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Atezolizumab Retains Cellular Binding to Programmed Death Ligand 1 Following Aerosolization *via* Mesh Nebulizer

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Abstract. *Background/Aim: Cytotoxic inhalable drugs were shown to be advantageous in treating malignancies of the respiratory tract. However, these drugs have not always presented a safe profile and were reported to induce local adverse events. Protein-based anticancer drugs, such as immune checkpoint and vascular endothelial growth factor inhibitors, do not induce tissue injury, nor do they exhibit vesicant properties upon direct contact with tissues. Protein drugs are susceptible to the heat and stress encountered during droplet generation for delivery by nebulization. The aim of this study was to investigate the capacity of atezolizumab, an antibody to programmed death ligand 1, to bind target cells after nebulization with a vibrating mesh (VM) nebulizer. Materials and Methods: We compared Fourier-transformed infrared (FTIR) and Raman spectra of native atezolizumab (60 mg/ml) and its nebulized form following 10-min nebulization in a piezoceramic VM nebulizer. The binding of atezolizumab to DU-145 prostate cancer cells was evaluated using competitive blocking of anti-CD274 staining. Results: Nebulization did not induce Raman or FTIR spectral modification nor did it affect the binding capacity of atezolizumab. Conversely, heat-inactivated atezolizumab lost its cell-binding capacity and did not reduce anti-CD274 immunostaining. Native and nebulized atezolizumab displayed identical spectra, whereas the FTIR spectra of the heat-inactivated drug was significantly altered. Conclusion: VM nebulization does not obliterate the functionality of the drug atezolizumab. The integrity of a nebulized form can be rapidly assessed by FTIR and Raman spectrometry.*

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Inhalation, or pulmonary drug delivery, is an advantageous route of administration to treat pulmonary diseases and some systemic disorders. It has become the main route of administration of treatment for asthma, chronic obstructive pulmonary disease, pulmonary hypertension, and cystic fibrosis (1). The inhaled drug allows a lower dose to have a rapid onset and to have the same effect as a higher dose delivered by systemic routes (2).

The rate and effectiveness of inhaled drug accumulated in the lungs, blood, and brain are well documented in tracking of radiolabeled nicotine by positron-emission tomography following a few puffs of conventional or e-cigarette smoke (3, 4). Under typical smoking conditions, brain ^{11}C-nicotine accumulation was found to begin approximately 7-15 s after the radioactivity was detected in the oral cavity (3). An inhaled dose of nicotine might require 3 to 5 min to reach maximal values. However, it is obvious that the inhalation route sometimes works as fast and as effectively as intra-arterial injection and can even bypass the blood–brain barrier (5). The advantages of inhalation therapy were effectively explored in the commercialization of inhalable insulin (Afrezza® and Exubera®). Inhalable insulin is as active as the injectable formulation and exerts hypoglycemic effects faster. Research to improve formulations of inhalable insulin is still ongoing (6). Another interesting application of intranasal insulin is the capability to elicit certain effects in cases of Alzheimer's disease, mild cognitive impairment, or dementia (7, 8).

The possibility to deliver inhalable protein or peptide drugs to the lungs and to the brain (including brain tumors) was recently reviewed (5). Nose-to-brain delivery is a non-invasive method that enables drug delivery to the central nervous system (CNS), bypassing the blood–brain barrier through the only point that directly connects the CNS with the external environment (5). By this route, drugs can rapidly access the CNS following a 'shortcut' from the nose to the brain, directly utilizing trigeminal or olfactory nerves located in the upper part of the nasal cavity.

