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Therapeutic potential of andrographolide-loaded nanoparticles on a murine asthma model

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Abstract

Corticosteroids commonly prescribed in asthma show several side-effects. Relatively non-toxic andrographolide (AG) has an anti-asthmatic potential. But its poor bioavailability and short plasma half-life constrain its efficacy. To overcome them, we encapsulated AG in nanoparticle (AGNP) and evaluated AGNP for anti-asthmatic efficacy on murine asthma model by oral/pulmonary delivery. AGNP had 5.47% drug loading with a sustained drug release *in vitro*. Plasma and lung pharmacokinetic data showed predominantly improved AG-bioavailability upon AGNP administered orally/by pulmonary route. Cell numbers, IL-4, IL-5, and IL-13 levels in broncho-alveolar lavage fluid and serum IgE content were reduced significantly after administration of AGNP compared to free-AG treatment. AGNP-mediated suppression of NF- κ B was predominantly more compared to free-AG. Further, pulmonary route showed better therapeutic performance. In conclusion, AGNP effectively controlled mild and severe asthma and the pulmonary administration of AGNP was more efficacious than the oral route.

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Key words: Andrographolide; Nanoparticle; Murine asthma model; Ovalbumin; Eosinophilia

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hyper-secretion, airway hyper-responsiveness followed by shortness in breathing¹ and approximately three hundred million people around the world suffer from asthma.² Many inflammatory cells such as Th2 cells, B-cells, mast cells and a number of cytokines are involved in the allergic cascade.^{1,3} Allergen induces crosslinking of IgE with IgE-receptor (Fc ϵ RI) on mast cells, releasing histamine, leukotrienes, interleukins (ILs) and other inflammatory mediators.^{4,5} Airway eosinophilia together with Th2 cytokines, IL-4, IL-5 and IL-13, may ultimately contribute to airway hyper-responsiveness in asthma.⁶ Treatment with corticosteroids is a common therapeutic strategy for asthma but has severe side-

effects.^{7–9} Novel therapeutic agents with their effective route of administration and prolonged action may be an alternative to corticosteroids for management of asthma.

Nanoparticles are frequently used for loading different types of drug due to the several advantages such as improved bioavailability, sustained release, tissue targeted delivery, improved permeability to biological membranes, and usefulness in different routes of administration.^{10–14} In the past few decades, biodegradable polylactide-co-glycolide (PLGA), approved by the US Food and Drug Administration and European Medicine Agency, has been extensively used to develop sustained release nanoparticles to treat various diseases including pulmonary diseases in animals^{15–17} and humans.^{18,19}

Andrographolide (AG), a labdane diterpene lactone,²⁰ attenuates ovalbumin (OVA) induced allergic asthma.^{21–24} But poor water solubility,²⁵ and very short biological half-life in gastrointestinal acidic and alkaline conditions due to its degradation²⁶ constrain its efficacy. *In vivo* efficacy of sparingly water soluble AG may be enhanced by encapsulating it in nanoparticle.^{12–14}

Here we prepared AG-encapsulated PLGA nanoparticle (AGNP) to improve its bioavailability and efficacy over free-

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Table 1
Composition, drug loading and entrapment efficiency of different experimental nanoparticle formulations.

Formulation code	Amount of Drug (mg)	Amount of PLGA (mg)	Amount of FITC (μ L)	Theoretical drug loading (%)	Actual drug loading (%) ^a	Entrapment efficiency (%) ^a
BNP	-	50	-	-	-	-
AGNP	5	50	-	9%	5.47 ± 0.075	60.18 ± 0.83
AGNP1	10	50	-	16%	5.49 ± 0.031	60.43 ± 0.34
FITC-AGNP	5	50	100	9%	5.07 ± 0.19	55.78 ± 2.09

NB:

BNP: Blank Nanoparticle.

AGNP: PLGA nanoparticle encapsulating AG.

AGNP 1: AGNP with 1:5 drug-polymer ratio.

FITC-AGNP: Fluorescein isothiocyanate labeled AGNP.

^a Each value represents mean ± SD (n = 3).

drug in OVA-induced murine asthma model. Further, effective route of administration for AGNP was investigated by comparing oral route and pulmonary route.

Methods

Animals

Male C57 BL/6 mice (6–8 weeks old) and male Sprague–Dawley rats (180–200 g) were used for the studies. All the experimental procedures were approved by the Animal Ethics Committee (AEC), Jadavpur University and carried out following the guidelines of AEC. The animals had free access of standard diet²⁷ and water *ad libitum*.

The detailed methodologies have been given in the Supplementary file.

Fourier-transform infrared spectroscopy (FTIR)

To detect any possible interaction between the drug and the selected excipients, FTIR study was performed as reported earlier.²⁸ Free-AG, PLGA, polyvinyl alcohol (MW 85,000–124,000) (PVA), physical mixture of PLGA and PVA (PM 1), physical mixture of PLGA, PVA and AG (PM 2), blank nanoparticle (BNP) and AGNP were scanned between 4000 cm^{-1} and 400 cm^{-1} using FTIR (IR affinity 1, Shimadzu, Japan).

Preparation and characterization of AGNP

Composition of AGNP, BNP and fluorescein isothiocyanate labeled AGNP (FITC-AGNP) was given in Table 1. The nanoparticles were prepared by multiple-emulsion solvent evaporation technique²⁸ (the detailed method given in the supplementary file). Briefly, AG (5 or 10 mg) pre-dissolved in dimethyl sulfoxide (100 μ L) was dissolved with 50 mg PLGA in 2 ml dichloromethane (DCM) and acetone (DCM: acetone, 1:1, v/v) mixture as the organic phase which was homogenized with 2.5% w/v PVA, and then homogenized with 1.5% w/v PVA, stirred overnight, sonicated, centrifuged and the precipitate was lyophilized.

Drug loading and entrapment efficiency were determined as described by Paul et al.²⁹ Drug loading was evaluated by

dissolving 2 mg nanoparticle formulation in acetonitrile:methanol (1:1), centrifuged and supernatant was subjected to UV spectroscopy at λ_{max} 223 nm. The drug loading and entrapment efficiency were calculated using the following equations²⁹:

$$\text{Drug loading (actual) (\%)} = \frac{\text{Amount of drug present in nanoparticles}}{\text{Amount of nanoparticles analyzed}} \times 100$$

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Drug loading (actual) (\%)}}{\text{Drug loading (theoretical) (\%)}} \times 100$$

The size and surface morphology of AGNP were studied by particle size analyzer and zeta sizer, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) following the methods.^{28–29} The details were given in the supplementary file.

In vitro drug release study

In vitro drug release study was carried out in phosphate buffer saline (PBS, pH 7.4) and simulated lung fluid (SLF, pH 7.4) as described earlier.²⁹ The details were given in the supplementary file. Lyophilized AGNP (2 mg) was suspended in 2 mL in each of the media and incubated at 37 °C in prelabeled microcentrifuge tubes. At specific time intervals (30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 30 h, 48 h, 96 h, 168 h, 336 h, 504 h, 840 h, 1176 h), the tubes were centrifuged and supernatant was taken and analyzed by UV spectrophotometer at λ_{max} 223 nm. For each time point three tubes were kept and three such sets were run.

In vitro aerodynamic property analysis of AGNP

The aerodynamic property (mass median aerodynamic diameter {MMAD} and geometric standard deviation {GSD}) of nebulized AGNP was analyzed by using an eight-stage Anderson Cascade Impactor.³⁰

Effect of AG and AGNP on histamine release from mast cells

A primary screening was conducted to evaluate the effect of AG/AGNP on mast cell degranulating agent, compound 48/80 (Sigma-Aldrich Co., St Louis, MO, USA)³¹ induced histamine

release from mouse peritoneal mast cells according to the method reported earlier.³² The details were given in the supplementary file.

Pharmacokinetic studies and lung accumulation of AG by LC–MS/MS

To compare the availability of AG in plasma and in lungs upon free-drug/AGNP treatment, pharmacokinetic profiles of the drug were performed in the respective tissues of mice. Mice were given free-AG/AGNP by oral route/pulmonary route. At predetermined time points (at 1 h, 2 h, 6 h, 10 h, 24 h and 48 h) post drug administration, the animals were sacrificed and blood and lungs were collected. The drug content in them was analyzed by LC–MS/MS. Diclofenac sodium was used as the internal standard.

Radiolabeling of AGNP, gamma scintigraphy, and biodistribution study of ^{99m}Tc-AGNP in rats

For gamma scintigraphic analysis, technetium-99 m (^{99m}Tc)-labeled PLGA nanoparticles containing AG formulation (^{99m}Tc-AGNP) were prepared according to Gaonkar et al.³³ To provide the evidence of localization of AGNP within the body, gamma scintigraphy and biodistribution study in male Sprague–Dawley rats were performed. Rats received ^{99m}Tc-AGNP orally / by pulmonary administration.

Development of asthma in mice along with treatments received

Male C57 BL/6 mice as mentioned earlier were divided into eight groups (each group had five animals). Eight different groups of mice received normal saline/ OVA-induced asthmatic mice³⁴/ OVA treated asthmatic mice received treatment of dexamethasone orally (DEX)/ AG orally (AG[O])/ AGNP orally (AGNP[O])/ budesonide by inhalation (BUD)/ AG by inhalation (AG[I])/ AGNP by inhalation (AGNP[I]). As dexamethasone is a commonly used systemic corticosteroid and budesonide is a widely-used inhaled corticosteroid (ICS) in acute asthma, we have chosen these two drugs as standard for this study for comparison of efficacy of AG from AGNP upon oral/pulmonary administration. Schematic diagram of generation of asthma model and treatment schedule is outlined in Figure 1. The details were given in the supplementary file.

Alveolar uptake study of AG loaded FITC nanoparticles (FITC-AGNP) in asthmatic mice

To investigate the deposition of nanoparticles in alveoli of asthmatic mice, mice were administered FITC-AGNP orally/ by inhalation.²⁹ The animals were sacrificed at predetermined time points and lungs were collected and fixed in formalin (10%).²⁹ The lung-sectioned was then observed under confocal microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany). Using Leicasuite software integrated density (ID) of the images was calculated to confirm the uptake.

Analysis of BALF and serum IgE

Bronchoalveolar lavage fluid (BALF) was isolated as mentioned earlier³⁵ and the total cell count and differential cell

count after Wright staining were performed.²¹ The levels of cytokines (IL-4, IL-5 and IL-13) were determined by ELISA kit. After cardiac puncture, blood was collected and total IgE level in serum was analyzed using ELISA (Catalogue no. BMS613 [IL-4], BMS610 [IL-5], BMS6015 [IL-13], Thermo Fischer Scientific, Massachusetts, USA).

Histological analysis

Lungs from each group of animals were removed and fixed in 10% formalin, embedded in wax and the tissue sections were stained with hematoxylin–eosin (H&E) and periodic acid–Schiff (PAS) after sectioning²² and observed under microscope (Carl Zeiss light microscope, Axiostar plus, Jena, Germany).

