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Original article

Development of a novel furocoumarin derivative inhibiting NF-κB dependent biological functions: Design, synthesis and biological effects

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ABSTRACT

Nuclear Factor kappaB (NF-κB) plays a very important role in the control of gene expression and is deeply involved in several human pathologies. Accordingly, molecules targeting NF-κB dependent biological functions are considered of great interest. Virtual screening of furocoumarin libraries against NF-κB p50 allowed to rank compounds in respect to their expected ability to bind NF-κB and the identified compound might be considered for the development of analogs to be tested for biological activity on inhibition of NF-κB/DNA complex formation. The data reported in the present paper suggest that, following this approach, the best ranked compounds identified by virtual screening (a) strongly bind *in silico* to NF-κB and (b) efficiently inhibit the molecular interactions between ^{32}P-labeled NF-κB double stranded DNA and p50 or p50/p65 complex. These data allowed to develop a novel lead of great interest for inhibiting NF-κB dependent biological functions. This novel molecule (compound **2**), bearing a methyl group in the 9 position of the psoralen nucleus, exhibits high efficiency in inhibiting NF-κB/DNA interactions. In addition, we found that compound **2** is a potent inhibitor of IL-8 gene expression in TNF-α treated IB3-1 cystic fibrosis cells. Taken together, our data indicate that compound **2** might find an important place in the set of molecules of interest for the development of pharmaceutical strategies against the inflammatory phenotype of cystic fibrosis.

1. Introduction

Nuclear factor kappaB (NF-κB) plays a critical role in several biological processes, including cell cycle regulation [1–3], expression of specific genes [4,5], regulation of cell differentiation [6,7] and apoptosis [8–11]. On the other hand, alteration of NF-κB activity is associated with several human pathologies, including osteoporosis [12], rheumatoid arthritis [13] and cancer [14,15]. In addition, NF-κB is one among the master transcription factors responsible for inflammation in cystic fibrosis (CF) cells infected with *Pseudomonas aeruginosa* [16–20]. Accordingly, targeting NF-κB appears to be a relevant therapeutic strategy, as recently reviewed [21–23]. Unfortunately, targeting NF-κB is not a simple task. First of all, several proteins belong to the NF-κB family, including RelA (also known as p65), RelB, cRel/Rel, p50 and p52, originating homo- and hetero-dimers, the most common of them being p50/p65 and p52/RelB. Moreover, the metabolic regulation of NF-kB biological functions involves several control levels, one of the most important being the interaction with inhibitory proteins belonging to the IκB (inhibitor of NF-κB) family [24,25]. Among these, IκBα plays a major role as recently reviewed by Ferreiro and Komives [24], generating a complex with the NF-κB homo- or hetero-dimers; this molecular interaction prevents NF-κB to translocate to the nucleus and exert its regulatory functions on transcription of target genes [24–26]. Activation of NF-κB is operated by the IκB kinase (IKK) complex, which is composed by the two catalytic subunits IKKα and IKKβ and a regulatory subunit, the NF-kappaB essential modulator (NEMO, also known as IKKγ) [27]. Upon different stimuli, IKK phosphorilates the N-terminal signal response domain of NF-κB-bound IκBα, causing subsequent

Abbreviations: VS, virtual screening; PSO, psoralen; ANG, angelicin; TMP, 4,5',8-trimethylpsoralen; TMA, trimethylangelicin; IL-8, interleukin-8; EMSA, electrophoretic mobility shift assay; CF, cystic fibrosis; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor kappaB; IκBα, inhibitory protein α of nuclear factor kappaB; PCR, polymerase–chain reaction; RT-qPCR, reverse transcription quantitative PCR; FBS, fetal bovine serum.

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polyubiquitinylation and proteasome-directed degradation, thus leading NF-κB to be translocated to the nucleus. At the nuclear level, NF-κB binds to the DNA target elements present in NF-κB regulated genes, as well as to co-activators of gene transcription [28]. In any case, the availability of NF-κB inhibitors is considered a major objective of therapeutic intervention.

In this respect, the concerted actions of researchers involved in bioinformatics, medicinal chemistry, cellular and molecular biology appear to be crucial for the development of novel drug candidates for important human pathologies [29–31]. In this context we have recently reported the possible use of a structured based virtual screening (VS) procedure to identify possible NF-κB binders [32–34], demonstrating that this *in silico* screening approach is suitable for the identification of low-molecular-weight compounds that inhibit NF-κB/DNA interactions and NF-κB dependent functions [32–34]. VS against NF-κB p50 using docking simulations was applied starting from a three-dimensional (3D) database containing more than 4.6 million commercially available structures. Docking simulations to p50 NF-κB were performed with a test set of six known inhibitors of NF-κB-DNA interactions [32,33]. In agreement with docking results, the highest-scored compound displayed a high level of inhibitory activity in EMSA experiments (inhibition of NF-κB/DNA interactions) and on biological functions dependent on NF-κB activity (inhibition of IL-8 gene expression in IB3-1 CF cells). In a more recent study, we constructed a focus library of differently substituted furocoumarins and analogs for an *in silico* screening against NF-κB with the aim of finding more potent NF-κB inhibitors belonging to the furocoumarin family [34]. We identified several furocoumarin derivatives expected to be active on NF-κB dependent functions: four of the five best ranked compounds (the commercially available ones) displayed interesting activities (inhibition of NF-κB/DNA interactions and IL-8 gene expression [34]), demonstrating the success of our VS approach.

