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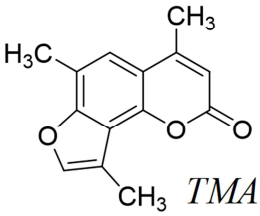
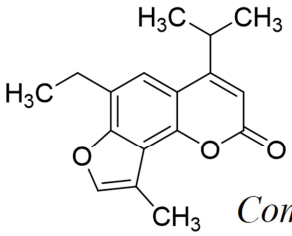


TMA

- Inhibitory activity on NF-kB/DNA interactions
- Mutagenicity
- Photoactivity



Compound ***23***

- Inhibitory activity on NF-kB/DNA interactions
- **No mutagenicity**
- **No photoactivity**

Design, Synthesis and Biological Evaluation of Novel Trimethylangelicin Analogues Targeting Nuclear Factor kB (NF-kB)

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Keywords: furocoumarin, angelicin, synthesis, NF-κB

Abstract

A series of trimethylangelicin (TMA) derivatives were designed and synthesized to overcome the unwanted effects of TMA, promising agent for treatment of inflammation-related diseases and other pathologies, such as cystic fibrosis. The new generation TMA analogues bore hindered substituents at the 4 position in order to minimize or avoid the photoreactions with DNA. Among them, the 4-isopropyl-6-ethyl derivative **23** exhibited TMA-like inhibitory activity on NF-κB/DNA interactions but it proved unable to photoreact with pyrimidine bases of DNA, nor to induce any other DNA damage. The isopropyl analogue **23** was proven to lack mutagenicity when assayed through Ames test and exhibited no anti-proliferative activity on cystic fibrosis IB3-1 cells, displaying at the same time inhibition of the TNF-α induced release of the NF-κB regulated PDGF-B chain, IL-10, IL-15, IL-17 and IFN-γ. Therefore compound **23** deserves further assay to determine its anti-inflammatory properties, since it lacks photoreaction properties and mutagenicity-related side effects.

INTRODUCTION

Nuclear Factor κB (nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) is a key transcription factor involved in several biological processes (such as inflammation and apoptosis) [1,2]. NF-κB is activated by several intra-cellular as well as extra-cellular stimuli [3]. Examples are bacterial or viral products [4], cytokines [5], oxidant-free radicals [6], ultraviolet irradiation [7]. After a complex, but well characterized process of activation [8] NF-κB translocates to the nucleus, where induces the transcription of target genes [3,9]. Inappropriate NF-κB activation/regulation causes inflammatory diseases [10], supporting the concept that NF-κB should be considered a target for therapeutic interventions [11]. In particular, NF-κB is a key transcription factor regulating a set of pro-inflammatory genes involved in the inflammatory status of patients affected by cystic fibrosis (CF) [12-14], a life-limiting autosomal recessive disorder caused by mutations in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride-conducting transmembrane channel that regulates anion transport and mucociliary clearance in the airways [15,16]. In these last years small molecule and gene-based therapies have been developed targeting the CFTR defect, in order to modify the disease and to improve the quality of life for CF patients [17,18].

With respect to the issues of modulating CFTR function and controlling inflammatory status of CF, TMA (4,6,4'-trimethylangelicin) (Fig. 1) is a small-molecule exhibiting both corrector and potentiator activities of CFTR [19]. TMA displays also anti-inflammatory properties by targeting NF-κB and reducing IL-8 expression [20], therefore demonstrating to be a promising agent for treatment of cystic fibrosis. Unfortunately, TMA was also shown to have potential drawbacks, such as phototoxicity and mutagenicity [21,22].

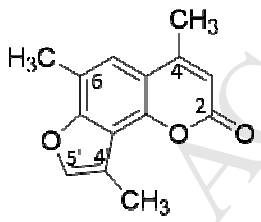


Figure 1. Structure of 4,6,4'-trimethylangelicin (TMA).

Recently we found that other TMA analogues/isosters shared the same anti-inflammatory properties [23]: indeed, they inhibited NF-κB/DNA interaction and they reduced NF-κB-mediated IL-8 gene expression in human bronchial epithelial CF IB3-1 cells, demonstrating again their potential interest for a possible development in the anti-inflammatory therapy. Due to our interest in this field [24-

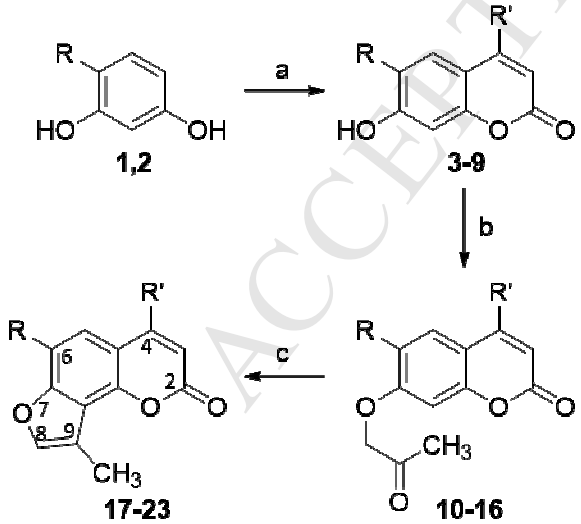
26], we went on modifying the TMA scaffold in order to identify new compounds with comparable biological properties in the respect of TMA but with reduced or abolished side effects.

RESULT AND DISCUSSION

Chemistry

Bearing in mind that the photoreaction of TMA with DNA is a photocycloaddition between a double bond of furocoumarin nucleus (lactone or furan double bond) and thymine (or cytosine) double bond [27], in order to minimize or avoid the covalent photoreactions with DNA we designed new analogues with substituents at the 4 position much more hindered than the classic methyl group. In the 6 position of furocoumarin backbone a methyl or an ethyl group were inserted, while the furan ring was not modified in the respect of the parent TMA, as the previous studies indicated that changes in this moiety were detrimental for activity [23].

The new TMA analogues were synthesized according to Scheme 1, adopting a well known synthetic strategy [28,29]. The starting 7-hydroxybenzopyran-2-ones (**3**-**9**) were synthesized condensing 4-methylresorcinol (**1**) or 4-ethylresorcinol (**2**) with the appropriate acetoacetic ester derivatives. Compounds **3**-**9** were functionalized with chloroacetone obtaining the O-α-ketoethers **10**-**16**. The intermediates ethers were finally cyclized in anhydrous alkaline medium affording the desired angelicins **17**-**23**.



Scheme 1. Synthesis of TMA analogues. Reaction conditions: a. Ethyl acetoacetate (for **3**) or ethyl propionylacetate (for **4**,**5**) or ethyl 3-oxohexanoate (for **6**,**7**) or ethyl isobutyrylacetate (for **8**,**9**), H_2SO_4, 1 h, 40-92%; b. Chloroacetone, K_2CO_3, reflux, 6 h, 74-96%; c. KOH, EtOH abs., reflux, 1 h, 17-47%. See Table 1 for R specification.

Table 1. R substituent for TMA analogues.

