CURCUMIN CROSS-LINKS CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) POLYPEPTIDES AND POTENTIATES CFTR CHANNEL ACTIVITY BY DISTINCT MECHANISMS

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Running title: CFTR cross-linking by curcumin.

Cystic fibrosis (CF) is caused by loss-of-function mutations in the CFTR chloride channel. Wild type and mutant CFTR channels can be activated by curcumin, a well tolerated dietary compound with some appeal as a prospective CF therapeutic. However, we show here that curcumin has the unexpected effect of cross-linking CFTR polypeptides into SDS-resistant oligomers. This effect occurred for CFTR channels in microsomes as well as in intact cells and at the same concentrations that are effective for promoting CFTR channel activity (5-50 μM). Both mature CFTR polypeptides at the cell surface and immature CFTR protein in the endoplasmic reticulum were cross-linked by curcumin, although the latter pool was more susceptible to this modification. Curcumin cross-linked two CF mutant channels (ΔF508 and G551D) as well as a variety of deletion constructs that lack the major cytoplasmic domains. In vitro cross-linking could be prevented by high concentrations of oxidant scavengers (i.e., reduced glutathione and sodium azide) indicating a possible oxidation reaction with the CFTR polypeptide. Importantly, cyclic derivatives of curcumin that lack the reactive β diketone moiety had no cross-linking activity. One of these cyclic derivatives stimulated the activities of wild type CFTR channels, Δ1198-CFTR channels and G551D-CFTR channels in excised membrane patches. Like the parent compound, the cyclic derivative irreversibly activated CFTR channels in excised patches during prolonged exposure (> 5 min). Our results raise a note of caution about secondary biochemical effects of reactive compounds like curcumin in the treatment of CF. Cyclic curcumin derivatives may have better therapeutic potential in this regard.

The CFTR chloride channel mediates electrolyte transport across a variety of epithelia (1). Mutations in the cftr gene that reduce the expression or function of this channel can cause cystic fibrosis (CF), the most common lethal disorder in Caucasians (2). CFTR is a member of the ATP Binding Cassette (ABC) transporter family (3) that, like other ABC transporters, contains two membrane spanning domains and two nucleotide binding domains (NBDs). The CFTR NBDs probably dimerize to form two composite ATP binding pockets (4), by analogy to the well characterized bacterial ABC transporters. CFTR channel opening is normally tightly linked to ATP binding to the NBDs. Additional regulation is provided by phosphorylation of the large cytoplasmic R domain typically by protein kinase A (5). Whether phosphorylation controls channel gating at the level of the NBDs or at a later step (or possibly both) is a matter of debate (6, 7).

The ΔF508 and G551D CFTR mutations are two of the most well studied CF-causing mutations. Both mutations reside in NBD1 but they have
fundamentally different effects on CFTR function. The ΔF508 mutation, which is the most common mutation in the CF population (8), primarily disrupts CFTR folding and export from the endoplasmic reticulum (ER) (9). ΔF508-CFTR also has deficits in channel gating and cell surface residence (10-12). The G551D mutation disrupts one of the ABC signature sequences that line the ATP binding pockets. This mutation has no apparent effect on channel synthesis or cell surface localization but nearly abolishes ATP-dependent channel opening (13). A growing number of small molecule activators of these mutant channels have been identified in recent years, some of which activate both types (14-17). Curcumin, the primary ingredient in turmeric, is one compound that promotes the channel activities of both ΔF508-CFTR and G551D-CFTR (18-20). This dietary ingredient initially was proposed to enhance the biosynthesis and ER export of the ΔF508 mutant by altering the ER luminal free calcium concentration via inhibition of the Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) pump (21). But, this biochemical effect has been called into question (21-25). On the other hand, multiple groups have reported a more direct stimulatory effect of curcumin on the single channel activities of ΔF508-CFTR and G551D-CFTR (18-20) in cells and in excised membrane patches. Given that this dietary ingredient is well tolerated by humans (26), curcumin might be worth considering as a prospective CF therapeutic.

CFTR channel activation by curcumin has several intriguing features including persistent (irreversible) activation of channels that occurs during extended exposure to this compound (> 5 min) (20). This persistent activation might be due to covalent modification of CFTR channels by curcumin; indeed, this compound is known to covalently react with nucleophilic amino acids in other proteins (27). In exploring this point we discovered to our surprise that curcumin cross-links CFTR polypeptides (wild type or CF mutants) into SDS-resistant oligomers. We examined this further given that: (i) other investigators had reported that CFTR polypeptides can exist as dimers based largely on the results of chemical cross-linking experiments (28-29) and (ii) there are several reports that CFTR dimerization might promote channel activity (30, 31). We show here, however, that the cross-linking effect of curcumin and its channel activating effect are separable features. The clearest evidence for this distinction is our identification of cyclic derivatives of curcumin that lack the ability to cross-link CFTR polypeptides but retain the ability to persistently activate CFTR channels in excised membrane patches.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs, Cell Culture, and Transfections**- Human wild type (WT) CFTR and the tested mutants were subcloned into the pCDNA3 mammalian expression vector (Invitrogen). Point mutants and deletion constructs (e.g. Δ1198-CFTR) were generated by PCR mutagenesis using appropriate mutagenic oligonucleotides and verified by DNA sequencing of the entire PCR product. In the HA-tagged Δ 1198-CFTR the tag portion was introduced at the amino-terminal tail.

HEK 293T/17 (Human Embryonic Kidney cells, ATCC# CRL-11268™) cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen) supplemented with 10% fetal bovine serum, 1 mM penicillin-streptomycin and 2 μg/μl puromycin (Sigma). The Calu-3 human airway epithelial cell line (derived from a lung adenocarcinoma, ATCC# HTB-55™) was cultured in MEM supplemented with 10% fetal bovine serum and 1 mM penicillin-streptomycin. All cells were grown at 37°C under a 5% CO2 atmosphere.

HEK-293T cells were transiently transfected with WT or Δ508-CFTR was provided by J. P. Clancy at the University of Alabama at Birmingham. CFBE41o- cells were cultured in MEM (Modified Eagle’s Medium, Invitrogen) supplemented with 10% fetal bovine serum, 1 mM penicillin-streptomycin and 2 μg/μl puromycin (Sigma). The Calu-3 human airway epithelial cell line (derived from a lung adenocarcinoma, ATCC# HTB-55™) was cultured in MEM supplemented with 10% fetal bovine serum and 1 mM penicillin-streptomycin. All cells were grown at 37°C under a 5% CO2 atmosphere.

