Trimethylangelicin reduces IL-8 transcription and potentiates CFTR function

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Cystic fibrosis (CF) is a severe genetic disease due to defects of the CF transmembrane conductance regulator (CFTR) gene (for review, see Ref. 39). CF affects several organs, with the chronic pulmonary disease being the major cause of reduction of the quality and expectancy of life. The hallmark of CF lung disease is chronic infection sustained by the gram-negative bacterium Pseudomonas aeruginosa and excessive lung inflammation with a huge infiltrate of neutrophils in the bronchial lumen, mainly due to the release of the chemokine interleukin (IL)-8 (2, 4, 5, 11, 24, 27, 36). The identification of novel drugs, to reduce the excessive lung inflammation in CF, is considered a key therapeutic target to circumvent progressive lung tissue deterioration (for review, see Ref. 25).

Psoralens (PSRs) are well-known furocoumarins belonging to the class of photosensitizers used for their activity in the treatment of various chronic inflammatory skin diseases (6, 7, 12, 13, 23, 30, 37), and they are characterized by a differently substituted tricyclic aromatic skeleton, derived from condensation of a coumarin nucleus with a furan ring. Among PSR-related compounds, the angular angelicin (ANG)-like isomers are both synthetic and natural compounds, e.g., derived from the medicinal plant Angelica archangelica, that could exhibit interesting pharmacological activity compared with linear PSRs, showing low toxicity and low DNA-binding activity (6, 23).

As far as lung inflammation in CF is concerned, we have preliminary indications that 10 M 5-methoxypsoralen inhibits IL-8 transcription in bronchial epithelial cells exposed to P. aeruginosa, thus suggesting the potential usefulness of PSRs in the regulation of proinflammatory genes and strengthening the idea of searching for more potent analogs for this application (33). The aim of the present study is to determine the activity of the four PSRs on the expression of IL-8 gene, the major chemokine released from CF cells infected by P. aeruginosa (19). The PSRs studied here, and described in Fig. 1, have been chosen as prototypes of linear vs. angular and of nonmethylated vs. trimethylated structures, namely PSR, ANG, 4,5’,8-trimethylpsoralen (TMP), 4,6,4’-trimethylangelicin (TMA). Since PSR-like structure might lead to alteration of the nuclear DNA-binding activity compared with linear PSRs, showing low toxicity and low DNA-binding activity (6, 23).

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DNA sequences was investigated by in silico docking analysis, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) of IL-8 promoter. Moreover, since PSRs have been shown to be potentiators of CFTR function (17), we have investigated the effect of the most effective PSR analog here described on the activation of CFTR-mediated chloride efflux. The present results here identify TMA as a compound acting both as a strong inhibitor of P. aeruginosa-dependent expression of IL-8 and as a potentiator of CFTR-mediated chloride efflux.

MATERIALS AND METHODS

Compounds. ANG (2-oxo-(2H)-furo-[2,3-h]-1-benzopyran) was purchased from Sigma/Aldrich (Milwaukee, WI). PSR, TMP, and TMA were synthesized at the Department of Pharmaceutical Sciences, University of Padova, Italy (Fig. 1).

Cell cultures. IB3–1 cells, derived from a CF patient with a F508del/W1282X mutant genotype, were grown as previously described (3, 8, 15, 16, 32); non-CF Calu-3, a cell line obtained from a human lung adenocarcinoma derived from submucosal gland of proximal bronchial airways, and CF CuFi-1 cell line, obtained from human bronchial epithelium derived from a CF patient with a F508del/F508del mutant genotype, were cultured on Transwell membranes (41). The cells were seeded at density of 600,000 cells/cm² onto collagen-coated Transwell polyester membranes (0.33 cm², 0.4-m pore size) (Becton Dickinson, Franklin Lakes, NJ). The experiments were performed when the confluent cell monolayers reached a transepithelial electrical resistance > 1,500 Ω × cm², CFBE41o– cells homozygous for the F508del allele (F508del/F508del) were a generous gift of Professor D. Gruenert, University of California at San Francisco, and CFBE41o–/sNHERF1 were CFBE41o– cells stably transfected with cDNA encoding wild-type NHERF1 (20). For chloride efflux experiments, the cells were seeded on 0.4-μM pore size polyethylene terephthalate filter inserts (Falcon Becton-Dickinson Labware).

