Physalins from *Witheringia solanacea* as Modulators of the NF-κB Cascade[⊥]

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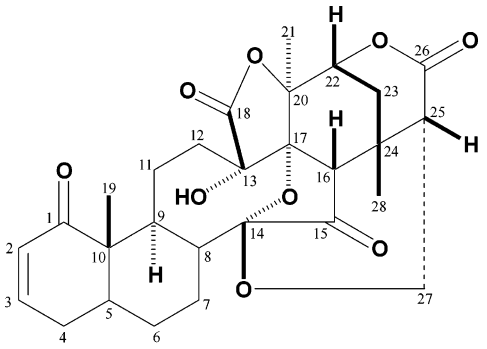
Crude extracts of *Witheringia solanacea* leaves showed inhibition of NF-κB activation at 100 μg/mL induced by phorbol 12-myristate-13-acetate (PMA) in HeLa cells stably transfected with a luciferase reporter gene controlled by the IL-6 promoter. Three physalins were isolated from an active fraction, namely, physalins B (**1**), F (**2**), and D (**3**). Of these compounds, **1** and **2** demonstrated inhibitory activities on PMA-induced NF-κB activation at 16 and 8 μM and induced apoptosis after 24 h in a cell-cycle analysis using a human T cell leukemia Jurkat cell line. Compound **2** also inhibited TNF-α-induced NF-κB activation at 5 μM through the canonical pathway, but was inactive in the Tet-On-Luc assay, indicating specificity of action, although it interfered with Tet-On-Luc at higher concentrations. It is suggested that the presence of a double bond and an epoxy ring between carbons 5 and 6 in compounds **1** and **2**, respectively (which are not present in compound **3**), are related to their anti-inflammatory activity.

The inducible transcription factor nuclear factor-kappaB (NF-κB) is one of the key regulators of genes involved in the immune/inflammatory response and in survival from apoptosis.[1,2] NF-κB is made up of homo- and heterodimers that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterized. In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. Stimulation with a variety of physiological or pathogenic stimuli leads to phosphorylation, ubiquitination, and the subsequent degradation of IκBα proteins, resulting in the translocation of NF-κB from the cytoplasm to the nucleus. IκB kinase (IKKs)-dependent phosphorylation of IκBα is a key step involved in the activation of NF-κB complexes.[3] NF-κB is implicated in the transcriptional regulation of several cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effector enzymes that participate in inflammatory diseases such as rheumatoid arthritis, asthma, and inflammatory bowel disease.[3] Moreover, there is evidence that NF-κB participates in the transcription of anti-apoptotic genes, cell regulation, cell invasion, and metastatic growth, suggesting it could be a potential target for cancer therapy.[2,4]

With around 3000 species, the cosmopolitan Solanaceae is one of the most economically important plant families due its wide variety of uses, for example as ornamentals, foods, medicines, and narcotics.[5] Few studies, however, have been performed with members of this family targeting NF-κB.[6] *Witheringia solanacea* L'Her. is a small shrub distributed widely from southern Mexico through Central America and the Antilles to South America.[7] In Panama, this species is known by the Kuna Indians as "Tinanguak'guid" and in Gnöbe-Buglé (formerly Guaymi) as "Diguima goi". The plant is used for body aches and skin diseases and as an antihelmintic.[8] In Mexico, *W. solanacea* is called "merengena" or "hierba cimarrona" in Spanish and "cuauhta tomatquilit" in Náhuatl and is used in the treatment of anemia, fungal infections, and acne.[9,10] While the phytochemistry of this species is unknown, the related species *W. coccoloboides* has been studied by Antoun et al., from which seven physalins were isolated.[11]

In the present study, the anti-inflammatory activity of *W. solanacea* leaves was evaluated by investigation of inhbition of the NF-κB cascade. Crude extracts and fractions at different concentrations showed significant inhibitory activities of NF-κB based upon the IL-6-dependent control of the luciferase gene. Of three compounds isolated, physalins **1** and **2** showed differential inhibitory activity of the NF-κB pathway. While compound **2** inhibited both PMA- and TNFα-induced NF-κB activation, compound **1** targeted only the NF-κB canonical pathway. Moreover, these two physalins were found to be inducers of apoptosis in the Jurkat leukemia cell line after 24 h of exposure.