CFBE41o- stably transduced with WT or Δ508-CFTR was provided by J. P. Clancy at the University of Alabama at Birmingham. CFBE41o- cells were cultured in MEM (Modified Eagle’s Medium, Invitrogen) supplemented with 10% fetal bovine serum, 1 mM penicillin-streptomycin and 2 μg/μl puromycin (Sigma). The Calu-3 human airway epithelial cell line (derived from a lung adenocarcinoma, ATCC# HTB-55™) was cultured in MEM supplemented with 10% fetal bovine serum and 1 mM penicillin-streptomycin. All cells were grown at 37°C under a 5% CO2 atmosphere.
DMEM plus 10% fetal bovine serum without antibiotic.

**Reagents**-The commercial curcumin preparation used for most experiments was purchased from Sigma (catalog number C7727; >94% curcuminoid content with >80% curcumin; the rest primarily desmethoxycurcumin). Synthetic curcumin, curcumin derived isooxazole BSc3596 and curcumin derived pyrazoles BSc3732 and BSc3736 were prepared as described previously (32). NPPB-AM was a generous gift from Dr. Gerald Schönknecht (Oklahoma State University). Disuccinimidyl suberate (DSS) was purchased from Thermo SCIENTIFIC. CFTR specific inhibitor CFTR(inh)-172 was purchased from Sigma. All stock solutions were prepared in DMSO so that the final concentration of DMSO did not exceed 1%.

**Experiments using microsomes**- The isolation of microsomal membrane vesicles (or microsomes) from HEK transfected cells followed a previously published protocol (33). Microsomal vesicle membranes were suspended in 140 mM N-methyl-D-glucamine-Cl, 3 mM MgCl₂, 1 mM EGTA, and 10 mM TES (pH 7.3) and, for each experimental condition, a volume of microsomes corresponding to 100 μg total protein was used, unless otherwise indicated. Experiments using microsomes were performed at 21-23 °C with the samples under constant rotation and were stopped by the addition of reducing Laemmli buffer (50 mM dithiothreitol, 1.2% β-mercaptoethanol) followed by incubation at 37 °C for 30 min. Samples were then resolved by SDS-PAGE on a 5% acrylamide gel (Criterion gel, Bio-Rad) and then transferred to polyvinylidene difluoride membrane (Immobilon-P transfer membranes; Millipore). The blots were successively incubated 1 h at room temperature with blocking buffer (5% milk in Tris (25 mM)-buffered saline (TBS)) followed by an overnight incubation at 4 °C with a monoclonal antibody directed against the HA-tag (monoclonal antibody sc-7392; Santa Cruz Biotechnology; diluted at 1:500) or against CFTR NH₂ tail (monoclonal antibody 3482; Chemicon; diluted at 1:1000), COOH tail (monoclonal antibody clone 24-1; R&D Sytems; diluted at 1:2000) or R-domain (monoclonal antibody clone 1660; R&D Sytems; diluted at 1:1000). Primary antibodies were diluted in 4% milk with TBS plus 133 mM glucose, 0.4% Tween 20, and 8% glycerol. After extensive washing steps in TBS plus 0.2% Tween 20, each blot was incubated 1 h at room temperature with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (ImmunoPure® antibody; Pierce) diluted at 1:10,000 in the same solution used for the primary antibody. Following extensive washing in TBS plus 0.2% Tween 20, each blot was incubated 5 min with the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and then exposed using XHR film (Hawkins X-Ray Supply). Quantification of signals was performed using the National Institutes of Health program ImageJ. The averaged data are presented as the means ± S.E. Statistical comparisons were made by performing unpaired t tests unless otherwise indicated.

**Cell surface biotinylation of HA-tagged Δ1198-CFTR in HEK cells**- Cell surface proteins were biotinylated using EZ-Link® Sulfo-NHS-SS-Biotin (Pierce) 48 h after transfection. After incubation with curcumin at 37 °C in PBS containing 1 mM MgCl₂ plus 0.1 mM CaCl₂, pH 7.4 (PBS-CM) cells were rinsed three times with PBS-CM pH 7.4 and once with PBS, pH 8.0. Cells were then incubated 30 min at 4 °C with EZ-Link® Sulfo-NHS-SS-Biotin (1 mg/ml) in PBS (pH 8.0) or with PBS only (control experiment). After the biotinylation step, the excess biotin was neutralized by incubating cells for 10 min at 4 °C with 1% bovine serum albumin in PBS. For chase experiments cells were warmed up to 37 °C for increasing period of times after the quenching of biotin with BSA followed by rinses in PBS-CM, pH 7.4. Biotinylated cells were lysed in 1% Triton X-100 in PBS supplemented with protease inhibitors (complete, EDTA-free; Roche Applied Science) (1 ml/dish) and cell lysates cleared by centrifugation (14,000 rpm for 15 min at 4 °C). The total protein content of supernatants was determined by the MicroBCA protein assay kit (Pierce). Part of each sample was incubated with streptavidin beads (streptavidin-agarose; Novagen) to isolate cell surface proteins and part reserved as total lysate for SDS-PAGE and immunoblotting. Streptavidin pulldowns were carried out overnight at 4 °C. The beads were then rinsed three times with 1% Triton X-100 in PBS, and proteins were eluted by incubation at 37 °C for 30 min with reducing Laemmli buffer (50 mM dithiothreitol, 1.2% β-
mercaptoethanol). After a brief centrifugation (1 min, 3500 rpm), proteins in the supernatants were resolved by SDS-PAGE on a 5 % acrylamide gel and then transferred to PVDF membrane. Blots were performed and analyzed as described above using the HA-tag antibody to probe for HA-tagged Δ1198-CFTR.

**Time-chase experiments-** WT-CFTR expressing HEK cells were incubated with curcumin (30 μM) for 15 min at 37°C in PBS-CM, pH 7.4. Cells were then rinsed twice with PBS-CM and once with DMEM supplemented with 10% Bovine Growth Serum (BGS) to remove curcumin. Cells were then incubated with DMEM supplemented with 10% BGS in absence or presence of cycloheximide (CHX, 25 μM) for 0 to 12h at 37°C. Cell lysates obtained after lysis with 1% Triton X-100 in PBS were analyzed by SDS-PAGE and immunoblotting as described above.