Cell infection, quantification of transcripts of inflammatory genes, and IL-8 protein. Cells were starved and treated with different PSRs for 20 h before infection, which was performed as previously described (3, 8, 15, 16, 32). Quantification of transcripts of inflammatory genes was performed by quantitative RT-PCR; proliferation and antibacterial assays were done as previously described (32, 33). IL-8 secretion was assessed at different doses of TMA added for 20 h, then media and TMA were freshly added and collected after 4 h of P. aeruginosa infection. IL-8 was measured by ELISA (Bender MedSystems, Wien, Austria), in duplicate, according to the manufacturer’s instructions.

Fluorescence measurements of apical chloride efflux. Chloride efflux was measured using the Cl⁻-sensitive dye N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) (20). Confluent Calu-3 cell monolayers were loaded overnight in culture medium containing 5 mM MQAE at 37°C in a CO₂ incubator and then inserted into a perfusion chamber that allowed independent perfusion of apical and basolateral cell surfaces. The apical Cl⁻ efflux measurements were performed when the confluent cell monolayers reached a transepithelial resistance > 1,500 Ω × cm² for Calu-3 and > 300 Ω × cm² for CFBE41o– and CFBE41o–/sNHERF1. Fluorescence was recorded with a Cary Eclipse Varian spectrofluorometer. To measure chloride efflux rate across the apical membrane, the apical perfusion medium was changed with a medium in which chloride was substituted with isosomotic nitrate. All experiments were performed at 37°C in HEPES-buffered bicarbonate-free media [in millimolars, Cl⁻ medium: 135 NaCl, 3 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 20 HEPES, 1 KH₂PO₄, 11 glucose; and Cl⁻ free-medium: 135 NaNO₃, 3 KNO₃, 0.8 MgSO₄, 1 KH₂PO₄, 20 HEPES, 5 Ca(NO₃)₂, 11 glucose]. We measured the apical CFTR-dependent chloride secretion, as previously described (20); CFTR-dependent chloride secretion was calculated as the difference in the rate of change of forskolin-stimulated fluorescence in the absence or presence of apical treatment with the specific CFTR inhibitor, CFTRinh-172 (28).

Phosphokinase array. The phosphorylation pattern of kinases was determined with the Human Phospho-MAPK Array Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instruc-
tions. Briefly, CuFi-1 cells were seeded into four petri dishes (2.5 × 10^6 in 6-cm diameter) to obtain 1 × 10^7 cells for each array. Serum-free medium was replaced, and 100 nM TMA or solvent alone was added to the cells for 20 h before the infection for an additional 30 min. Cell lysates (200 μg) were incubated with each array. After incubation of the arrays with anti-phospho-MAPK antibody cocktail, streptavidin-horseradish peroxidase, and washes, the membranes were exposed to chemiluminescent reagent and subjected to X-ray films. The signal of the spots developed on X-ray films were quantified by scanning the film on a high-resolution transmission-mode scanner and analyzing the array image file using the image analyses software Digitomer (MedCalc Software, Mariakerke, Belgium).

Signal transduction inhibitors. Triciribine hydrate (C_{13}H_{16}N_{6}O_{4}·xH_{2}O) was obtained from Sigma/Aldrich (Milwaukee, WI), and SL 0101–1 (3,4-di-O-acetyl-6-deoxy-α-L-mannopyranosyl)oxy]-5,7-dihydro-2-(4-hydroxyphenyl)-4H-1benzopyran-4-one from Tocris Bioscience. Stock solution of triciribine hydrate (10 mM) in dimethyl sulfoxide and SL 0101–1 (20 mM) in ethanol were stored at −20°C. The inhibitors were diluted into medium before addition to cells.

Docking of TMA into the DNA binding motif of NF-κB. TMA was docked into putative binding site of the NF-κB targets employing Glide software, as described elsewhere (35, 36a). TMA were docked again into DNA recognition site, using the central processing unit time-intensive and accurate extra-precision method.

