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Original Article

Therapeutic Assessment of Cytochrome C for the Prevention of Obesity Through Endothelial cell-targeted Nanoparticulate System

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Short Title: CytC as a Potential and Safe Antiobesity Drug

Abstract

Because the functional apoptosis-initiating protein, cytochrome c (CytC) is rapidly cleared from the circulation ($t_{1/2}$: 4 min), it cannot be used for *in vivo* therapy. We report herein on a hitherto unreported strategy for delivering exogenous CytC as a potential and safe anti-obesity drug for preventing diet-induced obesity, the most common type of obesity in humans. The functional activity of CytC encapsulated in prohibitin (a white fat vessel specific receptor) - targeted nanoparticles (PTNP) was evaluated quantitatively, as evidenced by the observations that CytC-loaded PTNP causes apoptosis in primary adipose endothelial cells in a dose dependent manner, whereas CytC alone did not. The delivery of a single dose of CytC through PTNP into the circulation disrupted the vascular structure by the targeted apoptosis of adipose endothelial cells *in vivo*. Intravenous treatment of CytC-loaded PTNP resulted in a substantial reduction in obesity in high-fat diet fed *wt* mice, as evidenced by the dose dependent prevention of the % increase in body weight and decrease in serum leptin levels. In addition, no detectable hepatotoxicity was found to be associated with this prevention. Thus, the finding highlights the promising potential of CytC for use an anti-obesity drug, when delivered through a nanosystem.

Introduction

Obesity, the deposition of excess fat in the body, is recognized as a growing medical problem worldwide[1]. The current pharmaco-therapeutic management of obesity is limited to one drug: Orlistat. This drug, however, is also associated with low efficacy (≤ 5%) and has numerous side effects, including severe diarrhea, acute kidney toxicity and malabsorption of vital vitamins[1-3]. Therefore, it is likely that it will not be possible to manage this growing epidemic disease using this drug, and there is great urgency and need for introducing a novel, safe and effective antiobesity drug.

White fat (WF) exhibits angiogenic activity[4, 5]. It was reported that the expansion and growth of WF is highly correlated with the formation of new adipose vessel[4-7]. Consequently, anti-antiangiogenic drugs such as TNP-470, matrix metalloproteinase inhibitors (MMPI), polyphenol compounds, proapoptotic peptide ($_D$(KLAKLAK)$_2$, KLA), etc have shown promise for weight reduction and adipose tissue loss in various models of obesity[8-12]. Their results clearly established that ablation or inhibition of neovascularization via apoptosis/necrosis of endothelial cells in WF has the potential to inhibit the formation of new adipocytes, adipocyte tissue hypertrophy and ultimately, the development of obesity. However, owing to the generally pleiotropic activity of conventional chemotherapeutics, the use of these drugs is associated with neurotoxicity, nephrotoxicity and cardiotoxicity, even though they are administered at their respective therapeutic dosage[9, 13, 14]. Therefore, the selective targeting of adipose endothelial cells by pharmacological manipulation through nanoparticulate-targeted system offers an attractive therapeutic avenue for the effective management of obesity.

Cytochrome c (CytC), either endogenous release or exogenous delivery into cell cytoplasm executes its apoptotic role through activation of a caspase cascade[15-17]. Thus, the use of this

biomacromolecule may be advantageous for the development of a potential novel therapeutic system; (i) CytC, itself triggers the release of it through stimulating the release of calcium from the endoplasmic reticulum and co-stimulates its apoptotic activity, (ii) it is non-toxic because of its impermeability to the cell membrane, and is readily cleared from the circulation ($t_{1/2}$: 4 min) [15-19]. However, to allow its eventual interaction with corresponding targeted moieties in the cytoplasm of cells, exogenous CytC must be shielded from external inactivating effects *in vivo* and subsequently be delivered to its site of action.

In an attempt to develop targeted therapy for the control of obesity, Kolonin, MG *et al*. previously originally reported on prohibitin as a vascular receptor for the circular peptide motif (CKGGRAKDC) in WF of wild type and $lep^{ob/ob}$ mice[12]. Regarding the adoption of an alternative adipose vascular-targeting strategy, we recently reported the preparation of an adipose endothelial cell-targeted nanoparticle *in vitro*[20, 21]. For *in vivo* applications, it was further optimized by the incorporation of three elements on its surface and this was regarded as prohibitin-targeted nanoparticle (PTNP). The PTNP system was designed in such way so that (i) a PEGylated lipid bilayer provided the protection of encapsulated therapeutic cargos against biodegradation, (ii) a prohibitin-homing peptide ligand specifically and potentially delivered its payload to the cytosol of endothelial cells in white fat vessel (WFV), and (iii) a long PEG-spacer between ligand and the surface of PEGylated nanoparticle assisted to reduce steric hindrance for multivalent active ligand-receptor recognition[22].

Considering that the PTNP system would be able to specifically and potentially deliver a therapeutic cargo to the endothelial cells of WFV, we propose herein a highly biodegradable protein, CytC as an effective and safe anti-obesity drug. Interestingly, CytC induced apoptosis of endothelial cells of WFV *in vitro* and *in vivo* when it was delivered through the PTNP system

and the CytC-loaded PTNP prevented diet-induced obesity (DIO) in a dose-dependent manner without any detectable liver toxicity. It can therefore be concluded that CytC is a potentially promising drug for the effective and safe prevention of DIO, the most common type obesity in humans.

Results

Physicochemical characterization of empty- and CytC-loaded PTNP

The average nanoparticle sizes and polydispersity index (PDI) of empty- and CytC-loaded PTNP were similar (around 100 nm, PDI<0.3) (**Table 1**). Nanoparticles with average diameter of around 100 nm may be suitable for the extended retention of them into the blood circulation for delivering the drug to its targeted cells[23]. The nanoparticles without or with CytC had shown almost neutral (0.3 ± 2.5 mV) and (2.5 ± 5.9 mV) zeta potentials, respectively (**Table 1**). The similar zeta potentials of empty- and CytC-loaded PTNP suggested that CytC retained maximally within the PTNP system as an aqueous phase since CytC with an isoelectric point (pI 10.5) and with an affinity to associate with neutral lipids might be facilitated its encapsulation into nanoparticles without electrostatic interaction with lipid membrane[24, 25].

Evaluation of the functional activity of CytC-loaded PTNP on primary endothelial cells derived from murine adipose tissue *in vitro*

In order to evaluate the functional activity of CytC via the cytoplasmic delivery of its active form by PTNP into primary endothelial cells isolated from murine adipose tissue (pcEC-IWAT), we performed a series of quantitative and qualitative *in vitro* apoptosis experiments. Treatment with various doses of CytC-loaded PTNP (3, 6 and 12 μg/ml of CytC) induced the activation of caspase 9, indicating the apoptosis of pcEC-IWAT cells in a dose-dependent manner, while the controls (free CytC at same doses and placebo treated cells) did not (**Fig. 1a**). In addition, apoptotic cells that were characterized by round shape, cell shrinkage, membrane blebbing, nucleus DNA fragmentation and condensation of pcEC-IWAT cells, were observed when CytC-loaded PTNP was used at a dose (6 μg/ml of CytC) whereas the controls did not (**Fig. 1b**). These

data indicate that CytC retained its active form into cytosol of pcEC-IWAT cells and that it caused apoptosis of these cells in a dose-dependent manner, only when CytC was delivered through the PTNP system.

Moreover, we evaluated the cell type specificity by the treatment of either the CytC-loaded PTNP system (6 μg/ml of CytC) or a physical mixture [empty-PNTP (0.65 μmol) + CytC (6 μg/ml)] on pcEC-IWAT and NIH3T3 to induce apoptosis for 3h. The CytC-loaded PTNP system significantly induced caspase 9 activation in pcEC-IWAT cells, compared to the NT control whereas, in the case of NIH3T3 cell, it did not. In addition, when both cells were treated with a physical mixture, no induction of caspase 9 activation was observed (**Suppl. Fig. 1**).