Western blot analysis for detection of NF- κ B in lung tissue

Protein p65 of the nuclear extract of lung tissue from each group of mice was determined according to the reported method.³⁶

Statistical analysis

The experimental data have been expressed as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey's test was performed for comparison between the groups. $P < 0.05$ was considered as statistical level of significance.

Results

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of AG, PLGA, PVA, their physical mixtures (PM1 and PM2), BNP and AGNP have been presented in Supplementary Figure 1 (Supplementary Figure 1 FTIR spectroscopy has depicted drug and other excipients interaction). The characteristic peaks of AG appeared at 1728 cm^{−1}, suggesting for lactone ring present in the structure and at 3396 cm^{−1} for O-H stretching vibration. The typical peaks at 2995.45 cm^{−1} and 2945.30 cm^{−1} for C-H stretching and at 1755.22 cm^{−1} for ester C-O stretching of PLGA were observed. In PM2, there were some minor shifts in the peaks within the range from wave no. 2945.30 cm^{−1} to 2943.37 cm^{−1} for PLGA, and 1728–1726.29 cm^{−1} in case of AG. This might be due to some physical interactions such as weak H-bonding, van der Waals force of attraction, dipole–dipole interaction, etc. The absence of characteristic peak of AG in AGNP and similarity of peaks between BNP and AGNP suggest that no free-drug was present on the nanoparticle surface.

Physicochemical characterization of AGNP

Initially, several formulations were prepared by varying drug-polymer ratio. Drug loading was found to be saturated at 1:10 drug-polymer ratio (AGNP). Further enhancement till 1:5 drug-polymer ratio (AGNP1) did not show any enhancement of drug loading. Drug loading percentage of AGNP and AGNP1 was found to be 5.47 $\pm$ 0.075 and 5.49 $\pm$ 0.031 (Table 1). Hence AGNP was chosen for further studies.

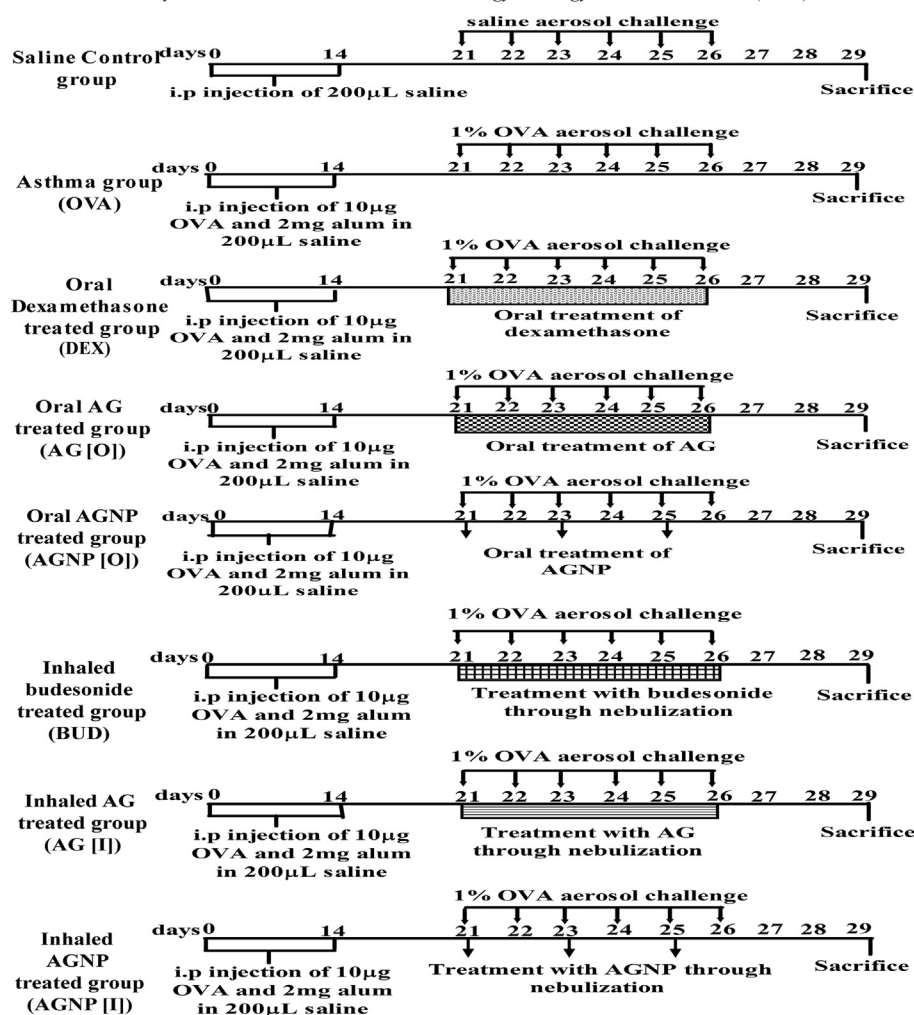


Figure 1. Experiment protocol and treatment schedule for mouse model of allergic asthma.

The average particle size and polydispersity index of AGNP were found to be 205 nm (Table 2) and 0.341 respectively. The zeta potential value of AGNP was obtained as -18.3 mV (Table 2).

SEM image of AGNP (Figure 2, A) has revealed that the particles were in nanosize range with a homogenous and thick distribution pattern. TEM photograph confirmed the homogenous distribution

Table 2

Average particle size, polydispersity index (PDI), zeta potential and data of various *in vitro* drug release kinetic equations obtained by fitting experimental drug release data to different release kinetic models along with corresponding R^2 values and release exponent (n) (Korsmeyer–Peppas model).

Formulation code	Average particle size (nm)	PDI ^a	Zeta potential (mV) ^a	Different kinetic model	R^2 value of corresponding kinetic model	
					Release in SLF (pH 7.4)	Release in PBS (pH 7.4)
AGNP	205	0.341 ± 0.008	-18.3 ± 0.01	Zero Order	$y = 0.0658x + 24.374$ $R^2 = 0.7456$	$y = 0.0450x + 12.260$ $R^2 = 0.8612$
				First Order	$y = -0.0007x + 1.8765$ $R^2 = 0.8953$	$y = -0.0003x + 1.9440$ $R^2 = 0.9048$
				Korsmeyer–Peppas	$y = 0.3548x + 0.9159$ $R^2 = 0.9702$ $n = 0.35$	$y = 0.3376x + 0.6996$ $R^2 = 0.9733$ $n = 0.33$
				Higuchi	$y = 2.4408x + 13.083$ $R^2 = 0.9300$	$y = 1.5885x + 5.4218$ $R^2 = 0.9715$
				Hixson–Crowell	$y = -0.0018x + 4.2205$ $R^2 = 0.8486$	$y = -0.0009 + 4.4450$ $R^2 = 0.8915$

SLF- Simulated lung fluid; PBS- Phosphate buffer saline.

^a Each value represents mean $\pm$ SD ($n = 3$).

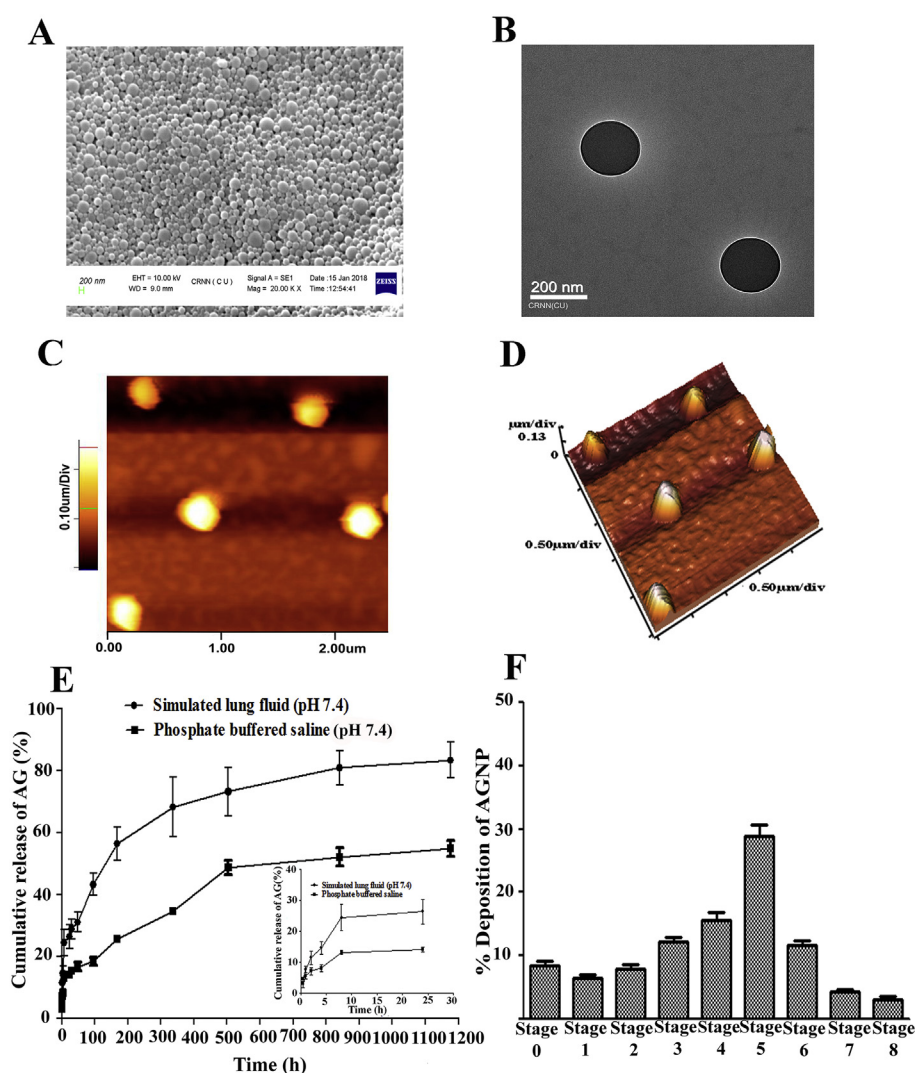


Figure 2. Characterization of AGNP. (A) SEM image of AGNP. (B) TEM image of AGNP. (C) AFM 2D image of AGNP. (D) AFM 3D image of particle. (E) *In vitro* release of AG from AGNP, release in SLF, pH 7.4 (—●—) and PBS, pH 7.4 (—■—). Within the large figure, the small figure indicates the drug release pattern from 0–24 h. (F) Percentage deposition of AGNP on different stages of Andersen Cascade Impactor. Data show mean $\pm$ SD ($n = 3$).

of drug within the particle (Figure 2, B). AFM images also showed the three dimensional (200 nm $\times$ 200 nm $\times$ 200 nm) structure of the particles with smooth surface (Figure 2, C and D).

