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Effect of budesonide in airway epithelial cell line Calu-3 with reduced CFTR function

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ABSTRACT

The purpose of this study was to evaluate the effect of inhaled corticosteroid budesonide in altering antibacterial and protein secretory properties of airway epithelial cell line Calu-3 with reduced cystic fibrosis transmembrane conductance regulator (CFTR) function. Calu-3 cells with reduced CFTR function (Calu-3A) was produced by stable transfection of cells with anti-sense CFTR oligonucleotides. The antibacterial activity and protein secretions were examined using the apical surface fluid (ASF) washings of monolayers grown at an air-liquid interface. At the basal level Calu-3A exhibited an increased protein secretion, decreased lysozyme secretion and no change in IL-8 secretion compared to Calu-3 cells. Both Calu-3 and Calu-3A exhibited a dose dependent increase in ASF antibacterial properties after culturing with budesonide (0.01 to 100 μg/ml). But budesonide (100 μg/ml) significantly increased total protein and decreased lysozyme and IL-8 secretion in Calu-3A. Our results suggested that the altered level of antibacterial activity was associated with the altered ASF protein secretion, and the increase or decrease in ASF antibacterial activity in Calu-3 and Calu-3A was independent of ASF lysozyme content. We demonstrated that budesonide treatment resulted in significant changes in protein secretion in Calu-3 cells when CFTR function was reduced. Above studies might have implications in understanding the effect of inhaled medication budesonide in cystic fibrosis airways.

Key words : Calu-3 cells, Budesonide, CFTR, Airway.

INTRODUCTION

Inhaled corticosteroids are being increasingly used in reducing lung damage arising from inflammation. In patients with CF lung disease, excessive inflammatory response relative to the bacterial burden leads to deteriorating lung function with an increased rate of morbidity and mortality. Budesonide is an inhaled glucocorticosteroid with an improved therapeutic index in the treatment of CF lung disease[1, 2]. Epithelial cells are considered to be among the most relevant cellular targets of topically delivered glucocorticoids.

It has been proposed that human airway epithelial cell line Calu-3, grown at an air-liquid interface culture are a suitable *in vitro* model of the airway epithelium to study inhaled glucorticosteroid (budesonide) transport, absorption and local metabolism[3]. Calu-3 cells possess cilia and secrete mucus and protein components associated with inflammation and host defense [4, 5]. The activity of MRP1 and P-glycoprotein efflux systems, which are relevant for drug delivery at the airway epithelium, have been examined in Calu-1 cells and budesonide has been shown to reduce Calu-1 MRP1 expression[6]. Budesonide binds to glucocorticoid receptor with high affinity resulting in activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-kB) [7]. These transcription factors are known to be involved in the up-regulation of several chemokines induced in epithelial cells, including RANTES and eotoxin [8, 9]. In Calu-1 and alveolar (A549) epithelial cells, budesonide regulate the expression vascular endothelial growth factor (VEGF) through its glucocorticoid receptor mediated action [10]. In other *in vitro* experimental models, budesonide has been shown to be potent in reducing eosinophil survival primed by epithelial cell secretions from nasal mucosa and GM-CSF release from cultured nasal mucosa epithelial cells[11, 12].

There is growing evidence to support a central role for CFTR in maintaining respiratory tract homeostasis due to its role in fluid and electrolyte transport, protein secretion and mucin production in the airway [13, 14]. Calu-3 cells expresses high levels of functional CFTR, hence it has extensively been used in understanding the CFTR-mediated ion transport defect in CF [15-17]. At an air-liquid interface culture Calu-3 cells secrete ASF, which contained significant amount of *in vitro* antibacterial activity to bacterial strains commonly found in CF airways and treatment of Calu-3 with budesonide resulted in 4- to 10-fold increase in ASF antibacterial activity, which suggested that treatment of epithelial cells with pharmacological agents could influence on ASF antibacterial properties[18]. Recently we identified measurable levels of proinflammatory mediator IL-8 and antibacterial component lysozyme in Calu-3 ASF secretions and these cells exhibited significant changes in the secretion of ASF proteins when treated with ion transport mediators known to alter CFTR activity, which suggested a possible involvement of CFTR in these processes [5].

