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ORIGINAL ARTICLES

# Technosphere® Insulin: Defining the Role of Technosphere Particles at the Cellular Level

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## Abstract

***Background:***

Technosphere® Insulin (TI) is a novel inhalation powder for the treatment of diabetes mellitus. Technosphere Insulin delivers insulin with an ultra rapid pharmacokinetic profile that is distinctly different from all other insulin products but similar to natural insulin release. Such rapid absorption is often associated with penetration enhancers that disrupt cellular integrity.

***Methods:***

Technosphere Insulin was compared to a panel of known penetration enhancers *in vitro* using the Calu-3 lung cell line to investigate the effects of TI on insulin transport.

***Results:***

Measures of tight junction integrity such as transepithelial electrical resistance, Lucifer yellow permeability, and F-actin staining patterns were all unaffected by TI. Cell viability and plasma membrane integrity were also not affected by TI. In contrast, cells treated with comparable (or lower) concentrations of penetration enhancers showed elevated Lucifer yellow permeability, disruption of the F-actin network, reduced cell viability, and compromised plasma membranes.

***Conclusions:***

These results demonstrate that TI is not cytotoxic in an *in vitro* human lung cell model and does not function as a penetration enhancer. Furthermore, TI does not appear to affect the transport of insulin across cellular barriers.

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**Abbreviations:** (ELISA) enzyme-linked immunosorbent assay, (FDKP) fumaryl diketopiperazine, (LDH) lactate dehydrogenase, (LOQ) limit of quantitation, (MTS) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), ($P_{app}$) apparent permeability, (SD) standard deviation, (TEER) transepithelial electrical resistance, (TI) Technosphere® Insulin

**Keywords:** Calu-3 cell monolayers, fumaryl diketopiperazine, inhaled insulin, Technosphere Insulin, Technosphere particles, tight junctions

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## Introduction

Protein and peptide pharmaceuticals are critical to the treatment of many conditions and represent an enormous potential for future therapeutics. Insulin therapy allows diabetes patients to lead relatively normal lifestyles with a disease that was once fatal. Other proteins, such as human growth hormone for Turners syndrome and salmon calcitonin for osteoporosis, also have tremendous therapeutic value.[1,2] Biomedical science continues to expand our understanding of protein function, protein involvement in normal cell function, and the potential use of proteins as targets and therapeutics for disease states. Thus the list of potential protein-based drug candidates is continuously growing. Unfortunately, the inherent instability of proteins has made it difficult to develop effective systems of administration other than injection.[3–8]

Pulmonary delivery is an emerging solution to these limitations.[9–12] The extensive surface area of the alveolar region of the lung and its close interface with the systemic blood supply allow for rapid absorption of proteins. Pulmonary administration could significantly improve delivery of intact protein therapeutics into the bloodstream by circumventing the harsh intestinal environment and avoiding digestive enzymes that contribute to the poor bioavailability typically associated with oral administration. Pulmonary drug absorption can occur by both the paracellular pathway, in which proteins diffuse through the tight junctions between cells, and via transcytotic pathways, in which the drug passes through the cells.[11–20]

Technosphere® Insulin (TI) is a novel inhalation powder for the treatment of diabetes mellitus.[21–24] The Technosphere technology platform is based on Technosphere particles (**Figure 1**), which are formed by the self-assembly of crystals of fumaryl diketopiperazine (FDKP),[25] a proprietary novel excipient. The median diameter of the primary particles is approximately 2–2.5 μm, and the aerodynamic diameter measured with an Andersen cascade impactor is also 2–2.5 μm. Technosphere particles have high internal porosity and high surface area for the adsorption of proteins or other drug substances. Technosphere Insulin powder consists of insulin that is adsorbed to Technosphere particles with a size range appropriate for delivery of drugs to the deep lung.

Technosphere Insulin has been shown to deliver insulin to the bloodstream with a pharmacokinetic profile characterized by a rapid rise in plasma insulin concentration that is much faster than subcutaneous insulin injection (**Figure 2**).[22] The pharmacokinetic profile of insulin administered as TI mimics the natural release of insulin by the pancreas and may provide a clinical advantage over standard subcutaneous insulin injections. An understanding of the mechanism by which Technosphere technology facilitates rapid insulin