In vitro drug release study

In vitro release pattern of AG from AGNP in PBS (pH 7.4)/SLF (pH 7.4) has been shown in Figure 2, E. Initially there was a burst release of drug up to 8 h and then the drug release was in a sustained manner in both PBS and SLF. After 21 days, 49% drug was released from the formulation in case of PBS. But there was different drug release pattern in SLF. The release of drug in SLF was much higher than the drug release in PBS at each time point. Drug release was found to be 43.15% within 96 h and followed sustained drug release pattern at least up to 50 days (duration of this study).

To find out the drug release kinetics the data were analyzed by using zero order, first order, Korsmeyer–Peppas, Higuchi and Hixson–Crowell plots. The plot of Korsmeyer–Peppas showed a good linearity in PBS and SLF, where the R^2 values were 0.9733 and 0.9702 in PBS and SLF respectively. The release exponent (n) values were 0.33 for PBS and 0.35 for SLF (Table 2). The data suggest that the release of AG from the formulation in both the media followed anomalous non-Fickian diffusion.³⁷

In vitro aerodynamic properties (MMAD and GSD) of AGNP

Particles were found to deposit on all the stages (Figure 2, F) of Andersen cascade impactor. Particle deposition was found to be less than 10% in stage 0–2 and 7–8. The stage 5 showed highest deposition (28.78%) among all the stages. MMAD and GSD of AGNP were found to be 2.18 μ m and 3.05 respectively. The data suggest that distribution of particles would take place in

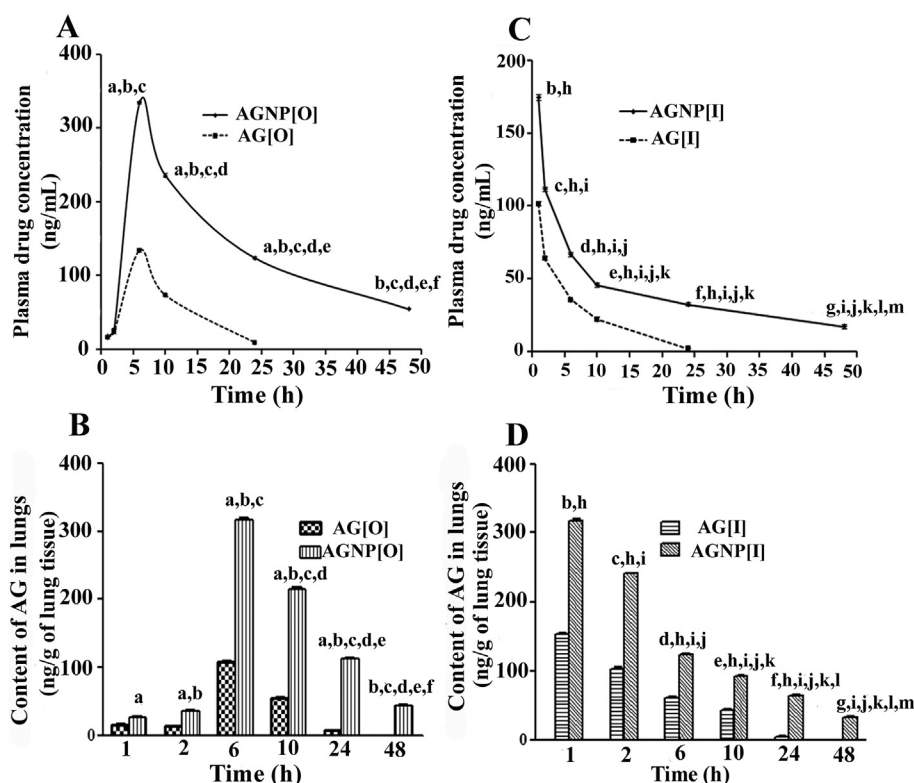


Figure 3. *In vivo* pharmacokinetic profiles of AG in plasma and in lungs upon administration of free-drug/AGNP by oral/ pulmonary route in mice. (A) Plasma concentration of AG after oral administration of AGNP/free-AG in suspension in mice. (B) Content of AG in lung after oral administration of AGNP/free-AG in suspension in mice. (C) Plasma concentration of AG after pulmonary administration of AGNP/free-AG in suspension in mice. (D) Content of AG in lung after pulmonary administration of AGNP/free-AG in suspension in mice. All values are represented as mean $\pm$ SD ($n = 3$). Significant difference from corresponding (i) AG[O] at each oral time intervals studied $^aP < 0.05$, (ii) AGNP[O] 1 h $^bP < 0.05$, (iii) AGNP[O] 2 h $^cP < 0.05$, (iv) AGNP[O] 6 h $^dP < 0.05$, (v) AGNP[O] 10 h $^eP < 0.05$, (vi) AGNP[O] 24 h $^fP < 0.05$, (vii) AGNP[O] 48 h $^gP < 0.05$, (viii) AG[I] at each inhalation time intervals studied $^hP < 0.05$, (ix) AGNP[I] 1 h $^iP < 0.05$, (x) AGNP[I] 2 h $^jP < 0.05$, (xi) AGNP[I] 6 h $^kP < 0.05$, (xii) AGNP[I] 10 h $^lP < 0.05$, (xiii) AGNP[I] 24 h $^mP < 0.05$.

all lobes of the lungs once nebulized and the particle size range was suitable for inhalation therapy.

Inhibitory effects of AG and AGNP on histamine release from mast cells

The amount of histamine release from mast cells by compound 48/80 treatment increased significantly than the normal mast cells. In contrast incubation with AG/AGNP suppressed the release of histamine from the cells-treated with compound 48/80, where dose dependent inhibition was observed. Inhibitory (50%) concentration (IC_{50}) values for AG and AGNP were found to be 23 μ M and 8.2 μ M respectively (Supplementary Table 1). Supplementary Table 1 shows IC_{50} values of AG and AGNP on compound 48/80 induced-histamine release from mice peritoneal mast cells.

In vivo studies

Pharmacokinetic study

In vivo plasma and lung pharmacokinetic profiles of AG after AGNP administration were compared to free-AG after oral/ pulmonary administration (Figure 3, Table 3). The data showed that at each time point plasma concentration of AG from animals-

treated with AGNP was higher than free-AG. Upon AGNP treatment, a steady blood level of AG was found to maintain up to 48 h (period of investigation) upon oral (Figure 3, A)/ pulmonary (Figure 3, C) administration. In case of oral administration of AG suspension/ AGNP suspension it took 6 h to reach maximum content of AG in both plasma and lung. Interestingly, upon pulmonary administration, content of drug in plasma/lung (Figure 3, C and D) was always higher upon AGNP treatment than free-AG treatment. Free-drug absorption from the absorption site might be less than AGNP. AUC_{0-t} values of AGNP were found to be much higher than free-AG for oral/pulmonary administration. Evaluation of lung accumulation of AG showed a higher deposition of AG from AGNP suspension in lung than free-AG. In case of pulmonary administration drug deposition was found to vary inversely with time. The values of t_{max} were found to be 1 h and 6 h for pulmonary administration and oral administration, respectively. Elimination half-life ($t_{1/2}$) of AG from AGNP in plasma was 3.93 fold and 6.38 fold higher than that of free-AG for oral administration and pulmonary administration, respectively (Table 3). After oral administration, the values of AUC_{0-t} of AGNP were found to be 5.37 times and 6.38 times higher than free-AG in plasma and lung, respectively. Again, upon pulmonary administration, AUC_{0-t} values were 3.2 times

Table 3

Plasma and lung pharmacokinetic parameters of AG from AGNP or free drug (AG) suspension after oral or pulmonary administration in mice.

Pharmacokinetic Parameters	Oral administration				Inhalation administration			
	Plasma profile*		Lung profile*		Plasma profile*		Lung profile*	
	AG	AGNP	AG	AGNP	AG	AGNP	AG	AGNP
Elimination $t_{1/2}$ (h)	4.6 ± 0.09	18.1 ± 1.05 ^a	4.5 ± 0.12	16.6 ± 2.02 ^d	4.2 ± 0.1	26.8 ± 1.92 ^{b,c}	4.9 ± 0.11	24.9 ± 2.3 ^{e,f}
C_{max} (ng/ml)	134.2 ± 3.1	334.0 ± 2.25 ^a	108.1 ± 2.13	317.0 ± 3.20 ^d	101.2 ± 1.5 ^a	174.2 ± 2.1 ^{b,c}	153.2 ± 2.45 ^d	317.0 ± 2.36 ^f
AUC _{0-t}	1177.6 ± 14.4	6331.6 ± 21.2 ^a	902.7 ± 7.8	5763.3 ± 23.3 ^d	631.1 ± 9.1 ^a	2044.2 ± 19.1 ^{b,c}	1093.5 ± 11.6 ^d	3984.8 ± 17.12 ^{e,f}
AUC _{0-inf}	1238.6 ± 9.2	7754.9 ± 21.52 ^a	947.3 ± 8.82	6807.8 ± 18.34 ^d	642.6 ± 7.1 ^a	2704.8 ± 12.22 ^{b,c}	1129.4 ± 10.84 ^d	5152.0 ± 24.32 ^{e,f}
CL (L/h/kg)	-	-	-	-	15.5 ± 2.2	3.69 ± 0.21 ^c	8.85 ± 1.8	1.94 ± .021 ^f
MRT _{last} (h)	9.5 ± 1.21	19.3 ± 2.11 ^a	9.5 ± 1.3	18.7 ± 3.1 ^d	5.5 ± 1.2	15.8 ± 2.05 ^c	6.2 ± 1.11	15.9 ± 2.15 ^f
V _{ss} (L/kg)	-	-	-	-	92.1 ± 3.33	122.3 ± 4.1 ^c	62.1 ± 3.1	60.8 ± 2.3

$t_{1/2}$: half-life; AUC_{0-t}: area under the concentration–time curve from time of dosing (t = 0) to a determined time point; CL: clearance; MRT: mean residence time; V_{ss}: steady state volume of distribution.

*Data were expressed as mean ± SD (n = 3).

Significant difference from corresponding (i) Oral AG administration (plasma profile) ^a $P < 0.05$, (ii) Oral AGNP administration (plasma profile) ^b $P < 0.05$, (iii) Inhalation AG administration (plasma profile) ^c $P < 0.05$, (iv) Oral AG administration (lung profile) ^d $P < 0.05$, (v) Oral AGNP administration (lung profile) ^e $P < 0.05$, (vi) Inhalation AG administration (lung profile) ^f $P < 0.05$.

Note: For CL and V_{ss} we conducted Student's *t* test.

and 3.6 times higher in case of AGNP than free-AG in plasma and lung respectively.