Nebulized inhalable anticancer drugs have been used in several clinical trials (9-11). The cytotoxic drugs tested in these clinical trials have been 5-fluorouracil, 9-nitrocamptothecin, cisplatin, doxorubicin, gemcitabine, and carboplatin (2). These drugs have not always presented a safe profile where the most severe toxicities have been related to the pulmonary tract. This first-pass toxicity is quite expected since extravasation of these drugs is known to cause severe tissue damage, sometimes resulting in long-term injury around the site of extravasation (12). Extravasation injury and inhaled drug-induced tissue toxicity to the nasopharyngeal and bronchial area might be comparable. Extravasation injuries can be extremely complicated with local recycling of absorbed drug, multiple surgical interventions, and even amputations in pediatric patients (13). At least in theory, upon direct contact with tissues, inhaled protein should not result in vesicant injury as compared to cytotoxic compounds. Cytotoxic inhalable drugs were however shown to induce pulmonary toxicities *e.g.* grade 4 respiratory distress/dyspnea and chemical pharyngeal mucositis.

Therefore protein-based anticancer drugs such as immune checkpoint inhibitors (ICI) (14) and vascular endothelial growth factor inhibitors (11) are expected to be the most appealing candidates for use in inhalation therapy. Interestingly, extravasation of ICI anticancer drugs does not result in local tissue damage. For instance, pembrolizumab extravasation caused no local irritation or tissue damage, and it was handled with observation only, resulting in complete resolution (15). Furthermore, new compositions of subcutaneous injectable drugs of anticancer proteins are being extensively investigated (16-18). Thus, recent results from a phase III study assessing atezolizumab, an antibody to programmed death ligand 1 (PD-L1) as a subcutaneous formulation were announced (16). The results show the formulation met its primary endpoints and demonstrated non-inferior levels of atezolizumab in the blood when it was injected subcutaneously. This clinical trial indirectly demonstrates that inhalable atezolizumab should not cause pharyngeal or pulmonary vesicant toxicities which accompany inhalable formulations of cytotoxic drugs. A known problem in the delivery of protein drugs by nebulization is their susceptibility to the heat and stress encountered during the droplet-generation procedure (19). Protein drugs can undergo degradation, inactivation, adsorption, unfolding, and aggregation due to the large air–liquid interface in the micron-sized droplets (20).

In this study, we examined if vibrating mesh (VM) nebulized forms of three anticancer protein drugs, namely atezolizumab, pembrolizumab, and bevacizumab, differ from their native forms as defined by Fourier-transformed infrared spectroscopy (FTIR) and Raman spectra.

Materials and Methods

Atezolizumab (Tecentriq®; Roche, Basel, Switzerland) is a humanized, non-glycated IgG1 monoclonal antibody, with a molecular weight of 145 kDa at a concentration of 60 g/l. Excipients are histidine, glacial acetic acid, sucrose, and polysorbate 20. Pembrolizumab (Keytruda®; Merck Sharp & Dohme, Rahway, NJ, USA,) is a humanized monoclonal antibody to programmed cell death protein 1 (PD1) (IgG4/kappa isotype with a stabilizing sequence alteration in the Fc region) with a molecular weight of 149 kDa at 25 mg/ml. Excipients include L-histidine, L-histidine hydrochloride monohydrate, Sucrose, and polysorbate 80. Bevacizumab (Avastin®; Roche) is a humanized recombinant monoclonal antibody with a molecular weight of 149 kDa at a concentration of 25 g/l. Excipients are trehalose dehydrate, sodium phosphate, and polysorbate 20.

Nebulization and thermal inactivation. Nebulization was performed with the help of Air Pro (Evolu, Forans International AG, Baar, Switzerland) VM nebulizer. This procedure is driven by oscillations (110+10 kHz) of piezoelectric ceramic which forces the liquid through 3 μm holes in metal mesh. The mass median aerodynamic diameter of produced aerosol at these conditions was 2.5 μm +30%. The fine particle fraction was 83.13% under these conditions. After opening the vial, each drug was placed into the reservoir of the device, and nebulization for 10 min at 20˚C was performed. One milliliter of nebulized mist was concentrated into liquid condensate and collected into 5 ml tubes. Temperature inactivation of each drug was performed by placing drug vials into a water bath at 85oC for 60 min. Nebulized and inactivated drugs were kept for 2 h at room temperature before testing for vibrational spectrometry and target cell binding.