Vascular disruption of adipose tissue with a single dose of CytC-loaded PTNP *in vivo*

In order to confirm the successful delivery of intact PTNP to WFV, double-labeled PTNP was *i.v*-injected into mice. After 6h, double-labeled PTNP was highly colocalized with vessels, as evidenced by the integration of NBD-DOPE (lipid membrane) and rhodamine (aqueous phase) in the same nanoparticle and with alexa647-labeled *G. simplicifolia* isolectin B4 (GS-IB4) (vessels), while it was not detected in the vessels of other organs (lungs, heart, kidneys and liver) (**Fig. 2a**). Meanwhile, confocal observations of plasma fractions in blood indicated that the double-labeled PTNP remained in an intact state [Colocalization of lipid membrane: NBD-DOPE and aqueous phase: rhodamine], as identified by yellow dots, which is analogous to long circulating double-labeled PEGylated nanoparticles (double-labeled NTNP) (**Suppl. Fig. 2a**). In addition, the spectra of NBD-DOPE and rhodamine appeared in the same position and the intensity was quite similar, like those of double-labeled NTNP (**Suppl. Fig. 2b**). Considering these results, the colocalization of the double-labeled PTNP to WFV indicates that PTNP remains intact in the

circulation and therefore shows promise for use in delivering an aqueous phase marker to a target. Thus, mice were given a single intravenous injection of a dose of 6 mg/kg of CytC in lieu of aqueous phase marker-loaded PTNP. At 24h postinjection, WFV stained with GS-IB4 was co-localized with the green fluorescence (FITC), indicating the existence of active caspase activity, whereas, in non-treated and empty-PTNP treated mice, only background fluorescence was observed (**Fig. 2b**). In addition, the delivery of CytC through the PTNP system did not induce the apoptosis of endothelial cells in other organs such as heart, lung, liver, spleen and kidney, whereas in the case of the physical mixture (empty-PTNP + CytC) or non-treated, apoptosis was not found in the WFV (**Suppl. Fig. 3**). These findings provide further evidence to show that the cytoplasmic delivery of active CytC via PTNP can cause adipose endothelial cell apoptosis *in vivo*.

Delivery of CytC-loaded PTNP decreases the progression of obesity burden

To determine whether the targeted delivery of CytC has any observable beneficial effects in preventing obesity, we then treated mice that had been exposed to a high-fat diet (HFD) at 3 day intervals for a total of 30 days with *i.v*-injections of 6, 1.2 and 0.25 mg/kg of the CytC-PTNP system or untreated [allowed to HFD and normal diet (ND)] as a pair of controls. The % increase in body weight for the HFD fed mice was significantly decreased when treated with the CytC-PTNP system respectively, compared to the HFD control group and the effect was dose-dependent (**Fig. 3**). In addition, the treatment of mice with the CytC-loaded PTNP system did not decrease the cumulative energy intake, compared to the physical mixture and the non-treated (ND and HFD) mice (**Suppl. Fig. 4a**) while the blood glucose concentration of all groups was comparable (**Suppl. Fig. 4b**). These results provide strong evidence to show that treatment with

CytC-loaded PTNP has the potential for use in preventing the development of obesity in mice that are fed HFD without any effect on food intake.

The prevention of diet-induced obesity is associated with ablation of fat mass

To observe whether the targeted apoptosis of adipose endothelial cells by treatment with CytC-loaded PTNP is associated with the ablation of fat mass, we excised the subcutaneous adipose tissue (SAT) and epididymal adipose tissue (EAT) on day 30 of the treatment. The size and length of SAT and EAT showed a substantial decrease and resembled excised organs obtained from the ND fed control group. In contrast, the size and length of these adipose tissues from the HFD control group were large (**Fig. 4a**). Dissected SAT and EAT also showed that the mass of fat depots from treated mice were significantly reduced compared to the HFD control group (**Fig. 4b**). These results indicated that targeted ablation of adipose endothelial cells via the CytC-PTNP system had the potential to reduce the increase in fat mass.

Plasma parameters related to anti-obesity

To further examine the effect of anti-obesity related to biomolecules, we measured serum parameters. The HFD fed control group showed high levels of leptin, whereas the levels for the ND fed control group were maintained at normal levels (**Fig. 5a**). Treatment with CytC-loaded PTNP suppressed the serum leptin levels in a dose-dependent manner (**Fig. 5a**). The treated mice also showed a tendency to have retrofitted serum levels of TNF-α compared with HFD controls, similar to the ND fed control group (**Fig. 5b**). The levels of triglycerides (TG), total cholesterol and free fatty acids (FFA) had a tendency to decrease in the treated mice compared with HFD

fed group (not statistically significant) (**Figs 5c-e**). These results suggested that CytC-loaded PTNP had an impact to retrofit the obesity-related parameters.

Toxicological study

To investigate the toxicities associated with this therapy, we further measured the serum parameters that are involved in eliciting the toxicities of liver. The serum levels of liver enzyme, alanine aminotransferase (ALT), remained normal in mice that were treated with CytC-loaded PTNP (**Fig. 6**).

Discussion

The current pharmaco-therapeutic management for obesity is limited only to the use of Orlistat, a lipase inhibitor. In the present study, the therapeutic potential of CytC as a novel antiobesity drug through prohibitin-targeted nanoparticulate system for the prevention of DIO was investigated.

CytC is a physiologically adaptable mitochondrial protein. It plays an important role in mediating apoptosis via the exertion of multiple stimuli such as Bax, Ca^{2+} overload, oxidants, chemotherapeutics, and DNA-damaging agents on mitochondria. Upon such conditions, a permeability transition pore was formed in the outer mitochondrial membrane, thus releasing CytC from the intermembrane space of mitochondria into the cytosol. The liberated CytC initiates the formation of an apoptosome consisting of apoptotic protease-activating factor-1 (APAF-1) oligomers. The APAF-1 apoptosome recruits and activates caspase 9, which, in turn, activates the executioner caspases, caspase 3 and 7. The active executioners kill the cell by the proteolysis of key cellular substrates[26-30]. In addition, since apoptosis is a physiological process in which apoptotic bodies are formed by degradation via the action of lytic enzymes, they can readily undergo clearance by lymphocytes and macrophages with minimal deleterious effects to the healthy surrounding tissue[26, 27]. Although an adequate level of cytoplasmic CytC can affirm apoptosis of that cell, the delivery of exogenous CytC to its active form in the cytoplasm of cell remains a great challenge *in vivo.*

In order to achieve the functional activity of this protein in obesity, it is necessary to deliver exogenous CytC into the cytoplasm of endothelial cells in adipose tissue after encapsulating it within nanoparticulate system, which can be targetable. PTNP system specifically accumulated into the vascular endothelial cells of adipose tissue in mice, while it was sparing the vessels of

other organs such as brain, heart, lungs, kidneys, liver and spleen[22]. Therefore, it may be feasible that PTNP has the ability to specifically deliver its associated therapeutic cargo in the WFV *in vivo*. We next encapsulated CytC into nanoparticles and the resulting products were characterized (**Table 1**). Since CytC is a water soluble physiological protein with an isoelectric point (pI 10.5), it may be assumed that it would be readily encapsulated as an aqueous internal core into a nanoparticle with a diameter of around 100 nm. It was reported that hydrophilic proteins, including CytC, Ovalbumin (OVA, pI 4.5) and Horseradish peroxidase (HPR, pI 7.2) are likely to be physically loaded into nanoparticles without using electrostatic interactions between carrier and proteins[24]. In addition, we found that the entrapped aqueous volume of CytC-PTNP estimated from the % recovery ratio of lipid (33.9%) and CytC (15.23%) after ultracentrifugation was 64.5 µl/mg of lipids, suggesting that this high volume of entrapment of CytC/mg by lipids (EPC and Chol) may be not only by the physical entrapment but also demonstrates the ability of CytC to interact with neutral lipids[24, 25]. However, thequestion of whether encapsulated CytC is an active or inactive state in nanocavities needs to be addressed. Therefore, we initially tested the feasibility of directly treating CytC with an organic solvent mixture [Chloroform: diisopropyl ethanol, 1:1 (vol/vol)] which was utilized in the preparation of the PTNP system. The ratio of $O.D_{400}$ and $O.D_{280}$ for the treated CytC solution along with probe sonication at the same condition indicated that most of the CytC (approximately 90%) remains in an active without lipids in this process (**Suppl. Fig. 5**). The active CytC-loaded PTNP was then applied on pcEC-IWAT for the quantitative evaluation of *in vitro* apoptosis, suggesting that cytoplasmic delivery of CytC through the PTNP system induced apoptosis of pcEC-IWAT in a dose-dependent manner (**Fig. 1a**). Furthermore, the apoptosis of pcEC-IWAT cells by the treatment of CytC-loaded PTNP was confirmed by the altered endothelial cell characteristics,

