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(+)-Altholactone exhibits broad spectrum immune modulating activity by inhibiting the activation of pro-inflammatory cytokines in RAW 264.7 cell lines

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GCS-F
IL-6
IFN-β

ABSTRACT

An evaluation of Indonesian plants to identify compounds with immune modulating activity revealed that the methanolic extract of an *Alphonsea javanica Scheff* specimen possessed selective anti-inflammatory activity in a nuclear factor-kappa B (NF-κB) luciferase and MTT assay using transfected macrophage immune (Raw264.7) cells. A high-throughput LC/MS-ELSD based library approach of the extract in combination with the NF-κB and MTT assays revealed the styryl lactone (+)-altholactone (**2**) was responsible for the activity. Compound **2**, its acetylated derivate (+)-3-*O*-acetylaltholactone (**3**), and the major compound of this class, (+)-goniothalmin (**1**), were further evaluated to determine their anti-inflammatory potential in the NF-κB assay. Concentration–response studies of **1**–**3** indicated that only **2** possessed NF-κB based anti-inflammatory activity. Compound **2** reduced the LPS-induced NO production, phosphorylation of IκBα, and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) using Western blot analysis. Further studies using qPCR indicated **2** reduced the expression of eight pro-inflammatory cytokines/enzymes (0.8–5.0 μM) which included: COX-2, iNOS, IP-10, IL-1β, MCP-1, GCS-F, IL-6 and IFN-β. These results indicated that **2** displays broad spectrum immune modulating activity by functioning as an anti-inflammatory agent against LPS-induced NF-κB signaling. Conversely the selective cytotoxicity and in vivo anti-tumor and anti-inflammatory activity previously reported for **1** do not appear to arise from a mechanism that is linked to the NF-κB immune mediated pathway.

1. Introduction

Styryl lactones are an important group of plant derived natural products from the family Annonaceae that exhibit a wide variety of biological activities. Selected examples, particularly from the genus *Goniothalamus*, include selectivity to cancer cell lines and against neglected tropical diseases (Chagas), as well as antifungal, antimicrobial, anti-inflammatory and immunosuppressive activity.[1–4] This has motivated a number of research groups to engage in the total synthesis of this class of compounds.[5] The majority of the research involving styryl lactones has revolved around the structure of (+)-goniothalmin (**1**) with over 100 derivatives reported in the primary literature isolated as natural products or derived from synthetic or semi-synthetic experiments.[1–6] Compound **1** has been reported to induce selective cytotoxicity to a variety of cancer cell lines,[1,4,6,7] and has been repeatedly isolated from the genus *Goniothalamus* which has a long history of use in folk medicine throughout South East Asia.[2,8] The extracts and leaves of Annonaceae plants have also traditionally been used for the treatment of rheumatism and edema.[5] Recent work has shown that racemic **1**

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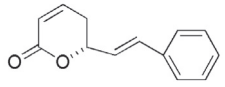
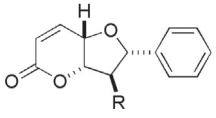
demonstrates encouraging antiproliferative activity in vivo using the Ehrlich solid tumor experimental model in mice with no reported toxicity after single or repeated doses.[9] A mechanism for the observed antiproliferative activity may be due to its ability to serve as an inhibitor of nucleo-cytoplasmic transport via blocking of CRM-1 mediated nuclear export.[4] Anti-edematogenic activity has also been observed using racemic **1** in a carrageenan edema model in vivo, which suggested a relationship between the anti-tumor and anti-inflammatory activities with the anti-inflammatory activity favoring the antiproliferative activity.[9] These results indicate further in vivo evaluations of **1** are warranted as a preclinical lead for the treatment of cancer and that its apparent anti-inflammatory activity could also be responsible for its anti-tumor activity. The immune modulating effects of **1** have been linked to its ability to inhibit the expression of both intercellular adhesion molecule and vascular adhesion molecule-1 (ICAM and VCAM-1) on the surface of murine endothelial cells (F-2).[10] However, the concentration reported for activity of 70 μM does not suggest it contains potent anti-inflammatory activity.

