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GS-5806 Inhibits Pre- to Postfusion Conformational Changes of the Respiratory Syncytial Virus Fusion Protein

Dharmaraj Samuel,[a]* Weimei Xing,[a] Anita Niedziela-Majka,[a] Jinny S. Wong,[b] Magdeleine Hung,[a] Katherine M. Brendza,[a] Michel Perron,[a] Robert Jordan,[a] David Sperandio,[a] Xiaohong Liu,[a] Richard Mackman,[a] Roman Sakowicz[a]

Gilead Sciences, Inc., Foster City, California, USA[a]; Gladstone Institutes, University of California, San Francisco, California, USA[b]

GS-5806 is a small-molecule inhibitor of human respiratory syncytial virus fusion protein-mediated viral entry. During viral entry, the fusion protein undergoes major conformational changes, resulting in fusion of the viral envelope with the host cell membrane. This process is reproduced *in vitro* using a purified, truncated respiratory syncytial virus (RSV) fusion protein. GS-5806 blocked these conformational changes, suggesting a possible mechanism for antiviral activity.

Respiratory syncytial virus (RSV) is an enveloped, single-stranded, negative-sense RNA virus that belongs to the *Pneumovirinae* subfamily of *Paramyxoviridae* (1). RSV infects the respiratory tract of infants, young children, and immunocompromised adults, causing severe disease (2–11). GS-5806 is a small-molecule inhibitor of RSV replication that is active against a diverse collection of RSV A and RSV B clinical isolates, with a mean 50% effective concentration (EC_{50}) of 0.43 nM (12, 13). GS-5806 blocks RSV fusion (F) protein-mediated cell-cell fusion, and mutations that confer drug resistance map to the RSV F gene, suggesting that the target of GS-5806 is the RSV F protein. Viral-cell membrane coalescence mediated by paramyxovirus fusion proteins involves several proteins, such as an attachment protein, cell surface receptors, and other cellular components that trigger conformational changes in the fusion protein that catalyze fusion of the two membranes (14–16). *In vitro*, triggering of pre- to postfusion conformational changes of RSV F proteins can be achieved by lowering the ionic strength of the buffer or by increasing the temperature (17–19). The conformational changes expose the buried hydrophobic fusion peptides, which interact with fusion peptides of neighboring molecules to form rosette-like structures (see Fig. S2, top panel, in the supplemental material). These macromolecular structures are distinct and easily observed by electron microscopy (EM). The conformational changes can also be triggered in the presence of liposomes prepared in low-ionic-strength buffer. The RSV F protein triggered in the presence of liposomes inserts into the lipid bilayer presumably mediated by the fusion peptides. To evaluate the effects of GS-5806 on the pre- to postfusion conformational changes of RSV F, we expressed the extracellular domain of RSV F protein (ΔTM-RSV F) in HEK293 cells. The protein was stored in high-ionic-strength buffer (500 mM NaCl, 250 mM imidazole, 20 mM Tris, pH 8.0) to keep it in pretriggered conformation. On exposure to low-ionic-strength buffer (10 mM HEPES, pH 8.0) (see Fig. S1 in the supplemental material), ΔTM-RSV F formed rosettes or inserted into liposomes that were easily observable by EM (see Fig. S2, bottom panel, in the supplemental material). These experiments were used to measure the effect of GS-5806 on the conformational changes of ΔTM-RSV F protein (18).

ΔTM-RSV F protein was triggered in the presence of GS-5806 (5-fold molar excess over protein), an inactive analog of GS-5806 (GSC-1), or 0.1% dimethyl sulfoxide (DMSO). The number of rosettes observed in 6 to 8 random EM images was quantified by visual inspection. The average number of rosettes per image in the DMSO- (control), GSC-1-, and GS-5806-treated samples was 108, 106, and 23, respectively. The decrease in the number of rosettes formed in the presence of GS-5806 was significant compared to that with the GSC-1-treated ($P < 0.002$) or DMSO-treated ($P < 0.002$) samples (Fig. 1A). The inhibitory effect of GS-5806 was dose dependent, with fewer rosettes observed with increasing concentrations of GS-5806 (Fig. 1B).

An RSV F resistance variant that contains a threonine-to-alanine amino acid change at position 400 of the RSV F protein was selected *in vitro* (12). ΔTM-RSV F T400A protein purified in the prefusion conformation was also triggered by low-ionic-strength buffer in a manner similar to that in the wild-type protein, but this process could not be inhibited by GS-5806. The numbers of rosettes formed in the presence of 0.1% DMSO (44 ± 9) and GS-5806 (46.6 ± 8) were similar (Fig. 1C), consistent with the reduced efficacy of GS-5806 observed in RSV variants expressing the T400A protein.