including round shape, DNA condensation, etc. that were consistent with the findings of apoptosis in other cell lines[16, 17] (**Fig. 1b**). It is likely that PTNP binds to this endothelial cell through the receptor-ligand interaction, internalizes and delivers its content into cell cytoplasm to be escaped from endosomes/lysosomes[21]. On the other hand, free CytC, due to its membrane impermeability and lack of appropriate machinery to enter into the cell, did not do (**Figs. 1a, b**). Moreover, in our preliminary examination, OVA, a non-apoptosis inducing protein, did not induce caspase 9 activation when it was delivered to pcEC-IWAT through the PTNP system (data not shown). These results demonstrate the functional ability of CytC to cause apoptosis of pcEC-IWAT in a dose-dependent manner.

CytC is a highly biodegradable protein with a biological half life of 4 min. It is easily adsorbed by blood proteins and the subsequent interaction with phagocyte results in the rapid clearance from the body[19]. Intactness of PTNP inside the circulation is one of the key features to deliver exogenous CytC as an aqueous internal core to the cytoplasm of vascular endothelial cells into adipose tissue since PTNP may offer a new identity of CytC by causing CytC take on the pharmacokinetic profiles of the carrier. Therefore, through double labeling of PTNP, we observed intact PTNP (an integrated lipid membrane and aqueous core in the same nanoparticle) on WFV (**Fig. 2a**). As mentioned above, cytoplasmic delivery of CytC via PTNP to endothelial cells could activate caspase cascade even at very minute doses. We then tested its functional activity *in vivo* after the systemic administration of CytC-loaded PTNP at one of these doses (6 mg/kg body weight). As a result, the vascular architecture in adipose tissue was destroyed via targeted apoptosis of endothelial cells (**Fig. 2b**). These findings provide further evidence to indicate that cytoplasmic delivery of CytC through intact PTNP inside the circulation is associated with adipose endothelial cell apoptosis *in vivo*.

The growth of adipose tissue is dependent on angiogenesis[4-7]. Ablation or inhibition of vascular endothelial cells in adipose tissue by therapeutic applications has established as a promising strategy to control obesity[8-12]. The intravenous administration of CytC-loaded PTNP prevented the % increase in body weight for the HFD fed mice in a dose-dependent manner (**Fig. 3**). The weight reduction can be explained by a significant loss of SAT and EAT mass which is related to the reduction in their size and length (**Figs. 4a, b**). Several antiangiogenic drugs (TNP-470, polyphenol compounds) prevented DIO with a decreased vasculature in adipose tissue. Ablation of vessels in adipose tissue promoted the loss of adipose tissue, as reported by the relation with adipocyte hypotrophy and adipocyte hypoplasia[8-12, 31]. In agreement, our present work also demonstrates that CytC prevents DIO in a dose-dependent manner via the targeted apoptosis of vascular endothelial cells in adipose tissue.

In developing a novel therapeutic protein, CytC for obesity, it may be feasible to investigate its effects on serum parameters which are related to obesity and the induction of toxicity that may limit the use of such protein. Treatment of the HFD fed mice with CytC-loaded PTNP significantly reduced serum leptin levels in a dose-dependent manner. A significant suppression of serum leptin levels is correlated with adipose tissue hypoplasia, reduction in weight and size of fat-depots[32] as alluded to above (**Fig. 5a**). The treated mice showed a tendency to have decreased serum levels of TNF-α (**Fig. 5b**). TNF-α is a proinflammatory cytokine that is mainly secreted by macrophages during obesity[33]. With the secretion, migration of several inflammatory cells such as macrophages and preadipocytes into adipose tissue is initiated and the subsequent result is the inflammation of adipose tissue that is highly associated with adipose tissue hypertrophy and insulin resistance[33, 34]. Thus, the treatment may also have an impact on a decrease in adipose tissue toxicity and an improvement in insulin sensitivity. The HFD treated

mice are known to have a tendency to a dyslipidemia characterized by a decrease in the levels of triglycerides (TG), total cholesterol (TC) and free fatty acids (**Figs 5c-e**). However, the finding of no significant levels of several serum parameters such as TG, FFA and TC were present after treatment with the CytC-loaded PTNP system for 30 days suggests that further investigations with the aim of assessing the improvement of blood glucose and insulin action by the administration of the CytC-loaded PTNP system for a long-term or to obese animals would be highly desirable. In addition, the evaluation of serum levels of liver enzymes, alanine aminotransferase (ALT) in the CytC treated mice by ALT assay indicated that no apparent hepatotoxicity develops as a result of treatment with CytC-loaded PTNP on day 30 (**Fig. 6**).

In conclusion, the findings reported here show the first therapeutic value of a highly biodegradable protein, CytC even at very low dosage after cytoplasmic delivery through prohibitin-targeted nanoparticles. With this strategy, CytC-loaded PTNP prevents diet-induced obesity in C57BL/6 mice in a dose-dependent manner, which is associated with the targeted ablation of vascular endothelial cells via apoptosis in adipose tissue. Such a physiological protein has the potential to improve the efficacy of obesity therapy while reducing the toxicities associated with both the drug and the carrier in other mouse models of obesity.

Materials and Methods

Materials and animals

N-[(3-maleimide-1-oxopropyl) aminopropyl polyethylene glycolcarbamyl] distearoyl-sn-Glycero-3-phosphoethanolamine (Maleimide-PEG-DSPE) was bought from Nippon Oil and Fat Co. (Tokyo, Japan). Peptide (Pep: GKGGRAKDGGC-NH2, Purity: 93.6%, MW: 1004.15) was synthesized by Kurabo Industries, Osaka, Japan. Cytochrome C from bovine heart (purity: 97%, Mol. Wt. 12327) was purchased from Sigma-Aldrich, USA.

Five week old male *wt* C57BL/6J mice were purchased from SLC Japan (Shizuoka, Japan). All animals were acclimatized for one week prior to use. Animal experiments involved standard procedures approved by the institutional animal care and research advisory committee of Hokkaido University, Sapporo, Japan.

Nanoparticle preparation and characterization

We conjugated a prohibitin-targeted peptide (GKGGRAKDGGC-NH2) with maleimide-PEG_{5kDa}-DSPE as described previously[21]. The incorporation of a prohibitin-targeting peptide lipid conjugate (1.25 mole% of Pep-PEG_{5kDa}-DSPE and 1 mole% of PEG_{2kDa}–DSPE of total lipids) or without the peptide, the addition of system PEG-density in egg York phosphatidylcholine (EPC) and cholesterol (Chol)-based nanoparticles were prepared by the reverse phase evaporation (REV) method as previously described[21]. In addition, rhodamine-DOPE (1 mol% of total lipids) or NBD-DOPE (1 mole% of total lipids) was attached on its surface and the aqueous phase with sulfo-rhodamine (0.5 mM) was loaded into nanoparticle. The sizes and zeta potentials of nanoparticles were measured by photon correlation spectroscopy on a Malvern Zetasizer (Malvern instruments, Malvern, UK).

