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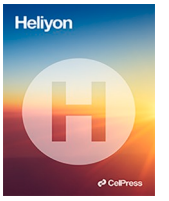

Research article

Selective pharmacological inhibition alters human carcinoma lung cell-derived extracellular vesicle formation

Ayodeji O. Ipinmoroti [a], Rachana Pandit [a], Brennetta J. Crenshaw [a], Brian Sims [b], Qiana L. Matthews [a,c,*]

[a] *Microbiology Program, Department of Biological Sciences, College of Science, Technology, Engineering, and Mathematics, Alabama State University, Montgomery, AL, 36104, USA*
[b] *Departments of Pediatrics and Cell, Developmental and Integrative Biology, Division of Neonatology, University of Alabama at Birmingham, Birmingham, AL, 35294, USA*
[c] *Department of Biological Sciences, College of Science, Technology, Engineering, and Mathematics, Alabama State University, Montgomery, AL, 36104, USA*

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ABSTRACT

Exosomes also termed small extracellular vesicles (sEVs) are important mediators of intercellular communication in many physiological and pathological processes such as protein clearance, immunity, infections, signaling, and cancer. Elevated circulating levels of exosomes have been linked to some viral infections, aggressive cancer, and neurodegenerative diseases. Some pharmacological compounds have been demonstrated to effectively inhibit exosome production pathways. There are very few studies on exosome inhibition and how they influence pathophysiological conditions.

Methods: In the current study, we examined how inhibition of extracellular vesicle release and/or uptake would impact the exosome formation pathway. Using a constellation of improved EV experimental approaches, we evaluated the concentration-based cytotoxicity effects of pharmacological agents (ketoconazole, climbazole, and heparin) on Human Lung Carcinoma (A549) cell viability. We investigated the effect of inhibitor dosages on exosome production and release. Analysis of exosome inhibition includes quantitative analysis and total protein expression of exosome release after pharmacological inhibition; we examined exosome protein level after inhibition.

Results: Selective inhibition of exosomes altered particle sizes, and heparin significantly reduced the total exosomes released. Climbazole and heparin undermined membrane-bound tetraspanin CD63 expression and significantly disrupted ALIX protein ($p \leq 0.0001$) and TSG101 ($p \leq 0.001$). Azoles and heparin also disrupt transmembrane trafficking by modulating Ras binding protein ($p \leq 0.001$).

Conclusion: These findings revealed that pharmacological inhibition of exosomes regulates the endocytic pathway and expression of endosomal sorting complex required for transport mediators, suggesting climbazole and heparin as effective inhibitors of exosome synthesis.

* Corresponding author. Department of Biological Sciences, College of Science, Technology, Engineering, and Mathematics, Alabama State University, Montgomery, AL, 36104, USA.
E-mail address: qmatthews@alasu.edu (Q.L. Matthews).

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1. Introduction

Extracellular vesicle (EV)-mediated cell-to-cell signaling and transport of biomolecules such as RNA, sRNA, miRNA, proteins, and other nucleic acids have been associated with viral-induced pathological conditions and cancer aggression [1]. Their mode of transfer and mechanism of action and how they influence pathological process is yet to be fully understood. Major classes of EVs include exosomes, microvesicles, and apoptotic bodies, these are classified based on their mode of formation and release. All classes of EVs play a vital role in cell-to-cell communication and signaling, but the most studied thus far are the exosomes [2–6].

Biogenesis and release of exosomes complete a cycle when bystander cells take up free vesicles either via endocytosis or passive surface receptor interaction. Exosomes are small EVs and are a subpopulation of heterogenous generated cell-derived membrane-bound EVs. Exosomes are present in a variety of biological fluids, including semen, urine, saliva, amniotic fluid, malignant ascites, breast milk, and blood [7]. They have been proven to be functional in various biological processes such as immunity, infection, protein clearance, and signaling [8,9]. Recently, exosomes derived from a patient diagnosed with prostate cancer have been shown to play a vital role in oncogenic reprogramming in stem cell therapy [10]. Firstly, exosomes are generated by the cell via invagination of the plasma membrane following the endosomal pathway to form multivesicular bodies (MVBs), and then the cycle is terminated via exocytosis of mature intraluminal vesicles (ILVs) into the extracellular space [5,11,12]. During MVB biogenesis, two distinct pathways are exploited, the first pathway is dependent on endosomal sorting complexes required for transport (ESCRT) which involves a complex of four main types of machinery associated with other topologically active membrane distortion proteins such as Vsp4. The ESCRT complex activity starts with endosomal microdomain formation while interacting with transmembrane cargos (ESCRT-0, -I) followed by vesicle scission and release, or they facilitate spiral formation for inward budding of membrane and scission of vesicles to form MVBs (ESCRT-III). A second pathway void of the ESCRT complex depends on cholesterol-rich lipid raft formation within the endosomal membrane. This pathway involves the activity of sphingomyelinase in converting sphingolipids to ceramide resulting in large microdomain formation followed by ILV formation within MVBs, these MVBs fuse with intracellular membrane to release ILVs as exosomes into the extracellular space [5,12]. Classical exosome biomarkers (CD9, CD63, and Alix) are important in sorting and screening exosomes and targeted uptake and/or release. An adjunct therapy approach targeted towards reducing the circulating level of human epidermal growth factor receptor 2 (HER2) positive exosomes has been reported in minimizing the severity of HER2-positive breast tumors. This study has suggested that the removal of EVs specifically exosomes derived from a tumor can provide an innovative approach to cancer therapeutics [10]. A comprehensive collection of pharmacological agents have demonstrated versatility and multifunctional ability which has enabled them to be considered “repurposing” agents in human disease studies, some of which have been termed “rare and neglected diseases”. Screening of pharmacological agents for repurposing without prior knowledge of function and mechanism has been demonstrated for activity in cell-based disease models [10,13].

However, there has been little information on how pharmacological agents could impact the formation of EVs, especially on intraluminal vesicle trafficking, membrane docking, and release. Given the pathological and immunological implication of exosomes in biological and chemical signaling in respiratory disorders, HIV, cancer, and tumor, they have been classified as essential mediators of cell-to-cell communication in many pathophysiological processes [14–17]. The potential of pharmacological agents in the inhibition of exosome biogenesis, secretion, and trafficking by infected, tumor cells and/or the tumor-associated stroma is presently under investigation. Useful findings from these studies could lead to identifying an efficacious therapeutic agent capable of suppressing the circulating level of tumor, cancer, and viral infection-derived exosomes. Also, these could help uncover other types of exosomes arising from pathologically challenged cells while simultaneously influencing effective prognostic outcomes. Studies have reported that ketoconazole and climbazole treatments inhibit exosome production in tumor and carcinoma cell lines. Therefore, in this study, we sought to evaluate and establish a time and concentration-dependent inhibitory effect of two imidazole derivatives and heparin on exosome synthesis and formation in human carcinoma lung cells (A549) for the first time. Ketoconazole and climbazole are imidazole derivatives and thus are anti-fungi agents. Ketoconazole has been shown to inhibit the biosynthesis of triglycerides and phospholipids, while climbazole regulates ergosterol synthesis which reduces membrane fluidity [10,12,18]. Climbazole inhibitory mechanism has been suggested to inhibit the ESCRT pathway via Alix and Rab27a regulation. Our third pharmacological agent is a heparan sulfate derivative, a naturally occurring glycosaminoglycan. It’s known to inhibit cellular proliferation and play a role in the alteration of receptor binding via modulation of some intracellular growth factors such as heparin-binding growth factors and fibroblast growth factors [19–22]. Although the inhibitors used in this study have been previously tested as inhibitors of exosome biogenesis, most studies on pharmacological agents of exosome inhibition made a deduction from a single time point [23] while some examined later time points, this study was designed to determine their time/concentration dependent exosome synthesis inhibitory capacities [10,18,23]. We believe this study is novel; at the end of this study, we can obtain effective exosome formation, inhibitory dosage, and time-points in human lung cells, this study will also add to the body of knowledge regarding the repurposing of drugs.

2. Materials and methods

2.1. Materials and drug sources

Dulbecco’s Modified Eagle Medium nutrient mixture (DMEM) (Fisher Scientific, Grand Island, NY, USA), penicillin/streptomycin solution (pen-strep) (Fisher Scientific, Grand Island, NY, USA), fetal bovine serum (FBS) (Fisher Scientific, Grand Island, NY, USA), and amphotericin B were purchased from Invitrogen (Camarillo, CA, USA). Heparin and ketoconazole were purchased from EMD Millipore Corp. USA (Burlington MA, USA). (Sigma-Aldrich, St. Louis, MO, USA). Climbazole was purchased from Tokyo Chemical Industry Co., LTD (Toshima, Kita-Ku, Tokyo, Japan). Unless otherwise indicated, all other drugs were purchased from commercial sources.