EMSA. EMSA was performed as previously described (8–10). Briefly, double-stranded synthetic oligodeoxynucleotides mimicking the NF-κB binding (NF-κB, sense: 5′-CGC TGG GGA CTT TTC ACG G-3′) have been employed. Oligodeoxynucleotides were labeled with [γ-32P]ATP using 10 units of T4-polynucleotide kinase (MBI Fermentas) in 500 mM Tris·HCl, pH 7.6, 100 mM MgCl₂, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA in the presence of 50 μM of [32P]-labeled oligonucleotide were added. Reaction temperature was increased to 100°C for 5 min and left diminishing to room temperature overnight. Binding reactions were set up as described elsewhere (9) in a total volume of 20 μl containing buffer plus 5% glycerol, 1 mM dithiothreitol, 10 ng of human NF-κB p50 protein, with or without 10 ng of NF-κB p65 protein (Promega), and different concentrations of compounds. After an incubation of 20 min at room temperature, 0.25 ng of 32P-labeled oligonucleotides was added to the samples for a further 20 min at room temperature and then they were electrophoresed at constant voltage (200 V) under low ionic strength conditions (0.25 × TBE buffer: 22 mM Tris-borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels were dried and subjected to standard autoradiographic procedures (10).

ChIP assay. ChIP assays were performed by using the ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY), as previously described (21, 22). Briefly, a total of 5 × 10⁶ IB3 cells (from two 6-cm-diameter petri dishes) were treated for 10 min at room temperature, with 1% formaldehyde culture medium. Cells were washed in phosphate-buffered saline, and then glycine was added to a final concentration of 0.125 M. The cells were then suspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris·Cl, pH 8.1) plus protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and the chromatin was subjected to sonication (using a Sonics VibraCell VC130 sonicator with a 2-mm probe). Fifteen 15-s sonication pulses at 30% amplitude were required to shear chromatin to 200- to 1,000-bp fragments. Aliquots (0.2 ml) of chromatin were diluted to 2 ml in ChIP dilution buffer containing protease inhibitors and then cleared with 75 μl of salmon sperm DNA/protein A-agarose 50% g gel slurry (Upstate Biotechnology) for 1 h at 4°C before incubation on a rocking platform with either 10 μg of NF-κB p65-specific antiserum (sc-372X, Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum (Upstate Biotechnology). Twenty microliters of diluted chromatin were saved and stored for later PCR analysis as 1% of the input extract. Incubations occurred overnight at 4°C and continued an additional 1 h after the addition of 60 μg protein A-agarose slurry. Thereafter, the agarose pellets were washed consecutively with low salt, high salt, and LiCl buffers. DNA/protein complexes were recovered from the pellets with elution buffer (0.1 M NaHCO₃ with 1% SDS), and cross-links were reversed by incubating overnight at 65°C with 0.2 M NaCl. The samples were treated with RNase A and proteinase K, extracted with phenol/chloroform, and ethanol precipitated. The pelleted DNAs were washed with 70% ethanol and dis-
Linear and angular PSRs inhibit IL-8 mRNA transcription in cells infected with P. aeruginosa. To determine the activity of the four PSR derivatives of this study (PSR, TMP, ANG, and TMA), we utilized CF (IB3–1, CuFi-1, CFBE41o–) and non-CF bronchial epithelial cell lines (Calu-3). None of the PSR derivatives had any effect on cell proliferation or bacterial growth (see Supplementary material, Supplemental Figs. S1 and S2; the online version of this article contains supplemental data). Figure 2 shows their effects on IL-8 transcription in the CF cells, IB3–1, that had been preincubated with PSRs for 20 h and then infected with P. aeruginosa (PAO1 laboratory strain) for a further 4 h. PSR reduced IL-8 mRNA accumulation by an average of 50%, starting from 0.1 μM concentration and reached 80% inhibition at 100 μM (Fig. 2A); TMP by an average of 40% inhibition, starting from 0.01 μM (Fig. 2B); ANG by an average of 50% inhibition, starting from 5 μM; and TMA by an average of 70% inhibition, starting from 1 nM. This first set of experiments suggests that methylation, which

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leads to an enhancement of lipophilicity, is a key feature to increase the inhibitory activity of both linear and angular PSRs, being the trimethylated angular compound TMA, the most potent derivative. To verify whether the inhibition of *P. aeruginosa*-dependent transcription of IL-8 mRNA observed in IB3–1 cells is reproducible in other bronchial epithelial cells and whether it is specific for CF-cells, linear PSRs were next tested in the non-CF cell line Calu-3, grown polarized on Transwell filters. Cells were treated on both the apical and basolateral sides with the linear PSR or TMP (10 μM for 20 h), and the cells were then exposed to PAO-1 (2 × 10⁷ colony-forming unit/ml) on the apical side for a further 4 h. Analysis of bacterial-evoked proinflammatory signaling was extended to other key genes involved in the chemotaxis of leukocytes besides IL-8, namely the adhesion molecule ICAM-1 and the chemokines growth-related oncogene (GRO)-α, GRO-γ, macrophage inflammatory protein-1α, and IFN-γ-inducible protein-10. As shown in Fig. 3, the inhibitory effect of PSR and