Compounds	R	R'
1	Me	-
2	Et	-
3, 10, 17	Et	Me
4, 11, 18	Me	Et
5, 12, 19	Et	Et
6, 13, 20	Me	Pr
7, 14, 21	Et	Pr
8, 15, 22	Me	*i*Pr
9, 16, 23	Et	*i*Pr

Biology

The angelicin derivatives were then tested for their inhibitory activity on NF-κB/DNA interactions. Moreover, since NF-κB is implicated in cell proliferation and survival [1-3], the effects of TMA analogues were tested also on the proliferation of cystic fibrosis IB3-1 cells [30]. The effects of TMA derivatives on NF-κB/DNA interactions were verified by electrophoretic mobility shift assay (EMSA), using purified NF-κB p50 and ^{32}P-labeled target NF-κB double-stranded oligonucleotide mimicking the NF-κB consensus sequence.

We first demonstrated that, as elsewhere published, TMA was able to inhibit the interactions between NF-κB p50 and a ^{32}P-labeled target NF-κB double-stranded oligonucleotide (Figure S1 in Supplementary Data). Second, the TMA analogues **17-23** were comparatively assayed to verify their ability to inhibit NF-κB/DNA interactions.

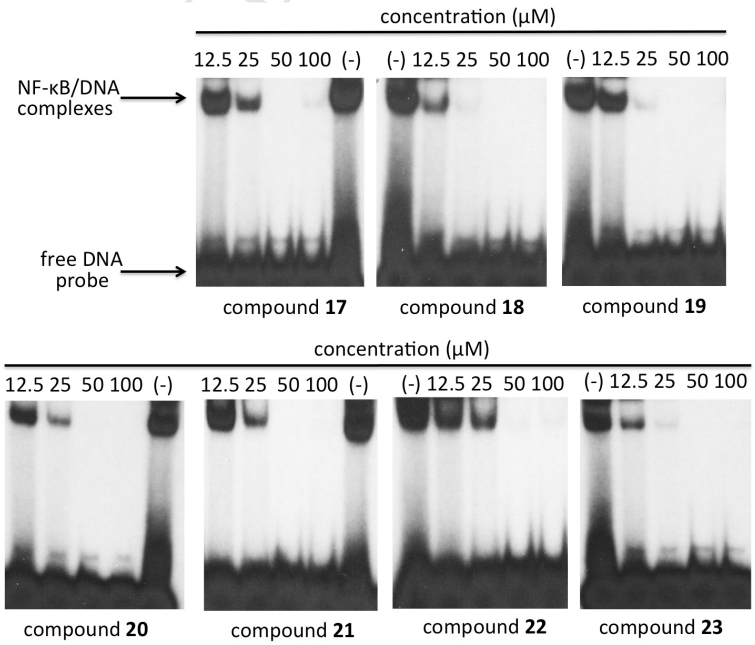


Figure 2. Representative EMSA experiment results depicting the effects of TMA analogues (**17-23**) at 12.5-100 μM concentrations on the molecular interactions between NF-κB p50 and ^{32}P-labeled target NF-κB double-stranded oligonucleotide. Arrows indicate NF-κB/DNA complexes and the free ^{32}P-labeled target NF-κB probe.

As shown in the representative example reported in Figure 2, all the TMA analogues exhibited inhibitory activity on NF-κB/DNA interactions, but compounds **18** and **23** were found to be the most effective, clearly showing a strong inhibitory effects when added at 12.5 μM.

A summary of EMSA data is reported in Table 2, together with the effects of TMA analogues on IB3-1 cell growth. For the analysis of the effects on cell proliferation, monolayers of 60% confluent IB3-1 cells (derived from a CF patient with a ΔF-508/W1282X mutant genotype) were seeded in 12-well plate, treated with different compound concentrations and the cell number/mL determined after 48 h of culture, obtaining the IC_{50} values reported in Table 2.

The results show that compounds **18** and **23** efficiently inhibit NF-κB/DNA interactions displaying low IC_{50} values (8.0 μM and 7.4 μM, respectively). However, while compound **18** exhibited some anti-proliferative activity on IB3-1 cells (IC_{50} = 50 μM), compound **23** was unable to inhibit IB3-1 cell growth even when added at 200 μM, thus proving to be an interesting NF-κB inhibitor without cytotoxic effects.

Table 2. Effects of the TMA analogues on inhibition of NF-κB/DNA complexes and proliferation of cystic fibrosis IB3-1 cells.

Compounds	Inhibition of NF-κB/DNA complex (IC_{50})	IB3-1 (IC_{50})
17	24.0 μM	>200 μM
18	8.0 μM	50 μM
19	17.8 μM	>200 μM
20	21.9 μM	200 μM
21	19.8 μM	162.5 μM
22	18.9 μM	>200 μM
23	7.4 μM	>200 μM

Inhibition of NF-κB/DNA interaction and cell growth are expressed as IC_{50} (concentration leading to 50% inhibition of NF-κB/DNA interaction and IB3-1 cell growth).

In this respect, we further demonstrated that 7.4 μM concentration of compound **23** was unable to exert cytotoxic effects on cystic fibrosis IB3-1 cells (Table 2) as well as in other in vitro cultured cell lines, such as K562 leukemia and U251 glioma cell lines (unpublished results). In conclusion on the basis of the effects on NF-kB/DNA interactions and on cell proliferation of IB3-1 cells (Table 2), compound **23** resulted the most interesting one. Therefore, we investigated its photochemical behavior in order to verify if the photosensitizing properties were abolished

introducing a bulky substituent in the 4 position. First of all the ability of compound **23** to form an intercalative molecular complex with DNA in the ground state has been investigated by linear flow dichroism (LD). Differently from TMA, that was efficiently complexed inside the macromolecule, compound **23** was unable to intercalate inside the double helix, even at the highest sensitivity (data reported in Supporting Information).

To exclude any further interaction with DNA, compound **23** was also evaluated for its ability to undergo covalent photocycloaddition with bases of DNA in the absence and in the presence of UVA light, taking TMA as reference compound. The photobinding properties of the compounds were tested in the presence of salmon testes DNA with and without UVA light irradiation.

The irradiated solutions were opportunely treated and analyzed to detect the formation of furocoumarin-pyrimidine bases photocycloadducts, which were then isolated (TLC) and analyzed (NMR). Differently from TMA, able to form cycloadducts with thymine characterized by a strong fluorescence (data not shown), with compound **23** we did not detect any significant band on the TLC plate and no band had the capacity to photoreverse under 254 nm light to the initial compound and thymine, typical of photocycloadducts between furocoumarins and DNA [31].

The presence of induced DNA damage in pBR322 plasmid DNA was studied by agarose gel electrophoresis. Open circular DNA, OC, is formed when single strand breaks are present at one strand. Linearization of the plasmid DNA (L) indicates that double strand breaks are induced by the damaging compounds. These forms of DNA have different macromolecular structures and can be distinguished as they behave differently in agarose gel electrophoresis.