**SyproRuby® staining and MALDI-TOF analysis-** After SDS-PAGE, 5 % acrylamide gels were incubated with the SyproRuby® Protein Gel Stain (Invitrogen) overnight under gentle agitation and washed with 10% methanol, 7% acetic acid. Stained gel bands were excised using the Genomic Solutions ProPic automated sample picker. After gel plugs were excised, the samples were processed for mass spectrometry. Samples were destained with three consecutive washes of (v:v) 50 mM ammonium bicarbonate (ABC) plus acetonitrile for 30 min. Reduction of cysteines was carried out by using a 20 mM solution of dithiothreitol (Fisher) dissolved in 50 mM ammonium bicarbonate, and incubated at 60 °C for 60 min. Alkylation of free cysteine residues was then carried out with 55mM iodoacetamide (Fisher) in a 50 mM ABC solution for 60 min at room temperature. Samples were washed for ten minutes with 50 mM ABC prior to digestion. Enzymatic digestion with trypsin (12.5 ng/μL) (Promega Gold Trypsin Mass Spectrometry Grade) was carried out for sixteen hours at 37°C. Peptide solutions were then extracted using two washes of 50 μl of a solution of (v:v) 0.1% trifluoroacetic acid and acetonitrile for 30 min. Supernatants were collected and dried down in a Savant SpeedVac. Samples were resuspended in 10 μl of 0.1% trifluoroacetic acid. C18 ZipTips (Millipore) were used to desalt peptide mixtures before applying samples to the Applied Biosystems MALDI-TOF 96-well target plate. Peptides in 1:10 dilutions were mixed with a solution of α-cyano-4-hydroxyphenylacetic acid (CHCA, Acros) matrix (5mg/mL). Samples were allowed to dry before performing MALDI-TOF MS utilizing the Applied Biosystems Voyager DE-Pro scanning from 900-4000 m/z in positive ion mode. Spectra were then processed and analyzed using Voyager Explorer software, and peptide masses were submitted to MASCOT database for protein identifications.

**Immunoprecipitations from airway epithelial cells and HEK transfected cells-** WT or ΔF508-expressing CFBE41o-, Calu-3 or HEK cells grown on 35 mm plastic dishes were incubated with curcumin, curcumin analogs or DSS at 37 °C in PBS-CM, pH 7.4. Cells were then lysed in 1% Triton X-100 in PBS supplemented with protease inhibitors (see above) and cell lysates cleared by centrifugation (14,000 rpm for 15 min at 4 °C). The total protein content of supernatants was determined by the MicroBCA protein assay kit (Pierce). For each experiment, equal amounts of total proteins between samples were subjected to immunoprecipitation by 1 μg of the anti-CFTR NH2 tail (mAb 3482; Chemicon), anti-CFTR COOH tail (mAb clone 24-1; R&D Systems) or anti-HA tag antibody (mAb sc-7392; Santa Cruz Biotechnology) covalently cross-linked to A/G agarose beads (Protein A/G Plus-Agarose; Santa Cruz Biotechnology). Immunoprecipitations were carried out overnight at 4 °C. Beads were rinsed three times with 1% Triton X-100 in PBS and proteins were eluted by incubation at 37 °C for 30 min with reducing Laemmli buffer (50 mM dithiothreitol, 1.2 % β-mercaptoethanol). After a brief centrifugation (1 min, 3500 rpm), proteins in the supernatants were resolved by SDS-PAGE on a 5 % acrylamide gel and then transferred to PVDF membrane. The anti-CFTR NH2 tail (diluted at 1:1000), the anti-CFTR COOH tail (diluted at 1:2000), the anti-HA tag (diluted at 1:500) or the anti-ubiquitin antibody (mAb UbP4D7, sc-8017 Santa Cruz Biotechnology; diluted at 1:200) were used as primary antibody to probe for CFTR signals. Blots were performed and analyzed as described above.
Patch Clamp Analysis- Macroscopic currents were recorded in the excised, inside-out configuration as described previously (19). Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1–2 MΩ (macroscopic recordings). CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to PKA catalytic subunit (PKA; 110 units/ml; Promega) plus MgATP (1.5 mM). CFTR currents were recorded in symmetrical solution containing 140 mM N-methyl-D-glucamine-Cl, 3 mM MgCl₂, 1 mM EGTA, and 10 mM TES (pH 7.3). Macroscopic currents were evoked using a ramp protocol from +80 to -80 mV with a 10-s time period, unless otherwise indicated. All patch clamp experiments were performed at 21–23 °C. Signals were filtered at 20 Hz. Data acquisition and analysis were performed using pCLAMP8 software (Axon Instruments). The averaged data are presented as the means ± S.E. Statistical comparisons were made by performing paired t tests unless otherwise indicated.

RESULTS

Curcumin cross-links CFTR polypeptides into SDS-resistant oligomers in microsomes and intact cells- We first tested the effects of curcumin on the SDS-PAGE mobility of a CFTR truncation mutant that lacks NBD2 and the carboxy terminal tail (Δ1198-CFTR). This channel construct is strongly and persistently activated by curcumin (EC₅₀ of 15-20 μM) in excised membrane patches (20). Fig. 1 and 2 show that over the same concentration range curcumin cross-linked Δ1198-CFTR polypeptides into SDS-resistant oligomers (molecular weight above 250 kD) in a time (Fig. 1B) and dose-dependent manner (Fig.1C and Fig. 2A). This cross-linking effect occurred both in microsomes (Fig. 1) and within intact transfected HEK cells (Fig. 2). Wild type (WT) CFTR also was cross-linked in microsomes (Fig. 1D) and intact cells (Figs. 2C and 3). Cross-linking was not reversed by high concentrations of reducing agents such as beta-mercaptoethanol or DTT (note: all gels were reducing gels; see Methods) and also was observed for a cysteine-free CFTR construct (results not shown). Thus, this apparent oligomerization is not due to disulfide cross-linking of CFTR polypeptides. CFTR cross-linking by curcumin was specific in the sense that no such effect was observed for two other compounds; (i) a second CFTR activator (NPPB-AM) that stimulates WT-CFTR and ΔF508-CFTR channels (but not G551D-CFTR) (19) and (ii) a half-curcumin-like molecule (Fig. 1C and D; see compound structures in Fig. 1A). In other microsome experiments (not shown) we observed no appreciable effects of ATP and/or PKA on cross-linking by curcumin.

The mobilities of the primary cross-linked products were consistent with CFTR dimers and indistinguishable from the cross-links induced by a standard chemical protein cross-linker (DSS (Fig. 1C and D); previously used by others to detect CFTR ‘dimers’ in membranes (29)). The time course of cross-linking in microsomes (Fig. 1B) was similar to the time course for the development of persistent channel activation by curcumin previously observed in excised membrane patches (20). Quantifying the degree of Δ1198-CFTR and WT-CFTR cross-linking in microsomes revealed that 42 ± 3 % (mean ± SEM; n= 8) and 42 ± 15 % (n = 3) of the total pools, respectively, were cross-linked following 30 min exposure to 30 μM curcumin.

