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Structural analogue of sildenafil identified as a novel corrector of the F508del-CFTR trafficking defect

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Running title : Sildenafil analogue corrects F508del-CFTR trafficking

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HTS, high throughput screening; ER, endoplasmic reticulum; PKA, protein kinase A; 4-PBA, 4-phenylbutyrate; 3HA, three haemaglutinin-epitope tags; BHK, baby hamster kidney

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Abstract

The F508del mutation impairs trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR) to the plasma membrane and results in a partially functional chloride channel that is retained in the endoplasmic reticulum and degraded. We recently used a novel High Throughput Screening (HTS) assay to identify small molecule correctors of F508del CFTR trafficking and found several classes of hits in a screen of 2000 compounds (Carlile et al., 2007). In the present study we have extended the screen to 42,000 compounds and confirmed sildenafil as a corrector using this assay. We evaluated structural analogues of sildenafil and found that one such molecule called KM11060 (7-chloro-4-{4-[(4-chlorophenyl) sulfonyl] piperazino}quinoline) was surprisingly potent. It partially restored F508del trafficking and increased maturation significantly when baby hamster kidney (BHK) cells were treated with 10 nM for 24 h or 10 μM for 2 h. Partial correction was confirmed by the appearance of mature CFTR in Western blots and by using halide flux, patch-clamp and short-circuit current measurements in unpolarized BHK cells, monolayers of human airway epithelial cells (CFBE41o$^-$), and intestines isolated from F508del-CFTR mice **(** *Cftr* ^{tm1Eur}**)** treated *ex-vivo*. Small molecule correctors such as KM11060 may serve as useful pharmacologic tools in studies of the F508del-CFTR processing defect and in the development of CF therapeutics.

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Introduction

Cystic Fibrosis (CF) is a common lethal genetic disease affecting Caucasians and is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Rommens et al., 1989; Riordan et al., 1989). CFTR is a cAMP-regulated anion channel expressed in airway, intestinal, pancreatic, and other epithelia that regulate luminal fluid volume and composition in the respiratory and gastrointestinal tracts. Airway mucus becomes viscous in people with CF, which disrupts normal mucociliary clearance of inhaled pathogens and leads to recurring airway infection. Intestinal epithelia are also abnormal and intestinal obstruction (meconium ileus) is common in CF newborns.

Over 1546 mutations have been documented in the CFTR gene (www.genet.sickkids.on.ca) but the most common by far is a phenylalanine deletion (F508del) in the first nucleotide binding domain, which is present on at least one chormosome in ~90% of people with CF (Bobadilla, 2002). This mutation impairs folding, trafficking, membrane stability, and channel gating (Cheng et al., 1990; Denning et al., 1992; Lukacs et al., 1993), leading to reduced CFTR expression and chloride conductance in the apical membrane and other abnormalities. Misfolded F508del-CFTR protein is retained in the endoplasmic reticulum (ER) but can be rescued by low temperature (<30°C) and by chemical chaperones such as phenylbutyrate or glycerol (Denning et al., 1992; Brown et al., 1996; Rubenstein et al., 1997), although the mutant has a shorter half-life in the plasma membrane (Lukacs et al., 1993) and is less responsive to cAMP stimulation (Hwang et al., 1997). Rescue of only 10-15% of the ER-retained F508del-CFTR may be sufficient to provide therapeutic benefit (Farmen et al., 2005), therefore identifying pharmacological agents which can partially correct the trafficking defect associated with this mutation could benefit most CF patients.

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High throughput screening (HTS) has recently been used to identify small molecules that increase F508del-CFTR activity (Galietta et al., 2001; Pedemonte et al., 2005; Van Goor et al., 2006; Carlile et al., 2007). Such molecules have been categorized according to whether they alleviate the folding/cellular processing defect (correctors), or increase the responsiveness of F508del-CFTR channels already present in the membrane to cAMP activation (potentiators). Two well characterized families of F508del-CFTR potentiators are the xanthines, notably 1,3-diallyl-8-cyclohexylxanthine and 3,7-dimethyl-1-isobutyl xanthine, and the flavones, such as genistein, apigenin and kaempferol (Drumm et al., 1991; Hwang et al., 1997; Lim et al., 2004). Other potentiators include tetrahydrobenzothiophene, phenylglycine, sulfonamide and derivatives of pyrrolo[2,3-b] pyrazines (Al-Nakkash and Hwang, 1999; Hwang and Sheppard, 1999; Yang et al., 2003; Pedemonte et al., 2005; Noel et al., 2006). Fewer correctors have been reported, and some of these (eg 4-phenylbutyrate and curcumin) partially correct the processing defect *in vitro* but are less efficacious when used *in vivo* (Rubenstein et al., 1997; Rubenstein and Zeitlin, 1998; Egan et al., 2004; Mall and Kunzelmann, 2005). Other families with corrector activity include the aminoarylthiazoles, bisaminomethylbithiazoles, the quinazoline corrector VRT-325, benzo[c]quinoliziniums such as MPB-07, and the alpha-glucosidase inhibitor miglustat (Dormer et al., 2001; Loo et al., 2005; Pedemonte et al., 2005; Norez et al., 2006; Van Goor et al., 2006). Sildenafil has also been shown to correct F508del-CFTR processing when used at high micromolar concentrations (Dormer et al., 2005; Poschet et al., 2007). Here we report the identification and characterization of a novel F508del-CFTR corrector that is related to sildenafil, but which rescues trafficking with much higher potency in several model systems including unpolarized cells, human airway epithelial cell monolayers, and freshly isolated intestines from CF mice.

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Materials and Methods

HTS assay

Screening was performed using BHK cells which stably express F508del-CFTR bearing three tandem haemagglutinin-epitope tags (3HA) and linker sequences in the fourth extracellular loop after amino acid 901 (Howard et al.,1995; Carlile et al., 2007). Rescue of the mutant by test compounds was monitored in a plate reader as antibody binding after fixing the cells with paraformaldehyde (for details see Carlile et al., 2007). All hits from the HTS assay are subsequently validated by immunoblot analysis for band C, and by measurement of cAMP-stimulated iodide effluxes to determine the functionality of rescued CFTR. Finally, cells are treated with the hit compounds and CFTR localization is observed by immunofluorescence microscopy.

Compounds

42,000 diverse small molecules were tested in the initial screen (2,000 compounds from Microsource Discovery Inc, and 40,000 compounds from ChemBridge Corp). A total of 89 commercially available analogues of hit compounds from the screen were then identified in a computational search based on structural similarity and were tested under the same assay conditions.

Immunoblot analysis

Cell lysates were quantified using the Bradford assay (BioRad, Hercules, CA), separated by SDS-PAGE (6% polyacrylamide gels) and analyzed by Western blotting. All samples were run with equal protein loading according to the Bradford assay. Western blots were blocked with 5% skimmed milk in PBS and probed overnight at 4^{o}C with a monoclonal primary anti-CFTR antibody (clone M3A7, Chemicon, Temecula, CA) diluted 1:1000. The blots were

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washed four times in PBS before adding the secondary HRP-conjugated anti-mouse antibody at a dilution of 1:5000 (Amersham, Piscataway, NJ) for one hour at room temperature. Blots were washed five times in PBS, visualized using chemiluminescence (Pierce, Rockford, IL) and analyzed by densitometry using the ImageJ program (National Institutes of Health). Optical density of bands on the immunoblots was determined as follows: Maximal and minimal optical densities for the blot were measured and the values normalized (range 0-10 in Fig 2, and 0-12 in Fig 3). The intensity of each band was then determined for each treatment and plotted against this scale to compare bands between treatments. Percent correction for any one treatment was determined from the proportion of signal in one band relative to the total signal for that treatment.

RNA extraction and quantitative real-time RT-PCR

We used a CFBE41o$^-$ airway epithelial cell line that was generously provided by Dr. J.P. Clancy (Univ. Alabama at Birmingham) and cultured as described previously (Bebok et al., 2005). The parental CFBE41o$^-$ cell line was originally developed from CF bronchial cells (F508del/F508del) by Dr. D. Gruenert and colleagues (Kunzelmann et al., 1993), and later transduced with wild-type or F508del-CFTR using the TranzVector lentivirus system (Wu et al., 2000). CFBE41o$^-$ cells were grown on 24 mm-transwell filters and exposed to 0.1% DMSO (untreated control) or 10 μM KM11060 (in DMSO) for 48 h at 37°C. Low temperature rescue of F508del-CFTR was carried out at 29°C for 48 h. RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's instructions, except for an extra acid phenol:chloroform extraction to remove contaminating genomic DNA prior to adding alcohol, and the addition of 10 μg of Glycoblue (Ambion, Austin, TX) as a carrier. All RNA samples were treated with DNase (Ambion, Austin, TX).