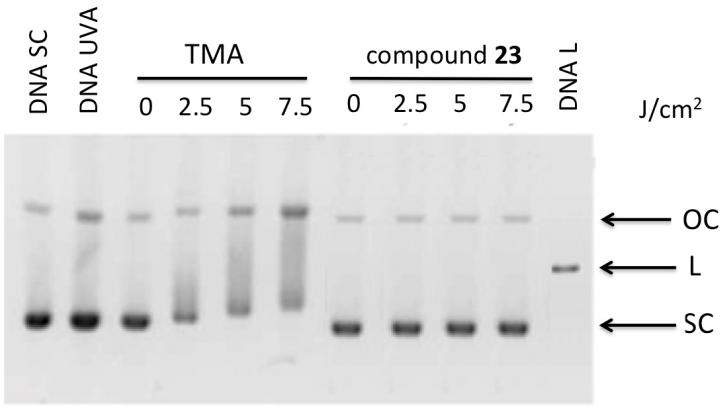


Figure 3. pBR322 DNA in the presence of TMA and **23** (100 μM) in the dark (0 J/cm^2) and irradiated with increasing doses of UVA light. Dark (DNA SC supercoiled), UVA irradiated DNA (at 7.5 J/cm^2, DNA UVA) and linear DNA (DNA L) samples were taken as controls. OC = open circular, L = linear, SC = supercoiled.

In Figure 3, as regard TMA samples, the increasing amount of single strand breaks (see the increasing darkening of the OC bands by increasing the radiant exposures) indicates a larger number of TMA induced lesions able to cut the DNA at one strand. At the same time, the SC DNA bands become lighter and the photo-addition products formed slow down SC DNA migration as irradiation augmented. None of these damages was visible for compound **23**, even when the experiments were performed under UVA light. Thus, compound **23** was unable to intercalate inside the double helix, nor to photoreact with pyrimidine bases of DNA, nor to induce any other DNA damage, providing evidence that this treatment is not able to exert any action of DNA, even when as an isolate substrate.

In order to verify the potential mutagenicity of compound **23**, it was assayed for genotoxicity evaluation through Ames test employing the histidine-requiring *Salmonella typhimurium* mutants TA 97A, TA98, TA100 and TA1535 strains. The histidine-requiring *Salmonella typhimurium* mutant strains described are particularly appropriate to preliminary check genotoxic potential of new chemical compounds because both of them have a plasmid that induce an increasing of error-prone repair of DNA damage (pKM101), and mutations that induce an increased permeability to larger molecules through a defective lipopolysaccharide layer (rfa mutations) and avoid the possibility to repair excision DNA damage (uvrB mutations) [32,33].

The genetic properties of the *Salmonella typhimurium* mutants TA 97A, TA98, TA100 and TA1535 strains allow to check frameshift mutation and base-pair substitution through the increasing number of revertant colonies in presence of mutagenic chemical compounds, with and without metabolic activation induced by S9 mix. The number of spontaneous revertants is relatively constant, but it dramatically increases - generally in a dose-dependent way - when a mutagen is added to the plate.

In general, the cut-off to discriminate between a mutagenic and non-mutagenic result is considered a 2-fold revertants increase over the response of the negative control (spontaneous revertants). For the above reported considerations, the Ames test is generally considered an appropriate preliminary screening tool to determine the mutagenic potential of new chemicals for different industrial applications, from agro-chemicals, pharmaceuticals and medical devices to health products (for e.g. personal cares, cosmetics, nutraceuticals, etc.). For the high significant predictive capacity, Ames test is required by the regulatory agencies for registration or acceptance of new compounds [56]. The results obtained for TMA and compound **23** using the *Salmonella typhimurium* mutants TA98 and TA100 are reported in Table 3.

Table 3. Mutagenicity of TMA and lack of mutagenicity of compound **23** on Salmonella typhimrium TA98 and TA100 strains.

TMA (4,6,4'-trimethylangelicin)						
	TA98 –S9			TA98 +S9		
Conc (µl/plate)	revertant colonies (average)	SD	t/c*	revertant colonies (average)	SD	t/c
DMSO	24.67	3.51	1.00	27.33	1.53	1.00
C+	576.50	119.50	**23.37**	502.50	20.51	**18.38**
1	25.50	7.78	1.03	20.50	4.95	0.75
5	15.50	3.54	0.63	52.00	26.87	1.90
10	28.50	13.44	1.16	123.00	84.85	**4.50**
50	24.00	5.66	0.97	225.50	45.96	**8.25**
100	30.00	1.41	1.22	187.50	37.48	**6.86**
	TA100 –S9			TA100 +S9		
Conc (µl/plate)	revertant colonies (average)	SD	t/c	revertant colonies (average)	SD	t/c
DMSO	213.67	30.35	1.00	210.33	23.97	1.00
C+	3871.00	272.94	**18.12**	2852.00	50.91	**13.56**
1	212.00	28.08	0.99	232.00	10.51	1.10
5	220.50	23.33	1.03	210.50	9.19	1.00
10	225.50	6.36	1.06	218.50	10.61	1.04
50	232.50	19.09	1.09	243.00	11.31	1.16
100	221.00	8.46	1.03	529.50	120.92	**2.52**
Compound **23**						
	TA98 –S9			TA98 +S9		
Conc (µl/plate)	revertant colonies (average)	SD	t/c	revertant colonies (average)	SD	t/c
DMSO	68.0	7.2	1.0	112.7	23.4	1.0
C+	1054.7	74.4	**15.5**	331.0	40.6	**2.9**
1	77.3	16.8	1.1	94.0	12.0	0.8
5	82.0	14.0	1.2	71.3	22.7	0.6
10	90.7	9.2	1.3	82.0	5.3	0.7
50	83.3	15.5	1.2	74.7	1.2	0.7
100	84.0	6.9	1.2	71.3	1.2	0.6
	TA100 –S9			TA100 +S9		
Conc (µl/plate)	revertant colonies (average)	SD	t/c	revertant colonies (average)	SD	t/c
DMSO	101.3	7.8	1.0	118.3	12.5	1.0
C+	478.4	26.9	**4.7**	285.0	5.4	**2.4**
1	76.0	2.6	0.8	135.0	27.8	1.1
5	99.3	1.2	1.0	126.7	12.6	1.1
10	83.0	11.5	0.8	120.0	8.7	1.0
50	88.7	10.1	0.9	138.3	2.9	1.2
100	87.3	6.1	0.9	180.0	17.3	1.5

In bold are values indicating mutagenicity (> 2.00). * t/c = treated/control.

While TMA displayed mutagenic activity (in bold are values indicating mutagenicity, i.e. the treated/control values > 2.00), compound **23** did not display genotoxic activity at all the concentrations tested with all the Salmonella strains. These data were confirmed using the *Salmonella typhimurium* mutants TA 97A and TA1535 strains (Table in Supplementary Data).

In order to verify whether compound **23** displays inhibitory effects on pro-inflammatory genes, IB3-1 were treated with 10 ng/ml TNF-α for 24 hours in the absence or in the presence of a 24-hours pre-treatment step with 1 μM compound **23** [23]. The secretome was then analysed using the Bio-plex approach using a 27-plex kit, analysing the pg/ml of 27 proteins present in the supernatant, including cytokines, chemokines and growth factors [23]. The 27-plex list was matched with the list of NF-κB regulated genes published by Pahl [3] (updated in https://www.bu.edu/nf-kb/gene-resources/target-genes/), including the recently validated IL-13 [34], IL-15 [35] and IL-17 [36]. The results Venn's diagram (shown in the upper part of Figure 4) demonstrates that 23 proteins were in common. From this list of 23 NF-κB regulated genes TNF-α was not analyzed, since TNF-α was exogenously added to induce IB3-1 pro-inflammatory genes. Further, 4 proteins were not considered (IL-13, GM-CSF, MIP-1α, MIP-1β) for the low level of released (< 5 pg/ml; data not shown). The results concerning the remaining 18 proteins are reported in the lower part of Figure 4 and demonstrated that treatment with compound **23** caused inhibition of release of 5 proteins (PDGF-B chain, IL-10, IL-15, Il-17 and IFN-γ) higher that 20%. The highest level of inhibition by compound **23** was found for IL-10 and IFN-γ.