The rationale of this report is based on the evidence that VS, in combination with molecular biology approaches, allows the identification of lead compounds that can be further modified to generate bioactive molecules useful in experimental therapy of human pathologies [29,34]. Following a recently published study [29], in the present work we have synthesized the most active compound identified through VS and a structurally-related derivative. Their activity on NF-κB/DNA interactions was determined by EMSA (Electrophoretic Mobility Shift Assay) and TNF-α induced expression of interleukin-8 (IL-8) in cystic fibrosis (CF) cells was evaluated by quantitative reverse transcription and polymerase–chain reaction (RT-PCR). This cellular system is very attractive, since it is well known that the hallmark in CF airway pathology is a characteristic elevated concentration of pro-inflammatory cytokines and chemokines, the most important of which is IL-8 [35–37]. On the other hand, it is firmly established that downstream activation of nuclear transcription factors, including NF-κB [17–19], is required for a cascade of pro-inflammatory cytokines and chemokines, first of all the NF-κB dependent IL-8, which is well known to play a role of master gene in PMN recruitment in CF lung [20,38,39]. Accordingly, candidate drugs interfering with NF-κB/DNA interactions and inhibiting IL-8 expression in CF cells are of great importance [38,40–45].

2. Results and discussion

2.1. Chemistry

Compound **1**, identified by VS as the best ranked ligand to p50-p50 dimer [34], was synthesized along with its closely related analog **2**, bearing a methyl group in the 9 position of the psoralen nucleus (Fig. 1). The rationale for synthesizing compound **2** was related with the observation that the introduction of a methyl group increases the inhibitory activity of furocoumarins (for instance psoralen and angelicin) on NF-κB/DNA interactions. As reported in Figs. 2 and 4, 5′,8-trimethylpsoralen (TMP) and trimethylangelicin (TMA) inhibit the interactions between p50 NF-κB and target ^{32}P-labeled DNA with higher efficiency in comparison to non-methylated psoralen and angelicin.

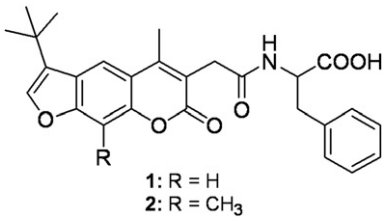


Fig. 1. Structures of synthesized compounds.

Methyl (7-hydroxy-2-oxo-2H-3-benzopyranyl)acetates **3**–**4** [46] were condensed with 1-chloropinacolone using a new MAOS (microwave-assisted organic synthesis) protocol to give the ethers **5**–**6** (Scheme 1). In this way, starting products were irradiated at 130 °C for 20 min in triethylamine and water to give the desired ethers with higher yield and reduced reaction times, if compared to previously reported methods [46]. Subsequent cyclization of the ketoalkyloxy side chain in alkaline medium afforded psoralenacetic acids **7**–**8**, which were finally reacted with DL-phenylalanine through activation of the carboxyl group *via* acyl chloride to give the amides **1a** and **2**. Special attention should be paid to this step because decarboxylation of psoralen acids can easily occur at temperature higher than room temperature. Finally, since a chiral center is present in the amide moiety of compound **1a**, the L-enantiomer **1b** was also synthesized starting from L-phenylalanine, in order to evaluate the influence of the stereochemistry on NF-κB-DNA interactions.

In order to obtain a first indication on biological activity, electrophoretic mobility shift assay (EMSA) was performed. This approach allows to rank even large set of newly synthesized compounds in respect to the effects on molecular interactions between NF-kB and target DNA sequences [32–34].

2.2. Biological activity of the compounds *1a–b* and *2*: EMSA studies

To determine biological activity of compounds **1a, 1b** and **2** in EMSA studies purified p50 NF-κB protein was first employed. The results obtained, shown in Fig. 3A, demonstrate that all the tested compounds are active in inhibiting NF-κB/DNA interactions, being compound **2** the most efficient. Accordingly, further analysis was performed on this compound (Fig. 3B) demonstrating an IC_{50} of about 30 μM and an efficiency in inhibiting the interaction of NF-κB-DNA to a reconstituted p50/p65 heterodimer (Fig. 3C). Interestingly, compound **2** is far more active than psoralen, TMP, angelicin and TMA (Table 1) in inhibiting NF-κB/DNA interactions. This set of experiments suggests that compound **2** might be a good candidate for developing molecules inhibiting the expression of NF-κB regulated genes. Interestingly, compound **2** shows docking activity to NF-κB (Fig. 4). Tyr57, Thr143, Lys144 and Lys145 are the amino acids apparently involved in the interactions between NF-κB and compound **2**.

On the basis of these observations, compound **2** was further characterized for its possible activity on the expression of interleukin-8 (IL-8) gene. It is firmly established that IL-8 gene expression is regulated by NF-κB [20–23]; therefore, since molecules inhibiting NF-κB/DNA interactions might exhibit inhibitory activities on NF-κB regulated genes [35–37], we were interested to determine the activity of compound **2** on IL-8 gene expression.