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cDNA was prepared using Superscript II (Invitrogen, USA), with 1 µg of RNA primed with 200 ng of random hexamers and 50 µM Oligod$T_{(18)}$. Real-time PCR primers were designed using qPrimer Depot (http://primerdepot.nci.nih.gov). The following primers were used to amplify CFTR; forward: 5'-ACAGAAGCGTCATCAAAGCA-3, reverse: 5'-CCACTCAG TGTGATTCCACCT-3'; HPRT; forward: 5'-ACACTGGCAA AACAATGCAG-3', reverse: 5'-ACACTTCG TGGGGTCCTTTT-3' and GAPDH; forward: 5'-CATCGCTCAGACACCAT-3', reverse: 5'-GGTCATTGATGGCAAC-3'. They were designed to span across exons to exclude genomic DNA, and a "no reverse transcriptase" (no RT) control was performed for each sample. 40-60 ng primer were used per 10 µl PCR reaction. Real-time PCR was performed using SYBR-green Superarray mix (Superarray, Frederick, MD) on the Rotor-gene real-time cycler (Corbett Life Sciences, Sydney, Australia) under the following cycling conditions: 1 cycle at 95°C, 10 min; 45 cycles 95°C, 10 sec, 58°C 20 sec and 72°C for 10 sec followed by a melt curve.

Relative expression of CFTR was quantified using a standard curve that spanned 5 - 6 logs, which was generated by serial dilution of cDNA from untreated CFBE41o$^-$ cells that had been reverse transcribed under the same conditions as the treated samples. Standard curves were included in every experiment for each gene analyzed. The efficiency was near 100% for all primer pairs used in this study.

Samples for each experiment were run in triplicate and experiments were repeated at least three times. The amount of CFTR transcript calculated from the standard curve was normalized by dividing by the level of GAPDH mRNA, although this did not vary between samples. Finally, the ratio of CFTR/endogenous reference gene was normalized against the CFTR/GAPDH ratio of the calibrator sample (designated as the untreated CFBE41o$^-$ F508del-CFTR sample), and the final value reported as a fold difference relative to the

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untreated control. An unpaired, 1-tailed student's t-test ($p<0.05$) was used to evaluate statistical significance.

Measurement of cytotoxicity

Toxicity of KM11060 was tested using the CellTiter-Glo assay from Promega, which monitors cell viability by detecting ATP levels as luciferin-luciferase bioluminescence (Crouch et al., 1993). Briefly, KM11060 was dissolved in DMSO, diluted in cell culture medium to a final concentration of 1-300 µM (DMSO < 1%), and dispensed into 96-well microplates as 50-µl aliquots containing 15,000 BHK cells/well. After incubation at 37°C in a 5% CO_2 incubator for 24 h, cells were incubated for 1 h with reagents from the kit as recommended by the manufacturer. Luminescence was detected using an automated microplate reader (FLUOstar OPTIMA, BMG Labtech, Germany) and normalized to the control group that received only vehicle (DMSO). Cytotoxicity was calculated as the % decrease in luminescence relative to controls without drug. Three independent cytotoxicity assays were carried out at each drug concentration on different days.

Halide flux assay

Iodide effluxes were performed using a robotic liquid handling system (BioRobot 8000, Qiagen, Valencia, CA) and Qiagen 4.1 software. Cells were cultured to confluence in 24-well plates. After treatment (or not) with a test compound, the medium in each well was replaced with 1 ml of iodide loading buffer: 136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, 20 mM Hepes, pH 7.4 with NaOH) and incubated for 1 h at 37^{o}C. At the beginning of each experiment, the loading buffer was removed by aspiration and cells were washed eight times with 300 µl efflux buffer (same as loading buffer except that NaI was replaced with 136 mM $NaNO_3$) to remove extracellular I^-. Efflux was measured by replacing the

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medium with 300 µl fresh efflux buffer every minute for up to 11 min. The first four aliquots were used to establish a stable baseline, then stimulation buffer containing 10 µM forskolin + 50 µM genistein was used. The iodide concentration of each aliquot (300 µl) was measured using an iodide-sensitive electrode (Orion Research Inc., Boston, MA). Relative iodide efflux rates were calculated from the difference between the maximal (peak) iodide concentration during stimulation and the minimum iodide concentration before stimulation (in µM/min). Data are presented as means ± SEM.

Patch-clamp recordings

Whole-cell patch-clamp experiments were performed at room temperature (22°C-25°C) using HEK293 cells stably transfected with F508del-CFTR. Currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Intruments, Foster City, CA) and I-V relationships were generated by stepping the membrane voltage from a holding potential of -60 mV to between -100 mV and +90 mV in increments of 10 mV. Data were filtered at 500 Hz (LP-902, Frequency Devices) and digitized at 4kHz using a Digidata 1440A interface (Axon Intruments, Foster City, CA). The pipette solution contained: 150 mM NMDG-Cl, 1.1 mM MgATP, 1.5 mM $CaCl_2$, 2 mM EGTA, 10 mM TES, pH 7.2 with NMDG. The bath solution contained: 140 mM NaCl, 1.6 mM K_2HPO_4, 0.4 mM KH_2PO_4, 5 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 with NaOH. Results were analyzed using pClamp 10 software (Molecular Devices Corp., Sunnyvale, CA). Liquid junction potentials were corrected before obtaining seals, and pipette capacitance was compensated in the cell-attached mode. Mean cell capacitance was 12.89 ± 0.49 pF for control cells (0.1% DMSO), 11.88 ± 1.26 pF for cells incubated at 29°C, and 14.39 pF ± 0.50 for cells treated with KM11060.

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Ussing chamber studies of CFBE41o- cell monolayers

5 x 10^5 CFBE41o$^-$ cells were seeded onto fibronectin-coated Snapwell 12-mm inserts (Corning Incorporated, Life Sciences, New-York, NY) and the apical medium was removed the following day. Transepithelial resistance was monitored using an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) and cells were used when the transepithelial resistance of the monolayer was 300-400 ohms.cm^2. In some experiments, F508del-CFBE41o$^-$ monolayers were grown at 29°C or treated with 20 µM KM11060 at 37°C for 48 h before being mounted in EasyMount chambers and voltage clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA). In the first set of experiments, the basolateral bathing solution contained 115 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM glucose (pH 7.4 with NaOH). The apical solution had low Cl^-: 1.2 mM NaCl, 115 mM Na-gluconate, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4 mM $CaCl_2$, 2.4 mM, KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM mannitol (pH 7.4 with NaOH) + 100 µM amiloride to block any sodium absorption through epithelial sodium channels. The $CaCl_2$ concentration was increased to 4 mM to compensate for chelation of calcium by gluconate. The apical solution contained mannitol instead of glucose to eliminate current mediated by Na^+-glucose cotransporters.
In the second set of experiments, apical membrane conductance was functionally isolated by permeabilizing the basolateral membrane with 200 µg/ml nystatin and imposing an apical-to-basolateral Cl^- gradient. For these experiments, the basolateral bathing solution contained 1,2 mM NaCl, 115 mM Na-gluconate, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4 mM $CaCl_2$, 2.4 mM, KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM glucose (pH 7.4 with NaOH) and the apical bathing solution contained 115 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM mannitol (pH 7.4 with NaOH). No amiloride was used in this set of experiments. Successful permeabilization of the basolateral membrane under

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these conditions was obvious from the reversal of I_{sc}. Solutions were continuously gassed and stirred with 95% O_2-5% CO_2 and maintained at 37°C. Ag/AgCl reference electrodes were used to measure transepithelial voltage and to pass current. Pulses (1-mV amplitude, 1 s duration) were imposed every 90 s to monitor resistance. Voltage clamps were connected to a PowerLab/8SP (ADInstruments, Colorado Springs, CO) for data collection. 10 µM forskolin + 50 µM genistein were added to the apical bathing solution to activate CFTR.

Ex-vivo experiments

Compounds were tested ex vivo using tissues from homozygous F508del/ F508del CFTR knockout mice (*Cftr*$^{tm1\ Eur}$; van Doorninck et al., 1995) and wild-type littermates controls. Mice were 14–17 weeks old, weighed 24-30 g, and were genotyped by standard PCR methods using tail DNA. The mice were kept in a pathogen-free environment in the animal facility at McGill University and fed a high protein diet constaining pork instead of beef (SRM-A, Hope Farms). All procedures followed Canadian Institutes of Health Research (CIHR) rules and were approved by the faculty Animal Care Committee. Ileal mucosa was stripped of muscle and incubated in William's E-Glutamax medium supplemented with insulin (10 µg/ml), 100 U/ml penicillin, and 100 µg/ml streptomycin and dexamethasone (1.6 ng/ml) containing 0.1% DMSO (control) or the appropriate concentration of KM11060 for 5 h. The tissue was rinsed repeatedly, then mounted in Ussing chambers to measure I_{sc}.

