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Rosiglitazone-loaded Nanospheres for Modulating Macrophage-Specific Inflammation in obesity

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Abstract

PPAR γ nuclear receptor agonists have been shown to attenuate macrophage inflammatory responses implicated in the metabolic complications of obesity and in atherosclerosis. However, PPAR γ agonists currently in clinical use, including rosiglitazone (RSG), are often associated with severe side effects that limit their therapeutic use. Here, 200 nm PLGA/PVA nanospheres were formulated for the systemic delivery of RSG specifically to macrophages. RSG was encapsulated with over 50% efficiency in the hydrophobic PLGA core and released specifically within the acidifying macrophage phagosomes. In bone marrow derived macrophages, RSG-loaded nanoparticles (RSG-NPs) induce a dose dependent upregulation (1.5 to 2.5-fold) of known PPAR γ target genes, with maximal induction at 5 μ M; and downregulate the expression of genes related to the inflammatory process, with maximum effect at 10 μ M. In *Ldlr*^{-/-} mice fed high fat diet, treatment with RSG-NPs alleviated inflammation in white adipose tissue and liver but, unlike treatment with free RSG, did not alter genes associated with lipid metabolism or cardiac function, indicating a reduction in the RSG side effect profile. These biocompatible, biodegradable RSG-NPs represent a preliminary step towards the specific delivery of nuclear receptor agonists for the treatment of macrophage-mediated inflammatory conditions associated with obesity, atherosclerosis and other chronic disease states.

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Keywords

PPAR γ agonists; macrophage targeting; PLGA/PVA nanospheres; inflammatory diseases

Introduction

Macrophages are critical determinants of metabolic disorders, including diabetes and obesity, and in atherosclerosis [1-3]. Obesity-induced macrophage inflammatory responses are associated with altered adipocyte function, insulin resistance and increased atherosclerosis. Therefore, controlling macrophage inflammation activities can prevent, attenuate, and possibly reverse such disorders (Figure 1). In this respect, the activation of nuclear receptors, such as the peroxisome proliferator activated receptor γ (PPAR γ), has been proved beneficial, in large part, by inhibiting macrophage inflammation [4, 5]. Molecular compounds have been designed to target PPAR γ , such as the clinically available Rosiglitazone (RSG) and Pioglitazone [6] for oral treatment of type 2 Diabetes. However, in addition to attenuating inflammation and promoting insulin sensitivity, these compounds are often associated with severe side effects including weight gain, edema, bone fracture, and congestive heart failure, which have severely limited their clinical application [7, 8].

Over the last two decades nanoparticles (NPs) have been demonstrated to be useful for systemic delivery and controlled release of therapeutic agents in a number of biomedical applications [9-11]. The NP geometrical and physico-chemical properties can be finely tuned during their synthesis process to optimize drug loading and enhance specificity in reaching the biological target [12-14], thus increasing the therapeutic efficacy and limiting side effects. The nuclear receptor PPAR γ is expressed in most cells and tissues, however, this ubiquitous distribution is problematic because of the unwanted side effects. Thus, specific activation of macrophage PPAR γ to induce a systemic anti-inflammatory response by down-regulating the secretion of cytokines would be highly desirable. Based on this rationale, the development of NPs for the preferential delivery of PPAR γ agonists, such as RSG, to macrophages represents a promising and novel therapeutic strategy for controlling the development and progression of many metabolic disorders and also in atherosclerosis.

Spherical NPs with a diameter of a few hundreds of nanometers are easily and rapidly engulfed by professional phagocytic cells, such as circulating monocytes and macrophages [15-22]. As such, a systemically injected NP loaded with RSG would be preferentially taken up by circulating monocytes, that eventually infiltrate developing vascular plaques and by macrophages, residing in the liver (Kupffer cells) and in the white adipose tissue (WAT). This targeted approach to specifically release RSG into monocytes and macrophages would be useful to control diverse inflammatory processes orchestrated by these cells, yet mitigate RSG accumulation in hepatocytes, cardiomyocytes, renal tubular and other cells causing the severe undesired effects [23]. Note that this approach is different, and possibly more general, than developing nanoparticles for the specific treatment of atherosclerotic plaques [24, 25], and it could be used for the effective treatment of any macrophage-mediated inflammatory disease [26].