1 $\Delta^{5,6}$
2 5β,6β-epoxy
3 5α-OH, 6β-OH

Results and Discussion

Crude extracts obtained from Soxhlet extraction of *W. solanacea* leaves were tested as inhibitors of NF-κB at 100 μg/mL. Hexane, EtOAc, and acetone extracts showed inhibitory activities of 27%, 21%, and 13.5%, respectively, of the positive control (mean data from three experiments), and no cytotoxicity was observed (Figure S1A, Supporting Information). Although the $CHCl_3$ extract was cytotoxic at 100 μg/mL, we focused on this extract because of the

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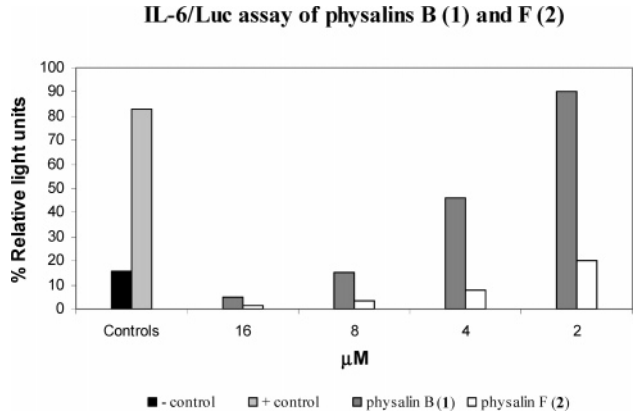

Figure 1. Inhibitory effect on NF-κB activation by compounds **1** and **2** from *Witheringia solanacea*. NF-κB was induced by PMA in HeLa cells stably transfected with a luciferase reporter gene controlled by the IL-6 promoter. Treatment values are given as a percentage of relative light units compared to the stimulated control (positive) (mean data of three experiments). Positive controls consisted of stimulated cells with PMA, whereas the negative control had resting cells without stimulation.

high yield obtained and its chemical complexity. When fractions obtained from the $CHCl_3$ extract by VLC were tested at different concentrations, fractions 7 and 8 were active at 50, 25, 12, and 6 μg/mL (Figure S1A, Supporting Information) and no physiological change was observed in cells after 7 h of exposure. By this fractionation step of the crude extract, it was partially possible to separate the cytotoxicity observed in the $CHCl_3$ extract from NF-κB inhibitory activity. The three compounds isolated from the active fraction 7 were identified as physalins B (**1**), F (**2**), and D (**3**), respectively. The three compounds were tested at 100 μg/mL (equivalent to 195.8 μM for **1**, 189.9 μM for **2**, and 183.7 μM for **3**), but at this concentration **1** and **2** were highly cytotoxic (data not shown). Compound **3** did not exhibit any inhibitory effect or cytotoxicity. Consequently, a MTT assay was carried out in order to establish the minimum micromolar concentration at which these compounds were not cytotoxic for HeLa cells after 72 h of exposure. It was found that their IC_{50} values were both approximately 2 μM (Figure S1B, Supporting Information). Compounds **1** and **2** were again tested as inhibitors of NF-κB using the IL-6/Luc assay at different micromolar concentrations obtained from the MTT assay. Both compounds were active at 16 and 8 μM, and no cytotoxicity was observed after 7 h of exposure of the HeLa cells in the IL-6/Luc assay (Figure 1). Compound **2** was also active at 4 and 2 μM (7% and 20% of the positive control, Figure 1), showing that **2** is active at lower concentrations than **1**. To see if there was any synergistic or additive effect, or improvement in the inhibitory activity among the three compounds, another assay was carried out using different combinations of the three physalins at 4 μM (noncytotoxic concentration), with no cytotoxic effects on HeLa cells after 7 h of exposure. The inhibitory effect was observed in samples where compound **2** was present, with the most potent combination being the one containing compounds **1** and **2** (1:1), which was 4% of the positive control (Figure S1C, Supporting Information).