Statistics

All results are expressed as the mean ± SEM of n observations. Sets of data were compared with either an analysis of variance (ANOVA) or Student's t-test. Differences were considered statistically significant when $p<0.05$. ns: non significant difference, *

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$p<0.05$, ** $p<0.01$, *** $p<0.001$. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (Graphpad Software).

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Results

Our HTS trafficking assay relies upon immunodetection of HA epitopes in the fourth extracellular loop of F508del-CFTR (for more details see Carlile et al., 2007). A screen of 42,000 compounds with this assay yielded 25 strong hits; i.e., giving surface fluorescence ≥ three standard deviations above the mean for untreated cells, and many other compounds that were arbitrarily classified as medium (2 x s.d.) or weak hits (1 x s.d.). Intrinsic fluorescence of potential hit compounds was measured and the fluorescent compounds were excluded from further study. One of the hits was the phosphodiesterase inhibitor sildenafil, which was also observed in our previous screen (Carlile et al., 2007) and had been reported to be a CFTR corrector (Dormer et al., 2005). In the present study, we used the HTS assay to test six structural analogues of sildenafil that carry the distinctive sulfonyl-piperazine group (Fig. 1). Five of these were also hits, with RJC03008 and KM11060 giving the strongest signals (Fig. 2A). Interestingly, KM11057 gave negligible correction whereas KM11060, which differs by only a single chlorine atom gave a strong positive signal. To further validate these hits, their effects were then analyzed by immunoblotting (Fig. 2B). Correction of F508del-CFTR was confirmed by the appearance of band C for all analogues except KM11057, consistent with the results of the trafficking assay (Fig. 2B). As shown in Fig. 2C, approximately 38% of the CFTR signal generated by cells following treatment with KM11060 was the band C glycoform. However the amount of band C was not strongly correlated with detection of 3HA F508del-CFTR at the plasma membrane for all correctors. For example RJC03008 produced less band C than expected based on the screen, whereas JFD01738 produced more. These results suggest there may be variable sequestering of F508del-CFTR in post-Golgi compartments, or some of the protein detected on the cell surface may be a different (eg band B) glycoform.

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Functionality of F508del-CFTR rescued by these compounds was examined using an automated iodide efflux assay (Fig. 2D, see Fig. 5A for representative time-course halide efflux curve). Treating cells with KM11060 (10 μM) for 24 h enhanced the cAMP-stimulated iodide efflux, indicating there was an increase in functional CFTR at the plasma membrane (Fig. 2D). Under the same conditions, JFD01738 and the other positive hits did not increase efflux responses despite inducing the appearance of band C (Fig. 2D). Since KM11060 gave the most band C and channel function in BHK cells, we investigated it in more detail.

To understand the dynamics of F508del-CFTR rescue by KM11060, we compared the time-course of correction and its concentration-dependence with those for sildenafil using the trafficking assay (Fig. 3A). 10 μM KM11060 induced a 1.3-fold elevation of surface expression within 2 h, which increased to 1.75-fold increase after 24 h and then declined. Significant correction was still detected after 96 h in the continued presence of drug. By contrast, 10 μM sildenafil did not correct noticeably until 6 h (1.5-fold increase), although it also peaked at 24 h and remained elevated after 96 h (Fig. 3A). Fig. 3B shows that a KM11060 concentration of 10 nM was sufficient to elevate F508del-CFTR surface expression when cells were treated for 24 h. Trafficking increased with concentration and reached a maximum at 10 μM. Sildenafil was much less potent; requiring 1 μM to increase surface expression significantly, and 100 μM for a maximal effect (Fig. 3B).

The time- and concentration-dependencies of KM11060 effects were analysed further by Western blotting, which confirmed the appearance of band C after 2 h when cells were treated with 10 nM (Fig. 3C). Densitometry of Western blots from two independent experiments revealed that 25% of the F508del-CFTR was in the mature form (band C) after 2 h exposure (Fig. 3D), and this increased to 50% after 24 h before declining to 43% after 72 h. Importantly, 20% of the F508del-CFTR was detected as band C after 24 h when cells were exposed to only 10 nM KM11060, and this increased further to 50% at 1 μM before declining

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to 27% at 100 μM. We used quantitative real time RT-PCR to assess the effect of KM11060 treatment on CFTR transcript levels in polarized CFBE41o$^-$ cells expressing F508del-CFTR (Fig. 4A). Relative expression of CFTR mRNA was 2 to 3.5-fold higher after low temperature (control, Fig. 4A). KM11060 (10 μM) probably acts at the post-transcriptional level since it had no effect on the total expression of F508del-CFTR. We tested KM11060 for cytotoxicity at a range of concentrations (1 to 300 μM) using BHK cells. As shown in Fig. 4B, KM11060 was not cytotoxic up to 100 μM, although cell viability decreased ~25 % after 24 h at the highest concentration (300 μM).

F508del-CFTR is less responsive to cAMP stimulation (Hwang et al., 1997; Bebok et al., 2005), therefore we examined whether F508del-CFTR channels rescued by KM11060 are active and determined the concentration at which functional correction is detectable. Similar results were obtained when iodide efflux assays were performed using BHK and human airway epithelial cells (CFBE41o$^-$), therefore only those for CFBE41o$^-$ cells are shown in Fig. 5. Treating CFBE41o$^-$ cells with 10 μM KM11060 for 24 h generated a significant iodide efflux response to 10 μM forskolin + 50 μM genistein whereas no response was observed in untreated CFBE41o$^-$ cells (Fig. 5A). Different times and concentrations of KM11060 treatment were also tested and the results are shown in Figs. 5B and C. Iodide efflux from CFBE41o$^-$ cells was detected after 2 h treatment, reached a peak at 6 h, and then declined, although there was still detectable rescue after 96 h (Fig. 5B). Treatment with KM11060 for 2 h partially restored cAMP stimulated iodide efflux from BHK cells, which then remained relatively constant (data not shown). The rescue of CFTR function required 10 μM KM11060 in BHK cells (data not shown) but only 10 nM in CFBE41o$^-$ cells (Fig. 5C). To explore how long functional correction by KM11060 persists, iodide efflux assays were performed in BHK and CFBE41o$^-$ cells at different times after washing out the compound. CFBE41o$^-$ cells were treated with KM11060 (10 μM) for 24 h, then washed with medium three times and

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assayed for cAMP-stimulated iodide efflux at different time points up to 24 h (Fig. 5D). cAMP-stimulated iodide efflux was detected 6 h after removing KM11060 but had disappeared by 24 h (Fig. 5D). In BHK cells, cAMP-stimulated iodide efflux remained relatively constant for 6 h after treatment, but also disappeared by 24 h (data not shown).

Iodide effluxes are convenient but provide only a qualitative assessment of CFTR activity since the driving force for iodide is unknown and declines with time. To examine functional rescue more rigorously, we measured the effect of KM11060 on whole-cell CFTR chloride currents in HEK293 cells stably transfected with F508del-CFTR. Recordings were compared before and after adding 10 μM forskolin + 30 μM genistein to control cells (0.1% DMSO) and to cells incubated at 29°C for 24 h and pre-treated with 10μM KM11060 for 48 h (Fig. 6A). *I-V* relationships were normalized to cell capacitance (Fig. 6B) and the stimulated current density (pA/pF) at +80 mV is shown in Fig. 6C. Fig. 6D shows the rapid activation of F508del-CFTR in a cell treated with KM11060 and its sensitivity to the CFTR blocker glibenclamide measured at +80 mV (100 μM; Hwang and Sheppard, 1999).