Fig. 5. Dose-response effect of TMA in CuFi-1 cells. A and B: cells were grown on 2-cm-diameter wells (2.5 × 10⁵ cells/well). After adhesion, fresh medium was replaced, and the cells were treated with the indicated concentrations of TMA, 20 h before bacterial exposure. IL-8 mRNA accumulation (A) and IL-8 protein release (B) are reported. C: similar to A and B, here TMA (100 nM) was added 20 h before infection or simultaneously with *P. aeruginosa*. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 6. Effects of TMA on proinflammatory gene expression in Calu-3 (A) and CuFi-1 (B) monolayers. The non-CF bronchial epithelial cells, Calu-3, and the CF bronchial epithelial cells, CuFi-1, were grown up to confluence on Transwell insert filters and exposed to PAO1 on the apical side in the presence of 100 nM TMA, as reported in MATERIALS AND METHODS. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 7. Effect of TMA on *P. aeruginosa*-dependent IL-8 transcription in CFBE41o⁻ and CFBE41o⁻/NHERF1. The cells were grown up to confluence on Transwell insert filters and exposed to PAO1 on the apical side in the presence of 100 nM TMA, as reported in MATERIALS AND METHODS. Values are means ± SE. **P < 0.01.
TMP was reproducible also in polarized, non-CF Calu-3 cells and was mainly restricted to IL-8 transcription. Angular PSRs were tested in both the non-CF Calu-3 and CF CuFi-1 cell lines, and, in both cases, ANG (10 μM) inhibited mainly the P. aeruginosa-dependent transcription of IL-8 mRNA, as shown in Fig. 4. Since the trimethylated angular PSR TMA was the most potent compound inhibiting IL-8 transcription in IB3–1 cells, a dose-response experiment of TMA was performed in CuFi-1 cells. As shown in Fig. 5, preincubation of TMA in CuFi-1 cells strongly inhibited IL-8 mRNA expression and release of IL-8 protein, starting from nanomolar concentrations. Conversely, simultaneous addition of 100 nM TMA with P. aeruginosa did not produce a significant inhibition. To test the effect of TMA in polarized bronchial epithelial cells, 100 nM TMA were preincubated in different CF and non-CF cells. The inhibitory effect of TMA was reproducible also in polarized cells grown on Transwell inserts, as Calu-3, CuFi-1, CFBE41o/H11002, and CFBE41o/sNH-ERF1 cell monolayers, where the mutated F508del CFTR has been rescued to the apical cell surface by NHERF1 stable overexpression (20), as shown in Figs. 6 and 7.

All of these results considered, trimethylation and angular structure confer the highest inhibitory potency to these PSR analogs. In particular, TMA is a strong inhibitor of P. aeruginosa-dependent IL-8 transcription, starting from nanomolar concentrations in at least four different CF and non-CF bronchial epithelial cell lines.

Effect of TMA on CFTR-mediated chloride efflux in bronchial epithelial cells. Since PSRs such as 5- and 8-methoxypsoralen, PSR, TMP, and ANG have been previously shown to potentiate CFTR-mediated chloride transport (17), the effect of TMA on CFTR-mediated chloride efflux was tested in Calu-3 polarized cell monolayers. Figure 8 illustrates typical experiments and the summaries of different experiments of chloride efflux performed in Calu-3 polarized cell monolayers treated (C and D) or not (A and B) with 250 nM TMA. As shown in Fig. 8D, a 15-min preincubation with TMA strongly increased the CFTR-dependent chloride efflux. Dose-response experiments were then performed to determine the minimum concentration of TMA that potentiates chloride efflux. As reported in Fig. 9A, TMA significantly increased the average forskolin stimulation of CFTR-dependent chloride efflux, starting from...
Moreover, to test whether TMA can also potentiate the transport activity of F508del CFTR that has been rescued to the apical membrane, we determined the effect of TMA on the chloride efflux in CFBE41o/H11002/sNHERF1 cell monolayers, where the mutated F508del CFTR has been rescued to the apical cell surface by NHERF1 stable overexpression (20). As shown in Fig. 9B, TMA (250 nM) significantly doubled F508del CFTR-dependent chloride efflux rescued in CFBE41o/H11002, while it was ineffective in CFBE41o cells. Altogether, these results indicate that TMA treatment is able to potentiate the apical chloride efflux mediated both by wild-type and by mutated CFTR rescued on the apical membrane of polarized bronchial epithelial cell monolayers.