In the preset investigation, spherical polymeric NPs with a diameter of about 200 nm were synthesized and characterized for the systemic, macrophage-selective delivery of RSG. These NPs consisted of an inner hydrophobic poly(lactic-co-glycolic acid) (PLGA) matrix, where the poorly water soluble RSG molecules [27, 28] are encapsulated, surrounded by an outer layer of polyvinyl alcohol (PVA), creating a hydration layer to reduce NP aggregation by steric repulsion and attenuate protein modification and opsonization in blood [29, 30]. The RSG-loaded NPs (RSG-NPs) were characterized for their geometrical and physico-chemical properties; loading and release performance; and macrophages internalization capabilities. Then, gene induction was studied in primary phagocytic cells, mouse bone marrow derived macrophages (BMDMs), and in the WAT, liver and heart of LDLR^{-/-} mice injected bi-weekly with RSG-NPs. As control experiments, the animals received dietary RSG daily for the whole duration of the study.

Experimental Section

Materials

PLGA (50:50, Carboxy Terminated, MW ~60 kDa) and PVA (MW ~50 kDa) were purchased by Sigma Aldrich (St. Louis, MO). Rosiglitazone was purchased from SST Corp. (Clifton, NJ) and used without further purification. Analytical grade dimethyl sulfoxide (DMSO), acetonitrile (ACN), chloroform and other solvents were obtained from Fisher Scientific. D-275 (3,3'-dioctadecyloxacarbocyanine perchlorate, DiOC18(3)) was purchased by Invitrogen. J-774 and RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Bethesda, MD) and cultured according to the manufacturer's protocol. Phosphate buffered saline (PBS) and cell culture media were purchased from Sigma Aldrich.

Synthesis of RSG-NPs

30 mg of PLGA (50:50) Carboxy terminated was dissolved in Chloroform and mixed with 6 mg of RSG dissolved in 600 µl of ACN to obtain a homogeneous solution. The single emulsion nanoparticle (NP) fabrication technique was adapted to synthesize RSG loaded nanoparticle. In a typical experiment, RSG-loaded PLGA nanoparticles were prepared by emulsifying the homogenous solution of PLGA and RSG with a solution of 2% PVA under sonication while maintaining the ratio between organic solvent and aqueous solution of 1:10. Thus formed emulsion was continuously stirred under reduced pressure to evaporate organic solvent. After the complete removal of organic solvent, the resulting nanoparticles were collected by centrifugation at 14000 × g for 20 minutes. Finally, the nanoparticles were further purified by centrifugation and resuspended in PBS. PLGA control nanoparticles without RSG were synthesized accordingly as described in the preparation of RSG loaded NPs in absence of RSG. DiOC18(3)-loaded NPs were prepared as RSG-loaded NPs, using in this case the 0.01% w/v of DiOC18(3) instead of RSG and using chloroform as solvent also for the lipophilic dye.

Characterization of RSG-NPs

Drug loaded nanoparticles were characterized by their hydrodynamic and morphological properties using dynamic light scattering (DLS, Malvern Zetasizer Nano S) and scanning electron microscopy (SEM, FEI Nova NanoSEM 230). DLS measurements were carried in

both water and PBS at 37 °C for the period of 11 days in order to understand the particle stability. Samples for SEM were prepared by drop casting and evaporation process in single side polished silicon wafer. Prior to SEM analysis the samples were coated with platinum and analyzed at an accelerating voltage of 10 KeV.

Drug Loading and Release Studies

RSG loading and release studies were performed by measuring RSG absorbance at 260 nm against that of a RSG standard curve to determine RSG concentration. The drug loading and encapsulation efficiency were calculated using freeze dried samples of known amount of nanoparticles. Drug loading was estimated as: $\text{Drug Loading (\%)} = (\text{Drug weight/NPs Weight}) \times 100$, while the encapsulation efficiency was measured following $EE = D_e/D_i$ equation, where D_e is the amount of drug encapsulated at the end of the synthesis process and D_i is the initial amount of drug used. Similarly, to measure the drug release kinetics of RSG from the NPs, NP solutions were loaded into Slide-A-Lyzer MINI dialysis microtubes with a molecular weight cutoff of 3.5 kDa (Pierce, Rockford, IL). The NPs were then dialyzed against pH 5.0 and 7.4 PBS buffer at 37 °C. PBS buffers were changed every 6 to 12 h during the whole dialysis process. At each predetermined time point, NP solutions from three mini dialysis units were collected separately for drug quantification.

