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The inhibition of TNF-α-induced NF-κB activation by marine natural products

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ABSTRACT

The deregulated activation of NF-κB is associated with cancer development and inflammatory diseases. With an aim to find new NF-κB inhibitors, we purified and characterized compounds from extracts of the Fijian sponge *Rhabdastrella globostellata*, the crinoid *Comanthus parvicirrus*, the soft corals *Sarcophyton* sp. nov. and *Sinularia* sp., and the gorgonian *Subergorgia* sp. after an initial screening of 266 extracts from different marine origins.

Results obtained show that selected purified compounds had a cytotoxic effect on the human leukaemia cell line K562, inhibited both TNF-α-induced NF-κB-DNA binding as well as TNF-α-induced IκBα degradation and nuclear translocation of p50/p65. Furthermore, we observed the inhibition of NF-κB activation induced by an overexpression of IKKβ. Interestingly, natural products inhibited IKKβ kinase as well as the 26S proteasome proteolytic activity.

1. Introduction

Nuclear factor κB (NF-κB) is an inducible transcription factor that regulates the transcription of genes involved in a wide range of immune and inflammatory cell functions [1,2]. While normal activation of NF-κB is required for cell survival and immunity, it is now well established that the deregulated activation of NF-κB is associated with cancer, inflammation, and a wide range of other pathologies [3–11]. Because of its severe implications in a large number of diseases, NF-κB has recently become a major target in drug discovery [4,7,8,12].

In non-stimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form, bound to the NF-κB inhibitor IκB [13]. Several factors are known to activate NF-κB, including the exposure to cytokines, to carcinogens, or to physical or oxidative stress [14]. The extra-cellular signals converge on the TNF-α receptor1 (TNFR1), and trigger the recruitment of the TNFR1-associated death domain protein (TRADD) and of the TNF-receptor-associated factor 2 protein (TRAF2) to TNFR1. This leads to the ubiquitination of the receptor-interacting protein (RIP) and to the activation of the transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) and of the IκB kinase complex IKK (kinase of IκB). Upon phosphorylation by IKK, IκB is rapidly degraded and NF-κB is released to translocate to the nucleus, where it binds to its target DNA sequences to initiate gene transcription [1,3,4,8,15–19].

A large number of natural and synthetic compounds are currently being investigated for NF-κB inhibitory activity [20–22]. Marine natural products, in particular, have recently been recognized as a promising source of NF-κB inhibitors [20,23,24]. With an aim to find new potent NF-κB inhibitors, we screened 220 extracts from Fijian marine organisms including algae, sponges, cnidarians, ascidians, and echinoderms collected by the Institute of Applied Sciences (IAS) Marine Collection (University of the South Pacific, Suva, Fiji) between 1997 and 2003. Since marine microorganisms have become widely recognized as a particularly promising source of novel bioactive natural products [25–28], we also screened 43 extracts from microalgae and cyanobacteria provided by the Culture Collection of Algae and Protozoa (CCAP) (Dunstaffnage Marine Laboratory, Oban, U.K.) and from bacteria isolated from Scottish and Costa Rican marine sediments and cultured in our laboratories. Notably, marine microorganisms are rich sources of polyunsaturated fatty acids (PUFA) and of carotenoids, which are likely to interfere with the activation of NF-κB through their strong antioxidant activity [29–34].

The relationship between the taxonomy, the habitat, and the metabonomics of the source organisms and the NF-κB inhibitory

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potential of the crude extracts included in our large-scale screening is discussed. This type of information can be of extremely high value in the development of more efficient and more sustainable sampling and screening strategies in NF-κB-related drug discovery. A first insight into the relationship between the metabonomics of marine organisms and the NF-κB inhibitory potential of the secondary metabolites produced by the latter was obtained by analyzing the fatty acid (FA) composition of the microalgae included in our screening by gas chromatography (GC).

Five extracts from Fijian marine organisms with strong NF-κB inhibitory activity were selected based on the availability of supplementary biomass and purified by assay-guided fractionation in order to identify the chemical structure and the mechanism of action of the compounds responsible for the bioactivity of the source extracts. The five selected samples include the extracts isolated from a crinoid (*Comanthus parvicirrus*, FJ01-199), from a sponge (*Rhabdastrella globostellata*, FJ97125D114), from two soft corals (*Sarcophyton* sp. nov., FJ03-147 and *Sinularia* sp., FJ01-111), and from a gorgonian (*Subergorgia* sp., FJ01-160). Preliminary results on the NF-κB inhibitory potential of the two naphtopyrones 6-methoxy-comaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) isolated from an extract of the crinoid *C. parvicirrus* (FJ01-199) were presented by Folmer et al. in 2008 [35]. As described in [35], both naphtopyrones (**1**) and (**2**) inhibit TNF-α-induced NF-κB activation in a dose-dependent manner (MIC = 300 μM), and their mechanisms of action involve the inhibition of the enzymatic activity of the kinase IKKβ and of the 26S proteasome. Additional results on the mechanism of action of (**1**) and (**2**) downstream of IκB degradation are included in the present paper, together with the results obtained on the mechanisms of action of the bioactive compounds isolated from the four remaining extracts FJ97125D114, FJ03-147, FJ01-111, and FJ01-160.

2. Materials and methods

2.1. Chemicals and biological material

Unless specified otherwise, all the chemicals were purchased from Sigma–Aldrich (Bornem, Belgium). RPMI 1640 cell culture medium and the antibiotic–antimycotic solution were purchased from Bio-Whittaker (Fisher Scientific) (Tournai, Belgium). Foetal calf serum (FCS) was purchased from Perbio (Erembodegem, Belgium). TNF-α was dissolved at 10 μg/mL in 1× PBS supplemented with 0.5% (w/v) BSA according to the manufacturer's instructions and stored at 4 °C until being used. Synthetic astaxanthin (Sigma–Aldrich (Bornem, Belgium)) (catalogue # A9335) was dissolved in DMSO at a concentration of 20 mg/mL and stored in the dark at 4 °C until being used. The chemical solvents (HPLC-grade) used for chromatography were purchased from Rathburn Chemicals (Glasgow, U.K.). NMR solvents were purchased from Sigma–Aldrich (Poole, U.K.).