Gamma scintigraphy

The accumulation of ^{99m}Tc-AGNP via oral administration and pulmonary administration in rats is shown in Figure 4. For better identification of radiolabeled compound in organs, rats were used for this experiment. Oral administration of ^{99m}Tc-AGNP had less hepatic accumulation at 1 h (Figure 4, A) than that at 4 h (Figure 4, B). At 1 h, no signal was detected in lungs upon oral administration of ^{99m}Tc-AGNP, but at 4 h, ^{99m}Tc-AGNP was found to accumulate in lung (Figure 4, B). As expected the accumulation of ^{99m}Tc-AGNP in lung at 1 h (Figure 4, C) was more for pulmonary administration than its oral delivery. At 4 h after the pulmonary administration the lung accumulation of ^{99m}Tc-AGNP was predominantly maintained as the signal of it at 1 h after pulmonary administration with some evidences of renal clearance (present in kidneys and urinary bladder) (Figure 4, D). Thus, ^{99m}Tc-AGNP was successfully delivered to lungs and also well-retained in lungs even after 4 h post treatment through oral/ pulmonary route as seen in the removed lungs (Figure 4, E and F).

Biodistribution study

Biodistribution (% of formulation distributed in lungs and other organs/tissues) study with radiolabeled AGNP has been given in Table 4. For oral administration, 4 h post treatment showed higher hepatic accumulation of ^{99m}Tc-AGNP (18.25% applied dose (AD) per g of organ) than 1 h post treatment (11.46% AD/g). Accumulation of ^{99m}Tc-AGNP in lung at 4 h (1.31% AD/g) was found to be more than that at 1 h (1.046% AD/g) by oral route. After oral administration, AGNP accumulated in lungs in a time-dependent manner. Predominant accumulation of ^{99m}Tc-AGNP was observed in lung at 1 h (4.14% AD/g) upon pulmonary administration and the value was also maintained at 4 h (3.37% AD/g) with some slow reduction of accumulated ^{99m}Tc-AGNP. Urinary excretion was also

observed. Distribution of ^{99m}Tc-AGNP in other organs is mentioned in Table 4.

Alveolar uptake study of FITC-AGNP

In vivo lung deposition of FITC-AGNP from oral /inhalation route at three different time points 1 h, 6 h, 24 h (Figure 5, A and B) was confirmed by confocal microscopy. At 1 h, uptake of nanoparticle in lungs was less when compared to the uptake of nanoparticles at 6 h by both the routes. There was no predominant change in intensity observed at 6 h and 24 h, for both the routes of administration. The images showed that the nanoparticles were internalized and well-retained in lung at least for 24 h in case of both the routes of administration. The integrated density (ID) of the images showed the time-dependent uptake up to 6 h (Supplementary Figure 2 showing quantification of integrated density (ID) for FITC-AGNP uptake in lung tissue of mice after oral/inhalation administration.) for both oral/ pulmonary administration of FITC-AGNP.

Effect of administration of AGNP on inflammatory cell recruitment and lung histology

To examine the anti-inflammatory effect of AGNP, total cell count and different inflammatory cell count in BALF were carried out (Figure 6, A and B). The total cell count and the count of different inflammatory cells such as eosinophil, macrophage, and neutrophil markedly increased after the exposure to OVA aerosol (in asthma control group of mice) as compared to normal saline (control) group of mice. Treatment with AGNP drastically decreased the above-mentioned cell counts and the effect of oral administration of AGNP was almost similar to its pulmonary administration. Further, histological evaluation revealed that airway inflammation was developed in asthma group of mice (Figure 6, C). In AG[O] group, no significant change compared to asthma group has been noticed whereas in AG[I] group, the OVA induced cellular infiltration was attenuated but at a low degree. A marked decrease in infiltration of inflammatory cells was noticed for AGNP[O]/AGNP[I] administration compared to asthma group. Lung sections stained with PAS (Figure 6, D) of

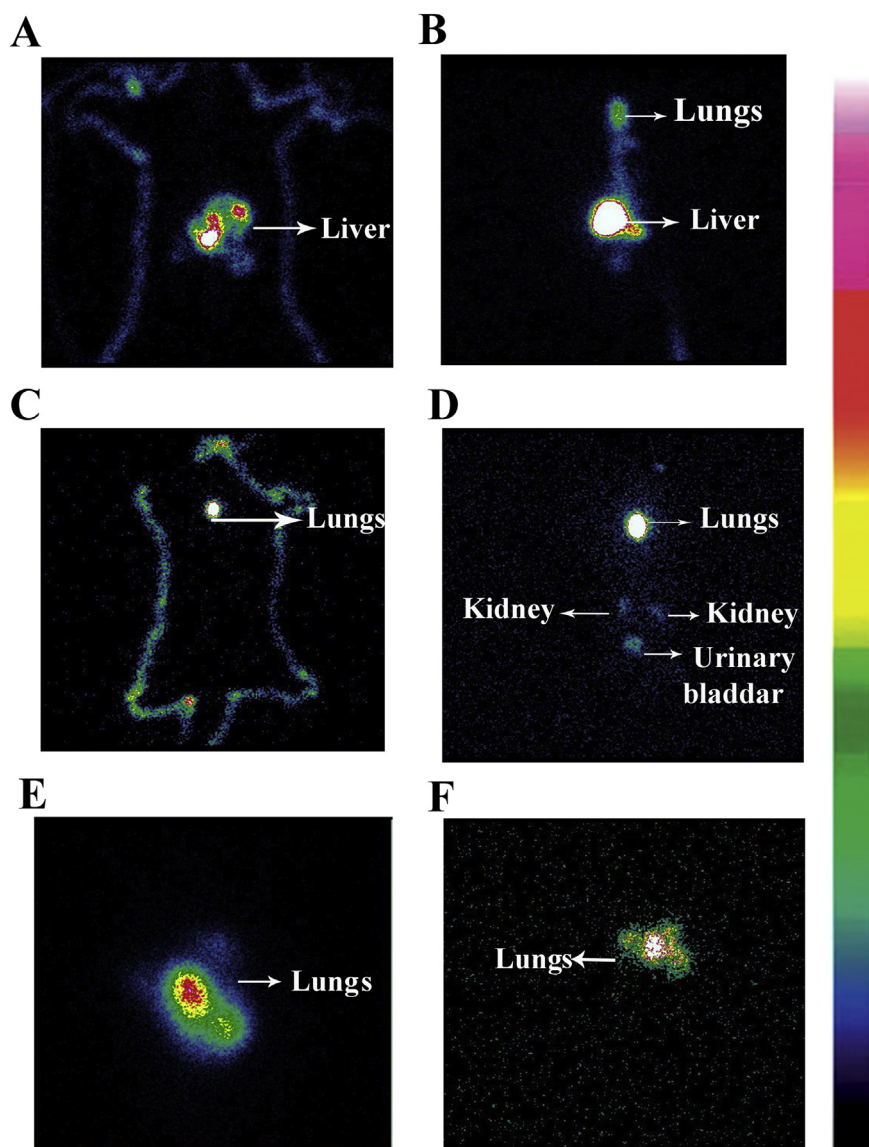


Figure 4. Gamma scintigraphy images of rats at 1 h and 4 h after receiving ^{99m}Tc labeled AGNP by oral administration and pulmonary administration. Images of post oral administration of ^{99m}Tc -AGNP at 1 h (A) and 4 h (B) and images of post pulmonary administration of ^{99m}Tc -AGNP at 1 h (C) and 4 h (D). Images of lungs at 4 h (E) oral administration of ^{99m}Tc -AGNP (F) Image of lungs after 4 h of pulmonary administration of ^{99m}Tc -AGNP.

asthma group showed goblet cell hyperplasia with over production of mucus in the bronchi. Treatment with AG through inhalation decreased the mucus production while AG treatment in oral group failed to produce the similar effect. Treatment with AGNP through oral/inhalation decreased the mucus production. Altogether the histological data of animals treated with AGNP [O]/AGNP[I] showed diminished OVA-induced cellular infiltration, thickening of airway wall and mucus production in lung tissue sections which were comparable to the results of standard drug groups received, DEX/BUD treatment, respectively.

Effect of AGNP administration on various cytokine levels in BALF and serum IgE

The cytokines responsible for inflammation associated with asthma (such as IL-4, IL-5 and IL-13 in BALF) and serum IgE were measured (Figure 7). After OVA challenge the cytokine

levels of mice of asthma group were elevated compared to those of normal saline (control) group. The elevated cytokine levels were found to be suppressed upon oral (AG[O])/pulmonary (AG[I]) administration of AG in AG [O]/AG[I] treated groups of mice. The inhibition became significantly ($P < 0.05$) more in case of AGNP. AGNP[O] treated mice showed more inhibition of IL-4 compared to those received standard drug (DEX) by oral route (Figure 7, A). The inhibitory effect of AGNP was more pronounced by pulmonary route than oral route. Pulmonary administration of AGNP even showed more inhibition of IL-4 than standard drug (BUD) administration in mice. Pulmonary administration of AGNP showed more reduction of IL-4 and IL-5 levels than its administration by oral route (Figure 7, A and B). AGNP by pulmonary administration predominantly reduced IL-13 level than that for AGNP by oral administration (Figure 7, C). Moreover, there was no significant difference in the level of the

Table 4

Biodistribution study of ^{99m}Tc labeled andrographolide loaded PLGA nanoparticles in rats after oral and pulmonary administration.

Organ/Tissue	Oral route of administration*		Pulmonary route of administration*	
	1 h	4 h	1 h	4 h
Heart	0.300 $\pm$ 0.040	0.481 $\pm$ 0.058 ^a	0.228 $\pm$ 0.034 ^b	0.338 $\pm$ 0.107
Blood	0.453 $\pm$ 0.029	0.255 $\pm$ 0.034 ^a	0.461 $\pm$ 0.036 ^b	0.340 $\pm$ 0.025 ^{a,b,c}
Liver	11.462 $\pm$ 0.840	18.259 $\pm$ 2.230 ^a	4.217 $\pm$ 0.406 ^{a,b}	3.423 $\pm$ 0.388 ^{a,b}
Lungs	1.046 $\pm$ 0.142	1.312 $\pm$ 0.325	4.143 $\pm$ 0.242 ^{a,b}	3.373 $\pm$ 0.314 ^{a,b,c}
Spleen	1.580 $\pm$ 0.392	4.224 $\pm$ 0.590 ^a	0.113 $\pm$ 0.021 ^{a,b}	0.133 $\pm$ 0.026 ^{a,b}
Stomach	10.862 $\pm$ 1.658	3.667 $\pm$ 0.880 ^a	0.372 $\pm$ 0.040 ^{a,b}	0.397 $\pm$ 0.027 ^{a,b}
Intestine	9.440 $\pm$ 0.758	12.796 $\pm$ 1.077 ^a	0.974 $\pm$ 0.093 ^{a,b}	1.266 $\pm$ 0.139 ^{a,b}
Kidney	3.495 $\pm$ 0.811	8.705 $\pm$ 0.996 ^a	2.000 $\pm$ 0.172 ^b	6.184 $\pm$ 0.389 ^{a,b,c}
Urinary bladder	9.609 $\pm$ 0.776	14.291 $\pm$ 2.372	4.028 $\pm$ 0.317 ^{a,b}	15.242 $\pm$ 2.931 ^{a,c}
Muscle	0.093 $\pm$ 0.024	0.129 $\pm$ 0.049	0.037 $\pm$ 0.006 ^b	0.089 $\pm$ 0.024
Brain	0.085 $\pm$ 0.015	0.172 $\pm$ 0.031	0.389 $\pm$ 0.065 ^{a,b}	0.309 $\pm$ 0.027 ^{a,b}

Note: One way ANNOVA followed by Tukey's test showed significant difference from corresponding (i) oral route of administration 1 h ^a P < 0.05, (ii) oral route of administration 4 h ^b P < 0.05, (iii) pulmonary route of administration 1 h ^c P < 0.05.