in vitro experiments

Isolated mouse bone marrow cells were cultured at 37°C in 5% CO₂ in DMEM supplemented with 20% FBS and 30% L929-cell conditioned medium for one week, then seeded into 24 well culture plates at a concentration of 2.5×10^4 BMDM per well. Cells were cultured overnight, then treated with empty NPs or RSG-NPs or free RSG for 24 hours, using concentrations of 0.5, 2.5, 5 and 10 µM RSG, RSG-NP dose-equivalent concentrations, or empty NP doses equivalent to the PLGA mass of the RSG-NPs. BMDMs were cultured for 24hrs or 48hrs with empty NPs, RSG-NPs or free RSG after which RNA was isolated for gene expression.

For the internalization study, J-774 cells were seeded in a 8 chamber culture slides at a concentration of 7×10^4 cell per well. After 24 hours, cells were treated with 3,3'-diocetadecyloxycarbocyanine perchlorate (DiOC18(3))-loaded NPs, in 3 different concentrations, providing the same polymer content as for the RSG-NPs loaded with 0.5, 2.5 and 10 µM RSG. After 2 hours of incubation, cells were washed three times with PBS to remove NPs that were not internalized and finally fixed and analyzed by confocal microscope

Mouse Studies

Male LDLR^{-/-} and C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were group-housed in microisolator cages with a 12 hour light and dark cycle. Twenty-month old LDLR^{-/-} mice were randomly assigned to 1 month of a high fat, high cholesterol Western Diet (HFD, Research Diets, New Brunswick, NJ) supplemented with RSG (1.2 g RSG/kg of HFD) or HFD and then retro-orbital bi-weekly injections of 100 µg of nanoparticles containing PLGA alone (empty NPs) or PLGA nanoparticles loaded with 50 µg RSG (RSG-NPs). Mice were sacrificed after 8 weeks of treatment, following an overnight fast, and

immediately dissected to obtain liver, heart and epididymal white adipose tissue samples that were snap frozen in liquid nitrogen and stored at -80 °C until processed for gene expression analyses. Bone marrow for cell culture studies was collected from C57BL/6J mouse femurs and tibia by syringe perfusion. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Methodist Hospital Research Institute.

Quantitative RT-PCR

RNA from BMDM and mouse tissue samples was isolated using RNeasy Mini kits (QIAGEN, Valencia, CA) and reverse-transcribed with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA). BMDM cDNA samples were then analyzed by quantitative RT-PCR using a LightCycler 480 II (Roche) using gene-specific primers purchased from IDT (Coralville, IA), and normalizing all gene expression to *Ppia* expression.

Statistical Analysis

Before conducting the statistical analysis of significance, all data were analyzed for assessing the integrity of collection and measurement procedures. Data were checked for the presence of outliers using graphical-based boxplot outlier detection as well as Dixon's Q test, to exclude the possibility that results were determined by only a few influential observations [31]. Statistical analysis of significance was conducted using ANOVA. Iterative weighted least squares ANOVA, was used wherever appropriate, to reduce the impact of heteroscedasticity. The equal-variance assumption was tested using the modified robust Brown-Forsythe Levene-type test for homogeneity of variance. The inverse of the residual variance was used as a weight for each observation in weighted least squares ANOVA. Multiple comparisons were carried out using the Tukey's HSD (honestly significant difference) test and adjusted p-values were computed to determine statistical significance in post-hoc pairwise comparisons. Comparisons with a p-value lower or equal to 0.05 were considered to show a statistically significant difference with respect to control. All analyses were conducted using the R software, version 2.15.1.

