Multiple Anticancer Effects of Damsin and Coronopilin Isolated from *Ambrosia arborescens* on Cell Cultures

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Abstract. *Terpenoids in plants are important sources for drug discovery. In this study, we extracted damsin and coronopilin, two sesquiterpene lactones, from Ambrosia arborescens and examined their anticancer effects on cell cultures. Damsin and coronopilin inhibited cell proliferation, DNA biosynthesis and formation of cytoplasmic DNA histone complexes in Caco-2 cells, with damsin being more potent than coronopilin. Further studies using the luciferase reporter system showed that damsin and coronopilin also inhibited expressions of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-3 (STAT3), indicating that these sesquiterpenes can interfere with NF-κB and STAT3 pathways. Finally, we examined the effects of two synthetic dibrominated derivatives of damsin, 11α,13-dibromodamsin and 11β,13-dibromodamsin. While bromination appeared to weaken the antiproliferative effects of damsin, the β epimer had strong inhibitory effects on STAT3 activation. In conclusion, the sesquiterpene lactones damsin and coronopilin have inhibitory effects on cell proliferation, DNA biosynthesis and NF-κB and STAT3 pathways, thus being potentially important for discovery of drugs against cancer.*

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The rates of colorectal cancer have increased significantly during the past decades, reaching the third position of the most common types of cancer in the world. The progression of normal colonic epithelium to the malignant phenotype occurs stepwise through a series of genetic events, which involve mutations of adenomatous polyposis coli (*APC*) gene, Kirsten rat sarcoma virus oncogen (*K-RAS*), deleted in colorectal carcinoma (*DCC*) and *p53* (1). The genetic events affect many signal transduction pathways such as β-catenin, cyclooxygenase-2 (COX-2) (2), phosphoinositol-3-kinase-Akt (PI3K/AKT) (3), nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-3 (STAT3) pathways (4), which regulate cell proliferation and apoptosis. Disturbances of the balance of cell proliferation and apoptosis are considered as an important factor leading to cancer development. Many drugs exert their anticancer effects by inhibiting cell proliferation and/or promoting apoptosis *via* various signal transduction pathways. Among these anticancer drugs, about 67% are of natural origin, indicating that medicinal herbs are important sources for discovery of drugs against cancer (5).

Terpenoids and their derivatives are phytochemicals. Due to their wide distribution in nature and multiple biological functions (6, 7), they may have particular importance for use in developing novel drugs. For example, ursolic acid and boswellic acids, two extensively studied triterpenoids, have been shown to inhibit cell proliferation and stimulate apoptosis of cancer cells (8, 9). Another group of natural terpenoids, sesquiterpene lactones (SLs), which are 15-carbon compounds consisting of three isoprene units and a lactone group, were recently proposed as a new source of drugs with potential values in the treatment of inflammation

and cancer (10-12). The SLs are well-known alkylating agents of cysteine residues in proteins through a Michael addition of their characteristic α-methylene-γ-lactone moiety (11-13). The NF-κB pathway is considered to be one of the most interesting targets of SLs (13, 14).

In this study we extracted two SLs, damsin and coronopilin (Figure 1) isolated from *Ambrosia arborescens* (15-17), and examined their biological effects on colon cancer cell lines and their influence on expression of NF-κB and STAT3. To evaluate the role of the α-methylene-γ-lactone moiety in the compounds, the effects of these two natural products were compared against those of two synthetic brominated derivatives, 11α,13-dibromodamsin and 11β,13-dibromodamsin (Figure 1).

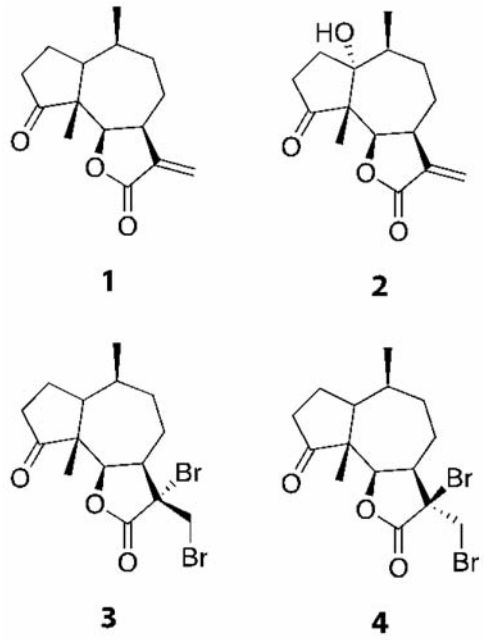

Figure 1. *Sesquiterpene lactones from Ambrosia arborescens and brominated derivatives. Damsin (**1**), coronopilin (**2**), 11α,13-dibromodamsin (**3**) and 11β,13-dibromodamsin (**4**).*