FTIR and Raman spectroscopy. Infrared absorption spectra were collected using Bruker Alpha FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a room-temperature deuterated triglycine sulfate detector. Drug samples (10 μl) were placed between CaF_2 windows and measured in transmission mode with a resolution of 4 cm^{-1} and 100 interferogram scans. The final spectra were obtained by subtracting the FTIR transmission spectrum of ultrapure water from the drug spectra. Spectral acquisition and manipulation were carried out using OPUS software version 7.5 (Bruker). Raman spectra of drugs were obtained using a HyperFlux PRO Plus spectrometer (Tornado Spectral Systems, Mississauga, ON, Canada) at 785 nm excitation equipped with fiber-optic cable to excite and collet Raman signals. Laser power was restricted to 100 mW and the Raman-probing took place in a 1-ml quartz cell. The overall accumulation time was between 150 and 1290 s for different samples. Raman spectra of water and the quartz cell were subtracted from the drug spectra. A polystyrene standard (ASTM E 1840) was used to confirm the calibration of the wavenumber axis.

Cell culture and flow cytometry. Human prostate cancer cell line DU-145 was obtained from American Type Culture Collection (Froceth, Vilnius, Lithuania). Cells were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin, and streptomycin. DU-145 cell lines were grown in a monolayer under standard culture conditions with 5% CO_2 in an incubator at 37˚C. For atezolizumab-binding experiments, cells were plated at a density of ~5,000/cm^2 into 100 cm^2 tissue culture dishes and allowed to adhere for 24 h. After 24 h, cell cultures were trypsinized, then suspensions were adjusted to 106 cell/ml and treated with atezolizumab (Tecentriq®; Roche), prepared in three different forms: a) Native, taken directly from the vial; b) nebulized; and c) heat-inactivated. Each of the three forms was tested in DU-145 cell-binding assays for 30-min incubation at 37˚C at the following concentrations: 0.001, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 μg/ml. Phosphate-buffered solution was used as a control. Following exposure cells, were washed twice in ice-cold phosphate-buffered solution. Cells were stained with fluorescein isothiocyanate-labeled mouse anti-human PD-L1 (CD274, clone MIH 1, 12-5983-41; Biosciences, San Diego, CA, USA). Antibodies were diluted 1:200 and staining were performed in fluorescence-activated sorting buffer for 15 min at room temperature. The same procedure was performed using isotype control antibodies (fluorescein isothiocyanate-labeled mouse IgG1 kappa; Biosciences). Cells were analyzed using a BD FACS LRDIII (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo 10.8.1 software (Tree Star, Ashland, OR, USA).

Statistical analysis. The statistical tests were performed using STATISTICA 12.0 (TIBCO Software Inc., Palo Alto, CA, USA). All the results are presented as means and standard error. Significance was considered at values of $p<0.05$.

Results

The nebulized drugs were easily condensed at room temperature into the collecting tube without any visible color, liquid turbidity, or viscosity change. Conversely, the heat-inactivation procedure resulted in visible coagulation and gel-like conversion of drugs. This form of drug was not further tested in target cell-binding assays since water solubility was not sufficient to compare the results with the nebulized form. Heat inactivation is also considered one of the causes of VM nebulizer-induced protein damage, and we detected significantly different FTIR spectra for heat-inactivated and nebulized drug forms (Figure 1). FTIR spectral analysis revealed that nebulization did not modify protein molecules compared to the native form. Conversely, heat inactivation significantly shifted the spectra compared to the intact and nebulized forms. To further investigate whether there were in fact any VM-induced protein molecule transformations, we performed Raman spectral analysis of all three drugs before and after nebulization (Figure 2). The effect of nebulization is shown as the line resulting from the subtraction of the spectra of the nebulized form of the drugs from the native forms. These isolines indicate the absence of molecular transformation or protein damage following the nebulization procedure. The only small effect was seen for atezolizumab (Figure 2C) at approximately 1200-1600 wavenumber/cm^{-1}.