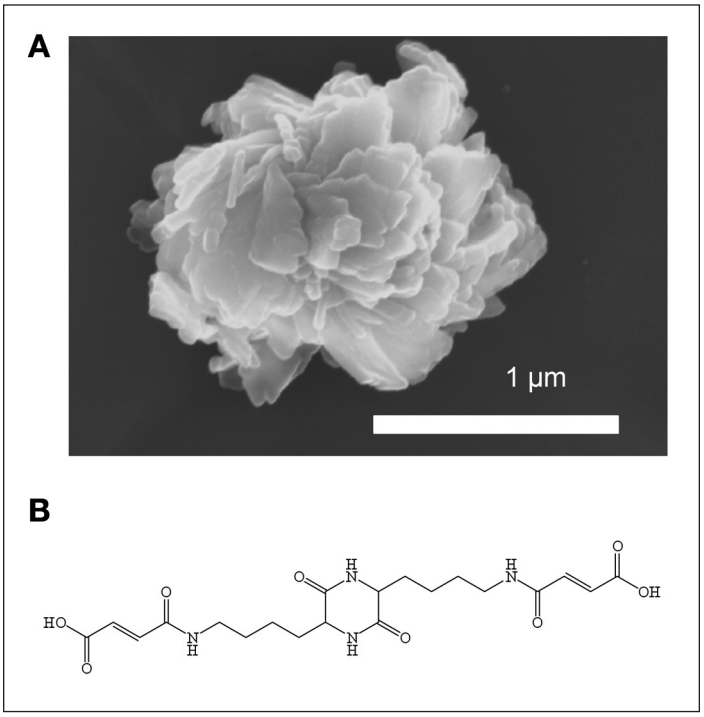


**Figure 1. (A)** Scanning electron micrograph of a Technosphere particle and **(B)** the chemical structure of FDKP used to form the particle.

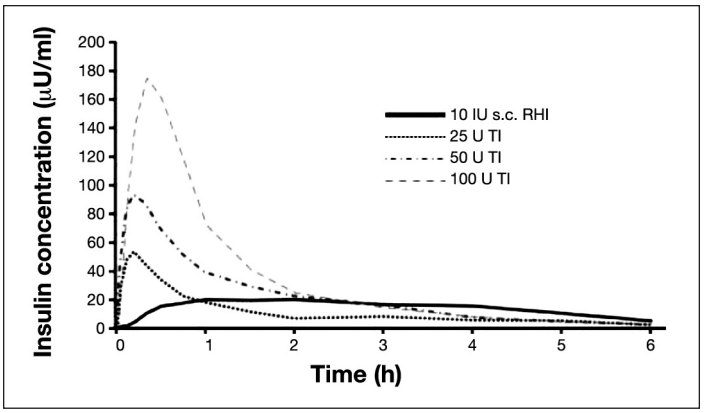


**Figure 2.** Pharmacokinetic absorption profile of TI in a euglycemic clamp study performed in healthy volunteers. Adapted with permission from Pfützner and colleagues.[22] The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers. s.c. subcutaneous; RHI, regular human insulin.

absorption could potentially lead to the development of an array of pulmonary formulations for the systemic delivery of insulin and other biopharmaceuticals.

The physicochemical basis of this observed pharmacokinetic profile is thought to result from the highly efficient delivery of TI particles to the deep lung, where they dissolve rapidly and provide high local concentrations of insulin and FDKP that drive diffusion across the membrane. However, rapid absorption of drugs is often associated with the use of penetration enhancers, and it is important to determine whether FDKP or any other component of TI acts as a penetration enhancer.

The mechanisms by which many penetration enhancers increase drug permeability and bioavailability are well documented.[26–33] These agents typically dilate tight junctions and open up the paracellular pathway between cells to enhance overall absorption. Enhancers such as detergents, medium chain fatty acids, and bile salts can disrupt the plasma membrane itself, enhancing transport by a potentially cytotoxic mechanism. Some penetration enhancers are also capable of increasing absorption by increasing the solubility/permeability characteristics of the drug article or by inhibiting processes that compete with absorption (e.g., efflux pumps).[34]

Upon inhalation, TI powder encounters bronchial and alveolar tissue. The most likely site for drug absorption in the lung is type I alveolar cells (type I pneumocytes), which constitute 90% of the lung surface area. Type I pneumocytes are wide, thin cells with a high surface area and what is often described as a "fried egg" shape. They form a monolayer of cells covered with a layer of surfactant, and their unique dimensions provide the very short diffusion path crucial for efficient gas exchange.[35] Primary type I-like pneumocytes can be generated in the laboratory as primary cultures; however, no established cell lines are available. For this reason, Calu-3 were used in this investigation. Calu-3 cells are bronchial cells that also have some features similar to type I cells, such as the ability to form polarized cells with intact tight junctions. Calu-3 cells grow as monolayers when cultured on Transwell® inserts and have been used as a representative model of the airway epithelium to model pulmonary absorption mechanisms.[36–41] To characterize the role of FDKP in insulin absorption, Calu-3 cells were exposed to insulin alone, solutions of dissolved TI powder, and a panel of known penetration enhancers. Stark differences were observed between the effects of TI (FDKP) and those of penetration enhancers on various cell properties.

## Methods

Technosphere Insulin and insulin-free Technosphere particles were produced by MannKind Corporation, as described previously.[23] Recombinant human insulin was purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488 carboxylic acid, tetrafluorophenyl ester, bis (triethylammonium salt), and Alexa Fluor 568 phalloidin were purchased from Invitrogen (Carlsbad, CA). All cell culture media and reagents were purchased from American Type Culture Collection (Arlington, VA). All other reagents were purchased from Sigma-Aldrich.

### *Dosing Solutions*

*I 10*

Solutions of insulin alone were prepared at a concentration of 10 U/ml (I 10) by dissolving insulin solids in transport buffer. The pH of the solution was typically 6.9 or adjusted to 6.2, 7.4, or 8.2 using HCl or NaOH as required for various studies.

*TI 10*

Dosing solutions of TI were also prepared at an insulin concentration of 10 U/ml by dissolving approximately 3.3 mg of TI powder per milliliter of solution. The pH of the TI 10 solutions was typically 6.4 or adjusted to 6.2, 7.4, or 8.2 using HCl or NaOH as required.

