal, and inguinal fat mass (Supplemental Fig. 4, A–C, respectively). Calorie restriction in hCES1 DIO mice trended to reduce total fat mass (Fig. 6E).

Similar to C57BL/6j mice, hCES1 DIO mice displayed elevated markers of acute phase and cardiovascular inflammation including fibrinogen (Supplemental Fig. 4D), sVCAM-1 (Supplemental Fig. 4E), uPAR (Supplemental Fig. 4F), leptin (Supplemental Fig. 4G), and adiponectin (Supplemental Fig. 4H). Dosing with NT-0796 significantly reduced circulating levels of fibrinogen, sVCAM-1, and suPAR to a greater extent than semaglutide or calorie restriction alone (Supplemental Fig. 4, D–F). Circulating levels of triglycerides were elevated in DIO mice, an effect completely reversed by treatment with semaglutide (Fig. 6F). Treatment of DIO mice with NT-0796 or calorie restriction marginally reduced plasma triglycerides, although this was not significant for NT-0796-treated animals (Fig. 6F).

In addition, NT-0796, semaglutide, and calorie restriction reduced circulating levels of total cholesterol (Fig. 6G), HDL (Fig. 6H), and leptin (Supplemental Fig. 4G). NT-0796 also lowered plasma LDL levels, although this did not reach significance (Fig. 6I). In addition to lowering cholesterol, circulating levels of PCSK9, an endogenous modulator of the receptor for LDL particles, were reduced by dosing with NT-0796 (Fig. 6J). Semaglutide treatment or calorie restriction of DIO mice failed to reduce PCSK9 (Fig. 6J). Reductions in plasma PCSK9 and cholesterol were not apparent in the C57BL/6j DIO mice treated with NT-0249 (data not shown).

The effect of semaglutide, NT-0249, or calorie restriction on glucose homeostasis at 25 days or insulin resistance at 27 days was assessed using the oral glucose tolerance test (OGTT) or insulin tolerance test (ITT), respectively. Relative to vehicle-treated DIO mice, NT-0249-dosed mice displayed improvements in glucose homeostasis following OGTT and trended to show an improvement in insulin resistance as assessed by ITT (Supplemental Fig. 5). The effect of NT-0249 on OGTT and ITT was of a similar magnitude to calorie restriction alone and carried less impact compared with semaglutide (Supplemental Fig. 5).

Terminal exposures of both NT-0249 and NT-0796 were determined in tissues at 2 hours post final dose on day 28 of each experiment. Both active species, that is NT-0249 (Fig. 7A) and NT-0796-delivered NDT-19795 (Fig. 7B), achieved free fraction concentrations at or above their respective in vivo IC50s (Supplemental Fig. 2) in both plasma and brain tissue. NT-0796, being a more tissue penetrant pro-drug, achieved an unbound brain-to-plasma partition coefficient of 0.4, higher than NT-0249s 0.1 (Fig. 7, A and B).

Hypothalamic GFAP, a marker of astrogliosis, was assessed by immunohistochemistry in DIO mice. Relative to normal chow fed animals, the percentage of strongly stained GFAP immunoreactivity in both the ARC and DMH was elevated in vehicle-treated DIO mice (Fig. 8, A–D), consistent with published literature. Dosing with NT-0249 or semaglutide for 28 days significantly reduced strong GFAP immunoreactivity in both the ARC (Fig. 8, A and C) and DMH (Fig. 8, B and D) relative to vehicle-treated DIO mice. Moderately stained GFAP regions were also reduced by NT-0249 or semaglutide (data not shown).