To test whether this change might indicate a loss of functional integrity, we explored competitive binding to cells using a PD-L1 assay. The prostate cancer cell line DU-145 exhibits characteristic spontaneous PD-L1 expression, which was detectable by fluorescence shift of stained cells as compared to the isotype control. The anti-CD274 staining intensity of DU-145 cells was modified by their preincubation with the native anti-PD-L1 drug atezolizumab (Figure 3). The concentration-depended inhibition of CD274 fluorescence was seen after preincubation of DU-145 cells with atezolizumab (Figure 3A). Testing native and nebulized atezolizumab revealed that nebulization did not result in any modification of PD-L1 binding (Figure 3B and C). A representative mean fluorescence intensity for the isotype control was 1,037 a.u., and for anti-PD-L1 staining was 3,135 a.u. Preincubation with 2 μg/ml of native atezolizumab in this sample reduced mean fluorescence intensity of anti-PD-l1 staining to 814 a.u., whereas 2 μg/ml of nebulized atezolizumab resulted in almost identical inhibition of PD-L1 staining, with a value of 822 a.u. Similarly, a low concentration (0.05 μg/ml) of native and of nebulized atezolizumab correspondingly resulted in intensities of 2,169 a.u. and 2,098 a.u. Tests were repeated at least three times with each concentration of nebulized or native atezolizumab, and differences in inhibition of PD-L1 staining were not significant. The VM nebulization of atezolizumab did not affect the target tumor cell-binding capacity at the range of concentrations tested.

FTIR and Raman spectral integrity of the other two anticancer drugs, pembrolizumab and bevacizumab, was also retained following VM nebulization

Discussion

We explored FTIR and Raman spectrometry to obtain data on the potential inactivation of atezolizumab, pembrolizumab, and bevacizumab following VM nebulization or forced thermal degradation.

FTIR and Raman spectroscopy are techniques used to authenticate the chemical structure of a material and reveal various parameters, namely identity, polymorphism, type of impurity, drug counterfeit (21), and drug stability (22). A molecule or material in a pharmaceutical product can be identified by a unique vibrational spectrum which provides a distinct chemical fingerprint for the molecule or material under investigation. Raman spectrometry and FTIR can be implemented during pharmaceutical development, in production for process monitoring, and in quality control. An attractive advantage to these techniques is that samples do not have to be extracted or prepared, and the analysis is non-destructive.

All three drugs tested are monoclonal antibodies. We used commercial products in their original packaging and concentrations formulated for the intravenous route of

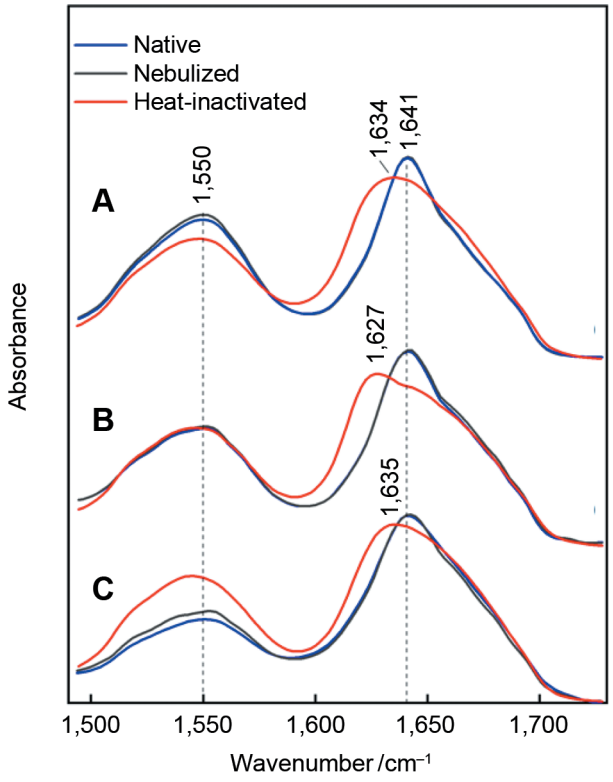

Figure 1. *Fourier-transform infrared absorbance of native, nebulized, and heat-inactivated atezolizumab (A), pembrolizumab (B) and bevacizumab (C).*