Next, the effects of compounds **1** and **2** were investigated on TNFα-induced NF-κB activation using the 5.1 cell line. As depicted in Figure 2A, **2** but not **1** significantly inhibited the luciferase expression driven by the HIV-1-LTR promoter in TNFα-stimulated cells in a concentration-dependent manner. The inhibitory effect of **2** was not due to an interference with the transcriptional machinery or with the in vitro activity of the luciferase enzyme at lower concentrations (5 μM; Figure 2B).

Finally, cell-cycle analysis was carried out for physalins **1** and **2** in order to see if these two compounds are inducers of apoptosis.

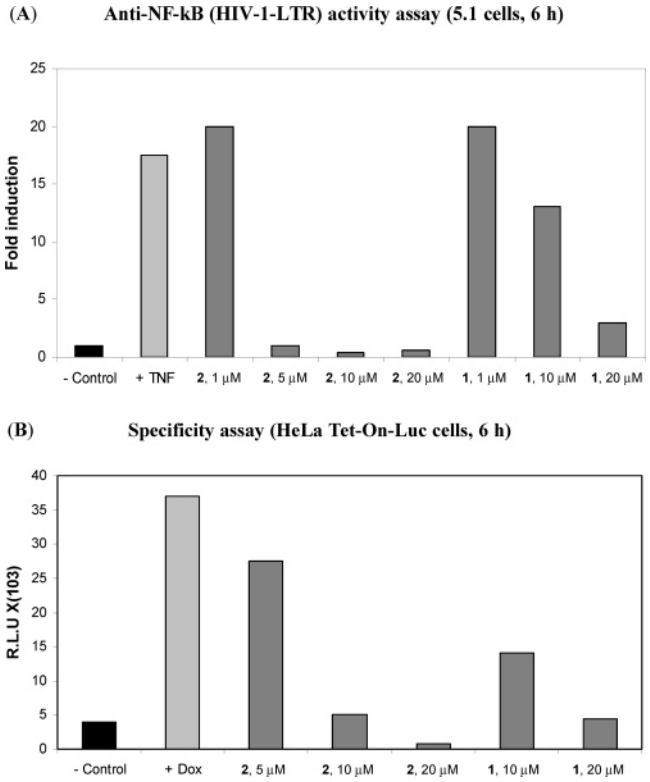

Figure 2. (A) Inhibition of NF-κB activation assay in 5.1 cell line preincubated with physalins B (**1**) and F (**2**) (5, 10, and 20 μM) for 30 min and then stimulated with TNFα (2 ng/mL) for 6 h. The specific transactivation was calculated as RLU/μg protein (relative light units) and expressed as a fold induction (mean of three experiments). (B) HeLa TET-ON-luc assay stimulated with doxycycline (2 μg/mL) in the presence or absence of the compounds for 6 h.

Jurkat cells were incubated with different concentrations of the compounds for 6 or 24 h and stained with propidium iodine, and the DNA content was analyzed by flow cytometry. This method determines the nuclear DNA loss as a marker of apoptosis. Neither compound **2** nor **1** was able to induce apoptosis after 6 h of treatment (Figure S2A, Supporting Information), further supporting the concept that the luciferase inhibitory activities of these physalins are not due to nonspecific cytotoxicity. In contrast, longer exposure of Jurkat cells to compound **2** resulted in a marked induction of apoptosis in a concentration-dependent manner (Figure S2B, Supporting Information).

So far, there have been no reports on actions against NF-κB activation, but physalins have been evaluated previously for cytotoxicity and antitumor activities, as well as for antimycobacterial properties.[11−14,16−18] Regarding the structure−activity relationship, in a cytotoxic activity study of physalins against HeLa cells, Kawai et al.[16] reported physalins B (**1**), C, and F (**2**) among the most active physalins. Reportedly, a conjugated 2-en-1-one moiety in ring A is essential for high activity, whereas the 2,3-saturated derivatives and the isomeric 3-en-1-one compounds are lower in activity. Chiang et al.[12] found that physalin F (**2**) showed cytotoxicity in vitro using DEA and MTT assays on eight cancer cell lines and three mammalian cancer lines, whereas physalin D was inactive. They attributed the activity of physalin F to the functional epoxy group located at carbons 5 and 6, and in the case of inactive physalin D this epoxide was replaced by OH-5α and OH-6β groups. Additionally, Chiang et al.[13] evaluated the effect of physalins B (**1**) and F (**2**) against various human leukemia cells in vitro, in which physalin F was more active. Further evidence reported for the related withanolides[19] indicated that the most potent compounds with cytostatic activity are those with a C-2, C-3 double bond in the A