2.2. Cell culture

The human lung carcinoma cell line (A549) used in this study was obtained from (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in DMEM containing L-glutamine supplemented with 10% FBS, 1% pen-strep, and 0.2% amphotericin-B (0.5 μg/mL) (Fisher Scientific, Grand Island, NY, USA). For routine maintenance, each cell line was grown as a monolayer at 37 °C with 5% carbon dioxide and 95% relative humidity (RH). For the collection of conditioned media (CM), the cells were trypsinized, plated, and allowed to attach overnight in complete media, following which the media were changed to 2% exosomes-free media for overnight incubation and the supernatants were collected for exosomes isolation. For treatment with the drug compounds, the cells were trypsinized, plated, and allowed to attach overnight in complete media. Overnight culture media was discarded and cells were washed with (PBS) twice. Cells were treated with various concentrations of the compounds in 2% exosomes-free FBS (Gibco, Fisher Scientific, Grand Island, NY, USA) supplemented media and then harvested at the indicated times for subsequent analysis. Cell viability was measured by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) cell cytotoxicity assay according to the manufacturer's protocol as we described.

2.3. Dose-response and cytotoxicity assays

2.3.1. MTT kit assay

We performed dose-response experiments to evaluate the cytotoxicity of compounds on cells, using the compounds at different concentrations. The assays included viability screening, to determine whether any decrease in cell viability was primarily due to drug concentration. Cell viability was assessed relative to drug concentration using an MTT assay. A549 cells were seeded independently in 96-well tissue culture plates (10,000 cells/well) and maintained in culture for 24 h (h) before treatment. Consequently, the growth medium was discarded and replaced with a 2% exo-free medium containing the selected compound. Cells were stimulated with ketoconazole (1 and 5 μM), climbazole (5 and 10 μM), and heparin (0.176 and 0.88 μM), while PBS and DMSO (0.1%) were used as a control. Treated cells were incubated at different time points (6, 12, 24, or 48 h respectively). Cells were treated with 50 μL of 5 mg/mL tetrazolium salt, MTT/1× phosphate buffer saline (PBS), and incubated for 4 h at 37 °C in a 5% CO_2 humidified incubator. After incubation, 100 μL of stop solution (DMSO) was added to each well. The colorimetric assay was read at 570 nm and a reference wavelength of 630 nm.

2.3.2. Calcein acetoxymethyl ester (calcein AM) fluorescence staining

To further establish the effect of inhibitor dosages on cell viability, we performed a Calcein AM assay. Cells were seeded (5×10^5 cells/well) in confocal dishes and incubated for 6, 12, 24, and 48 h at 37 °C and 5% CO_2 humidified atmospheric conditions. Spent media was removed from cells and washed with PBS. This was followed by the addition of 500 μL Calcein AM prepared solution (8 mL PBS: 4 μL Calcein AM (reconstituted in 1 mmol/L, DMSO) to each well and incubated in the dark at 37 °C for 30 min. Calcein AM solution was removed and washed with PBS. We added 500 μL of PBS to the cells before observing and images were captured using the GFP channel on the fluorescence microscope (Life Technologies EVOS FLc Imaging System, Carlsbad, CA USA).

2.4. Exosomes inhibitory compound treatment

Cells were maintained as previously described [6,24]. Overnight culture media was discarded, and cells were washed with PBS. Cells were stimulated with ketoconazole, climbazole, and heparin at different concentrations as previously mentioned, and incubated for 6, 24, and 48 h at 37 °C in a 5% CO_2 humidified incubator. Media was collected after incubation and processed for exosome isolation.

2.5. Isolation and purification of exosomes

Exosomes were isolated and purified from culture media. Exosomes were isolated as previously described [6]. In brief, media was collected after inhibitor treatment and spun down at 400×*g* at 4 °C for 10 min, using a Sorvall RT 6000 refrigerated centrifuge. The supernatant was collected, and the pellet was discarded. The collected supernatant was spun again at 2000×*g* at 4 °C for 10 min using a Sorvall RT 6000 refrigerated centrifuge and then filtered through a 10 mL syringe with a 25 mM syringe filter, with a porosity of 0.22 μM. The filtered supernatant was further centrifuged at 14,000×*g* for 45 min in an SW41T1 swinging bucket rotor at 4 °C using a Beckman Coulter Optima L-70K Ultracentrifuge. The supernatant was collected and centrifuged for 120,000×*g* for 70 min in an SW41T1 swinging bucket rotor at 4 °C using a Beckman Coulter Optima L-70K Ultracentrifuge. Approximately 500 μL of purified exosomes were collected below the meniscus of the centrifuge tube. Collected exosomes were quantified using Bradford Lowry protein quantification assay and stored at −80 °C for further analysis.

2.6. Analysis of EV particles by zeta-view particle tracking

Assessment of isolated exosome particles was carried out as previously described [6]. To evaluate the size distribution and concentration of isolated exosomes (particle per mL), nano size particle tracking evaluation was performed using a zeta-view particle metrix tracking analyzer instrument, (Inning am Ammersee, Germany). Exosomes particle sizes were analyzed based on Brownian motion and light scattering. The samples were prepared at a dilution of 1:75 in PBS (1×) and loaded in a 1 mL disposable syringe. The

NTA assesses particles based on the size and concentration of samples. The mean values of the 11 positions were recorded and processed for each reading frame of the four independent experiments.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified exosomes were added to 1× reducing buffer (Pierce Lane marker, Thermo Fisher Scientific, MA, USA) in a 1:1 ratio and boiled for 10 min at 95 °C. Approximately 30 μg of total protein in samples were loaded in a 4–20% 1.5 mm Bio-Rad precast gel and allowed to migrate at 100 V. The gel was washed and stained in Coomassie blue dye following multiple wash steps and the image was developed using a Coomassie gel filter on Bio-Rad ChemiDoc XRS + System.

2.8. Immunoblot analyses

Exosome proteins or cell lysates were blotted on a nitrocellulose membrane (5 μg) and were blocked in 5% nonfat dry milk prepared in 0.2% Tween-20 and 1× Tris Buffer Saline (TBS) and were subjected to immunoblot analysis using antibodies against Flotillin-1 (1:250, Thermo Fisher Scientific, MA, USA), TSG101 (1:250, Invitrogen, Thermo Fisher Scientific, MA, USA), ALIX, (1: 250, Cell Signaling Technology, Danvers, MA, USA), CD63, (1: 250, Cell Signaling Technology, Danvers, MA, USA), GAPDH (1: 250, DSHB, Iowa City, IA, USA), and Rab27a (1: 250, Proteintech, Chicago, IL, USA). Immune complexes were detected with appropriate goat anti-mouse (1:1000, Cell Signaling Technology, Danvers, MA, USA) and goat anti-rabbit (1:1000, Santa Cruz Biotechnology, Texas) secondary antibodies, the signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate and imaged using Bio-Rad ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA) as we described [6,24]. The Immunoblot signal was captured and analyzed via densitometric analysis on ImageJ software (NIH, Bethesda, MD, USA).