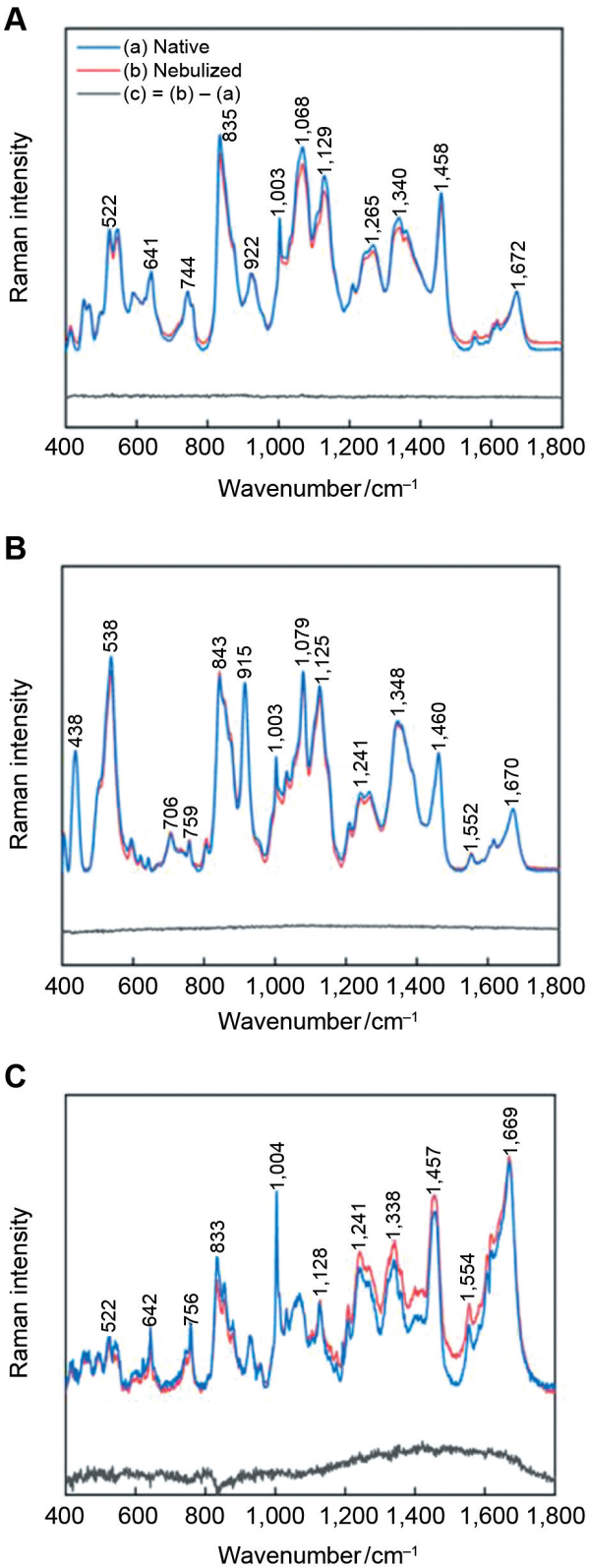

Figure 2. *Raman spectroscopy of pembrolizumab (A), bevacizumab (B) and atezolizumab (C). Nebulization did not significantly affect the Raman intensity of these drugs.*