The effect of GS-5806 on ΔTM-RSV F protein conformational change was further evaluated by a liposome binding experiment. In order to increase the chances of insertion into the lipid bilayer and to avoid rosette formation, the liposome concentration was kept high (~8 mM, 3,000-fold excess relative to RSV F). During the triggering process, RSV F molecules inserted into a few liposomes rather than partitioning evenly across all of the liposomes. The number of liposomes containing ΔTM-RSV F protein mole-

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Address correspondence to Dharmaraj Samuel, dharmaraj.samuel@pfizer.com.

* Present address: Dharmaraj Samuel, Centers for Therapeutic Innovation, Pfizer Inc., San Francisco, California, USA.

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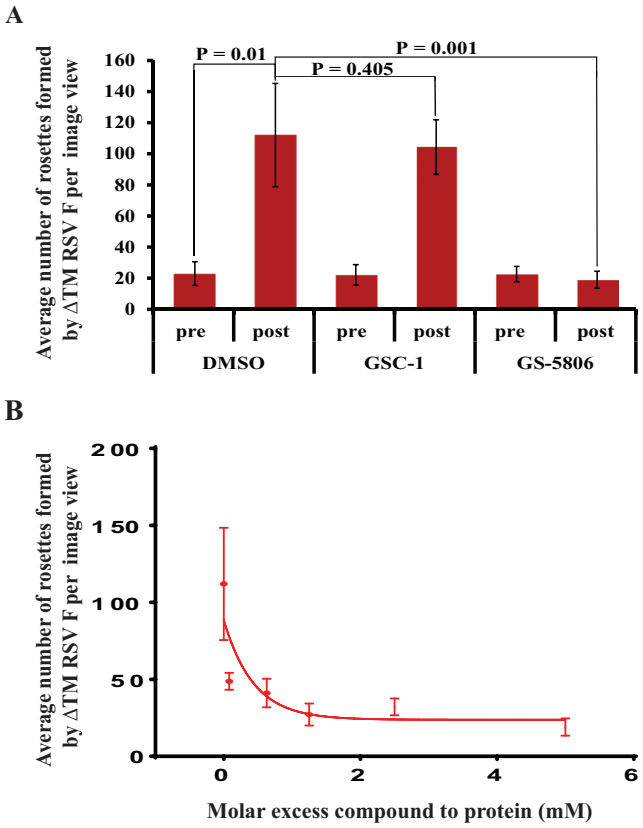

FIG 1 (A) GS-5806 inhibits pre- to posttriggered conformational changes of RSV F protein. Pre- to posttriggered conformational change was initiated by dialyzing ΔTM-RSV F protein overnight at 4°C in low-ionic-strength buffer (10 mM HEPES, pH 8.0) in the presence of a 5-fold molar excess of GS-5806, GSC-1 (an inactive analog), or DMSO (~0.1%). The mean number of rosettes observed per grid view for different samples was calculated from at least 6 randomly selected EM images. The plot shows the mean values, with error bars representing the standard deviation. (B) The formation of rosettes in GS-5806-containing samples decreased in a dose-dependent manner. The mean number of rosettes observed per grid view for different samples containing various concentrations of GS-5806 was calculated from 6 to 10 randomly selected EM images and plotted as a function of GS-5806 concentration. The error bars represent the standard deviation of the mean values. (C) The ΔTM-RSV F protein containing the T400A amino acid change is associated with reduced susceptibility to GS-5806. The effects of GS-5806 on ΔTM-RSV F T400A protein rosette formation were evaluated. The mean number of rosettes observed per grid view for different samples was calculated from 6 randomly selected EM images. The plot shows the mean values, with error bars representing the standard deviation.