10 μM forskolin + 30 μM genistein stimulated only small whole cell F508del-CFTR currents when cells were treated with vehicle (0.1% DMSO) for 24 h (n = 3; Figs 6A, B and C). The mean current density at +80 mV was 24.84 ± 10.17 pA/pF, comparable to that measured before cAMP stimulation (Fig. 6A; DMSO). Incubating cells at 29°C for 24 h increased CFTR chloride current measured at +80 mV by 4-fold to 108.12.±.0.97 pA/pF, n = 2, Figs. A-C). The macroscopic I/V relationship was weakly outwardly rectifying (Fig. 6B), which is not uncommon for CFTR-mediated whole-cell current. The rectification may be due to flickery gating at negative potentials or incomplete cell dialysis with high-Cl^- pipette solution, since outward rectification is much more pronounced in the cell attached configuration compared to excised patches (see Tabcharani et al., 1991). Indeed, inward rectification of CFTR has been reported in excised patches, and was ascribed to voltage-

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dependent changes in both single channel conductance and gating kinetics (Cai *et al.*, 2003). The same agonists stimulated much larger F508del-CFTR-mediated currents (167.42 ± 29.77 pA/pF (n = 5) at +80mV) when cells were incubated with 10 μM KM11060 at 37°C for 48 h. Taken together, the activation by forskolin + genistein, linear current-voltage relationship, and sensitivity to glibenclamide provide strong evidence that the whole cell currents and iodide effluxes are mediated by F508del-CFTR, and indicate that KM11060 increases cAMP-stimulated channel activity by ~6-fold in this cell type.

To test the corrector activity of KM11060 using a more physiologically relevant cell type, we studied monolayers of the CF airway epithelial cell line CFBE41o$^-$ (F508del/F508del) cultured on permeable supports (Bebok et al., 2005). To monitor CFTR-dependent I_{sc}, a basolateral-to-apical Cl^- gradient was imposed for 15-30 min and 100 μM amiloride was added to the apical side to block apical Na^+ conductance. Fig. 7A-C shows representative recordings of I_{sc} obtained from F508del-CFBE41o$^-$ monolayers that had been incubated without compound at 37 ^{o}C or 29 ^{o}C, or with 10 μM KM11060 at 37 ^{o}C for 48 h, respectively. Forskolin and genistein had no effect on the untreated cells kept at 37 ^{o}C (Fig. 7A) but did stimulate current across monolayers that had been incubated at low-temperature, and these were sensitive to the CFTR channel blocker $CFTR_{inh}$-172 (10 μM; Ma et al., 2002). KM11060 treatment (20 μM for 48 h) increased the forskolin + genistein-stimulated I_{sc} by about 37-fold compared with DMSO controls (Fig. 7C, D). The compound-corrected I_{sc} was blocked by $CFTR_{inh}$-172, suggesting that the entire stimulation was mediated by rescued F508del-CFTR (Fig. 7C). Correction by KM11060 was 26% (n = 6) of that induced by low-temperature (n = 5, Fig. 7D), which was significant but less than noted above for HEK 293 cells in patch clamp experiments. The basolateral membrane was permeabilized using nystatin in some experiments to confirm that the stimulated I_{sc} was mediated by apical Cl^- conductance. When assayed under these conditions, 20 μM KM11060 restored 28% (n = 4)

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of the current relative to low-temperature incubation (Fig. 7E), similar to that seen using unpermeabilized monolayers.

Finally, we measured the effect of KM11060 treatment on intestinal tissue isolated from CF mice that were homozygous for F508del-CFTR. The I_{sc} response to forskolin and its sensitivity to $CFTR_{inh}$-172 were examined after *ex-vivo* exposure to 20 μM KM11060 for 5 h. This treatment increased the forskolin + genistein-stimulated current ~16-fold compared to ileum treated with vehicle alone (compare Figs 8C,D and 8B,D, n = 5), and represents restoration of ~51% of the secretory response seen using ileum from wild-type mice (n = 5, Fig. 8A, D). *Ex-vivo* correction of F508del-CFTR in mouse ileum agrees well with the gain of function observed in BHK and human airway epithelial cells after correction of F508del-CFTR. These results indicate that KM11060 rescues F508del-CFTR trafficking in cultured cells and native epithelial tissues.

Discussion

Defective F508del-CFTR folding and trafficking can be overcome by small molecules (Loo et al., 2005; Pedemonte et al., 2005; Van Goor et al., 2006; Wang et al., 2007), however the correctors identified to date have relatively low potency. In this screen of 42,000 chemically diverse molecules we obtained several distinct groups of "hit" compounds. One of these was the PDE5 inhibitor sildenafil, which has been reported previously to correct F508del-CFTR trafficking (Dormer et al., 2005; Carlile et al., 2007). A computional search for similar structures yielded many analogues of sildenafil carrying the distinctive sulfonyl-piperazine group, but of those tested, only the quinoline KM11060 was able to rescue cAMP-stimulated channel activity in both BHK and $CFBE41o^-$ cells, suggesting that other analogues may inhibit channel function in addition to promoting surface expression. Previous studies have demonstrated that CFTR potentiators like genistein can also inhibit CFTR at some

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concentrations (Moran et al., 2005). It has been proposed that CFTR potentiators may bind at the NBD1-NBD2 interface, stabilising the dimerisation of these domains and consequently the open state of the channel (Moran et al., 2005). At high potentiator concentrations, genistein might also bind to NBD2 before dimerisation and interfere with the normal interaction between NBD1 and NBD2. By analogy, we might speculate that the sildenafil analogues (other than KM11060) correct F508del-CFTR folding whilst inhibiting gating. If correct, they are more likely bind to NBD2 than NBD1 since interaction of KM11060 to NBD1 was not detected biochemically in preliminary experiments (data not shown). Correction of CFTR channel activity by KM11060 was further validated using multiple assays and cell lines, and by *ex-vivo* experiments using tissues from CF mice heterozygous for the F508del mutation. We found that KM11060 partially corrects F508del-CFTR processing and increases surface expression to ~75% of that observed in cells incubated at low-temperature. Up to 50% of the F508del-CFTR in cells treated with KM11060 was complex-glycosylated, indicating passage through the Golgi. Although we used a combination of glycerol and low temperature in biochemical studies, glycerol had a deleterious effect on CFTR function according to iodide efflux assays, hence only low temperature rescue was used in functional studies. Interestingly, KM11060 and low temperature incubation had different effects on unpolarized vs epithelial cells, suggesting that polarized cells may have another level of protein trafficking control that is not apparent in non-polarized cells. The mechanim of KM11060 action is not known, but may involve differential phosphorylation of CFTR or the folding machinery. Regardless, since there was no increase in the total amount of CFTR, KM11060 apparently corrects trafficking at the level of the ER, consistent with quantitative RT-PCR results showing that KM11060 treatment does not affect the amount of F508del-CFTR mRNA expressed. Rescue was accompanied by partial correction of iodide efflux and whole cell conductance in different

cell types. KM11060 restored ≈26% of the Cl^- current obtained by low-temperature correction in human CF airway epithelial cells and ≈51% of the normal cAMP response in CF mouse intestine. Much evidence suggests that even partial correction (6-10%) should provide therapeutic benefit for CF patient (Johnson et al., 1992; Farmen et al., 2005). Thus KM11060, which is effective at 10 nM, and other analogues of sildenafil are suitable starting points for further optimization of potency, efficacy, and selectivity, and should be evaluated for their pharmacokinetic and toxicological properties.

Sildenafil is a potent inhibitor of cGMP-specific phosphodiesterase 5 (PDE5) and is marketed as a treatment for erectile dysfunction (Viagra®). A previous study demonstrated improved CFTR processing and conductance in nasal cells from CF patients after they were treated with 150 μM sildenafil (Dormer et al., 2005), and sildenafil also hit in a recent HTS screen for CFTR correctors (Carlile et al., 2007). In the present study we identified a structural analogue of sildenafil which is much more potent and is also efficacious in native tissue. The cellular mechanism remains to be elucidated, however the results imply a role for PDEs and cGMP in CFTR processing. Interestingly, increasing cGMP levels in CF respiratory epithelial cells may correct several aspects of CF pathology including abnormalities in protein glycosylation, bacterial adherence and proinflammatory responses (Poschet et al., 2007). Consistent with that report, we found in preliminary experiments that KM11060 inhibits PDE5 activity and transiently increases cGMP levels (data not shown). Such increases in cGMP level could transiently increase cGMP-dependent protein kinase (GK) activity, which may phosphorylate and reduce ER retention of F508del-CFTR. It remains uncertain whether sildenafil analogues that restore trafficking but not function differ from KM11060 in their effect on CFTR phosphorylation. Further studies are also needed to establish if PDE5 mediates the actions of KM11060 on F508del-CFTR trafficking, and explore why rescue varies among different cell types.

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In summary, the present results show that KM11060, a structural analogue of sildenafil, rescues the trafficking of functional F508del-CFTR in several cell types including a human bronchial epithelial cell line and native epithelial tissue from F508del mice. The findings suggest KM11060 as a promising compound for further development of CF therapeutics.