Results and Discussion

Synthesis and physico-chemical characterization of the RSG-NPs

In order to determine the effects of nanoparticle-mediated delivery of PPAR γ agonists, RSG-NPs were synthesized using a single emulsion technique [30, 32]. Briefly, RSG-NPs were prepared by emulsifying a solution of PLGA and RSG with a homogenous solution of PVA 2% under sonication. After synthesis, the resulting NPs were analyzed by scanning electron microscopy (SEM) to assess their morphological characteristics. As shown in Figure 2a, SEM micrographs clearly reveal unimodal spherical NPs with diameters of ~180 nm. Dynamic light scattering (DLS) measurements detected a hydrodynamic NP diameter of 235 $\pm$ 3.7 nm, with a narrow polydispersity index (PDI = 0.16) (Figure 2b). Note that the DLS data revealed a slightly larger diameter as compared to the SEM analysis reflecting the different NP response under anhydrous (SEM) and aqueous (DLS) conditions. In order to address the NP stability during a freeze/thawing process, DLS measurements were

performed with freeze-dried samples after resuspension in water and PBS, respectively at 37 °C. The resulting DLS analysis of these NPs found no change in size or shape under either condition (See Supplementary Materials). Next, the long-term stability of RSG-NPs was estimated in pH 7.4 PBS at 37°C over an extended time course of 11 days. As shown in Figure 2b, the diameter and PDI of these NPs were found to be highly stable, under physiological conditions, with no significant variation over time.

The pharmacokinetic properties of the proposed RSG nanoformulation were assessed via UV spectrophotometry for drug loading and encapsulation efficiency. This analysis revealed a RSG loading efficiency of ~45% of the total NP weight, and encapsulation efficiency >50% of the available drug (Figure 2c). Such high drug loading demonstrates that this approach holds promise for the systemic delivery of RSG and similar compounds. It also suggests that low injected doses of NPs may be sufficient for delivering high doses of RSG directly to the target tissue and specific cellular compartments. Finally, the RSG release from the NPs was studied over time at pH 7.4 and 5.0, values selected to mimic the conditions encountered in the circulation and in an acidifying endosomal environment, respectively (Figure 2d). The total NP load of RSG was released within 2 hours incubation at pH 5.0; whereas NPs incubated at neutral pH required 48 hours for the same amount of drug release. Most importantly, only 10% of the loaded RSG was released within 2 hours of neutral pH exposure (inset of Figure 2d). The rapid drug release observed under acidic condition (i.e. cell endosome) should be ascribed to the dissolution of the stabilizing outer PVA layer and the well-known progressive degradation of the PLGA core, starting from the inner side of the NP. The exposed PLGA surface undergoes hydrolysis, upon removal of the PVA layer, and experiences a progressive increase in porosity which is responsible for the release process. The progressive decrease in particle size and removal of the stabilizing PVA layer has been documented via electron microscopy in the Supplementary Materials (Supplementary Figure S2). Moreover, the RSG solubility in water, very poor at physiological pH, increases with an acidic environment, thus supporting even more the observed rapid release [33-36]. This differential release kinetics is instrumental to the specific macrophage delivery of RSG. Indeed, 200 nm spherical NPs are known to be rapidly taken up by macrophages within the first few hours post injection [18, 37]. As such, the RSG-NPs would be predicted to release most of their therapeutic payload directly within the target cells with very little RSG lost in the circulation. Consequently, high intracellular doses of RSG would be directly delivered to macrophages resulting in a strong induction of PPAR γ target genes.

NPs internalization in J-774 macrophages

To characterize the macrophage uptake of the NPs, J-774 cells were incubated with DiOC18(3)-loaded NPs for 2 hours. After fixation, it was possible to clearly prove that NPs were internalized by cells, with an accumulation in the perinuclear region, in a concentration-dependent manner, in the absence of any stimulation (i.e. LPS treatment) (Figure.3).