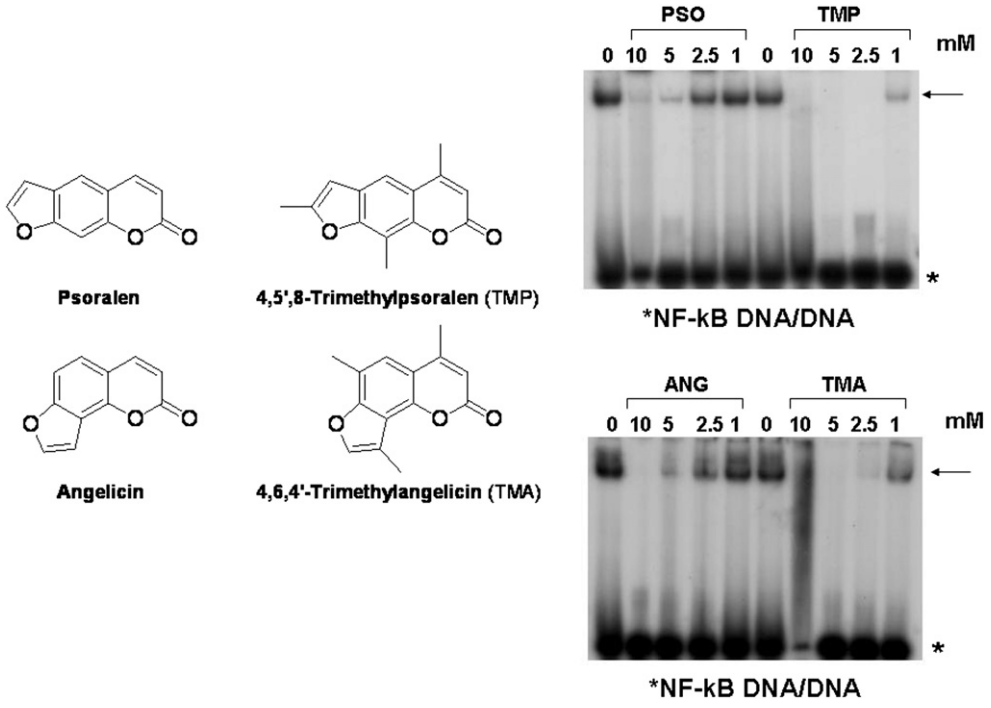


Fig. 2. Structures of psoralen, TMP, angelicin and TMA and their effects on the molecular interactions between NF-κB p50 and ^{32}P-labeled target NF-κB double stranded oligonucleotide. Compounds were first incubated with NF-κB and then the ^{32}P-labeled target NF-κB oligonucleotide was added. NF-κB/DNA complexes were analyzed by polyacrylamide gel electrophoresis. Arrows indicate NF-κB/DNA complexes; asterisks indicate the free ^{32}P-labeled target NF-κB probe.

2.3. Biological activity of compound **2**: inhibition of IL-8 mRNA accumulation in TNF-α treated IB3-1 CF cells

In order to determine the biological activity of compound **2** on IL-8 gene expression we therefore employed IB3-1 CF cells treated with 80 ng/ml of TNF-α in the presence of 10, 20 and 50 μM compound **2**. In the IB3-1 CF cellular system NF-κB dependent genes, including the gene coding for the pro-inflammatory protein IL-8 are activated following infection with *P. aeruginosa*, or treatment with TNF-α of IL-1β [20,38,39]. This feature is very important in the pathophysiology of CF, since several clinical complications are caused by exacerbation of this inflammatory response. Cells were incubated overnight in the presence of compounds **2** and then treated with TNF-α (80 ng/ml). After 1 day of incubation, cellular

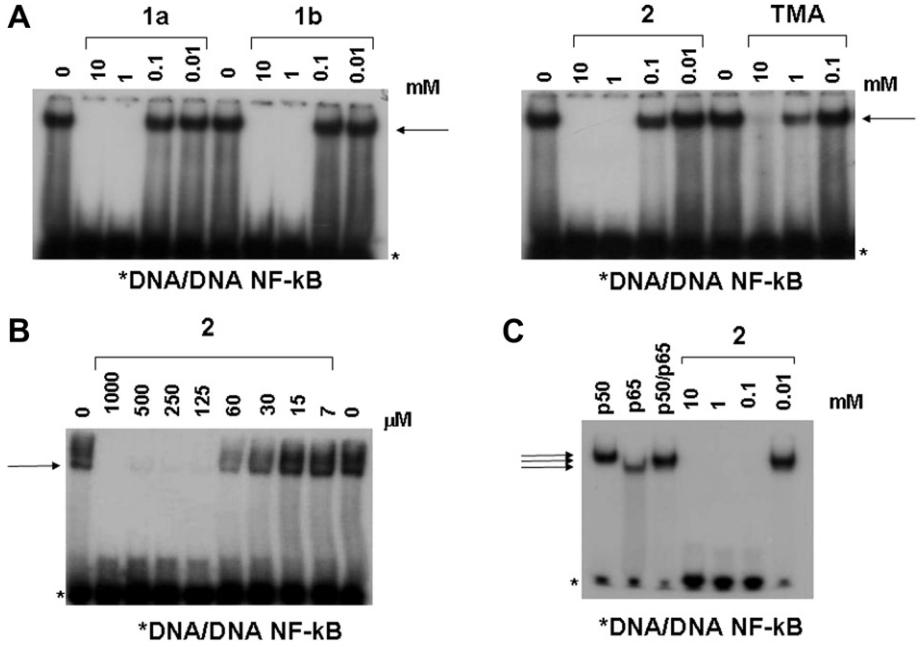


Fig. 3. Effects of compounds **1a**, **1b**, **2** and TMA on the molecular interactions between NF-κB p50 (A and B) and NF-κB p50/p65 (C) and ^{32}P-labeled target NF-κB double stranded oligonucleotide. Compounds were first incubated with NF-κB and then the ^{32}P-labeled target NF-κB oligonucleotide was added. NF-κB/DNA complexes were analyzed by polyacrylamide gel electrophoresis. Arrows indicate NF-κB/DNA complexes; asterisks indicate the free ^{32}P-labeled target NF-κB probe.