In the present study we evaluated the effect of budesonide in Calu-3 cells when CFTR function is defective. We tested the hypothesis that reduced level of CFTR function in Calu-3 could result in altered Calu-3 ASF properties and that budesonide significantly alter the Calu-3 protein secretion in ASF. To generate Calu-3 cells with reduced CFTR function (Calu-3A), we stably transfected Calu-3 with expression vectors that generate anti-sense CFTR mRNA. We assessed Calu-3 and Calu-3A antibacterial activity and protein secretions in ASF at the basal level and after culturing cells with budesonide.

EXPERIMENTAL SECTION

Methodology

Calu-3 and Calu-3A cell culture at an air-liquid interface

Calu-3 and Calu-3A cells were grown at an air-liquid interface culture as described previously [18, 19]. Calu-3A cells with reduced levels of CFTR protein was generated by expressing anti-sense mRNA [20, 21]. Calu-3 cells were stably transfected with expression vector (pcDNA3.1) containing CFTR nucleotides 951 to 1850 in the anti-sense orientation. We used LipoTAXI mammalian transfection kit (Stratagene,La Jolla,CA) for transfection of Calu-3 cells and stable neomycin resistant clones with antisense mRNA were selected by RT-PCR. Calu-3A with reduced level of CFTR function was confirmed by the reduction of forskolin-mediated iodide-efflux assay. Both Calu-3 and Calu-3A cells were cultured at 37°C and 5% CO_2 in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) that was supplemented with 15% fetal calf serum (FCS), 500 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained in 75-cm^2 tissue culture flasks and split when 80-90% of confluency was reached. Air-liquid interface culturing of Calu-3 cells was carried out by plating cells at 2 X 10^6 cells/cm^2 onto Costar transwell inserts (0.4 µm pore size, 12 mm diameter, clear polycarbonate membrane, Costar plates; Corning Inc., Corning, NY) that had been coated with human placental collagen. Culture medium within the insert (apical side) was removed on the second day after plating cells and medium was changed basolaterally on alternate days. After approximately 11 days of culture at an air-liquid interface, confluent monolayers with a transepithelial resistance (TER) value of greater than 300ohm·cm^2 were basolateraly treated with budesonide (100 µg/ml) and air-liquid interface culture was continued. Cells were treated with budesonide for 96 hr, because we expect a significant amount of basal level fluid and protein accumulation by then. Sham treated monolayers were used as controls.

Antibacterial assay with ASF washings

Calu-3 and Calu-3A ASFs were collected by washing the apical surface of each new monolayer three times with 60 µl of sterile water and combining the washes and in vitro antibacterial activity was carried out [5, 18, 22, 23]. In ASF washings in ~ 180 µl of water were centrifuged for 15 minutes at 13,000 g at 4°C to remove cell debris. The supernatant referred to as the ASF washings were assayed at the time of collection or stored in aliquots (-20°C) until used in assays. For antibacterial assay, *Escherichia coli* M15 (E. *coli*) were washed three times with sterile, non-bacteriostatic water and resuspended to 10^4 or 10^6 cells/µl. Serial dilutions were used to obtain samples with the desired number of colony-forming units (CFU) per microliter. In this fashion, known amounts of bacteria assayed by plating on luria broth (LB) agar plates were added to 30 µl of ASF washings and incubated overnight at 37°C. The bacteria-ASF mixture was then plated onto LB plates and CFU were counted after 24 hr. The antibacterial activity in ASF washings was performed at the basal level and after culturing Calu-3A and Calu-3 cells with budesonide.

Measurement of ASF lysozyme levels by *Micrococcus lysodeikticus* assay

Calu-3 and Calu-3A cells were treated with CFTR channel agonist forskolin and lysozyme levels in ASFs were measured by turbidimetric assay based on the enzymatic hydrolysis of *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell wall and measuring a fall in optical density at 450 nm [23, 24, 25]. The above method was employed because we have previously observed forskolin dependent changes in Calu-3 lysozyme secretion, which suggested a possible

involvement of CFTR in lysozyme or protein secretion [23]. In the present study we expect forskolin dependent increase in lysozyme secretion when CFTR function is defective in Calu-3A cells. Cells were basolateraly treated with forskolin (50μM) for 24 hr and ASF lysozyme levels were measured by incubating 20 μl of ASF washing in a suspension of *M. lysodeikticus* (0.3 mg/ml) for 18 hrs at 37°C. Lysozyme standards (0–500 ng/ml) and *M. lysodeikticus* suspensions were prepared in 50 mM sodium phosphate buffer pH 7.4 containing sodium azide (1mg/ml) and bovine serum albumin (1mg/ml). A standard curve was derived and the concentration of lysozyme in each sample was determined.