Methanol extracts from 220 marine organisms collected around the Fiji islands (18°00′N, 175°00′E) between 1997 and 2003, including 102 sponges, 9 ascidians, 12 echinoderms, 81 cnidarians, and 16 algae, were kindly provided by the IAS Marine Collection of the University of the South Pacific, Suva (Fiji). The extracts (1–5 mg, D.W.) were prepared in DMSO at a concentration of 20 mg/mL for the biological assays. Strains of bacteria were isolated from marine sediment collected in 2004–2005 at 0–20 m depth along the coast of Dunstaffnage Bay, Oban (U.K.) (56°27′N, 5°25′W) and along the coast of Costa Rica (Gandoca-Manzanillo Wildlife Reserve, Caribbean Sea (9°37′N, 82°36′W), Cahuita National Park, Caribbean Sea (9°43′N, 82°48′W), Las Baulas Marine National Park, Pacific Ocean (9°34′N, 85°07′W), and Cabo Blanco Natural Absolute Reserve, Pacific Ocean (10°18′N, 85°50′W)), using a grab sampler or a shovel depending on the collection depth. The sediment samples were maintained on ice during the transportation to the laboratory and stored at 4 °C until processing. The sediment samples were inoculated in 10^{-1} to 10^{-3} dilutions on marine agar (Scottish samples) or on M1 agar (10 g soluble starch, 4 g yeast extract, 2 g peptone, 18 g agar, 33 g Instant Ocean® sea salt (Spectrum Brands, Inc. (Salzbach, Germany)) per litre of distilled water, pH 7.6) supplemented with 10 μg/mL nalidixic acid (Sigma–Aldrich (Louisville, MO, U.S.A.)) and 50 μg/mL nystatin (Sigma–Aldrich (Louisville, MO, U.S.A.)) (Costa Rican samples) and cultured at room temperature. The phylogenetic analysis of the bacteria was performed by Gram staining and by 16S rDNA sequencing followed by FASTA (European Bioinformatics Institute) database search [36]. The 16S rDNA sequencing was performed by Dr. David Green at the SAMS Dunstaffnage Marine Laboratory (Oban, U.K.) by Macrogen Inc. (Seoul, Korea) (Scottish samples), and by Godofredo Solano at INBio (Santo Domingo de Heredia, Costa Rica) (Costa Rican samples), using the 27f primer (5′-AGAGTTTGATCMTGGCTCAG-3′) (INBio (Santo Domingo de Heredia, Costa Rica)) and an ABI BigDye™ terminator system on an Applied Biosystems® sequencer (Applied Biosystems (Foster City, CA, U.S.A.)). Strains of the marine cyanobacterium *Phormidium foveolarum* originating from Corsica, France (CCAP 1446/8) and of the freshwater cyanobacterium *Fischerella muscicola* originating from Allahabad, India (CCAP 1427/1) were obtained from the Culture Collection of Algae and Protozoa (CCAP) at the SAMS Dunstaffnage Marine Laboratory (Oban, U.K.) and cultured in Provasoli-enriched artificial seawater (ASW) medium and in BG11 medium, respectively (protocols from the CCAP online catalogue available at www.sams.ac.uk). Strains of the microalgae *Chlorococcum submarinum* (Chlorophyta) originating from brackish water in Merseyside, U.K. (CCAP 213/10), *Chlamydomonas raudensis* (Chlorophyta) originating from Lake Bonney, Antarctica (CCMP 1619, from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (West Boothbay Harbor, ME, U.S.A.), *Chlamydomonas noctigama* (Chlorophyta) originating from a meadow lake in the former Czechoslovakia (CCAP 11/17), and *Chlamydomonas hedleyi* (Chlorophyta) originating from seawater in the Florida Keys, U.S.A. (ATCC 50216, from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) were obtained from CCAP. CCMP 1619 is identical to SAG 49.72 at the Culture Collection of Algae (SAG) of the University of Göttingen (Göttingen, Germany) and to UW0241 at the University of Washington Culture Collection (UW) in Seattle, WA (U.S.A.), and CCAP 11/17 is also known as UTEX 2289 at the Culture Collection of Algae of the University of Texas, Austin (UTEX) in Austin, TX (U.S.A.). The microalgae were cultured in 3N-BBM+W culture medium (protocol from the CCAP catalogue) at 20 °C, except for CCMP 1619, which was cultured at 4 °C.

2.2. Analysis of the fatty acid composition of the microalgae included in the large-scale screening

The fatty acids present in the methanol extracts from the microalgae included in our screening for NF-κB inhibitors were converted to fatty acid methyl esters (FAMEs) as described in [37]. Briefly, 5 mg of extract was dissolved in 1 mL of toluene. 2 mL of 1% (v/v) sulfuric acid in methanol was added, and the samples were heated for 2 h at 100 °C in sealed tubes. On cooling, 5 mL of water with 5% (w/v) NaCl was added, and the formed esters were extracted in hexane. Prior to gas chromatography (GC) analysis, the esters were purified by thin layer chromatography on silica G60 plates (Merck Sharp & Dohme Ltd. (Hoddesdon, U.K.)), using hexane/ether/glacial acetic acid 78:20:2 as solvent. The GC analysis was performed on a Shimadzu GC-2014 gas chromatograph (equipped with a split injector and flame ionization detector (Shimadzu Europe, Ltd. (Manchester, U.K.)). Separation was carried

out on a polar Zebron ZB-WAX column (30 m × 0.25 mm, 0.25 μm) (Phenomenex, Macclesfield, U.K.), using helium (BOC (Aberdeen, U.K.)) as the carrier gas. The oven temperature was programmed to increase from 160 °C to 240 °C at a rate of 4 °C min^{-1} and then to hold for 10 min at 240 °C. Data acquisition and analysis were performed using the Shimadzu GC Solution software (Shimadzu Europe, Ltd. (Manchester, U.K.)). Individual fatty acids were identified by reference to authentic GC standards (Supelco 37 component FAME mix (Sigma–Aldrich, Poole, U.K.)).

2.3. Purification and structure elucidation of natural products isolated from methanol extracts from Fijian marine invertebrates

Approximately 1.5 g each of extract from five Fijian marine invertebrates, including the crinoid *C. parvicirrus* (FJ01-199), the sponge *R. globostellata* (FJ97125D114), the soft corals *Sarcophyton* sp. nov. (FJ03-147) and *Sinularia* sp. (FJ01-111), and the gorgonian *Subergorgia* sp. (FJ01-160) was provided by the Institute of Applied Sciences Marine Collection (University of the South Pacific, Suva, Fiji). The extracts were purified by assay-guided fractionation using liquid–liquid partitioning (water–methanol–dichloromethane–hexane) followed by high-performance liquid chromatography (HPLC). HPLC separations were carried out using a Gemini reversed-phase HPLC column (C_{18}, 250 × 10 mm, 5 μm) (Phenomenex, Macclesfield, U.K.) and an HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) with diode array detector, semi-preparative binary pump system, methanol/water gradients). The chemical structures of the isolated compounds were elucidated by nuclear magnetic resonance (NMR) (^1H, ^{13}C, ^1H–^1H COSY, HMBC, and HSQC; 400 MHz; $CDCl_3$) (Varian Ltd. (Oxford, U.K.)), UV, and low and high resolution mass spectroscopy (MS) (Agilent Technologies, Waldbronn, Germany). The purified compounds were stored as dry powder or oil at 4 °C and prepared in DMSO at a concentration of 20 mg/mL for the biological assays.

2.4. Cancer cell culture

K562 (human chronic myelogenous leukaemia) and Jurkat (T cell leukaemia) cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) Braunschweig, Germany) were cultured in RPMI 1640 cell culture medium supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic–antimycotic solution at 37 °C and 5% of CO_2.

2.5. Transient transfection and luciferase reporter gene assay

Transient transfections of K562 cells were performed as described previously [38]. 5 μg of luciferase reporter gene construct containing 5 repeats of a consensus NF-κB site (Stratagene (La Jolla, CA, U.S.A.) and 5 μg *Renilla* luciferase plasmid (Promega, Leiden, Netherlands) were used for each pulse. Following electroporation, the cells were resuspended in growth medium (RPMI 1640 with 10% (v/v) FCS) and incubated at 37 °C and 5% of CO_2. Twenty hours after transfection, the cells were harvested and resuspended in growth medium (RPMI 1640 with 10% (v/v) FCS) to a final concentration of 10^6 cells/mL and treated for 2 h with the crude extracts or the isolated natural products at different test concentrations. The cells were then challenged with 20 ng/mL TNF-α for 2 h. 75 μl Dual-Glo™ Luciferase Reagent (Promega, Leiden, Netherlands) was added to the cells for a 10 min long incubation at 22 °C before firefly luciferase activity was measured. Then, 75 μl Dual-Glo™ Stop & Glo® Reagent (Promega, Leiden, Netherlands) was added for 10 min at 22 °C in order to assay *Renilla* activity. Firefly and *Renilla* luciferase activities were measured using an Orion microplate luminometer (Berthold, Vilvoorde, Belgium) by integrating light emission for 10 s. The results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to *Renilla* luciferase activity. The luciferase reporter gene assays were performed as double-blind (*i.e.* without knowing the nature and the origin of the test samples).

2.6. Cytotoxicity assay

The effects of the crude extracts and of the purified natural products on the viability of K562 test cells during the reporter gene assay were assessed using a CellTiterGlo® kit (Promega, Leiden, Netherlands) as described in the manufacturer's protocol.

2.7. Effects of the test compounds on the enzymatic activity of luciferase

The effects of the crude extracts and of the purified natural products on the enzymatic activity of luciferase were assessed by incubating 100 μL of QuantiLum Recombinant firefly luciferase (Promega, Leiden, Netherlands), diluted 10^5 times in a 10% BSA solution, with the test compounds at the concentration used in the luciferase assays, for 1 h at room temperature. The enzymatic activity of luciferase at the end of the treatment was measured on a luminometer after an addition of 100 μL of luciferin substrate (SteadyGlo® reagent, Promega, Leiden, Netherlands).