Gene induction efficiency of RSG-NPs on primary phagocytic cells

In order to evaluate the effect of RSG-NPs on the gene expression in macrophages, mouse bone marrow derived macrophages (BMDMs) were incubated with increasing concentrations of free RSG, dose-equivalent RSG-NPs and empty NPs. Known PPAR γ regulated genes involved in lipid transport (CD36, FABP4 and ABCG1) and inflammation (IL-1 β , iNOS and TNF- α) were examined for altered expression. Figure.4a shows the expression of inflammatory genes in BMDMs after 18 hours of stimulation with 10 ng/ml of LPS. IL-1 β and iNOS inhibition in response to RSG treatment showed a clearer dose-dependent response than for TNF- α . Free RSG and RSG-NPs were equally effective in attenuating LPS-induced iNOS expression at all concentrations, while RSG-NPs were more effective than free RSG in decreasing TNF- α expression. BMDMs were also incubated with free RSG, dose-equivalent RSG-NPs and empty NPs at different concentration for 48 hours to induce expression of genes involved in lipid transport. As shown in Figure.4b, all RSG target genes were induced by 48 hours treatment with either free RSG or RSG-NPs. Similar results were also obtained after 24 hours incubation for CD36 and ABCG1, while FABP4 expression was not induced at this time point. (See Supplementary materials). ABCG1 expression, however, no longer demonstrated dose responsiveness, being equally induced at all RSG concentrations, albeit less so than at 24 hours of treatment ($\sim$ 1.3- vs. $\sim$ 2.5-fold increase). Note that free compounds in vitro are in general expected to perform better than the corresponding nanoformulations because of faster cell uptake. However, for almost all the considered concentrations, RSG and RSG-NPs provide comparable levels of gene induction at 24 and 48h demonstrating the potential of the RSG nanoformulation.

Gene induction efficiency of RSG-NPs in mice

The in vivo performance of RSG-NPs was tested in male LDLR $^{-/-}$ mice and compared to the efficacy of freely administered RSG. The animals were fed a high-fat diet (HFD) for 1 month to induce obesity and then treated with dietary RSG; RSG-NPs and empty NPs. The treatment lasted 8 weeks for all experimental groups during which the mice were fed HFD and injected bi-weekly with 100 μ g of empty NPs or RSG-NPs for 8 weeks, or fed HFD supplemented with 1.2 g RSG/kg HFD for 8 weeks which is known to produce an almost constant plasma RSG concentrations of $\sim$ 25 μ g/ml (data from Daiichi Sankyo, Inc.), which already accounts for the first-pass effect losses, typical of any orally administered agents. RSG-NPs were administered intravenously every three days with a total RSG dose equivalent to 25 μ g/ml (50 μ g of RSG per mouse in 2 ml of blood). Indeed, given the injection schedule, the RSG amount provided with the NPs is significantly lower than for the oral administration, here used as a control.

After the 8 weeks of treatment, no difference in weight gain was detected among the three experimental groups (Figure.5), confirming the absence of any major adverse reaction of the animals.

Animals injected with empty NPs were used as controls. RT-PCR analysis was performed on three tissues, namely white adipose tissue (WAT), liver and heart, and on different classes of genes, known targets of PPAR γ which regulate lipid metabolism and inflammation. In WAT, which has the highest concentration of PPAR γ , the RSG-NPs did

not induce any significant induction in genes involved with lipid metabolism, including the fatty acid transporter CD36, the fatty acid binding protein FABP4, the reverse cholesterol transport proteins ABCA1 and ABCG1, and the adipocyte-specific protein adiponectin (Adipoq). In contrast, all these genes were up-regulated in mice that received dietary RSG. Importantly, both RSG and RSG-NP treated mice showed a reduction in the expression of pro-inflammatory genes. Free RSG was observed to attenuate iNOS and the macrophage chemoattractant OPN ($p=0.07$); whereas RSG-NPs had the greatest effect on IL-1 β , which may be predominantly expressed by adipose tissue macrophages that accumulate with obesity. This data suggests that RSG-NPs primarily have effects on phagocytic cells without altering the intracellular lipid metabolism of adipocytes. On the other hand, dietary RSG appears to affect all adipose tissue cells, including adipocytes, altering the lipid accumulation, transport and metabolism in whole tissue (Figure 6).