administration, assuming that this would be the most straightforward to apply in experiments and clinical trials in the future. These antibody isotypes of these drugs are known to be relatively thermostable – IgG1 and IgG4 (23). However, overheating is not the only destructive factor involved in the process of nebulization. We chose the VM-type nebulizer to produce an inhalable drug form. VM nebulizers are rapidly becoming the first choice for achieving medication efficiency in the development of new nebulized pharmaceutical drugs (24). The higher nebulization rate of VM nebulizers, and their reproducibility over other types, *e.g.*, jet and ultrasonic nebulizers, were also reported (25, 26). VM nebulizers can prevent aerosol recirculation and maintain consistent drug concentrations during the procedure (27, 28). Finally, VM nebulizers are handy, easy to use, and pocket size. The specific design of VM nebulizers reduces reservoir heating and likely eliminates thermal degradation of the protein during the operation (29). The manufacturer of the VM nebulizer we used claims the temperature at the mesh is less than 40˚C. Considering the future development of inhalable proteins, there are additional ways to protect protein during VM nebulization: *e.g.* by making a gelatin-modified RBD-62 formulation (30).

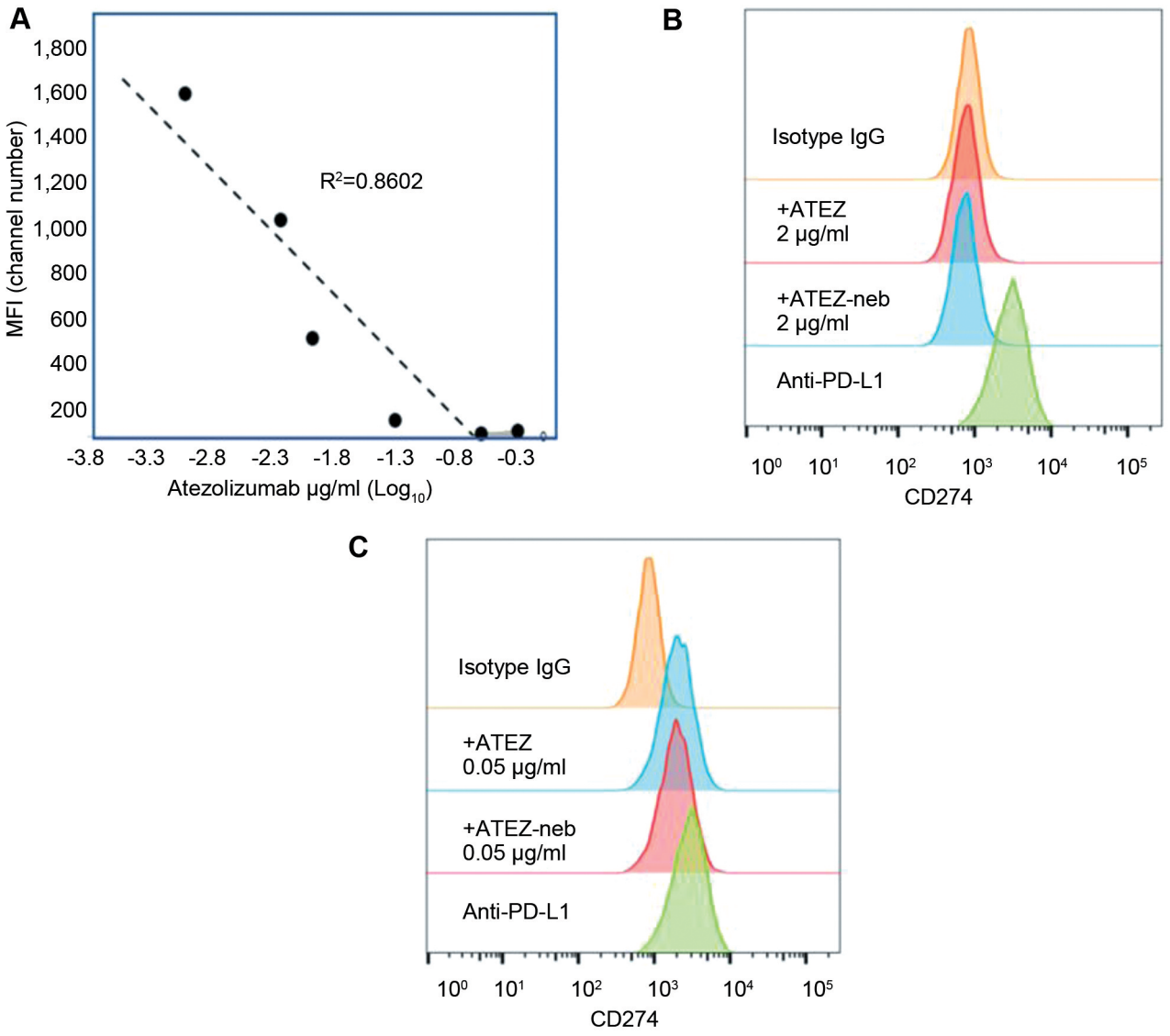

Figure 3. *Flow cytometry of PD-L1 staining (CD274) of DU-145 cells following their incubation with atezolizumab. A: Mean fluorescence intensity (MFI) by flow cytometry of PD-L1 staining (CD274) of DU-145 cells after incubation with different concentrations of native atezolizumab. B: A representative example comparing CD274 blockade with 2 µg/ml atezolizumab as the native or nebulized form. C: CD274 blockade following the addition of the lowest dose of atezolizumab tested (0.05 µg/ml) in its native and nebulized forms.*

Dégardin K *et al*. reported Raman spectrometry to be remarkably effective in performing reliable and fast analysis of counterfeit medicines (21). The method was effective in identifying generics, counterfeits, and placebos – all compounds which exhibit minuscule differences compared to their genuine counterparts. We hypothesized that a similar approach can be used in screening protein drugs for their ability to retain (or lose) functionality following nebulization. Currently, only one protein drug – nebulized DNAse (dornase alfa) is approved by the US