2.8. Electrophoretic mobility shift assay (EMSA)

Jurkat cells were resuspended in growth medium (RPMI 1640 with 10% (v/v) FCS) to a final concentration of 10^6 cells/mL and treated for 2 h at 37 °C and 5% of CO_2 with the test compounds at various concentrations. The cells were then challenged with 20 ng/mL TNF-α for 2 h at 37 °C and 5% of CO_2. Nuclear extracts were prepared using the method described by Müller et al. [39] and stored at −80 °C. The oligonucleotide NF-κB c (consensus NF-κB site) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and its complementary sequence (Eurogentec, Seraing, Belgium) were used as probe. The probe was hybridized and labeled with ^{32}P ATP (MP-Biomedicals, Brussels, Belgium) and the EMSA was performed as published before [39]. Briefly, 10 μg of nuclear extract was incubated in binding buffer with the ^{32}P ATP labeled probe for 20 min. The DNA–protein complexes were analyzed by electrophoresis on a 4% native polyacrylamide gel using Tris–glycine buffer, and visualized by autoradiography.

In order to investigate the direct interference, within the nucleus, of the test compounds with the binding of activated NF-κB to its DNA binding site, 10 μg of nuclear extract from Jurkat cells treated for 2 h with 20 ng/mL TNF-α at 37 °C and 5% of CO_2 were incubated for 20 min on ice with the test compounds prepared in binding buffer at a 10^3 fold dilution compared to the MIC observed in the EMSA. The nuclear extracts were then incubated with the ^{32}P ATP labeled probe for further 20 min on ice, and the DNA–protein complexes were analyzed by electrophoresis as described above.

In the immuno-depletion experiments, the nuclear extracts and labeled probes were incubated in the reaction mixture for 30 min on ice prior to a 30 min incubation on ice with 2 μg of antibodies. All antibodies (anti-p50, anti-p65, anti c-Rel and anti-RelB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

2.9. Western blot analysis

To determine the effects of the test compounds on TNF-α-dependent IκBα degradation and on TNF-α-dependent translocation of p65 and p50 into the nucleus, cytosolic and nucleic protein extracts were prepared as previously described [38]. Briefly, 2 × 10^6 cells/mL were pre-treated with the test compounds at a

concentration of 100 μg/mL for 2 h and then exposed to 20 ng/mL of TNF-α for various amounts of time. 10 μg of protein extract was resolved on a 10% SDS-PAGE gel, transferred onto a membrane, blocked with 5% non-fat milk, and probed with specific antibodies against IκBα, p65, and p50 (Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.)). Specific antibodies against lamin C (Vision Biosystems Novacastra (Zaventem, Belgium)) and against tubulin-α (Calbiochem (San Dieo, CA, U.S.A.)) were used to verify the purity of the cytoplasmic and nuclear extracts and to confirm equal protein loading in each well. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and finally detected by ECL reagent (GE Healthcare, Diegem, Belgium). The levels of protein expression (band area × mean band intensity) were quantified using the software Adobe Photoshop Elements version 2.0 (Adobe Systems Inc. (U.S.A.)).

2.10. Fluorescence microscopy

Jurkat cells were prepared at a concentration of 10^6 cells/mL in RPMI 1640 cell culture medium with 10% (v/v) FCS. The test

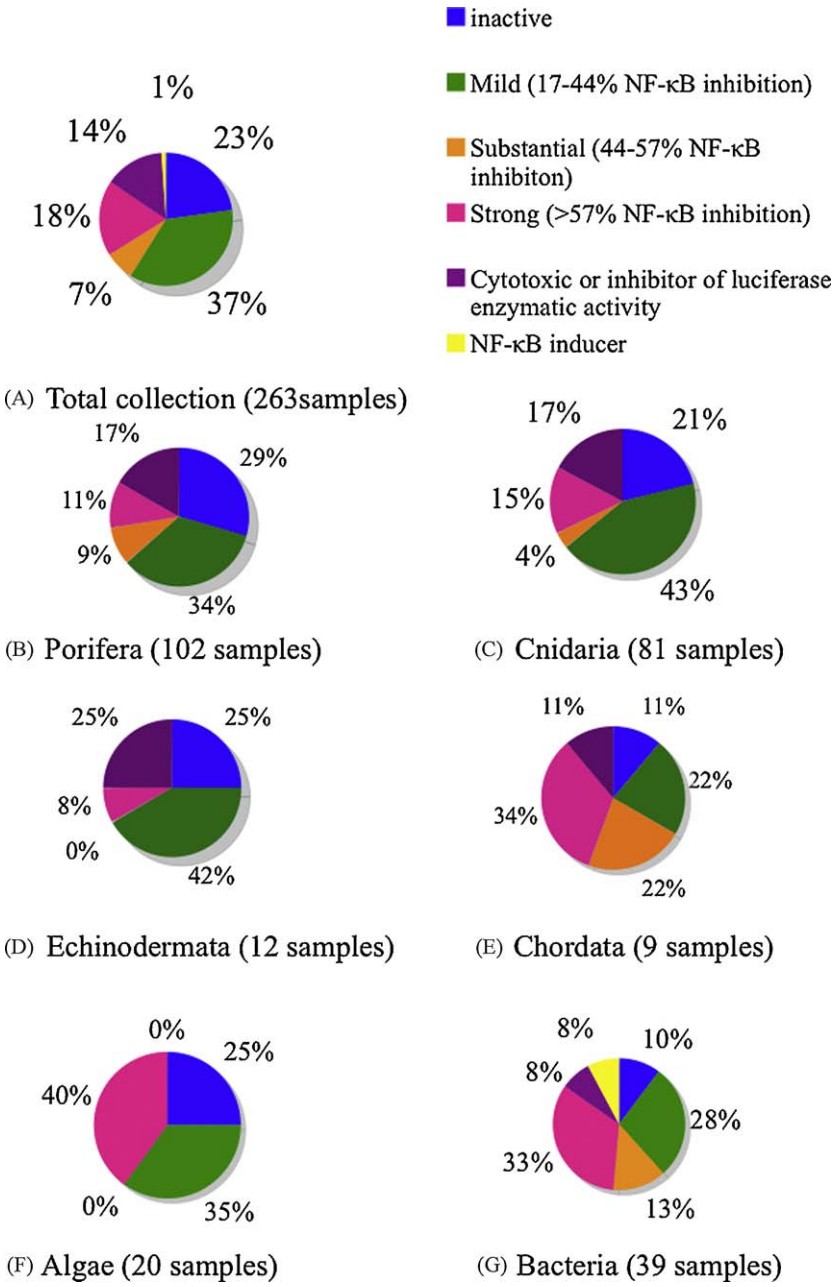


Fig. 1. Distribution of NF-κB inhibitory activity and cytotxicity of crude extracts across different phyla of marine organisms, as listed in Table 2. The screening for NF-κB inhibitory activity, using a luciferase reporter gene assay, includes a total of 263 crude extracts (A) isolated from marine organisms belonging to the phyla Porifera (102 representatives) (B), Cnidaria (81 representatives) (C), Echinodermata (12 representatives) (D), Chordata (9 representatives) (E), Chloro-, Phaeo-, and Rhodophyta (grouped under Algae) (20 representatives) (F), or Firmicutes, Bacteroidetes, Cyano-, Actino-, or Proteobacteria (grouped under Bacteria) (39 representatives) (G). Each extract was assayed in duplicates, at a single test concentration of 100 μg/mL. The cytotoxicity and the effects of the extracts on the enzymatic activity of luciferase were assessed at the same test concentration. Strong NF-κB inhibition is defined as ≥2.3 fold decrease in NF-κB-induced luciferase activity, in comparison with control cells. Substantial NF-κB inhibition refers to a 1.8–2.3 fold decrease in luciferase activity. Mild NF-κB inhibition refers to a 1.2–1.8 fold decrease in luciferase activity. Neutral in terms of NF-κB inhibitory activity refers to a ≤1.2 fold decrease in luciferase activity. A cytotoxic sample is defined as a sample inducing ≥25% cell death, in comparison with the control. An inhibitor of the enzymatic activity of luciferase is defined as an inducer of ≥25% decrease in enzymatic activity of luciferase, in comparison with the control. Extracts that enhanced the TNF-α-induced NF-κB activity by ≥25% are classified as NF-κB inducers.

compounds were prepared in DMSO at a concentration of 100 mg/mL. 1 μL of the solution was added to 1 mL of cell suspension to achieve a final concentration of 100 μg/mL. The mixtures were incubated for 2 h at 37 °C, with 5% CO_2. After the incubation period, the cells were centrifuged for 5 min at 1.2 rcf, washed twice with PBS 1×, and resuspended in 1 mL of PBS 1× supplemented with 5 μL of Hoechst staining solution (bisbenzimide H33258 fluorochrome trichloride) (BD Biosciences (Erembodegem, Belgium)). After 10 min of incubation with the Hoechst staining solution in the dark and at room temperature, the cell suspensions were placed onto glass slides and analyzed under an inverted fluorescence microscope (Leica Microsystems (Wetzlar, Germany)) (1000× magnification; excitation filters: excitation/emission filter cubes—340–380 nm (UV excitation), 400–425 nm (blue emission) and 450–490 nm (blue excitation), 510–515 nm (green emission)). The background fluorescence of Jurkat cells was assessed using non-treated cells. The images taken with the different filters were merged using the ImageJ software [40].