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Footnotes

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Legends for figures

Fig. 1. Chemical structures of sildenafil analogues tested. 1. sildenafil; 1-[[3-(4,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)ethoxyphenyl]sulfonyl]-4-methyl-piperazine. 2. RJC03008; 1-[(4-chlorophenyl)sulfonyl]-4-pyrimidin-2-piperazine. 3. JFD01738; 1-(2-methoxyphenyl)-4-[(methylphenyl)sulfonyl] piperazine. 4. HTS05921; 2-[(1,2-dimethyl-1H-imidazol-4-yl)sulfonyl]-6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline. 5. TB00008; 1-(2-phenoxyethyl)-4-(phenylsulfonyl)piperazine. 6. KM11060; 7-chloro-4-{4-[(4-chlorophenyl)sulfonyl]piperazino}quinoline. 7. KM11057; 7-chloro-4-[4-(phenylsulfonyl)piperazino]quinoline.

Fig. 2. Effect of sildenafil and its analogues on the surface expression of CFTR. (A) BHK cells expressing F508del-CFTR were pretreated for 24 h with 0.1% DMSO (control, n = 3) or with 10µM of each analogue prior to monitoring the surface expression by immunofluorescent high throughput screening assay (n = 3 for each). Data are presented as mean ± SD (B) Immunoblot showing F508del-CFTR in lysate of cells treated with sildenafil analogues at 10µM for 24 h. As a positive control, cells were treated with 10% glycerol at 29^{o}C for 48 h. “Control” cells were treated with vehicle alone (0.1% DMSO). Band C corresponds to the mature CFTR and band B to core-glycosylated CFTR. Tubulin is shown as a loading control. (C) Densitometry of three independent immunoblots to monitor the relative amounts of band C and band B present. (D) Measurement of CFTR channel function by iodide efflux from BHK cells expressing F508del-CFTR and treated for 24 h with 0.1% DMSO (control, n = 33), low temperature (29°C, n = 9) or with 10µM of the following molecules : sildenafil (n = 11), RJC03006 (n = 15), KM11060 (n = 24), KM11057 (n = 7), HTS05921 (n = 10), TB00008 (n = 10) and JFD01738 (n = 14). Iodide efflux was stimulated

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by addition of 10µM forskolin + 50µM genistein. The iodide efflux shown is the largest peak value measured after subtracting the basal rate prior to stimulation. Data are presented as mean ± SEM and compared to the control. ns: non significant difference, * $p<0.05$, *** $p<0.001$.

Fig. 3. Time course and concentration dependence of trafficking correction by sildenafil and KM11060. (A) BHK cells expressing F508del-CFTR were treated with compounds (10 µM) for various times prior to assaying surface expression at various times prior to assaying with 10µM of compound (SD, n=3). "Control", cells were treated with vehicle alone (0.1% DMSO). (B) BHK cells expressing F508del-CFTR were treated with various concentrations of compound for 24 h prior to analysis with the corrector assay (SD, n=3). "Control", cells were treated with 0.1% DMSO. (C) Immunoblot to monitor correction of F508del-CFTR in cells treated with 10 µM KM11060 for different times and at different concentrations for 24 h. The positive control shows F508del-CFTR from cells treated with 10% glycerol at 29^{o}C for 48 h. Tubulin is shown as a loading control. (D) Densitometry of 3 independent immunoblots to quantify the relative amounts of correction as determined by the appearance of band C. Note the earlier and more potent effect of KM11060 on the levels of band C.

Fig. 4. Effect of KM11060 on mRNA level and cell viability. (A) F508del-CFTR mRNA levels quantified in polarized CFBE41o^{-} cells under control conditions and after treatment with 10 µM KM11060 or incubation at 29°C for 48 h. Control cells were exposed to vehicle alone (0.1% DMSO). mRNA levels were normalized to GADPH expression level. (B) Relationship between the viability of BHK cells expressing F508del-CFTR and concentration of KM11060 after 24 h treatment. Cytotoxicity was calculated as the % decrease in luminescence relative to controls receiving vehicle alone (0.1% DMSO).

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Fig. 5. Functional rescue of F508del-CFTR by KM11060 in CFBE41o$^-$ cells. (A) Iodide efflux assay of rescued F508del-CFTR at the plasma membrane in CFBE41o$^-$ cells treated with 10µM KM11060 (n = 6) for 24 h prior to assay. Stimulation was evoked by 10µM forskolin + 50µM genistein. Control cells received vehicle alone (0.1% DMSO, n = 6). (B) Time-course of CFTR function after KM11060 treatment. CFBE41o$^-$ cells expressing F508del-CFTR were treated with 10µM of KM11060 for varying times up to 96 h and subsequently monitored for cAMP-stimulated iodide efflux (n = 3 for each time point). (C) Dependence of functional rescue on the concentration of KM11060. CFBE41o$^-$ cells were treated for 24 h prior to analysis by iodide efflux (n = 3 for each concentration). (D) Washout experiments to determine the persistence of CFTR function after the removing KM11060. CFBE41o$^-$ cells were treated for 24 h with 10µM of KM11060, then washed three times and cultured for various times prior to analysis by iodide efflux (SEM, n = 3 for each time after washout). "Control" indicates F508del-CFTR cells treated with vehicle alone (0.1% DMSO). Data are presented as mean cAMP-stimulated peak and are compared statistically to untreated control cells. ns: non significant difference, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 6. Activation of CFTR chloride currents in HEK293 cells after treatment with KM11060. Whole-cell patch-clamp experiments performed using HEK293 cells stably transfected with F508del-CFTR. (A) Representative traces of whole-cell chloride currents recorded in control cells treated with 0.1% DMSO at 37°C and incubated at low-temperature (29°C) for 24 h, or incubated with 10µM KM11060 at 37°C for 48 h. The traces show currents recorded before (left panel) and after (right panel) the addition of 10µM forskolin + 30µM genistein. (B) Current-voltage (I/V) relationships for the currents shown in A and after addition of 10µM forskolin + 30µM genistein in the three experimental conditions. Please

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note that currents before addition of forskolin and genistein have been subtracted from those recorded after the addition of the agonists. Data are means ± SEM (control, n = 3; 29°C, n = 5; KM11060, n = 5). (C) Histogram showing the current density at +80 mV in the three conditions. (D) Time course of whole-cell current shown in A, after 48 h treatment with 10μM KM11060. 100μM of glibenclamide caused a rapid inhibition consistent with CFTR channels.

Fig. 7. Rescue of F508del-CFTR in human bronchial epithelia (CFBE41o$^-$). (A-C) Representative traces of the short-circuit current (I_{sc}) responses to 10μM forskolin, 50μM genistein and 10μM CFTRinh-172 in CFBE41o$^-$ cells treated with (A) 0.1% DMSO; (B) incubated at 29°C for 48 h; and (C) exposed to 20μM KM11060 for 48h. (D) Histogram showing the change in I_{sc} (ΔI_{sc}) after addition of forskolin + genistein, defined as the difference between the sustained phase of the current response after stimulation and the baseline immediately before stimulation. Data are presented as mean ± SEM (n = 4 for control, n = 6 for KM11060 and n = 5 for 29°C). ** p<0.01. (E) Bar graph showing the effect of adding forskolin + genistein on apical current measured after permeabilizing the basolateral membrane with 200μg/ml nystatin and imposing an apical-to-basolateral Cl^- gradient. Data are presented as mean ± SEM (n = 3 for control, n = 4 for KM11060 and 29°C). * p<0.05, ** p<0.01.

Fig. 8. *Ex-vivo* rescue of F508del-CFTR in mouse ileum by KM11060. (A) Representative traces of short-circuit current (I_{sc}) response to 10μM forskolin, 50μM genistein and 10μM CFTRinh-172 using ileum from wild-type CFTR mice. (B) Ileum from F508del-CFTR mice incubated *ex-vivo* with vehicle alone (0.1% DMSO). Stimulation of electrogenic Na^+-glucose co-transport with 10mM glucose (apical solution normally contained mannitol) was used to

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confirm tissue viability at the end of each experiment. (C) Rescue of forskolin/genistein-activated I_{sc} response in ileal mucosa from F508del-CFTR mice by KM11060 treatment (20 μM for 5 h). (D) Bar graph showing stimulation by forskolin + genistein under the three conditions. Data are presented as mean ± SEM (n = 5 for each). ** $p<0.01$.