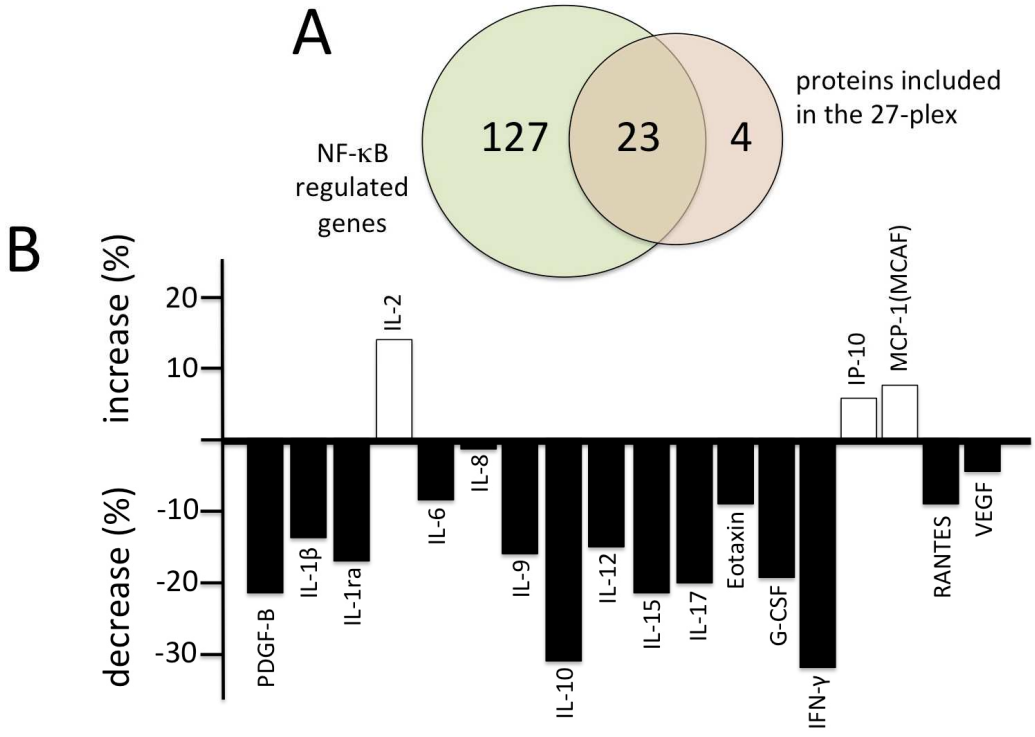


Figure 4. A. Venn's diagram comparing the list of NF-κB regulated genes published by Pahl [3] (implemented with IL-13, IL-15 and IL-17) [34-36] with the 27-plex proteins. B. Changes of release caused by treatment of TNF-α induced IB3-1 cells with 1 μM compound **23**. The following proteins were not considered: TNF-α (since TNF-α was used for IB3-1 induction) and IL-13, GM-CSF, MIP-1α, MIP-1β (since they release was lower that 5 pg/ml; data not shown).

CONCLUSIONS

In conclusion we have synthesized and validated a TMA derivative, compound **23**, which exhibits TMA-like inhibitory activity on NF-κB/DNA interactions without displaying TMA unwanted effects. First of all, compound **23** was unable to intercalate inside the double helix, nor to photoreact with pyrimidine bases of DNA, nor to induce any other DNA damage. In addition compound **23** was proven to lack mutagenicity when assayed through Ames test employing the histidine-requiring *Salmonella typhimurium* mutants TA 97A, TA98, TA100 and TA1535 strains. On the other hand, compound **23** exhibited inhibitory effects on some NF-κB regulated genes (PDGF-B chain, IL-10, IL-15, Il-17 and IFN-γ) induced in cystic fibrosis IB3-1 cells by TNF-α treatment. Therefore, in consideration of the role of NF-κB in regulating inflammation in cystic fibrosis [12-14,37,38], compound **23** can be proposed for further characterization as a possible anti-inflammatory agent to be used in combination with other anti-inflammatory molecules used at sub-optimal concentrations.

EXPERIMENTAL SECTION

Chemistry

General Information. All commercial chemicals and solvents used were analytical grade and were used without further purification. Microwave assisted reactions were performed on a CEM Discover monomode reactor in closed devices with the temperature monitored by a built-in infrared sensor and the automatic control of the power. Melting points (uncorrected) were determined using a Gallenkamp MFB-595-010M capillary melting point apparatus. Analytical thin layer chromatography (tlc) was performed on pre-coated silica gel plates (Merck 60-F-254, 0.25 mm), which were developed on a mixture of $CHCl_3$/MeOH (9/1). The NMR spectra were recorded on a Bruker 300-AMX spectrometer with TMS as internal standard. Coupling constants are given in Hz. HRMS spectra were acquired using a XEVO G2-S Qtof (Waters) mass spectrometer with direct injection of the sample and collecting data in positive ion mode. Purity of compounds was determined by elemental analysis. Elemental analysis were performed on a Perkin-Elmer 2400 analyser and all values were within ± 0.4 % of the theoretical values.

Synthesis of alkyl-7-hydroxycoumarins 3-9.

General procedure. To a solution of **1** or **2** (10.0 mmol) in the appropriate acetoacetic ester (10.0 mmol), H_2SO_4 (7 mL) was added dropwise and the mixture was stirred at room temperature for 1 h. The solution was poured into an ice/water mixture (100 g) and the obtained precipitate was filtered and washed with water to give the corresponding alkyl-7-hydroxycoumarin.

6-Ethyl-7-hydroxy-4-methylbenzopyran-2-one (3). From **2**: yield 92%: mp 197 °C. ^{1}H-NMR (DMSO-*d6*): 10.52 (broad s, 1H, 7-OH), 7.44 (s, 1H, 5-H), 6.72 (s, 1H, 8-H), 6.09 (q, *J* = 1.1 Hz, 1H, 3-H), 2.59 (q, *J* = 7.5 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 2.37 (d, *J* = 1.1 Hz, 3H, 4-Me), 1.15 (t, *J* = 7.5 Hz, 3H, -CH$_2$-C<u>H</u>$_3$).

4-Ethyl-7-hydroxy-6-methylbenzopyran-2-one (4). From **1**: yield 73%: mp 151 °C. ^{1}H-NMR (DMSO-*d6*): 10.52 (broad s, 1H, 7-OH), 7.52 (s, 1H, 5-H), 6.72 (s, 1H, 8-H), 6.05 (t, *J* = 1.2 Hz, 1H, 3-H), 2.76 (qd, *J* = 7.4, 1.2 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 2.18 (s, 3H, 6-Me), 1.21 (t, *J* = 7.4 Hz, 3H, -CH$_2$-C<u>H</u>$_3$).