2.11. Co-transfection experiments

The effects of the test compounds on TNFR1-, TRADD-, TRAF2-, and IKKβ-induced NF-κB activation were analyzed by luciferase reporter gene assay as described above, using K562 cells transfected with the reporter gene plasmids pNF-κB-consensus and pRhLuc, as well as with either of the pTNFR1, pTRADD, pTRAF2, or pIKKβ expression vector plasmids. The pTNFR1, pTRADD, pTRAF2, and pIKKβ expression vector plasmids were generous gifts from Prof. Bharat Aggarwal (University of Texas M.D. Anderson Cancer Research Centre, Houston, TX, U.S.A.). Each compound was tested in two independent experiments.

2.12. IKKβ protein kinase assays

The effects of the test compounds on the kinase activity of IKKβ were investigated using a K-LISA IKKβ inhibition assay kit (Calbiochem, San Diego, CA, U.S.A.) as described in the manufacturer's protocol. The IKKβ inhibitor Calbio IV provided with the K-LISA kit was used (at a concentration of 1 μM) as a positive control. Each compound was tested in three independent experiments.

2.13. Effects of the test compounds on the chymotrypsin-like protease activity of the 26S proteasome

The effects of the test compounds on the chymotrypsin-like protease activity of the 26S proteasome were evaluated using a Proteasome-Glo™ assay kit (Promega, Leiden, Netherlands). 100 μL of K562 leukaemia cells prepared at a concentration of 2×10^5 cells/mL in RPMI 1640 culture medium supplemented with 10% FBS was treated for 2 h at 37 °C with 100 μg/mL of the test compounds. 100 μL of Proteasome-Glo™ reagent containing luciferase and the proteasome substrate succinyl-leucine-leucine-valine-tyrosine-aminoluciferin was then added to each well, and after 10 min of shaking at room temperature the luminescence was measured on a luminometer. The proteasome-inhibiting peptide aldehyde MG-132 (Calbiochem (San Diego, CA, U.S.A.)) was used as a positive control. A viability assay using the Cell-Titer Glo® kit (Promega, Leiden, Netherlands) as described in the manufacturer's protocol was run in parallel with the proteasome assay in order to standardize the results of the proteasome assay to the number of live cells in each sample at the end of the assay. Each compound was tested in four independent experiments.

2.14. Statistical analysis

The results presented in this paper were obtained from at least two independent experiments. The data were analyzed for statistical significant differences using the Student's *t*-test. The results are expressed as the mean ± SD. *p*-Values below 0.05 (*) or 0.01 (**) were considered as statistically significant.

3. Results

3.1. Large-scale screening of marine natural products for NF-κB inhibitory activity

The large-scale screening of extracts from marine organisms was performed using luciferase reporter gene assays. In parallel with the luciferase reporter gene assays, all the samples were screened for their cytotoxicity on the K562 test cells over the incubation period of the luciferase reporter gene assays. All the experiments were performed at a single test concentration of 100 μg/mL, and in a double-blind manner. Taxonomic and bioactivity data were only exchanged after completion of the screening assays. The quantitative results of the large-scale screening were arbitrarily translated to a qualitative scale, based on observations made during the investigation of the NF-κB inhibitory potential of kava derivatives [41]. Extracts achieving a ≤1.2 fold (17%) decrease in NF-κB-induced luciferase activity, compared to the negative control, are classified as neutral in terms of NF-κB inhibitory activity. Extracts achieving a 1.2–1.8 fold (17–44%) decrease in NF-κB-induced luciferase activity, compared to the negative control, are classified as mild inhibitors of NF-κB activation. Extracts achieving a 1.8–2.3 fold (44–57%) decrease in NF-κB-induced luciferase activity, compared to the negative control, are classified as substantial inhibitors of NF-κB activation. Extracts achieving a ≥2.3 fold (57%) decrease in NF-κB-induced

Table 1
Fatty acid composition of the microalgae included in the large-scale screening for NF-κB inhibitors. Values given as percentage of total FAMEs in each algal extract, based on the peak areas on the GC chromatograms. CCAP 213/10, *Chlorococcum submarinum* strain isolated from brackish waters in Merseyside (U.K.); CCAP11/17, *Chlamydomonas noctigama* strain isolated from a meadow lake in the former Czechoslovakia; CCMP 1619, *Chlamydomonas raudensis* strain isolated from Lake Bonney, Antarctica (freshwater); ATCC 50216, *Chlamydomonas hedleyi* strain isolated from seawater in the Florida Keys (U.S.A.).

	Algal strain			
	CCAP 213/10	CCAP 11/17	CCMP 1619	ATCC 50216
Myristic acid (14:0)	7.47%	3.47%	3.51%	4.18%
Palmitic acid (16:0)	69.34%	38.81%	25.01%	47.16%
Heptadecanoic acid (17:0)	1.66%	<1%	<1%	14.43%
Stearic acid (18:0)	3.95%	16.31%	3.06%	1.06%
Oleic acid (18:1) (ω-9 fatty acid)	<1%	<1%	17.40%	3.88%
Eicosenoic acid (20:1) (ω-9 fatty acid)	4.99%	2.48%	3.58%	6.48%
Nervonic acid (24:1) (ω-9 fatty acid)	<1%	9.77%	<1%	<1%
Linoleic acid (18:2) (ω-6 fatty acid)	<1%	1.11%	4.21%	<1%
α-Linolenic acid (18:3) (ω-3 fatty acid)	<1%	<1%	12.31%	<1%
Others	13%	28%	31%	23%