Materials and Methods

Materials. Caco-2 and HeLa cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). A luciferase activity assay kit was obtained from Berthold Technologies (Bad Wildbad, Germany). Cell death detection kit and the cell proliferation reagent (WST-1) were obtained from Roche Diagnostics GmbH (Mannheim, Germany). [^{3}H]Thymidine was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). The cell culture mediums used were obtained from Sigma-Aldrich (Stockholm, Sweden). Luciferase activity was measured using an Autolumat LB 9501 (Berthold Technologies, Bad Wildbad, Germany). All other chemicals were obtained from commercial suppliers of analytical grade. High resolution mass spectrometry (HMRS) electrospray ionization (ESI) spectra were recorded with a micromass quadrupole-time of flight (Q-TOF) micro spectrometer. Nuclear magnetic resonance (NMR) spectra (in $CDCl_3$) were recorded with a Varian Gemini at 300 Mhz (^{1}H) and at 75 MHz (^{13}C). Chemical shifts are given in ppm relative to the residual $CHCl_3$ in $CDCl_3$ (7.25 ppm ^{1}H and 77.00 ppm ^{13}C). All flash chromatography was performed with 60 Å 30-75 μm Silica gel. Thin layer chromatography (TLC) analyses were carried out on Silica Gel 60 F_{254} plates (Merck, Darmstadt, Germany).

Isolation of damsin and coronopilin. Damsin and coronopilin were isolated from A. arborescens. The dry plant (164 g) was extracted in a Soxhlet extractor with petroleum ether (20-40˚C). The crystals of damsin were filtered directly from the petroleum ether extract and purified using a silica gel 60 column chromatography by elution with petroleum ether (40-60˚C): ethyl acetate (7:3 v/v), yielding 1.17 g (0.7%) of pure damsin in the fractions 6-10. The remaining plant material was extracted with dichloromethane in the Soxhlet extractor, producing 6.35 g of crude extract. Then 1.04 g of the extract were fractionated using Sephadex LH-20 column chromatography by elution with methanol:dichloromethane (1:1 v/v) followed by a silica gel 60 column chromatography with petroleum ether (40-60˚C):ethyl acetate (3:2 v/v) as eluent, yielding 0.20 g (0.7%) of coronopilin in the fractions 11-20. The purity of the compounds was confirmed by TLC and NMR.

Bromination of damsin with trimethylammonium perbromide (TMPAP)/Na_2CO_3. To a solution of damsin (100 mg, 0.40 mmol) in dioxane (10 ml), a solid mixture of TMPAP (191 mg, 0.51 mmol) and Na_2CO_3 (855 mg, 8.07 mmol) was added. After 20 h, 30 ml of diethyl ether was added and the resulting white precipitate was filtered, and then the solvent was evaporated under vacuum and the crude reaction products were separated by column chromatography with a gradient of petroleum ether (40-60˚):ethyl acetate yielding 11α,13-dibromodamsin (122 mg, 74%) and 11β,13-dibromodamsin (20 mg, 12%).

Culture of Caco-2 cells. The Caco-2 cells were cultured in Dulbecco's minimal essential medium (DMEM) with L-glutamine, containing 100 IU/ml penicillin, 10 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum (FCS) as previously described (18). They were maintained at 37˚C in a humidified incubator containing 95% air and 5% CO_2. For assays of cell proliferation, DNA replication and apoptosis, the cells were detached with 0.02% EDTA/0.05% trypsin resuspended to a density of 1×10^5/ml and seeded into 96-well plates. The phytochemicals tested were dissolved in dimethyl sulfoxide (DMSO). The concentration of DMSO in the cell culture was 0.1%, and medium containing 0.1% DMSO only was used as the control.