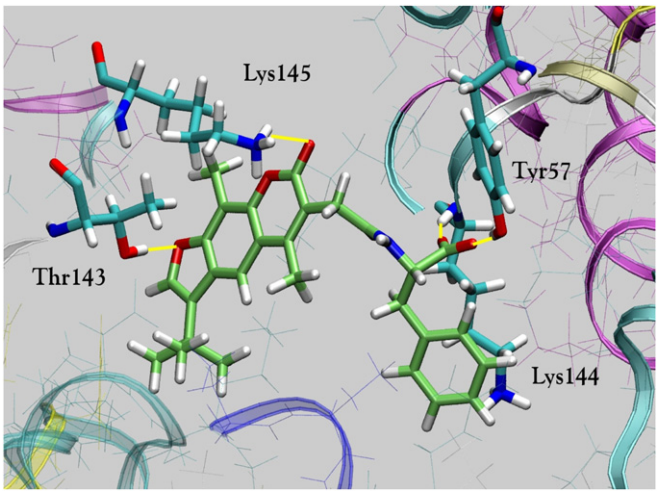


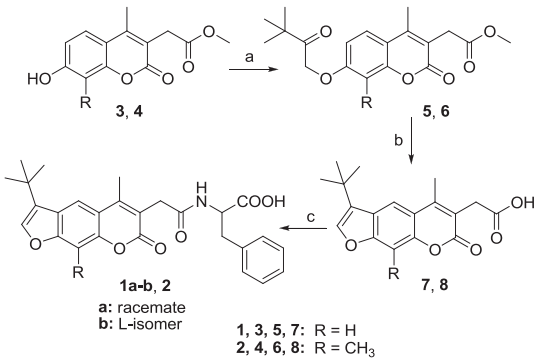
Fig. 4. Stereo view of the complex formed by NF-κB p50 homodimer and the docked compound **2** (bottom side of the panel). The amino acids Tyr57, Thr143, Lys144 and Lys145, involved in the interactions with compound **2**, are highlighted.

RNA was isolated for RT-qPCR analysis and the cellular supernatants isolated for determination of the release of IL-8.

The results obtained are reported in Fig. 5, which demonstrates that compounds **2** inhibits TNF-α induced IL-8 mRNA accumulation (Fig. 5A) and IL-8 protein release (Fig. 5B) exhibiting fair dose–response effects. While statistically-significant differences were not observed when compound **2** was used at 10 μM concentration, the highest effects were observed at 50 μM, being 30 μM compound **2** still active (Fig. 5, panels A and B). Interestingly, compound **2** inhibits IL-8 gene expression without major effects on IB3-1 cell growth (compare panels A and B of Fig. 5 to panel C). These results demonstrate that treatment of IB3-1 CF cells with compound **2** significantly reduces the TNF-α-dependent transcription of the pro-inflammatory mediator IL-8 and the relative release of IL-8.

3. Conclusions

The data reported in the present paper suggest that the best ranked compound(s) identified through VS approach (compounds **1a**–**b**) efficiently inhibit the molecular interactions between ^{32}P-labeled NF-κB double stranded DNA and p50 or p50/p65 complex. These data demonstrate that docking simulations are suitable for predictive studies on binding affinity of small compounds to a difficult target as NF-κB homodimer and heterodimer.



Scheme 1. Reagents and conditions: (a) 1-chloropinacolone, TEA/H_2O, MW, 130 °C, 20 min; (b) 1 M NaOH, propan-2-ol, reflux, 4 h; (c) (i) $SOCl_2$, r.t., 3 h; (ii) DL-Phe or L-Phe, TEA/H_2O, r.t.

Table 1
Inhibitory activity on NF-κB/DNA interactions of compounds **2** in respect to other furocoumarins.

Compound	IC_{50}[a]
2	0.03 ± 0.002
Psoralen	3 ± 0.2
TMP	0.5 ± 0.1
Angelicin	2.5 ± 0.4
TMA	1 ± 0.2

[a] IC_{50} is the inhibitory concentration leading to 50% inhibition of NF-κB/DNA interactions.

Moreover our data allow to develop a novel lead of great interest for inhibiting NF-κB dependent biological functions. This novel molecule (compound **2**), bearing a methyl group in the 9 position of the psoralen nucleus, exhibits higher efficiency in