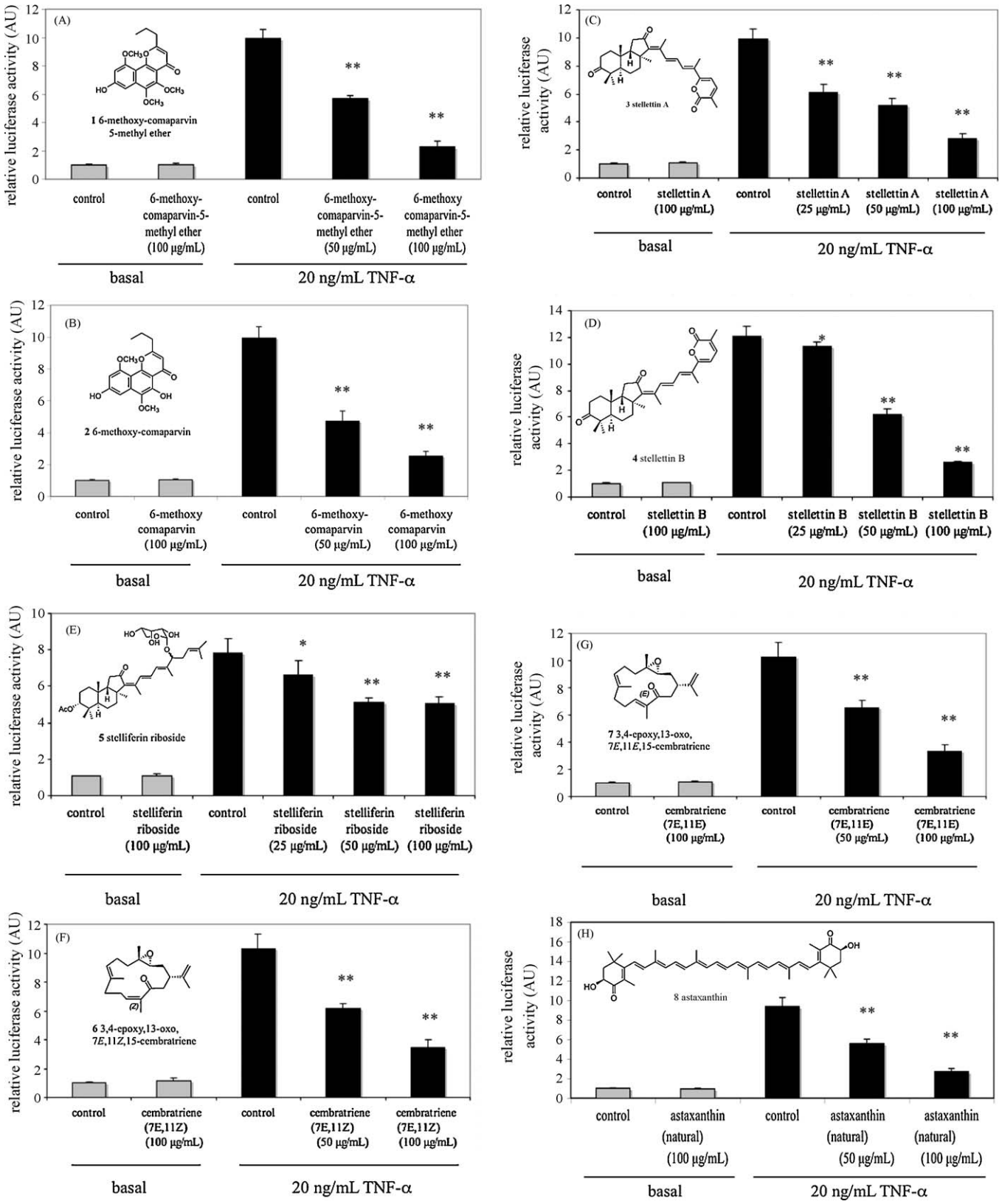


Fig. 2. NF-κB inhibitory potential of marine natural products isolated from Fijian extracts shown to inhibit TNF-α-induced transcriptional activity of NF-κB. Luciferase activity of pNF-κB-Luc K562 cells pre-treated for 2 h with different concentrations (in µg/mL) of 6-methoxy comaparvin-5-methyl ether (ComME) (A), 6-methoxy comaparvin (Com) (B), stellettin A (StA) (C), stellettin B (StB) (D), stelliferin riboside (E), 3,4-epoxy,13-oxo,7*E*,11*Z*,15-cembratriene (CemEZ) (F), 3,4-epoxy,13-oxo,7*E*,11*E*,15-cembratriene (CemEE) (G), natural/synthetic astaxanthin (AstN/AstS) (H/I), or jasplakinolide (Jasp) (J), and treated for 2 h with 20 ng/mL of TNF-α. The results in (A–J) are given as a ratio of the luminescence measured for the firefly luciferase divided by the luminescence measured for the *Renilla* luciferase. The negative control in (A–J) refers to the transcription activity of NF-κB in absence of any test compound. Effects of the test compounds, at a concentration of 100 µg/mL, on the viability of the K562 test cells and on the enzymatic activity of luciferase over the incubation period of the luciferase reporter gene assay, in comparison with the effects of the compounds on NF-κB-induced luciferase gene expression under the same treatment conditions as for A–J (K). Results shown as mean ± SD of eight individual measurements. * and ** refer to $p < 0.05$ and $p < 0.01$, respectively, in comparison with the control.

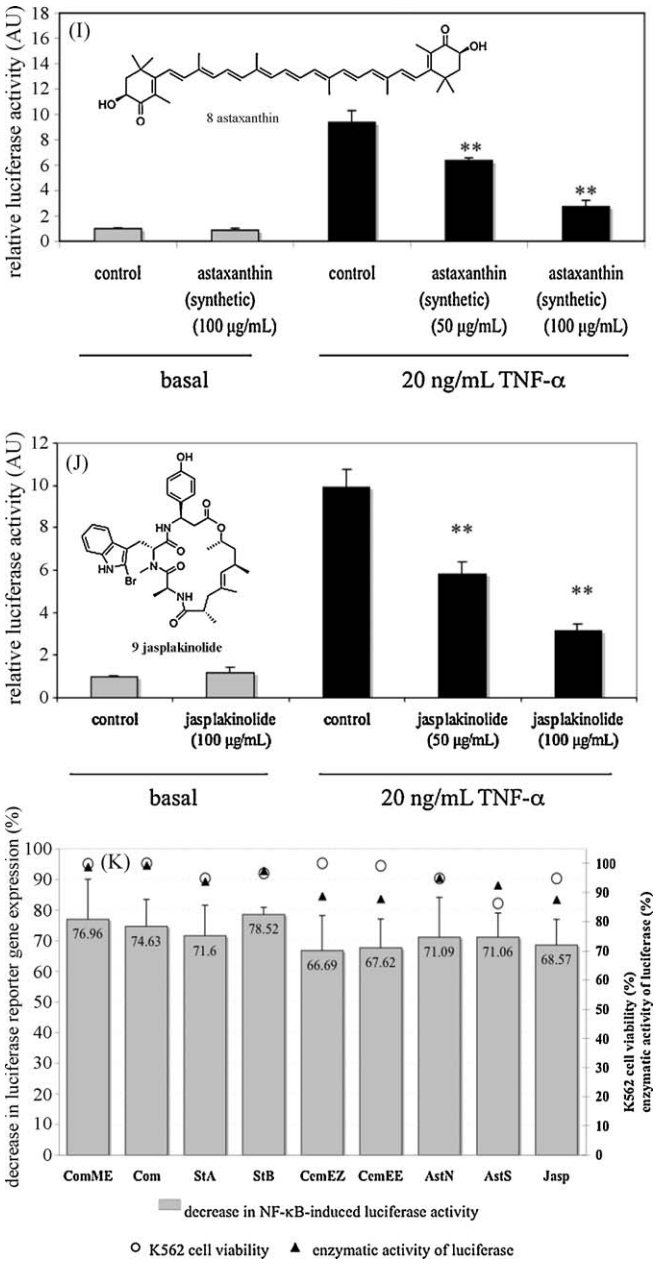


Fig. 2. (*Continued*).

luciferase activity, compared to the negative control, are classified as strong inhibitors of NF-κB activation. Samples classified as strong NF-κB-inhibitors were evaluated for their *in vitro* effects on the enzymatic activity of luciferase. The extracts that, during the time frame of the screening assays, were shown to induce ≥25% cell death or to provoke ≥25% decrease in enzymatic activity of luciferase, in comparison with the negative control, have been grouped together as samples leading to false positive results in the luciferase reporter gene assay used for the large-scale screening. Samples enhancing the TNF-α-induced activation of NF-κB by ≥25% are classified as inducers of NF-κB. The results of the large-scale screening, at a phylum level, are shown in Fig. 1. The genus- and strain-specific results of the screening can be found in the supplementary material. As shown in Fig. 1, 18% of the tested extracts strongly inhibited TNF-α-induced NF-κB activity at a concentration of 100 μg/mL. The most active groups of marine organisms included in the screening were the algae (40% hit rate of strong (≥2.3 fold decrease) NF-κB inhibitory activity), the chordates (34% hit rate), and the bacteria (33% hit rate). Three of the bacterial extracts, all isolated from *Pseudomonas* sp. strains, were shown to stimulate TNF-α-induced NF-κB activity at the test concentration of 100 μg/mL.

3.2. Fatty acid composition of the microalgae screened for NF-κB inhibitory activity

The fatty acid (FA) composition of the microalgae included in the large-scale screening for NF-κB inhibitors is presented in Table 1.

As shown in Table 1, there is little difference in FA composition between the extracts from the *Chlorococcum* strain and the extracts from the *Chlamydomonas* strains. All the extracts contain high amounts (up to 81% of the organisms' total FA composition) of the three saturated fatty acids palmitic acid (16:0), stearic acid (18:0), and myristic acid (14:0). The only noticeable difference is that the *Chlorococcum* extract is proportionally richer in myristic (14:0) and palmitic (16:0) acid than the *Chlamydomonas* extracts.