4,6-Diethyl-7-hydroxybenzopyran-2-one (5). From **2**: yield 83%: mp 108 °C. ^{1}H-NMR (DMSO-*d6*): 10.51 (broad s, 1H, 7-OH), 7.48 (s, 1H, 5-H), 6.73 (s, 1H, 8-H), 6.05 (t, *J* = 1.1 Hz, 1H, 3-H), 2.78 (qd, *J* = 7.4, 1.1 Hz, 2H, 4-C<u>H</u>$_2$-CH$_3$), 2.59 (q, *J* = 7.5 Hz, 2H, 6-C<u>H</u>$_2$-CH$_3$), 1.21 (t, *J* = 7.4 Hz, 3H, 4-CH$_2$-C<u>H</u>$_3$), 1.15 (t, *J* = 7.5 Hz, 3H, 6-CH$_2$-C<u>H</u>$_3$).

7-Hydroxy-6-methyl-4-propylbenzopyran-2-one (6). From **1**: yield 90%: mp 166 °C. ^{1}H-NMR (DMSO-*d6*): 10.52 (broad s, 1H, 7-OH), 7.53 (s, 1H, 5-H), 6.73 (s, 1H, 8-H), 6.05 (t, *J* = 1.1 Hz, 1H, 3-H), 2.71 (td, *J* = 7.5, 1.1 Hz, 2H, -C<u>H</u>$_2$-CH$_2$-CH$_3$), 2.18 (s, 3H, 6-Me), 1.63 (sest, *J* = 7.5 Hz, 2H, -CH$_2$-C<u>H</u>$_2$-CH$_3$), 0.97 (t, *J* = 7.5 Hz, 3H, -CH$_2$-CH$_2$-C<u>H</u>$_3$).

6-Ethyl-7-hydroxy-4-propyl-benzopyran-2-one (7). From **2**: yield 90%: mp 127 °C. ^{1}H-NMR (DMSO-*d6*): 10.52 (broad s, 1H, 7-OH), 7.48 (s, 1H, 5-H), 6.73 (s, 1H, 8-H), 6.04 (t, *J* = 1.1 Hz, 1H, 3-H), 2.72 (td, *J* = 7.6, 1.2 Hz, 2H, -C<u>H</u>$_2$-CH$_2$-CH$_3$), 2.59 (q, *J* = 7.5 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 1.63 (sest, J = 7.6 Hz, 2H, -CH$_2$-C<u>H</u>$_2$-CH$_3$), 1.15 (t, *J* = 7.5 Hz, 3H, -CH$_2$-C<u>H</u>$_3$), 0.97 (t, *J* = 7.6 Hz, 3H, -CH$_2$-CH$_2$-C<u>H</u>$_3$).

7-Hydroxy-4-isopropyl-6-methylbenzopyran-2-one (8). From **1**: yield 40%: mp 147 °C. ^{1}H-NMR (CDCl$_3$): 7.41 (s, 1H, 5-H), 6.96 (s, 1H, 8-H), 6.17 (d, *J* = 1.1 Hz, 1H, 3-H), 3.27 (sept/d, *J* = 7.2 H, 1.1 Hz, 1H, -C<u>H</u>-(CH$_3$)$_2$), 2.31 (s, 3H, 6-Me), 1.31 (d, *J* = 7.2 Hz, 6H, -CH-(C<u>H</u>$_3$)$_2$).

6-Ethyl-7-hydroxy-4-isopropylbenzopyran-2-one (9). From **2**: yield 52%: mp 168 °C. ^{1}H-NMR (CDCl$_3$): 7.41 (s, 1H, 5-H), 7.05 (s, 1H, 8-H), 6.17 (d, *J* = 1.1 Hz, 1H, 3-H), 3.30 (sept/d, *J* = 7.2 H, 1.1 Hz, 1H, -C<u>H</u>-(CH$_3$)$_2$), 2.71 (q, *J* = 7.5 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 1.51 (d, *J* = 7.2 Hz, 6H, -CH-(C<u>H</u>$_3$)$_2$), 1.26 (t, *J* = 7.5 Hz, 3H, -CH$_2$-C<u>H</u>$_3$).

Synthesis of alkyl-7-O-(2'-oxopropyl)ethers 10-16.

General procedure. A mixture of alkyl-7-hydroxybenzopyran-2-one (5.0 mmol), chloroacetone (0.5 mL, 5.9 mmol) and anhydrous K_2CO_3 (2.1 g, 15.0 mmol) in acetone (75 mL) was refluxed 6 h. After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated

from the combined filtrate and washings to give the corresponding alkyl-7-(2'-oxopropyloxy)-2*H*-1-benzopyran-2-one.

6-Ethyl-4-methyl-7-(2'-oxopropyloxy)benzopyran-2-one (10). From **3**: yield 87%: mp 142 °C. ^{1}H-NMR (DMSO-*d6*): 7.52 (s, 1H, 5-H), 6.91 (s, 1H, 8-H), 6.19 (q, *J* = 1.4 Hz, 1H, 3-H), 4.99 (s, 2H, -OC$\underline{H}_2$), 2.68 (q, *J* = 7.6 Hz, 2H, -C$\underline{H}_2$-CH_3), 2.40 (d, *J* = 1.4 Hz, 3H, 4-Me), 2.19 (s, 3H, -COC$\underline{H}_3$), 1.20 (t, *J* = 7.6 Hz, 3H, -CH_2-C$\underline{H}_3$).

4-Ethyl-6-methyl-7-(2'-oxopropyloxy)benzopyran-2-one (11). From **4**: yield 76%: mp 127 °C. ^{1}H-NMR ($CDCl_3$): 7.39 (s, 1H, 5-H), 6.59 (s, 1H, 8-H), 6.15 (t, *J* = 1.1 Hz, 1H, 3-H), 4.62 (s, 2H, -OC$\underline{H}_2$), 2.77 (qd, *J* = 7.4, 1.1 Hz, 2H, -C$\underline{H}_2$-CH_3), 2.34 (s, 3H, 6-Me), 2.32 (s, 3H, -COC$\underline{H}_3$), 1.21 (t, *J* = 7.4 Hz, 3H, -CH_2-C$\underline{H}_3$).

4,6-Diethyl-7-(2'-oxopropyloxy)benzopyran-2-one (12). From **5**: yield 74%: mp 89 °C. ^{1}H-NMR (DMSO-*d6*): 7.56 (s, 1H, 5-H), 6.92 (s, 1H, 8-H), 6.13 (t, *J* = 1.0 Hz, 1H, 3-H), 4.99 (s, 2H, -OC$\underline{H}_2$), 2.81 (qd, *J* = 7.4, 1.0 Hz, 2H, 4-C$\underline{H}_2$-CH_3), 2.68 (q, *J* = 7.5 Hz, 2H, 6-C$\underline{H}_2$-CH_3), 2.19 (s, 3H, -$COCH_3$), 1.23 (t, *J* = 7.4 Hz, 3H, 4-CH_2-C$\underline{H}_3$), 1.19 (t, *J* = 7.5 Hz, 3H, 6-CH_2-C$\underline{H}_3$).