*TI 25 and TI 50*

Dosing suspensions of TI were prepared at an overall insulin concentration of 25 or 50 U/ml (TI 25, TI 50). The solutions were saturated with FDKP and contained undissolved particles.

### *Cell Culture*

Calu-3 cells were obtained from American Type Culture Collection. Calu-3 cells were seeded on 0.45 μm pore size Transwell cell culture inserts ($5 \times 10^5$ cells/cm$^2$) and cultured for 14 to 16 days (37 °C at 5% $CO_2$/95% relative humidity). Cells were cultured in Earle's minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). Transepithelial electrical resistance (TEER) was measured using a using voltohmmeter (World Precision Instruments) with an STX-2 electrode.

### *Permeability Studies*

All permeability studies were carried out in transport buffer, which consisted of Earle's Balanced Salt Solution containing 0.5% bovine serum albumin, 0.1 mg/ml $CaCl_2$, and 0.1 mg/ml $MgCl_2 \cdot 6H_2O$. Six- or 12-well plates

containing 14-day Calu-3 cell monolayers plated on Transwell filters were preincubated with transport buffer by slowly shaking on an orbital mixer for 30 min at 37 °C at 5% $CO_2$/95% relative humidity. For insulin and Lucifer yellow permeability experiments, dosing solutions were added to the dosing (apical) chamber, and 100 µl samples were collected from the receiver (basolateral) chamber every 30 min for 2 h. The volume of the receiver chamber solution was maintained by adding 100 µl transport buffer after each sample was removed. Insulin permeability experiments were repeated using an alternative sampling method, in which individual monolayers were transferred to new basolateral chambers at each time point. The entire contents of each basolateral chamber was then available for analysis. Insulin concentrations were determined by enzyme-linked immunosorbent assay (ELISA; Linco). For studies involving fluorescent species (Lucifer yellow and Alexa Fluor 488 labeled insulin), concentrations were determined by fluorescence using a SPECTRAmax Gemini EM fluorescent plate reader. The apparent permeability ($P_{app}$) was calculated from

$$P_{app} = \frac{dQ/dt}{AC},$$

where $dQ/dt$ is the steady-state rate of accumulation of insulin in the basolateral solution, $A$ is the area of the filter, and $C$ is the concentration of insulin in the apical chamber. This equation is based on the assumption that so little insulin is transported across the membrane that the concentration on the apical side does not change, and the concentration of insulin in the basolateral chamber (initially zero) remains insignificant compared to the initial concentration.

### *Enzyme-Linked Immunosorbent Assay*

The accumulation of insulin in the basolateral chamber was measured using the Linco Insulin ELISA (Millipore) as described by the manufacturer. Standard curves were generated using transport buffer as the matrix: Limit of Quantitation = 35 pM.

### *Microscopy*

Imaging was performed using a Zeiss Axioplan II upright fluorescent microscope (Zeiss, Germany) using a 10 ×/0.30, 40 ×/0.75, or 100 ×/1.30 (oil emersion) objective.

For actin staining experiments, cells grown on Transwell inserts were treated for up to 2 h with apical dosing solutions containing TI 10, I 10, buffer alone, or saturated solutions of TI. The insert was washed three times with transport buffer or Hanks Balanced salt solution and fixed for 10 min at room temperature in 4% paraformaldehyde. The cells were permeabilized by treatment with 0.1% Triton X-100 (5 min at room temperature) and washed again as before. Samples were treated with 0.5 µM Alexa Fluor 568 phalloidin (37 °C, 30 min), washed, excised, and mounted on slides for imaging.

### *Alexa Fluor Labeling*

Human insulin was labeled using the Alexa Fluor 488 Protein Labeling Kit, according to the manufacturer's instructions. Dialysis was performed to remove unbound label. The labeling was confirmed by reverse-phase high-performance liquid chromatography using differential absorption at 495 and 220 nm.

### *Cytotoxicity Assays*

*Lactate Dehydrogenase Assay*

Lactate dehydrogenase (LDH) release was measured using the Cytotox 96 kit (Promega, Madison, WI). The 50 µl samples from permeability experiments were analyzed according to the manufacturer's instructions. Relative LDH leakage was determined against 0.4 mM Triton X-100.

*3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) Assay*

Cell proliferation was monitored using the CellTiter 96® Aqueous Nonradioactive Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's instructions.

## Results

### *Comparison of Technosphere Insulin to Classical Penetration Enhancers*

*Fumaryl Diketopiperazine Is Not a Penetration Enhancer in Calu-3 Cell Monolayers*

A direct comparison of TI to various classical penetration enhancers/surfactants was performed on Calu-3 cell monolayers. As shown in **Figure 3**, penetration enhancers such as capric acid and deoxycholate caused significant disruption of the actin cytoskeleton. Various ionic and nonionic detergents also disrupted the actin cytoskeleton. In contrast, TI had no visible impact on the actin staining pattern in Calu-3. Direct exposure to a suspension equivalent to 30 mM TI also had no apparent effect on the actin structure of Calu-3 monolayers.