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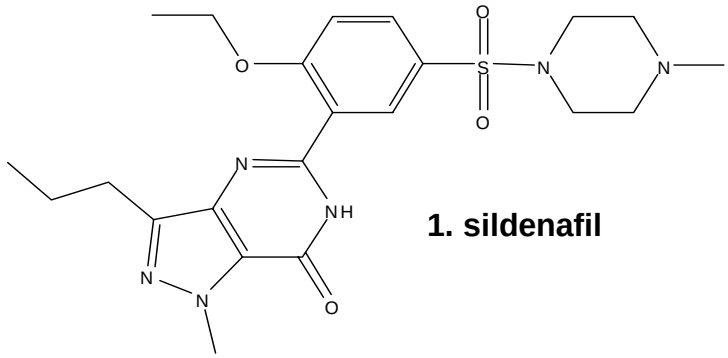
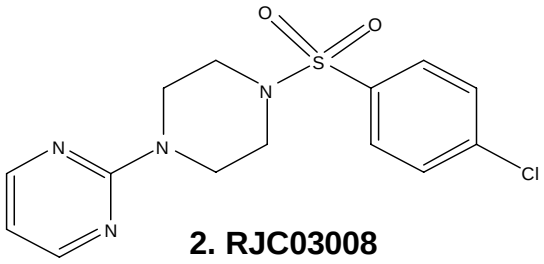
Figure 1



1. sildenafil



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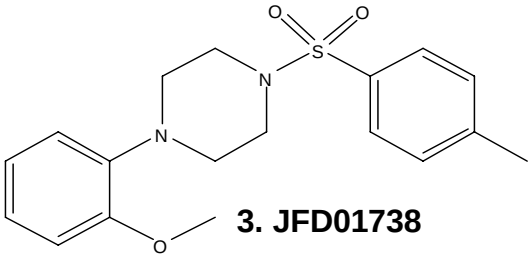

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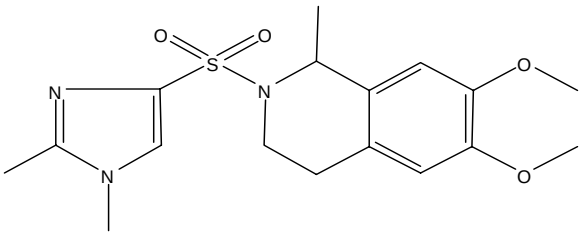

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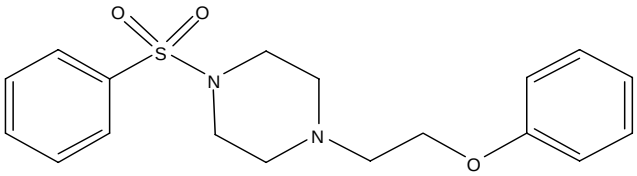

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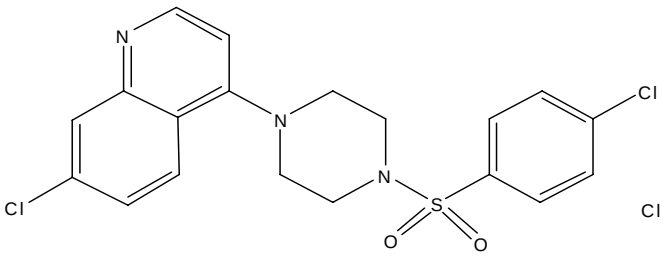

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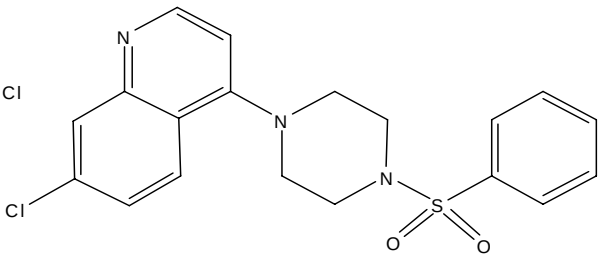