Measurements of ASF total protein, IL-8 and lysozyme by immunoassay

Calu-3 and Calu-3A ASF washings were collected at the basal level and after treating with budesonide. The total protein concentration in each ASF washing was measured using a DC protein assay kit obtained from Bio-Rad Laboratories (Bio-Rad, Hercules, CA). Quantitative immunoassays were used to measure IL-8 and lysozyme levels in ASF washings. The values were determined and the concentration of IL-8 and lysozyme was normalized against the total protein of respective ASF washing. The IL-8 kit (sensitivity 10 pg/ml) was obtained from R & D systems Inc (Minneapolis,MN) and the lysozyme kit (sensitivity 0.5 ng/ml) was obtained from ALPCO Diagnostics (Windham,NH).

Statistical Analysis

The protein values are expressed as mean ± standard error of the mean (S.E.M). One-way and repeated analysis of variance with post hoc analysis by the Student-newman-Keuls method was used with $P< 0.05$ considered significant.

RESULTS

In CF airways, loss of functional CFTR is known to cause reduced level of antibacterial activity. We tested if culturing Calu-3 and Calu-3A with budesonide would result in altered ASF antibacterial properties. Cells were basolateraly treated with budesonide for 72 hrs and ASF washings were collected and results were compared with controls. Both Calu-3 and Calu-3A ASF washings exhibited an increased level of ASF antibacterial activity to E.*coli* after culturing cells with budesonide (100 μg/ml) (Fig.1). To extend these findings, dose-response studies were carried out by culturing Calu-3 and Calu-3A cells with budesonide at concentrations between 0.01 to 100 μg/ml. The antibacterial activity was tested in ASF washing by incubating with 10^4 CFU of E.*coli*. As shown in Fig 2, a dose dependent inhibition of bacterial growth was observed in both Calu-3 and Calu-3A ASF washings. In all cases, the addition of drug directly to ASF washings from control cultures was without effect on ASF antibacterial activity.

To define changes in ASF protein composition between Calu-3A and Calu-3 cells, we analysed total protein, IL-8 and lysozyme content in ASF washings after culturing cells in the presence of budesonide (100 μg/ml). Quantitative immunoassays were used to measure IL-8 and lysozyme levels in ASF washings (Fig.3). When Calu-3 and Calu-3A were treated with sham or budesonide, the total protein concentration recovered in ASF washings of Calu-3A was greater than Calu-3 ASF washings, but contained significantly reduced levels of lysozyme compared to Calu-3 ($P< 0.001$). There was no significant change in IL-8 level in ASF washings of Calu-3 and Calu-3A in sham treatment. However, budesonide treatment resulted in a significant increase in

IL-8 levels in ASF washing of Calu-3 cells compared to sham. In contrast, budesonide treatment of Calu-3A resulted in significant decrease in IL-8 levels in ASF washings compared to sham treatment ($P< 0.001$). These results indicated that under basal conditions, Calu-3A ASF had significantly higher total protein and reduced lysozyme level. Budesonide significantly increased Calu-3 IL-8 secretion and influenced Calu-3A protein secretion.

DISCUSSION

In the present study we used inhaled corticosteroid budesonide, which is routinely used in treating CF patients [1, 2, 25, 26]. We demonstrated that budesonide had a significant effect in increasing ASF antibacterial activity in both Calu-3 and Calu-3A cells. At the basal level Calu-3A exhibited an increased protein secretion, decreased lysozyme secretion and no change in IL-8 secretion compared to Calu-3 cells. Calu-3 A cells responded to budesonide by increasing total protein and decreasing lysozyme and IL-8 secretion. We demonstrated above changes in protein secretion of Calu-3 and Calu-3A ASF, when these cells were grown at an air-liquid interface, wherein cells were in direct contact with air, simulating *in vivo* condition of respiratory epithelium. Further the collection of ASF washings from apical surface enabled us to evaluate the protein secretory properties of respiratory epithelium in response to budesonide treatment.