Confocal observation of double labeled prohibitin-targeted nanoparticle *in vivo*

Mice (6-wk-old male *wt* C57BL/6J) were administered intravenously with double labeled PTNP (lipid membrane: 1 mole % NBD-DOPE and aqueous phase: 0.5 mM Rhodamine in 10 mM HEPES) to a total lipid dose of 0.1 mmol/kg. After 6h, the mice were anesthetized and as much blood as possible removed by cardiac puncture. Tissues from the the adipose inguinal region, lungs, heart, kidney and liver were collected and washed 3 times with Hank's Buffered Salt Solution (HBSS) and then cut into small pieces. The pieces, after washing with HBSS, were transferred to light-protected disposable microcentrifuge tubes (1.5 ml) containing HBSS (1 ml) and then placed on ice until use. The pieces that were transferred to glass-base dishes were viewed under a confocal laser scanning microscopy (A1; Nikon).

Meanwhile, blood was collected and centrifuged at 400x g to remove blood cells. The plasma fractions that were obtained from the double-labeled PTNP system and the double-labeled non-targeted PEGylated nanoparticles (double-labeled NTNP) after transferring into glass-base dishes were observed by confocal microscopy (A1; Nikon).

Measurement of encapsulated CytC into nanoparticles

CytC was dissolved in HEPES buffer (pH 7.4) at the concentration of 1.2 mg/ml. The solution was applied as an aqueous phase to be encapsulated into nanoparticles. Then, free CytC (non-encapsulated) was washed away by carrying out two times ultracentrifugation for 30 min at 85000x g for each wash. Precipitation and BCA assays were performed for protein measurement without affecting interfering substances, as described previously with minor modification[35]. Briefly, after washing the pellets with 2 ultracentrifugations as described above, the pellets

containing various concentrations of CytC-loaded nanoparticles were dispersed in deionized water (0.1 ml). The final volume in each suspension (0.1 ml) was transferred to separate disposable microcentrifuge tubes (1.5 ml) with the addition of 1 µl sodium dodecyl sulfate (SDS) (5% w/v) containing 0.1 N NaOH. The optical density (O.D) of samples and controls (empty nanoparticle) as a blank was measured at 562 nm using a Beckman coulter DU-640 spectrophotometer. We measured the recovery ratio of encapsulated CytC according to the formula: % Recovery Ratio (%RR) = P1 / P0 X 100, Where P0= O.D of the total CytC used in nanoparticle (free + encapsulated), P1= O.D of CytC (encapsulated). In addition, we also measured the encapsulation of rhodamine, as described previously[22]. The % recovery ratio of CytC and rhodamine using a 1.2 mg/ml of CytC and a 0.5 mM solution of rhodamine was determined to be approximately 15.2±0.3% and 2.8±0.5% respectively, and were chosen in all the proceeding experiments.

Moreover, we directly added the CytC solution (1.2 mg/ml) in a 1:1 (chloroform: diisopropyl ether) mixture (vol/vol) without lipids (EPC and Cholesterol) and the preparation was then probe sonicated at the same conditions as described previously[21] to observe the impact on the integrity of CytC in this process. With or without probe sonication, the water phase (CytC) and the organic solvent phase were immiscible **(Suppl. Fig. 5a)**. In addition, after probe sonication, we measured the optical density (O.D) of the treated CytC solution, to confirm its active state. The absorbance of the CytC solution with or without treatment with an organic solvent was measured at 400 nm for the heme moiety and at 280 nm for the protein moiety due to the aromatic amino acids in the protein chain. As a result, the O. D_{280} of the treated CytC solution was quite similar to that of the non-treated CytC solution, indicating that the CytC is not removed by the organic solvent (**Suppl. Fig. 5b**). Furthermore, the $O.D_{400}$ of the treated CytC solution was decreased

(around 18%) in comparison to the non-treated sample, indicating that the majority of the CytC protein appeared to retain its intactness (**Suppl. Fig. 5b**). The ratio of the absorbance of heme and protein in the treated CytC after probe sonication was decreased but the majority of the CytC (around 90%) remained in its active state (**Suppl. Fig. 5c**).

Quantitative evaluation of apoptosis activity through an *in vitro* apoptosis assay

Primary endothelial cells were isolated from murine adipose tissue (pcEC-IWAT) as described previously[21]. pcEC-IWAT cells ($5x10^4$ cells/well) were cultured overnight in the presence of 1 ml EGM-2MV media (Lonza) in 48 well plates. The cultured cells were incubated with 3, 6 and 12 mg/l CytC-loaded PTNP after adding 0.5 ml EGM-2MV whereas the final applied lipid contents (0.65 μmol) were adjusted to that of the placebo (empty-PTNP), placebo and free CytC (3, 6 and 12 μg/ml) for 3 hours. After rinsing with 0.5 ml (-) PBS, the cells were treated with 0.1 ml of lysis buffer in each well. The well plate was kept at -80°C for 3h and the cells detached from the wells with cell scrappers. The cell suspension was centrifuged for 2 min at 450 ×g. The cell lysate, mixed with an equal volume (1:1) of Caspase-Glo 9 reagent (G8212, Promega, USA), was incubated at room temperature for approximately 1h. The expression of caspase 9 was recorded as luminescence (RLU) with a bio-instrument atto luminometer (AB-2250, Osaka, Japan).

In addition, both pcEC-IWAT and NIH3T3 cells were also prepared as described above. These cells were treated with the CytC-PTNP system (6 μg/ml of CytC) and the physical mixture [empty-PTNP (0.65 μmol) + CytC (6 μg/ml)], followed by incubation for 3h for induction of apoptosis. The expression of caspase 9 was recorded as luminescence (RLU), as described above.

Characterization of apoptotic primary adipose endothelial cells by confocal microscopy

pcEC-IWAT cells were seeded on sterile 35 mm glass-base dishes in the presence of 1 ml EGM-2MV media. The cell density was 2×10^5 cells/well. The cells were incubated for 24 h to 50% confluence and then incubated with 6 μg/ml of CytC-loaded PTNP while the final applied lipid contents (0.65 μmol) were adjusted to that of the placebo (empty-PTNP), free CytC (6 μg/ml), empty-PTNP and non-treated cells after adding 1 ml EGM-2MV for 6h. The cell nuclei were stained with Hoechst 33342 (final concentration: 2.5 μg/ml) for 15 min and the cells were observed by CLSM with Plan Apo 20 x/NA objective lenses.

Apoptosis assay *in vivo*

Mice (6-wks-old male *wt* C57BL/6J) received a single dose of placebo (empty-PTNP) or CytC-loaded PTNP (6 mg/kg body weight) intravenously via the tail vein or remain untreated. At 24h postinjection, the excised SAT was stained with a Green FLICA (fluorescent-labeled inhibitors of caspase) Caspase 9 Assay Kit (Immunochemistry Technology, LLC, Bloomington) and Alexa647-labeled GS-IB4.

Moreover, mice (6-wks-old male *wt* C57BL/6J) were intravenously injected with a single dose of a physical mixture [empty-PTNP (0.1 mmol/kg) + CytC (6 mg/kg)] and the CytC-loaded PTNP system (6 mg/kg of CytC) or remain untreated. At 24h postinjection, tissues from several organs inguinal region, heart, liver, spleen, lung and kidney were collected, stained as described above and then observed by confocal microscopy (A1: Nikon).