**Discussion**

Obesity is a major public health issue, having tripled worldwide since 1975 and with the World Health Organization now declaring that at least 60% of Europeans are either obese or overweight (Hepatology, 2021; Boutari and Mantzoros, 2022). Recent success in the field of obesity treatment has come with incretin mimetic drugs, although their adverse event profile is now established (Baden et al., 2023). Consequently, alternative or complimentary therapeutic strategies may be required. Previously, studies in NLRP3−/− mice (Stienstra et al., 2011) and prophylactic dosing with a peripherally restricted NLRP3 inhibitor MCC950 (Pavillard et al., 2017) implicated a pathogenic role for this inflammasome in obesity initiation. However, we show for the first time, using two structurally distinct clinical candidates, that NLRP3 inhibition reverses pre-established obesity in mouse models. Comparison with the effects of either semaglutide or calorie restriction also reveals novel insights into weight loss-independent effects of this strategy.

Importantly, efficacy on pre-established obesity appears correlated to the degree of brain target cover achieved by NLRP3 inhibitors. Using an E_max model to evaluate target cover, significant anti-obesity effects of NLRP3 inhibition could be achieved with brain target covers of < 70% but greater than ≥ 50%. Furthermore, significant weight reductions were apparent when NT-0796- or NT-0249-dosed mice achieved free brain compound exposure levels equivalent to or greater than their respective IC50s. The anti-obesity effect of NT-0796 appeared more pronounced than that of NT-0249, which may reflect the highly tissue-penetrant ester chemotype of NT-0796, facilitating enhanced target cover within tissues (Smolak et al., 2024). However, given the differing backgrounds of mice in the NT-0249 and NT-0796 studies, we cannot rule out a
contributing role for a genetic component in the apparently superior efficacy of NT-0796.

Reversal of obesity by NLRP3 inhibition appears to have been mediated largely by reduced food intake, since roughly comparable calorie restriction yielded comparable weight loss in the DIO mouse model. Therefore, although not specifically measured, we infer that there is no independent effect of NLRP3 inhibition on energy expenditure in this study. Semaglutide likely achieves enhanced weight loss by increasing energy expenditure, as previously reported (Gabery et al., 2020). Combined with the lack of any significant effect of our NLRP3 inhibitor compounds on either food intake or body weight in normal mice fed standard chow, we interpret our findings to suggest that 1) inflammatory responses involving NLRP3 activation play a key role to drive obesity in the DIO mouse model and 2) these compounds must be dosed to achieve coverage in brain for these effects to be observed. Stated differently, NLRP3 activation in the brain is implicated in the pathogenesis of DIO.

NLRP3-derived products such as IL-1β, IL-18, and the downstream mediator IL-6 are implicated in obesity-associated cardiovascular risk (Ridker et al., 2017, 2020). We provide data to support a direct role for saturated fatty acids, such as palmitate, upregulated in obesity, as effective damage-associated molecular patterns, capable of fully activating the NLRP3 inflammasome. In contrast to our NLRP3 inhibitors, GLP-1RAs in our hands fail to substantially impact NLRP3 activity. These observations led us to compare the impact on cardiovascular risk biomarkers associated with obesity of a specific NLRP3 inhibitor versus semaglutide.

In C57BL/6 and hCES1 DIO mice, lowering of the acute phase cardiovascular risk factor fibrinogen by NLRP3 inhibition was notably more robust than was achieved by either semaglutide treatment or calorie restriction. This is expected, since hepatic fibrinogen expression is under tight control of the IL-1β-sensitive cytokine IL-6 (Fuller and Zhang, 2001). This is further supported by the inhibition of DIO-induced elevations in circulating IL-1RA by NT-0249. IL-1RA is a member of the IL-1 family and a further acute phase protein under inflammatory control. Thus, IL-1RA and fibrinogen are sensitive biomarkers of NLRP3 activity in the DIO model (compared with IL-1β or IL-6, which remained low in plasma). In addition, obesity-induced increases in circulating sVCAM-1, itself a predictive marker of cardiovascular mortality and morbidity (Troncoso et al., 2021), were suppressed by NLRP3 inhibitors. This is concordant with sVCAM-1’s vascular expression being strongly regulated by inflammatory cytokines, especially IL-1β (Tamaru et al., 1998). Notably, neither semaglutide nor calorie restriction were able to reverse DIO-driven increases in sVCAM-1. suPAR, a further IL-1β-sensitive predictive marker of cardiovascular events (Hayek et al., 2023), was also specifically reduced by NLRP3 activation inhibitors but notably unchanged by semaglutide. Thus, robust inhibition of fibrinogen, sVCAM-1, and suPAR by NLRP3 inhibitors has the potential to reduce cardiovascular risk in obese populations.

