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Gold nanorods inhibit respiratory syncytial virus by stimulating the innate immune response

Swapnil S. Bawage, MSc[a], Pooja M. Tiwari, PhD[a,1], Ankur Singh, MD[b], Saurabh Dixit, PhD[a], Shreekumar R. Pillai, PhD[a], Vida A. Dennis, PhD[a], Shree R. Singh, PhD[a,*]

[a]*Center for NanoBiotechnology Research, Alabama State University, Montgomery, AL, USA*
[b]*College of Medicine, University of South Alabama, Mobile, AL, USA*

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Abstract

Respiratory syncytial virus (RSV) causes severe pneumonia and bronchiolitis in infants, children and older adults. The use of metallic nanoparticles as potential therapeutics is being explored against respiratory viruses like Influenza, Parainfluenza and Adenovirus. In this study, we showed that gold nanorods (GNRs) inhibit RSV in HEp-2 cells and BALB/c mice by 82% and 56%, respectively. The RSV inhibition correlated with marked upregulated antiviral genes due to GNR mediated TLR, NOD-like receptor and RIG-I-like receptor signaling pathways. Transmission electron microscopy of lungs showed GNRs in the endocytotic vesicles and histological analyses indicated infiltration by neutrophils, eosinophils and monocytes correlating with clearance of RSV. In addition, production of cytokines and chemokines in the lungs indicates recruitment of immune cells to counter RSV replication. To our knowledge, this is the first *in vitro* and *in vivo* report that provides possible antiviral mechanisms of GNRs against RSV.

Key words: Gold nanorods; Bronchiolitis; Pneumonia; Nanomedicine; Antiviral; Nanoparticles

Recent developments in the field of nanomedicine have led to development of novel nanomaterials with potential applications in diagnosis and treatment of infectious and non-infectious diseases.[1] Nanoparticles, unmodified or functionalized with biomolecules, can be used for anti-viral drug delivery,[2] virus detection[3–6] and as anti-viral therapeutic agents.[7–12] Metallic nanoparticles have shown anti-viral activity against respiratory viruses like Influenza,[10,13,14] Parainfluenza[15] and Adenovirus.[16] Gold nanomaterials are gaining popularity due to their surface structure, low toxicity and their potential for a wide range of biomedical applications. Gold nanorods (GNRs) are currently being investigated for treatment of cancer,[17–19] HIV,[20] Respiratory Syncytial Virus (RSV),[21] and for various biomedical applications.[22]

RSV, a negative-sense, single-stranded RNA virus causes severe pneumonia and bronchiolitis resulting in an estimated annual global mortality of 160,000–600,000.[23] In the United States, almost all children below the age of two acquire RSV infection and over 100,000 children are hospitalized every year. The geriatric and immunocompromised populations are at the high risk end of developing severe RSV disease.[24,25] Nevertheless, there is no effective vaccine or treatment for RSV. There is limited success for treatment of RSV using palivizumab (prescribed for high-risk individuals) and ribavirin.[26–29] Current research efforts to develop effective treatments against RSV are focused on using fusion inhibitors, subunit vaccine, attenuated RSV, DNA vaccine, siRNA molecules and virus-like particles.[30,31] Despite a high potential for use of nanomaterials as anti-RSV therapeutic, this option remains minimally explored.

In this study, we investigated the ability of GNRs to inhibit RSV infection in HEp-2 cells and in BALB/c mice. In addition we examine the impact of GNR on antiviral genes and cytokines expression levels both in HEp-2 cells and in mice. Our results

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* Corresponding author at: Center for NanoBiotechnology Research, Alabama State University, Montgomery, AL, USA.
E-mail address: ssingh@alasu.edu (S.R. Singh).
[1] Current address: Wallace H. Coulter Department of Biomedical Engineering, Georgia Tech College of Engineering and Emory School of Medicine, Georgia Institute of Technology, Atlanta, GA, USA.

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show that GNRs are potent inhibitors of RSV primarily *via* inducing the innate immune response. Thus, GNRs have the potential for treatment against RSV and other viruses with similar infection mechanisms.

Methods

Cells virus and reagents

HEp-2 cells and RSV long strain (VR-26) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Minimum Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), PKS [penicillin (75 U/mL), kanamycin (100 μg/mL) and streptomycin (75 μg/mL)] designated as MEM-10. DMEM supplemented with 2% FBS (designated as DMEM-2) was also used in other experiments.