Cell proliferation assay. The cell proliferation of Caco-2 cells was assayed by use of the reagent WST-1 (4-[3-84-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazoliol]-1,3-benzene disulfonate), which is metabolized by mitochondrial dehydrogenases to a formazan staining dark red (19). The formation of formazan is proportional to the number of viable cells. Briefly, 2×10^4 Caco-2 cells in 200 μl medium were incubated with the compounds tested at concentrations from 25 to 100 μM for 24 h, followed by adding 20 μl WST-1 reagent. After incubation for 1 h, the optical density was read at 405 nm using 655 nm as background. The cell

proliferation rate was expressed as a percentage of the control. All determinations were carried out in triplicate in three different experiments.

DNA replication. DNA synthesis was measured by the incorporation of [^{3}H]thymidine as described previously (20). Briefly Caco-2 cells were pre-incubated for 20 h with the compounds at different concentrations. [^{3}H]Thymidine (0.5 μCi/well) was added, followed by incubation for 4 h. Cells were washed with phosphate buffered saline, treated with 5% trichloroacetic acid for 20 min at 4˚C, and fixed with methanol. The cells were lysed in 0.5 M sodium hydroxide and 0.1% sodium dodecyl sulphate for 15 min and the radioactivity in the lysate was counted by liquid scintillation. DNA synthesis was expressed as a percentage of the non-treated cells.

Assay of apoptosis. Apoptosis was assayed by a cell death detection ELISA^{PLUS} kit (Roche Diagnostics GmbH) (9) according to the instructions. Briefly, 1×10^4 cells were seeded in 96-well plates and treated with damsin and coronopilin at different concentrations. The cells were lysed and centrifuged and the formation of cytoplasmic histone-associated DNA fragments was measured by a plate reader at 415 nm using 490 nm as background. The enrichment factor was calculated as fold increase comparing with that of control (cells without treatment).

NF-κB and STAT3 luciferase assays. To study the NF-κB-dependent transcription, the 5.1 Jurkat cell line (21) was used. The cells are stably transfected with luciferase gene driven by the HIV-1-LTR promoter, which contains two NF-κB-binding sites that are required to induce the transactivation. The cells were preincubated for 30 min with the compounds and stimulated with tumor necrosis factor-α (TNFα) (5 ng/ml) for 6 h. Then the cells were lysed in 25 mM Tris–phosphate, pH 7.8, containing 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1% Triton X-100, and 7% glycerol. Luciferase activity was measured following the instructions of the assay kit. Basal luciferase activity was subtracted from that of TNFα-induced activity. To determine STAT3 transcriptional activity, HeLa cells were transiently transfected with the plasmid p4xM67-tk-Luc, in which the luciferase gene is driven by a promoter containing four copies of the STAT binding site. Twenty-four hours after transfection, the cells were stimulated with interferon gamma (IFNγ) (10 μM) for 6 h. The luciferase activity was measured as described above. Basal luciferase activity was subtracted from IFNγ-induced activity. The inhibitory activity of the compounds is expressed as a percentage of those treated with only TNFα and IFNγ, respectively. Half maximal inhibitory concentration (IC_{50}) activity was calculated using SigmaPlot software (Systat Software GmbH, Germany).

Results and Discussion

Isolation and derivatization of SLs from Ambrosia arborescens. The dried leaves of *Ambrosia arborescens* were extracted continuously with petroleum ether. Crystallized damsin was purified using silica gel chromatography. Coronopilin was isolated from the dichloromethane extract using molecular exclusion chromatography (Sephadex LH-20) and purified by silica gel chromatography. Both damsin and coronopilin were obtained as white crystals, and the compositions were confirmed by ^{1}H NMR and ^{13}C NMR with comparison of authentic samples and data in the literature (15, 22, 23). The chemoselective bromination of damsin was carried out with TMPAP in basic medium, only affecting the double bond of the lactonic ring, giving the epimers 11α,13-dibromodamsin and 11β,13-dibromodamsin with an overall yield of 86% with an epimeric excess of 62% for 11α,13-dibromodamsin (Figure 1). Other brominating conditions using TMPAP/*p*-toluenesulphonic acid, bromine and *N*-bromosuccinimide did not have the desired selectivity and the α-carbonyl position was also affected. The ^{1}H NMR spectrum showed the nucleophilic 1,2 addition of bromine by the perbromide reagent to the double bond (Table I), the signals of the exocyclic double bond of damsin (24-26) were shifted to high fields in both products as doublets. This was confirmed by HRMS with an ion $(M+Na)^+$ at 428.9677 for 11α,13-dibromodamsin and an ion $(M+H)^+$ at 406.9857 for 11β,13-dibromodamsin. The absolute stereochemistry of each epimer was established through ^{1}H NMR analysis (Table I), nuclear overhauser effect (NOE) difference experiments and comparison with computational models (Figure 2). In the case of 11α,13-dibromodamsin, the α disposition of bromine at C-11 was inferred from the deshielded chemical shift (+0.45 ppm) observed in the signal of H-6 (Table I), with respect to the same signal in compound 11β,13-dibromodamsin. The NOE correlations and the interatomic distances are shown in Figure 2.