Permeability experiments with Lucifer yellow were consistent with the observations in the microscopy study. Lucifer yellow $P_{app}$ values obtained from monolayers

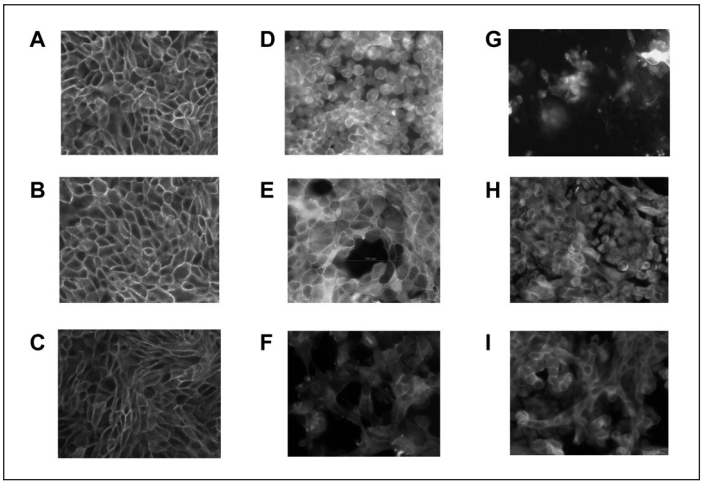


**Figure 3.** Effect of TI and penetration enhancers on the actin cytoskeleton. Fourteen-day Calu-3 cell monolayers were treated and stained for F-actin and viewed by fluorescent microscopy, as described in Methods. Cells were treated with **(A)** control buffer, **(B)** 10 U/ml (6 mM) TI, **(C)** 50 U/ml TI suspension (30 mM saturated solution), **(D)** 25 mM ethylenediaminetetraacetic acid, **(E)** 17 mM capric acid, **(F)** 1.3 mM deoxycholate, **(G)** 0.4 mM Triton X-100, (H) 64 μM digitonin, or **(I)** 1 mM SDS, sodium dodecyl sulfate.

treated with known penetration enhancers were orders of magnitude higher than those obtained from monolayers treated with TI (**Figure 4**).

### *Effect of Technosphere Insulin on Monolayer Integrity*

*Technosphere Insulin Does Not Affect Monolayer Integrity*

The $P_{app}$ of Lucifer yellow through monolayers exposed to TI 10, FDKP alone (approximately 3 mg/ml dissolved insulin-free Technosphere particles), and insulin alone (I 10) was comparable by one-way analysis of variance, $p = 0.66$ (**Figure 5A**). No significant drop in TEER value was recorded by the end of the 2 h experiments for insulin ($p = 0.08$) or TI ($p = 0.15$) as determined by the *t*-test, (**Figure 5B**). These results were consistent with observations of the actin staining patterns (**Figures 3A and 3B**).

Lactate dehydrogenase leakage, which monitors cytotoxicity by measuring the amount of enzyme leaking out of the cells, was determined at the end of