Fig. 2 indicates that the surface pool of CFTR in intact cells is susceptible to cross-linking by curcumin as deduced from surface biotinylation experiments (panels A and B). Surface-accessible HA-tagged Δ1198-CFTR in HEKs was biotinylated at 0 °C after exposure to the indicated concentrations of curcumin for 10 min at 37 °C. Curcumin efficiently cross-linked both the surface pool (streptavidin pulldown) and the total pool (lysate). In the experiment shown in Fig. 2A, 13%, 37% and 58% of the biotinylated pool was cross-linked after exposure to 7, 15 and 30 μM curcumin, respectively.

In initial chase experiments we detected no apparent disappearance of the cross-linked CFTR polypeptides from the cell over a 60 min chase period at 37 °C (Fig. 2B); thus, these adducts are not rapidly degraded by the cell. However, in subsequent chase experiments performed over
longer time periods we observed that the cross-linked complexes gradually disappeared from the cells (e.g., Fig. 2C). For example, 44 ± 16 % and 8 ± 2 % of the cross-linked WT-CFTR adducts remained in the cells 4 and 12h following curcumin washout (n = 2; see Fig. 2C for example time course). The disappearance of the cross-linked complexes was unaffected by treating the cells with a protein synthesis inhibitor (cycloheximide; Fig. 2C) or with inhibitors of the proteasome (MG132) or lysosomal-dependent degradation (chloroquine) (results not shown). As expected, the monomeric band C form of WT-CFTR (band C is the mature, fully glycosylated form) was more stable under these conditions; i.e., in the same chase experiments performed in the presence of cycloheximide 95 ± 12 % and 66 ± 1 % of the monomeric band C remained 4 and 12 h after curcumin washout, n = 2). Interestingly, the monomeric band B signal (immature, ER form) transiently increased at the first time point after curcumin washout even in the presence of cycloheximide (3 out of 3 experiments; e.g., Fig. 2C) before returning to its initial level (see also Fig. 3 and below). Taken together these results imply that the gradual disappearance of the curcumin-induced cross-linked product over time is not due to degradation by the common degradative pathways but rather may be due to slow reversibility of this modification within the cell.

MALDI-TOF analysis of the high molecular weight adduct also was performed to rigorously confirm the presence of CFTR polypeptides in the apparent dimer. Fig. 2D shows a SYPRO® Ruby-stained gel resolving the monomeric and high molecular weight forms of HA-tagged Δ1198-CFTR isolated from untreated and curcumin-treated HEK cells by immunoprecipitation. The indicated bands were excised and analyzed by peptide mass fingerprinting. CFTR-derived peptides were identified in each band with total sequence coverage of 16% (band 1) and 13% (bands 2 and 3). No other proteins were detected by MALDI-TOF analysis of any of the three bands.

Curcumin cross-links WT-CFTR and, to a greater extent, ΔF508-CFTR in airway epithelial cell lines- Cystic fibrosis is primarily a disease of the large airways; accordingly, treatment strategies must be directed toward rescuing mutant CFTR function in lung epithelial cells. Fig. 3 shows that curcumin cross-linked both WT-CFTR and ΔF508-CFTR in a CF bronchial epithelial cell line (CFBE) transduced with either construct. As reported previously for these and other cell types, the monomeric ΔF508-CFTR polypeptide migrates primarily as the immature, ER form (band B) as opposed to the mature band C form that comprises the majority of the WT-CFTR monomer. Both forms were cross-linked by curcumin (or by DSS). However, the band B forms of either ΔF508-CFTR or WT-CFTR were more efficiently cross-linked than the mature band C WT-CFTR polypeptide (Figs. 3B and D; 96 ± 1% of ΔF508-CFTR, 76 ± 13% of WT-CFTR band B vs. 22 ± 8% of WT-CFTR band C (n = 5 each)). Cross-linking of the wild type CFTR protein by curcumin (and by DSS) was less efficient but detectable in another human airway epithelial cell line which endogenously expresses WT-CFTR (Calu-3; Fig. 3C), which indicates that the degree of cross-linking may be cell type-specific.

The amino terminal tail of CFTR is buried in the cross-linked oligomer, but is not necessary for cross-linking by curcumin- We observed that a monoclonal antibody that recognizes the amino terminal of CFTR (mAb 3482; Chemicon) negligibly immunoprecipitated the cross-linked oligomer. These immunoprecipitation (IP) experiments were performed using curcumin-treated WT or ΔF508 CFBE cells that were lysed in Triton X-100 to minimize effects on native protein structure (Fig. 3D; compare to the IP result obtained with a carboxy terminal tail antibody (mAb clone 24-1; R&D Systems)). This anti-CFTR NH₂ tail antibody does effectively IP monomeric CFTR (Fig. 3D) and labels the cross-linked oligomer in immunoblots (Fig. 1B). To explore if the CFTR amino-terminal tail was required for the cross-linking induced by curcumin we tested if this compound could cross-link a deletion construct that lacks the entire amino terminal tail (Δ2-79-CFTR). Fig. 3E shows that curcumin also cross-links this construct in transfected HEK cells as well as a deletion construct that lacks most of the R domain (ΔR-CFTR). Thus, the amino terminal tail is not required for cross-linking by curcumin but somehow is buried in the oligomer once formed.
Cross-linking possibly involves an oxidation reaction and clearly requires the \(\beta\)-diketone moiety of the curcumin molecule. We tested two possible mechanisms that could account for this biochemical effect of curcumin: (i) polyubiquitination of CFTR polypeptides induced by curcumin and (ii) an oxidation reaction involving the reactive diketone group of this compound. The rationale for exploring the first mechanism was based on a published report that curcumin induces ubiquitination of the ErB2 protein kinase receptor in a carcinoma cell line (34). We found no evidence, however, for this modification when we probed immunoprecipitated CFTR samples (monomers and curcumin-induced oligomers) with an anti-ubiquitin antibody. No ubiquitin signal was observed for the CFTR IPs even when the cells were first treated with a proteasome inhibitor (MG132) to accumulate ubiquitinated polypeptides (accumulation verified by immunoblotting with the anti-ubiquitin antibody; Fig. 4A). Conversely, Fig. 4B shows that CFTR cross-linking by curcumin in microsomes was prevented by two oxidant scavengers; reduced glutathione (scavenges diverse oxidants via its free thiol group; (35)) and sodium azide (scavenges singlet oxygen; (36)). The latter results are consistent with an oxidation reaction, although it is also possible that these compounds reduced the effective curcumin concentration by chemical transformation (especially in the case of glutathione, which might bind curcumin via its free thiol (37)).