Several studies have also reported the selective in vitro cytotoxicity of (+)-altholactone (**2**) and its derivatives against a variety of human tumor cell lines.[11–13] Mechanistic research has shown that **2** may function to inhibit human HL-60 or CEM leukemia cells by inducing apoptosis through either oxidative stress, based on its ability to serve as a mitochondria complex I inhibitor[14] or synergism with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[13] Also noteworthy is that acetylated **2**, (+)-3-*O*-acetylaltholactone (**3**), was reported to have five fold greater NADH oxidase inhibitory activity compared to **2**.[15] The above reports suggested that evaluating **1**–**3** for the first time in the nuclear factor-kappa B (NF-κB) mediated immune pathway to examine their anti-inflammatory activities could prove noteworthy because dysregulation of NF-κB has been implicated in many inflammatory diseases including rheumatoid arthritis and cancer.[16–18] Our hypothesis was that an assessment of their immune modulating activity could provide insights as to whether their selective cytotoxicity to cancer cell lines was in part due to their anti-inflammatory activity based on the close links observed between inflammation and tumorigenesis.[19,20] We have recently observed this occurrence from our work with the bengamides, where we discovered their potent anti-inflammatory activity involving NF-κB appears to be responsible at least partially for their significant anti-cancer activity.[21]



(+)-goniothalamin (**1**)



(+)-altholactone R = OH (**2**)
(+)-3-*O*-acetylaltholactone (**3**) R = OAc

During the course of our International Cooperative Biodiversity Group (ICBG) collaboration to screen 114 Indonesian plants for their immune modulating and cytotoxic activity against tumorigenic and nontumorigenic cell lines,[22] we identified an extract from the family Annonaceae (*Alphonsea javanica Scheff*, coll. no. UHA 105) that had noticeable anti-inflammatory activity. Using an LC/MS-ELSD based library approach on the extract in combination with our NF-κB luciferase and MTT cytotoxicity assays to define anti-inflammatory leads from plants,[18] we discovered that (+)-altholactone (**2**) was responsible for the activity. A perusal of the styryl lactone literature indicated that our discovery was the first example to report **2** from specimens of *A. javanica*.[1–4] We then conducted concentration-dependent evaluations of the styryl lactones **1**–**3**, using our NF-κB and MTT assays, to investigate their anti-inflammatory and cytotoxic activities side by side. Our results indicated that only **2** deserved further investigation to: (a) confirm its anti-inflammatory activity by nitrite production and Western blotting analysis, (b) evaluate its activity by qPCR to probe its target(s) in the NF-κB pathway, and (c) examine its selective cytotoxicity against breast and prostate cancer cell lines. The results of our experiments outlined above are reported here in.

2. Results and discussion

To rapidly identify the metabolite(s) responsible for the observed anti-inflammatory activity from the *A. javanica* extract (coll. no. UHA 105 M), we used our previously reported high throughput LC–MS/ELSD based peak-library approach in conjunction with an NF-κB luciferase and MTT assay.[21] This data set is shown in Figure 1 and indicated three fractions (H27, H28 and H31), obtained from the crude extract, displayed potency in the NF-κB luciferase assay. Also noteworthy was that each of these fractions showed weak cytotoxicity to macrophage (RAW 264.7) immune cells in the MTT assay (data not shown) indicating selectivity. Analytical LC-MS/ELSD data indicated these fractions possessed *m*/*z* ions which corresponded to a pure major metabolite H27 (233 *m*/*z*), and what turned out to be an inseparable mixture of metabolites in H28 (233, 282 and 235 *m*/*z*) and H31 (205, 249, 233 and 367 *m*/*z*) as seen in Figures S1–S3 in the Supplementary data. Scale up reverse phase HPLC of the parent methanol extract (100.0 mg) was needed to provide greater quantities of the major metabolite and minor components for further structural and bioassay analysis. Fraction H27 (13.2 mg) contained pure (+)-altholactone (**2**) (syn. goniothalenol)[2,23] and was de-replicated based on the molecular formula of $C_{13}H_{12}O_4Na$ $[M+Na]^+$ established from HRESITOFMS along with ^1H and ^{13}C NMR data (see Figs. S4–S5) that matched previous reports.[23]

Based on the distinct anti-inflammatory activity we observed for the fraction containing (+)-altholactone (**2**), we decided to investigate more thoroughly its effects on key targets in the immune pathway, including NF-κB signaling, nitrite production, and expression of a series of pro-inflammatory cytokines. It also seemed logical to obtain or generate and further evaluate the anti-inflammatory activity of (+)-goniothalamin (**1**) and (+)-3-*O*-acetylaltholactone (**3**), versus **2** due to the wide variety of biological activities observed for **1**[1] and the fact that **3** was reported to possess NADH oxidase inhibitory activity five times greater than **2**.[15] The first step involved determining the IC_{50} inhibitory values observed for **1**–**3** shown in Table 1, on lipopolysacharide (LPS) mediated induction of the NF-κB reporter gene when compared with their cytotoxicity in RAW 264.7 macrophage immune cells in the NF-κB luciferase and MTT assays.[21] Compound **1** displayed moderate NF-κB inhibitory activity (IC_{50} = 27.4 μM) with comparable cytotoxicity in the MTT assay (IC_{50} = 34.2 μM). Alternatively, (+)-altholactone (**2**), exhibited selective NF-κB inhibitory activity (IC_{50} = 6.7 μM) with weak cytotoxicity in the MTT assay (IC_{50} = 43.4 μM). Further analysis indicated compound **2** also inhibited nitrite production (IC_{50} = 0.8 μM). Interestingly, compound **3** exhibited much weaker NF-κB inhibitory activity (IC_{50} = 34.3 μM) versus **2** and had comparable cytotoxicity in the MTT assay (IC_{50} = 36.1 μM). Overall, these data indicated that only **2** possessed potential anti-inflammatory activity in our assays and was on par with other immune modulating plant-derived natural products reported by others.[24]