Despite a lack of activity of semaglutide on the IL-1β-sensitive markers fibrinogen, sVCAM-1, and suPAR, beneficial effects of GLP-1RAs on cardiovascular function in obese patient populations are apparent (Marx et al., 2022). The lowering of cholesterol and triglycerides by GLP-1RAs likely contributes to their cardioprotective functions, and we observed robust reduction in

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**Fig. 7.** Terminal plasma and brain exposures of NLRP3 inhibitors, dosed 100 mg/kg by mouth, three times a day in DIO hCES1 mice. Two hours post final dose on day 28, blood was collected into tubes containing EDTA with esterase inhibitors then plasma prepared and brains homogenized immediately after dissection. Resulting samples were analyzed by liquid chromatography and tandem mass spectrometry for NT-0249 (A) or, for NT-0796-dosed animals, both the ester pro-drug NT-0796 and active acid metabolite NDT-19795 were measured (B). Free fraction compound concentrations calculated in each matrix are shown (A and B). Data are expressed as mean ± S.E.M. (A, n = 20 per group or B, n = 10 per group). The unbound brain-to-plasma partition coefficient is highlighted for each compound. Dashed lines indicate in vivo assessed IC₅₀ values for respective compounds (see Supplemental Fig. 2).
Fig. 8. Effects of NT-0249, semaglutide, or calorie restriction on GFAP expression in the hypothalamus of DIO mice. GFAP immunohistochemistry within (A) the ARC or (B) DMH of control or DIO mice treated with NT-0249 (100 mg/kg by mouth, three times a day), semaglutide (0.01 mg/kg s.c., once a day), or calorie restriction. Quantification of strong GFAP immunoreactivity within the ARC (C) or DMH (D) is expressed as percent of the respective region and with respect to different treatment interventions. Data are expressed as mean ± S.E.M. (n = 10 per group) and analyzed by one-way ANOVA with Dunnett’s multiple comparisons test using GraphPad Prism v10.0.2. *P = 0.0335, **P = 0.0104 versus DIO/vehicle (B); *P = 0.0182, **P = 0.0087 versus DIO/vehicle (C).
triglycerides of DIO mice treated with semaglutide. We also investigated the plasma levels of PCSK9, a clinically relevant, IL-1β-sensitive (Ding et al., 2020) lipid metabolism biomarker of cardiovascular risk. PCSK9 was marginally elevated in DIO mice, but, importantly, we show that NT-0249 (and not semaglutide) robustly lowers levels of PCSK9 in DIO mice. Concordant with the modulation of PCSK9, NT-0796 also trended to lower circulating levels of LDL. The additional control of PCSK9 levels by NT-0796 may enable enhanced improvements in hyperlipidemia by NLRP3 inhibition in obese states (over semaglutide and calorie restriction). Future studies in murine models of atherosclerosis are warranted to determine the extent to which obesity-associated vascular disease can be ameliorated by NLRP3 inhibition.