ring, as well as the presence of a carbonyl and a hydroxyl at position C-1 and C-4, respectively. The presence of an epoxide ring at C-5 or C-6 or a halogen atom in the second ring (B) is a less important prerequisite. Jayaprakasam et al.[20] have also reported that in withaferin A an α,β-unsaturated ketone functionality at C-2, C-3 in ring A and the C-27 hydroxyl contributed significantly to its antiproliferative activity against lung, breast, colon, and CNS human tumor cell lines. In a recent study, it was reported that physalins B, F, and G have immunomodulatory activity in macrophages and in lipopolysaccharide-induced shock.[21] The authors suggested that the inhibitory effect of physalins B, F, and G could be associated with the suppression of other cytokines such as IL-6 and IL-12, but no structure−activity relationships were discussed.

In this study, we were able to establish that the inhibitory activity of physalins B (**1**) and F (**2**) against NF-κB, under stimulation with PMA and TNF-α, is related to the double bond and epoxy ring at positions C-5 and C-6, respectively, which has been linked with antitumor activity previously.[12,13] The conjugated 2-en-1-one moiety in ring A apparently is less important in anti-inflammatory activity in our models. This structure−activity relationship was observed when the three physalins (**1**−**3**) were tested at different combinations to see if the inhibitory activity would improve upon the addition of a second compound. As seen in Figure 1A, compound **2** is presumably the most active compound in the series of the three isolated compounds, and our results in the HIV-1-LTR-Luc assay suggested that the inhibitory activity of **2** is through the NF-κB canonical pathway, which is triggered by TNFα, among other inducers.[2]

The present study provides molecular evidence that the anti-inflammatory activity of *W. solanacea* and its physalin constituents (**1**−**3**) is likely to be mediated via the NF-κB pathway. Bioassay-guided fractionation resulted in the identification of physalins F (**2**) and B (**1**) as the major bioactive compounds mediating NF-κB inhibition and apoptosis.

Experimental Section

General Experimental Procedures. Chromatographic separations were performed using solid-phase extraction (SPE) (Strata SI-1 silica (55 μm, 70A), 10 g /60 mL Giga tubes, normal-phase cartridges) and by vacuum-liquid chromatography (VLC) (Kieselgel 60 PF 254 + 366 silica gel for preparative layer chromatography, Merck; 60 mm × 120 mm glass columns). Preparative TLC (Merck Si gel 60 with F_{254} indicator on aluminum sheets, 20 × 20 cm; 4% vanillin in H_2SO_4 and heating) was used to isolate compounds. Mass spectra were recorded on a VG ZAB-SE instrument (FAB and EISMS) and Finnigan navigator (EISMS); IR spectra on a Nicolet 360 FT-IR spectrophotometer. Positive and negative EISMS were performed on pure compounds. NMR experiments (one-dimensional and two-dimensional) were performed on a Bruker AVANCE 500 MHz spectrometer.

Plant Material. Leaves of *Witheringia solanacea* L'Her. (Solanaceae) were collected from Cerro Trinidad (08°41′ N and 079°55′ W), Capira, Panama, and were identified by C. Galdames and S. Knapp at the Centro de Investigaciones Farmacognósticas de la Flora Panameña (CIFLORPAN), Panama. A voucher specimen (No. 1149, PMA) is deposited at the herbarium of the Centre for Pharmacognosy and Phytotherapy, School of Pharmacy, University of London.