The next step required using Western blotting analysis, shown in Figure 2, to measure the effects of (+)-altholactone (**2**) in the presence of LPS, on the phosphorylation of IκBα, and the expres-

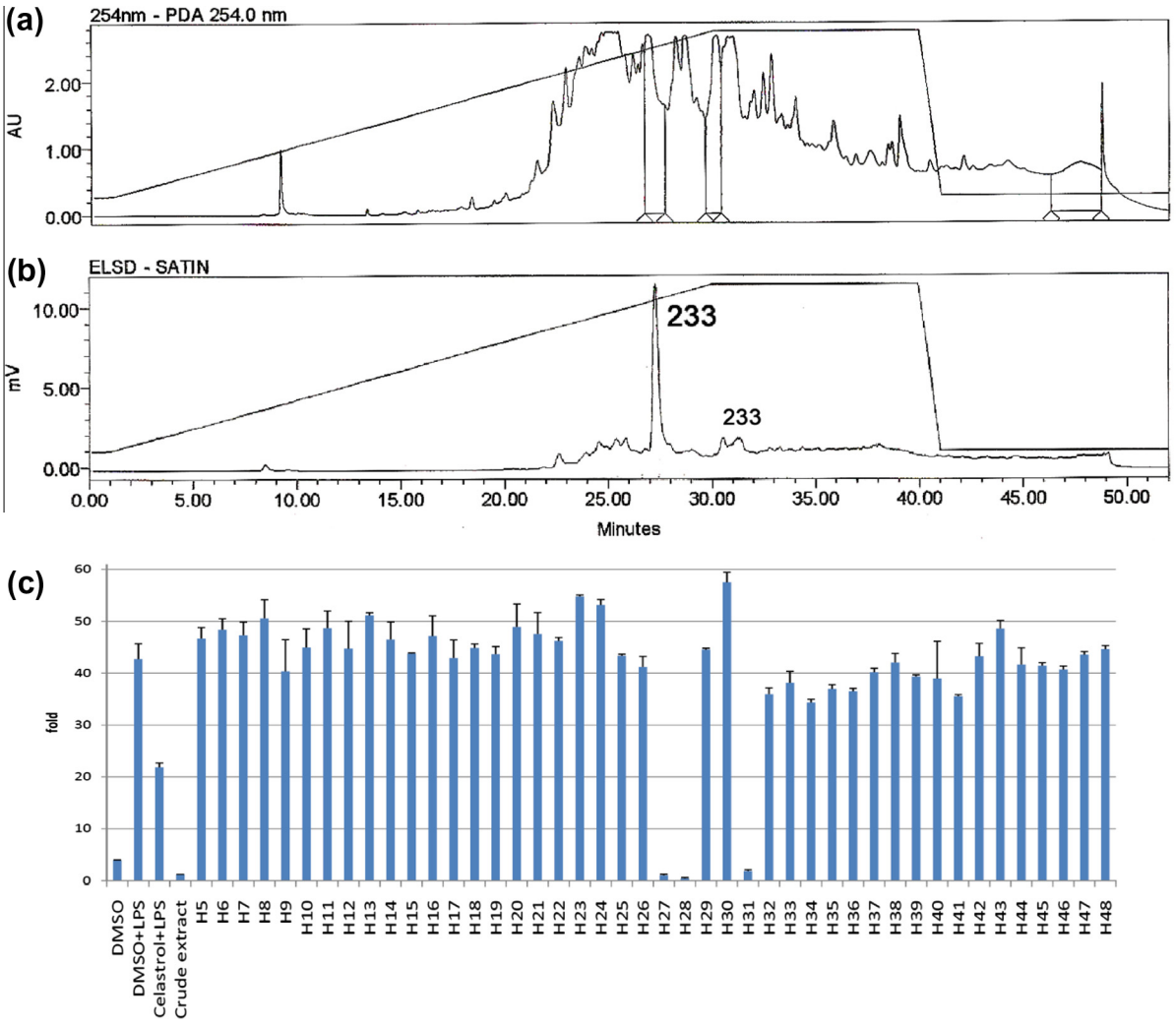


Figure 1. Observed LC–MS/ELSD library trace of coll. no. UHA 105 M including: (a) UV data at 254 nm; (b) ELSD data with selected annotations including *m*/*z* ions and (c) NF-κB luciferase assay data stimulated by the LPS for the standard celastrol (250 nM), the MeOH crude extract (20 μg/mL) and LC library fractions (H5-H48, ~10 μg/mL).