6-Methyl-7-(2'-oxopropyloxy)-4-propylbenzopyran-2-one (13). From **6**: yield 74%: mp 109 °C. ^{1}H-NMR (DMSO-*d6*): 7.61 (s, 1H, 5-H), 6.91 (s, 1H, 8-H), 6.13 (t, *J* = 1.1 Hz, 1H, 3-H), 4.99 (s, 2H, -OC$\underline{H}_2$), 2.74 (td, *J* = 7.6, 1.1 Hz, 2H, -C$\underline{H}_2$-CH_2-CH_3), 2.26 (s, 3H, -COC$\underline{H}_3$), 2.19 (s, 3H, 6-Me), 1.64 (sest, *J* = 7.6 Hz, 2H, -CH_2-C$\underline{H}_2$-CH_3), 0.98 (t, *J* = 7.6 Hz, 3H, -CH_2-CH_2-C$\underline{H}_3$).

6-Ethyl-7-(2'-oxopropyloxy)-4-propylbenzopyran-2-one (14). From **7**: yield 96%: mp 118 °C. ^{1}H-NMR (DMSO-*d6*): 7.57 (s, 1H, 5-H), 6.92 (s, 1H, 8-H), 6.14 (t, *J* = 1.1 Hz, 1H, 3-H), 4.99 (s, 2H, -OC$\underline{H}_2$), 2.74 (td, *J* = 7.6, 1.2 Hz, 2H, -C$\underline{H}_2$-CH_2-CH_3), 2.68 (q, *J* = 7.3 Hz, 2H, -C$\underline{H}_2$-CH_3), 2.19 (s, 3H, -COC$\underline{H}_3$), 1.65 (sest, J = 7.6 Hz, 2H, -CH_2-C$\underline{H}_2$-CH_3), 1.19 (t, *J* = 7.3 Hz, 3H, -CH_2-C$\underline{H}_3$), 0.98 (t, *J* = 7.6 Hz, 3H, -CH_2-CH_2-C$\underline{H}_3$).

4-Isopropyl-6-methyl-7-(2'-oxopropyloxy)benzopyran-2-one (15). From **8**: yield 73%: mp 118 °C. ^{1}H-NMR ($CDCl_3$): 7.44 (s, 1H, 5-H), 6.60 (s, 1H, 8-H), 6.15 (d, *J* = 1.1 Hz, 1H, 3-H), 4.62 (s, 2H, -OC$\underline{H}_2$), 3.26 (sept/d, *J* = 7.4, 1.1 Hz, 1H, -C$\underline{H}$-$(CH_3)_2$), 2.35 (s, 3H, -COC$\underline{H}_3$), 2.33 (s, 3H, 6-Me), 1.31 (d, *J* = 7.4 Hz, 6H, -CH-(C$\underline{H}_3$)$_2$).

6-Ethyl-4-isopropyl-7-(2'-oxopropyloxy)benzopyran-2-one (16). From **9**: yield 92%: mp 80 °C. ^{1}H-NMR ($CDCl_3$): 7.44 (s, 1H, 5-H), 6.61 (s, 1H, 8-H), 6.18 (d, *J* = 1.1 Hz, 1H, 3-H), 4.62 (s, 2H, -OC$\underline{H}_2$), 3.28 (sept/d, *J* = 7.1, 1.1 Hz, 1H, -C$\underline{H}$-$(CH_3)_2$), 2.77 (q, *J* = 7.6 Hz, 2H, -C$\underline{H}_2$-CH_3), 2.32 (s, 3H, -COC$\underline{H}_3$), 1.32 (d, *J* = 7.1 Hz, 6H, -CH-(C$\underline{H}_3$)$_2$), 1.27 (t, *J* = 7.6 Hz, 3H, -CH_2-C$\underline{H}_3$).

Synthesis of alkyl-2*H*-furo[2,3-*h*]-1-benzopyran-2-ones 17-23.

General procedure. To an ethanolic solution (50 mL) of alkyl-7-(2'-oxopropyloxy)-2*H*-1-benzopyran-2-one (1.0 mmol), a 4% ethanolic potassium hydroxide solution (15 mL) was added and the mixture was refluxed in the dark for 1 h. The solution was cooled, diluted with water (50 mL) and acidified with diluted HCl. The ethanol was removed under reduced pressure and the solution extracted with $CHCl_3$ (3 x 50 mL). The organic phase was evaporated under reduced pressure and the residue was purified by column chromatography to give the corresponding furobenzopyranone.

4,9-Dimethyl-6-ethyl-2H-furo[2,3-h]-1-benzopyran-2-one (17). From **10**: yield 47%: mp 148 °C. ^{1}H-NMR (DMSO-*d6*): 7.87 (q, *J* = 1.1 Hz, 1H, 8-H), 7.47 (s, 1H, 5-H), 6.32 (q, *J* = 1.3 Hz, 1H, 3-H), 2.89 (q, *J* = 7.6 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 2.48 (d, *J* = 1.1 Hz, 3H, 9-Me), 2.41 (d, *J* = 1.3 Hz, 3H, 4-Me), 1.29 (t, *J* = 7.6 Hz, 3H, -CH$_2$-C<u>H</u>$_3$). HRMS (ESI-TOF) for $C_{15}H_{15}O_3$ $[M + H]^+$: calcd.: 243.1021, found: 243.1059. Anal. calcd. for $C_{15}H_{14}O_3$: C, 74.36; H 5.82; found: C, 74.10; H, 5.95.

6,9-Dimethyl-4-ethyl-2H-furo[2,3-h]-1-benzopyran-2-one (18). From **11**: yield 30%: mp 96 °C. ^{1}H-NMR (DMSO-*d6*): 7.88 (q, *J* = 1.3 Hz, 1H, 8-H), 7.55 (s, 1H, 5-H), 6.27 (t, *J* = 1.0 Hz, 1H, 3-H), 2.89 (qd, *J* = 7.6, 1.0 Hz, 2H, -C<u>H</u>$_2$-CH$_3$), 2.50 (s, 3H, 6-Me), 2.43 (d, *J* = 1.3 Hz, 3H, 4-Me), 1.27 (t, *J* = 7.6 Hz, 3H, -CH$_2$-C<u>H</u>$_3$). HRMS (ESI-TOF) for $C_{15}H_{15}O_3$ $[M + H]^+$: calcd.: 243.1021, found: 243.1123. Anal. calcd. for $C_{15}H_{14}O_3$: C, 74.36; H 5.82; found: C, 74.61; H, 5.92.

4,6-Diethyl-9-methyl-2H-furo[2,3-h]-1-benzopyran-2-one (19). From **12**: yield 40%: mp 115 °C. ^{1}H-NMR (DMSO-*d6*): 7.87 (q, *J* = 1.1 Hz, 1H, 8-H), 7.52 (s, 1H, 5-H), 6.27 (t, *J* = 1.1 Hz, 1H, 3-H), 2.95-2.85 (m, 4H, 4 and 6-C<u>H</u>$_2$-CH$_3$), 2.41 (d, *J* = 1.1 Hz, 3H, 9-Me), 1.29 (t, *J* = 7.5 Hz, 3H, 4 or 6-CH$_2$-C<u>H</u>$_3$), 1.19 (t, *J* = 7.5 Hz, 3H, 4 or 6-CH$_2$-C<u>H</u>$_3$). HRMS (ESI-TOF) for $C_{16}H_{17}O_3$ $[M + H]^+$: calcd.: 257.1178, found: 257.1246. Anal. calcd. for $C_{16}H_{16}O_3$: C, 74.98; H 6.29; found: C, 75.10; H, 6.51.