**Effect of TMA on phosphoprotein phosphorylation in CuFi-1 cells.** To investigate the potential effect of TMA on phosphoproteins involved in the proinflammatory signaling leading to IL-8 transcription, extracts of CuFi-1 cells preincubated with TMA and exposed to *P. aeruginosa* were analyzed with a Human phospho-MAPK array assay. Figure 10 indicates that *P. aeruginosa* induces the phosphorylation of several known protein substrates, such as the MAP kinases p38, ERK-1/2, JNK1/2/3, and, newly reported here, of ribosomal S6 kinase (RSK)1/2, mitogen- and stress-activated kinase 2, heat shock protein 27, glycogen synthase kinase-3, and AKT1/2/3. As far as the effect of TMA is concerned, the results presented in Fig. 10, A and B, indicate that preincubation of TMA further increases the phosphorylation on MAPK ERK and JNK, whereas it reduces that of RSK1 and AKT2/3. To obtain preliminary insights on whether the inhibitory effect of TMA on IL-8 expression and release could be mediated by the
reduced phosphorylation of RSK1 and AKT, we tested the effect of known pharmacological inhibitors of these kinases on P. aeruginosa-dependent transcription of IL-8 gene. As shown in Fig. 10C, both triciribine hydrate, which is known to inhibit AKT1/2/3, and SL 0101–1, which is known to inhibit RSK, reduced IL-8 mRNA accumulation by an average of 35–45%, suggesting that RSK1 and AKT could be involved in the reduction of IL-8 gene expression mediated by TMA.

Effect of TMA on the interaction of NF-κB with IL-8 gene promoter. Transcription of IL-8 gene in human bronchial epithelial cells exposed to P. aeruginosa is known to be regulated by the interaction of the nuclear transcription factor NF-κB with the IL-8 gene promoter, as evidenced by previous mechanistic studies and confirmed by interfering experiments utilizing transcription factor decoy oligodeoxynucleotides (3). Therefore, we reasoned whether the inhibitory effect of TMA could be, at least in part, related to a reduction in the NF-κB-dependent regulation of the IL-8 gene transcription. To explore this possibility, we performed experiments based on three complementary approaches: 1) in silico docking analysis, 2) EMSA, and 3) ChIP. The results of the first two approaches are depicted in Fig. 11, which clearly indicates that TMA is able to bind to NF-κB (in silico docking analysis, A) and inhibits the NF-κB/DNA interactions (EMSA studies, B–D).

The inhibitory effects on NF-κB/DNA interactions were reproducibly obtained using NF-κB p50 (Fig. 11B), reconstituted p50/p65 heterodimer (Fig. 11C), and unfractionated nuclear factors from IB3–1 cells (Fig. 11D). No major differences were found when TMA was first added to the target NF-κB oligonucleotide (Fig. 11B, top) or to NF-κB p50 (Fig. 11B, bottom). In addition, ChIP analysis demonstrates that TMA strongly reduces recruitment of NF-κB to the IL-8 gene promoter. IB3–1 cells were treated with PAO-1 or PAO-1 plus TMA, nuclear/DNA interactions stabilized by formaldehyde treatment, and the shared chromatin immunoprecipitated with antibodies against NF-κB. In parallel, the same procedure was performed with control untreated IB3–1 cells. The DNA from immunoprecipitates was isolated and PCR-amplified with primers specific for the IL-8 promoter (Fig. 12A). Quantitative real-time PCR profiles for the amplification of the IL-8 promoter are shown in Fig. 12, C and D, and demonstrate that treatment of IB3–1 cells with PAO-1 induces a fast and sharp recruitment of NF-κB to the IL-8 gene promoter. This effect is inhibited by in the presence of TMA.