GNRs (45 nm × 10 nm) were purchased from Nanopartz Inc. (Loveland, CO, USA) (Supplementary Figure S1). For *in vitro* assays, HEp-2 cells were incubated for 24 h at 37 °C, 5% CO_2. Next day, cells were treated with either 2.5 μg/mL GNR (**GNR**) and 100 PFU/mL RSV (**RSV**) or pre-incubation of GNRs (2.5 μg/mL) with 100 PFU/mL RSV for 30 min (**GNR–RSV**). The cells were incubated for 1 h at 37 °C and processed for immunofluorescence and other *in vitro* studies.

Immunofluorescence microscopy

RSV inhibition was studied using immunofluorescence microscopy as described previously.[32] Briefly, following treatment with GNR and GNR–RSV, cells were incubated for another 48 h. After 48 h, cells were fixed with 10% trichloroacetic acid and washed successively with 70%, 90% and 100% ethanol. Cells were then blocked with 3% dry milk powder and incubated for 1 h with 1:500 goat anti-RSV antibody (Millipore, Temecula, CA, USA) followed by anti-goat antibody (FITC-conjugated IgG H + L, 1:1000). Finally, ProLong® Gold antifade mountant with DAPI was applied and visualized using fluorescence microscope.

Animal experiments

Animal experiments were performed according to the National Institutes of Health (NIH) guidelines for animal use and were approved by Alabama State University's Institutional Animal Care and Use Committee (IACUC). Female 4 to 6 week-old BALB/c mice (Charles River Laboratories Inc., Wilmington, MA, USA) were housed under standard approved conditions and provided daily with sterile food and water *ad libitum*.[33] Four groups of mice (n = 3 per group) were administered intranasally either with PBS, RSV (6×10^5 PFU), GNR (20 μg) or GNR–RSV (20 μg GNR and 6×10^5 PFU of RSV). On day 5, mice were sacrificed and blood, bronchoalveolar lavage (BAL), lungs and spleens were collected. RSV titers were quantified from lungs (using plaque assay and qPCR) and BAL (plaque assay). Transmission electron microscopy (TEM), histopathology and antiviral PCR array analysis were done for lung tissues. Cytokine analysis of lung homogenate, BAL and serum was performed.

Plaque assay

The ability of GNRs to inhibit RSV *in vitro* was determined by plaque assay as described previously.[32] HEp-2 cells were infected with RSV and treated with GNRs (1.25 and 2.5 μg/mL). Lungs were collected in DMEM and processed to determine the RSV titers using gentleMACS dissociator (Miltenyi Biotec Inc., Auburn, CA, USA) following the manufacturer's procedures. Similarly, BAL samples collected from mice were used to determine RSV titers in BAL.

Real-time PCR

Lungs collected from control and treatment group mice were used to extract total RNA using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using SuperScript® II reverse transcriptase following the manufacturer's protocol (Invitrogen, Life Technologies, Carlsbad, CA, USA) using an oligo-dT primer. Master mix and PCR conditions were prepared as described previously.[34,35]

Transmission electron microscopy

Lungs from each mice group were collected and fixed with paraformaldehyde–glutaraldehyde and osmium tertraoxide followed by ethanol washes. Tissue sections were polymerized in Embed812 resin and ultrathin sections were collected on copper grids. The tissue sections were stained with uranyl acetate, lead citrate and then imaged using a Zeiss EM10 TEM microscope.

Histopathology

Lungs were harvested from PBS, RSV, GNR and GNR–RSV groups of mice on day 5 after treatment and tissues were fixed in formalin, sectioned, stained with hematoxylin–eosin and analyzed at Nationwide Histology (Veradale, WA, USA).

Antiviral PCR array

Antiviral gene response was studied using HEp-2 cells either untreated (control) or treated with RSV or GNR–RSV for 24 h. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) followed by cDNA synthesis and PCR for human antiviral response PCR arrays (Qiagen SA Biosciences, Valencia, CA, USA) following the manufacturer's protocol. The RT^2 profiler PCR array data analysis version 3.5 (available on vendor's web portal) was used for data analysis. Fold changes in gene regulation were calculated for RSV and GNR–RSV treated cells by comparing them with untreated cells.

Similarly, for *in vivo* mouse anti-viral gene response, lungs (PBS, RSV, GNR and GNR–RSV groups) were harvested at day 5 after treatments and total RNA was extracted using RNeasy fibrous tissue mini kit. cDNA synthesis, PCR and data analysis were performed for mouse antiviral response PCR arrays (Qiagen SA Biosciences, Valencia, CA, USA) as described above. Only genes that were significantly up- or down-regulated (P value <0.05) from both *in vitro* and *in vivo* experiments were included in the final analysis. The gene network displays were generated using gene regulation data to predict the functional protein associations for *in vitro* and *in vivo* experiments with the help of STRING version 10.[36]