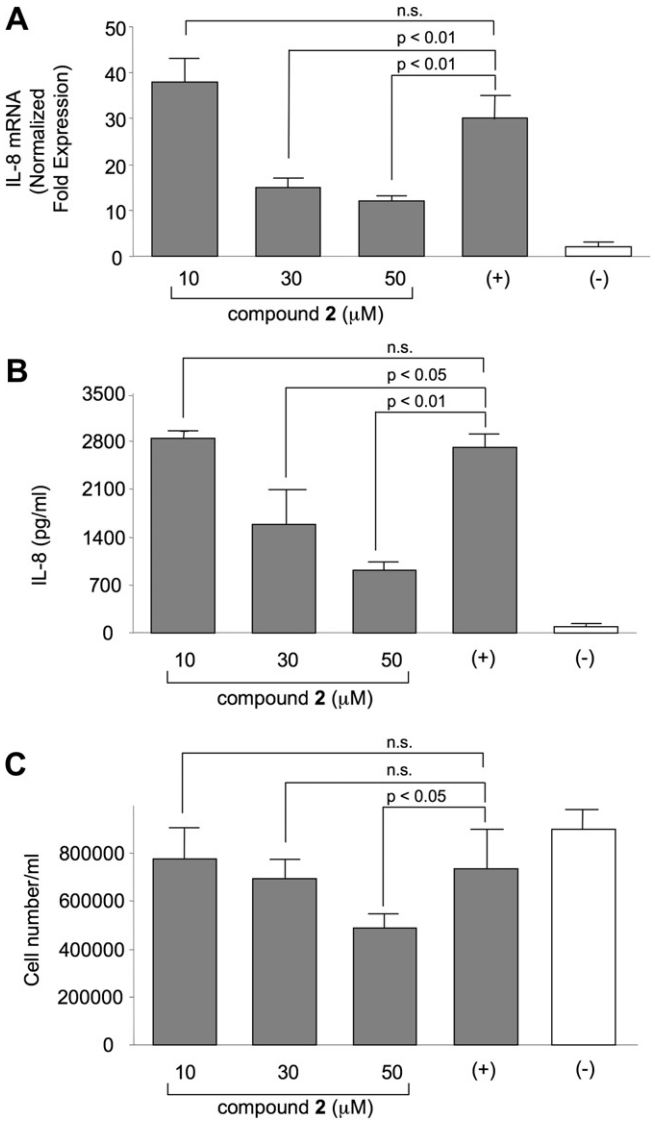


Fig. 5. Effects of compound **2** on IL-8 gene expression in TNF-α treated cells. IB3-1 cells were exposed for 24 h to the indicated concentrations of compound **2** in the presence of TNF-α as described in methods. After this incubation time, RNAs (A) and cellular supernatants (B) were isolated. Quantitative RT-PCR was performed and IL-8 mRNA content was quantified in respect to control uninduced IB3-1 cells (A). The pg/ml of IL-8 were quantified by Bio-plex analysis [52,53] and the results obtained are shown in panel B. Panel C shows the effects of compound 2 on cell proliferation. (−) = untreated IB3-1 cells; (+) = TNF-α treated IB3-1 cells ($n = 3$). The results represent the average ± SD. $p < 0.001$, highly significant; $p < 0.005$, significant; n.s., not significant.

inhibiting NF-κB/DNA interactions and strongly binds *in silico* to NF-κB. Very good agreement does exist between docking studies (showing high binding efficiency of compound **2** to NF-κB) and EMSA experiments (showing higher inhibitory activity). These data are consistent with the observation that psoralens-like molecules bearing methyl groups (TMP and TMA) are more active in inhibiting NF-κB/DNA interactions in respect to the reference compounds psoralen and angelicin. About potency in inhibiting NF-κB/DNA interactions, compound **2** is the most active furocoumarin derivative recently studied by our research group.

The results presented in this study identify compound **2** as a lead compound for generating libraries of structurally-related molecules, whose synthesis and evaluation will allow to establish structure-activity relationships (SAR) for this class of compounds. Works are in progress about design and synthesis of novel structurally-related furocoumarins bearing modifications in the points of molecular diversity (such as the substituents of furan ring or benzene ring, the length of the alkyl chains in 3 position of the pyrone ring, the aminoacid condensed on the carboxylic function of the 3-side chain) in order to define the role of each functional group.

With respect to possible biomedical applications, our data suggest that compound **2** might be of interest for experimental therapy of CF. In fact, compound **2** inhibits NF-κB/DNA interactions and IL-8 gene expression in TNF-α treated IB3-1 CF cells. Interestingly, and relevant to possible biomedical applications of our data, NF-κB inhibitors affecting IL-8 gene expression are employed in clinical trials for CF. For instance Saadane et al. [40] demonstrated that treatment of with parthenolide and stimulation with IL-1β and/or TNF leads to inhibition of IL-8 secretion induced by these cytokines and prevents NF-κB activation, IκBα degradation, and IκB Kinase complex activity. Therefore parthenolide inhibits IκB kinase, resulting in stabilization of cytoplasmic IκBα, which in turn leads to inhibition of NF-κB translocation and attenuation of subsequent inflammatory responses. IκB kinase may be a good target, and parthenolide might be promising treatments for the excessive inflammation in CF. In addition, Cigana et al. [41] demonstrated azithromycin as powerful inhibitor of NF-κB and AP-1 DNA binding, leading to anti-inflammatory effects in CF cells, indicating inhibition of transcription of pro-inflammatory genes as possible mechanism, thus providing a rationale for the possible use of specific TF inhibitors for therapy. Finally, Tchilibon et al. [42] in a systematic search for candidate drugs that might be used therapeutically to suppress IL-8 secretion in CF, identified a potent and efficacious series of amphiphilic pyridinium salts. The mechanism of action of these compounds appears to involve inhibition of signaling of the NF-κB and AP-1 transcription factors to the IL-8 promoter.

Taken together, our data indicate that compound **2** might find an important place in the set of molecules of interest for the development of pharmaceutical strategies against the inflammatory phenotype of CF.

4. Experimental section

4.1. Chemistry

All commercial chemicals and solvents used were analytical grade and were used without further purification. Analytical thin layer chromatography (TLC) was performed on pre-coated silica gel plates (Merck 60-F-254, 0.25 mm). Melting points were determined on a Gallenkamp MFB-595-010 M melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker 300-AMX spectrometer with TMS as an internal standard. Coupling constants are given in Hz and the relative peaks area were in agreement with all assignment. Elemental analysis were performed on a Perkin–Elmer 2400 analyzer. Mass spectra were performed on a Applied Biosystem Mariner System 5220 with direct injection of the sample. Microwave-assisted reactions were performed on a CEM Discover® monomode reactor with the temperature monitored by a built-in infrared sensor and the automatic control of the power; all the reactions were performed in closed devices with pressure control. Compounds **3** and **4** were prepared according to literature methods [46]. Purity for all the tested compounds was determined by elemental analyses and was found equal or more than 95%.