An earlier study suggested that budesonide treatment of Calu-1 at an air-liquid interface culture did not cause changes in the cytotoxicity of cells, as verified by colorimetric MTT assay and also no change observed in tight junctional properties of Calu-3 monolayers as measured by transepithelial resistance (TER) which indicated the integrity of epithelial monolayers in culture [3, 10]. Similarly, in the present study we observed no difference in the TER values between Calu-3 and Calu-3A monolayers at the basal level or after treating with budesonide. Moreover, the reduced level of CFTR expression in Calu-3A had no effect in altering the integrity of monolayer (data not shown). Our results indicated that Calu-3A cells with reduced CFTR function contained reduced level of ASF antibacterial activity and reduced lysozyme secretion. The reduced level of Calu-3A lysozyme secretion was confirmed by enzymatic degradation of M.*lysodeikticus* assay as well as using an immunoassay.

We have earlier shown that Calu-3 ASF antibacterial activity has a number of similarities to previously characterized antibacterial activity of ASF from primary cultures of airway cells [18]. In the present study, the reduced level of antibacterial activity seen in Calu-3A ASF washings is analogous to the reduced level of antibacterial activity seen in CF airway disease [13, 14]. However the mechanisms involved in reduced CFTR function in CF airways leading to reduced antibacterial activity and Calu-3A with reduced antibacterial properties remain unclear. In the human airway, the protein composition of ASF displays a remarkably high complexity and various antimicrobial components of innate immunity act in concert to protect the lung from infection [14, 27-29]. For example, the antibacterial activity of lysozyme and LL37/hCAP-18, a cathelicidin, have previously been reported to be synergistic *in vitro* [29]. However in this study, the presence of other antibacterial factors such as β-defensins, LL-37/hCAP-18, lactoperoxidase were not excluded as the crucial components contributing to antibacterial properties of Calu-3 or Calu-3A ASF. The mechanisms of synergistc actions of these components need to be delineated.

Based on our observations, we hypothesize that the alteration in antibacterial activity of Calu-3 and Calu-3A ASF could be due to altered protein secretion in the ASF. When Calu-3A cells were treated with forskolin, we observed an increased lysozyme secretion and this finding is in agreement with our previous observation that forskolin decreased lysozyme in wild type Calu-3 cells [23], suggesting that CFTR might play a role in lysozyme secretion. Similarly, studies of Duszyk et al suggested a role for functional CFTR in regulation of lysozyme secretion in human airways [30]. Another study suggested that pharmacological correction of the defective CFTR-mediated protein secretion is promising for the development of a rational drug therapy for CF patient[31,32]. Therefore, increasing evidence suggest that CFTR plays a role in airway protein and mucin secretion [23, 31-33]. The pharmacological effects of synthetic drugs and steroids have previously have previously been tested in Asthma and other airway diseases [34,35].