Anti-obesity study

The high-fat diet (58Y1 (5.10 kcal/g) composed of 34.9% fat, 23.1% protein and 25.9% carbohydrates) and the normal diet (EQ 5L37 (3.12 kcal/g) with 4.5% fat, 25.0% protein and 49.3% carbohydrates) were obtained from the TestDiet (Division of Land O'Lakes Purina Feed, LLC, USA). CytC-loaded PTNP was prepared as described above at a concentration of 1.2 mg/ml CytC solution. We considered the Recovery Ratio of encapsulated CytC (15.2%) in the dose calculation. Six-wk-old male *wt* C57BL/6J mice were randomly divided into 5 groups. Three groups at least three mice per group along with an exposure of high fat diet (HFD) were *i.v*-injected by an intravenous injection of 6, 1.2 and 0.25 mg/kg of CytC-loaded PTNP at 3 day intervals for 30 days whereas the other two groups (n=3) were allowed HFD and ND during this period, remaining untreated as

a pair of controls. After the treatment, the mice (five groups) were sacrificed and blood was drawn by cardiac puncture under anesthesia. The blood containing tubes were allowed to stand at room temperature for 3h and serum was then separated by centrifugation for 2 min at 300 ×g. Whole serum portions were immediately stored at -20°C for measurement of blood parameters. Epididymal adipose tissue (EAT) and subcutaneous adipose tissue (SAT) were removed, images obtained using a digital camera and the weights were determined.

In addition, we further measured the amount of food intake after the treatment of mice that were fed with HFD with the CytC-loaded PTNP system which was intravenously injected at the highest dose (6.0 mg/kg). The physical mixture [empty-PTNP (0.1 mmol/kg) + CytC soln. (6.0 mg/kg)]-treated (HFD), non-treated (HFD) and non-treated (ND) were also taken as controls. The amount of food intake of the CytC-loaded PTNP-treated mice (n=3) was measured every 24h during a 3-day internal. Moreover, while the food intake was measured, we also analyzed the

blood glucose concentration of all groups during this feeding condition at 24 h intervals, using Breeze2 (Bayer health care, Germany).

Measurement of blood parameters

The serum samples from the five groups were defrosted at room temperature and then analyzed. Leptin and TNF-α in serum were determined by ELISA assay (R&D systems). Serum triglycerides (TG) were measured using a Triglyceride Quantification Kit (BioVision). Serum free fatty acid content, total cholesterol and alanine aminotransferase (ALT) were determined using a commercially available kit (Wako Pure Chemicals).

Statistical analyses

All statistical analyses were performed using the JMP6 statistical package (SAS Institute, Cary, USA). Student's t test used to determine the significance of the difference between means of two groups. Dunnett's multiple comparison test was used to evaluate statistical significance between each group and HFD control group. A P value of < 0.05 was considered to be significant.

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Tables

Table 1 Physicochemical properties of Empty- and CytC-loaded PTNP

Aqueous phase	Size (nm)	ζ-potential (mV)
Empty	100.9 ± 8.0	0.3 ± 2.5
CytC	120.1 ± 15.4	2.5 ± 5.9

Nanoparticles were prepared by reverse phase evaporation (REV) method. The sizes and zeta potentials of empty- and CytC-loaded PTNP were measured by a Malvern Zetasizer. Data are represented as the "mean ± SD" of three independent experiments.

Figure legends

Figure 1. Evaluation of functional activity of CytC-loaded PTNP on primary endothelial cells derived from murine adipose tissue *in vitro*.

(**a**) pEC-IWAT cells (5 x10^4 cells/well) were treated with CytC-loaded PTNP (3, 6 and 12 μg/ml of CytC) and controls (Empty-PTNP and free CytC at the same doses) for 3h to induce apoptosis. A mixture of equal volumes of Caspase-Glo 9 reagent and cell lysate was incubated for approximately 1h and the activity of caspase 9 was measured based on luminescence (RLU) (n=4). **P<0.01 (CytC-loaded PTNP compared to free CytC) (Student's *t* test). (**b**) pcEC-IWAT cells (2 x10^5 cells/well) were treated with CytC-loaded PTNP (6 μg/ml of CytC) and controls (non-treated, empty-PTNP and free CytC at the same dose) for 6h for the induction of apoptosis. Nuclei (blue) were also stained with Hoechst 33342 and observed by CLSM. Scale bar = 20μm.

Figure 2. Destruction of adipose vascular endothelial cell with a single dose of CytC-loaded PTNP *in vivo*.

(**a**) Delivery of aqueous phase marker to adipose vessels. Double-labeled PTNP (lipid membrane: NBD-DOPE and aqueous phase: Rhodamine) was *i.v*-injected with a total lipid dose of 0.1 mmol/kg body weight into mice. After 6h, tissue pieces of inguinal adipose tissue, lungs, heart, kidney and liver were observed by confocal microscopy. Scale bar = 20 μm. (**b**) Vascular disruption by adipose endothelial cell apoptosis. Mice were untreated or received a single dose of empty or CytC-loaded PTNP (6 mg/kg) intravenously. We identified endothelial cells as middle panel (red) and apoptotic cells, left panel (green) in unfixed SAT. Cells stained with both markers appear yellow (right panel) indicate endothelial cell apoptosis. Scale bar = 100 μm.

Figure 3. Delivery of CytC-loaded PTNP decreases the progression of obesity burden.

Mice were exposed to a high fat diet (HFD) along with an *i.v*-injection of 6, 1.2 and 0.25 mg/kg of CytC-loaded PTNP at 3 day intervals for 30 days respectively whereas the other two groups were left untreated and allowed access to a HFD and a normal diet (ND) as a pair of controls. The body weights of each mouse were measured every 3 days before injections and the % increase in body weight in treated mice as compared to untreated controls starting from the day of treatment (black arrow) are shown. Data are expressed as the mean ± SD. (n=3); **P<0.01, *P<0.05 and NS, not significant versus HFD controls (Dunnett's multiple comparison test).

Figure 4. The prevention of diet-induced obesity is associated with the ablation of fat mass.

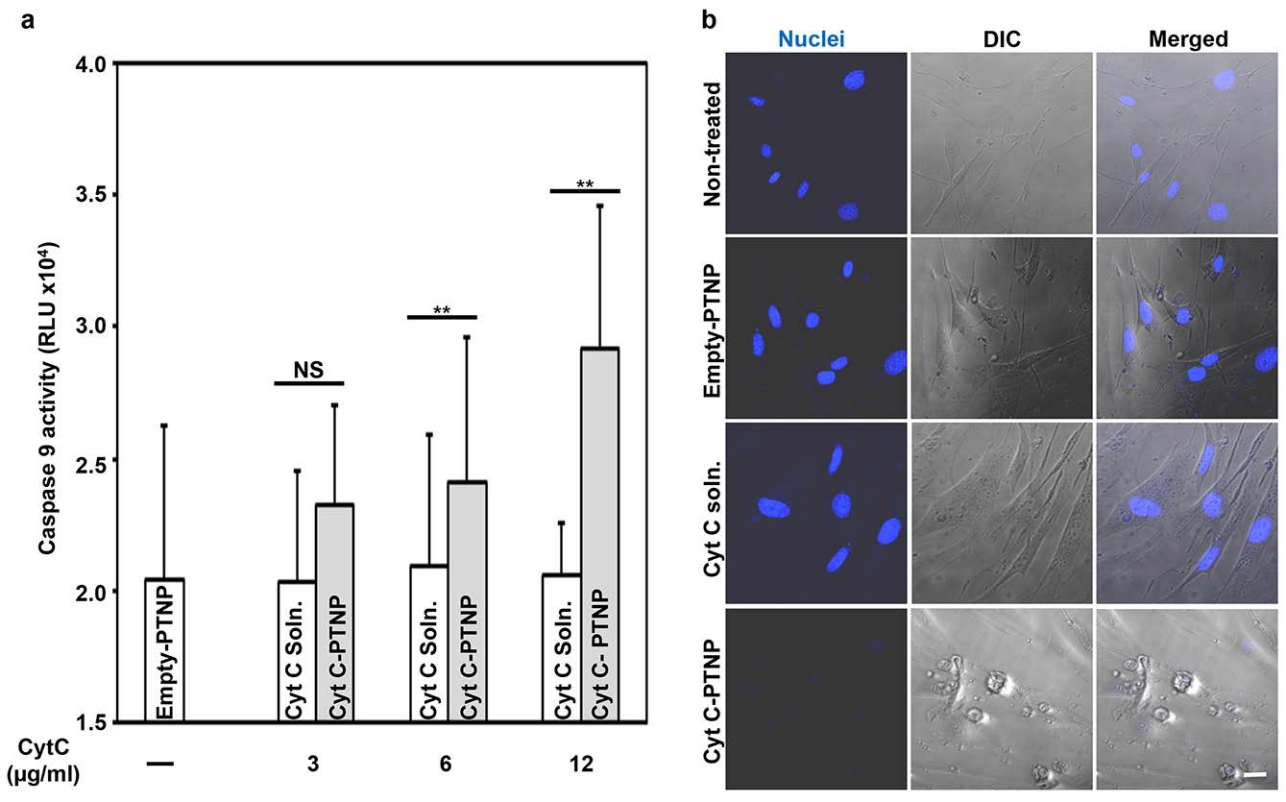
On 30 day of the treatment, representative mice and adipose tissues were taken snaps and measured weights of excised subcutaneous (SAT) and epididymal (EAT) adipose tissues. (**a**) The appearance of representative treated (6 mg/kg/3days), HFD and ND control mice and their fat depots excised from SAT and EAT regions. (**b**) Percentages of fat mass per body weight. Weights of EAT and SAT tissues were normalized by individual body weights. Data are expressed as the mean ± SD. (n=3); **P<0.01, *P<0.05 and NS, not significant versus HFD controls (Student *t*-test).