4.1.1. General procedures for methyl [7-(3′,3′-dimethyl-2′-oxobutoxy)-2-oxo-2H-benzopyran-3-yl]acetates **5**–**6**

A mixture of **3** or **4** (10.0 mmol) and 1-chloropinacolone (17.0 mmol) in triethylamine (3.6 mL, 26.0 mmol) and water (3.6 mL) was microwave irradiated at 130 °C (power set point 200 W; ramp time 2 min; hold time 20 min). After cooling the reaction mixture was poured into 1 M HCl (300 mL) and the resulting precipitate was filtered off to give **5**–**6**.

4.1.2. Methyl [4-methyl-7-(3′,3′-dimethyl-2′-oxobutoxy)-2-oxo-2H-benzopyran-3-yl]-acetate (**5**)

Yield 98%; mp: 158 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55 (d, J = 8.9 Hz, 1H, 5–H), 6.91 (dd, J = 8.9, 2.6 Hz, 1H, 6–H), 6.68 (d, J = 2.6 Hz, 1H, 8–H), 4.96 (s, 2H, $OCH_2COC(CH_3)_3$), 3.71 (s, 5H, CH_2COOCH_3 and CH_2COOCH_3), 2.39 (s, 3H, 4-CH_3), 1.27 (s, 9H, $C(CH_3)_3$). Anal. calcd. for $C_{19}H_{22}O_6$: C, 65.88; H, 6.40; found: C, 65.85; H, 6.47. HRMS (ESI-TOF) for $C_{19}H_{23}O_6$ $[M + H]^+$: calcd.: 347.1495, found: 347.1558.

4.1.3. Methyl [4,8-dimethyl-7-(3′,3′-dimethyl-2′-oxobutoxy)-2-oxo-2H-benzopyran-3-yl]-acetate (**6**)

Yield 94%; mp: 144 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40 (d, J = 8.9 Hz, 1H, 5–H), 6.59 (d, J = 8.9 Hz, 1H, 6–H), 5.00 (s, 2H, $OCH_2COC(CH_3)_3$), 3.73 (s, 2H, CH_2COOCH_3), 3.70 (s, 3H, CH_2COOCH_3), 2.39 (s, 3H, 4-CH_3), 2.36 (s, 3H, 8-CH_3), 1.27 (s, 9H, $C(CH_3)_3$). Anal. calcd. for $C_{20}H_{24}O_6$: C, 66.65; H, 6.71; found: C, 66.69; H, 6.72. HRMS (ESI-TOF) for $C_{20}H_{25}O_6$ $[M + H]^+$: calcd.: 361.1651, found: 361.1698.

4.1.4. General procedures for (3′-tert-butyl-7′-oxo-7H-furo[3,2-g][**1**]benzopyran-6′-yl)acetic acids **7**–**8**

A mixture of **5** or **6** (10.0 mmol) in propan-2-ol (40 mL) and 1 M NaOH (60 mL) was refluxed for 4 h. After cooling the mixture was acidified with 5 M HCl, the resulting precipitate was filtered off and crystallized from ethyl acetate to give **7**–**8**.

4.1.5. (3′-tert-butyl-5′-methyl-7′-oxo-7H-furo[3,2-g] [**1**] benzopyran-6′-yl)acetic acid (**7**)

Yield 94%; mp: 264 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.94 (s, 1H, 2′–H), 7.44 (s, 1H, 4′-H or 9′–H), 7.40 (s, 1H, 4′–H or 9′–H), 3.83 (s, 2H, OCH_2COOH), 2.57 (s, 3H, 5′–CH_3), 1.46 (s, 9H, $C(CH_3)_3$). Anal. calcd. for $C_{18}H_{18}O_5$: C, 68.78; H, 5.77; found: C, 68.73; H, 5.80. HRMS (ESI-TOF) for $C_{18}H_{17}O_5$ $[M–H]^-$: calcd.: 313.1076, found: 313.1129.

4.1.6. (3′-tert-butyl-5′,9′-dimethyl-7′-oxo-7H-furo[3,2-g] [**1**] benzopyran-6′-yl)acetic acid (**8**)

Yield 83%, mp: 222 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (s, 1H, 2′–H), 7.41 (s, 1H, 4′–H), 3.83 (s, 2H, OCH_2COOH), 2.59 (s, 3H, 2′–CH_3 or 5′-CH_3), 2.54 (s, 3H, 2′-CH_3 or 5′–CH_3), 1.45 (s, 9H, $C(CH_3)_3$). Anal. calcd. for $C_{19}H_{20}O_5$: C, 69.50; H, 6.14; found: C, 69.54; H, 6.18. HRMS (ESI-TOF) for $C_{19}H_{19}O_5$ $[M–H]^-$: calcd.: 327.1232, found: 327.1297.