2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey post hoc analysis. Statistical

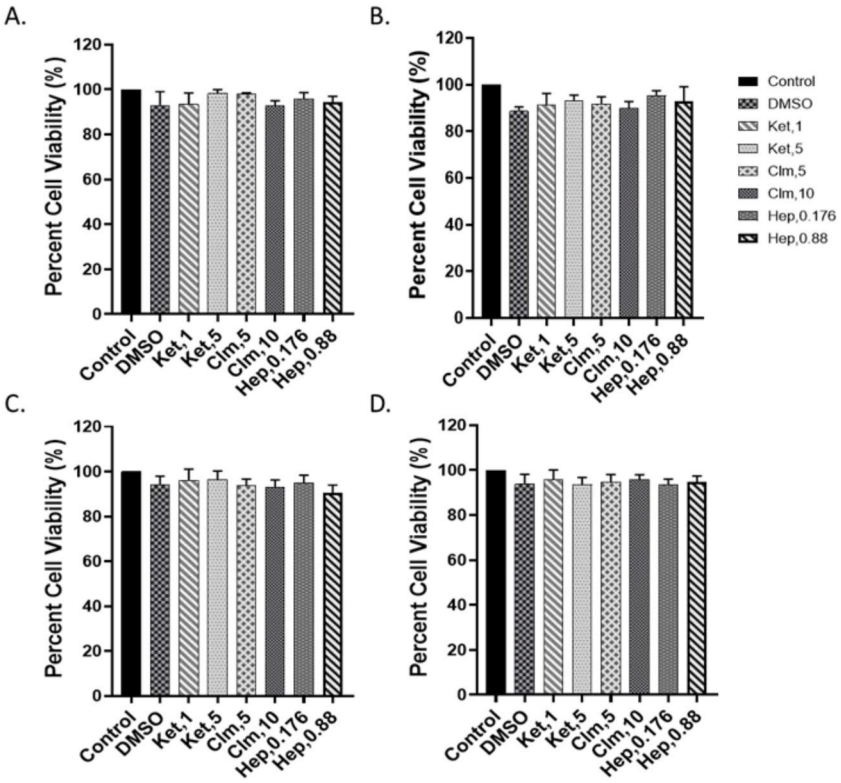

Fig. 1. Cytotoxicity evaluation of selected drug concentrations: Analysis of selected inhibitory concentration activities. Bar graphs showing the response of A549 cells (as percent (%) viability) to ketoconazole (1 and 5 μM), climbazole (5 and 10 μM), and heparin (0.176 and 0.88 μM) activities after (A) 6, (B) 12, (C) 24, and (D) 48 h of treatment. Cells were cultured overnight in DMEM and replaced with 2% exosome-free media and then treated with the inhibitors or PBS for 6, 12, 24, and 48 h. MTT dye was added to each well and incubated for 3–4 h. A detailed description of this procedure is described in the "Materials and Methods" section. The graph depicts % viability change compared to untreated controls and represents the mean ± SD of 4 separate experiments.

significance is indicated by the mean ± SD as follows: for multigroup comparisons, one-way ANOVA was used. Statistical significance was established to be *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3. Results

3.1. Drug cytotoxicity effect via dose response

We performed a dose-response assay using ketoconazole (1, 5 μM), climbazole (5, 10 μM), and heparin (0.176, 0.88), all treatments were analyzed at 6, 12, 24, and 48 h time points. The assay includes viability screening, to determine whether any decreases in cell viability was primarily due to drug concentration. Ketoconazole, climbazole, and heparin are currently approved pharmacological agents in the United States [10]. The purpose of this screening was to obtain an optimum concentration that will induce negligible or no effect on cell viability. The viability of ketoconazole-treated cells was not significantly affected at both concentrations relative to untreated cells at all treatment time points (Fig. 1). Climbazole and heparin treatments also induced no significant effects at 5, 10, and 0.176, 0.88 μM concentrations respectively, which enables the selection of compounds based on potency without cytotoxic effect (Fig. 1A–D). To further confirm no cytotoxic activity and to generate more accurate values, we evaluated esterase activity in cells treated with ketoconazole (1 and 5 μM), climbazole (5 and 10 μM), and heparin (0.176 and 0.88 μM) concentrations at different time points as previously mentioned. Fig. 2A shows the fluorescence images of viable cells after treatment with inhibitors at the chosen concentrations. Esterase activity shows that there were no significant differences in all inhibitor concentrations relative to controls (uninfected and DMSO treated) (Fig. 2B).

3.2. Azoles positively regulate total exosomes production in a time-dependent approach

We employed a standard procedure of exosome isolation based on our modified exosome isolation methods and quantified them via a standard protein quantification assay. Fig. 3 showed the effect of ketoconazole and climbazole treatment (belonging to imidazole) on

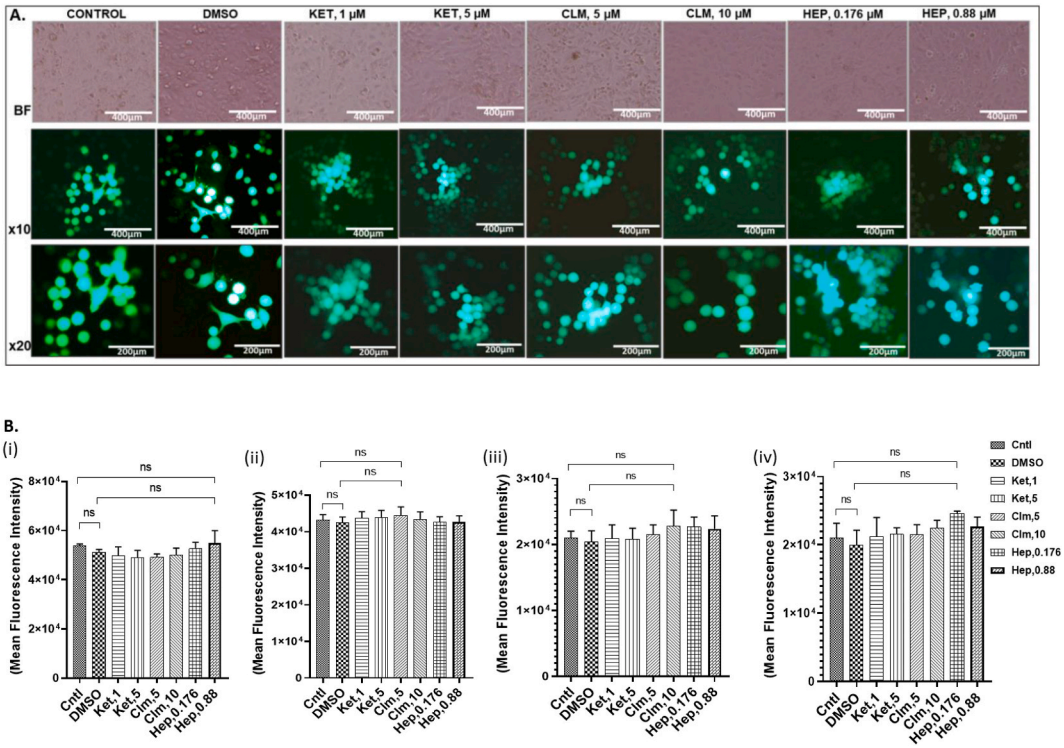

Fig. 2. Cytotoxicity evaluation of concentrations of pharmacological agents. (A) Bright field and fluorescence images of A549 cell viability after treatment with ketoconazole, climbazole and heparin. Calcein is retained within live cells due to esterase activity emitting green calcein fluorescence. The cell viability graph is depicted by mean fluorescence intensity. (B) Graphs showing the response of A549 cells to ketoconazole (1, 5 μM), climbazole (5, 10 μM), and heparin (0.176, 0.88 μM) activities after (i) 6 h, (ii) 12 h, (iii) 24 h, and (iv) 48 h of treatment. Cells were cultured overnight in DMEM and replaced with 2% exosome-free media and then treated with the inhibitors or PBS (as control) for 6, 12, 24, and 48 h, cells were stained by using Calcein AM dye, where live cells appear as green fluorescence. A detailed description of this procedure is described in the "Materials and Methods" section. The graph depicts % viability change compared to untreated controls and represents the mean ± SD of 4 separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

total exosome protein concentration. Untreated cells and DMSO (0.1%, vehicle-treated control) were used as controls. Ketoconazole and climbazole dosages had no significant effect on total exosome protein concentration after 6 h of treatment when compared to the controls (Fig. 3B). However, ketoconazole induced a significant decrease in the concentration of exosomes in A549 cells after 48 h of treatment relative to control and vehicle treatment at 5 μM concentration, climbazole was found to negatively regulate total exosomes proteins compared to the control treatments after 24 h and 48 h treatments ($p < 0.001$) (Fig. 3C and D). Climbazole treatments reduced total exosome proteins at all time points. An observable reduction in total exosome proteins in heparin and azole-treated cells relative