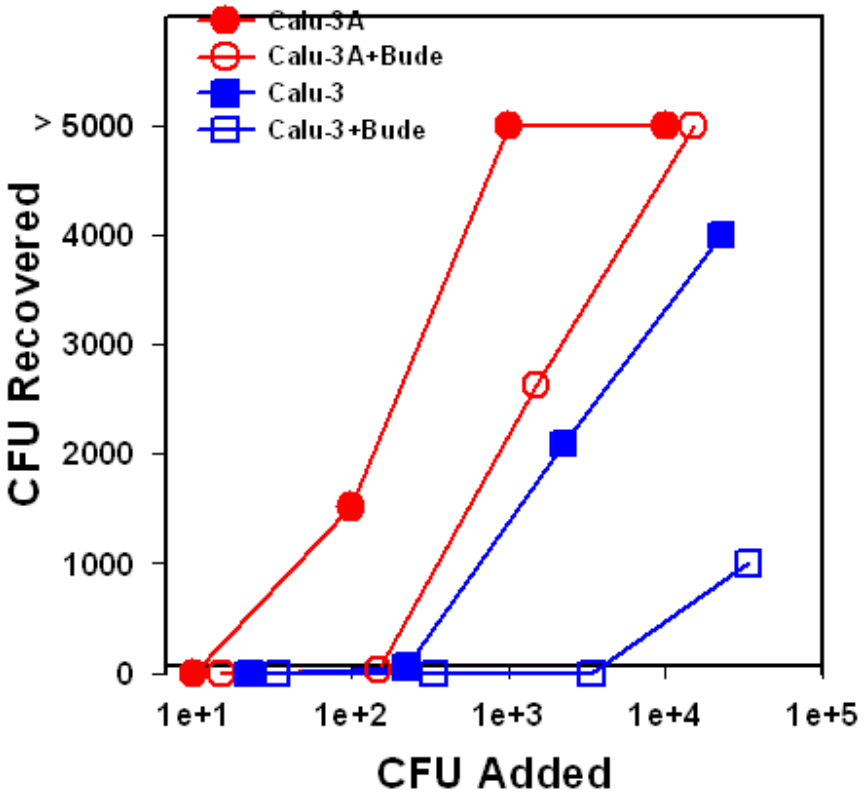

Figure 1: Response of Calu-3A and Calu-3 cells to budesonide (100 µg/ml) treatment for 48 hr. The indicated number of E. *coli* CFU were incubated overnight in 30µl of each ASF washing. Then ASF washings were plated onto LB agar and recovered CFU counted after 24 hr. Controls were sham treatment with sterile water. Data are representative of 3 experiments for each inoculum of E. *coli*.

We observed that Calu-3 and Calu-3A cells secreted different basal levels of total protein and lysozyme. There was an increase in ASF total protein concentration in Calu-3A compared to Calu-3 ASF. However this increase was not due to increased lysozyme or IL-8 secretion, because Calu-3A exhibited significantly decreased lysozyme secretion in sham treatment, which might contribute to decreased level of antibacterial activity. Interestingly, budesonide treatment exhibited a significant effect in altering the protein composition of Calu-3A ASF. IL-8 is a potent

neutrophil chemo-attractant that has been implicated in a number of inflammatory airway diseases such as CF, and has recently been shown to influence on antibacterial protein secretion [32,33,36]. Our studies indicated that budesonide exhibited opposite effects in Calu-3 and Calu-3A IL-8 secretion, possibly suggesting a putative role of CFTR in the release of this proinflammatory mediator by epithelial cells. The identity of other Calu-3A proteins, increased due to budesonide treatment, remain undetermined in this study and altered protein composition of ASF might be due to altered CFTR function. Our studies suggest that the altered level of antibacterial activity was associated with the altered ASF protein components, and the increase or decrease in ASF antibacterial activity in Calu-3 and Calu-3A was independent of ASF lysozyme content.