Curcumin has a broad range of pharmacological activities linked to its structure which displays two phenolic rings and a \(\beta\)-diketone reactive group. Curcumin is known to establish covalent bounds with nucleophilic amino-acid side chains by oxidation or alkylation (27). Conceivably, curcumin could cross-link CFTR channels by covalently binding to nucleophilic residues in adjacent polypeptides. To further explore the underlying mechanism of curcumin-induced cross-linking we tested three cyclic derivatives of this compound that lack the reactive \(\beta\)-diketone group for their abilities to cross-link CFTR channels. These derivatives were synthesized initially as part of an analysis of the effects of curcumin and its derivatives on beta amyloid aggregation. Fig. 5 shows that none of the cyclic derivatives were able to cross-link \(\Delta1198\)-CFTR, WT-CFTR, \(\Delta F508\)-CFTR or G551D-CFTR either in microsomes (Fig. 5B and D) or in intact cells (Fig. 5C). For positive controls we used synthetic curcumin in addition to the purified curcumin preparation obtained commercially, both of which robustly crosslinked CFTR polypeptides (e.g. Fig. 5B).

**CFTR channels are activated by cyclic curcumin analogs that cannot cross-link CFTR polypeptides**- The three cyclic analogs that cannot cross-link CFTR were tested for their abilities to promote CFTR channel activity in excised membrane patches. The oxazole (BSc3596) was found to be more potent than the others in pilot experiments and was selected for further study. Fig. 6 shows that BSc3596, like the parent compound, activated \(\Delta1198\)-CFTR channels, G551D-CFTR channels and partially phosphorylated WT-CFTR channels. Excised inside-out patches were first treated with PKA catalytic subunit to phosphorylate CFTR and then with the PKA inhibitory peptide (PKI) to block further phosphorylation (the PKI-induced decrease in WT-CFTR-mediated current (Fig. 6A) presumably represents partial dephosphorylation mediated by membrane-associated phosphatases). WT-CFTR currents were stimulated by BSc3596 at a dose as low as 0.5 \(\mu\)M with maximal stimulation at 10 \(\mu\)M compound (3.0 ± 0.8 fold stimulation above post-PKI current (n = 4), p < 0.05). The currents stimulated by BSc3586 were inhibited by a specific CFTR channel inhibitor, CFTR(inh)172.

Persistent (i.e., irreversible) activation of CFTR channels during extended exposure to curcumin (> 5 min) in the absence of ATP is an intriguing feature of this compound. Given that this time-dependent mode of activation might be related to the time-dependent cross-linking that we observed, we determined if CFTR channel activation by BSc3596 (which cannot cross-link CFTR polypeptides) also becomes irreversible over time. Fig. 6B and C show that this is the case. Prolonged (> 15 min) exposure of G551D-CFTR or \(\Delta1198\)-CFTR-containing excised membrane patches to 30 \(\mu\)M BSc3596 in the absence of ATP irreversibly stimulated the current (54 ± 17% (n=5) of the BSc3596-stimulated current remained after washout of BSc3596 for \(\Delta1198\)-CFTR). This
result confirms that irreversible channel activation is unrelated to the cross-linking of CFTR polypeptides into SDS-resistant oligomers. G551D-CFTR and Δ1198-CFTR normally have very low activity because of their defective activation by ATP. The fact that the BSc3596 analog robustly stimulated G551D-CFTR and Δ1198-CFTR activity in the absence of ATP indicates that, like curcumin, the BSc3596 analog likely acts downstream of ATP binding and NBD dimerization.

DISCUSSION

Our study raises a note of caution about unexpected biochemical effects of reactive compounds like curcumin on CFTR channels. We observed curcumin-induced cross-linking of a wide variety of CFTR constructs both in vitro and in multiple cell types including airway epithelial cells. This effect occurred at concentrations of curcumin and exposure times that are effective for stimulating CFTR channel activity and that are typically used in other applications (20, 38, 39). We consider it unlikely that such cross-linking has a positive influence on the function of CFTR, although it is interesting to note that cross-linking does not obviously inhibit CFTR channel function over relatively short time periods (< 60 min). In this regard, macroscopic CFTR-mediated currents in excised membrane patches are stably stimulated by curcumin over this time frame (no ‘run down’ following the initial rapid stimulation (20)). Up to 50 % of the CFTR polypeptides become cross-linked under the same conditions over this time period (e.g., compare Fig. 1B and 2A). Thus, cross-linking per se has no noticeable effect on macroscopic CFTR channel function at least in this time frame. On the other hand, the ability of curcumin to rapidly and persistently stimulate CFTR channels is unrelated to this cross-linking activity as best illustrated by our results with the cyclic curcumin derivatives.

Nature of the cross-link- We interpret the high molecular weight complexes that are induced by curcumin to be CFTR dimers (and higher order oligomers to a lesser extent) based on: (i) indistinguishable SDS-PAGE mobilities of the curcumin-induced high molecular weight bands and those bands induced by a standard chemical cross-linker (DSS) and (ii) the fact that CFTR was the only polypeptide in the curcumin-induced high-molecular weight complex identified by MALDI-TOF analysis. Of course, this does not completely rule out the possibility of an interaction between CFTR and another membrane-associated protein (small enough to not apparently change CFTR SDS-PAGE mobility).

Every CFTR construct that we tested could be cross-linked by curcumin including a variety of deletion constructs lacking the amino and carboxy terminal tails and all major cytoplasmic domains (R domain (Fig. 3E), NBD2, (Fig. 1B and C) and NBD1 (data not shown)). The transmembrane domains or cytoplasmic loops would appear to be the most likely sites for cross-linking by default, although it is possible that cross-linking occurs at multiple sites in the polypeptide. It is of potential interest that the NH2-tail of CFTR is buried within the curcumin-induced oligomers; i.e., inaccessible for immunoprecipitation. However, this cytoplasmic tail clearly is not necessary for cross-linking per se. Curcumin-induced cross-linking can occur at the plasma membrane and in the ER as shown by the cross-linking of the cell surface localized WT CFTR and the ER-retained mutant ΔF508 CFTR. The ΔF508 CFTR findings also indicate that the immature form of CFTR is more efficiently cross-linked. Increased cross-linking efficiency similarly was observed for the band B (ER) form of WT CFTR. Whether this is due to conformational differences that are permissive for cross-linking or to other factors in the ER (e.g., protein crowding) cannot be determined by the present data.