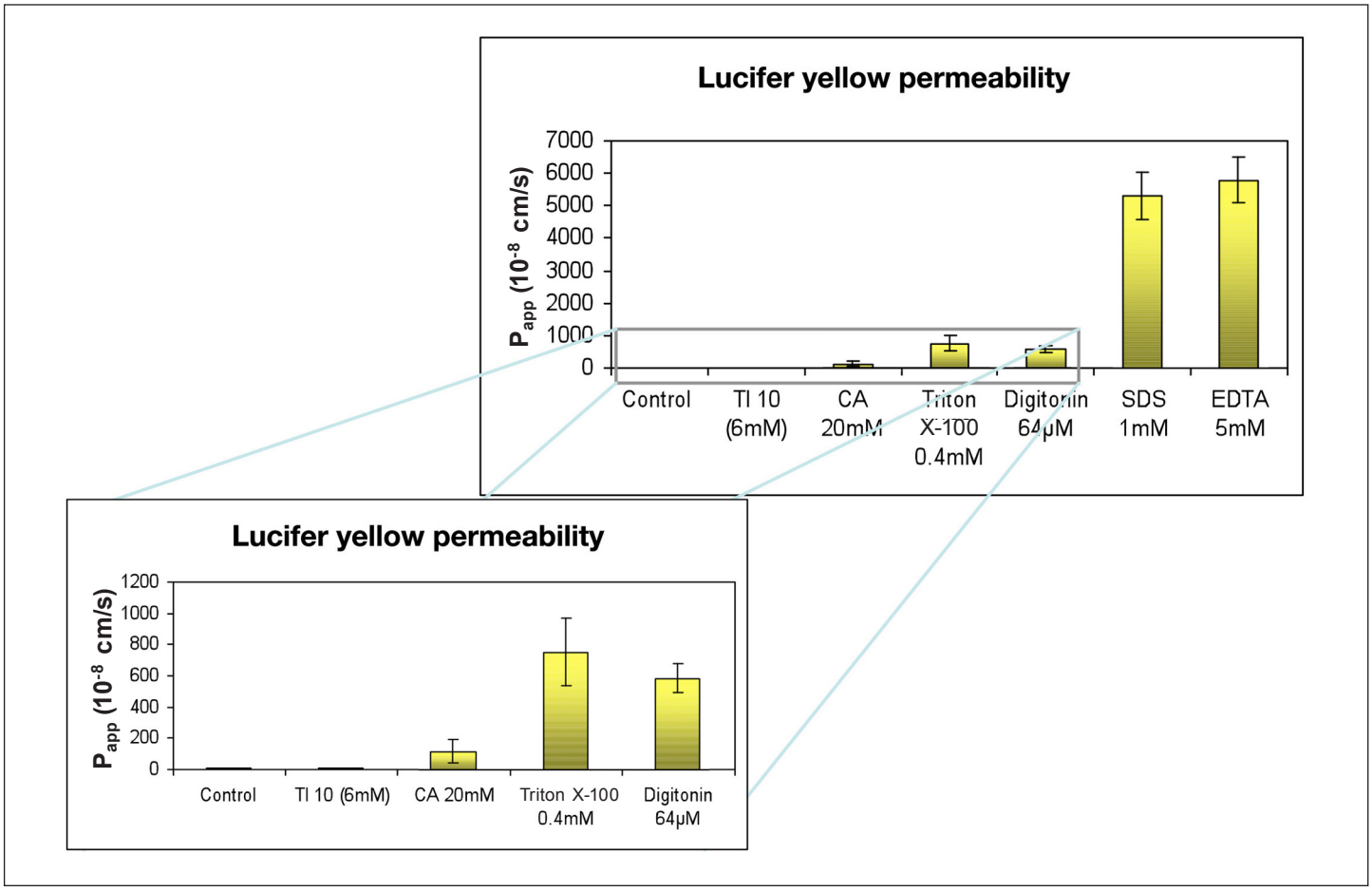


**Figure 4.** The effect of TI and penetration enhancers on Lucifer yellow $P_{app}$. Fourteen-day Calu-3 cell monolayers were treated for 2 h with TI or various penetration enhancers (Lucifer yellow $P_{app}$ mean ± standard deviation [SD], $n = 2$). CA, capric acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

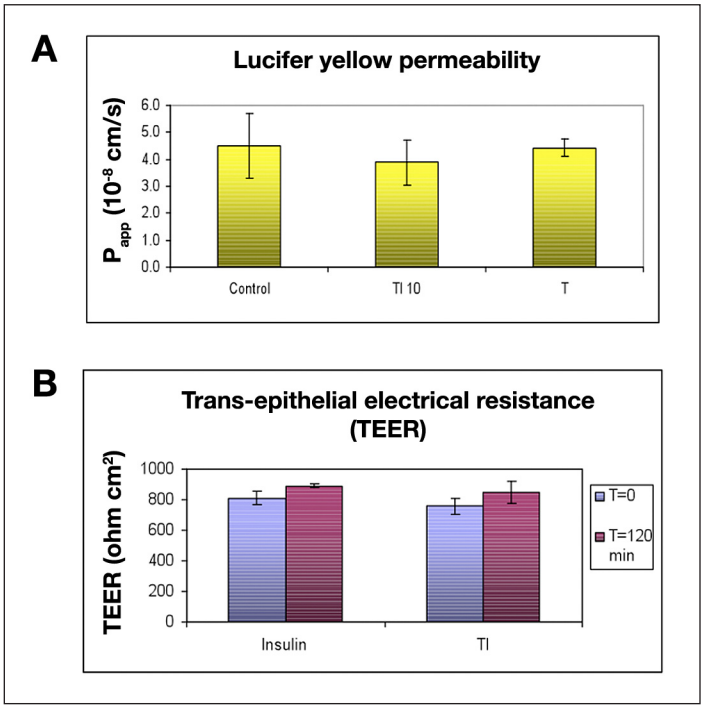


**Figure 5.** Effect of TI on monolayer integrity. (**A**) Lucifer yellow $P_{app}$ of 14-day Calu-3 cell monolayers (mean ± SD, $n$ = 3) treated for 2 h with Lucifer yellow alone (4.48 x $10^{-8}$ ± 1.2 x $10^{-8}$ cm/s), 10 U/ml TI (3.88 x $10^{-8}$ ± 8.5 x $10^{-9}$ cm/s), or an equivalent amount of insulin-free Technosphere particles (4.43 x $10^{-8}$ ± 3.3 x $10^{-9}$ cm/s). (**B**) No drop in TEER was observed between $T$ = 0 and 120 min for 14-day Calu-3 cell monolayers treated with 10 U/ml insulin or 10 U/ml TI (mean ± SD, $n$ = 3).

the Lucifer yellow permeability experiments. In contrast with a positive control (Triton X-100), no significant LDH accumulation was observed in cells treated with insulin or TI (**Figure 6A**). MTS assays showed no significant effect on the ability of the cells to proliferate after exposure to TI (**Figure 6B**). Capric acid, a known penetration enhancer, significantly inhibited proliferation in Calu-3 cells, as did the nonionic detergent Triton X-100. Propidium iodide, a membrane impermeant dye often used to assess compromised plasma membranes and/or cell death, revealed no cell damage upon TI treatments (**Figure 6C**).