Similar trends were observed for the liver. Dietary RSG increased expression of the lipid metabolism genes FASN and FABP4, but not CD36, and decreased DGAT1 expression. This implies a phenotype of increased lipid synthesis and intracellular transport but not extracellular uptake, in which less of the newly synthesized lipids are converted into triglyceride. However, dietary RSG treatment had no effect on SREBP1C, a major regulator of glucose metabolism and de novo fatty acid and lipid synthesis; ACC2, which regulates mitochondrial uptake of fatty acid; ABCA1 or ABCG1, which regulate the efflux of intracellular cholesterol to lipoprotein acceptors. Moreover, the livers of male *Ldlr*^{-/-} mice fed a high-fat and cholesterol diet have marked increases in gene expression of cytokines and markers of macrophage accumulation, and decreased expression of several anti-oxidant genes, indicative of a pro-inflammatory tissue environment associated with increased liver injury (Figure.7). Dietary RSG induced a decrease in hepatic expression of the macrophage chemoattractants MCP-1 and OPN, without significantly altering the expression of the macrophage marker CD68 or the pro-inflammatory cytokines IL-1 β and TNF α ; whereas RSG-NP treatment attenuated MCP-1 expression without similarly altering OPN. Taken together, this data suggest that both dietary RSG and RSG-NP treatments modestly attenuate diet-induced liver inflammation, but dietary RSG alone appears to stimulate liver free fatty acid synthesis without altering fatty acid uptake, storage, export or consumption resulting in the progressive accumulation of endogenously synthesized lipid that could lead eventually to worsening fatty liver disease.

RSG is well known to have detrimental effects on the heart and has been linked to increased risk of fatal cardiac arrhythmia [8, 38]. This may be partially associated with a reduction in cardiac expression of the voltage-dependent K⁺ channel proteins (KCNA2 and KCNA5) and the gap junction protein CX43. PCR analysis of samples from the heart showed that dietary RSG significantly increased cardiac expression of the lipid metabolism gene FABP4, but not CD36; whereas the RSG-NPs had no detectable effect on either genes, in line with the observations in the other tissues. More importantly, dietary RSG increased the expression of the pro-fibrotic cytokine TGF β 1 and extracellular matrix protein collagen II, indicative of a potential RSG-mediated increase in cardiac remodeling and fibrosis. Also, dietary RSG was associated with significant decreases in KCNA2 and KCNA5, but not Cx43 and a trend towards increased expression of BNP, a marker of increased strain-induced cardiomyocyte stress and calcium signaling. None of the above genes were induced by RSG-NPs (Figure.

8). This would indicate that RSG-NPs do not induce any significant alteration in genes associated with cardiac arrhythmia, that are largely responsible for the severe side effects of RSG in patients.

NP-mediated delivery of other PPAR γ ligands has been shown to attenuate other inflammatory conditions supporting the feasibility of this approach, although these reports used site-directed delivery of rapidly degraded NPs to attenuate acute inflammatory responses. For example, Nagahama et al [39], used a single intramuscular injection of PLGA NPs loaded with the PPAR γ agonist pioglitazone to improve blood flow in a mouse model of hind limb ischemia. Similarly, Alves et al [40] used a single injection of PLGA NPs to administer the PPAR-g agonist 15d-PGJ2 to attenuate neutrophil migration and inflammation in mouse models of acute peritonitis. Both studies found that NP delivery greatly increased the effective dose of the administered drugs. Our results complement these studies, indicating that long-term NP-direct drug therapy can attenuate chronic disease.

Conclusions

Spherical nanoparticles with an average diameter of 200 nm were formulated for the macrophage-selective delivery of RSG. The polymers PLGA and PVA were used as constituents because of their known biodegradability and biocompatibility. The size, shape and composition combination supported the rapid accumulation of the systemically injected nanospheres in circulating monocytes and resident macrophages. RSG was then released in the acidic endosomal environment of these phagocytic cells. Experiments with BMDMs found that these RSG-NPs induced PPAR γ target genes with the same, or higher, induction efficiency as free RSG. Further, HFD-fed Ldlr $^{-/-}$ mice intravascularly injected with RSG-NPs showed alleviation of macrophage inflammation in white adipose tissue and liver, with no effect on lipid metabolism genes or cardiac genes associated with cardiac side effects of systemic RSG delivery. This macrophage-selective delivery of RSG represents a means of attenuating inflammation without incurring in known side effects associated with systemic RSG exposure. Thus, this nanopatform provides a novel approach for the targeted delivery of agonists for nuclear receptor ligands, such as PPAR γ , into monocytes and macrophages to attenuate chronic disease states mediated by macrophage inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure.1.