* Each value represents % applied dose (AD) per gram of organ/tissue $\pm$ SD (n = 3).

cytokines upon AGNP[I] administration and by inhalation of the standard anti-asthmatic drug (BUD). Elevated serum IgE level was also significantly (P < 0.05) reduced by AGNP upon oral / pulmonary administration (Figure 7, D).

Inhibition of ovalbumin induced NF- κ B signaling by AGNP in allergic airway inflammation

A report suggests that NF- κ B rapidly translocates from cytoplasm to nucleus in the sensitized mice.²³ In the OVA-treated asthma group of animals, NF- κ B (p65) activation markedly increased in the nuclear extract of lung tissue as compared to that of lung of normal saline (control) group of mice (Figure 7, E and F). There was no change in NF- κ B expression upon oral administration of andrographolide (Figure 7, E). In contrast, a marked suppression of the protein (p65) was observed in case of pulmonary administration of AG (Figure 7, F). Administration of AGNP by oral route as well as pulmonary route markedly suppressed NF- κ B expression which was comparable to dexamethasone and budesonide respectively.

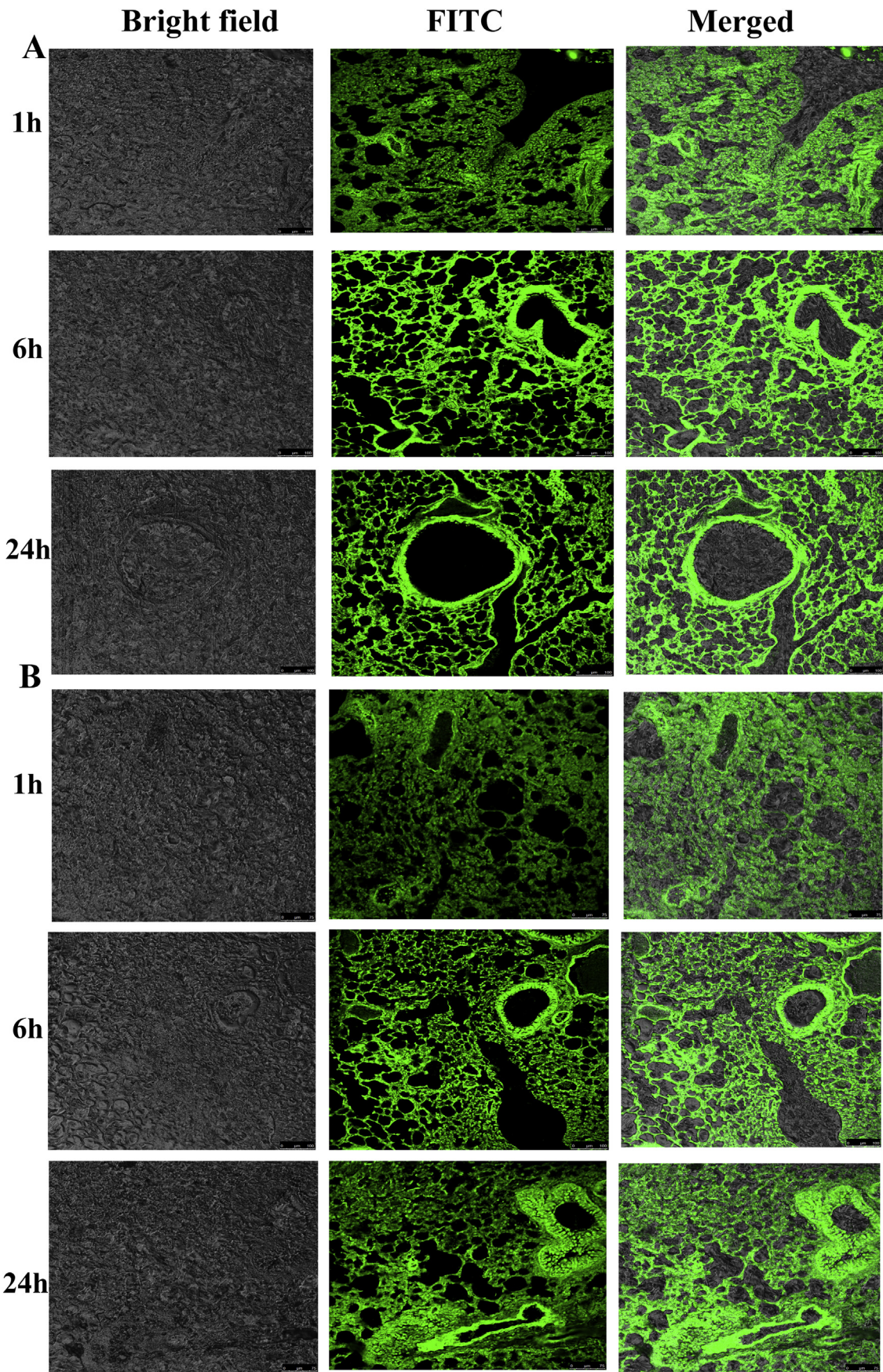
Discussion

In the present study we have first investigated the physicochemical parameters of the particles. FTIR data showed that no chemical interaction existed between the drug and the excipients. However, some physical interactions (such as weak hydrogen bond, van der Waals force of attraction, dipole moment etc.) might play a role in formation of nanoparticle structures.³⁸ The prepared nanoparticles were found to be spherical with no visual crevices on their surface. Lower PDI value indicates that the AGNP had a narrow range of distribution.³⁹ The zeta potential value of AGNP (−18.3 mV) indicates that the particles had good amount of surface charge not to conglomerate in a suspended state.²⁸ *In vitro* release of AG from nanoparticle showed an initial burst release over a period of 8 h followed by a sustained drug release pattern in PBS/SLF. This could be probably due to the loosely bound AG present closed inside the peripheral region of the nanoparticle surface. Further, a sustained

release pattern of AG in both SLF and PBS indicates a slower diffusion of AG from polymeric core. The aerosol droplets generated from nebulized AGNP were in the ideal MMAD range (2.18 μm) that could deliver drug into the alveoli of lungs.⁴⁰

Mast cells play a key role in the allergic inflammation cascade by releasing mediator such as histamine.^{4,5} AGNP inhibited histamine release 2.8 times more from mast cells than AG. Less permeability of AG into the cells might attribute to its lower effect on the mast cells compared to AGNP.

AGNP was administered by oral/pulmonary route to identify better route of administration. Drug levels were more in blood and lung upon AGNP administration by both the routes. Encapsulation of the drug in the nanoparticle might protect it from the gastro-intestinal pH-dependent degradation²⁶ that produced more plasma concentration and longer residence time of the drug administered through nanoparticle. In case of pulmonary administration particle size plays an important role.⁴¹ Free-drug might have undergone muco-ciliary clearance in the upper respiratory tract,⁴² causing them to be swallowed and was degraded by stomach acidic pH and intestinal alkaline pH.²⁶ Particles with size around 200 nm are not easily recognized by alveolar macrophages due to smaller size^{41,42} and thus, the administered nanoparticles were rapidly taken up by epithelial cells (AGNP had average particle size 205 nm). Drug molecules present close to the surface of polymeric matrices of AGNP initially released in lung fluid (as our data showed Figure 2, E in SLF) which were absorbed to the systemic circulation leading to higher plasma concentration of AG from AGNP than free-AG upon their pulmonary delivery.⁴² In gamma scintigraphy, ^{99m}Tc -AGNP was detected in lung at 4 h after oral administration/pulmonary administration. For pulmonary administration, the uptake was predominantly higher in lungs at the initial time point (at 1 h). Retention of nanoparticle in lung for prolonged efficacy has been achieved for ^{99m}Tc -AGNP upon pulmonary administration. However, smaller nanoparticles might have been eliminated faster and were detected in kidneys and urinary bladder at 4 h after ^{99m}Tc -AGNP pulmonary administration. Biodistribution study showed time-dependent accumulation of ^{99m}Tc -AGNP in lung after oral administration. Pulmonary