## Effect of Fumaryl Diketopiperazine on Insulin Apparent Permeability

### *Technosphere Insulin Does Not Alter Insulin Apparent Permeability in Calu-3 Cells*

To determine whether FDKP increases insulin permeability, dosing solutions of I10 and TI10 were applied to the apical chambers of 14-day Calu-3 Transwell monolayers. These concentrations were estimated to be comparable to but higher than the average concentration of insulin *in vivo* after a typical dose of TI powder (**Table 1**). Basolateral samples were collected at fixed time intervals, and $P_{app}$ was calculated as described in Materials and Methods. As shown in **Figure 7**, the insulin $P_{app}$ from TI

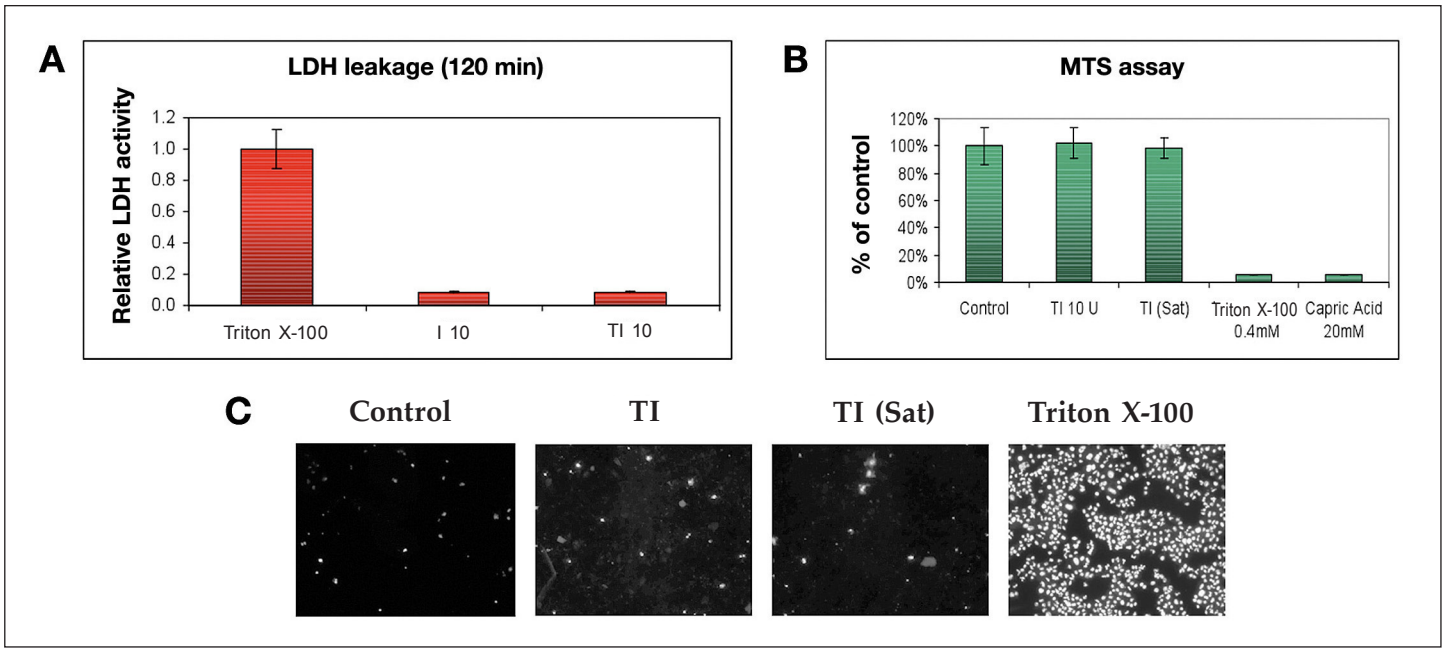


**Figure 6.** Effect of TI on cytotoxicity and plasma membrane integrity. (**A**) Relative LDH leakage for 14-day Calu-3 cell monolayers treated for 2 h in the presence of 0.4 mM Triton X-100, 10 U/ml insulin (I10), or 10 U/ml TI (TI10) (mean ± SD, $n$=3). (**B**) MTS assay results for 3-day Calu-3 cells treated with 0.4 mM Triton X-100, 2 mM capric acid, 10 U/ml TI, or saturated solutions of TI containing precipitate (mean ± SD, $n$ = 12). (**C**) Fourteen-day Calu-3 cell monolayers were treated for 2 h with 10 U/ml TI, 25 U/ml (saturated solution) TI, or 0.4 mM Triton X-100 in the presence of propidium iodide. Cells were imaged using fluorescence microscopy.

**Table 1.**
**Calculations of Average Concentration of Technosphere Insulin and Insulin in the Lung after a Typical Dose of Technosphere Insulin Powder**

| | | |
|---|---|---|
| Liquid volume in lung | | |
| Surface area: | | 70 $m^2$ |
| Thickness of hypophase: | | 0.1 µm |
| Volume of hypophase: | | 7 ml |
| Dose parameters | | |
| Estimated dose to lung: | TI powder | Insulin |
| | 5 mg | 500 µg<br>14.4 U |
| Average concentration in lung: | 0.71 mg/ml | 71 µg/ml<br>2 U/ml |