7. KM11057

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Figure 2

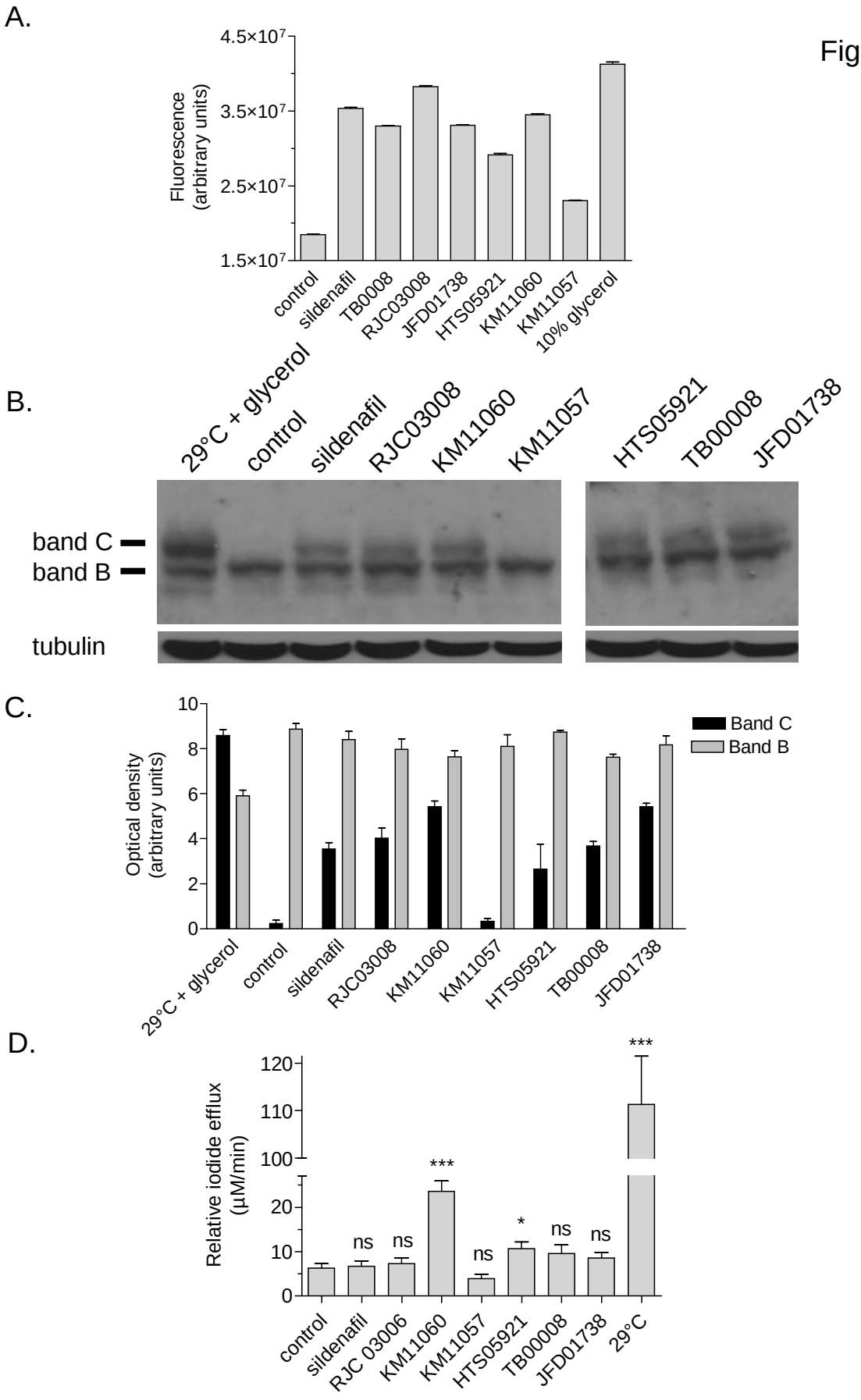

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Figure 3

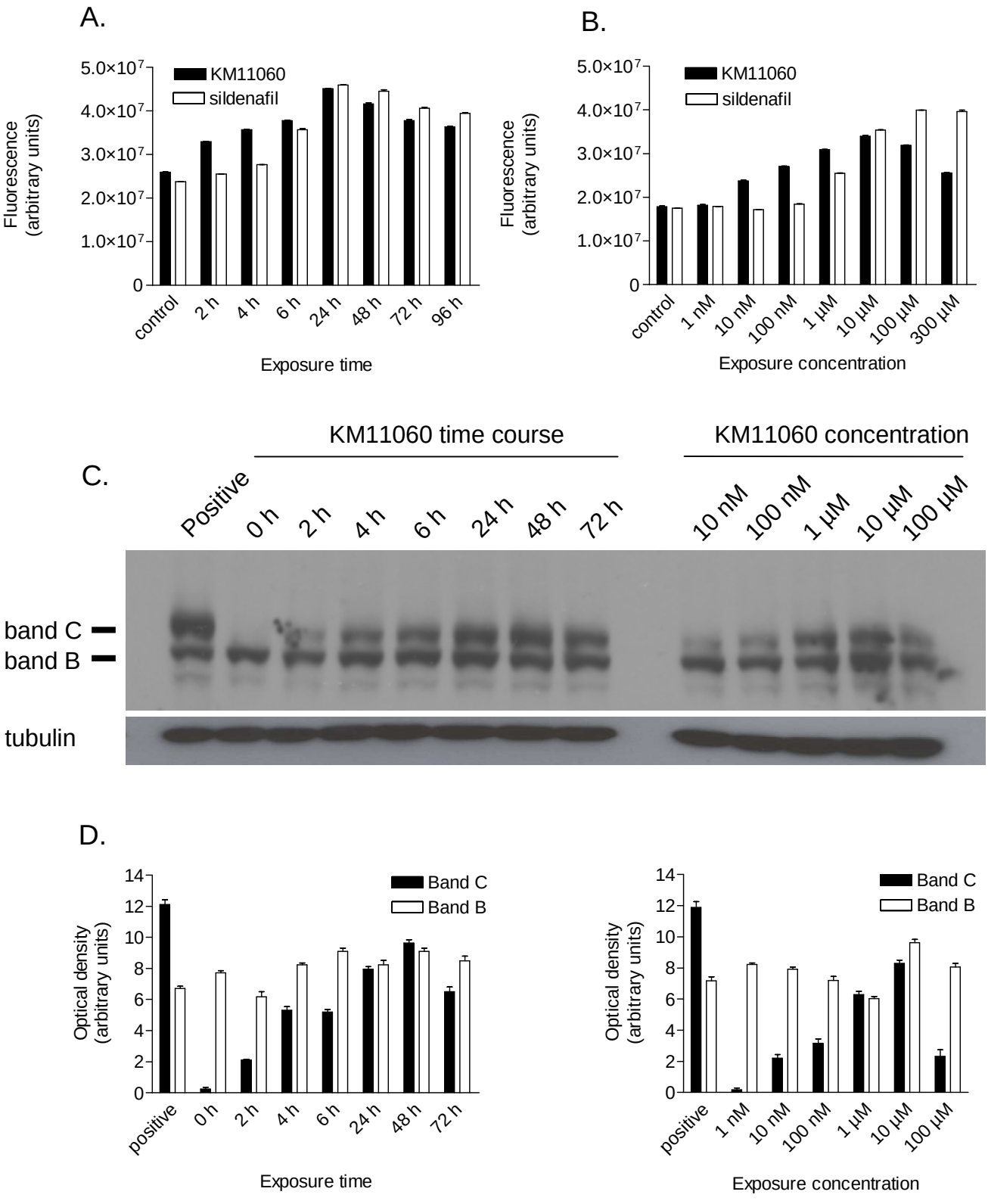

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Figure 4

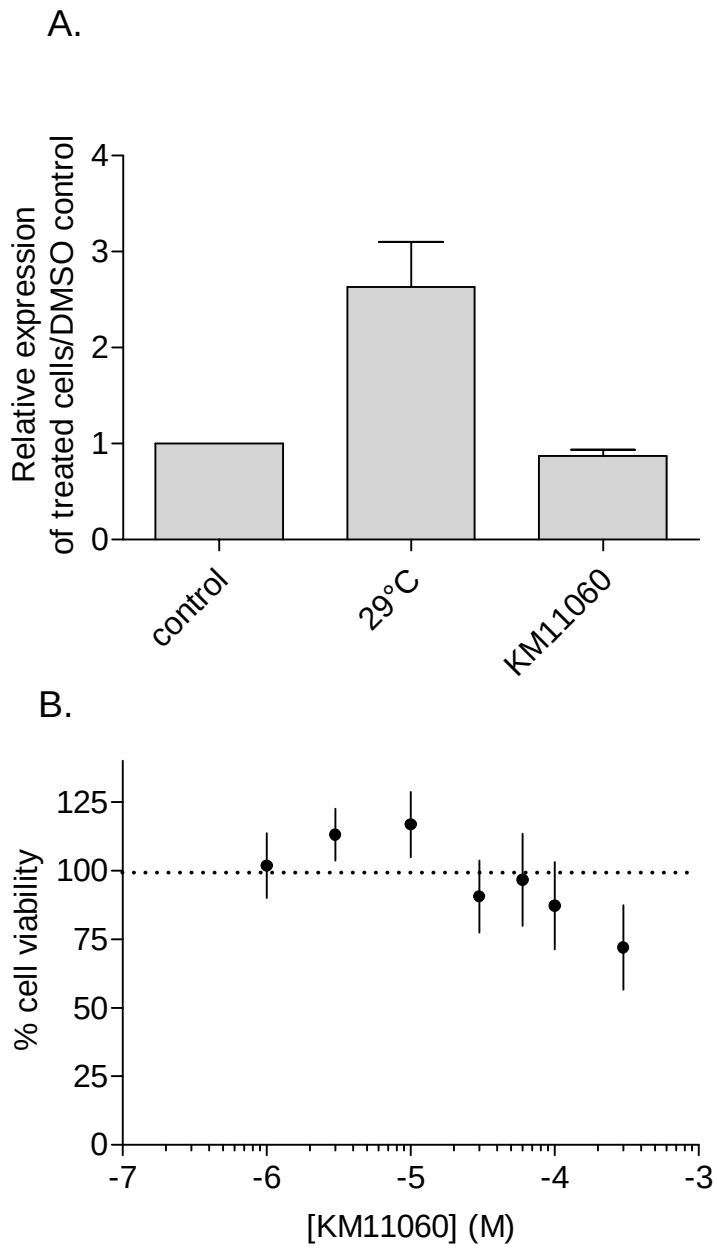