Table 1
Comparative IC_{50} values of compounds **1**–**3**

Compound	Raw 264.7[a]	IC_{50} (μM)	
		NF-kB[b]	NO[c]
1	34.2	27.4	nt
2	43.4	6.7	0.8
3	36.1	34.3	nt

[a] Cytotoxicity measured against murine macrophage (RAW 264.7) cell lines −18 h.
[b] NF-kB inhibition measured using an LPS-induced NF-kB luciferase assay with RAW 264.7 cells −18 h.
[c] Nitric oxide (NO) production. Not tested (nt). The values (IC_{50}) represent the mean ± SE for $n = 3$.

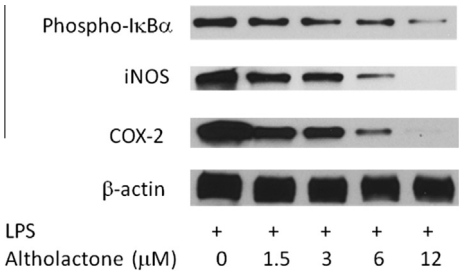


Figure 2. Western blot analysis of (+)-altholactone (**2**) at varying concentrations and its inhibition of LPS-induced phosphorylation of IκBα, and expression of iNOS and COX-2 with β actin as a control.

sion of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) compared to β-actin as a positive control. With increasing concentrations of **2** (1.5–12.0 μM), there is a clear decrease in the observed phosphorylation of IκBα, which has been established as a true indicator of inhibition of the NF-κB mediated pathway.[25] There was also a noticeable decrease in the expression of iNOS and COX-2 at 5.0 μM. This data set indicated that **2** exhibits potential immune modulating activity by inhibiting several key downstream targets in the immune pathway (NF-κB, iNOS, COX-2) and that further investigations of **2** using qPCR analysis on a series of pro-inflammatory cytokines could provide insights into the upstream targets responsible for this observed anti-inflammatory activity.

In an effort to locate the specific immune modulating target(s) of (+)-altholactone (**2**) we examined its inhibition of LPS-induced mRNA expression of eight pro-inflammatory cytokines/enzymes measured using qPCR analysis and these results are shown in Figure 3. The inhibition of **2** on the expression of iNOS and COX-2 shown using Western blotting in Figure 2 was further substantiated by its ability to inhibit the mRNA expression of these pro-inflammatory cytokines as shown in Figure 3a (iNOS,