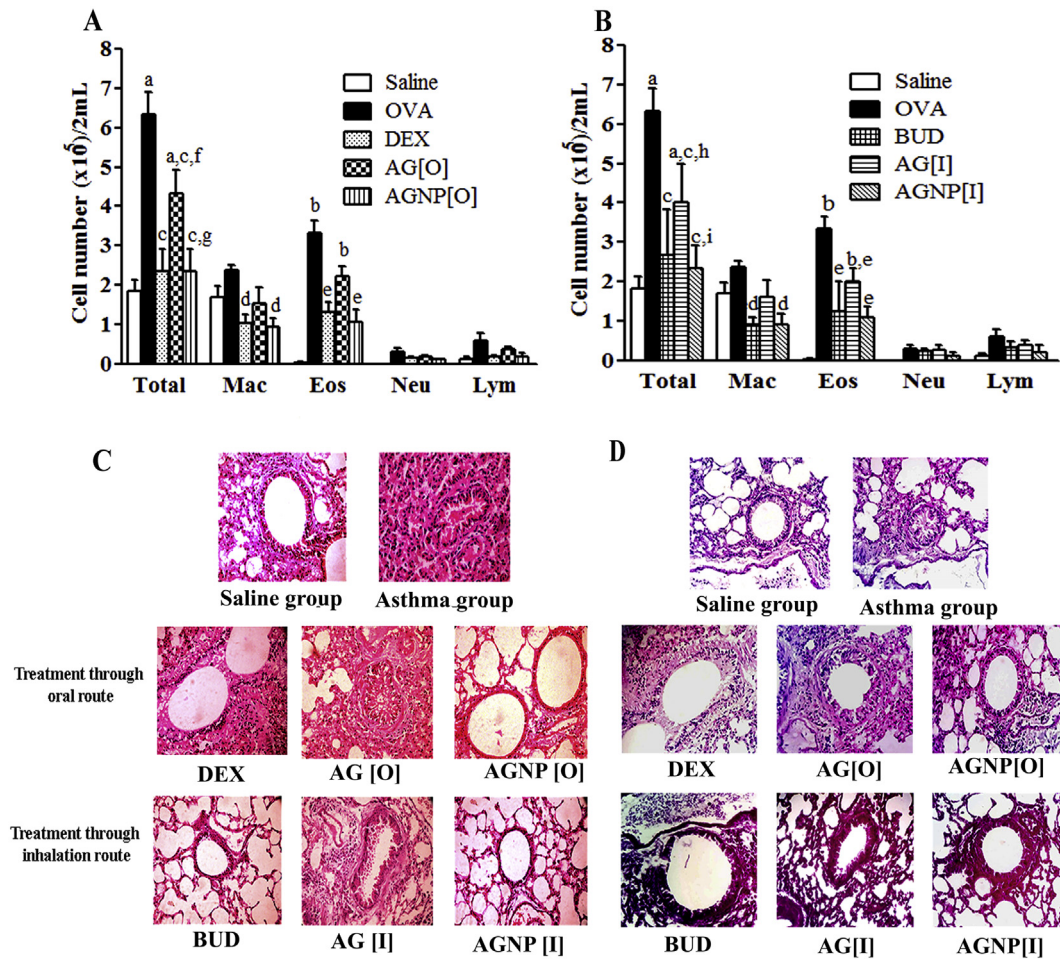


Figure 6. Therapeutic effect of AGNP on airway inflammatory cell recruitment and histological analysis of lung tissue. (A) Total cell count and inflammatory cell count in BALF obtained from saline, OVA (asthma group), oral dexamethasone, oral AG and oral AGNP treated mice. Each bar represents mean $\pm$ SD of five mice. (B) Total cell count and inflammatory cell count in BALF obtained from saline (control), OVA (asthma group), inhaled budesonide, inhaled AG and inhaled AGNP treated mice. Each bar represents mean $\pm$ SD of five mice. (C) Histological examination of lung tissue sections by H&E staining (magnification 40 $\times$). (D) Histological examination of lung tissue sections by PAS staining. Significant difference corresponding from (i) saline (Total) $^aP < 0.05$, saline (Eos) $^bP < 0.05$, (ii) OVA (Total) $^cP < 0.05$, OVA (Mac) $^dP < 0.05$, OVA (Eos) $^eP < 0.05$, (iii) DEX (Total) $^fP < 0.05$, (iv) AG[O] (Total) $^gP < 0.05$, (v) BUD (Total) $^hP < 0.05$, (vi) AG[I] (Total) $^iP < 0.05$. In figures A and B, "Total" indicates total cell count, "Mac" indicates macrophage count, "Eos" indicates eosinophil count, "Neu" stands for neutrophil count, and "Lym" indicates lymphocyte count.

administration showed higher percentage ^{99m}Tc -AGNP accumulation in lung than its oral administration. FITC-AGNP deposition in asthmatic lung after oral/ pulmonary administration was found to be time dependent up to 6 h which retained till 24 h. Thus, AGNP successfully delivered drug in lung by both the routes of administration for a prolonged period of time.

Eosinophilia is an indication of allergic diseases.^{43,44} The inflammatory cell counts became significantly less after the treatment with AGNP by oral/pulmonary route in OVA-treated mice and the findings were similar to those obtained upon the administration of dexamethasone/budesonide. The results might be due to the higher deposition of AGNP in lung compared to AG. Histological analysis suggests that AGNP upon oral/ pulmonary administration lowered the number of inflammatory cells and

reduced the thickened airway walls. Earlier findings have indicated that IL-4, IL-5 and IL-13 play a critical role in mucin Muc5ac gene, protein expression and induction of goblet cell hyperplasia.^{45,46} We have found that AGNP reduced the elevated IL-4, IL-5 and IL-13 levels significantly by pulmonary route than oral route. These results altogether denote that AG released from AGNP might have better interfered with cytokines Th2, which is a key mediator in eosinophilic inflammation in asthma,²² due to its sustained release in lungs. Elevated IgE levels in serum are the clear evidence of Th2 immune response. In our study, the data showed that oral/ pulmonary administration of AGNP markedly decreased the serum IgE levels. Further, crosslinking of IgE on Fc ϵ RI is known to trigger the allergic cascade resulting in activation of NF- κ B and secretion of different cytokines.^{5,47} We, therefore, examined NF-

Figure 5. Confocal images of asthmatic lung sections of mice treated with FITC-AGNP by oral/ pulmonary routes. (A) Images of lung sections were taken at 1 h, 6 h and 24 h after oral administration of FITC-AGNP. (B) Images of lung sections at 1 h, 6 h and 24 h after inhalation of FITC-AGNP. Magnification of the images was 20 $\times$. Scale bar "—" equivalent to 100 μm .

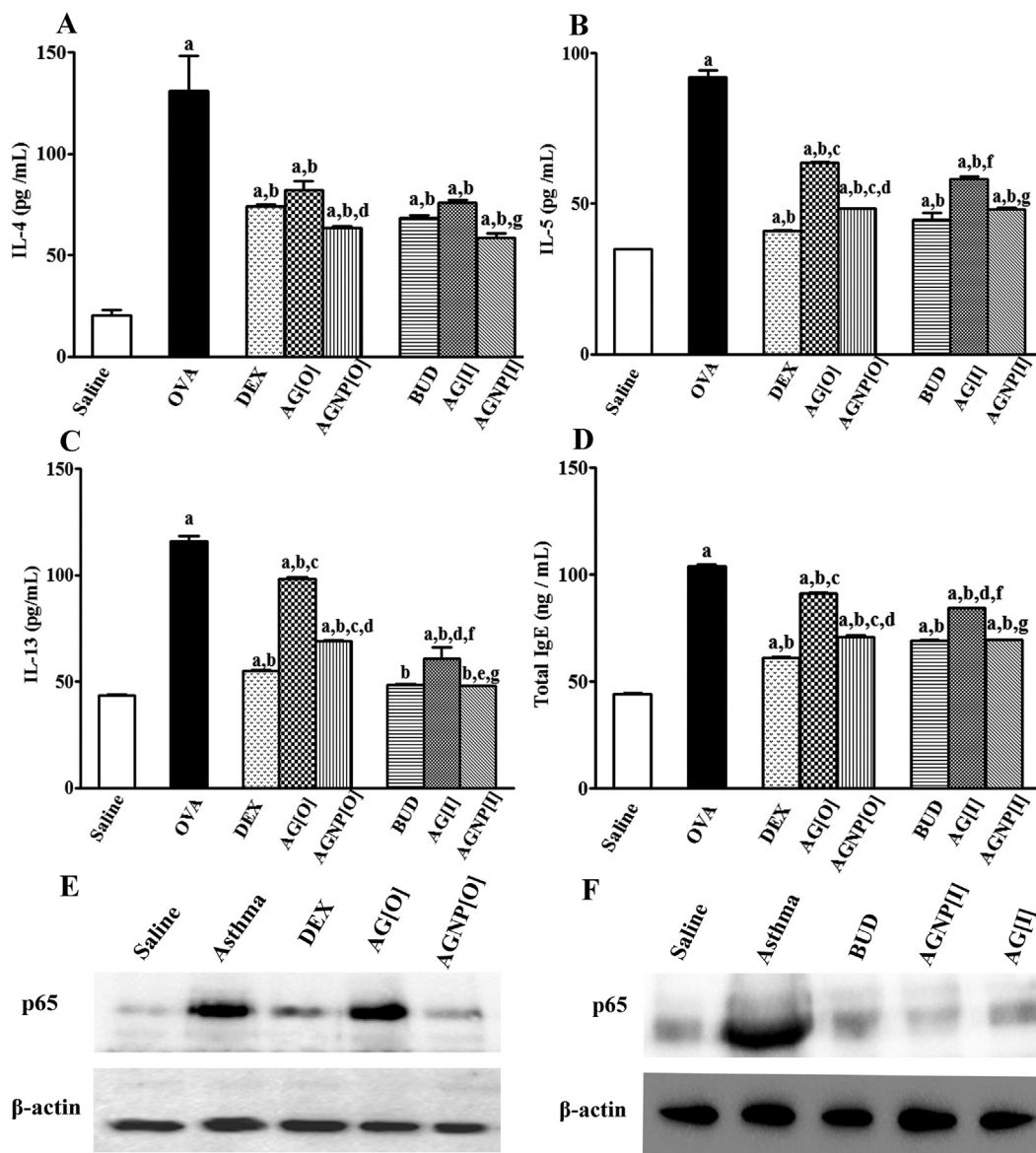


Figure 7. Modulation of cytokines levels in BALF, serum IgE level and NF- κ B (p65) expression among the experimental groups. (A) IL-4 level in BALF (values shown mean $\pm$ SD; n = 3). (B) IL-5 level in BALF (values shown mean $\pm$ SD; n = 3). (C) IL-13 quantification in BALF (values shown mean $\pm$ SD; n = 3). (D) IgE quantification in serum (values shown mean $\pm$ SD; n = 3). (E) Nuclear fractions were extracted from the lungs upon oral treatments of AG, AGNP and DEX in mice and were immunoblotted for p65. (F) Nuclear fractions were extracted from the lungs upon AG, AGNP and BUD treatments administration by pulmonary route in mice and were immunoblotted by p65 of NF- κ B. Significant difference from corresponding (i) saline (control) ^a*P* < 0.05, (ii) OVA ^b*P* < 0.05, (iii) DEX ^c*P* < 0.05, (iv) AG[O] ^d*P* < 0.05, (v) AGNP[O] ^e*P* < 0.05, (vi) BUD ^f*P* < 0.05, (vii) AG[I] ^g*P* < 0.05.

κ B protein expression where we found a dramatic inhibition of NF- κ B upon AGNP[Oral]/AGNP[pulmonary] administration similar to the effects obtained from the treatment of anti-asthmatic drugs, dexamethasone and budesonide respectively. Pulmonary administration of AG inhibited NF- κ B expression but surprisingly no such inhibition was found in oral treatment of AG (in PBS). This might be due to the instability of AG in extreme acidic and alkaline conditions in gastrointestinal tract.²⁶ All the results discussed in this study clearly showed better effectiveness of AG while administered in the form of AGNP through inhalation route. AGNP can be useful in a mild asthma as well as for prolonged treatment. AGNP may be a promising therapeutic agent also in

severe asthma clinically. To the best of our knowledge this is possibly the first ever report on inhibitory effect and the preferable route of administration of andrographolide (nanoparticle) in OVA-induced asthma in mice. Further studies are warranted to translate the findings for clinical investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2019.04.009>.

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Supplementary file

Therapeutic potential of andrographolide-loaded nanoparticles on a murine asthma model

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Detailed Methodology, Supplementary Table 1, Supplementary Figure 1, Supplementary Figure 2 and additional References

Materials and Methods

Materials

Andrographolide (AG) (TCI Chemicals, Tokyo, Japan), polyvinyl alcohol (PVA, MW 85,000-124,000; S.D. Fine Chem. Pvt. Ltd., Mumbai, India), and fluorescein isothiocyanate 98% (FITC) (HiMedia Laboratories, Mumbai, India) were procured. PLGA {MW 50,000–75,000; poly (lactide-co-glycolide) ratio 75:25}, ovalbumin, alum, dexamethasone, budesonide and compound 48/80 were procured from Sigma-Aldrich Co., St Louis, MO, USA.