Figure 5. Plasma parameters related to anti-obesity.

After the treatment, serum samples from five groups were collected. (**a**) Serum leptin levels were determined using ELISA assays. (**b-e**) Tumor-necrosis factor-alpha (TNF-α) (b), Triglyceride (c), total cholesterols (d) and non-esterified free fatty acid (e) in plasma of treated mice (6 mg/kg/3days CytC-loaded PTNP) and ND controls were compared to the high fat controls. Data are expressed as the mean ± SD (n=3); **P<0.01, *P<0.05 compared to the high fat diet controls (Dunnett's multiple comparison test).

Figure 6. Toxicological study.

Serum alanine aminotransferase (ALT) in plasma of treated mice (6 mg/kg/3days CytC-loaded PTNP), HFD and ND controls were compared to the high fat controls. Data are expressed as the mean ± SD (n=3); NS (non-significant), compared to the high fat diet controls (Dunnett's multiple comparison test).


a
Caspase 9 activity (RLU x10⁴)
4.0
3.5
3.0
2.5
2.0
1.5
NS
**
**
Empty-PTNP
Cyt C Soln.
Cyt C-PTNP
Cyt C Soln.
Cyt C-PTNP
Cyt C Soln.
Cyt C- PTNP
CytC (µg/ml)
—
3
6
12
b
Nuclei
DIC
Merged
Non-treated
Empty-PTNP
Cyt C soln.
Cyt C-PTNP