Emerging data suggest that reactive gliosis in the hypothalamus and elsewhere contributes to the maintenance of obesity (Huwart et al., 2022; Sa et al., 2022). In line with this, weight-reducing efficacy of NLRP3 inhibition in DIO mice was greatest when NT-0249 or NT-0796 dosing strategies achieved significant brain exposure. In further support of this, the dosing strategies used for NT-0249 reversed DIO-induced GFAP expression, which is also known to be sensitive to the NLRP3-dependent cytokine IL-1β (Sticcozi et al., 2013). The inhibition of hypothalamic GFAP expression by NT-0249 is demonstration of its anti-inflammatory potential and supports previous evidence highlighting a direct role of NLRP3 in cerebral GFAP expression following diverse inflammatory challenges (Jha et al., 2010; Youm et al., 2013; Tejera et al., 2019; She et al., 2022). Indeed, microglial NLRP3 is a key regulator of astrocyte activation, an effect thought to rely, at least in part, on microglial release of and astrocyte sensing of IL-1 cytokines (Li et al., 2022). Furthermore, specific astrocytic deletion of the IL-1 receptor signaling adaptor MyD88 inhibits obesity pathogenesis, whereas astrocytic enhancement of IKKβ/IL-1β signaling supports the development of obese states in mice (Zhang et al., 2017; Jin et al., 2020). Although not specifically measured in the current study, our demonstration of GFAP reduction via a centrally penetrant NLRP3 inhibitor is consistent with a potential role for MyD88/NF-kB-dependent astrocyte activation in the maintenance of obesity. Emerging data indicates that extra-cerebral GABA release from reactive astrocytes can modulate hypothalamic neuronal activity to control energy expenditure (Sa et al., 2023). Whether a similar astrocytic mechanism contributes to the reduction in calorie intake and modulation of body weight we observe via NLRP3 inhibition requires additional work. However, it is noteworthy that the NLRP3-dependent cytokine IL-1β is associated with increased GABAergic tone and astrocytic GABA release in rodents (Patel et al., 2019; Shim et al., 2019).

In addition to the effects of NT-0249 and NT-0796 on adiposity and gliosis, we show that lower doses are sufficient to mediate peripheral anti-inflammatory effects in DIO mice. Therefore, NLRP3 activation within peripheral tissues, such as the liver, contributes to obesity-associated systemic inflammation that can be ameliorated by NLRP3 inhibitors independently of NLRP3. Previous work has also indicated a pathogenic role for NLRP3 in the initiation of insulin resistance during obesity. In particular, HFD-fed NLRP3−/− mice were less insulin resistant and more glucose tolerant than HFD-fed wild type mice (Vandanmagsar et al., 2011). We detected improvements in glucose homeostasis and insulin resistance in mice treated with NLRP3 inhibitor NT-0249, but these improvements were similar to that of calorie restriction alone. Thus, further studies are required to disentangle the contribution of weight loss to the effect of NT-0249 on glucose homeostasis and insulin resistance.

In summary, we demonstrate that NLRP3 inhibitors are uniquely positioned (over GLP-1RAs) to block the initiation of cellular inflammatory responses to obesity-related damage-associated molecular patterns, such as palmitate. Furthermore, using two novel inhibitors, NT-0249 and NT-0796, we demonstrate that NLRP3 inhibition curtails systemic inflammation and astrogliosis in preestablished obese states in mice. Importantly, we show that IL-1β-responsive, systemic inflammatory markers, including the acute phase protein fibrinogen and cardiovascular risk factors eCAM-1, uPAR, and PCSK9 are particularly sensitive to NLRP3 inhibition during obese states. In addition, when NLRP3 inhibitors are dosed to levels achieving brain exposure, we demonstrate for the first time their capacity to reverse preestablished obesity with similar efficacy to the GLP-1RA semaglutide.

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Data Availability
The authors declare that all the data supporting the findings of this study are available within the paper and its Supplementary Material.

Authorship Contributions
Participated in research design: Thornton, Reader, Smolak, Lindsay, Harrison, Clarke, Watt.
Conducted experiments: Smolak.
Performed data analysis: Thornton, Digby, Smolak, Watt.
Wrote or contributed to the writing of the manuscript: Thornton, Digby, Smolak, Harrison, Watt.

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