6,9-Dimethyl-4-propyl-2H-furo[2,3-h]-1-benzopyran-2-one (20). From **13**: yield 17%: mp 146 °C. ^{1}H-NMR (DMSO-*d6*): 7.87 (q, *J* = 1.3 Hz, 1H, 8-H), 7.54 (s, 1H, 5-H), 6.26 (s, 1H, 3-H), 2.82 (t, *J* = 7.6, 1.0 Hz, 2H, -C<u>H</u>$_2$-CH$_2$-CH$_3$), 2.50 (s, 3H, 6-Me), 2.42 (d, *J* = 1.3 Hz, 3H, 9-Me), 1.68 (sest, *J* = 7.6 Hz, 2H, -CH$_2$-C<u>H</u>$_2$-CH$_3$), 1.00 (t, *J* = 7.6 Hz, 3H, -CH$_2$-C<u>H</u>$_3$). HRMS (ESI-TOF) for $C_{16}H_{17}O_3$ $[M + H]^+$: calcd.: 257.1178, found: 257.1246. Anal. calcd. for $C_{16}H_{16}O_3$: C, 74.98; H 6.29; found: C, 75.06; H, 6.45.

6-Ethyl-9-methyl-4-propyl-2H-furo[2,3-h]-1-benzopyran-2-one (21). From **14**: yield 32%: mp 138 °C. ^{1}H-NMR (DMSO-*d6*): 7.88 (q, *J* = 1.2 Hz, 1H, 8-H), 7.53 (s, 1H, 5-H), 6.27 (t, *J* = 1.1 Hz, 1H, 3-H), 2.90 (q, *J* = 7.5 Hz, 2H, 6-C<u>H</u>$_2$-CH$_3$), 2.84 (td, *J* = 7.5, 1.2 Hz, 2H, 4-C<u>H</u>$_2$-CH$_2$-CH$_3$), 2.42 (d, *J* = 1.2 Hz, 3H, 9-Me), 1.68 (sest, *J* = 7.5 Hz, 2H, 4-CH$_2$-C<u>H</u>$_2$-CH$_3$), 1.29 (t, *J* = 7.5 Hz, 3H, 4-CH$_2$-

CH_2-C<u>H</u>$_3$ or 6-CH_2-C<u>H</u>$_3$),1.00 (t, *J* = 7.6 Hz, 3H, 4-CH_2-CH_2-C<u>H</u>$_3$ or 6-CH_2-C<u>H</u>$_3$). HRMS (ESI-TOF) for $C_{17}H_{19}O_3$ $[M + H]^+$: calcd.: 271.1334, found: 271.1390. Anal. calcd. for $C_{17}H_{18}O_3$: C, 75.53; H 6.71; found: C, 75.37; H, 6.45.

6,9-Dimethyl-4-isopropyl-2H-furo[2,3-h]-1-benzopyran-2-one (22). From **15**: yield 35%: mp 124 °C. ^{1}H-NMR ($CDCl_3$): 7.42 (q, *J* = 1.2 Hz, 1H, 8-H), 7.33 (s, 1H, 5-H), 6.28 (s, 1H, 3-H), 3.36 (sept, *J* = 6.9 Hz, 1H, -C<u>H</u>-$(CH_3)_2$), 2.54 (s, 3H, 6-Me), 2.53 (d, *J* = 1.2 Hz, 3H, 9-Me), 1.35 (d, *J* = 6.9 Hz, 6H, -CH-(C<u>H</u>$_3)_2$). HRMS (ESI-TOF) for $C_{16}H_{17}O_3$ $[M + H]^+$: calcd.: 257.1178, found: 257.1214. Anal. calcd. for $C_{16}H_{16}O_3$: C, 74.98; H 6.29; found: C, 74.72; H, 6.10.

6-Ethyl-4-isopropyl-9-methyl-2H-furo[2,3-h]-1-benzopyran-2-one (23). From **16**: yield 35%: mp 101 °C. ^{1}H-NMR (Acetone-*d6*): 7.64 (s, 1H, 8-H), 7.52 (s, 1H, 5-H), 6.18 (s, 1H, 3-H), 3.46 (sept, *J* = 6.4 Hz, 1H, 4-C<u>H</u>-$(CH_3)_2$), 2.91 (q, *J* = 7.3 Hz, 2H, 6-C<u>H</u>$_2$-CH_3), 2.44 (s, 3H, 9-Me), 1.37-1.29 (m, 9H, 6-CH_2-C<u>H</u>$_3$ e -CH-(C<u>H</u>$_3)_2$). ^{13}C-NMR (Acetone-*d6*): δ 164.80 (CO), 162.00 (C4), 157.75 (C6a), 149.89 (C9b), 144.17 (C8), 126.21 (C6), 120.70 (C5), 118.58 (C9a or C9), 117.79 (C9a or C9), 114.90 (C4a), 109.98 (C3), 30.60 ($(CH_3)_2$<u>C</u>), 24.21 (CH_3<u>C</u>H_2), 23.15 ((<u>C</u>$H_3)_2$C), 15.54 (<u>C</u>H_3CH_2), 10.71 (9-Me). HRMS (ESI-TOF) for $C_{17}H_{19}O_3$ $[M + H]^+$: calcd.: 271.1334, found: 271.1424. Anal. calcd. for $C_{17}H_{18}O_3$: C, 75.53; H 6.71; found: C, 75.34; H, 6.56.

Biology

EMSA (Electrophoretic Mobility Shift Assays). Electrophoretic mobility shift assays were performed using double stranded ^{32}P-labeled oligonucleotides as target DNA [23]. Binding reactions were set up in binding buffer (10% glycerol, 0.05% NP-40, 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10 mM $MgCl_2$), in the presence of 0.1 µl/20 µl of NF-κB-p50 (50 gsu) (PROMEGA, Madison, WI, USA) and 0.25 ng of labeled oligonucleotide, in a total volume of 20 µl. After 30 min binding at room temperature samples were electrophoresed at constant voltage (200 V for 30 min) through a low ionic strength (0.25 TBE buffer) (1 TBE/40.089 M Tris–borate, 0.002 M EDTA) on 6% polyacrylamide gels until the tracking dye (bromophenol blue) reached the bottom of a 16 cm slab. Gels were dried and exposed for autoradiography with intensifying screens at 80°C. Images were scanned and analyzed with the Image Lab ChemiDoc (Biorad). In these experiments, DNA/protein complexes migrate through the gel with slower efficiency. In studies on the inhibitors of protein/DNA interactions, addition of the reagents was as follows: (i) nuclear factors; (ii) active principles; (iii) binding buffer; (iv) labeled oligonucleotides mimicking the binding sites for TF to be modulated. The nucleotide sequence of double-stranded target DNA utilized in these experiments was 5'-CGC TGG GGA CTT TCC ACG G-3' (sense strand, NF-κB)

[50]. The synthetic oligonucleotides utilized in this study were purchased from Sigma Genosys (Sigma Genosys, Cambs, UK).