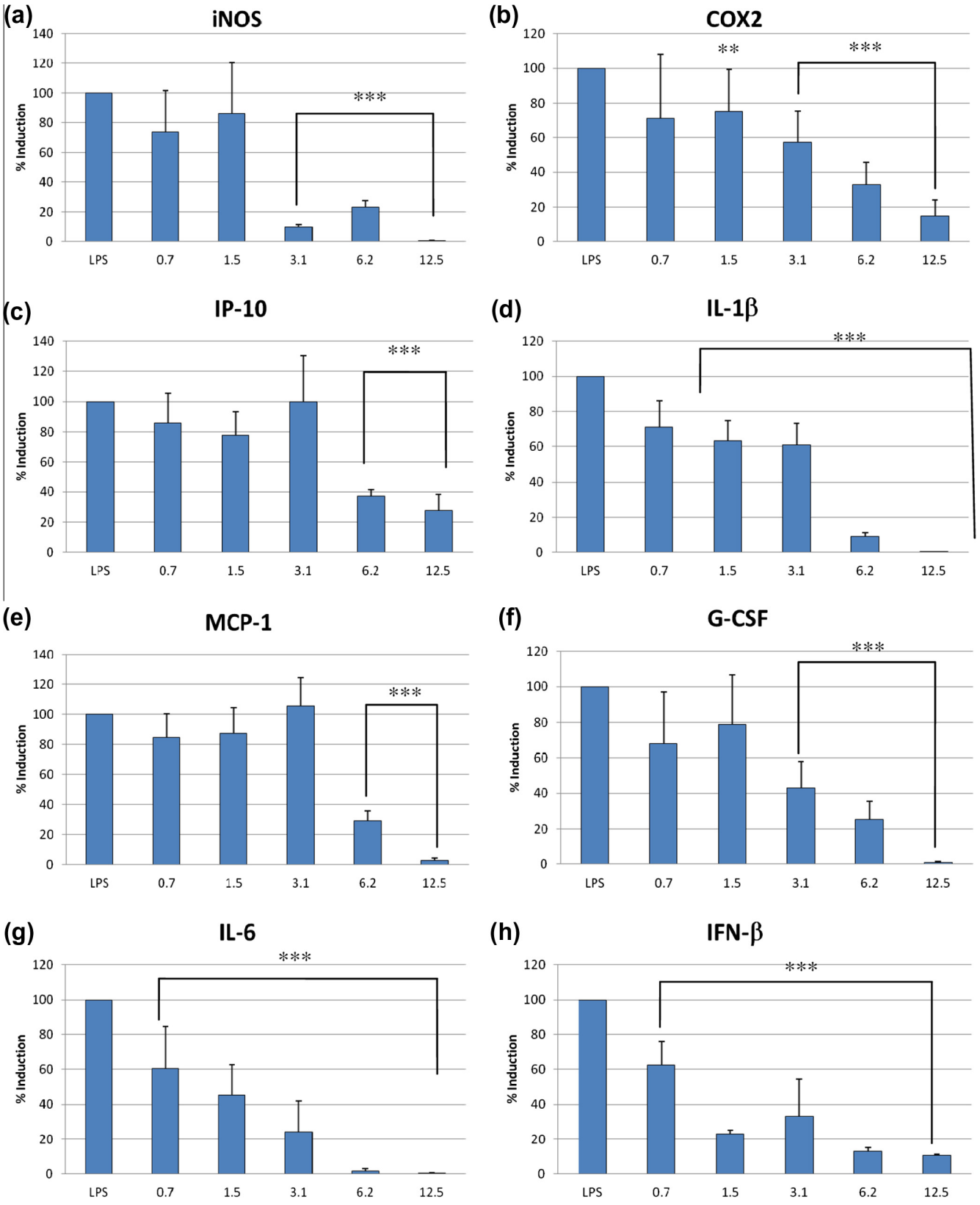


Figure 3. Effects of (+)-altholactone (**2**) on the inhibition of LPS-induced mRNA expression of pro-inflammatory cytokines and enzymes measured by qPCR including: (a) inducible nitrous oxide synthase (iNOS); (b) cyclo-oxygenase-2 (COX-2); (c) interferon gamma-induced protein 10 (IP-10); (d) interleukin-1 beta (IL-1β); (e) monocyte chemotactic protein-1 (MCP-1); (f) granulocyte colony-stimulating factor (G-CSF); (g) interleukin-6 (IL-6) and (h) interferon beta (IFN-β). LPS (100 ng/mL) is used as a positive control and added to (0.7–12.5 μM) samples. Data are presented as mean ± SD of three independent experiments. $^{**}p$ <0.05, $^{***}p$ <0.001.

IC_{50} = 2.0 μM) and Figure 3b (COX-2, IC_{50} = 3.0 μM). Similar results were observed for **2** in Figures 3c–f regarding its inhibition of interferon gamma-induced protein 10 (IP-10, IC_{50} = 5.0 μM), interleukin-1 beta (IL-1β, IC_{50} = 3.0 μM), monocyte chemotactic protein-1 (MCP-1, IC_{50} = 4.0 μM) and granulocyte colony-stimulating factor (G-CSF, IC_{50} = 4.0 μM). The greatest potency was observed against interleukin-6 (IL-6, IC_{50} = 0.9 μM) and interferon beta (IFN-β, IC_{50} = 0.8 μM). Although we were unable to determine a specific target fully responsible for the immune modulating activity, our results indicate **2** functions as a broad spectrum anti-inflammatory agent by inhibiting multiple targets in the NF-κB immune mediated pathway.

Based on the selective anti-inflammatory activity we observed for (+)-altholactone (**2**), and previous reports indicating that it displays cytotoxcity to several tumor cell lines,[1,11–13] we decided to evaluate its cytotoxicity against both non-tumorigenic breast (MCF-10A) and tumorigenic breast (MCF-7, MDA-MB-231) and prostate (PC3, LNCaP) cell lines. Our screening results of **2** against these cell lines are shown in Figure S6 in the Supplementary data. No inhibitory effects were seen at concentrations up to 100 μM against any of the cell lines and these results are consistent with one report that **2** does not display potency against tumorigenic breast cancer cell lines.[13] Alternatively, **2** has shown between six and 10-fold selectivity to human colorectal colon (Sw480), lung carcinoma (COR-L23) and leukemic T-lymphocytes (CEM) cells versus human non-tumorigenic (HT1080, MRC-5, and MCF-12) cell lines.[11–13] Its structural analogs, including **3**, have also reportedly exhibited low micromolar activity (2.0–20.0 μM) against mouse leukemia (P-388, L1210), human lung (A549), colon (HT-29), epidermoid carcinoma (KB) and (COR-L23) cancer cell lines.[1,12]