Extraction and Isolation. Powdered leaves (500 g) were exhaustively extracted by Soxhlet with solvents of increasing polarity: hexane, chloroform, ethyl acetate, acetone, and methanol. Chlorophyll was removed from the $CHCl_3$ extract using activated carbon. The resulting mix was vacuum filtered through a bed of Celite, where the activated carbon plus chlorophyll was retained. The residue was dried under vacuum and fractionated by VLC. The compounds were identified by one-dimensional (^1H, ^{13}C, DEPT-135) and two-dimensional (HMQC, HMBC, COSY, and NOESY) NMR experiments and by comparison of the physical data with those previously reported in the literature (Tables S1 and S2, Supporting Information).[11,13−15,22] Compound **1** (9 mg): the EISMS showed a molecular ion at *m*/*z* 511.2 [+H], which was consistent with the molecular formula of physalin B, $C_{28}H_{30}O_9$ (MW 510.532). Compound **2** (6 mg): the molecular formula obtained by EISMS showed an intense ion peak at *m*/*z* 527 [+H], consistent with the molecular formula of physalin F, $C_{28}H_{30}O_{10}$ (MW = 526.531). Compound **3** (4 mg): the EISMS showed an intense ion peak at *m*/*z* 561 [$-H_2O$][−H], which corresponded to the molecular formula of physalin D, $C_{28}H_{32}O_{11}$ (MW = 544.547).[11,14,15,22]

IL-6/Luciferase Assay. To test for the activity of fractions and compounds as inhibitors of NF-κB, a luciferase assay was employed using HeLa cells. The cells were stably transfected with a luciferase reporter gene controlled by the IL-6 promoter. The stimulant phorbol 12-myristate-13-acetate (PMA) was added to a final concentration of 50 ng/mL, and the plates were incubated for 7 h. To be considered active, the luciferase value must be at least 30% of the positive control. Readings were taken with an Anthos Lucy 1 luminometer/photometer. Toxicity was evaluated visually under magnification (40×) by morphological changes in the cells. The methodology followed in this work for the IL-6/luciferase assay is described in detail elsewhere.[23]

HIV-1-LTR Luciferase Assay. To determine the effects of physalins B (**1**) and F (**2**) on TNFα-induced NF-κB-dependent specific transcription, the 5.1 cell line was used. This cell line is a Jurkat-derived clone stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-1-LTR promoter. Due to this promoter being highly dependent on NF-κB activation induced by TNFα, high expression of luciferase activity reflects NF-κB activation through the canonical pathway. The cells were preincubated with the different compounds for 30 min and then stimulated with TNFα (2 ng/mL) for 6 h. Then, the cells were lysed in 25 mM Tris phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9510 following the instructions of the luciferase assay kit (Promega), and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted from each experimental value, and the specific transactivation was calculated as RLU/μg protein (relative light units) and expressed as a fold induction.[24]

HeLa Tet-On-Luc Assay. The construction and culture conditions of the HeLa-Tet-On-Luc cell line were previously described.[25] The cells (5×10^5 cells/mL) were seeded the day before the assay and then stimulated with doxycycline (2 μg/mL) in the presence or absence of the compounds for 6 h. The cells were then washed twice in PBS and lysed, and the luciferase activity was measured as described before in the TNF-α luciferase assay.

Determination of Nuclear DNA Loss (Apoptosis) and Cell Cycle Analysis. The human T cell leukemia Jurkat was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL). The cells (10^6) were treated with different concentrations of the compounds for 6 or 24 h. The percentage of cells undergoing apoptosis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4 °C). Cells were then washed twice with PBS containing 4% glucose and subjected to RNA digestion (Rnase-A, 50 U/mL) and PI (20 μg/mL) staining in PBS for 1 h at room temperature, then analyzed by cytofluorimetry. With this method, low molecular weight DNA (fragmented DNA) leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows the determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).[24]

Cytotoxicity Assay (MTT). A standard MTT (tetrazolium salt, 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide, Sigma) cytotoxicity assay was employed as described previously.[26] Cells were exposed for 2 h to 0.5 mg/mL MTT solution (200 μL) at 37 °C and solubilized in 90% 2-propanol with DMSO. Readings were conducted after 72 h using the photometric mode of the Anthos Lucy 1 luminometer/photometer at 570 nm.

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Supporting Information Available: Inhibitory effect on NF-κB activation of crude extracts, fractions, and different combinations of

the three physalins isolated and flow cytometry graphs. Tables containing the ^{1}H and ^{13}C NMR data for physalins B (**1**), F (**2**), and D (**3**). This information is available free of charge via the Internet at http://pubs.acs.org.

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