Expected effects of macrophage-targeted RSG delivery in inflammatory processes in different tissues. After adhering to macrophage cell membrane, NPs are entrapped in endocytic vesicles and progress through endosome maturation until endosome acidification causes rapid degradation of the NP, resulting in RSG release from the NP polymeric matrix and its diffusion to the cytosol and nucleus where it can bind PPAR γ to regulate gene expression. The higher magnification shows a schematic of a RSG-loaded NP consisting of an internal hydrophobic core, which entraps the RSG cargo, covered by a hydrophilic PVA layer. In WAT, RSG-NPs would normalize adipose function by decreasing macrophage-mediated inflammation and cytokine secretion, thereby decreasing macrophage recruitment and excess lipolysis to improve normal lipid storage. Improved WAT lipid storage would reduce pro-inflammatory lipid accumulation in the liver and vascular macrophages, reducing liver inflammation and atherosclerosis development. Monocyte/macrophage phagocytosis of RSG-NPs would also attenuate cytokine secretion by vascular macrophages to inhibit further recruitment of macrophages to existing atherosclerotic plaques and thus attenuate atherosclerosis progression.

Figure 2. Physico-chemical and pharmacokinetic characterization of RSG-NPs

a. Representative SEM image of RSG-NPs, showing a monodisperse population with an average ~ 200 nm diameter. **b.** DLS measurements of RSG-NPs over time at 37 °C and pH 7.4 (upper line: diameter; bottom line: polydispersity index PDI). **c.** Loading efficiency (RSG weight/RSG-NP weight) and encapsulation efficiency (RSG-NP drug cargo/input drug) of RSG-NPs. **d.** RSG release profiles under physiological (pH 7.4, white squares) and acidic (pH 5, black squares) conditions. The inset shows the same release curves over the first 6h.

Figure.3. NPs internalization in J-774 macrophages

a. Confocal microscopy images of DiOC18(3)-loaded NPs internalized into J-774 macrophages after 2 hours of incubation. 3 different NPs concentration, defined as 0.5, 2.5 and 10 (see text for more details), were compared with control. **b.** Confocal Z-stack of cells treated at the highest concentration of DiOC18(3)-NPs, that clearly shows the NPs localization inside the cell

Figure 4. *in vitro* gene expression analysis on Bone Marrow Derived Macrophages

a. Before LPS stimulation, BMDMs were treated with different concentrations of free RSG and RSG-NPs, namely 0.5, 2.5 and 10 μ M. **b.** Cells were exposed to free RSG and RSG-NP and empty NP equivalent concentrations (0.5, 2.5, 5.0 and 10.0 μ M), and RT-PCR analyzed for PPAR γ target gene expression 2 days after treatment. Samples were analyzed by weighted least squares Anova followed by Tukey's HSD with statistical differences indicated as follows: + $p < 0.05$ vs. LPS-stimulated control group, * vs. NPs group or ** for free RSG vs. the RSG-NPs

Figure.5. Body weight comparison among the three animal group used

a. Schematics of the animal experiments with the time points and RSG doses. **b.** Body weight after 8 week treatment for the three experimental groups: empty NPs, RSG-NPs and dietary RSG. No statistically significant difference is observed among the groups. Only for the empty NP group, a slight increase in overall weight at the end of the treatment period is observed, compared to the other two groups

Figure 6. RSG and RSG-NP mediated changes in WAT gene expression

RT-PCR analysis of gene expression in WAT of mice treated with oral RSG or injected with RSG-NPs or empty NPs. Samples were analyzed by weighted least squares ANOVA with Tukey's HSD post-hoc tests and statistical differences are indicated as **p<0.05 vs. the indicated groups

Figure 7. RSG and RSG-NP mediated changes in liver gene expression

RT-PCR analysis of gene expression in livers of mice treated with oral RSG or injected with RSG-NPs or empty NPs. Samples were analyzed by weighted least squares ANOVA with Tukey's HSD post-hoc tests and statistical differences are indicated as **p<0.05 vs. the indicated groups

Figure 8. RSG and RSG NP mediated changes in cardiac gene expression

RT-PCR analysis of gene expression in livers of mice treated with oral RSG or injected with RSG-NPs or empty NPs. Samples were analyzed by weighted least squares ANOVA with Tukey's HSD post-hoc tests and statistical differences are indicated as ** $p < 0.05$ vs. the indicated groups.