In summary our results indicate that (+)-altholactone (**2**) exhibits broad spectrum immune modulating activity and functions as an anti-inflammatory agent by inhibiting a wide variety of targets in the NF-κB immune mediated pathway. Compound **2** was not cytotoxic against tumorigenic breast and prostate cancer cell lines in our assays however its selective cytotoxicty against other cell lines[11–13] may arise from its anti-inflammatory activity due to the close link that exists between inflammation and tumorigenesis.[19,20] NF-κB has been shown to be a key protein favoring cell proliferation and inhibiting apoptosis in various cell types.[26] To the best of our knowledge this is the first report describing **2** or any of the other hundreds of reported sytryl lactones as demonstrating definitive NF-κB based immune modulating activity. Alternatively, (+)-goniothalmin (**1**), the parent styryl lactone in this class, along with the acetylated derivative of **2** (+)-3-*O*-acetylaltholactone (**3**), do not exhibit NF-kB based anti-inflammatory activity. These results suggest the modes of action of **1** and **3** involving their cytotoxicity against tumor cell lines appear to arise from mechanisms independent of targets in the NF-κB immune mediated pathway.

3. Experimental section

3.1. General experimental procedures

Analytical LC–MS analysis was performed on samples at a concentration of approximately 5 mg/mL, using a reversed-phase 150 × 4.60 mm 5 μm C_{18} Phenomenex Luna column in conjunction with a 4.0 × 3.0 mm C18 (Octadecyl) guard column and cartridge (Holder part number: KJ0-4282, Cartridge part number: AJ0-4287, (Phenomenex, Inc., Torrance, CA). Samples were injected onto the column using a volume of 15 μL, with a flow rate of 1 mL/min that was monitored using a Waters model 996 photodiode array (PDA) UV detector. The elution was subsequently split (1:1) between a S.E.D.E.R.E. model 55 evaporative light scattering detector (ELSD), and an Applied Biosystems Mariner electrospray ionization time of flight (ESI-TOF) mass spectrometer. Optical rotation was performed on a Jasco P-2000 Polarimeter. All NMR experiments were run on a Varian Unity spectrometer (500 and 125 MHz for ^{1}H and ^{13}C, respectively).

3.2. Biological material, collection and identification

Specimens of the *Alphonsea javanica* Scheff. (coll. no. UHA 105) were collected at 978 m (S 03.63951, E 121.15631) from the Mekongga mountainous regions South East Sulawesi province, Indonesia.

3.3. Extraction, LC–MS/ELSD library fraction collection and isolation

Specimens of *A. javanica* (coll. no. UHA 105) were dried (200.0 g) and extracted first using methanol and subsequently with hexanes to generate 7.40 and 1.57 grams of oil, respectively. A portion of the methanol extract (187.3 mg) and hexanes extract (193.6 mg) was provided to UC Berkeley to include in a 96-well plate crude extract library that was initially screened in the NF-κB luciferase and MTT assays. The active methanol extract (sample coded UHA 105 M) was further prepared as a [15.0 mg/150 μL] sample in methanol to undergo a standard LC–MS/ELSD library for bioassay to pinpoint the active component(s) using methods reported previously.[22] Gradient conditions involved using H2O:CH_3CN (with 0.1% formic acid) consisting of 10:90 → 100% over 30 min at 2 mL/min into a 5 μ RP-HPLC C-18 column—250 × 10 mm (Phenomenex, Inc., Torrance, CA) that was then fractionated into a 96-well plate. The well fractions were monitored using a Waters model 996 photodiode array (PDA) UV detector. The elution was subsequently split (1:1) between a S.E.D.E.R.E. model 55 evaporative light scattering detector (ELSD), and an Applied Biosystems Mariner electrospray ionization time of flight (ESI-TOF) mass spectrometer. A duplicate 96-well plate library was then made using a 12-channel pipette creating an exact copy and counter balance for analytical reference and centrifugal drying. Plates were dried and concentrated using a Savant AES2010 SpeedVac. Scale up RP-HPLC of (+)-altholactone (**2**) involved repeated automated injections using the same conditions reported for the LC–MS/ELSD library with fractions instead being partitioned into 20 mL scintillation vials. The vials were dried and concentrated using a Savant AES2010 SpeedVac. A total of 100.0 mg of UHA 105 M was used to make ten injections ([10.0 mg/150 μL]) that were purified using the aforementioned conditions which resulted in 48 fractions. The HPLC fraction H27 (13.2 mg) contained pure (+)-altholactone (**2**). Compound **2** was used to provide (+)-3-*O*-acetylaltholactone (**3**) by semi-synthesis. A sample of **2** (5.0 mg, isolated from *A. javanica*) was dissolved in dry pyridine (300 μL) and acetic anyhydride (1000 μL). The mixture was stirred for 24 h and the reaction was quenched with water and extracted with CH_2Cl_2. The organic layer was then dried with $NaSO_4$ and yielded pure (+)-3-*O*-acetylaltholactone (**3**, 4.2 mg). (+)-Goniothalaman (**1**) was prepared using synthetic methods described previously.[1]