DISCUSSION

The most important finding of this paper is that the trimethylated angular PSR TMA is a strong inhibitor of the expression of the IL-8 gene in bronchial epithelial cells in which the inflammatory response has been challenged with P. aeruginosa, the most common bacterium found in the airways of patients affected by CF. This effect 1) was obtained at low (nanomolar) concentrations of the molecule; 2) is not accompanied by a reduction of cell growth; 3) was independent of the expression of wild-type or mutated CFTR; and 4) appeared to be relatively specific for IL-8, as little or no inhibitory effect was observed on the accumulation of other proinflammatory transcripts, such as those encoded by ICAM-1, GRO-α, GRO-γ, MIP-1α, and IP-10. Moreover, TMA strongly potentiates chloride efflux through both wild-type and mutated CFTR that has been rescued on the apical membrane. Altogether, these results indicate that TMA is a promising dual-acting compound to be investigated both as a modulator of the critical step of the excessive IL-8-mediated inflammatory response and as potentiator of the function of chloride transport through mutated CFTR protein.

The effect of TMA on IL-8 gene transcription is of great interest, considering that IL-8 is the most abundant chemokine found in the bronchoalveolar fluid of CF patients (5). As a matter of fact, IL-8 drives the recruitment of the excessive amount of neutrophils found in CF bronchial lumens, which unceasingly contributes to the progressive lung tissue damage and respiratory insufficiency of these patients, mainly on release of different kinds of proteases. Therefore, the development of drugs targeting IL-8 expression is of great interest, since traditional drugs, such as corticosteroids or ibuprofen, have limited effect in CF. Importantly, in a study evaluating the effect of anti-inflammatory drugs on the CF pulmonary pathophysiology, the nonsteroidal anti-inflammatory drug ibuprofen was reported to partially block
CFTR-mediated chloride transport at pharmacologically relevant concentrations, thus potentially worsening the basic lung ion transport defect in CF (18). On the contrary, here we report that TMA, in addition to its anti-inflammatory effect, is able to potentiate CFTR-dependent chloride efflux, suggesting that it already shows a dual-action that could be beneficially employed in CF in conjunction with other therapeutic strategies designed to increase mutated CFTR expression on apical membrane of bronchial cells and, in this way, help to both correct the ion transport defect and mitigate the excessive inflammation.

As far as the potential side effects of TMA are concerned, we should underline that TMA belongs to a class of furocoumarins (including ANG and related molecules) applied for their antiproliferative activity in the therapeutic treatment of various skin diseases, because of their photosensitivity to UV light (23, 37). In this respect, it is known that the planar structure of furocoumarins helps them to intercalate between nucleic acid base pairs. Interestingly, in the case of linear furocoumarins, the PSR monoaducts formed in the DNA can further react photochemically with a pyrimidine base on the complementary strand of the DNA, thus leading to interstrand cross-links (ICL). On the other hand, and relevant to biomedical applications, angular PSRs, such as TMA, allow only monofunctional DNA binding upon irradiation with UV light, thus reducing undesirable side effects, especially long-term ones, such as genotoxicity and risk of skin cancer (6, 7, 13, 23, 30). It is well known indeed that PSRs cause cell damage by covalent binding to DNA following UVA irradiation, and these molecules exhibit a planar tricyclic structure with two photoreactive sites (3,4-pyrone and 4\(^2\)=5\(^2\)-furan double bonds). The initial intercalation and interaction with double-stranded DNA is not characterized by covalent bonds, but, on absorption of a photon of UVA by furocoumarins moiety, a cyclo-addition with the 5,6-double bond of pyrimidine residue (preferentially a thymine) of the DNA takes place. The resulting monoadduct can form a diaduct by absorbing a second photon, if a new pyrimidine on the opposite strand of DNA is available for an ICL. On the contrary,
ANG and its angular analogs are monofunctional isoprenoid isomers and cannot create ICLs because of the angular geometric structure (26, 31, 34). On the other hand, several recently published papers demonstrate biological activities of furocoumarins in the absence of UVA irradiation (1, 14). However, interactions with other molecules cannot be excluded and should be further explored. In any case, the activity of TMA here reported is not dependent on UVA irradiation, and data available so far predict that drug concentrations suitable for IL-8 inhibition can be reached with no side effects, although thorough investigation on efficacy and safety in animal models will be required to support the concept that TMA can be proposed for anti-inflammatory treatment. Therefore, the inhibition of IL-8 expression by TMA encourages studies on in vivo experimental mouse models, since it predicts that TMA might inhibit in vivo IL-8-dependent recruitment of neutrophils in the bronchial lumen.