At an *intra*-genus level, on the other hand, more important differences in FA composition can be observed. Amongst the extracts from the three *Chlamydomonas* strains, the Antarctic strain CCMP 1619 is the only one that accumulates the ω-3 poly-unsaturated fatty acid (PUFA) linolenic acid in high concentrations (12% of the organism's total FA composition). CCMP 1619 and CCAP 11/17 accumulate large amounts of ω-9 mono-unsaturated FAs oleic acid (18:1) and nervonic acid (24:1), respectively, while ATCC 50216 is the only one to accumulate high levels (14%) of saturated heptadecanoic acid (17:0). The three *Chlamydomonas* strains included in the study accumulate similar concentrations of ω-6 PUFA linoleic acid (18:2) (0–4% of the organisms' total FA composition) and of ω-9 mono-unsaturated FA eicosenoic acid (20:1) (2–6% of the organisms' total FA composition).

3.3. Chemical identity and NF-κB inhibitory potential of marine natural products isolated from Fijian extracts shown to inhibit TNF-α-induced transcriptional activity of NF-κB

Extracts from five Fijian marine invertebrates, including the crinoid *C. parvicirrus* (FJ01-199), the sponge *R. globostellata* (FJ97125D114), the soft corals *Sarcophyton* sp. nov. (FJ03-147) and *Sinularia* sp. (FJ01-111), and the gorgonian *Subergorgia* sp. (FJ01-160), were kindly provided by the IAS Marine Collection (University of the South Pacific, Suva, Fiji) for further investigation of the chemical structure and mechanism of action of the bioactive compounds. Following the bioassay-guided purification of the extracts by chromatography, the chemical structures of the major bioactive compounds were elucidated by NMR, UV, and mass spectrometry. The extract from the crinoid *C. parvicirrus* yielded two major bioactive compounds, namely the naphtopyrones 6-methoxycomaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) previously identified by Smith and Sutherland [42]. The extract from the sponge *R. globostellata* yielded the three bioactive triterpenoids stellettin A (**3**), stellettin B (**4**), and stelliferin riboside (**5**) previously isolated from various species of marine sponges [43–47]. The two known cembranoids 3,4-epoxy,13-oxo,7*E*,11*Z*,15-cembratriene (**6**) and 3,4-epoxy,13-oxo,7*E*,11*E*,15-cembratriene (**7**) [48] were identified as the major bioactive compounds of the extract isolated from the soft coral *Sarcophyton* sp. nov. The major bioactive compound isolated from the extract of the gorgonian *Subergorgia* sp. was identified as astaxanthin (**8**), a carotenoid produced by several marine bacteria and algae and accumulated from their diet by wide range of marine organisms [31,49,50]. Because of the difficulty to isolate pure astaxanthin (**8**) from the *Subergorgia* extract, synthetic astaxanthin (**8**) was tested in

parallel with the impure natural astaxanthin (**8**). The extract from the soft coral *Sinularia* sp. yielded the cyclodepsipeptide jasplakinolide (**9**) as the major bioactive compound. Jasplakinolide (**9**) is normally isolated from sponges, rather than form soft corals [51], suggesting that the *Sinularia* sp. extract included in our study was probably contaminated with spongean material on which the soft coral had been encrusted.

The effects of the marine natural products **1**–**9** on TNF-α-induced transcriptional activity of NF-κB was examined using a luciferase reporter gene assay on pNF-κB-Luc K562 cells. The results are presented in Fig. 2. As shown in Fig. 2, none of the tested compounds has any effect on basal NF-κB transcription. All the compounds reduce TNF-α-induced NF-κB activation in a concentration-dependent manner. All the compounds, except (**5**), achieved a strong decrease in the transcriptional activity of NF-κB at a concentration of 100 μg/mL, which is equivalent to 216 μM for (**3**) and (**4**), 331 μM for (**6**) and (**7**), 168 μM for both natural and synthetic (**8**), and 141 μM for (**9**). (**5**) showed little NF-κB inhibitory activity, even at the highest test concentration of 100 μg/mL, which corresponds to 159 μM. Due to its poor bioactivity in the luciferase reporter gene assay, (**5**) was discarded from the remaining bioassays.

3.4. Cytotoxic effect of NF-κB-inhibiting natural products isolated from Fijian marine organisms on the human leukaemia cell line K562

The viability of K562 cells exposed to 100 μg/mL of the Fijian marine natural products **1**–**4** and **6**–**9** over the 4 h long test period of the luciferase reporter gene assay is shown in Fig. 2K. The results indicate that none of the tested compounds severely affects the viability of K562 leukaemia cells in the test conditions of the luciferase reporter gene assay, and cytotoxicity of the test compounds can be excluded as a potential source of false positive results.

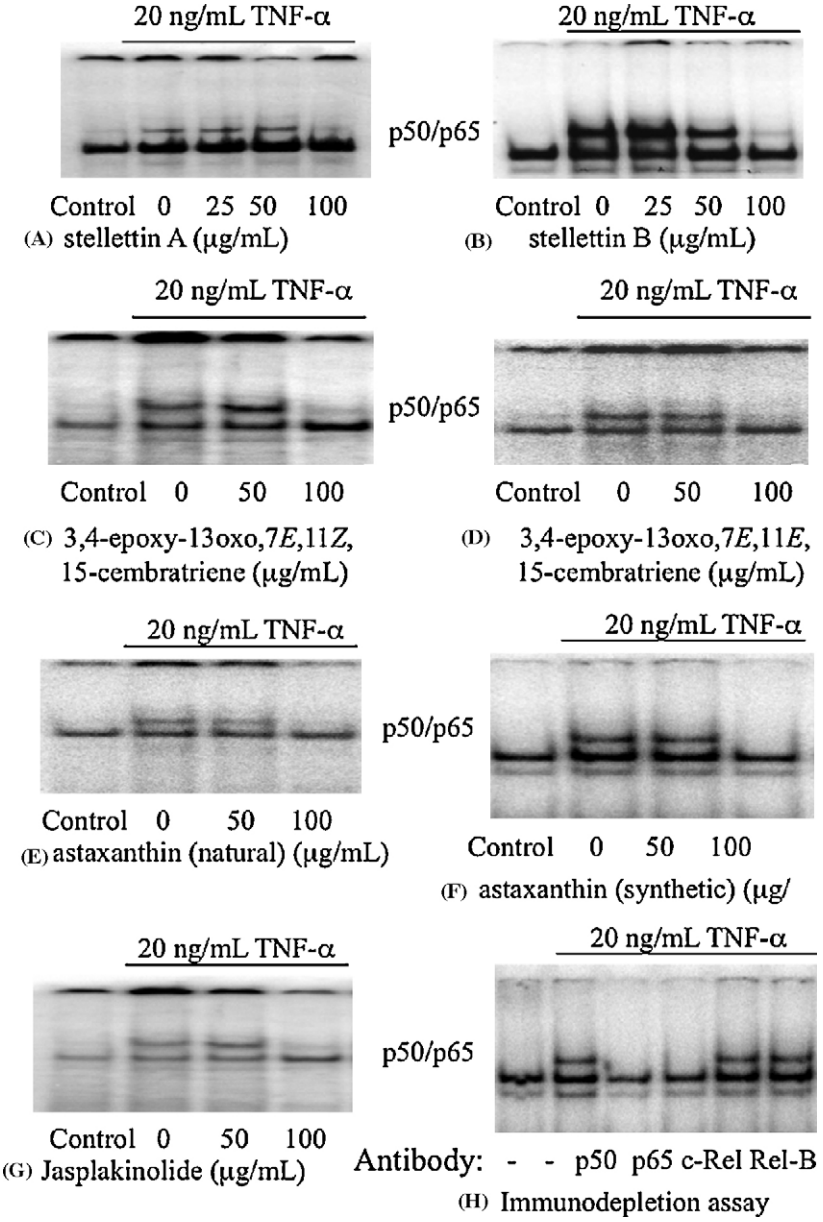


Fig. 3. Inhibition of TNF-α-induced NF-κB-DNA binding by NF-κB-inhibiting natural products isolated from Fijian marine organisms. Jurkat cells were pre-treated for 2 h with different concentrations (in μg/mL) of stellettin A (A), stellettin B (B), 3.4-epoxy,13-oxo,7*E*,11*Z*,15-cembratriene (C), 3,4-epoxy,13-oxo,7*E*,11*E*,15-cembratriene (D), natural astaxanthin (E), synthetic astaxanthin (F), or jasplakinolide (G), before being treated for 2 h with 20 ng/mL of TNF-α. For the immunodepletion assay, the nuclear extracts and labeled probes were incubated in the reaction mixture for 30 min on ice with 2 μg of anti-p50, anti-p65, anti-c-Rel, and anti-Rel-B antibodies (H).