3.4. NF-κB luciferase assay

Extracts and pure compounds were tested in an NF-κB luciferase reporter assay in mouse macrophage (RAW264.7) immune cells to determine NF-κB activity. The RAW264.7 cells were kindly provided by Dr. Greg Barton (Dept. of MCB, UCB) along with the NF-κB luciferase reporter construct which has been described elsewhere.[27] Stably transfected RAW 264.7 cells with the NF-kB reporter gene were plated in 96-well plates (5 x 10^4 cells per well). Following a 24 h recovery period, the cells were treated with the

extract(s) or compound(s) for an additional 18 h in the presence of LPS (100 ng/mL). To check NF-κB luciferase activity, the Luciferase Reporter Assay System purchased from Promega (Madison, WI) were used. Cell lysates (15 μL) from treated RAW264.7 cells were placed in opaque 96-well plates. Luciferase Assay Reagent (50 μL) was injected and read by a fluorometer (LMAX 2, Molecular devices). The values (IC_{50}) represent the mean ± SE for $n = 3$. The anti-inflammatory standard compound celastrol was obtained from Cayman Chemical Company, Ann Arbor, MI, USA.

3.5. Determination of phosphorylation of IkBα by Western blot analysis

RAW264.7 cells were cultured in 6-well plates to near confluency, treated with increasing concentrations of compound in the presence of LPS and incubated at 37 °C for 4 h. Cells were immediately harvested in 200 μL of loading buffer (10% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.125 M Tris–HCl pH 6.7, 0.15% bromophenol blue), sonicated for 15 s, heated to 99.9 °C for 5 min and fractionated by electrophoresis on 4–15% polyacrylamide, 0.1% SDS resolving gels (BioRad). Proteins were electrically transferred to Immobilon-II membranes (Millipore, Billerica, MA), and blocked at room temperature for 1 h with 5% non-fat dry milk in wash buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Blots were subsequently incubated with antibodies against mouse phosphor-IκBα and IκBα (Cell Signaling, 1:1000). Immunoreactive proteins were detected after 1 h of incubation at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:3000 dilution). Blots were incubated with Western Lighting Chemiluminecense Reagent Plus (PerkinElmer Life Sciences) and chemiluminescence was detected using CL-X Posure clear blue X-ray film (Thermo Scientific).

3.6. Griess (nitrite oxide) assay

Murine macrophage cells (RAW264.7) were plated in a 96-well plate in RPMI1640 and incubated at 37 °C for 24 h. The medium was changed, allowing cell induction by addition of compound and lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (LPS; Sigma–Aldrich) in the medium. After incubation, 50 μL of the supernatant was removed and incubated with the Griess reagent [150 μL; 0.5% sulfanilamide, 0.05% (*N*-1-naphthyl) ethylenediamine dihydrochloride, 2.5% H_3PO_4 and 97% H_2O by weight] for 30 min at room temperature in the dark. The absorbance is measured at 530 nm on a Dynex MRX II microplate spectrophotometer and calibrated using a standard curve constructed with sodium nitrite to yield NO_2^- concentration.

3.7. In vitro quantitative real-time PCR analysis

(+)-Altholactone (**2**) was evaluated in triplicate ($n = 3$) for its effects on inducible nitrous oxide synthase (iNOS), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), interferon γ inducible protein 10 (IP-10), interferon beta (IFN-β), granulocyte colony-stimulating factor (GCS-F) and monocyte chemotactic protein-1 (MCP-1). The results are presented as means ± standard deviation of the mean (STD). Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) using GraphPad Prism software (version 3.02 for Windows, GraphPad Software Inc., La Jolla, CA, USA). The statistical significance of mean differences was based on a p value of <0.05. A total RNA of 0.5 μg from RAW264.7 cells after treatment was exposed to DNase I treatment (Aurum™ Total RNA Mini Kit, BioRad, Hercules, CA, USA). cDNA was synthesized from DNase-treated RNA by reverse transcription (iScript™ RT Supermix, BioRad). cDNAs from each experimental condition were pooled and qPCR analysis was performed according to the manufacturer protocol with the iTaq™ Universal SYBR® Green Supermix (BioRad). Quantitative real-time PCR was performed on Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expression for each gene was normalized using the expression of GAPDH as a control housekeeping gene.