The efficient cross-linking of ΔF508-CFTR was unexpected because curcumin was originally reported to promote the maturation and ER export of ΔF508-CFTR (although this result is in dispute) (21- 25). It is conceivable that cross-linking of ΔF508-CFTR polypeptides within the ER could promote export by burying ER retention signals or hydrophobic surfaces (40). Indeed, we and others reported previously that ΔF508-CFTR export from the ER could be promoted by co-expressing this processing mutant with certain fragments of the
wild type CFTR protein (i.e., by transcomplementation) (41). For curcumin, however, we observed no induction of the classical band C form of the ΔF508-CFTR protein, only a nearly complete (approximately 90%) conversion of the band B form to a putative dimer. We are surprised that others who have tested the effects of curcumin on ΔF508-CFTR processing have not reported a similar finding (although some published blots indicate a marked reduction in the band B form of ΔF508-CFTR following curcumin treatment (e.g., see Fig. 1A (40 μM curcumin treatment for 18 h at 37 °C) and Fig. 1D (50 μM curcumin treatment for 3 h at 37 °C) in (23)). This apparent disparity unlikely is due to the use of different cell types or curcumin preparations, given that we observed efficient ΔF508-CFTR cross-linking in two cell types (HEK and bronchial epithelial cells (CFBE)) with two curcumin preparations (including synthetic curcumin).

Curcumin-induced CFTR cross-linking likely occurs via a reaction involving its α, β-unsaturated β-diketone reactive group since removal of this moiety by cyclization abolished this effect. This moiety of curcumin can react with nucleophilic side chains of cysteine, histidine, arginine and lysine via a Michael-type addition reaction (27) which potentially leads to protein cross-linking (42). The inability of reducing agents like DTT and β-mercaptoethanol to disrupt curcumin-induced CFTR oligomers, as well as the ability of curcumin to cross-link a cysteine-free CFTR construct (data not shown), indicate that curcumin cross-links CFTR without reacting with cysteine sulfhydryl groups. We attempted to determine which other amino acid residues could be involved in the curcumin-induced CFTR cross-linking by pretreating CFTR-containing microsomes with nucleophilic amino acid modifiers like N-acetylsuccinimide (lysine modifier), phenylglyoxal (arginine modifier) (43) and DEPC (histidine modifier) (44) before curcumin treatment. None of these modifiers prevented CFTR cross-linking (data not shown) indicating either that curcumin interacts with CFTR via a different reaction than the Michael addition or that the modifiers were unable to block all reactive groups. Curcumin also can behave as a photosensitizer via its ketone groups, which can absorb light and lead to the production of reactive species like singlet oxygen (45, 46). These reactive oxygen species can oxidize nucleophilic amino acid residues which in turn can react with other residues to create new inter- or intra-molecular bonds (47). Such a photo-oxidation reaction might contribute to CFTR cross-linking by curcumin since i) this compound absorbs light in the visible spectrum (46) and ii) NaN₃, which is a potent quencher of singlet oxygen (48), reduced curcumin-induced CFTR oligomerization. However, we observed no cross-linking by a “half-curcumin” analog that has one β-ketone group and therefore should retain its capacity as a photosensitizer. It appears that the symmetrical structure of curcumin is required to induce CFTR cross-linking. This argues for a direct interaction of each β-ketone of curcumin with one CFTR molecule although it is also conceivable that a change in membrane fluidity following curcumin treatment (49) might allow spatially adjacent CFTR polypeptides to engage in strong hydrophobic and/or van der Waals interactions that may promote CFTR oligomerization. Whatever the mechanism this modification appears to be gradually reversible within the cell as indicated by the slow disappearance of the cross-linked adducts (and concomitant increase in the monomeric CFTR pool) after curcumin washout (see Fig. 2C and Results).

Lessons for drug design- The lesson for CFTR drug design is that highly reactive compounds such as curcumin can have unexpected biochemical effects on the CFTR polypeptide. This caveat probably is not unique to curcumin. There are other examples in the literature of protein cross-linking by aromatic compounds. The underlying mechanisms may vary among compounds; for example, erbstatin (a tyrosine kinase inhibitor) reportedly induces protein cross-linking via an oxidation reaction involving its aromatic hydroxyls (50). The fact that such gross biochemical changes can be induced by multiple compounds and reaction mechanisms emphasizes the importance of fully characterizing the biochemical effects of prospective drugs.

Protein cross-linking by curcumin also may not be unique to CFTR. Curcumin is under investigation in the treatment of a wide variety of disorders (e.g., as an anti-tumorigenic and
antioxidant compound (37, 51). Given its high degree of chemical reactivity it seems plausible that other proteins could be cross-linked by this compound as well.

A cyclic curcumin derivative robustly and persistently activates CFTR channels without cross-linking CFTR polypeptides. On a positive note, one of the cyclic curcumin derivatives (BSc3596) potently activated wild type CFTR, G551D-CFTR and Δ1198-CFTR channels in excised membrane patches. This curcumin derivative, like two other cyclic analogs that lack the β-diketone moiety, failed to cross-link CFTR into SDS-resistant oligomers. Thus, it is possible to design curcumin derivatives that retain the ability to activate CF mutant channels without this secondary biochemical effect.

In a previous study we reported that CFTR Cl- channels in excised membrane patches become persistently (irreversibly) activated by curcumin during extended exposure to this compound (> 5 min; (20)). We considered the possibility that this persistent activation could be related to the CFTR cross-linking that occurs with a similar time course. This was an intriguing possibility because it had been argued by some investigators that CFTR channel activity can be enhanced by maneuvers that promote CFTR dimerization (29, 52). However, we observed that BSc3596, like the parent compound, irreversibly activated CFTR channels in spite of its inability to induce cross-linking. This finding clearly separates the property of curcumin to cross-link CFTR polypeptides into SDS resistant dimers from its ability to irreversibly potentiate channel function. Conceivably, the irreversible activation by BSc3596 and curcumin reflects a different type of covalent interaction with the CFTR polypeptide; one that does not require the β-diketone group.

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**FOOTNOTES**

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The abbreviations used are: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; NBD, nucleotide-binding domain; ABC, ATP-binding cassette; WT, wild type; PKA, protein kinase A; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DSS, disuccinimidyl suberate; DTT, dithiotreitol. TES, 2-[[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

**FIGURE LEGENDS**

**Figure 1:** Curcumin cross-links Δ1198-CFTR and WT CFTR in microsomes in a time and dose-dependent manner- Fig. 1A gives the structures of curcumin, the “half-curcumin” analog 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one) and the CFTR potentiator NPPB-AM. Fig. 1B: Δ1198 CFTR-containing microsomes (60 μg total protein per condition) were incubated at room temperature for the indicated time periods. The control vehicle corresponds to 60 min incubation with DMSO only. After SDS-PAGE the blot was probed with the anti-CFTR NH2 tail antibody (mAb 3482; Chemicon). Fig1. C and D: Δ1198 or WT CFTR-containing microsomes were treated with the indicated concentrations of curcumin for 40 min at room temperature. To assess the specificity of the curcumin cross-linking effect Δ1198 or WT CFTR containing microsomes were also treated with the “half-curcumin” analog (30 μM; see Fig. 1A) or NPPB-AM (200 μM). Microsomes also were treated with the chemical cross-linker DSS as indicated. After SDS-PAGE the blot was probed with the anti-CFTR NH2 tail antibody to detect Δ1198 CFTR or the anti-CFTR COOH-tail antibody to probe for WT CFTR.