Inhibitory effect of damsin and coronopilin on the growth of Caco-2 cells. After incubation of Caco-2 cells with damsin and coronopilin at different concentrations, we found that damsin dose-dependently inhibited the cell proliferation, with 36% inhibition occurring at 50 μM and 45% at 100 μM. Compared to damsin, coronopilin was less effective and 100 μM coronopilin only led to 19% inhibition (upper panel of Figure 3). We further examined the effects of the compounds on DNA replication and found that both damsin and coronopilin strongly reduced [^{3}H]thymidine incorporation into DNA in a dose-dependent manner (lower panel of Figure 3). The effect of damsin was also greater than that of coronopilin as 50 μM damsin almost completely blocked DNA biosynthesis, whereas a higher concentration (100 μM) of coronopilin was required to achieve similar effects. Since DNA synthesis occurs in the S phase and mitosis in the M phase of the cell cycle, our results indicate that both damsin and coronopilin have inhibitory effects on the transition of the cells from the S to the M phase, thus blocking cell proliferation. It is well-known that cyclins and different cyclin-dependent kinases (CDKs) are key molecules that regulate the transition of the cell cycle. Whether damsin and coronopilin inhibit the expression of cyclins and activities of CDKs is an interesting question for further investigation.

Table I. *Nuclear magnetic resonance (NMR) spectroscopic data for 11α,13-dibromodamsin and 11β,13-dibromodamsin (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) in $CDCl_3$.*

Pos	11α,13-dibromodamsin			11β,13-dibromodamsin		
	δ ^{1}H (J in Hz)	δ ^{13}C	NOE	δ ^{1}H (J in Hz)	δ ^{13}C	NOE
1	2.04 m	45.3 d		1.92 m	48.6 d	
2a	2.07 m	24.1 t		2.03 m	23.7 t	
2b	1.86 m			1.86 m		
3a	2.53 ddd (19.1, 7.4, 1.5)	34.7 t		2.48 ddd (18.9, 8.4, 1.0)	35.9 t	
3b	2.24 dd (19.5, 10.2)			2.24 m		
4		220.5s			216.9	
5		53.8 s			54.3	
6	4.96 dd (5.1, 1.0)	80.8 d		4.51 d (9.6)	83.4 d	
7	2.72 ddd (1.5, 10.0, 5.0)	56.1 d	H-6, H-13a, H-13b, H-1, H-8α, H-8β	3.29 ddd (12.0, 9.7, 5.1)	44.8 d	H-6, H-13a, H-13b, H-8α, H-9α, H-8β, H-9β
8β	1.78 m	19.2 t		1.73 m	25.9 t	
8α	1.65 m			2.11 m		
9a	1.93 m	36.8 t		1.64 m	33.8 t	
9b	1.68 m			1.93 m		
10	2.23 m	34.4 d		2.22 m	34.1 d	
11		61.8 s			62.3	
12		170.6 s			172.0	
13a	3.92 d (12.6)	35.4 t	H-13b	4.22 d (103)	35.7 t	H-13b
13b	3.68 d (12.6)		H-13a, H-8α, H-8β	3.80 d (10.3)		H-7, H-8α
14	1.07 d (7.6)	16.6 q		1.12 d (7.5)	14.8 q	
15	1.18 s	16.0 q		1.21 s	13.9 q	

Pos: Position; NOE: Nuclear Overhauser Effect.