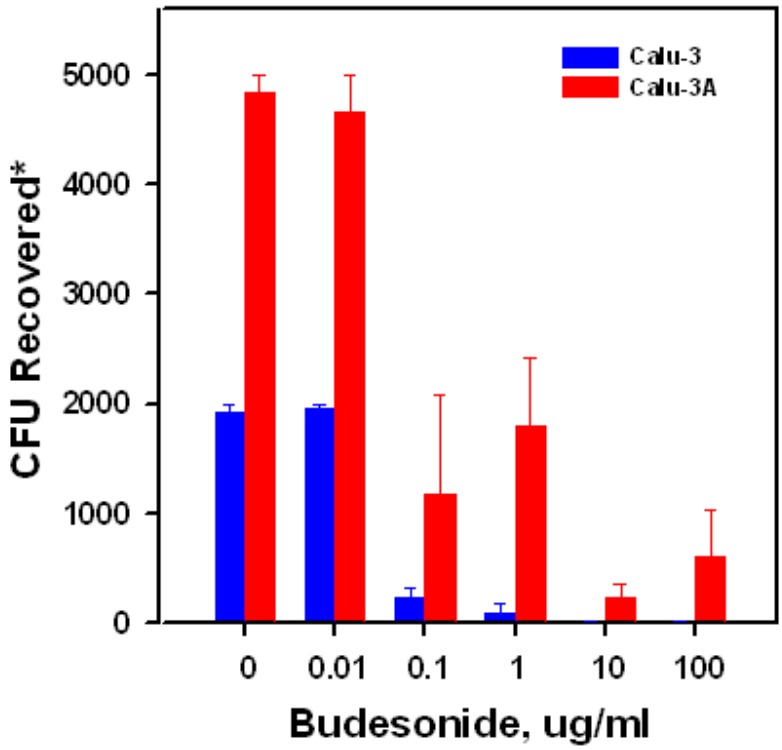

Figure 2: Dose response relationship for ASF antibacterial activity in Calu-3A and Calu-3 cells after culturing cells with budesonide for 48 hr. Each ASF washing was assayed with 10^5 E.*coli* CFU and the recovered CFU plotted as a function of budesonide concentration. Data are representative of 3 experiments at each condition.

In summary, we demonstrated both Calu-3 and Calu-3A responded to budesonide by increasing the ASF antibacterial activity, but the secretion of proteins by Calu-3A with reduced CFTR function was significantly altered. Though increasing evidence suggest a role for CFTR in airway protein secretion, the mechanisms of altered CFTR function leading to chronic bacterial infections in CF airways remain incompletely understood. The present study may be useful in understanding the role of inhaled medication in altering airway antibacterial properties and protein secretions, which may find clinical implications in understanding the defective CFTR function in CF airways.

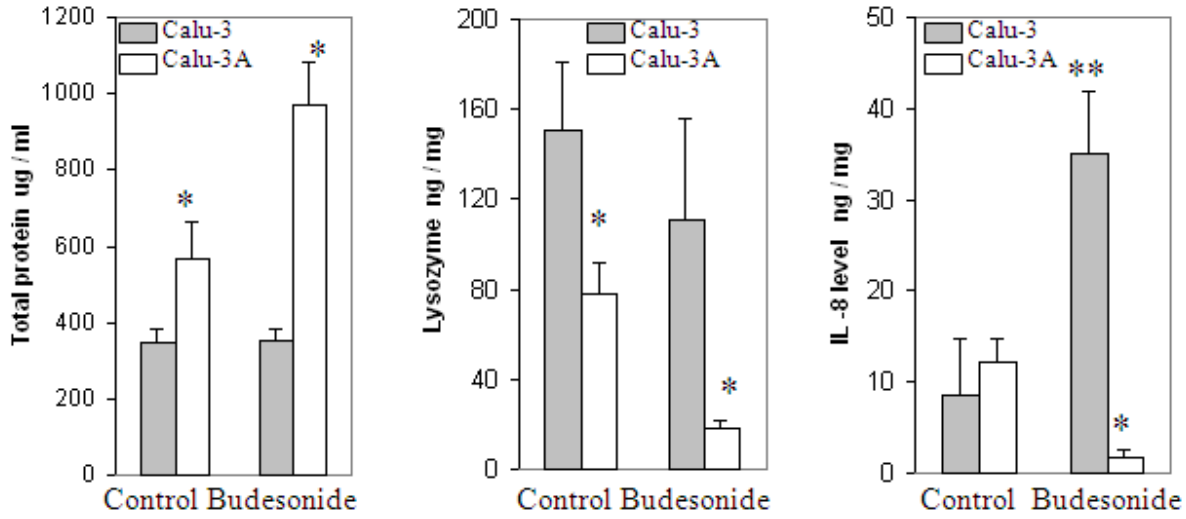

Figure 3. The effect of budesonide on Calu-3 and Calu-3A protein secretion. Cells were treated with budesonide (100 µg/ml) for 72 hrs and apical surface fluid (ASF) washings were used to measure total protein, lysozyme and IL-8 levels. The total protein was measured using DC protein assay (Bio-Rad Laboratories). Lysozyme and IL-8 levels were measured with quantitative immunoassays (R&D Laboratories & ALPCO Diagnostics respectively) and the concentrations were normalized to the total protein concentration of respective ASF washing. Controls were sham treatment with sterile water. (n=9 for each condition). *P<0.001 signficant difference of Calu-3A was compared with respective Calu-3 in each group and **P<0.0001 budesondie treated Calu-3 was compared with Calu-3 control.

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