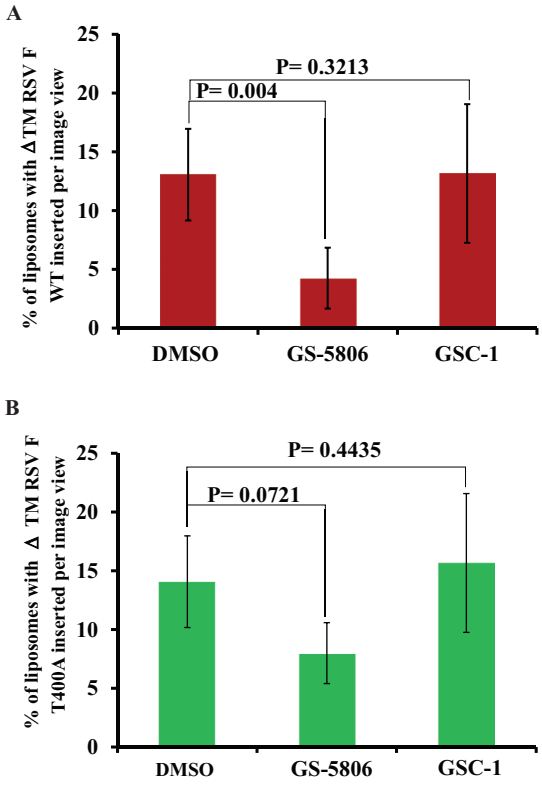

FIG 2 (A) GS-5806 inhibits the deposition of the ΔTM-RSV F protein into liposomes. The ΔTM-RSV F protein conformational change was triggered by mixing with liposomes prepared in low-ionic-strength buffer. The number of liposomes containing ΔTM-RSV F molecules in GS-5806-, GSC-1-, or DMSO-treated samples was quantified from 7 randomly selected EM images and represented in the plot as a percentage of the total liposomes in the sample. The error bars represent the standard deviations of the mean values. (B) GS-5806 does not affect the deposition of RSV F T400A protein on liposomes. The number of liposomes containing ΔTM RSV F T400A molecules in GS-5806-, GSC-1-, or DMSO-treated samples was quantified from 7 randomly selected EM images and represented in the plot as a percentage of the total liposomes in the sample. The error bars represent the standard deviations of the mean values.

cules was quantified by inspection of seven randomly selected EM images for each experiment. Very few ΔTM-RSV F-inserted liposomes were observed in the GS-5806-treated sample compared to those observed in the DMSO- or GSC-1-treated samples (Fig. 2A). On average, 4% ± 3% of ΔTM-RSV F-containing liposomes were observed in the GS-5806-treated sample, whereas 13% ± 4% (DMSO) or 13% ± 6% (GSC-1) of ΔTM-RSV F-containing liposomes were observed in control samples. Interestingly, the average number of ΔTM-RSV F molecules per liposome in the GS-5806-treated sample was 7 ± 3, versus 25 ± 10 in the DMSO-treated or GSC-1-treated samples. Similarly, the number of liposomes with ΔTM-RSV F T400A molecules in GS-5806-treated samples was 8% ± 3%, versus those in the DMSO-treated (14% ± 3%) or GSC-1-treated (16% ± 2%) samples (Fig. 2B). Unlike ΔTM-RSV F, the average numbers of ΔTM-RSV F T400A molecules deposited per liposome (~30) were similar for all three treatments. These observations are supported by liposome flotation experiments (see Fig. S3 in the supplemental material). Rosette formation and liposome association experiments show that GS-5806 interferes with the pre- to posttriggered conformational changes

of ΔTM-RSV F protein, similar to the conformational changes inhibited by influenza virus entry inhibitor (19).

Several RSV entry inhibitors representing diverse chemical classes (VP-14637, TMC-353121, and BMS-433771) have been reported in the literature (20–23). In the presence of these inhibitors (5-fold molar excess over protein), the number of rosettes formed was reduced by 2- to 5-fold compared to those with the DMSO control (see Fig. S4 in the supplemental material). The binding sites for two of these entry inhibitors, TMC-353121 and BMS-433771, were identified by X-ray crystallography and chemical cross-linking methods and found to be close to the six-helix bundle of the RSV F protein (24, 25). However, GS-5806 did not influence the formation of six-helix bundles when isolated peptides were mixed in the presence of GS-5806 (see Fig. S5A in the supplemental material). In addition, differential scanning calorimetry, isothermal titration calorimetry, and circular dichroism did not detect direct interaction of GS-5806 with isolated six-helix bundles (see Fig. S5B in the supplemental material). These results suggest that GS-5806 does not interact with the isolated six-helix bundle the same as TMC-353121 or BMS-433771 compounds but still interferes with the transition of the RSV F protein from the pre- to posttriggered conformation to elicit that it is antiviral activity.

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D.S. performed protein purification, characterization, and assays; analyzed the data; and wrote the manuscript. W.X. expressed RSV F proteins in HEK293 cells, purified protein, prepared samples for assays, and analyzed data. A.N.-M. prepared samples for liposome assays and analyzed data. J.S.W. performed EM analysis. M.H. cloned RSV F genes. K.M.B. performed mass spectrometry analysis of the RSV F variants. M.P. performed cell culture experiments with the compounds. R.J. helped to prepare the manuscript. D.S. prepared compounds used in the assays. X.L., R.J., R.M., and R.S. provided scientific insights and critical comments.

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