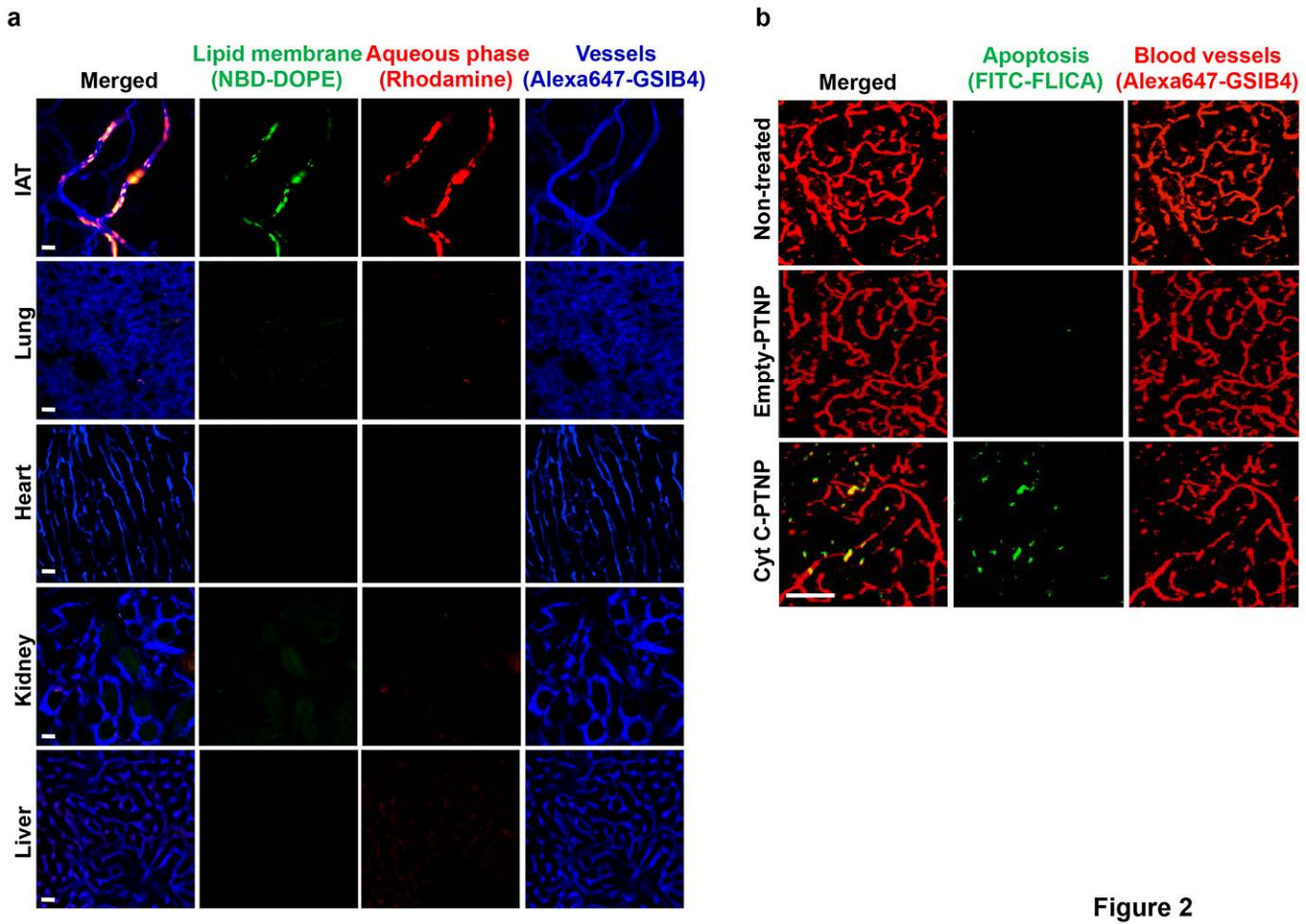
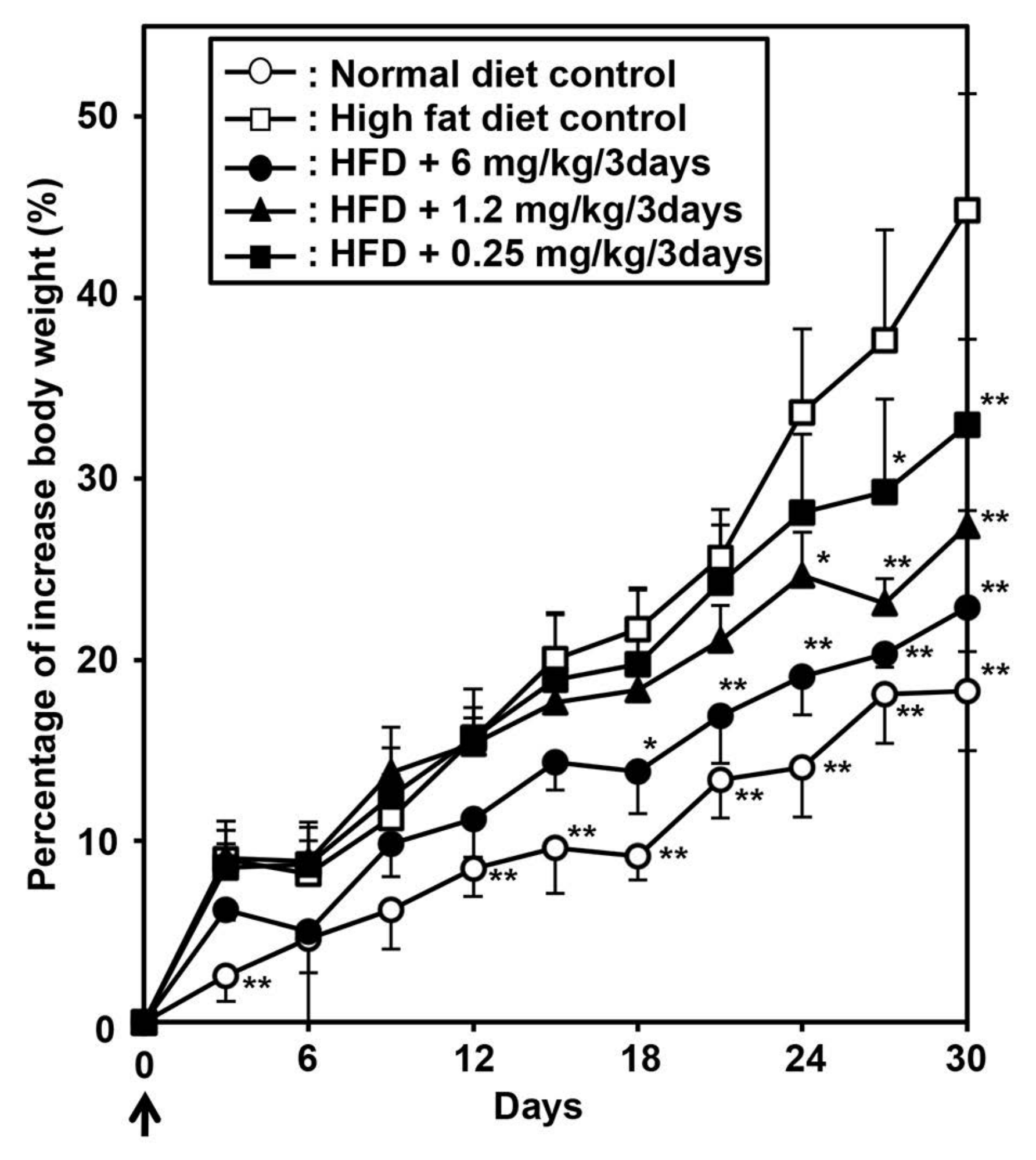
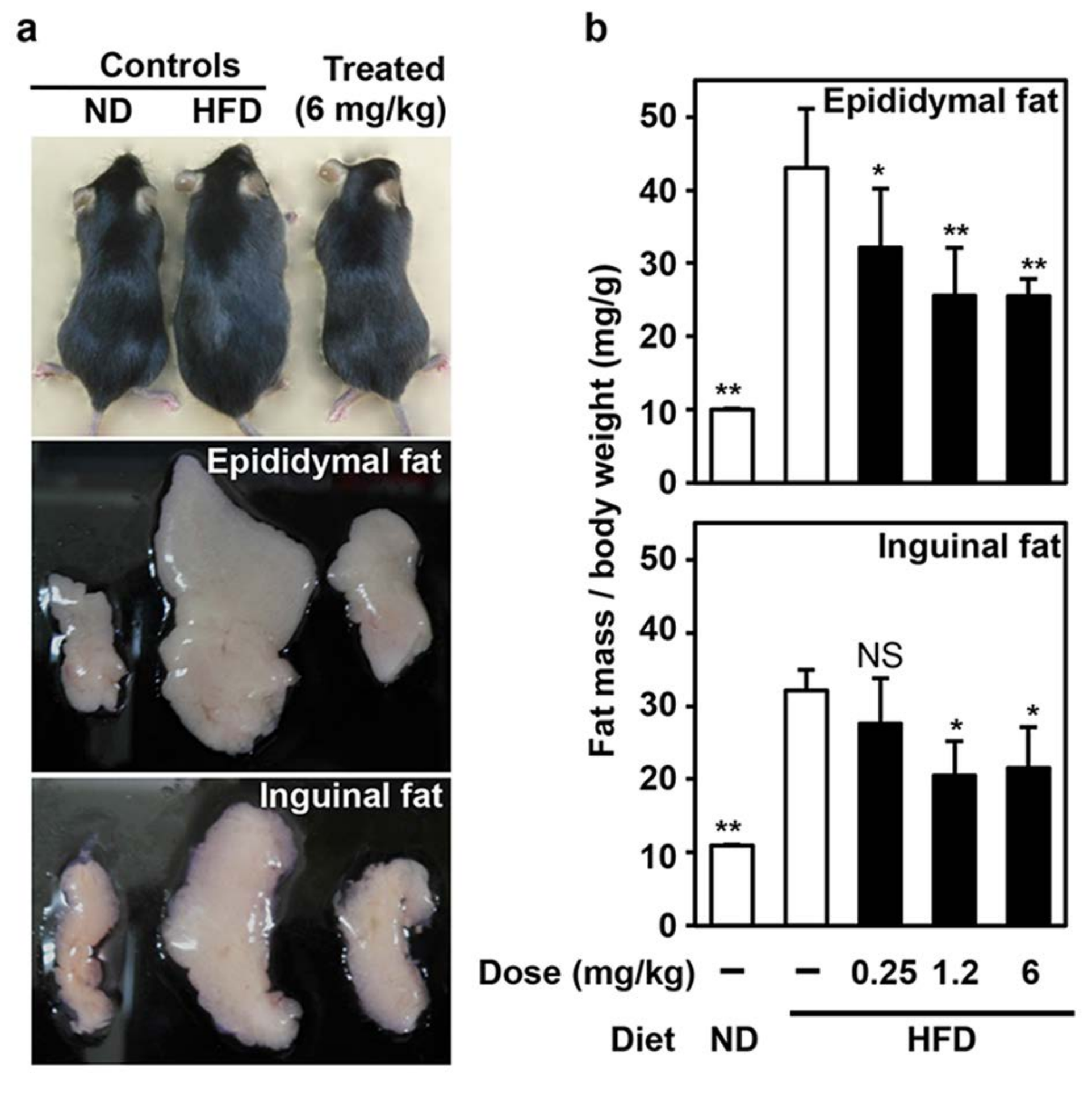

a
Merged
Lipid membrane (NBD-DOPE)
Aqueous phase (Rhodamine)
Vessels (Alexa647-GSIB4)
IAT
Lung
Heart
Kidney
Liver
b
Merged
Apoptosis (FITC-FLICA)
Blood vessels (Alexa647-GSIB4)
Non-treated
Empty-PTNP
Cyt C-PTNP

Figure 2


: Normal diet control
: High fat diet control
: HFD + 6 mg/kg/3days
: HFD + 1.2 mg/kg/3days
: HFD + 0.25 mg/kg/3days
Percentage of increase body weight (%)
0
10
20
30
40
50
0
6
12
18
24
30
Days
*
**


a
Controls
Treated
ND
HFD
(6 mg/kg)
Epididymal fat
Inguinal fat
b
Epididymal fat
Inguinal fat
Fat mass / body weight (mg/g)
0
10
20
30
40
50
**
*
**
**
NS
*
*
Dose (mg/kg) – – 0.25 1.2 6
Diet ND HFD