3.5. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on the enzymatic activity of luciferase

The effects of the Fijian marine natural products **1**–**4** and **6**–**9** on the enzymatic activity of luciferase are shown in Fig. 2K. The results indicate that none of the tested Fijian marine natural products severely affects the enzymatic activity of luciferase at the concentrations at which a strong decrease in luciferase activity was observed in the luciferase reporter gene assays (100 μg/mL), confirming that the results obtained in the luciferase assay are unbiased by effects of the test compounds on the enzymatic activity of luciferase.

3.6. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on TNF-α-induced NF-κB-DNA binding

The inhibition of TNF-α-induced NF-κB activation by the Fijian marine natural products **3**–**4** and **6**–**9** was confirmed by EMSA in Jurkat T-cells. As shown in Fig. 3, all the tested compounds achieve complete inhibition of TNF-α-induced binding of NF-κB to its DNA binding site at a concentration of 100 μg/mL, which corresponds to 216 μM for (**3**) and (**4**), 331 μM for (**6**) and (**7**), 168 μM for (**8**), and 141 μM for (**9**). At a concentration of 50 μg/mL, the inhibition is either very weak, or completely absent.

In order to investigate the direct interferences, at the nuclear level, of compounds **1**–**4** and **6**–**9** with the binding of activated NF-κB to DNA, Jurkat cells were treated with TNF-α in order to induce the activation of NF-κB. The nuclear extracts were incubated with the test compounds prepared in binding buffer. The nuclear extracts from the treated cells were incubated with the ^{32}P ATP-labeled NF-κB c probe, and the DNA–protein complexes were analyzed by electrophoresis as described above. As shown in Fig. 4, **1**–**4**, (**6**), (**7**), and (**9**), but not (**8**) (natural or synthetic) have a direct inhibitory effect on the binding of NF-κB to its DNA binding site. Since inhibiting the transcriptional activity of NF-κB by targeting the binding of NF-κB to its DNA binding site requires the access of the bioactive molecule into the cells, and into the nuclei, we analyzed whether the NF-κB-inhibiting Fijian marine natural products were capable of penetrating into the nucleus of Jurkat cells, using fluorescent microscopy. As shown in Fig. 5, all the marine natural products tested in the present survey are naturally fluorescent. Yellow or green fluorescence is, as a matter of fact, a widespread property of marine natural products [52]. In terms of chemical ecology, the fluorescence of marine secondary metabolites provides cellular and tissue protection against UV radiation, and fluorescent natural products are frequently used by numerous marine organisms for underwater signaling [52]. The fluorescence of the compounds was used to illustrate that the test compounds are able to permeate Jurkat leukaemia cells and to penetrate into the latter's nuclei in less than 2 h. Background fluorescence of untreated cells was negligible compared to the fluorescence emitted by the treated cells (Fig. 5).

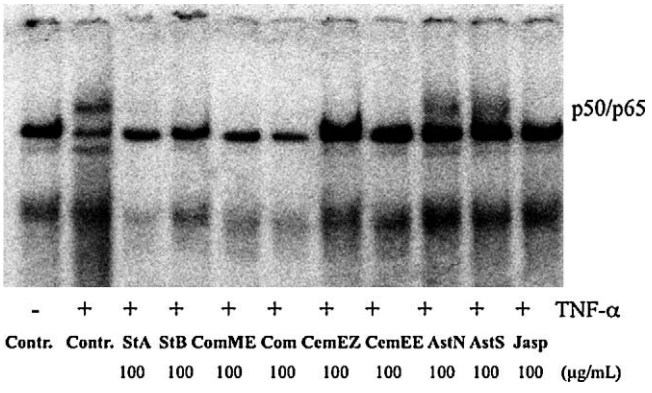


Fig. 4. Direct interferences of stellettin A (StA), stellettin B (StB), 6-methoxy-comaparvin-5-methyl ether (ComME), 6-methoxy-comaparvin (Com), 3,4-epoxy,13-oxo,7*E*,11*Z*,15-cembratriene (CemEZ), 3,4-epoxy,13-oxo,7*E*,11*E*,15-cembratriene (CemEE), natural astaxanthin (AstN), synthetic astaxanthin (AstS), and jasplakinolide (Jasp) with the binding of activated NF-κB to DNA. Jurkat cells were treated with 20 ng/mL of TNF-α for 2 h. 10 μg of nuclear extract was incubated for 20 min with 5 μL of binding solution containing 100 ng/mL of test compound, followed by a 20 min long incubation with the radioactively labeled probe. The control (Contr.) refers to nuclear extracts treated with no test compound.

3.7. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on TNF-α-induced IκBα degradation and on TNF-α-induced nuclear translocation of p50/p65

In order to further investigate the effects of the Fijian marine natural products **3**, **4**, and **6**–**9** on the TNF-α-induced activation of NF-κB, Western blot analyses of cytosolic and nucleic protein extracts from Jurkat cells pre-treated with the Fijian marine natural products for 2 h and treated with TNF-α for different amounts of time were performed. The blotted membranes were analyzed using primary antibodies against IκBα, p50, and p65. The results show that all the tested Fijian marine natural products prevent TNF-α-induced degradation of IκBα and inhibit the nuclear translocation of p50 and p65 (Fig. 6).

3.8. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on NF-κB activation induced by an overexpression of TNFR1, TRAF2, TRADD, or IKKβ

In order to further investigate the mechanism of action of the marine NF-κB inhibitors **1**–**4** and **6**–**9**, co-transfection-based luciferase reporter gene assays were performed. K562 leukaemia cells were co-transfected with the pNF-κB-Luc and phRL-TK plasmids, together with either one of the pTNFR1, pTRAF2, pTRADD, or pIKKβ plasmids. As shown in Fig. 7A, at the MIC value observed in the EMSA (100 μg/mL), none of the tested Fijian marine natural products significantly inhibits the NF-κB activity induced by the overexpression of the TNF-α receptor TNFR1, or of the TRAF2 or TRADD proteins. However, at the same test concentration of 100 μg/mL, (**1**) and (**7**) significantly ($p < 0.01$) inhibited the NF-κB activity induced by the overexpression of IKKβ. (**2**), (**4**), (**6**) and (**9**) were shown to be weak ($p < 0.05$) inhibitors of IKKβ-induced NF-κB activation. (**8**) did not have any substantial effect on the NF-κB activation induced by an overexpression of IKKβ.

3.9. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on the kinase activity of IKKβ

The potential of the NF-κB inhibitors **1**–**4** and **6**–**9** to inhibit the kinase activity of IKKβ was assessed using an IKKβ kinase assay. The results indicate that all the tested compounds inhibit the kinase activity of IKKβ in a concentration-dependent manner. Nevertheless, the IKKβ inhibitory potential of (**3**) and (**4**), and of (**8**) was very weak, even at the highest test concentration (200 μg/mL). (**2**), (**6**), and (**9**) only induced a significant ($p < 0.01$) inhibition of IKKβ at, and above the test concentrations of 200 μg/mL (606 μM), 100 μg/mL (331 μM), and 200 μg/mL (282 μM), respectively. (**1**) and (**7**) were the strongest IKKβ inhibitors amongst the tested compounds. (**1**) and (**7**) inhibited IKKβ in a significant ($p < 0.01$) manner at a concentration of 50 μg/mL, which corresponds to 145 μM for (**1**) and to 165 μM for (**7**) (Fig. 7B). These results are in agreement with the results obtained in the luciferase reporter gene assay performed with K562 cells co-transfected with a pIKKβ expression vector plasmid (Fig. 7A).