**Figure 2:** Curcumin cross-links CFTR channels within intact HEK cells including the cell surface pool- Fig. 2A: HA-tagged Δ1198 CFTR expressing HEK cells were incubated with the indicated concentrations of curcumin for 10 min at 37 °C before biotinylation of cell surface proteins at 0 °C. Cell surface CFTR expression was assessed by isolating biotinylated proteins by streptavidin pulldown (9.5 mg total protein were subjected to streptavidin pulldown for each sample) and the total CFTR pool was assessed by direct SDS-PAGE/immunolotting of cell lysates (250 μg total protein directly resolved by SDS-PAGE for each sample). The blot was probed with the anti-HA tag antibody. Fig. 2B: HA-tagged Δ1198 CFTR expressing HEK cells were incubated with curcumin (30 μM) for 10 min at 37 °C before biotinylation of cell surface proteins at 0 °C. After quenching of the biotin with BSA and extensive washing steps, cells were warmed to 37 °C for the indicated period of times to follow the stability of the curcumin-induced
CFTR complexes. In this experiment, for each sample, 5.4 mg total protein were subjected to strepavidin pulldown and 150 μg were directly resolved by SDS-PAGE. The blot was probed with the anti-HA tag antibody. Fig. 2C: WT-CFTR expressing cells initially treated with curcumin (30 μM for 15 min at 37°C) were incubated in curcumin-free media (DMEM plus 10% BGS) with or without cycloheximide (CHX, 25 μM) for the indicated period of time. The graph shows the band D signal intensity (cross-linked dimer) at t(h) after curcumin washout in presence or absence of cycloheximide normalized to the initial band D signal at time zero. Fig. 2D: vector alone transfected cells (left lane) or HA-tagged Δ1198 CFTR expressing cells were treated with curcumin (50 μM) or DMSO for 10 min at 37 °C. For each sample, after cell lysis, 10 mg of total proteins were immunoprecipitated by 5 μg of the anti-HA tag Ab. After extensive washing steps and SDS-PAGE of the samples the acrylamide gel was stained with the SyproRuby® fluorescent dye overnight at room temperature. Bands 1, 2 and 3 stained by the dye were excised from the gel and their protein content analyzed by MALDI-TOF. Insets give for each band the number of peptides identified, examples of three of those peptides and the Mowse scores. Protein identification by peptide mass fingerprinting is considered significant (p < 0.05) for a score > 67.

Figure 3: DSS and curcumin cross-link WT and ΔF508 CFTR in airway epithelial cells- Fig. 3A, B and C: CFBE41o- cells stably transduced with WT or ΔF508 CFTR and Calu-3 cells which endogenously express WT CFTR were treated with DSS (1 mM) or curcumin (50 μM) for 15 min at 37 °C. The control (-) corresponds to 15 min incubation with DMSO only. Cell lysates were then subjected to immunoprecipitation by the anti-CFTR COOH tail Ab before reducing SDS-PAGE. The blots were probed with the same antibody. Bands B and C are ER and mature forms of CFTR, respectively. Panel B was a longer exposure to increase the ΔF508 signal. Fig. 3D: CFTR NH2-tails are buried within the curcumin-induced CFTR complexes. WT or ΔF508 CFTR expressing CFBE41o- cells were treated with curcumin (50 μM) for 15 min at 37 °C prior to lysis and immunoprecipitation by 1 μg of the anti-CFTR COOH or the anti-CFTR NH2 tail antibody. After reducing SDS-PAGE the blots were probed with the anti-CFTR COOH tail antibody.

Figure 4: The curcumin-induced CFTR complexes do not result from CFTR ubiquitination but can be prevented by antioxidants- Fig. 4A: HA-tagged Δ1198 CFTR expressing HEK cells were incubated with curcumin (50 μM) or DMSO for 10 min at 37 °C. As a positive control for ubiquitination some of the HA-tagged Δ1198-CFTR expressing HEK cells were treated with the proteasome inhibitor MG132 (55 μM) for 2h at 37 °C prior to curcumin addition. Cell lysates were subjected to immunoprecipitation by the anti-HA tag antibody before reducing SDS-PAGE. Duplicate samples were then probed with the anti-HA tag antibody or with an anti-ubiquitin antibody. Fig. 4B: Curcumin-induced CFTR cross-linking is inhibited by reduced glutathione (GSH) or NaN3. Δ1198 CFTR-containing microsomes were incubated with GSH (20 mM) or NaN3 (100 mM) for 10 min at room temperature before incubation with curcumin (30 μM, 30 min at room temperature). Controls correspond to Δ1198 CFTR-containing microsomes incubated with GSH or NaN3, only. After reducing SDS-PAGE, CFTR was probed with the anti-NH2 tail antibody.

Figure 5: Cyclic curcumin analogs that lack the β-diketone reactive group do not cross-link CFTR- Fig. 5A: structures of synthetic curcumin and of three curcumin analogs (BSc3596, BSc3732 and BSc3736) for which β-diketone reactive group has been cyclized. Fig. 5B: Δ1198 CFTR-containing microsomes were incubated with 30 μM curcumin, synthetic curcumin, BSc3596, BSc3732 or BSc3736 for 30 min at room temperature. After reducing SDS-PAGE, Δ1198 CFTR was probed with the anti-CFTR R-domain.
antibody. Fig. 5C: WT, ΔF508 or G551D CFTR-expressing HEK cells were treated with 30 μM curcumin or BSc3596 or with 1 mM DSS for 15 min at 37 °C. The control vehicle corresponds to a similar incubation with DMSO only. Cell lysates were then subjected to immunoprecipitation by the anti-CFTR COOH tail antibody and the same antibody was used to probe for CFTR after reducing SDS-PAGE. Fig. 5D: WT, ΔF508 or G551D CFTR-containing microsomes were incubated with 30 μM curcumin or BSc3596 or with 1 mM DSS for 30 min at room temperature. The control vehicle corresponds to DMSO only. Before exposure to curcumin, BSc3596, DSS or DMSO, microsomes were pre-treated with PKA catalytic subunit (110 U/ml) plus ATP (1.5 mM) for 10 min at room temperature to achieve CFTR phosphorylation. Phosphorylation was then stopped by adding PKI (1.4 μg/ml) to each sample for another 10 min at room temperature. After reducing SDS-PAGE, CFTR was probed using the anti-CFTR COOH tail antibody. Right panel is longer exposure of the ΔF508-CFTR blot.