Preliminary results on the mechanism(s) by which TMA reduces IL-8 gene expression have been obtained by analyzing the effect of TMA on phosphoprotein kinases and on the interaction of NF-κB with IL-8 gene promoter. Here, we confirm that P. aeruginosaa activates MAP kinases ERK, JNK, and p38 in bronchial epithelial cells, together with previously uncharacterized kinases, such as RSK1/2, mitogen- and stress-activated kinase-2, heat shock protein-27, glycogen synthase kinase-3, and AKT1/2/3. Interestingly, TMA further increases the phosphorylation of the MAP kinases, which are expected to play a proinflammatory role, the only reduction being observed for RSK1 and AKT2/3. Although their pharmacological inhibition with triciribine hydrate and SL 0101–1 seems to confirm that RSK1 and AKT2/3 are indeed relevant in the signaling pathway activating IL-8 gene transcription, further investigation is necessary to consolidate RSK1 and AKT2/3 as molecular targets of TMA. Many laboratories, including ours, proposed NF-κB as a key transcription factor activating IL-8 gene transcription. Here, we found by EMSA experiments that TMA is able to bind NF-κB (Fig. 11A) and reduces the in vitro interaction of NF-κB with oligonucleotides designed on its consensus sequence identified in the promoter of IL-8 gene only at millimolar concentration (Fig. 11, B–D), which is many orders of magnitude higher than the nanomolar concentrations inhibiting IL-8 gene transcription in whole cell experiments. Despite the fact that discrepancies between in vitro activities and effects on whole cellular systems are expected (22, 35), these results suggest that TMA could intervene on other molecular targets, leading to the sharp inhibition of IL-8 gene expression observed at nanomolar concentration. Interestingly, in relation to this specific issue, ChIP assays allow the proposal of a very intriguing observation: 1) P. aeruginosaa infection leads to a sharp recruitment of NF-κB to the IL-8 gene promoter, supporting the role of NF-κB in the IL-8 gene transcription and IL-8 mRNA accumulation; and 2) TMA treatment strongly inhibits the NF-κB recruitment. Despite the fact that these data do not allow us to identify univocally the level(s) of TMA activity of the very complex NF-κB pathway, they support the hypothesis that, at least in part, IL-8 gene transcription is involved through interference on the positioning of NF-κB on the NF-κB binding sites present in the IL-8 gene promoter. Further experiments using primers amplifying other gene promoters carrying NF-κB binding sites will clarify whether this effect is restricted to a specific class of NF-κB dependent genes.

Among additional cross talks affected by TMA treatment, the effects on CFTR functions should be considered. Recently published observations, indeed, suggest a mechanism that links CFTR channel function to intracellular NF-κB signaling and inflammatory response (38). These studies support the concept that small molecules or therapeutic compounds that rescue optimal biological functions of mutant can attenuate the NF-κB mediated chronic inflammation. Our results could be considered in general agreement with the report by Vij et al. (38), as we found that TMA potentiates CFTR function and, in parallel, inhibits NF-κB activity and IL-8 transcription. However, a new thorough investigation needs to be pursued to confirm this potential mechanism of action.

The mechanism by which TMA potentiates CFTR function is completely unknown. Besides the original papers on the effect of PSRs on CFTR function (17), a larger panel of coumarin compounds from Chinese medicinal herbs has recently been published, both confirming the effect of ANG or isoprenoid and identifying imperatorin and osthole as the two most effective potentiating compounds, with $K_d$ values around 10 μM (40). In principle, TMA, as reported here, seems more effective than imperatorin and osthole. Molecular modeling and docking studies of TMA into CFTR as protein target could help explaining the action mechanism of this potentiator and thus contribute to even better drug design for potentiating CFTR function.

In conclusion, in our opinion, TMA deserves great attention and warrants further analyses to determine the mechanism of action on the modulation of the immune response, on the molecular mechanism of potentiation of CFTR function, and on the efficacy and safety in preclinical studies, to confirm its usefulness as an innovative therapeutic approach in CF lung disease.

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DISCLOSURES

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