4.1.7. General procedures for 2-[2′-(3”-tert-butyl-7”-oxo-7H-furo [3,2-g] [1]benzopyran-6”-yl)-acety-lamino]-3-phenylpropionic acids **1a**–**b** and **2**

A solution of acids **7**–**8** (1.0 mmol) in thionyl chloride (25 mL) was stirred at room temperature until a clear solution was formed. The solvent was evaporated under nitrogen stream and the residue was dissolved in tetrahydrofuran (10 mL). The solution was added dropwise to a mixture of DL-phenylalanine or L-phenylalanine (0.3 g, 2.0 mmol) in triethylamine (0.3 mL, 2.1 mmol) and water (5 mL). After reaction completion, the mixture was diluted with water (40 mL) and acidified with 1 M HCl to pH = 3. The resulting precipitate was filtered off and crystallized from propan-2-ol to give **1a**–**b** and **2**.

4.1.8. DL-2-[2′-(3”-tert-butyl-5”-methyl-7”-oxo-7H-furo[3,2-g] [**1**] benzopyran-6”-yl)-acetyl-amino]-3-phenylpropionic acid (**1a**)

Yield 45%; mp: 244 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.91 (s, 1H, 2”–H), 7.45 (s, 1H, 4”–H or 9”–H), 7.43 (s, 1H, 4”–H or 9”–H), 7.16–6.75 (m, 5H, H Ar Phe), 4.80–4.71 (m, 1 H, 2–H), 3.66 (d, J = 14.1 Hz, 1H, 2′–H), 3.55 (d, J = 14.1 Hz, 1H, 2′–H), 3.24 (dd, J = 14.1, J = 4.9 Hz, 1H, 3–H), 3.03 (dd, J = 14.1, J = 8.0 Hz, 1H, 3–H), 2.55 (s, 3 H, 5”–CH_3), 1.47 (s, 9 H, C(CH_3)$_3$); ^{13}C NMR (75 MHz, acetone-d_6): δ 172.8, 168.7, 160.9, 156.2, 149.5, 149.4, 141.8, 137.6, 129.7, 129.0, 128.0, 126.2, 123.4, 118.2, 117.2, 116.0, 99.14, 53.5, 36.6, 33.6, 30.6, 29.6, 15.4. Anal. calcd. for $C_{27}H_{27}NO_6$: C, 70.27; H, 5.90; N, 3.03; found: C, 70.25; H, 5.93; N, 3.04. HRMS (ESI-TOF) for $C_{27}H_{26}NO_6$ [M–H]$^-$: calcd.: 460.1760, found: 460.5102.

4.1.9. L-2-[2′-(3”-tert-butyl-5”-methyl-7”-oxo-7H-furo[3,2-g] [1] benzopyran-6”-yl)-acetyl-amino]-3-phenylpropionic acid (**1b**)

Yield 27%; mp: 158 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (s, 1H, 2”–H), 7.42 (s, 1H, 4”-H or 9”–H), 7.41 (s, 1H, 4”-H or 9”–H), 7.14–6.95 (m, 5H, H Ar Phe), 4.80–4.71 (m, 1 H, 2–H), 3.65 (d, J = 14.1 Hz, 1H, 2′–H), 3.54 (d, J = 14.1 Hz, 1H, 2′–H), 3.23 (dd, J = 14.1, J = 4.9 Hz, 1H, 3–H), 3.02 (dd, J = 14.1, J = 8.0 Hz, 1H, 3–H), 2.53 (s, 3 H, 5”–CH_3), 1.47 (s, 9 H, C(CH_3)$_3$); ^{13}C NMR (75 MHz, acetone-d_6): δ 172.8, 168.6, 160.9, 156.2, 149.5, 149.4, 141.8, 137.7, 129.7, 129.0, 128.0, 126.1, 123.4, 118.2, 117.2, 116.0, 99.15, 53.6, 36.6, 33.7, 30.6, 29.6, 15.4. Anal. calcd. for $C_{27}H_{27}NO_6$: C, 70.27; H, 5.90; N, 3.03; found: C, 70.28; H, 5.88; N, 3.03. HRMS (ESI-TOF) for $C_{27}H_{26}NO_6$ [M–H]$^-$: calcd.: 460.1760, found: 460.5214.

4.1.10. DL-2-[2′-(3”-tert-butyl-5”,9”-dimethyl-7”-oxo-7H-furo[3,2-g] [1]benzopyran-6”-yl)-acety-lamino]-3-phenylpropionic acid (**2**)

Yield 45%; mp: 120 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (s, 1H, 2”–H), 7.43 (s, 1H, 4”–H), 7.11–6.85 (m, 5H, H Ar Phe), 4.77–4.70 (m, 1 H, 2–H), 3.66 (d, J = 14.1 Hz, 1H, 2′–H), 3.56 (d, J = 14.1 Hz, 1H, 2′–H), 3.24 (dd, J = 14.1, J = 5.5 Hz, 1H, 3–H), 3.02 (dd, J = 14.1, J = 8.5 Hz, 1H, 3–H), 2.61 (s, 3 H, 5”-CH_3 or 9”–CH_3), 2.54 (s, 3 H, 5”–CH_3 or 9”–CH_3), 1.46 (s, 9 H, C(CH_3)$_3$); ^{13}C NMR (75 MHz, acetone-d_6): δ 172.8, 168.8, 160.9, 155.2, 149.8, 147.1, 141.5, 137.6, 130.1, 129.0, 128.0, 126.2, 122.4, 117.8, 115.9, 114.3, 108.1, 53.5, 36.5, 33.6, 30.6, 29.7. Anal. calcd. for $C_{28}H_{29}NO_6$: C, 70.72; H, 6.15; N, 2.95; found: C, 70.77; H, 6.16; N, 2.93. HRMS (ESI-TOF) for $C_{28}H_{28}NO_6$ [M–H]$^-$: calcd.: 474.1917, found: 474.1879.

