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Effect of protease inhibitors on pulmonary bioavailability of therapeutic proteins and peptides in the rat

Kiran Prakash Amancha [1], Alamdar Hussain *

Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of Louisiana at Monroe, 700 University Ave., Monroe, LA 71209, USA

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ABSTRACT

The objective of the present study was to evaluate the effect of protease inhibitors on the pulmonary absorption of therapeutic peptides and proteins with varying molecular weights. Dry powder formulations of leuprolide (1.2 kD), salmon calcitonin (3.4 kD), human insulin (5.8 kD), human leptin (16.0 kD), and human chorionic gonadotropin (HCG) (36.5 kD) were prepared with or without protease inhibitors; aprotinin and bestatin. The formulations were administered intrapulmonary to anesthetized rats. The pharmacokinetics of these proteins were assessed by measuring serum drug concentrations. In addition, *in vitro* stability of these proteins in rat lung homogenate was assessed using the trifluoroacetic acid method. Bioavailability of leuprolide following pulmonary administration was 75% higher compared to subcutaneously administered leuprolide. Protease inhibitors had little or no effect on the pulmonary bioavailability of leuprolide. However, protease inhibitors (1 mg/kg) increased the bioavailability of calcitonin by more than 50%. Similarly, the bioavailabilities of leptin and HCG in the presence of bestatin were increased by 1.9 and 2.1-fold, respectively. Leuprolide was stable both in the lung cytosol and subcellular pellets with about 10% degradation at the end of the study period (4 h). In contrast, calcitonin, insulin, leptin and HCG were significantly degraded in the lung cytosol and subcellular pellets. Presence of protease inhibitors in formulation could improve the stability of protein drugs. The results of this study demonstrate that the pulmonary absorption of proteins may be enhanced by the selection of optimal concentration and type of protease inhibitor.

1. Introduction

Drug delivery to the lungs is an effective way of targeted drug delivery for the treatment of local and systemic ailments. Lung delivery is gaining substantial interest as an alternative route for the administration of systemically acting drugs that are poorly absorbed from GIT such as polypeptides, proteins, genes, vaccines and diagnostic agents (Stegemann et al., 2013). Successful delivery of pulmonary protein formulations depends mainly on (1) preventing biotherapeutic structure and activity modifications during the formulation and manufacturing processes, (2) avoiding physical instability of the drug product during the shelf-life, (3) well-designed device for optimum delivery of the medication and (4) inhalation technique and patient co-ordination (Labiris and Dolovich, 2003). In general, dry powder formulations and devices are recognized to be more efficient and convenient for patients compared to nebulizers. They are more compatible with proteins than metered dose inhaler formulation components and propellants. Protein formulations when delivered in the form of dry powders have better stability and also prevent the structure activity modifications. This is particularly true for protein molecules which are unstable in solution formulations and tend to aggregate to form sub-visible particles resulting in safety and efficacy issues. For these reasons, inhalation powders delivered by dry powder inhalers are promising dosage forms for pulmonary delivery of therapeutic proteins (Mack et al., 2012).

Several small molecules were approved by the FDA for pulmonary delivery using dry powder inhalers and examples of these products include Advair®, Foradil®, Spiriva®, Asmanex®, Symbicort®, and Dulera® (Hickey, 2013). There were several attempts made for pulmonary delivery of dry powder protein formulations. Some of the examples include Exubera®, AIR® Afrezza®, Aerovant®, Fludase®, and MKC253®. Exubera® was pulled from the market due to safety and device related issues. Failures of Exubera® led to phase III termination of AIR® by Eli Lilly/Alkermes. Very recently, inhalable insulin product Afrezza® was approved by the FDA

* Corresponding author at: Department of Pharmaceutical Sciences, College of Pharmacy, The University of Oklahoma, 1110 N. Stonewall Ave., Oklahoma City, OK 73117, USA. Tel.: +1 405 271 6593x47472; fax: +1 405 271 7505.
E-mail address: alamdar-hussain@ouhsc.edu (A. Hussain).
[1] Address: Insys Therapeutics, Inc., 444 S. Ellis St. Chandler, AZ 85224, USA.

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following a positive recommendation from the FDA advisory panel. Dance Biopharm is a clinical stage company focused on development of inhaled insulin, Dance-501, which is a liquid formulation of insulin. This product has completed phase-2 clinical trials and the company is in the preparation for conducting pivotal clinical trials. Other protein products mentioned above are in different phases of clinical development. On the other hand, Pulmozyme® is the FDA approved inhalation product (solution for oral inhalation) for the treatment of cystic fibrosis. Several attempts were made to deliver dry powder proteins formulations of r-human-DNase; IgG1, lysozyme, anti-CD4 antibody, alpha-1-antitrypsin, trypsinogen, and salmon calcitonin (Cape et al., 2008; Clark et al., 2008; Maa et al., 1999; Schule et al., 2008). Nevertheless, development of inhalation formulations of protein drugs has lagged behind other delivery routes in terms of the number of marketed products.

Despite inherent advantages associated with pulmonary delivery of biotherapeutics, it is often noted that the bioavailability of these drugs from the pulmonary route is still poor when compared with the subcutaneous route. One of the reasons for poor bioavailability of these drugs following pulmonary delivery is due to the degradation of these drugs by proteases in the lung (Shinzo et al., 1996). Different types of proteases present in the lungs include serine proteases, cysteine proteases, metalloproteases, aminopeptidases, leu-aminopeptidases, trypsin, and cathepsin B. Among these proteases, serine proteases and aminopeptidases constitutes majority of the proteases present in the lungs (Baginski et al., 2011; Forbes, 2000; Forbes et al., 1999; Yang et al., 2000). Hence, pulmonary absorption of protein drugs may be improved by the use of protease inhibitors. For instance, pulmonary absorption of insulin was significantly improved by these additives (Yamamoto et al., 1996). In their study the authors concluded that the enhanced absorption of insulin by protease inhibitors may be related to their inhibitory action on the activity of various proteases in the lung resulting in increased stability and absorption of the drug from the lung. Moreover, pulmonary absorption of calcitonin was markedly improved by various protease inhibitors, and these compounds reduced the degradation of calcitonin in the rat lung homogenate (Baginski et al., 2012; Takahiro et al., 1994). However, the effect of protease inhibitors on the pulmonary absorption of therapeutic peptides and proteins with varying molecular weights has not been thoroughly investigated.

