Original Study

Toxicity and Pharmacokinetic Studies of Aerosolized Clinical Grade Azacitidine

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Abstract

Aerosolized clinical grade azacitidine (Aza) was tested for toxicity and pharmacokinetics in mice. No systemic or pulmonary toxicity was observed at a therapeutic dose. Reversible lung inflammation was identified at a 3-fold higher dose. Aerosolized Aza localizes the drug to the target site (lungs) with a little drug release into the circulation. These studies provide some essential details to support upcoming clinical trials.

Background: Azacitidine as an effective epigenetic therapeutic agent has not been used as an aerosol form to treat lung cancer patients. We aerosolized clinical grade azacitidine (Aza), optimized the formulation, and studied its pharmacokinetics and toxicity in mice. **Methods:** Extrusion-precipitation method and DNA methyltransferase inhibition rate were used to measure the aerodynamic size and aerosolized Aza activity. In the single dose pharmacokinetic study, Aza concentrations in peripheral blood and lungs were measured by LC-MS method. In the multiple-dose toxicity studies, histo-pathological evaluation was used to determine the organ and bone marrow toxicities. **Results:** In pharmacokinetic study, aerosolized Aza was found to deposit mainly into the lung with very little drug detected in the circulation. In contrast, intravenously injected (IV) Aza resulted in a high Aza concentration in the peripheral blood, with trace amounts of drug in the lung, and it was associated with significant myelosuppression. No significant myelosuppression, pulmonary toxicity, hepatotoxicity, or nephrotoxicity were observed at a daily dose of 2.5 mg/m^2 for 7 days. Reversible lung inflammation was found in mice treated with 7.5 mg/m^2 aerosolized Aza at 3 but not 6 weeks after treatment. **Conclusions:** Aerosol Aza aerodynamic size favors deposition of the drug to the human lower airways. The aerosol process do not compromise the drug activity. Aerosolized Aza has higher lung deposition and much less systemic toxicity than IV drug. The safe starting dose for clinical phase I trials should be 2.5 mg/m^2 for 5 to 7 days.

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Introduction

The majority of lung cancer occurs as a result of cumulative damage in the bronchial epithelium caused by inhaled carcinogens.[1-5] Additionally, damaged airway epithelium is prone to develop second primary malignancies during the life span of individuals with the diagnosis of primary lung cancer. Therefore, it is necessary to develop an early therapeutic or preventive method to specifically treat the abnormal airway epithelium. In theory, this method should have definite advantages over systemic treatment.

The initial abnormalities in the airway epithelium consist of reversible epigenetic changes before the genetic changes occur. For example, the epigenetically mediated silencing of tumor suppressor genes (TSGs) by CpG island hypermethylation in promoter regions appears dominant in transcriptional repression and is tightly associated with the premalignant[6] and malignant phenotypes.[1-5,7-9] Reversing aberrant DNA hypermethylation in the airway epithelium using an airway-targeted therapy is a promising strategy to prevent primary or secondary lung cancers and inhibit the growth of localized lung cancers.

Azacytidine (Aza) is an inhibitor of DNA methyltransferases (DNMTs) and a prototypical demethylation agent that has been shown in vitro and in vivo to induce repeated expression of genes silenced through promoter hypermethylation.[10-13] Aza has been approved to treat myelodysplastic syndrome. Clinical grade Aza (Vidaza; Celgene, Summit, NJ) is delivered to patients by subcutaneous or intravenous (i.v.) administration, and the primary toxicity of systemic delivery of this drug is myelosuppression. However, laboratory findings have revealed that the concentration of drug needed to induce gene demethylation and reexpression is much lower than that required to produce cytotoxicity.[14]

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We have previously reported the therapeutic advantages of intratracheal and aerosol administration of Aza compared with i.v. Aza in orthotopic human lung cancers in a mouse xenograft model.[10,15] We have also demonstrated that aerosolized Aza can (1) inhibit human orthotopic lung cancers in mouse xenograft models, (2) reduce the promoter methylation of several TSGs and increase their protein expression in xenograft models, and (3) be better tolerated than with i.v. administration. The use of aerosolized clinical grade Aza (Vidaza) and evaluation of its potential systemic and pulmonary toxicity has not been previously studied. In these studies, we examined the suitability of clinical grade Aza for aerosol delivery, with particular emphasis on lung deposition and pulmonary and systemic toxicity in mice.

Materials and Methods

The Drug

All Aza used in the toxicity and pharmacokinetic studies was clinical grade Aza (Vidaza). Vidaza was purchased from the Department of Oncology pharmacy at Montefiore Medical Center as vials of lyophilized power containing 100 mg Aza and 100 mg mannitol in each vial for injection.