Cell cultures and cell proliferation assay. IB3-1 cells [30], derived from a CF patient with a DF508/W1282X mutant genotype and immortalized with adeno12/SV40, were grown in LHC-8 supplemented with 5% FBS in the absence of gentamycin, at 37°C/5% CO_2 [23]. The effects of compounds (TMA derivatives) on the IB3-1 cell proliferation were analyzed as elsewhere described (RS1). In order to determine IC_{50} values the following concentrations were used 12.5, 25, 50 µM, 100, 200 and 800 µM. Cells were counted with a Z2 Coulter Counter (Coulter Electronics, Hialeah, FL, USA). The cell number/ml was determined as IC_{50} after 2 days of culture, when untreated cells are in log phase of cell growth. For induction of pro-inflammatory genes, monolayers of 60% confluent IB3-1 cells were seeded in 6- or 12-well plates in LHC-8 medium and after 24 hours compound **23** was added, 5 hours before stimulation with 80 ng/ml TNF-α (ORF Genetics, Kopavogur, Iceland), and incubated for a further 24 hours.

Bioplex analysis. Cytokines, released from cells into tissue culture supernatants, were measured by Bioplex cytokine assay (Bio-Rad Laboratories, Hercules, CA) as suggested by the manufacturer (Luminex technology) [39]. The Bioplex 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 multiplex beads (27-plex) of the Bioplex human cytokines 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 washed by vacuum filtration three times with 100 µl of Bioplex 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 were 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 Bioplex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bioplex Suspension Array System and Bioplex Manager software (Bio-Rad Laboratories, Hercules, CA).

Interaction with salmon testes DNA

Linear flow dichroism. Linear flow dichroism spectra were measured as described in Supplementary Data.

Irradiation procedure. UVA irradiation was carried out by using Philips HPW 125 lamps, mainly emitting at 365 nm. Blue The total energy hitting the sample was monitored by means of a

radiometer (Variocontrol, Waldmann, Villingen-Schwenningen, Germany), equipped with a Variocontrol UV Sensor (Waldmann). The radiant power emitted by the UVA lamp was about 8 mW/cm^2. The samples were maintained at room temperature during irradiation.

Isolation of photoadducts. Ethanol concentrated solutions of the compounds (2 mg/mL) were added dropwise to *salmon testes* DNA (Sigma-Aldrich) in 10 mM NaCl and 1 mM EDTA solution (1.5×10^{-3} M) to achieve a DNA/compound ratio of about 40.The mixture was irradiated with 15 J/cm^2 UVA light in a glass dish. After irradiation, the DNA was precipitated with 1 M NaCl and 2 volumes of cold ethanol; the precipitated DNA was collected, washed with 80% ethanol, dried and then dissolved in water. The final solutions were hydrolyzed with 0.5 M HCl, heated at 100°C for 1 h, neutralized with NaOH and extracted three times with chloroform. Then, the organic layers were collected, dried under high vacuum and dissolved in ethanol. The hydrolyzed mixtures were separated by thin-layer chromatography (TLC; F_{254} plates, 0.25 mm, Merck, Darmstadt, Germany) eluting with ethyl acetate/ethanol (9:1). Bands of interest, visualized with a Mineral Light Lamp VL-4LC VILBERT-LOURMAT, were scraped off and the expected photoproducts extracted with chloroform and ethanol and then analyzed by NMR. ^{1}H-NMR were performed in deuterated acetone, deuterated DMSO or $CDCl_3$ using a Bruker AMX300 spectrometer (300 MHz).

pBR322 DNA strand breaks. Each pBR322 DNA sample (150 ng) (Life Technologies, Monza, Italy) dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was irradiated with increasing BL and UVA radiation exposures (0, 2.5, 5.0, 7.5, J/cm^2) in the presence of 300 μM of the compounds. The samples were loaded on 1% agarose gel, after addition of 1 μL of gel loading buffer (0.25% Bromophenol blue, BPB; 0.25% xylene cyanol; and 40%, w/v, sucrose) to each of them. The electrophoretic run was carried out in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 25 V for 6 h, using the GNA-100 electrophoretic apparatus (Pharmacia, Uppsala, Sweden). After staining with ethidium bromide (1 μg/mL in TAE buffer) for 20 min, the DNA bands were visualized with a UV transilluminator and detected with Bio-Rad GelDoc EZ (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Samples of DNA alone in the dark and after irradiation were used as controls.

Mutagenicity assay (Ames test). Mutagenicity assay was performed for TMA and compound **23**, following the plate incorporation method with the histidine-requiring *Salmonella typhimurium* mutant TA97A, TA98, TA100 and TA1535 strains purchased by Molecular Toxicology Inc. (Boone, NC, USA; moltox.com) [32,33]. All strains (100 μl per plate of fresh overnight cultures) were checked with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic activator (S9 mix). The lyophilized post-mitochondrial supernatant S9 mix (Aroclor 1254-induced, Sprague–Dawley male rat liver in 0.154 M KCl solution), commonly used for the activation of pro-mutagens

to mutagenic metabolites (Molecular Toxicology, Inc., Boone, NC, USA) was stored at - 80 °C before use. The concentration tested for all the samples were 1, 5, 10, 50, 100 µg/plate (see following table for molar concentrations). A fully-grown culture of the appropriate tester strain (0.1 ml) was added to 2 ml molten top agar (0.6% agar, 0.5% NaCl, 0.5 mM L-histidine/biotin solution) at 46 °C, together with 0.1 ml of each sample solution at different concentrations, and 0.5 ml S9 mix for assays with metabolic activation. The ingredients were thoroughly mixed and poured onto minimal glucose agar plates (1.5% agar in 2% Vogel–Bonner medium E with 5% glucose solution). DMSO was used as a negative control (100 µl/plate). Positive controls were prepared as follows: 2-aminoanthracene (2 µg/plate) and 2-nitrofluorene (2 µg/plate) for TA 97A, TA98 and TA1535 with and without metabolic activator (S9 mix) respectively; 2-aminoanthracene (2 µg/plate) and sodium azide (2 µg/plate) for TA100, with and without metabolic activator (S9 mix) respectively. The plates were incubated at 37 °C for 72 h and then the his+ revertants were checked and counted using a Colony Counter 560 Suntex (Antibioticos, Italy). A sample was considered mutagenic when the observed number of colonies was at least twofold over the spontaneous level of revertants [32,33]. All determinations were made in triplicate. Relative standard deviations were computed using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

Author contributions

A.C., G.Marzaro and C.V. synthesized the compounds. G.Miolo performed the photoactivity measurements. I.L., E.D., G.S., G.C. and MC. D. performed the other biological assays. A.C. and R.G. conceived the idea and wrote the main manuscript text. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Data

Detailed analytical data for compound **23**; HRMS for compounds **17-22**; EMSA experiment on TMA; LD measurement; mutagenicity of compound **23** on *Salmonella typhimrium* TA97A and 1535 strains.

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Highlights

- TMA (trimethylangelicin) is a promising agent for treatment of inflammation-related diseases, unfortunately showing unwanted side effects (photoreactivity and mutagenicity).
- New generation TMA analogues with hindered substituent at the 4 position were designed and synthesized.
- All the title compounds inhibited NF-κB/DNA interactions.
- A lead compound was selected for the good inhibitory activity on NF-κB/DNA interactions, for its inhibitory effects on some NF-κB regulated genes and for the absence of TMA side effects.