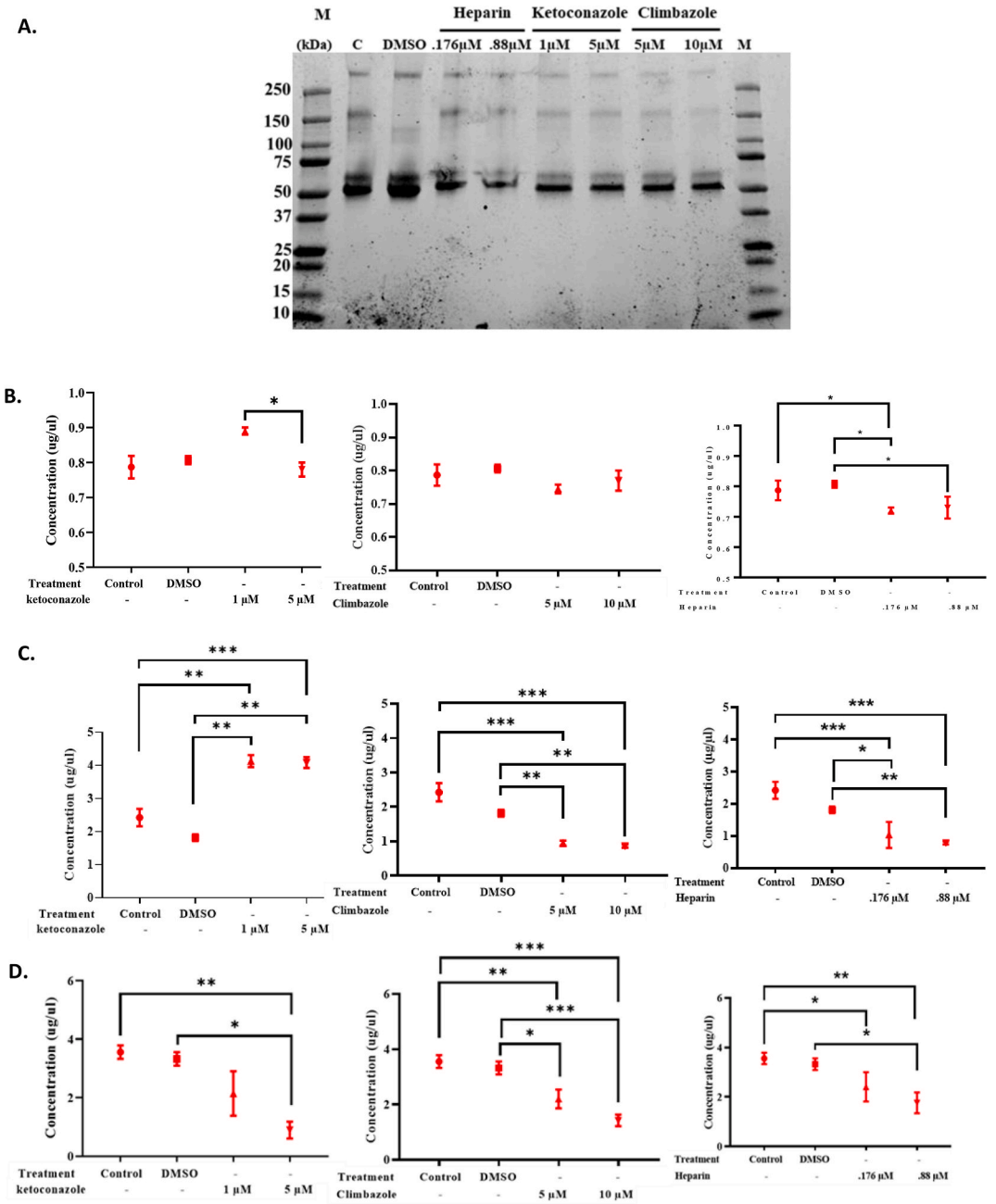

Fig. 3. Ketoconazole, heparin, and climbazole inhibit total exosome protein concentration in a dose-dependent approach after 24 and 48 h of treatment. (A) Exosome proteins were separated according to their molecular weight via SDS-PAGE (Also see Supplementary Fig. 3). Graphs showing exosome protein concentration after (B) ketoconazole, climbazole, and heparin treatments after 6 h, (C) 24 h, and (D) 48 h. Concentration of exosome protein was measured via Bradford Lowry Assay. Heparin significantly inhibited total exosome protein concentration at both concentrations and all time points relative to untreated cells. Statistical mean ± SD was derived from four independent experiments. Asterisks (*) denote significance levels at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to controls and were computed using GraphPad Prism.

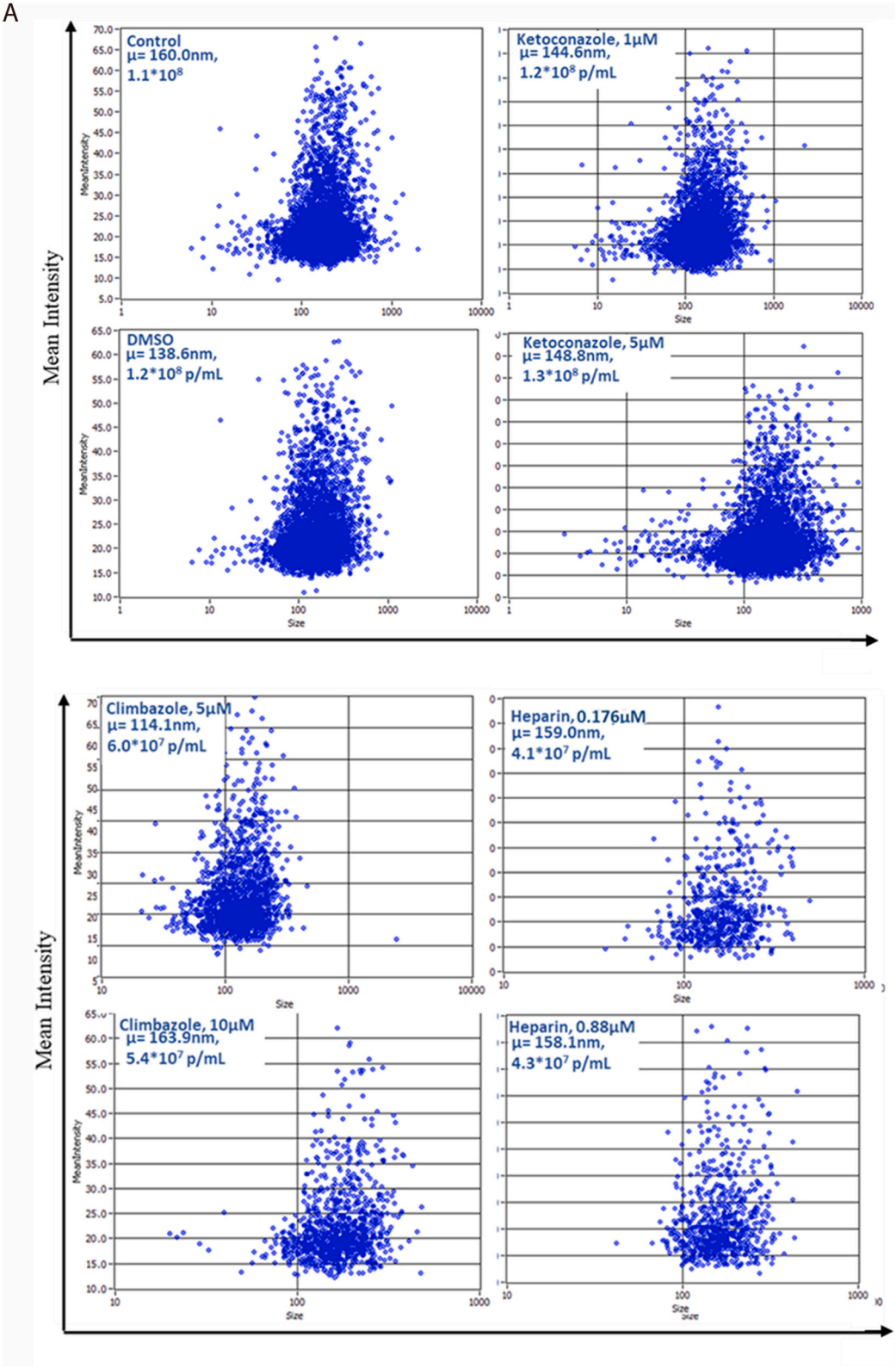

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Fig. 4a. Evaluation of exosome particles size distribution and concentration using Zeta particle tracking analyzer and the quantitative particle analysis: Scatter plot of exosome distribution in response to activities of inhibitors ketoconazole (1 μM and 5 μM), climbazole (5 μM and 10 μM) and heparin (0.176 μM and 0.88 μM) after (A) 6 h, (See 24 and 48 h scatterplot data in Supplementary Fig. 1). Untreated A549 cell-derived exosomes were used as positive control while DMSO (0.1%) was used as a drug vehicle and thus serve as a control.