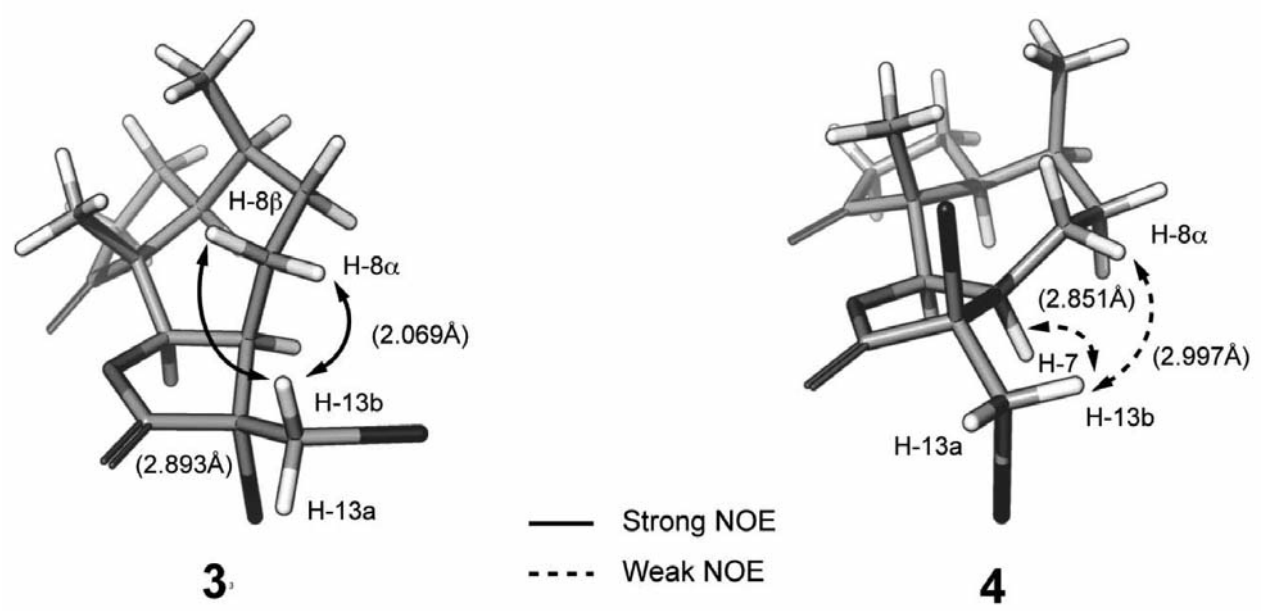

Figure 2. *Nuclear Overhauser Effect (NOE) correlations used to confirm the relative stereochemistry of compounds 11α,13-dibromodamsin (**3**) and 11β,13-dibromodamsin (**4**). The interatomic distances are specified in parentheses.*