Food and Drug Administration for aerosolized use as a mucolytic to treat pulmonary disease (the major cause of morbidity and mortality) in cystic fibrosis (31). It reduces the viscosity of mucus in the lungs, promoting improved clearance of secretions. The reduction of viscosity is due to a decrease in the length of DNA derived from human neutrophils, which infiltrate the airways to eliminate pathogenic bacteria. The trials showed that dornase alfa is well tolerated, significantly improves lung function, and reduces the risk of pulmonary exacerbation (31). Evaluation of dornase alfa aerosols produced by various nebulizers, including VM nebulizers, showed no overall loss of enzymatic activity or protein content and no increase in aggregation or degradation (1).

Following the success of dornase alpha, it seems that the idea of inhaled immunotherapy to treat pulmonary cancer is quite feasible (14). Interestingly, this concept was raised by a group of investigators who previously demonstrated skill and extensive knowledge in the field of inhalation therapy using conventional cytotoxic anticancer drugs (9-11). Exploring monoclonal antibodies designed to interact with the PD-1/PD-L1 axis can have several important implications.

Firstly, this treatment can not only trigger immune responses at the point of drug-binding sites (at the tumor–immune cell locations) but can also accelerate systemic reactions eliciting general immune recognition and memory mechanisms. This effect was demonstrated *via* local injection of ICI drugs to patients with melanoma (32). Furthermore, we assume that inhalation of ICIs makes the tumor become 'visible' to the immune system, and consequently the tumor tissue can be reached though routes other than vascularization. Some regions of tumors are poorly vascularized, which renders them hypoxic (33). A hypoxic environment favors invasive and resistant cancer cells responsible for tumor tissue repopulation (33, 34). Moreover, as these regions are more distant from blood vessels, the cells are exposed to a lower drug concentration from systemic routes (35). Drug deposited into the lung *via* inhalation is mainly absorbed into the local bloodstream and can also be drained by the lymphatic system, as was demonstrated for nebulized cisplatin (9). Therefore, lung-deposited drugs can follow the same routes of spread as potential invasive cancer cells from a solid lung tumor (*i.e.*, micrometastases).