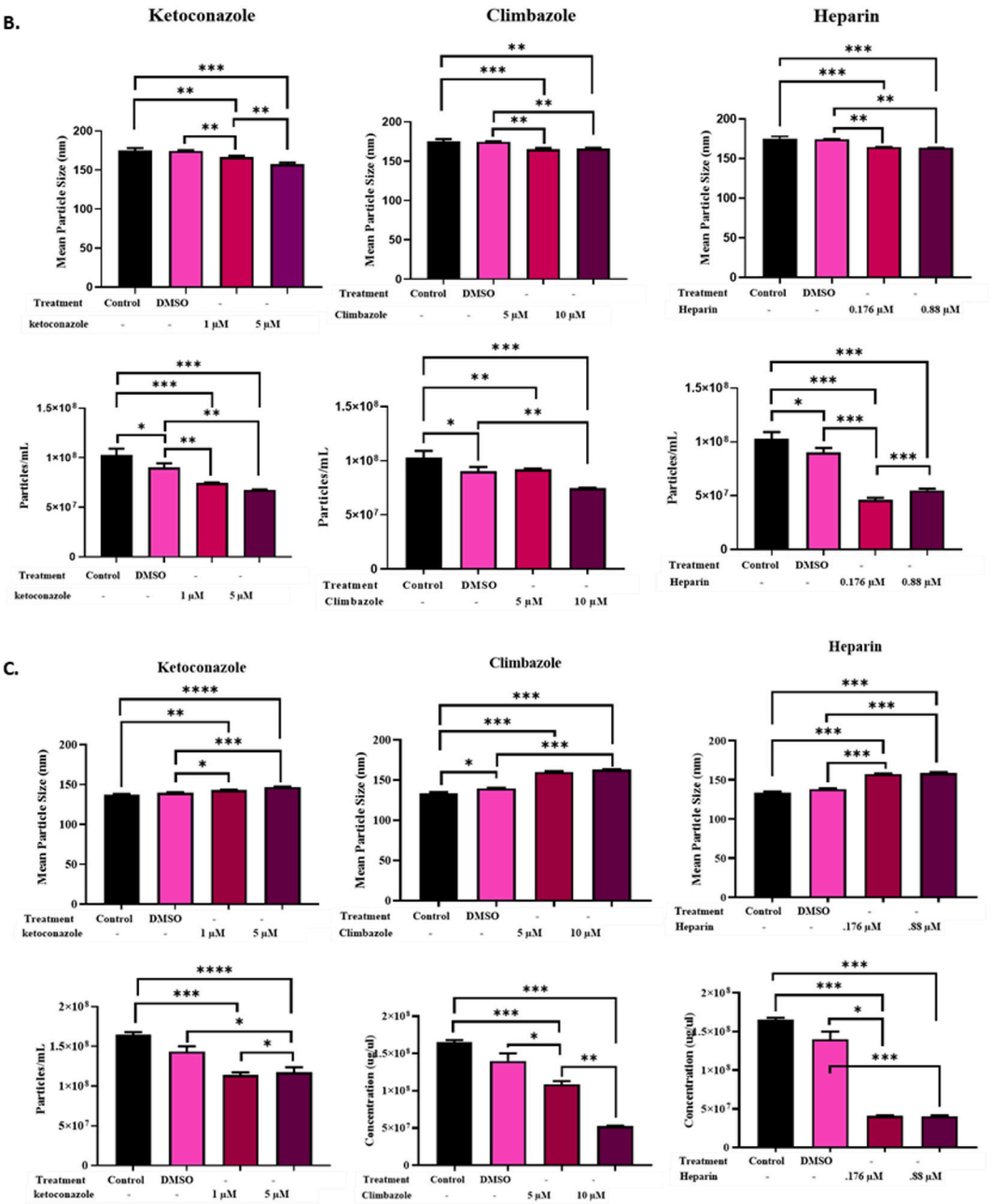

Figure 4bc. A graph showing exosome particle sizes and concentration/mL per dose inhibitory compound after (B) 6 h (C) 24 h. The exosome in the conditioned media was isolated, filtered through a 0.22 μM filter, and analyzed by the particle tracking analysis in 4 separate experiments. Each graph represents the mean ± SD of 11 frame positions obtained from 4 separate experiments. Data for exosome was captured using a particle metrix zeta-view analyzer (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ***$p < 0.0001$).

to the controls was further confirmed via SDS-PAGE gel imaging (Fig. 3A). These results provide a time-dependent assessment of ketoconazole and climbazole on exosome production.

3.3. Heparin potently suppressed total exosomes production by A549 cells

Heparin's inhibitory mechanism is slightly different from that of the imidazole mentioned in this study. Heparin blocks the transfer of exosomes from a donor cell to recipient cells by binding to their surfaces, it was detected to efficiently block the transfer of brain tumor exosomes into bystander cells via direct interaction and co-localization between exosomes and heparin [25]. After exosome isolation for heparin-treated A549 cells, total exosome protein quantification revealed that heparin demonstrated potent inhibitory activity on total exosome protein isolated, which was further substantiated in Fig. 3A. This result indicates that heparin could efficiently down-regulate the total exosome content derived from lung cells.

3.4. Azole and heparin treatment altered cell-derived exosomes particles concentration

We determined the exosome particle concentration and size distribution using a particle tracking analyzer, and the data were represented as the mean ± standard deviation of five repeated trials. Each sample trial count was obtained as the mean of eleven reading frames and positions as measured by the particle viewer. Samples were diluted at a dilution factor (*df*) of 0.01 and results were obtained as scatter plot distribution of particles, concentration per mL, and real-time particle imaging.

Exosomes particle sizes are usually in the range of 50 to approximately 200 nm, The mean size distribution of our isolated exosomes particles was in the range of approximately 50–200 nm (Supplementary Fig. 1). The mean sizes and particle concentrations of exosomes derived from both dosages of ketoconazole, climbazole and heparin were significantly altered when compared to untreated and DMSO treated cell-derived exosomes after 6, 24 and 48 h of treatment (Fig. 4B, C and Fig. 4D). The average particle concentration was significantly reduced after 48 h treatment in all concentrations of ketoconazole (1 μM; mean = 7.5×10^7 particles/mL, 5 μM; mean = 6.8×10^7 particles/mL) climbazole (5 μM; mean = 9.2×10^7 particles/mL, 10 μM; mean = 7.5×10^7 particles/mL) and heparin (0.176 μM; mean = 4.8×10^7 particles/mL, 0.88 μM; mean = 9.6×10^7 particles/mL) treatments when compared to untreated cells (mean = 1.3×10^8 particles/mL) ($p < 0.01$) (Figs. 4A–4C, also see Supplementary Fig. 1). We found that heparin-treated cell-derived exosomes were approximately 1.3-fold lower than the control. Particle tracking analysis of ketoconazole and climbazole-treated cell-derived exosomes has shown that they readily inhibit exosome production at different concentrations [10,23,26]. Our findings suggest that heparin appears to be a more potent inhibitor when compared independently with azole treatments.

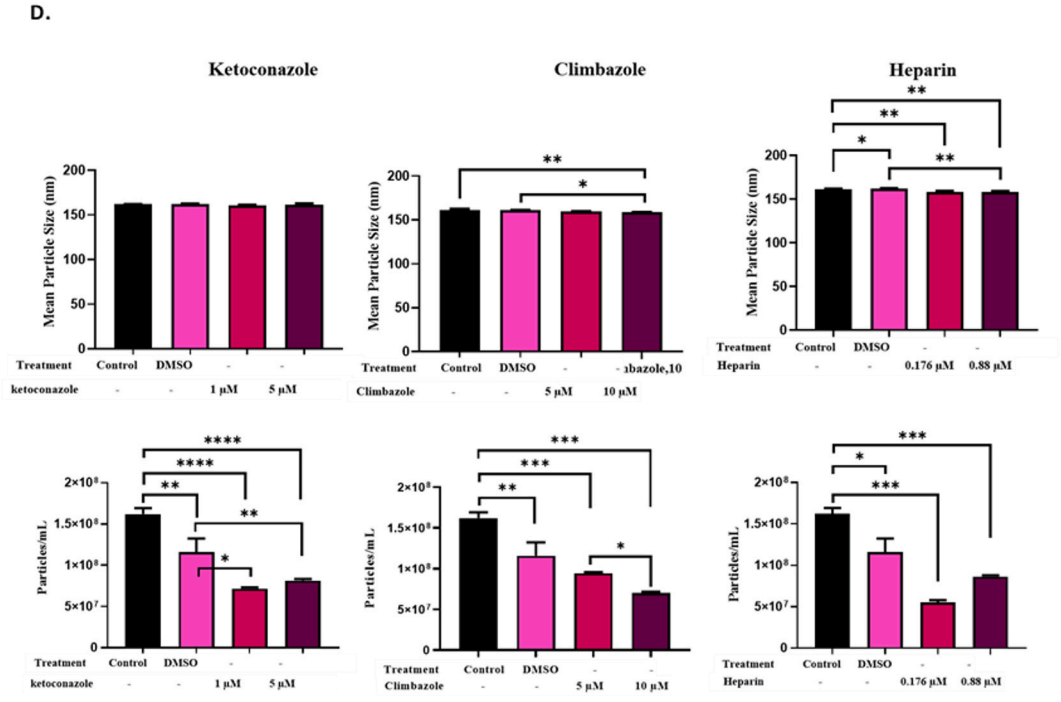

Fig. 4d. A graph showing exosome particle sizes and concentration/mL per dose inhibitory compound after (D) 48 h. The exosome in the conditioned media was isolated, filtered through a 0.22 μM filter, and analyzed by the particle tracking analysis in 4 separate experiments. Each graph represents the mean ± SD of 11 frame positions obtained from 4 separate experiments. Data for exosome was captured using a particle metrix zeta-view analyzer (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ***$p < 0.0001$).