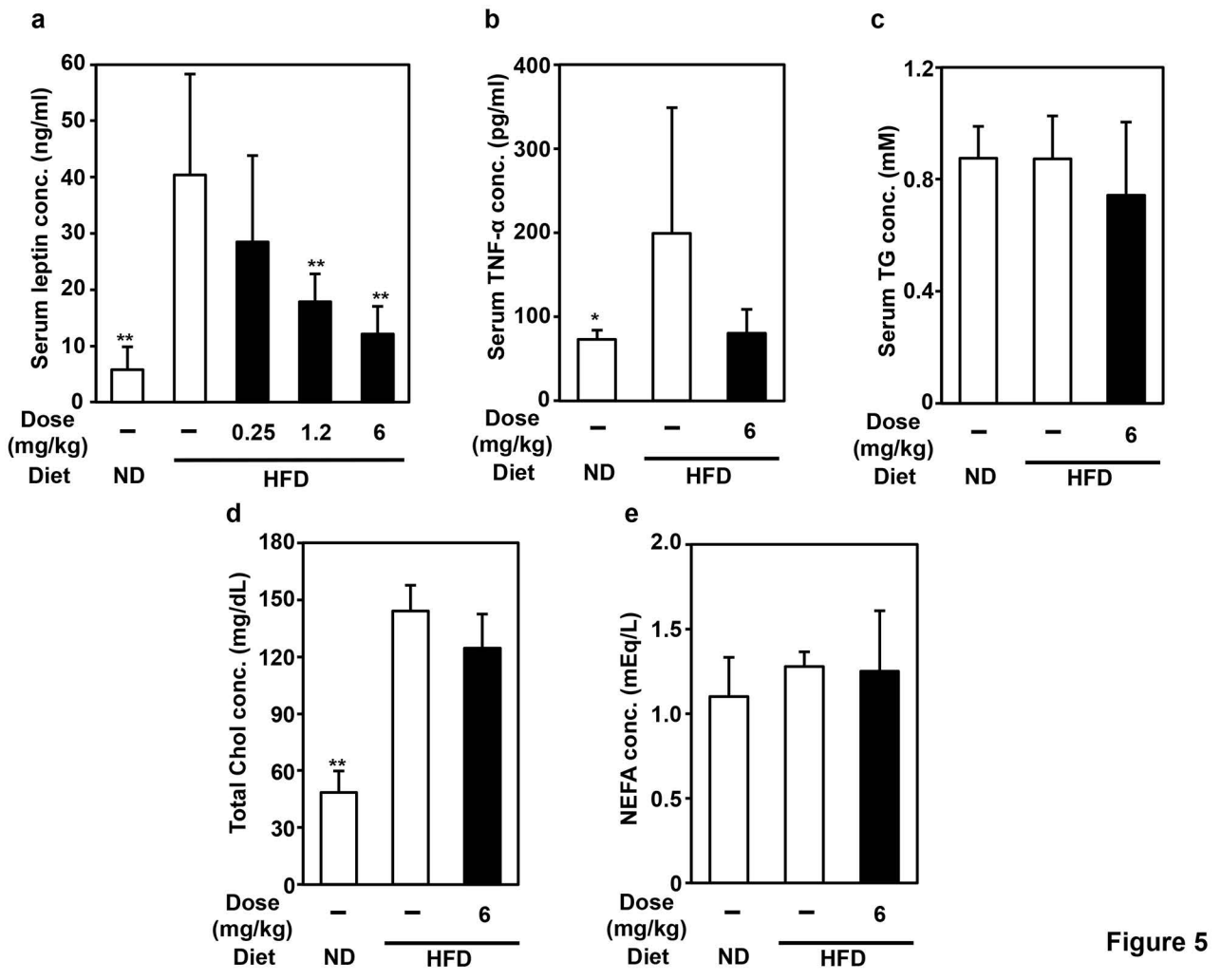
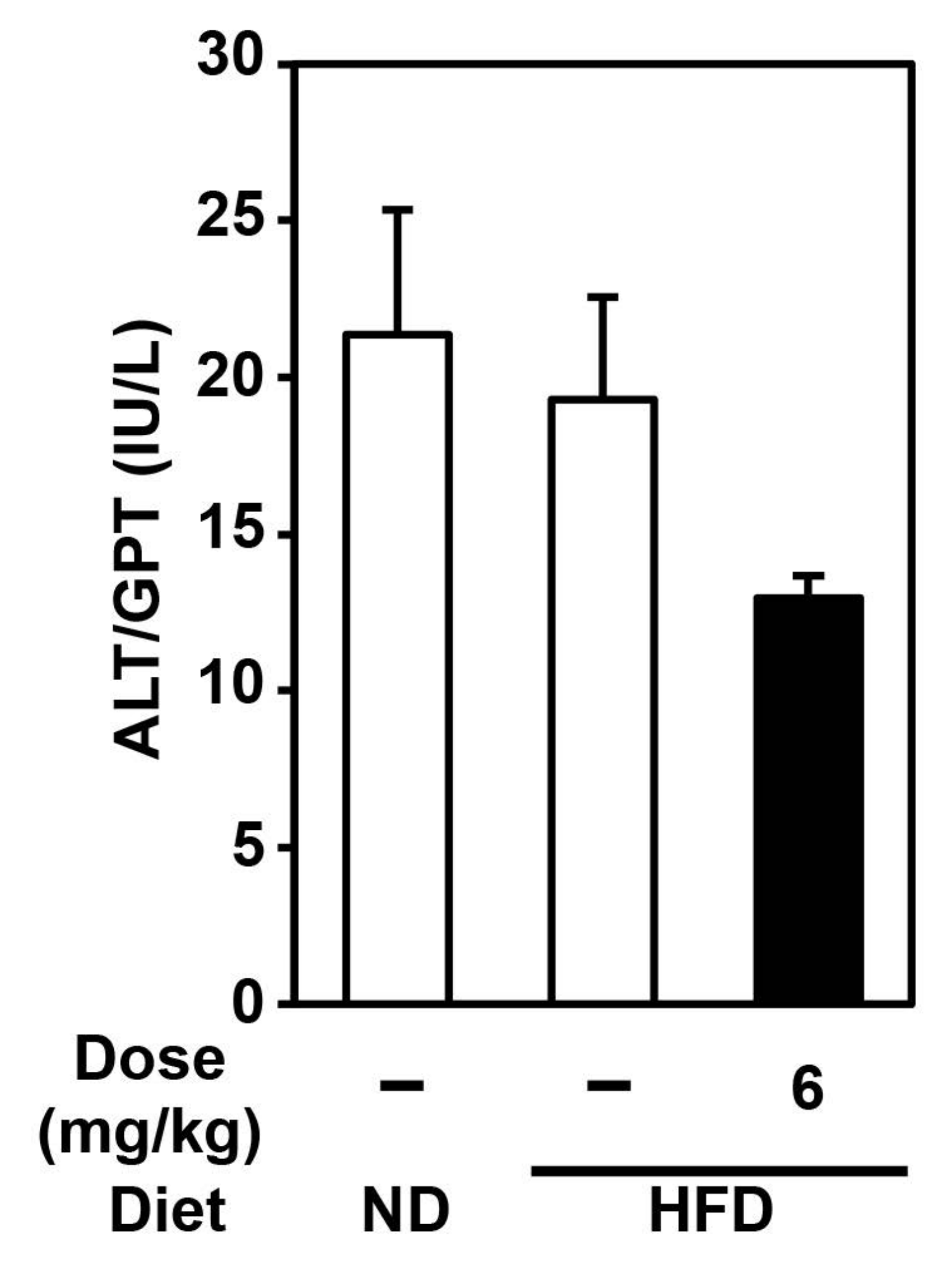

a
Serum leptin conc. (ng/ml)
60
50
40
30
20
10
0
**
**
**
Dose (mg/kg)
–
–
0.25
1.2
6
Diet
ND
HFD
b
Serum TNF-α conc. (pg/ml)
400
300
200
100
0
*
Dose (mg/kg)
–
–
6
Diet
ND
HFD
c
Serum TG conc. (mM)
1.2
0.8
0.4
0
Dose (mg/kg)
–
–
6
Diet
ND
HFD
d
Total Chol conc. (mg/dL)
180
150
120
90
60
30
0
**
Dose (mg/kg)
–
–
6
Diet
ND
HFD
e
NEFA conc. (mEq/L)
2.0
1.5
1.0
0.5
0
Dose (mg/kg)
–
–
6
Diet
ND
HFD

Figure 5


30
25
20
15
10
5
0
ALT/GPT (IU/L)
Dose
(mg/kg)
Diet
–
–
6
ND
HFD