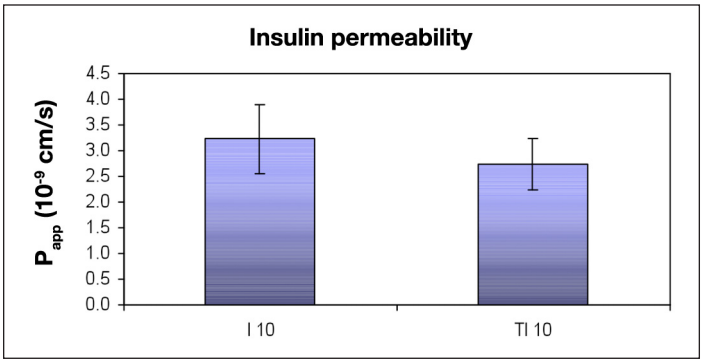


**Figure 7.** Insulin $P_{app}$ in Calu-3 cell monolayers. No significant difference in $P_{app}$ (mean ± SD, $n$ = 3, $p$ = .36) was observed in 14-day Calu-3 cell monolayers treated for 2 h with 10 U/ml insulin ($3.23 \times 10^{-9} \pm 6.7 \times 10^{-10}$ cm/s) or 10 U/ml TI ($2.73 \times 10^{-9} \pm 5.0 \times 10^{-10}$ cm/s).

was comparable to that obtained from the insulin control samples ($p$ = 0.36). Similar values of $P_{app}$ were obtained in experiments conducted at pH 6.2 and 8.2, close to the extremes of pH that could be tolerated by the Calu-3 cells (data not shown). These results indicate that the transport of insulin was not affected by the presence of FDKP or other components of TI.

### *Fluorescent Insulin Uptake Studies*

*Alexa Fluor 488 Insulin Does Not Accumulate Within Calu-3 Cells Treated With Technosphere Insulin*

Calu-3 cell monolayers cultured for 14 days were treated with insulin labeled with Alexa Fluor 488 in the presence or absence of TI and its various components (Technosphere particles, FDKP, and polysorbate 80). No accumulation of fluorescently labeled insulin was detected under any condition tested (**Figure 8**), indicating that the cell membranes were intact. Cells exposed to a saturated suspension of TI also showed no visible signs of insulin accumulation. In contrast, positive controls using 17 mM capric acid or 64 µM digitonin showed significant accumulation of the labeled insulin in the compromised cells. These results confirm those obtained with propidium iodide (**Figure 6C**) and demonstrate that the plasma membrane was not compromised. Identical results (data not shown) were obtained in experiments performed with fluorescently labeled insulin formulated as TI powder that was then dissolved and applied to the cells.

## Discussion

Penetration enhancers act by damaging cell membranes and/or loosening tight junctions.[26–33] Results of these cell-based experiments demonstrated that TI does not function as a penetration enhancer. The actin staining patterns of Calu-3 monolayers exposed to TI were the same as those of control monolayers, while monolayers exposed to known permeation enhancers exhibited gaps in the monolayer and rounding of individual cells. Studies of the transport of Lucifer yellow confirmed the microscopic observations: the permeability of Lucifer yellow through membranes subjected to permeation enhancers was orders of magnitude higher than the permeability measured in monolayers exposed to insulin alone or TI.

Studies in Calu-3 monolayers comparing insulin with TI demonstrated that neither FDKP nor the other components of TI have an effect on actin staining patterns, Lucifer yellow permeability, TEER, or insulin permeability. There was no evidence that TI opened tight junctions, permeabilized the plasma membrane, or otherwise compromised cell monolayer integrity. Furthermore, solutions of dissolved TI powder (FDKP and polysorbate 80) did not induce cytotoxicity.

Studies with fluorescently labeled insulin in Calu-3 indicate that insulin is not transported by a transcellular route and that TI does not compromise the cell membrane. Studies in A549 cells, a transformed cell line that resembles type II pneumocytes but does not form sufficient tight junctions for $P_{app}$ studies,[38,42,43] also show no intracellular accumulation of insulin (data not shown). This provides some evidence that differences in plasma membrane proteins between Calu-3 and one alveolar cell model are not significant enough to change the route of insulin transport. Given the extremely low $P_{app}$ of insulin, it is possible that insulin does accumulate in the cells, but the levels are too low to detect with fluorescence