Finally, another class of protein drugs, antiangiogenic compounds, can be applied in nebulized form in combination with ICIs. Bevacizumab was one of the drugs extensively investigated in non-small cell lung cancer trials in combination with other drugs. The combination of atezolizumab and bevacizumab as a first-line therapy for the intravenous route (36) clearly demonstrates that protein drugs can play a significant role in daily clinical practice – the therapeutic niche which was formally attributed to cytotoxic compounds. The tissue-sparing effect of protein drugs (including preservation of intratumoral immune cells) offers an outstanding opportunity to explore them in locoregional, intra-arterial, and inhalation therapies.

Recent trials of subcutaneous formulations of trastuzumab (18), rituximab (17), and atezolizumab (16) have shown that patients and healthcare providers favored subcutaneous injections at home for the treatment of malignancies. We can envisage a time when inhalable protein formulations might also be safely used at home. Here, we explored FTIR and Raman spectrometry as a rapid test to screen the quality and functional integrity of inhalable formulations of protein drugs. The idea of applying Raman spectroscopy and FTIR to the analysis of pharmaceutical aerosols, including dry-powder inhalable particles, was suggested by Mansour *et al*. (37). Interestingly, FTIR spectrometry has already been applied for the characterization of inhalable paclitaxel designed to treat lung cancer (38). The concept of applying Raman spectroscopy to the quality control of protein drugs (including bevacizumab and atezolizumab) through a perfusion bag wall appeared to be a rapid and precise technique applicable to centralized hospital workflow (39). FTIR and Raman spectroscopy are non-invasive and non-destructive techniques delivering molecular fingerprints and drug concentrations. Here we suggest the use of this rapid and cost-effective technique in the preparation of personalized inhalable anticancer protein aerosols. Interestingly, anti-PD-L1 molecules (analogous to human atezolizumab) assembled into chitosan nanoparticles were shown to significantly inhibit lung metastases in mouse models when used as an inhalation therapy (40). Aerosolized delivery may also offer great opportunities in gene therapy (41). Furthermore, the cost-effectiveness of life-years saved by localized therapies, such as inhalation therapy, should not be underestimated. For instance, bevacizumab and other anti-vascular endothelial growth factor drugs are being routinely used in oncology as well as in ophthalmology (wet macular degeneration). Comparing ophthalmological and oncological guidelines reveals that local intravitreal injection requires roughly 1,000 times a lower dose per individual. For example, the recommended intravitreal dose of bevacizumab is 0.63-2.5 mg per person per month, whereas the commercial bevacizumab drug information leaflets recommend 15 mg/kg of the drug per 3 weeks in patients with non-small cell lung cancer. This results in 1,050 mg of a drug per 70 kg person per 3 weeks. This high systemic dose results in a potentially high enough drug concentration in the vicinity of retinal cells but ophthalmologists prefer localized injection. Yet not only biomedical but also economic issues of protein drugs matter. The benefits of systemic ICI treatment were clearly revealed in a 'financial toxicity' study by Giuliani and Bonetti in which they combined pharmacological costs with measures of efficacy (42). The advantages of inhalable nebulized ICI and anti-vascular endothelial growth factor proteins have not yet been explored in clinical trials. These advantages include not only the realization of higher drug doses at the sites of tumorous lesions but also significantly reduced treatment cost (inhalation offers a reduction of total dose), ease of administration, stable pharmacokinetics due to multiple daily drug applications, possibly daily home use, and better patient compliance.

Conclusion

The results of the present study demonstrate that VM nebulization does not affect the functionality of the anti-PD-L1 drug atezolizumab and the integrity of a nebulized format can be rapidly assessed by FTIR and Raman spectrometry.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

GZ, DCh and VU participated in the study design, and drafted the article. MT performed and interpreted vibrational spectrometry results. PB, AD, LS and JJ designed the experiment and participated in nebulization, drug mist collection, and heat-inactivation procedures. LZ, ZS, JJ and MZ carried out cell culture treatments and flow cytometry. LZ and ZS performed the statistical analyses. All Authors read and approved the final article.

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