Aerosol Administration

The aerosol equipment and the administration method were the same as described previously.[10] Briefly, the aerosol was generated with PARI's Proneb Ultra compressor and LC Star nebulizer (PARI Respiratory Equipment, Midlothian, VA). The nose-only exposure system (CH Technologies, Westwood, NJ) linked with PARI's aerosol system in a closed chemical hood was used for aerosol administration to mice (Figure 1C), and the aerosol time was strictly controlled. All aerosol doses used in the experiments reported in this article are "lung-deposited doses" calculated as previously reported by our group.[10]

The Aerodynamic Size

Two formulations were tested separately. The suspension formulation was made by adding 4 mL of sterile water for injection USP (Abbott Laboratories, East Windsor, NJ) to 100 mg Vidaza lyophilized powder for a final Aza concentration of 25 mg/mL. The solution formulation was made by adding 10 mL of sterile water to 100 mg Vidaza powder for a final Aza concentration of 10 mg/mL. The aerodynamic diameters of aerosol droplets were determined by the extrusion-precipitation method using a 7-stage cascade impactor (In-Tox Products, Moriarty, NM) linked to PARI's aerosol system. The condensed aerosol samples were collected at 3 different periods: from 3.5 to 4 minutes, from 7.5 to 8 minutes, and from 11.5 to 12 minutes. Aerodynamic size and fraction of aerosol with a particular size range were measured and calculated according to the manufacturer's protocol. The mean and standard deviation were obtained from 3 independent experiments.

Aerosolized Aza Activity

Clinical grade Aza was aerosolized with PARI's aerosol system for more than 1 hour, and the aerosol fog from 58 minutes to 62 minutes was condensed into a sterile tube (Aero Vidaza). Aerosolized mannitol (Sigma-Aldrich, St. Louis, MO) using the same procedure (Aero mannitol), and nonaerosolized Aza (Vidaza) at the same concentration were used as negative and positive controls. The condensed liquids were used to treat the human non–small-cell lung cancer (NSCLC) cell line H226 at 0.5 μM for 10 days; the medium and drug were changed every other day. Ten days later, the nuclear proteins of the cells were extracted. EpiQuik DNA methyltransferase (DNMT) activity assay kit (Epigentek, Farmingdale NY) was used to measure DNMT activity quantitatively per the manufacturer's instructions.

Animals

Male and female ICR mice 6 to 8 weeks old (Harlan) were housed in the animal facility at Albert Einstein College of Medicine. All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (No. 20130312) was approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. During the studies, animals were observed daily. Humane end points were used during this study: moribund animals were euthanized by CO_2 in an inhalation chamber followed by exsanguination. We used 2 criteria to identify the moribund animals based on our animal use protocol: (1) a mouse had difficulty breathing, eating, or drinking and (2) a mouse lost $\geq$ 15% body weight over a period of 4 days. Anesthesia (isoflurane) was used to minimize discomfort during blood sampling.

Pharmacokinetics of the Aerosolized Aza in Mice

Twelve ICR mice (male and female) were divided into 2 groups and given a single dose of either aerosolized or i.v. Aza at 2.5 mg/m^2 using the method described previously. At 20 minutes, 2 hours, 6 hours, and 24 hours, 3 mice in each group were euthanized and blood was taken from the abdominal vena cava. The mice were then perfused with saline through the right ventricle and the lungs were excised. Aza in the blood and the lungs was extracted and quantitatively measured by a previously reported method using a liquid chromatography–mass spectrometry system.[16] The quantitative detection was performed by Millis Scientific (Baltimore, MD). The sensitivity was 1 ng Aza/mL of sample. The Aza concentration in blood/tissue versus time data were analyzed and simulated with the best fit (the highest R^2 value). The equation of the simulated curves C = f(t) were used to calculate AUC from 0 to 24 hours as AUC = $\int_{0\text{-}24}$ f(t) dt. Peaking time (T) and peak concentration were obtained directly from observation of the curves.

White Blood Cell Count

ICR mice were distributed into 3 groups of 12 mice (6 male and 6 female) each. The groups were treated once daily for 7 days with either aerosolized Aza at 2.5 mg/m^2 (0.83 mg/kg) or 7.5 mg/m^2 (2.5 mg/kg) or tail vein injection of Aza at 7.5 mg/m^2. The aerosol dose was calculated and controlled as described previously.[10,17] Blood samples (50 μL) were obtained from the facial vein[18] under isoflurane anesthesia 1 week before and 1, 3, and 6 weeks after treatment. Red blood cells were removed from the blood samples using RBC lysis buffer (eBioscience, San Diego, CA) and white blood cells (WBCs) were collected according to the manufacturer's protocol and counted using a hemocytometer.