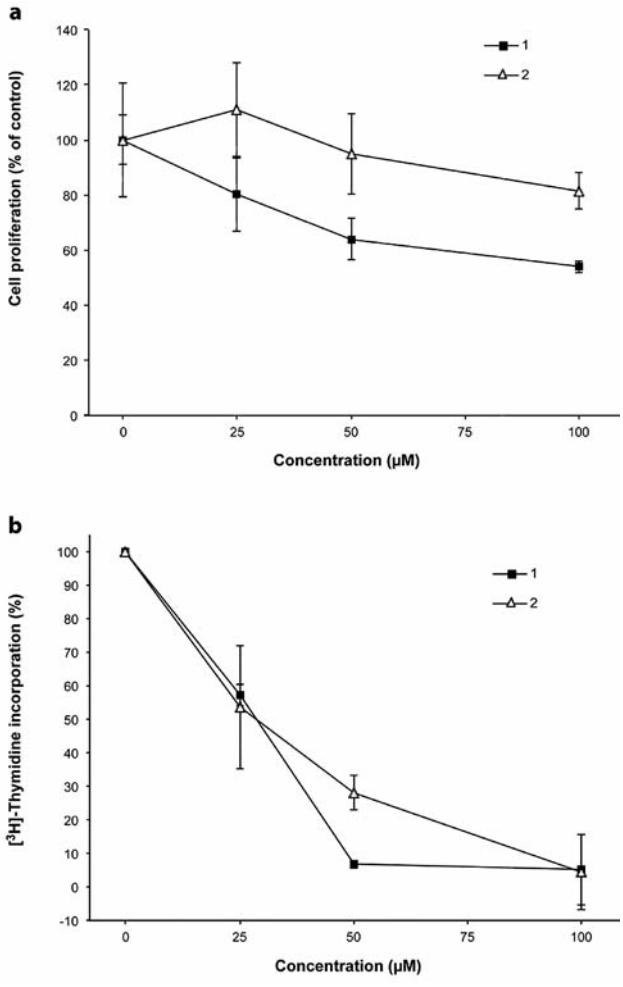

Figure 3. *a: Antiproliferative activity of damsin (**1**) and coronopilin (**2**) on Caco-2 cells. Results are the means (±SD) of three independent experiments performed in triplicate. The p-values (Friedman test) for the effect of (**1**) is 0.022, and for that of **2**, 0.048. b: Effects of **1** and **2** on DNA synthesis in Caco-2 cells are expressed as a percentage of the control. The results are the means (±SD) of three separate experiments performed with sextuplicate samples. The absolute thymidine incorporation in control cell was 0.4 fmol/h/10^6 cells.*

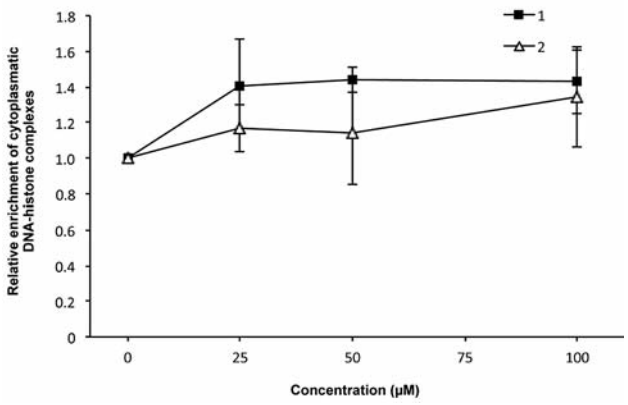

Figure 4. *Effect of damsin (**1**) and coronopilin (**2**) on apoptosis. Caco-2 cells were incubated with the compounds for 6 h and the formation of DNA histone complexes was determined. The results are the means (±SD) of three separate experiments performed in triplicate.*

Effects of damsin and coronopilin on apoptosis. Proliferation and apoptosis are two physiological processes affecting cell fates. Disturbance of the balance of cell proliferation and apoptosis predisposes for cancer development. We therefore examined whether damsin and coronopilin affected apoptosis by measuring the formation of cytoplasmic DNA histone complexes, a biological marker of apoptosis. After treating the cells with the compounds for 6 h, a mild increase (about 40%) of cytoplasmic DNA histone complexes by damsin was observed, but the dose-dependent pattern was poor (Figure 4). Coronopilin was less effective. Longer stimulation of the cells for 24 h did not lead to positive results (data not shown). The results do not indicate a strong pro-apoptotic effect of damsin and coronopilin, and their anticancer effects may mainly be mediated by their inhibitory effects on cell proliferation.

Inhibitory effects on NF-κB and STAT3 activities by damsin and coronopilin. NF-κB and STAT3 are two important pathways involved in cell survival and proliferation of cells. NF-κB signalling is important for expression of several inflammatory genes and anti-apoptotic genes. NF-κB has been considered to be an important link between inflammation and cancer. In order to gain insight into their mechanisms of action, the effects of damsin and coronopilin on NF-κB and STAT3 transcriptional activities were investigated in Jurkat cells and HeLa cells using the luciferase reporter system. The expression of NF-κB and STAT3 were induced by TNFα and IFNγ, respectively. We found that damsin and coronopilin significantly inhibited the TNFα-induced expression of NF-κB (Figure 5) and IFNγ induced expression of STAT3. The IC_{50} values for the two compounds are shown in Table II. In both cases, the IC_{50} values of damsin were lower than those of coronopilin, indicating again that damsin is more effective than coronopilin. NF-κB signalling can be inhibited by multiple mechanisms, including inhibition of inhibitor of NF-κB kinase (IKK) activity, inhibitor of NF-κB (IκB) phosphorylation, IκB degradation, and DNA binding. Some SLs, such as ergolide, artemisinin, costunolide and zerumbone, have been reported to have inhibitory effects on the NF-κB pathway (13). Our findings that both NF-κB and STAT3 could be inhibited by damsin and coronopilin are interesting, as NF-κB and STAT3 are constitutively activated in neoplastic cells in response to autocrine and paracrine factors that are produced within the