Figure 6: BSc3596 activates CFTR channels in excised membrane patches- Fig. 6A, B and C: Macroscopic currents recorded in inside-out configuration for WT, G551D or Δ1198-CFTR channels. Current were recorded using a ramp protocol (-80 to + 80 mV). PKA (110 U/ml) and ATP (1.5 mM) were present in the bath solution at the beginning of the recording to achieve channel phosphorylation. Phosphorylation was then inhibited by adding PKA inhibitory peptide (PKI; 1.4 μg/ml) to the bath before exposure of the membrane patch to the BSc3596 analog. Fig. 6A WT-CFTR activation: WT-CFTR containing patches were exposed to increasing concentrations of the BSc3596 analog (0.5, 1, 5, 10 and 30 μM) before inhibition of the CFTR mediated-Cl− current by 10 μM of the specific CFTR inhibitor CFTR(inh)172. Inset, titration data with best fit to the Hill equation (Hill coefficient = 0.3; n = 4). Fig. 6B G551D-CFTR activation: G551D-CFTR containing membrane patches were exposed to 30 μM BSc3596 after ATP washout over a 15 min time period. The analog was perfused from the chamber and the current inhibited by 10 μM CFTR(inh)172. Right, scatter plot of BSc3596 data obtained from 4 individual G551D experiments. The variable degree of activation is due presumably to variable numbers of G551D-CFTR channels in these 4 patches. Fig. 6C Persistent activation of Δ1198-CFTR channels by BSc3596. Same conditions as for Fig. 6B.
Figure 1

A

Curcumin

Half curcumin

NPPB-AM

B

Δ1198 CFTR (microsomes)

Curcumin (30 μM) DMSO

3 6 12 20 30 45 60 60 (min)

kD

C

Δ1198 CFTR (microsomes)

Curcumin (μM)

0 0.5 1 5 10 20 30

kD

D

WT CFTR (microsomes)

Curcumin (μM)

0 0.5 1 5 10 20 30

kD
Figure 2

A  

| Streptavidin pulldown (surface CFTR) | Lysate (total CFTR) | Biotin (μM) | Curcumin (μM) |
|--------------------------------------|---------------------|-------------|---------------|
| + + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + + | 0 1 3 7 15 30 50 50 0 1 3 7 15 30 50 50 |

B  

| Streptavidin pulldown (surface CFTR) | Lysate (total CFTR) | Chase time (min) |
|--------------------------------------|---------------------|------------------|
| + + + + + + + + + + + + + + + + + + | + + + + + + + + + + + + + + + + + + | 0 0 3 7 15 35 45 60 25 0 0 3 7 15 35 45 60 25 |

C  

| 0 3 6 12 | Time back in DMEM + 10% BGS (h) | CHX (25 μM) | Curcumin (30 μM) |
|----------|---------------------------------|-------------|------------------|
| - - - +  | - - - + - - - + - - - + - - - + | - - - + - - - + - - - + - - - + |

D  

| HA-Δ1198 CFTR (HEK cells) | Curcumin (50 μM) |
|---------------------------|------------------|
| + - + + + + + + + + + + + | kD 250 |

Band 1:  
- 14 CFTR peptides identified (3 shown as examples): K.VSLAPQANL.TELDIYSR.R R.KVSLAPQANL.TELDIYSR.R K.FAEKDNIVL.GEGGTLSGGQR.A  
- Mowse score: 149

Band 2:  
- 16 CFTR peptides identified (3 shown as examples): R.GQLLAVAGSTAGK.T K.LMGCDSDQQFSAERR.N K.FAEKDNIVL.GEGGTLSGGQR.A  
- Mowse score: 116

Band 3:  
- 17 CFTR peptides identified (3 shown as examples): K.ENIFGVS.DEYR.Y R.LVITSEMIELQSVK.A K.FAEKDNIVL.GEGGTLSGGQR.A  
- Mowse score: 125

Band 4:  
- WT-CFTR

Band D t(h)/Band D t(0) (%) 
- CHX  
- + CHX 

WT-CFTR 

0 5 10 15 

Time (h)
Figure 3

A  WT CFTR (CFBE cells)
    DSS       Curcumin
    -       -
    +       +
    kD
    250
    150
    100

B  ΔF508 CFTR (CFBE cells)
    DSS       Curcumin
    -       -
    +       +
    kD
    250
    150
    100

C  WT CFTR (Calu-3 cells)
    DSS       Curcumin
    -       -
    +       +
    kD
    250
    150
    100

D  WT CFTR (CFBE cells)
    COOH IP   NH2 IP
    -       -
    +       +
    kD
    250
    150
    100

E  ΔF508 CFTR (CFBE cells)
    COOH IP   NH2 IP
    -       -
    +       +
    kD
    250
    150
    100

HEK cells

ΔF508  Δ2-79  ΔR
    -       -       -
    +       +       +
    kD
    250
    150
Figure 4

A

|          | HA-Ab | Ubiquitin-Ab |
|----------|-------|-------------|
| IP       | -     | -           |
| Lysate   | +     | -           |
|          | -     | +           |
|          | +     | -           |
|          |       | +           |

MG132 (55 μM)
Curcumin (50 μM)

HA-Δ1198 CFTR (HEK cells)

B

|          |          |
|----------|----------|
|          | NaN₃ (100 mM) |
|          | GsH (20 mM) |
|          | Curcumin (30 μM) |

Δ1198 CFTR (microsomes)
Figure 5

A

Synthetic curcumin

BSc3596

BSc3732

BSc3736

HEK cells

DMSO

Curcumin

BSc3596

BSc3732

BSc3736

C

D

WT   ΔF508   G551D

vehicle curcumin  BSc3596  DSS  vehicle curcumin  BSc3596  DSS  vehicle curcumin  BSc3596  DSS

vehicle curcumin  BSc3732  DSS  vehicle curcumin  BSc3732  DSS  vehicle curcumin  BSc3732  DSS

vehicle curcumin  BSc3736  DSS  vehicle curcumin  BSc3736  DSS  vehicle curcumin  BSc3736  DSS

Δ1198 CFTR (microsomes)
Figure 6

A) WT-CFTR

B) G551D-CFTR

C) Δ1198-CFTR

Table: EC50 = 0.8 ± 0.6 μM
Curcumin cross-links cystic fibrosis transmembrane conductance regulator (CFTR) polypeptides and potentiates CFTR channel activity by distinct mechanisms
Karen Bernard, Wei Wang, Rajeshwar Narlawar, Boris Schmidt and Kevin L. Kirk

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