3.5. Heparin and climbazole negatively regulate the expression of classical exosomes biomarkers

We determined the effect of inhibitors on the expression of exosome classical markers and modulators of exosome formation pathways, Slot-blot immunodetection analysis was used to confirm the expression of a few well-established markers of exosomes, including CD63, Alix, TSG101, Rab27a, and flotillin-1.

Studies have revealed that factors such as TSG101, CD63, and Alix loading into the intraluminal vesicle are associated with endosomal pathway complex activity, and they could facilitate exosome cargo sorting and loading [11,27]. Expression of exosome-associated tetraspanin CD63 expression level on exosome was significantly decreased in exosomes derived from cells treated with 5 μM ketoconazole, and 10 μM climbazole but was increased significantly in 0.88 μM heparin treatment ($p < 0.01$) when normalized to GAPDH after 6 h of treatment (Figs. 5A and 5B). On the other hand, there was no significant effect of all inhibitor concentrations on flotillin-1 and Rab27a levels after 6 h of treatment, however, we observed a significant decrease in TSG101 level in 0.176 and 0.88 μM heparin treated cell-derived exosomes after 6 h of treatment. After 24 h of incubation CD63 expression significantly increased in both climbazole and heparin treatments but was not affected by ketoconazole treatments (Fig. 5A). We observed no changes in the expression of Rab27a and ILV trafficking protein, Rab27a after the first 6 h of treatment in all concentrations of inhibitors used. However, expression of Rab27a in exosomes reduced significantly at higher concentrations of azoles and heparin treatments after 24 and 48 h treatments ($p < 0.001$) (Figs. 5B and 5C). We further evaluated how inhibition of exosomes affects membrane trafficking and cell adhesion protein flotillin, while flotillin level was not affected after 6 h of inhibitor treatment, it was negatively regulated at higher concentrations of climbazole and heparin after 24 and 48 h, which suggests that membrane trafficking activities could have been impacted (Figs. 5B and 5C). TSG101 expression in exosomes was further downregulated significantly at 5 μM ketoconazole, 10 μM of climbazole treatments after 24 and 48 h, and 0.88 μM heparin treatments after 48 h. We also found a differential reduction in the levels of CD63, Rab27a, and TSG101 in the cytoplasmic fraction of cells treated with ketoconazole and heparin, mostly after 24 h relative to the controls (Supplementary Fig. 2). This implies that the effect of climbazole and heparin on TSG101-associated exosome synthesis machinery strengthened with time (Fig. 5). A significant increase in the levels of CD63, Rab27a, and flotillin was observed in climbazole and heparin-derived exosomes compared to untreated cell-derived exosomes after 48 h of treatment (Fig. 5C). We observed alteration in the expression levels of Alix in climbazole and heparin-derived cell-derived exosomes after 6 h, however after 24 and 48 h treatment, Alix expression was found to be significantly downregulated in ketoconazole-derived exosomes ($p < 0.001$, $p < 0.005$) (Fig. 5D). These results suggest that ketoconazole, climbazole, and heparin inhibitory effects could be time-dependent rather than concentration-dependent.

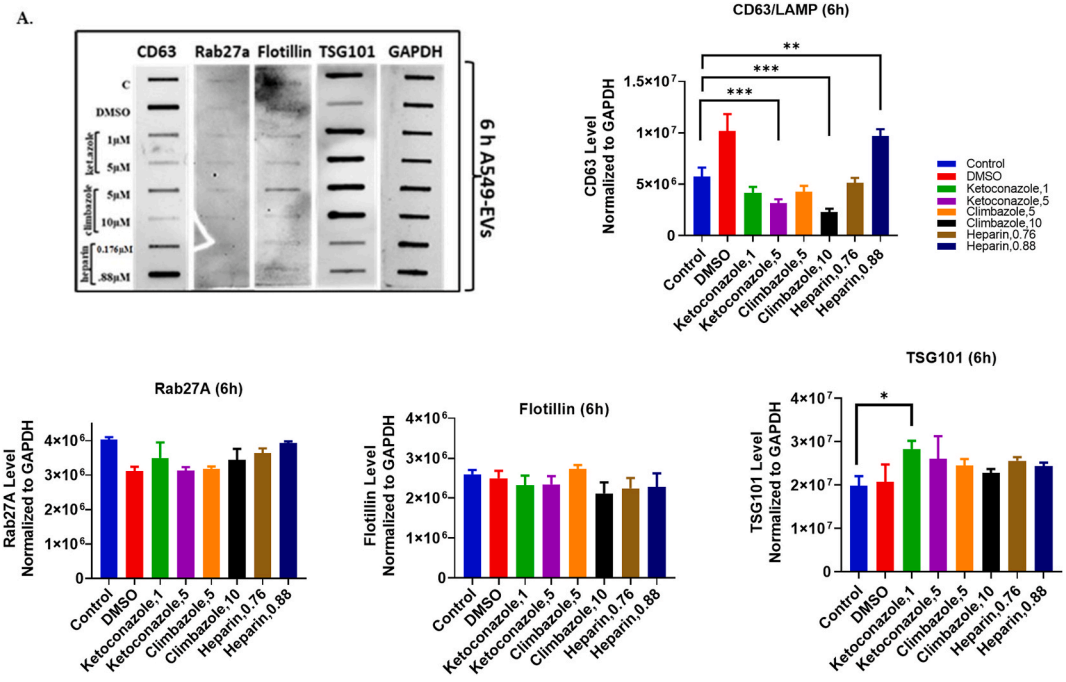

Fig. 5a. Climbazole and heparin inhibit exosome biogenesis and secretion via alteration of Rab protein and exosome classical markers. A549 cells were plated overnight in exosome-free DMEM media and then treated with ketoconazole, climbazole, heparin, or DMSO (vehicle control) for (A) 6 h. Expression levels of all proteins were normalized to GAPDH. Ketoconazole and climbazole at 5 μM and 20 μM concentrations significantly inhibited the expression levels of CD63 in A549 cell-derived exosome after 6 h treatment. Flotillin and TSG101 levels were significantly downregulated in exosomes derived from cells treated with climbazole, ketoconazole, and heparin at all time points of treatment. Rab27a protein level was downregulated by ketoconazole and heparin treatments (Also see Supplementary Fig. 3). (*) Asterisk denotes significance at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to controls and was computed using GraphPad Prism.