Fourier-transform infrared spectroscopy (FTIR)

The pure drug andrographolide (AG), PLGA, PVA, physical mixture of PLGA, PVA (PM1) and a physical mixture of AG with PLGA, PVA (PM2), blank nanoparticle (BNP) and andrographolide nanoparticles (AGNP) were mixed separately, with infrared (IR) grade potassium bromide (KBr) in the ratio of 1:100. About 5.5 metric ton pressure was applied with a hydraulic press to prepare corresponding

pellets of each sample. The pellets were subjected to scanning over a wave number range of 4000–400 cm^{-1} in an inert atmosphere using FTIR instrument (IR affinity 1, Shimadzu, Japan).

Preparation of PLGA nanoparticles containing andrographolide (AGNP)

AGNP were prepared by multiple emulsion solvent evaporation technique. Briefly, 50 mg of PLGA (75:25) was solubilized in 2 ml of organic phase containing dichloromethane (DCM) and acetone (DCM: acetone was in 1:1, v/v ratio). Weighed amount of AG (5mg or 10mg) was then dissolved in 100 μL of dimethyl sulfoxide (DMSO) and it was then added dropwise to the organic phase containing polymer with stirring by using magnetic stirrer at 600 rpm. Two aqueous solutions of PVA of concentrations 2.5% w/v and 1.5% w/v were prepared parallelly. The organic phase was then emulsified by dropwise addition of a total 1.2 mL volume of 2.5% w/v PVA solution using a high speed homogenizer (IKA Laboratory Equipment, Model T10B Ultras-Turrax, Staufen, Germany) at 15,000 rpm for 10 min. The primary emulsion was then added slowly to 75 mL of 1.5% w/v PVA solution under the same homogenization condition to obtain secondary emulsion. The final emulsion thus obtained was placed in bath sonicator (Trans-O-Sonic, Mumbai, India) with ice bath for 30 min to break the large particles, if any. After sonication the emulsion was kept on magnetic stirrer for overnight at room temperature to remove organic solvent and also for solidification of particle. Then the emulsion was centrifuged at 5,000 rpm for 5 min and the pellet was discarded to remove the larger particles. The supernatant was taken and again centrifuged at 16,000 rpm to get the pellet of nanoparticles. The pellet thus obtained was washed three times with Milli Q water to remove excess PVA adhered on the surface of particles and was finally dispersed in very small amount (1mL) of Milli Q water. The sample was frozen first at -40°C and was then lyophilized (Laboratory Freeze Dryer, Instrumentation India Ltd., Kolkata, India) for 8 h to get solid nanoparticles. Blank nanoparticles (BNP) were prepared using the same method without any addition of drug.

FITC nanoparticles (FITC-AGNP) were prepared by following the same technique as described above where a stock solution of FITC (0.4% w/v) in ethanol: chloroform (1:3) was prepared and 100 µL of this solution was added in the organic phase containing drug and polymer before homogenization.

Physicochemical characterization of nanoparticles

Determination of % drug loading and entrapment efficiency

For determination of drug loading, 2mg of AGNP and 2mg of BNP were dissolved separately in 2 ml acetonitrile and methanol (1:1 ratio v/v) mixture and were vortexed. The solutions were kept in incubator shaker for 1 h and were then centrifuged at 16000 rpm at 4°C for 15 min to separate any undissolved material. The supernatants were collected and analyzed by UV spectrophotometer at λ_{max} 223 nm using the solvent mixture as blank. The drug loading and entrapment efficiency were calculated using the following equations

$$\text{Drug loading (actual) (\%)} = \frac{\text{Amount of drug present in nanoparticles}}{\text{Amount of nanoparticles analyzed}} \times 100$$

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Drug loading (actual) (\%)}}{\text{Drug loading (theoretical) (\%)}} \times 100$$

Particle size, polydispersity index and zeta potential

Average particle size, polydispersity index and zeta potential of different formulations were measured by dynamic light scattering technique using Malvern Zetasizer Nano-ZS 90 (Malvern Instruments, Malvern, UK) at 25°C. Weighed amount of formulations were dispersed in Milli Q water and sonicated for 15 min before being analyzed by Zetasizer.

Surface morphology study

The surface morphology of nanoparticles was studied by scanning electron microscopy (SEM) (Zeiss, EVO18 Special edition, Oberkochen, Germany). The lyophilized sample was placed on specimen stubs which was then coated with platinum by (Quorum, Q150T ES, Lewes, UK) sputter coater before analysis. The nanoparticles were further characterized by transmission electron microscopy (TEM) (Jeol, JEM 2100, Tokyo, Japan) using copper grid on which the dispersion of nanoparticles was placed. Nanoparticles were again subjected to atomic force microscopy (AFM) (Veeco, dcp-II, AP-0100, USA) to confirm the morphology.

In vitro drug release study

For *in vitro* drug release study, phosphate buffer saline (PBS, pH 7.4) and simulated lung fluid (SLF, pH 7.4) were used as the drug release media. Briefly, suspensions of 2 mg of lyophilized AGNP in 2 mL of each media were placed in microcentrifuge tubes (triplicate in number). The tubes were then kept in BOD incubator shaker (Somax Incubator Shaker; ShenjhenPango Electronic Co. Ltd., Shenzhen, China) at 37°C at 120 rotation/min. At specific time intervals (30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 30 h, 48 h, 96 h, 168 h, 336 h, 504 h, 840 h, 1176 h), the tubes were centrifuged and 2 mL supernatant was taken from each tube and same volume was replaced by fresh buffer/SLF. To quantify AG, the collected aliquots were analyzed by UV spectrophotometer (Model Intech-295, Gentaur GmbH, Aachen, Germany) at λ_{max} 223 nm.

The drug release data were applied to different kinetics models such as zero order, first order, Higuchi, Korsmeyer-peppas and Hixson crowell model. The equation with highest R^2 value was selected to show the kinetic pattern of drug release in both PBS and SLF.

In vitro aerodynamic analysis of AGNP

The aerodynamic property of AGNP was analyzed by using an eight stage Anderson Cascade Impactor (Thermo Fisher Scientific, MA, USA) equipped with a vacuum pump. Briefly, AGNP was nebulized for 10 min into the cascade impactor. The nanoparticles deposited on each plate and the filter was collected in

microcentrifuge tubes by washing with acetonitrile: methanol (1:1). The tubes were then centrifuged at 16,000 rpm for 10 min and collected supernatants were analyzed for quantification of AG by UV spectrometer at λ_{max} 223nm. The nanoparticle deposition was calculated from the amount of AG deposited on each plate. Using mass median aerodynamic diameter (MMAD) calculator, MMAD and geometric standard deviation (GSD) were calculated from the weight of nanoparticle deposited on the different stages. The flow rate of the apparatus was 28.3 L/min.

Effect of AG and AGNP on histamine release from mast cell

A primary screening was conducted to evaluate the effect of AG/AGNP on compound 48/80 induced histamine release from mice peritoneum mast cells. Mast cells were isolated from mice peritoneum and purified. Purified mice peritoneal mast cells (2×10^5 cells/mL) were preincubated for 10 min in carbondioxide incubator (MCO-15AC; Sanyo, Tokyo, Japan). A definite quantity (5-40 μM) of AG/AGNP containing equivalent quantity of AG suspended in PBS and passed through 0.22 μ membrane filter aseptically was added to the cells as pretreatment and the cells were incubated for 24 h. After washing the cells thrice with PBS, the cells were subjected to degranulation by incubation with compound 48/80 (4 $\mu\text{g/mL}$). The reaction was stopped by keeping the test tubes in ice. The supernatant was then collected by centrifugation. The cell pellet was collected. The cell pellet was then treated with 0.05% w/v Triton X to lyse and centrifuged to estimate the residual histamine. The histamine content in supernatant and cell pellet was estimated by procedure of Shore et al [1]. Spontaneous release of histamine was estimated by the same procedure without addition of samples. The percentage of histamine release was calculated by the following equation:

$$\text{Histamine release (\%)} = [(\text{Supernatant-spontaneous}) / \{(\text{Supernatant} + \text{cell pellet}) - \text{spontaneous}\}] \times 100\%$$

In vivo studies

Pharmacokinetic studies

To compare the availability of AG from free-drug suspension/AGNP suspension in plasma and in lung, pharmacokinetic profiles of the drug were performed in the respective tissues. Mice were divided into four groups. In one group, AG (free-drug) was administered through oral route as per dose (Group I). The next group received AGNP as suspension with an equivalent amount of AG through oral route (Group II). AG was administered through nebulization to the third group (Group III) where the dose was same as oral administration and AGNP suspension (containing equivalent amount of AG) was administered to the fourth group through nebulization (Group IV). At a predetermined time points (at 1 h, 2 h, 6 h, 10 h, 24 h and 48 h) post drug administration, the animals were sacrificed. For plasma pharmacokinetic study, blood was collected by cardiac puncture from at least three animals at the above-mentioned time points from each group and lungs were separated to analyze the AG availability. Blood collected in EDTA solution was then centrifuged at 6000 rpm for 5 min to separate the plasma and stored at -80°C for further analysis [2]. Thawed lung tissue was homogenized before analysis [3].

LC-MS/MS study

AG content in plasma/ lung tissue homogenate was measured by LC-MS/MS. Briefly, 100 µL of plasma or lung homogenate was mixed with 300 µL of ice cold acetonitrile containing internal standard (Diclofenac sodium). The sample was mixed by vortexing and after centrifugation at 10,000 rpm for 10 min and supernatant was collected. AG stock was prepared in methanol and working stocks (WS) were prepared by serial dilution in 1:1 methanol/water mixture. Calibration control (CC) and quality control (QC) samples were prepared by spiking WS samples in blank plasma and blank lung homogenate separately. Next, 100 µL of CC/QC plasma/lung homogenate sample was precipitated with 300 µl ice cold acetonitrile containing internal standard. The samples were mixed using vortexer and it was followed by centrifugation at 10,000 rpm for 10 min. The supernatants were collected and loaded into LC-MS/MS. Liquid chromatography with Triple quad mass spec (HPLC, Model: 20AC, Shimadzu, Japan and MS

from AB-Sciex Model: API4000, Foster city, CA) was used for analysis. Using YMC Triart C18 column (dimension 30 × 2.1mm) and gradient elution technique of two solvents (Solvent A: 0.1% formic acid in water and Solvent B: 80:20 acetonitrile/water with 0.1% formic acid), the analysis was done. Total run time was 3.0 min.

Radiolabeling of AGNP

For gamma scintigraphic analysis, technetium-99m (^{99m}Tc)-labeled PLGA nanoparticles containing AG formulation (^{99m}Tc -AGNP) was prepared. Briefly, AGNP was dispersed in 1mL nitrogen purged water. After that aqueous ^{99m}Tc -pertechnetate ($^{99m}\text{TcO}_4^-$) (74–148 MBq) was added to it, followed by addition of freshly prepared stannous chloride dihydrate (SnCl_2) solution in a concentration of 1mg/mL. After 15 min of incubation, it was subjected to thin layer chromatography to analyze radiolabeled efficiency where silica gel strips were the stationary phase and acetone was mobile phase. The strips were cut after developing the spots and was subjected to analysis by a well-type gamma scintillation counter (Electronic Corporation of India, model LV4755, Hyderabad, India) at 140keV. Due to the solubility problem of AG, ^{99m}Tc -labeling could not be done in free-drug.