3.8. MTT cytotoxicity assay

The methanol crude extract LC-MS/ELSD library well fractions were tested at 20 μg/mL, respectively based on assumed weights of 0.15 mg/well estimated from a 15 mg injection divided into two library plates (7.5 mg each) equally fractionated/50-well ~0.15 mg/well. (+)-Altholactone (**2**) was tested using concentrations of 6.25, 12.5, 25, 50 and 100 μM, respectively against the non tumorigenic murine macrophage (RAW264.7) and breast (MCF-10A) cell lines as well as against tumorigenic breast (MCF-7, MDA-MB-231) and prostate (PC3, LNCaP) cell lines. Cells in 96-well plates in the required growth medium were treated with extracts dissolved in DMSO for 20 h. After incubation, MTT solution was added to wells and incubated for another 2 h. Media were removed and DMSO was added to dissolve purple precipitates. Then plates were read at 570 nm using a plate reader. The values (IC_{50}) represent the mean ± SE for $n = 3$. The cytotoxic standard control compound doxorubicin was obtained from Cayman Chemical Company, Ann Arbor, MI, USA.

3.8.1. (+)-Goniothalamin (1)

LRESITOFMS *m*/*z* 201.1 $[M+H]^+$ see Figure S10 in the Supplementary data. This compound contained spectroscopy data equivalent to previous reports.[1]

3.8.2. (+)-Altholactone (2)

Brown oil: $[\alpha]_D^{20} + 127.5$ (*c* 0.2, $CHCl_3$); LRESITOFMS *m*/*z* 233.1 $[M+H]^+$; HRMS $[M+Na]^+$: $C_{13}H_{12}O_4Na$ calcd 255.06278, found: 255.06575. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.35 (5H, m), 7.01 (1H, dd, *J* = 5.0, 10.0), 6.23 (1H, d, *J* = 9.5), 4.93 (1H, dd, *J* = 2.0, 5.0), 4.75 (1H, d, *J* = 6.0), 4.64 (1H, t, *J* = 5.0), 4.45 (1H, dd, *J* = 2.5, 6.0); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.6, 140.6, 138.2, 128.8,128.8, 128.5, 126.3,126.3 123.8, 86.6, 86.1, 83.8 and 68.3. Additional ^1H and ^{13}C NMR data is provided in Figures S4–S5 (in the Supplementary data) and is equivalent to spectroscopic data reported previously.[23]

3.8.3. (+)-3-*O*-Acetylaltholactone (3)

Clear oil: $[\alpha]_D^{20} + 36.7$ (*c* 0.12, EtOH; LRESITOFMS *m*/*z* 275.1 $[M+Na]^+$; HRMS $[M+Na]^+$: $C_{15}H_{14}O_5Na$ calcd. 297.0733, found: 297.0727. ^1H NMR ($CDCl_3$, 500 MHz): 7.35 (5H, m), 7.04 (1H, dd, *J* = 5.5 9.5), 6.27 (1H, d, *J* = 9.5), 5.39 (1H, dd, *J* = 1.5, 3.5), δ 4.97 (1H, d, *J* = 3.5, H-2), 4.95 (1H, d, *J* = 1.0, 4.0), 4.93 (1H, dd, *J* = 2, 5 Hz), 4.62 (1H, dd, *J* = 4.0, 5.5), 2.14 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 169.5, 160.6, 139.3, 137.6, 128.8, 128.8, 128.6, 126.3, 126.3, 124.8, 86.2, 83.7, 70.7, 69.2 and 20.9. Additional ^1H and ^{13}C NMR data is provided in Figures S8–S9 (in the Supplamentary data) and is equivalent to spectroscopic data reported previously.[15]

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Supplementary data

Supplementary data (ten figures are provided and include: (a) LC–MS/ELSD data of library fractions, (+)-goniothalamin (**1**), (+)-altholactone (**2**) and (+)-3-*O*-acetylaltholactone (**3**), (b) ^{1}H and ^{13}C NMR data of **2**–**3** and (c) the MTT cytotoxicity assay data against one non tumorigenic and four tumorigenic cell lines for (**2**). These data sets associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bmc.2013.04.055.

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