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Figure 5

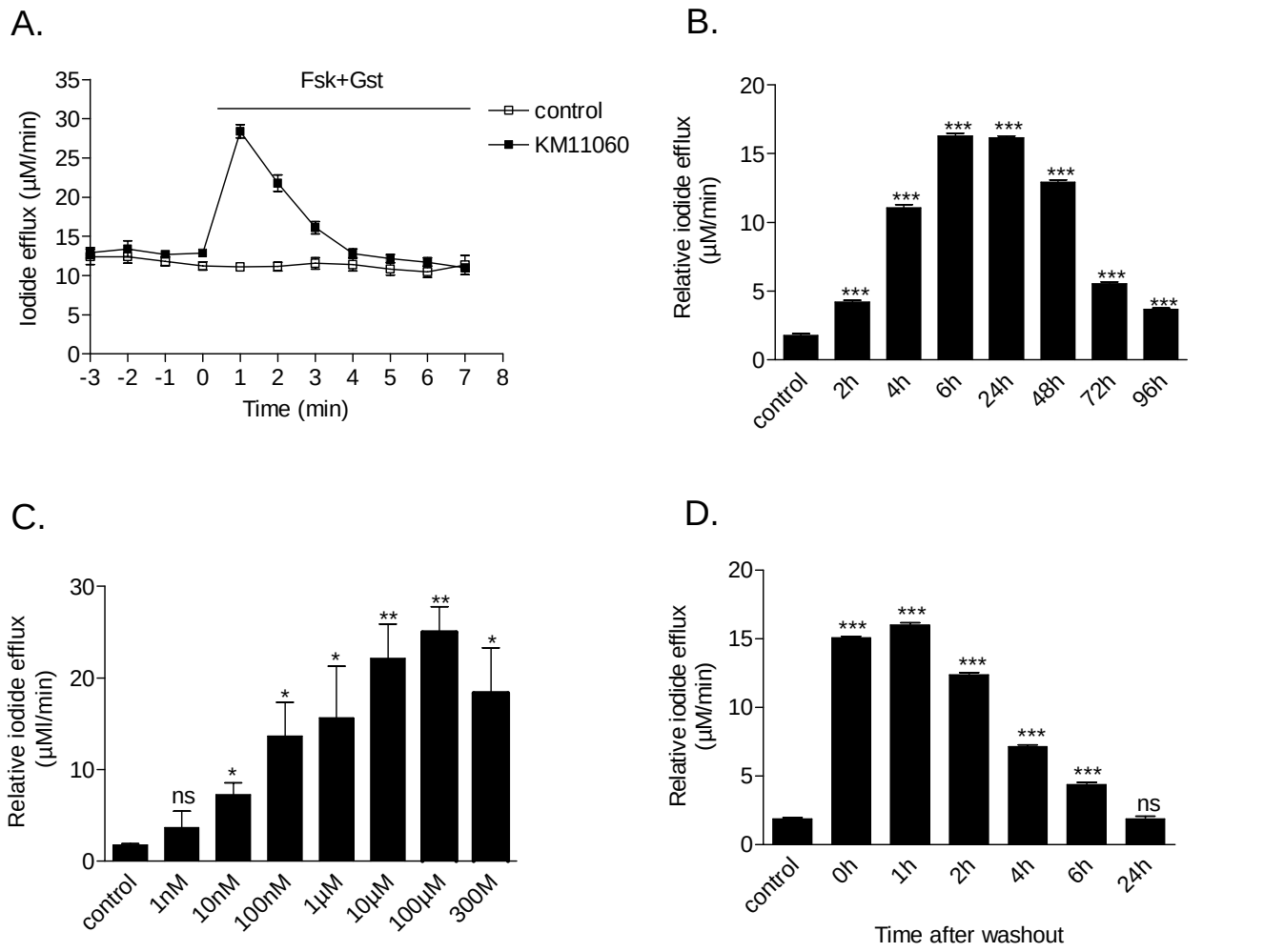

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Figure 6

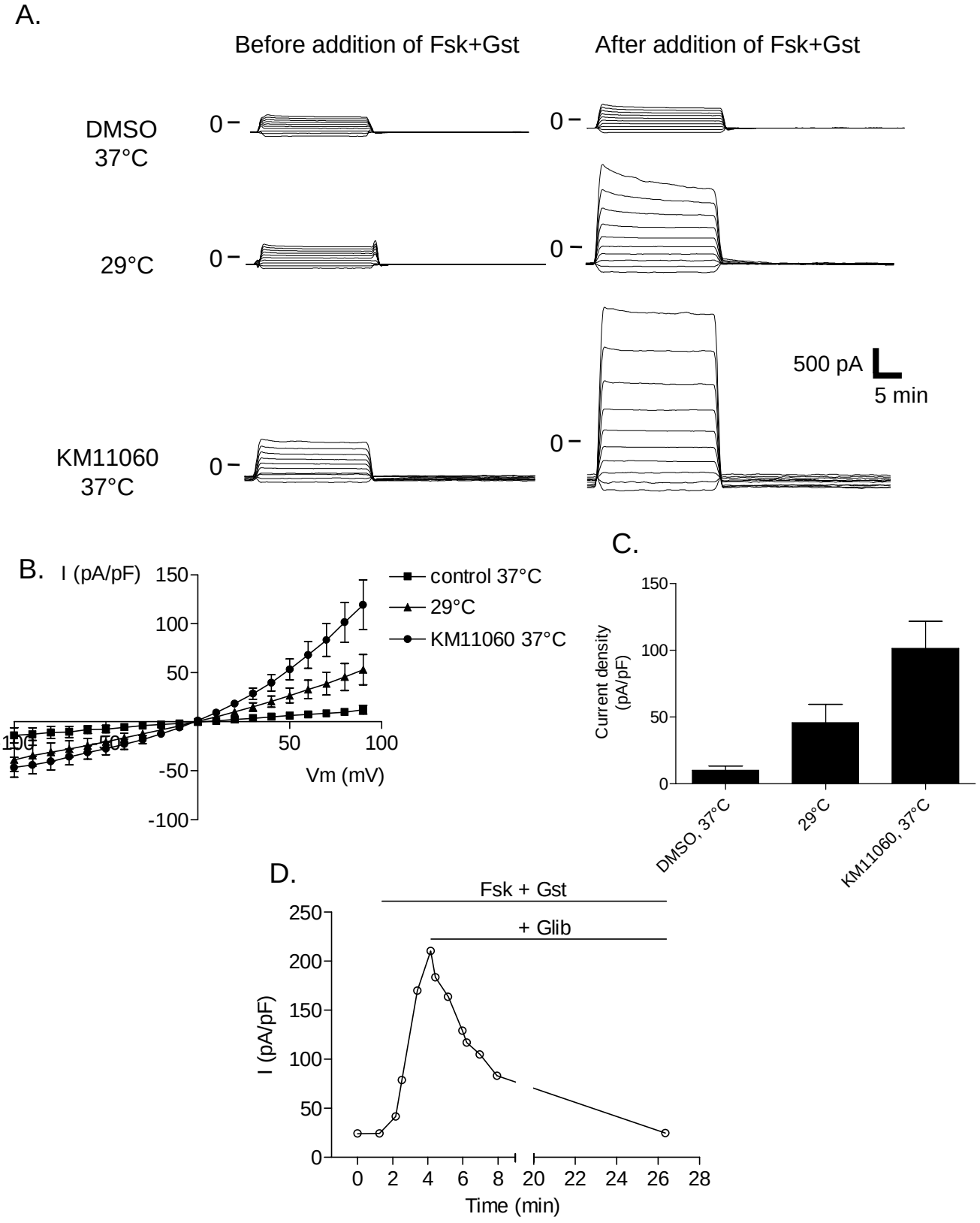

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Figure 7

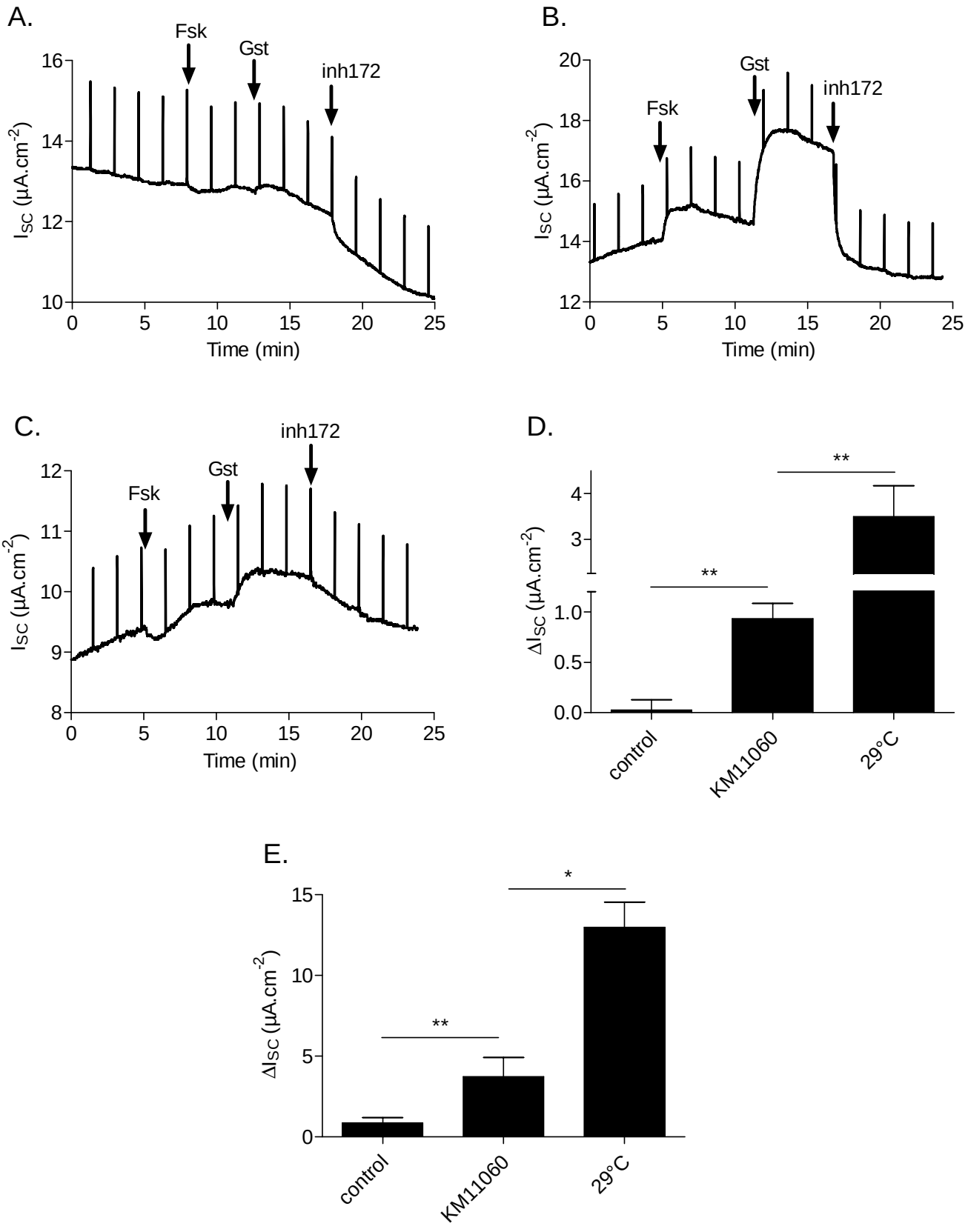

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Figure 8