4.2. Docking of compounds into DNA binding of NF-κB

All the compound library were docked into putative binding site of the NF-κB targets employing Glide software [47]. The 3D structures of the NF-κB–DNA complexes were retrieved from the Protein Data Bank (PDB codes: 1NFK and 1LE9) [33,34]. Starting from the co-crystallized complexes, the NF-κB p50–p50 homodimer (from 1NFK), p50 monomer chain A (p50a from 1NFK), p50 monomer chain B (p50b from 1NFK; p50b from 1LE9) for docking were prepared using Maestro graphical interface, as described in detail elsewhere [32–34]. The Standard-Precision (SP) method as implemented in Glide, was employed as first for p50 NF-κB. The maximum number of poses per ligand to pass to the grid-refinement calculation was set to 10,000. Only one good pose for each molecule was retained. Subsequently, all possible redundant structures were eliminated from the database using the table project implemented in Glide. Based on the G-score function, the highest-ranking 1000 docked ligands were selected and collected in a new multifile for further docking runs. After a minimization process in the OPLS_2001 force field by premin script, the picked out compounds were docked again into DNA recognition site, using the more CPU time-intensive and accurate Extra-Precision (XP) method.

Finally, the first ten molecules with the highest-ranking poses (XP G-score value) against the most protein target were individuated as promising. The molecules were docked into the protein targets applying the accurate XP method as described above but in this case the total number of docked retained poses for each compound was set to 100. The subsequent selection of the putative bioactive conformation within a cluster of poses for each ligand, were based on both G-score and E-model values.

4.3. Electrophoretic mobility shift assay (EMSA)

Electrophoretic Mobility Shift Assay (EMSA) was perfomed as previously described [48–50]. Briefly, double stranded synthetic oligodeoxynucleotides mimicking the NF-κB binding (NF-κB, sense: 5′-CGC TGG GGA CTT TCC ACG G-3′) have been employed. Oligodeoxynucleotides were labeled with γ^{32}P-ATP using 10 Units of T4-polynucleotide-kinase (MBI Fermentas) in 500 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, (1 mM EDTA in the presence of 50 mCi γ^{32}P-ATP) in a volume of 20 μl for 45 min at 37 °C. Reaction was brought to 150 mM NaCl and 150 ng complementary oligodeoxynucleotide was 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 [49] in a total volume of 20 μl containing buffer TF 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 a incubation of 20 min at room temperature, 0.25 ng of ^{32}P-labeled oligonucleotides were added to the samples for 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 [49].

4.4. Cell cultures

IB3-1 cells (LGC Promochem), derived from a CF patient with a ΔF508/W1282X mutant genotype and immortalized with adeno12/SV40 [51], were grown in LHC-8 basal medium (Invitrogen, Carlsbad, CA), supplemented with 5% FBS in the absence of gentamycin, at 37 °C/5% CO_2.

4.5. Proliferation assay

IB3-1 cells were seeded at a density of 100,000 cells in 24 wells plates in LHC-8 medium in presence of 5% FBS. After adhesion, cells were starved in serum free LHC-8, drug was added at serial dilutions (as indicate in figure) and incubated for further 3 days. After this period cells were washed with PBS and detached with trypsin/

EDTA. Cells were resuspended in DMEM medium and counted with a Sysmex XE-2100 Cytometer (Dasit, Milan, Italy).

4.6. Quantitation of transcripts of pro-inflammatory genes

This was carried out as described previously [20]. Briefly, total RNA was extracted using TRIzol Reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. Reverse transcription (RT) was performed using Reverse Transcription System kit (Promega, Madison, WI): 1 mg of total RNA was reverse trascribed in the presence of 5 mM $MgCl_2$, 1× Reverse transcription Buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTPs, 20 U recombinant Rnasin Ribonuclease Inhibitor, 15 U AMV Reverse Transcriptase, 0.5 mg Oligo$(dT)_{15}$ primers in a total volume of 20 ml for 10 min at 70 °C and 60 min at 42 °C. The resulting cDNA was quantified by relative quantitative real-time PCR (real-time qPCR). For the Real-time qPCR, 1 ml of cDNA were used for each Sybr Green real-time PCR to quantify the relative IL-8 expression. Each 25 μl of total reaction volume contained 1 μl of cDNA, 10 pmol of primers, 1 × iQ™ SYBR®Green Supermix (Bio-Rad Laboratoires Inc., Hercules, CA). Real-time PCRs were performed for a total of 40 cycles (95 °C for 10 s, 68 °C for 30 s, and 72 °C for 60 s) using an iCycler IQ® (Bio-Rad Laboratoires Inc., Hercules, CA). Primer sequences were: IL-8 forward: 5′-GTG CAG TTT TGC CAA GGA GT-3′; IL-8 reverse: 5′-TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC-3′.

4.7. Bio-plex analysis

Cytokines in tissue culture supernatants released from the cells under analysis, were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) [52,53] as described by the manufacturer. The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 μl of sample. In our experiments, the premixed singleplex beads of the Bio-Plex human cytokines IL-8 were used. 50 μl of cytokine standards or samples (supernatants recovered from treated cells and diluted to 2 μg/μl) were incubated with 50 μl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed by vacuum filtration three times with 100 μl of Bio-Plex wash buffer, 25 μl of diluted detection antibody were added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 μl of streptavidin-phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA).

4.8. Statistics

Results are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made by using paired Student's t test and a one-way analysis of variance (ANOVA). Statistical significance was defined as significant with $p < 0.05$, and highly significant with with $p < 0.01$.

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