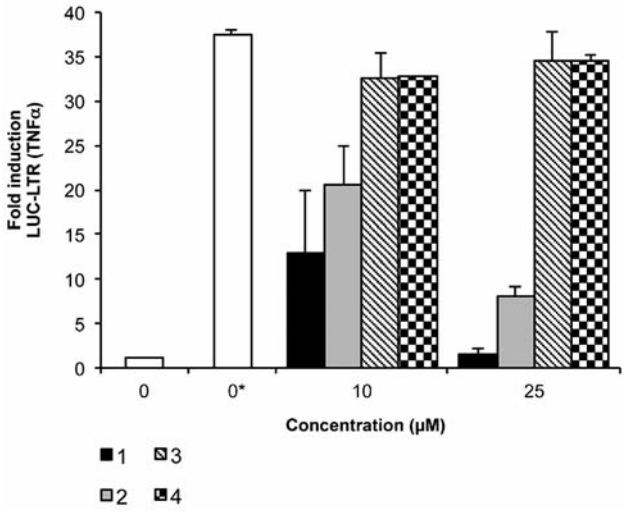

Figure 5. *Inhibitory activity of damsin (**1**), coronopilin (**2**), 11α,13-dibromodamsin (**3**) and 11β,13-dibromodamsin (**4**) on nuclear factor κB (NF-κB) expression in luciferase-transfected 5.1 cells. The cells were stimulated with tumor necrosis factor-α (TNFα) in the presence or absence (0*) of the compounds. 0 is the negative control.*

tumour microenvironment (27). STAT3 and NF-κB in a cooperative manner regulate several pro-inflammatory genes, such as interleukin 6 (*IL-6*), *IL-11*, chemokines, growth factors and *COX2*, that are crucial for maintaining a pro-carcinogenic inflammatory environment (6, 7). Therefore, STAT3 and NF-κB establish a feed-forward loop between cancer cells and the tumour microenvironment. The inhibitory effects of damsin and coronopilin on both NF-kB and STAT3 indicate that they could be candidates for developing potent anticancer drugs.

Anticancer effects of two brominated derivatives of damsin. To gain insight of the structure-function relationship of damsin, we further studied the anticancer effects of two brominated derivatives of damsin: 11α,13-dibromodamsin and 11β,13-dibromodamsin. 11α,13-dibromodamsin inhibited cell proliferation but dose-dependent effects were not demonstrated, whereas the 11β,13-dibromodamsin did not show a significant effect. Differing from damsin and coronopilin, these two brominated derivatives had no effects on the expression of NF-κB induced by TNFα (Figure 5). As for the effects on STAT3, while 11α,13-dibromodamsin failed to show any effect, 11β,13-dibromodamsin was found to be the strongest inhibitor against STAT3 activation, with the IC_{50} being 9.7 μM, which is even lower than that for damsin (12.4 μM) and coronopilin (18.3 μM) (Table II). The negative effects of 11α,13-dibromodamsin and 11β,13-dibromodamsin on NF-κB might be explained by the loss of the Michael acceptor moiety and thereby by the alkylating capacity of cys-38 in p65/p50 (NF-κB) (28).

Table II. *Half -maximal inhibitory concentration (IC_{50}; μM) values of damsin, coronopilin and dibrominated derivatives 11α,13-dibromodamsin and 11β,13-dibromodamsin toward nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) activation.*

Compound	NF-κB	STAT3
Damsin	7.2	12.4
Coronopilin	10.1	18.3
11α,13-dibromodamsin	>25[a]	>25a
11β,13-dibromodamsin	>25[a]	9.7

[a]Inhibition not detected at the concentration tested.

However, for the effects on STAT3 the different effects cannot be explained in the same way, and rather highlight the importance of stereoselective bromidation of the α-methylene-γ-lactone moiety for the specific biological activities.

In summary, we identified two SLs, damsin and coronopilin, that have antiproliferative activity towards the Caco-2 cell line. Damsin was the most active. The antiproliferative effects may be related to its inhibitory effects on NF-κB and STAT3 pathways. In addition, the inhibitory effect of damsin on STAT3 can be significantly enhanced by dibromination to form 11β,13-dibromodamsin.

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