Gamma scintigraphy

To provide the evidence of localization of AGNP within the body, gamma scintigraphy was performed. Male Sprague-Dawley rats (of body weight 180-200 g) were used for this study. Rats were divided in two groups. One group received ^{99m}Tc -AGNP orally and the other one received ^{99m}Tc -AGNP by pulmonary administration. In the both groups the dose of ^{99m}Tc -AGNP was same. The animals were then anaesthetized with ketamine hydrochloride and fixed on a wooden board for imaging. The static images were snapped at 1 h and 4 h of post treatment, with the help of planar gamma camera (GE Infinia Gamma Camera equipped with Xeleris Workstation, GE, Cleveland, OH).

Biodistribution study of ^{99m}Tc -AGNP in rats

All the experimental animals were well hydrated by injecting 2mL of normal saline prior to ^{99m}Tc -AGNP administration through oral route and pulmonary route (as mentioned above). After 1 h and 4 h of administration, the animals from both groups were sacrificed. The organs and tissues were then collected, washed with saline and dried with blotting paper. Using a well-type gamma scintillation counter radioactivity was measured along with an injection standard (equal volume of ^{99m}Tc -AGNP given to rat).

Murine asthma model

Male C57 BL/6 mice of 6-8week age, weighing between 20-22g were divided into eight groups (each group had five animals) for induction of asthma according to the protocol with some modifications. Briefly, each mouse was sensitized on day 0 and 14 by i.p injection of 10 μg OVA and 2 mg aluminum hydroxide in 200 μL of saline (except the saline control group). On days 21-26, the animals were challenged for 30 min with 1% OVA aerosol in saline or only saline. After 72 h of last aerosol challenge, the mice were sacrificed and Bronchoalveolar lavage (BAL) fluids, lungs and blood samples were collected.

Alveolar uptake study of FITC loaded nanoparticles (FITC-AGNP) in asthmatic mice

To investigate the deposition of nanoparticles in alveoli of asthmatic mice, the study was performed. Briefly, after induction of asthma in mice according to above protocol, mice were divided into two groups. The experiment was performed at three time points (1 h, 6 h, and 24 h). One group of animals was treated with FITC-AGNP (dose equivalent to 1mg AG /kg of body weight) orally and another group was treated with FITC-AGNP (same dose as oral administration) through inhalation. At the predesigned time points, at least three animals were sacrificed from each group. The lungs were isolated, washed and fixed with formalin solution (10%). To confirm the lung deposition of nanoparticles, the tissue sections were prepared and observed under confocal microscopy (TCS SP8; Leica Microsystems, Wetzlar, Germany, equipped with Leicasuitelas X software) at an excitation wavelength of 494 nm and emission wavelength

of 521 nm and photographs were taken with a magnification 20X. Using Leicasuite software further integrated density (ID) of the images was calculated to confirm the uptake.

Treatment

On days 21-26, the animals (treatment groups) were treated with dexamethasone (DEX) (1mg/kg in PBS through oral administration), budesonide (BUD) (1mg/kg in PBS through nebulization), AG {1mg/kg in PBS orally (AG[O])} to one group and through nebulization to another group(AG[I]) [4] 1 h prior to aerosol allergen exposure (Figure 1). Between 21st -26th day, AGNP was administered on every alternate day [dose equivalent to 1mg of AG /kg in PBS, orally to one group (AGNP[O]) and through nebulization to another group (AGNP[I])], 1 h prior to aerosol allergen exposure (Figure 1). As dexamethasone is a commonly used systemic corticosteroid and budesonide is a widely-used inhaled corticosteroid (ICS) in acute asthma, we have chosen these two drugs as standard for this study for comparison of route of administration of AGNP.

Isolation and analysis of bronchoalveolar lavage fluid (BALF)

For isolation of BALF, the trachea of each mouse was cannulated and lungs were washed with 1 ml of sterile PBS using 24 gauge cannula. The procedure was repeated thrice and BALF was collected. The total cell count was performed using haemocytometer. For differential cell count the fluid was centrifuged at 3000 rpm for 10 min and was counted after Wright staining. The level of cytokines (IL-4, IL-5 and IL-13) were determined by ELISA kit (Catalogue no. BMS613 [IL-4], BMS610 [IL-5], BMS6015 [IL-15], Thermo Fischer Scientific, Massachusetts, USA).

Measurement of IgE in serum

After sacrifice, blood was collected from heart of each mouse by cardiac puncture and the serum was separated by centrifugation after clotting of blood. Total IgE level in serum was analyzed using ELISA (Catalogue no. 88-50460-22, Thermo Fischer Scientific, Massachusetts, USA).

Histological analysis

Lungs from each group of animals were isolated and fixed in 10% formalin, embedded in wax and were sectioned. To observe the histologic changes, the lungs sections were stained with haematoxylin and eosin stain (H & E) and for the mucus production examination periodic acid Schiff (PAS) staining was done before microscopical analysis.

Western blot analysis for detection of NF- κ B in lung tissue

The lung tissues were washed with PBS multiple times and then lysed with lysis buffer and nuclear proteins were separated by nuclear extraction reagents (NE-PER 78833, Pierce, Rockford, IL, USA) as per manufacturer's protocol. The total protein concentration was determined using the BCA protein assay reagent (Thermo, Rockford, IL, USA). Proteins were separated by 12% SDS-PAGE and after electrophoresis, the separated proteins were transferred to polyvinylidenedifluoride (PVDF) membranes, using the wet transfer method and nonspecific sites were blocked with 5% BSA for an hour and incubated with primary antibodies overnight at 4°C. After washing three times, the membranes were incubated with HRP- labeled secondary antibodies and blots were tested by ECL plus Western Blotting Detection System (Amersham Life Sciences, Amersham, UK) reagents and binding of antibody was visualized in Bioradchemi doc imaging system.

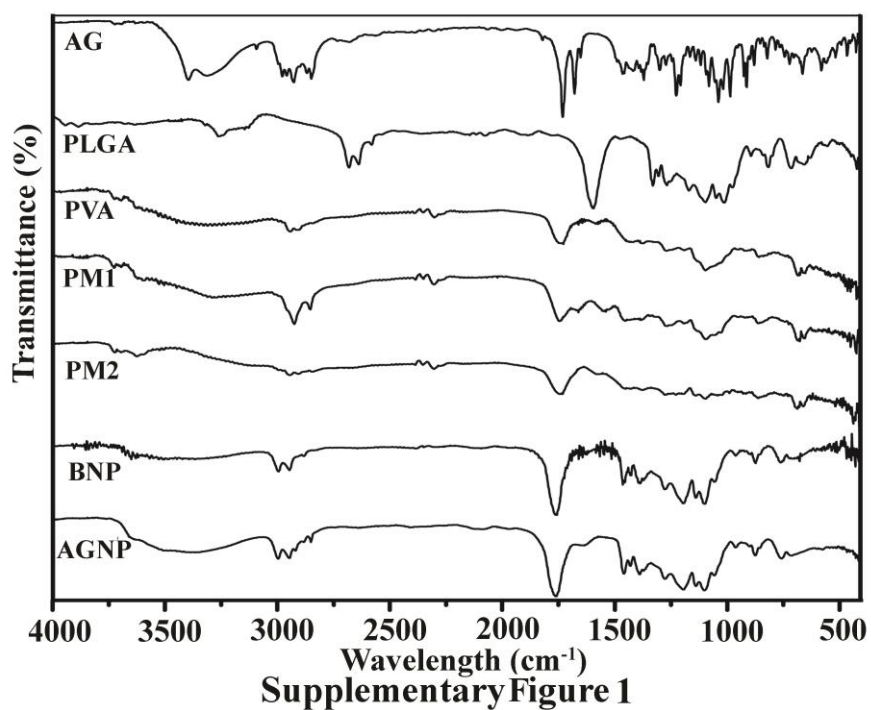
Results

Supplementary Table 1 IC₅₀ values of AG and AGNP on compound 48/80 induced-histamine release from mice peritoneal mast cells^a

Treatment	IC ₅₀ (Mean $\pm$ SD, n=3) (μ M)
AG	23 $\pm$ 1.2
AGNP	8.2 $\pm$ 0.52 ^b

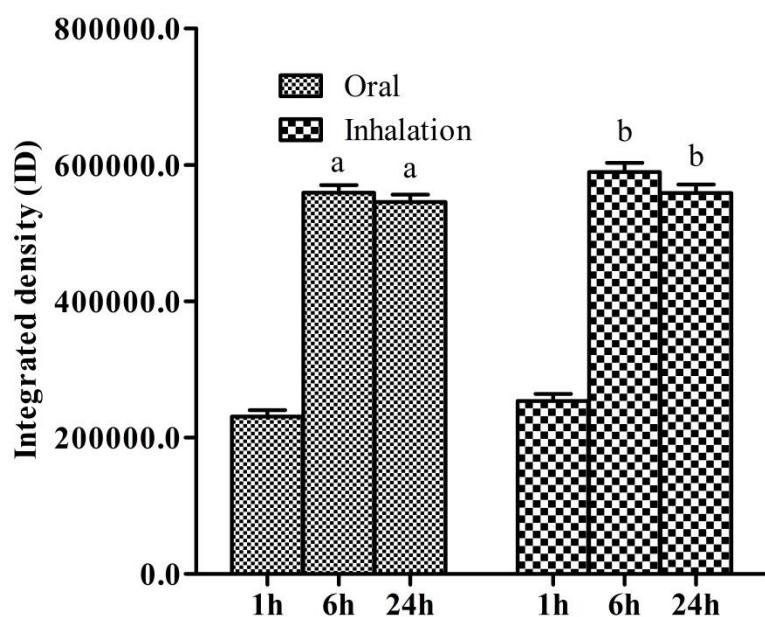
^a Assays are described in Material and methods

^b $p < 0.05$, significant difference compared to AG



Supplementary Figure 1 FTIR spectroscopy depicting drug and other excipients interaction.

Notes AG: Andrographolide; PLGA: poly lactide co glycolic acid; PVA: polyvinyl alcohol; PM1: physical mixture of PLGA and PVA; PM2: physical mixture of PLGA, PVA and AG, BNP: blank nanoparticle; AGNP: andrographolide nanoparticle.



Supplementary Figure 2

Supplementary Figure 2 Quantification of integrated density (ID) for FITC-AGNP uptake in lung tissue of mice after oral/inhalation administration. Data show mean $\pm$ SD (n=3). Significant difference from corresponding (i) oral route of administration 1 h ^a p <0.05, (ii) pulmonary route of administration 1 h ^b p <0.05

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