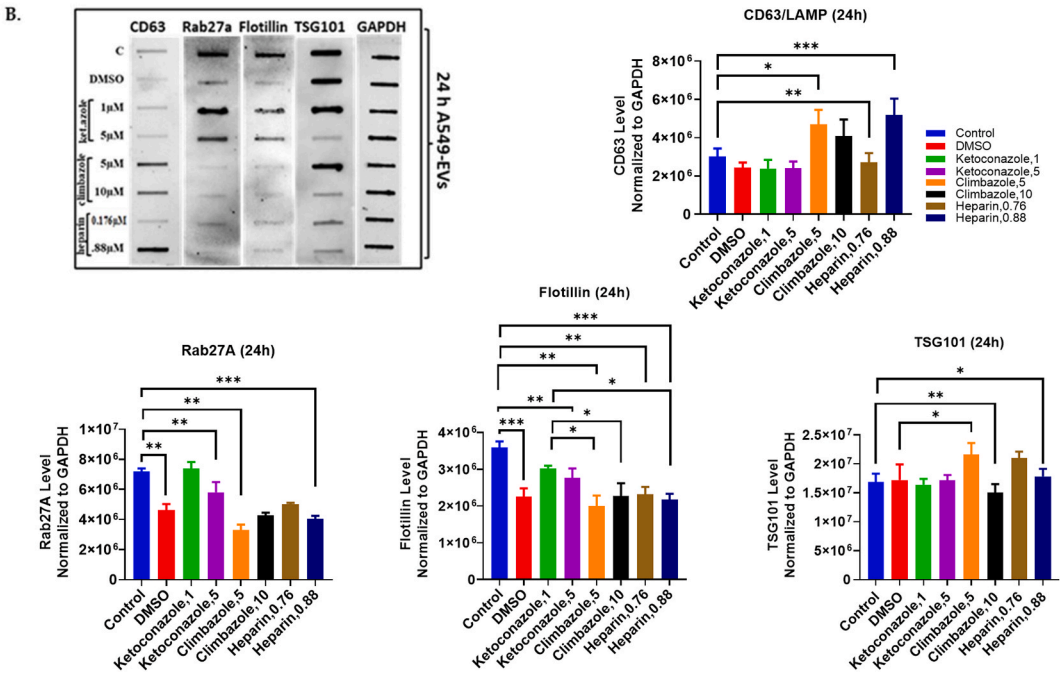

Fig. 5b. Climbazole and heparin inhibit exosome biogenesis and secretion via alteration of Rab protein and exosome classical markers. A549 cells were plated overnight in exosome-free DMEM media and then treated with ketoconazole, climbazole, heparin, or DMSO (vehicle control) for (B) 24 h. Expression levels of all proteins were normalized to GAPDH. Ketoconazole and climbazole at 5 μM and 20 μM concentrations significantly inhibited the expression levels of CD63 in A549 cell-derived exosome after 24 h treatment. Flotillin and TSG101 levels were significantly down-regulated in exosomes derived from cells treated with climbazole, ketoconazole, and heparin at all time points of treatment. Rab27a protein level was downregulated by ketoconazole and heparin treatments (Also see Supplementary Fig. 3). (*) Asterisk denotes significance at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to controls and was computed using GraphPad Prism.

4. Discussion

The rate of occurrence of viral disease outbreaks and some cancers in past years has increased rapidly with limited satisfactory improvement in therapeutic advancement. Inadequate screening and prognostic measures coupled with limited observable clinical symptoms remain a few of the main causes of the upsurge. The effectiveness of available measures such as surgical procedures, radio/chemotherapy, immunotherapy, or cryoablation is limited in the metastatic phase of some cancers [23]. Therefore, it is important to use a concerted approach to unravel the mechanism of spread and resistance. EVs are heterogeneously released by all cell types including healthy and cancer cells. They may be a vital part of cell-to-cell signaling under normal physiological conditions or infection states [28–30]. Out of the three major classes (exosomes, micro-vesicle, and apoptotic bodies) of EVs that are known to be released by cells, exosomes are released more often as they take part in major signaling pathways in intercellular communication in cancers, and viral infections [31,32]. Exosomes are known to shuttle cytosolic components via intercellular transport, they play a vital role in cancer and tumor stem cell reprogramming progression. Activities of exosomes include the promotion of tumorigenesis, angiogenesis, and drug resistance which has been recently at the forefront of EV research [7,32–36]. We have recently shown the effect of viral infection on exosome biogenesis and biomarker expression and cargo loading, our findings showed that the expression of various biomarkers and immune modulators in exosomes was vastly affected by viral particle concentration [6]. Exosome-mediated signaling between cells specifically aimed toward a specific therapeutic purpose will offer a significant therapeutic breakthrough in some cancers, neurodegenerative diseases, and viral diseases. Currently, the estimated cost of new drug discovery or bringing in new medication through the federal drug administration (FDA) is more than 1 billion U.S dollars [10,37], moreover, approval odds are high, because new drug approval by a regulatory body such as FDA takes up to 20 years before they are allowed for public consumption [23]. Therefore, repositioning already established and approved compounds that have been proven to be safe for human use is an ideal means of innovative therapeutics.

Expanding the spectrum of drug purposes is a strategy that allows time and cost management in new therapeutic drug discovery. The application of some imidazole derivatives and their role in exosome biogenesis has opened a new paradigm for cancer therapy [10, 13,23]. Combining a repurposed drug such as ketoconazole with other established compounds has been shown to exert a degree of anticancer properties and has been shown in the management of prostate and hepatocellular cancer [38,39]. A remarkable case report suggests that heparin possesses certain anti-cancer properties in humans, this has also been shown in animal model studies, where heparin treatment decreased metastasis, and has been suggested to inhibit the interaction between cells and platelets in tumor progression, the mechanism of action involves cell-exosome uptake inhibition by interference [20,25].

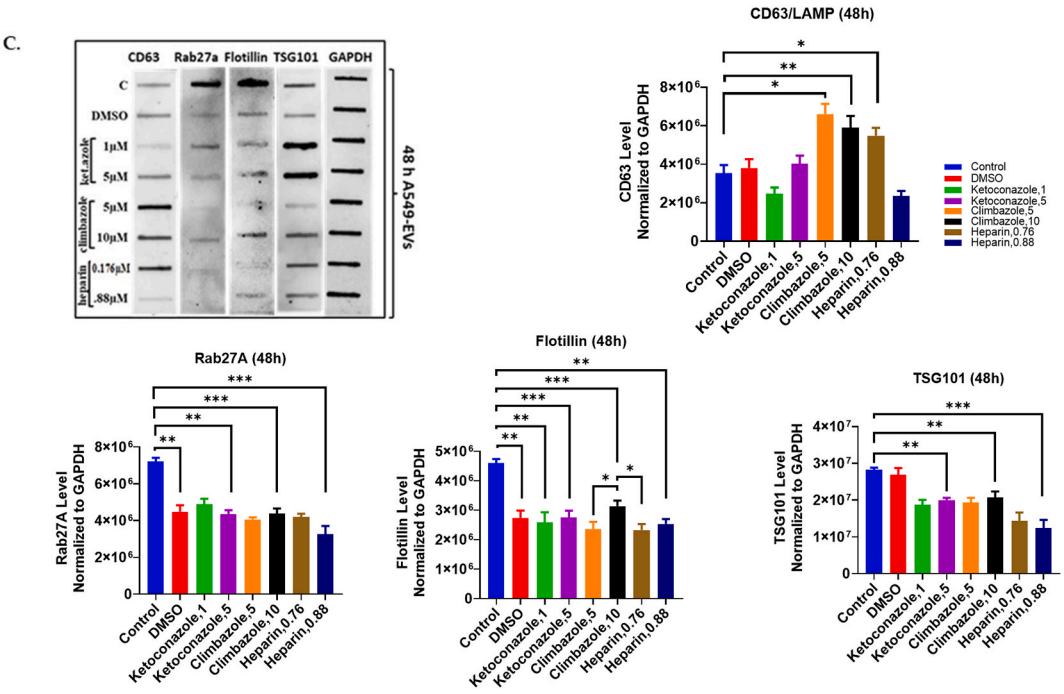

Fig. 5c. Climbazole and heparin inhibit exosome biogenesis and secretion via alteration of Rab protein and exosome classical markers. A549 cells were plated overnight in exosome-free DMEM media and then treated with ketoconazole, climbazole, heparin, or DMSO (vehicle control) for (C) 48 h. Expression levels of all proteins were normalized to GAPDH. Ketoconazole and climbazole at 5 μM and 20 μM concentrations significantly inhibited the expression levels of CD63 in A549 cell-derived exosome after 48 h treatment. Flotillin and TSG101 levels were significantly down-regulated in exosomes derived from cells treated with climbazole, ketoconazole, and heparin at all time points of treatment. Rab27a protein level was downregulated by ketoconazole and heparin treatments (Also see Supplementary Fig. 3). (*) Asterisk denotes significance at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to controls and was computed using GraphPad Prism.