In the present study, we have investigated the pulmonary absorption of therapeutic peptides and proteins with molecular weights ranging from 1.2 kD to 36.5 kD. The therapeutic peptides and proteins included in this study are leuprolide (1.2 kD), calcitonin (3.4 kD), insulin (5.8 kD), leptin (16.0 kD), chorionic gonadotropin (HCG, 36.5 kD); and protease inhibitors were aprotinin (a serine protease inhibitor) and bestatin (an aminopeptidase inhibitor). Commercial significance of these proteins is as follows: Leuprolide is used in the treatment of prostate and breast cancer (Wilson et al., 2007). Calcitonin is used in the treatment of osteoporosis and hypocalcaemia (Woodrow et al., 2006). It is worthwhile to mention that FDA has recommended stopping long-term usage of salmon calcitonin for the treatment of osteoporosis in women who are at least five years past menopause citing lack of benefit and concerns about a possible cancer risk. In 2012, the European Medicines Agency recommended that salmon calcitonin not be used to treat osteoporosis after determining that the risk of developing cancer was 2.4% higher in patients using the nasal spray formulation compared with in those who took placebo. However, salmon calcitonin drug products are still prescribed for other indications. Insulin is used in the treatment of diabetes mellitus. Leptin regulates food intake and energy metabolism, and is used in the treatment of obesity (Phillip and Oksana, 2003). HCG is used as a fertility medication and in the treatment of hypogonadism (Tsampalas et al., 2010). In addition, we have also examined the effect of these protease inhibitors on *in vitro* degradation of these proteins in the lung homogenate and subcellular fraction.

2. Materials and methods

2.1. Materials

Human recombinant insulin (28.8 IU/mg; purity ⩾97%; HPLC), salmon calcitonin (purity ⩾97%; HPLC), human recombinant leptin (purity ⩾97%; SDS–PAGE), trifluoroacetic acid, aprotinin, and bestatin were obtained from Sigma Aldrich (St. Louis, MO). Human chorionic gonadotropin (purity ⩾98%; SDS–PAGE) was purchased from Cell Sciences (Canton, MA). Leuprolide (purity ⩾99%; HPLC) was obtained from Bachem, Inc. (Torrance, PA). Inhalation grade lactose monohydrate was purchased from Kerry Bio-Science (NY). Leuprolide radioimmunoassay (RIA) kit and salmon calcitonin RIA kit were purchased from Bachem, Inc. (Torrance, PA) and Phoenix Pharmaceuticals, Inc. (Burlingame, CA), respectively. Human insulin specific RIA kit and human leptin RIA kit were purchased from Linco Research, Inc. (St. Charles, MO). Human chorionic gonadotropin (HCG) enzyme-linked immunosorbent assay (ELISA) kit was obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX). Anesthetics ketamine and xylazine were purchased from Henry Schein, Inc. (Melville, NY).

2.2. Preparation of subcellular fractions

Male Sprague–Dawley rats were obtained from Harlan laboratories (Houston, TX). Rats ($n = 4$) weighing 250–350 g were housed in a 12 h light–dark cycle and a constant temperature environment of 21 °C, provided with standard diet *ad libitum*. According to the reported procedure, rats were anesthetized with an overdose of sodium pentobarbital (150 mg/kg) by intraperitoneal injections (Liu et al., 1992). The lungs were surgically isolated and washed with ice-cold PBS (pH 7.4). Lung homogenates were prepared with a lab homogenizer. The resulting mixture was centrifuged for 10 min, 3500 rpm, at 4 °C. The supernatant was collected and centrifuged for 40 min, 35,000 rpm, at 4 °C in a Beckman Ultracentrifuge (Brea, CA). The final supernatant was used as cytosol, and the precipitate fraction was used as subcellular pellets, a mixture of organelles. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Monroe and all surgical and treatment procedures were consistent with the IACUC policies and procedures.

2.3. Degradation of proteins in lung cytosol and subcellular pellets

Degradation of proteins in lung cytosol and subcellular pellets was determined using the trifluoroacetic acid (TFA) method (Duckworth et al., 1972). The assay system consisted of 450 µl of protein solution in the presence or absence of aprotinin (1 mg/ml) or bestatin (1 mg/ml) and 350 µl of lung cytosol or subcellular fraction. The individual protein concentrations were maintained at 0.1 µmol/ml. The assay system was incubated at 37 °C for 4 h. Triplicate samples of each 25 µl were taken and mixed with 50 µl of 10% TFA solution. The resulting mixture was then centrifuged at 10,000 rpm for 5 min to remove the precipitated protein. The protein concentration in the supernatant was analyzed as using commercially available RIA/ELISA kits. Concentrations of both the protease inhibitors were used at 1 mg/ml, as this concentration has been reported to be effective in inactivating lung protease enzymes when utilized in other pulmonary formulations (Sang-Ha et al., 2007).

According to protocols from suppliers, leuprolide RIA has sensitivity of 0.2 ng/ml and range was between 0.01 and 1.28 ng/ml. Salmon calcitonin RIA has sensitivity of 43.7 pg/ml and the range