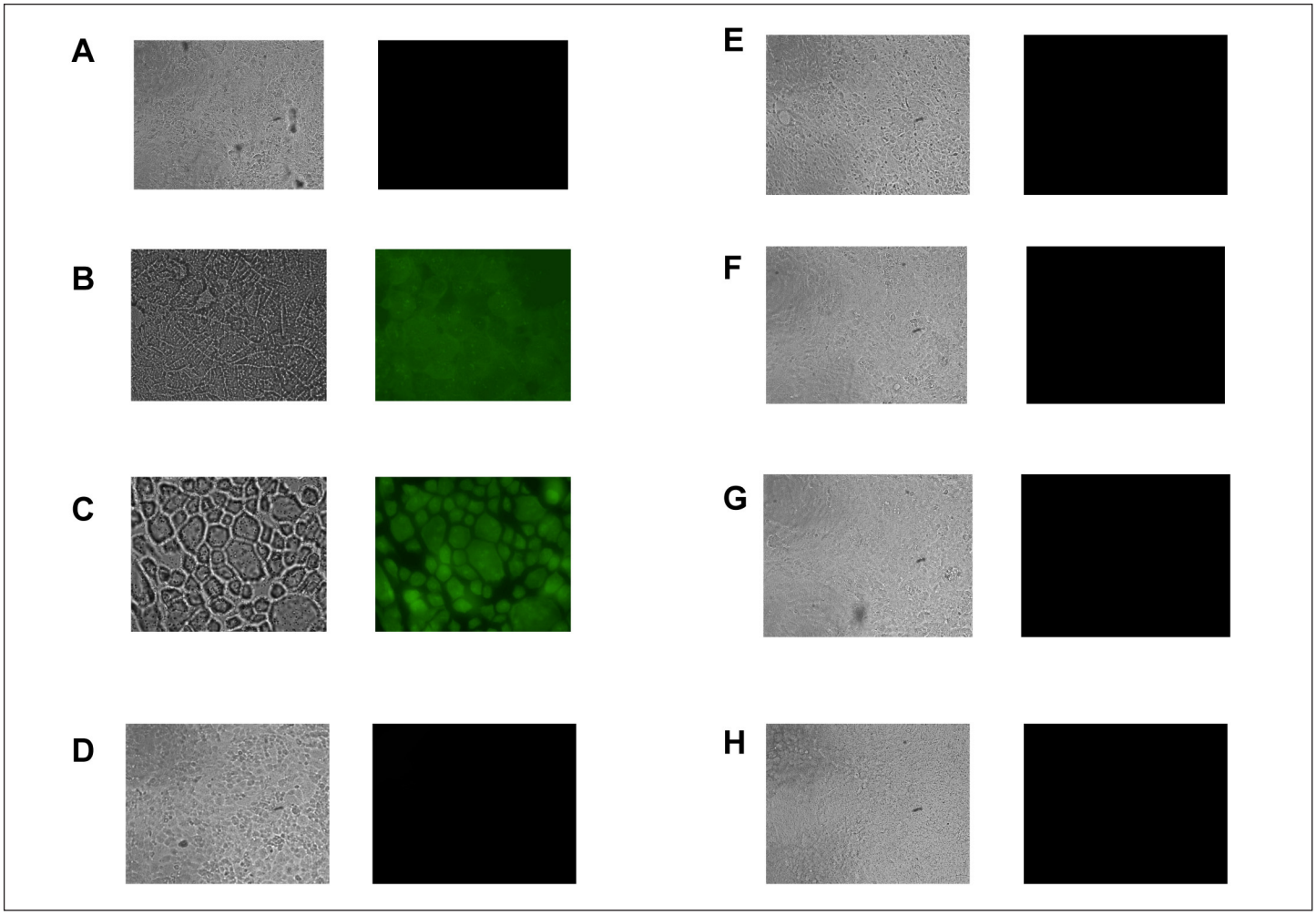


**Figure 8.** Intracellular accumulation of fluorescently labeled insulin. Fourteen-day Calu-3 cell monolayers were treated for 2 h with Alexa Fluor 488 labeled insulin and **(A)** buffer alone, **(B)** 64 μM digitonin, **(C)** 17 mM capric acid, **(D)** 10 U/ml TI, **(E)** 25 U/ml (saturated solution) TI and proportional fractions of 10 U/ml TI components, **(F)** FDKP, **(G)** insulin-free Technosphere particles, and **(H)** polysorbate 80. Cells were imaged by fluorescence microscopy. **(B)** Digitonin and **(C)** capric acid images are at increased magnification (1000x total magnification), illustrating intracellular accumulation of insulin.

microscopy. However, the absence of detectable propidium iodide accumulation in cells exposed to TI suggests that there is no nonspecific disruption of the plasma membrane. With the general effects of TI now established, future studies with type I-like pneumocytes would be the logical next step.

These results demonstrate the safety of the TI platform and provide valuable information about the mechanism of action. Even though the pharmacokinetic profile of insulin administered as TI is characterized by rapid absorption of insulin, neither FDKP nor other components of TI was found to have an effect on insulin permeation through individual cells or cell monolayers. The rapid absorption of insulin may result from "upstream" processes such as highly efficient delivery powder to the deep lung and rapid dissolution. The current experiments, which used pre-equilibrated dosing solutions and suspensions, could not address these phenomena. Transport studies that incorporate the distribution and dissolution of TI powder are a challenging but critical next step for investigation.

These studies raise interesting questions about how TI provides such a rapid pharmacokinetic profile without directly affecting the $P_{app}$ of insulin. One possibility is that, due to the high solubility of TI at physiological pH, rapid dissolution paired with a steep concentration gradient established by depositing powder on the alveolar surfaces facilitates a rapid increase in insulin flux into the bloodstream. The concentration gradient decreases as insulin is absorbed and the powder is diluted by the lung's natural clearance mechanism, so the rate of absorption decreases to produce the ultrarapid pharmacokinetic profile observed in human subjects.

## Conclusions

Technosphere Insulin, FDKP, and the other components of TI powder are not penetration enhancers in Calu-3 cells. Technosphere Insulin does not disrupt the tight junctions between cells, permeabilize or damage the plasma membrane, or induce cellular toxicity. These findings, combined with observation that neither TI nor FDKP increase insulin $P_{app}$, support the hypothesis that FDKP plays a passive role at the site of insulin absorption and that its function in TI is to act as a particle substrate that delivers insulin to the deep lung, where natural mechanisms of absorption then occur.

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**Disclosure:**

All authors are employed by MannKind Corporation.

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