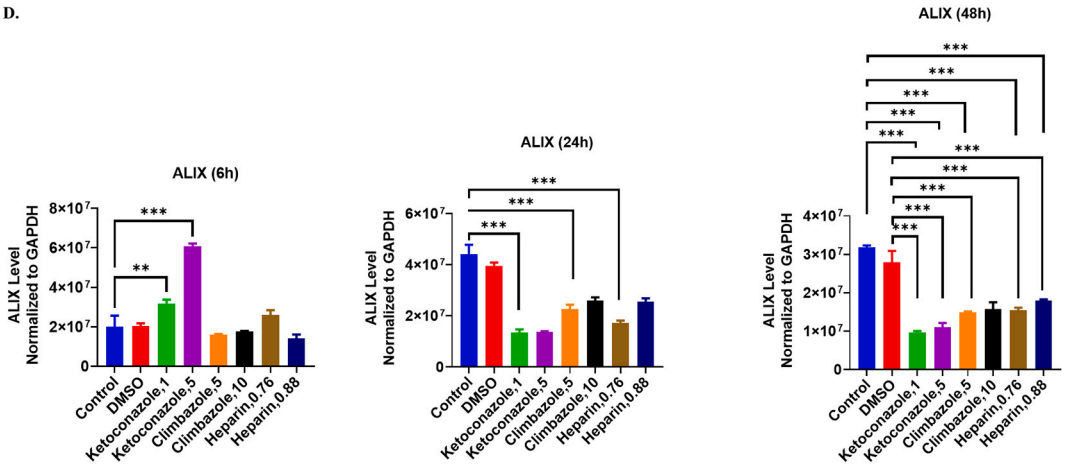

Fig. 5d. (D) Alix level was upregulated in ketoconazole-treated cell-derived exosome compared to untreated after 6 h but was significantly downregulated in ketoconazole, climbazole, and heparin treatment when compared to untreated after 24 and 48 h. Mean values ± SD were derived from four independent experiments. (*) Asterisk denotes significance at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to controls and was computed using GraphPad Prism.

Our aim in this study is to evaluate the inhibitory effect of certain exosome inhibitors on exosome formation and uptake or release, taking into consideration our previous studies on exosome biogenesis. The goal of this study is to develop an effective pharmacological inhibitor for exosome regulation, thereby enhancing therapeutic development in cancer and viral diseases. We used two antifungal agents and an anticoagulant that has previously been reported as potential inhibitors of exosome synthesis. A549 cell response to drug dosage assay revealed that the effect of drug compound on cell viability was non-toxic at low concentrations. This was necessary

because we aimed to establish the optimum dose that would induce a negligible or no toxic effect on cell viability. Studies have shown that potent inhibition of exosome biogenesis was more efficient at physiological dosing that is non-toxic to cells [25]. We visualized total exosome protein isolated from all treatments using SDS-PAGE technique and Coomassie staining imaging was done using the Chemidoc MP imaging system. We further quantified total exosome protein isolated from all treatments. Our chosen concentrations for climbazole and heparin significantly reduced total isolated protein relative to untreated cell and vehicle controls. Ketoconazole on the other hand had no negative effect on total exosome protein until after 48 h of treatment. Although ketoconazole has been shown to inhibit certain exosome formation pathways, a report has also shown that a combination of ketoconazole and compounds such as sunitinib, a tyrosine kinase inhibitor used in cancer treatment was more effective in the inhibition of tumor-specific exosomes than ketoconazole alone [23]. Our chosen azole derivatives have demonstrated suppressive capabilities in the biosynthesis of ergosterol and cellular proliferation. This result suggests that the inhibitory activity of ketoconazole might require more time to take effect. In addition, this is the first study where A549 cells have been evaluated in an exosome inhibition study, this outcome might be cell-specific. All drug compounds employed in this study have been previously tested in other cell lines and their potencies in exosome formation and release regulation have been reported. The outcome of our particle metrix analysis of isolated exosomes revealed that there were variations in exosome particle sizes in all drug treatments relative to the control which may be a result of changes in cargo packaging, formation, and particle aggregation. Heparin treatment of glioma-derived exosomes led to the aggregation of exosomes in a study carried out in 2013 [25]. We demonstrated that ketoconazole, climbazole, and heparin significantly reduced exosome particle concentrations after 6, 24, and 48 h of treatment even at low concentrations. Interestingly, compared to ketoconazole and climbazole, heparin treatment reduced total exosome particles per mL relative to untreated cell-derived exosomes after each treatment period (Refer to Fig. 4 and Supplementary Fig. 1). Another remarkable finding was that, although there was a notable reduction in exosome particle numbers in cells treated with all three inhibitors, nevertheless, there was no significant difference in cell viability of all cells treated with our chosen inhibitory compound concentration. This could rule out the possibility that reduced-cell viability might directly correlate to reduced-exosome particle numbers. Along these same lines, a study in 2016 showed that exosome levels in squamous carcinoma OSC-4 cells were suppressed by 10 μg/mL heparin treatment [22].

Furthermore, we investigated the effect of our chosen pharmacological agents on classical exosome markers (CD63, Alix, TSG101, Flottilin-1, and Rab27a) which are indicators of the endosomal sorting complex required for transport pathways. We quantified protein expression levels via Bio-Rad image-lab densitometry and normalized expression levels to GAPDH, we observed that ketoconazole and climbazole inhibited CD63 in exosomes after all selected hours of treatment. TSG101, flotillin-1, and Rab27a levels were significantly reduced in climbazole and heparin-derived exosomes, suppression of these proteins could lead to inhibition of multiple pathways in exosome biogenesis and secretion. For instance, TSG101 also known as Vps23 is known to be associated with transmembrane protein ubiquitination by the ESCRT-I complex, this study suggests that ketoconazole and climbazole might have altered the exosome synthesis pathway which could be observed in the reduction of TSG101 signaling in climbazole and heparin treated cell-derived exosomes. The reduction of flotillin-1 in exosomes after 24 and 48 h treatment could also suggest that both azoles and heparin treatments negatively regulate the exosome formation machinery. These findings imply that climbazole and heparin may play dual functions in exosome formation and secretion. We also found significant changes in Rab27a and flotillin levels in DMSO-treated cell-derived exosomes compared to untreated, this might be because of regulated exocytosis or other related mechanisms. Studies have shown that DMSO treatment impacts cell membrane which could in turn alter exocytosis [39]. One of our important findings was the reduction of Alix expression in ketoconazole and climbazole-derived exosomes after 24 and 48 h treatment. Alix protein is also known to be involved in the ESCRT pathway where they sort membrane proteins into vesicles that bud into the lumen creating multivesicular bodies, which supports the effect of azole on ESCRT machinery. Previous studies on the use of drug agents as exosome inhibitors employed mainly prostate, renal, and kidney cell lines [10,23]. Since the effects of pharmacological inhibitors of exosome synthesis could vary based on cell type. It is also important to know how lung cell-derived exosomes are impacted by inhibitory agents, this would add to the needful information necessary for the development of exosome-related respiratory illness/disease therapies. Thus, the novelty of this study includes the use of our selected drug concentrations in human carcinoma lung cells (A549). The uniqueness of this study also includes the time and concentration-dependent effects of our chosen drugs on viability, exosome formation, and synthesis in human carcinoma lung cells whose outcome will provide us with the optimum and most appropriate concentrations that would be non-toxic to cells in the advent of other studies. Ketoconazole, climbazole, and heparin have been proven to be potent inhibitors of exosome synthesis in a few cell lines and may be applicable in translational studies in drug repurposing and cancer therapy. Our findings address some unanswered time-related questions, which could be applicable in evaluating detrimental exosomes in respiratory disease therapy development.

5. Conclusion and perspectives

At end of this study, we demonstrated that time-dependent pharmacological inhibition of carcinoma lung cell-derived exosomes led to a reduction of total exosome particle concentrations mostly in climbazole and heparin-derived exosomes. We observed significant alteration in the levels of classical marker tetraspanin CD63 in all inhibitor treatments and concentrations. The mean particle sizes of isolated exosomes also vary significantly between treatment groups and controls. Evaluation of major proteins in the exosome formation pathway (Alix, TSG101, and flotillin) showed that the inhibitory effect of ketoconazole, climbazole, and heparin led to negative regulation of the exosome synthesis accessory proteins. The expression of Rab27a, a key player in the membrane transport and trafficking of intraluminal vesicles to the plasma membrane was reduced significantly after 24 and 48 h treatments. Since exosomes are known to be major carriers and transporter of cytosolic proteins, miRNA, and mRNA along with some chemoresistance mechanisms, these results are preliminary and are indicators that pharmacological inhibition of exosomes can be a good tool for regulating exosome

formation and transport in the development of novel secondary target for adjunct therapy.

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Author contribution statement

Ayodeji O. Ipinmoroti: Conceived, designed and executed the experiments; Analyzed and interpreted the data; Wrote the paper. Rachana Pandit, Brennetta J. Crenshaw: Performed the experiments; Analyzed and interpreted the data. Brian Sims: Revised and edited manuscript; Interpreted the data; Qiana L. Matthews: Conceived and designed the experiments; Analyzed and interpreted the data; Edited the manuscript; Contributed reagents, materials, analysis tools or data. All authors contributed to the article and approved the submitted version.

Data availability and statement

The original data will be maintained by the corresponding author. Information pertaining to the datasets will be made available upon written request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16655.

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