3.10. Effects of NF-κB-inhibiting natural products isolated from Fijian marine organisms on the proteolytic activity of the 26S proteasome

The last component of the NF-κB activation pathway investigated as a potential target for the NF-κB-inhibiting natural products isolated from Fijian marine organisms was the 26S proteasome, which is known to degrade poly-ubiquitinated IκB, thereby releasing the active p50/p65 dimer for nuclear translocation. The results indicate that, at the MIC value observed in the EMSA (100 μg/mL), (**1**) was the only tested compound shown to significantly ($p < 0.01$) inhibit the proteolytic activity of the 26S proteasome. The proteasome-inhibiting potential of (**1**) (1.6 fold decrease in proteolytic activity at a test concentration of 303 μM) is, however, very weak in comparison with the commercially available proteasome-inhibiting peptide aldehyde MG-132 [53], which was shown decrease the proteolytic activity of the 26S proteasome by a 10 fold at a concentration of 10 μM) (Fig. 7C).

4. Discussion

The aim of the present study was to search for novel inhibitors of NF-κB, a transcription factor that has recently become a major target in drug discovery due to its severe implications in cancer development, inflammation, and many other diseases [7,8,54,55]. Out of the 263 extracts from marine organisms screened for their potential to inhibit TNF-α-induced NF-κB activation, 18% had a strong NF-κB inhibitory potential at a concentration of 100 μg/mL. This high hit rate does not come as a surprise, as marine natural products have recently been recognized as promising candidates for NF-κB inhibition [20,56,57]. The two NF-κB-inhibiting marine natural products macrolide bryostatin 1 isolated from a bryozoan [56] and salinosporamide A isolated from a bacterium [24] are currently in clinical trials [58]. Several ecological reasons why marine natural products may inhibit NF-κB have been established [20,21,59–69].

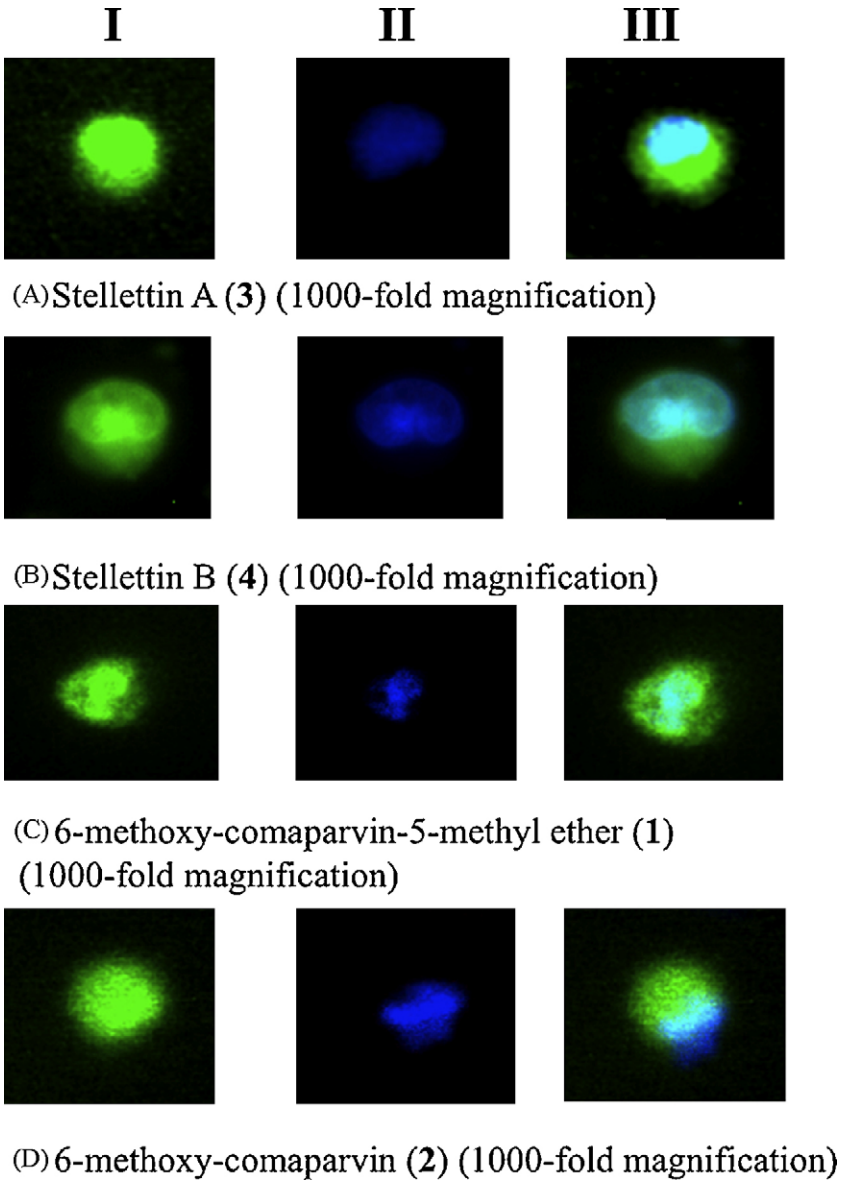
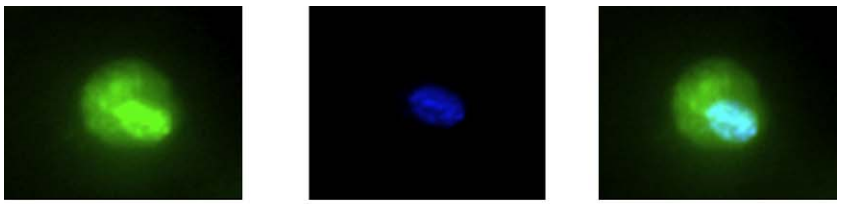
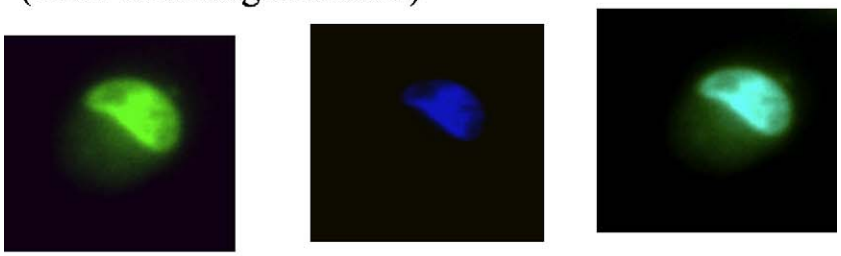


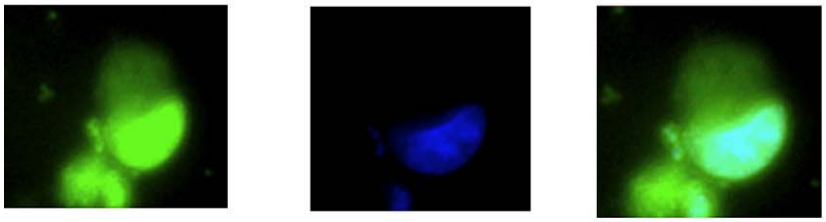
Fig. 5. Fluorescent microscopy based visualization of the distribution of stellettin A (A), stellettin B (B), 6-methoxy-comaparvin-5-methyl ether (C), 6-methoxy-comaparvin (D), 3,4-epoxy,13-oxo,7*E*,11*Z*,15 cembratriene (E), 3,4-epoxy,13-oxo,7*E*,11*E*,15 cembratriene (F), and jasplakinolide (G) in Jurkat leukaemia cells after a 2 h long incubation with the test compounds at a concentration of 100 μg/mL. The background fluorescence of untreated Jurkat cells is shown as a reference (H). Column I: Jurkat cells permeated by the naturally fluorescent test compounds (340–380 nm excitation filter, 400–425 nm emission filter). Column II: Visualization of the Hoechst-stained cellular nuclei (450–490 nm excitation filter, 510–515 nm emission filter). Column III: Merge of the image in column I with the image in column II. Each slide corresponds approximately to an area of 10 μm × 10 μm.



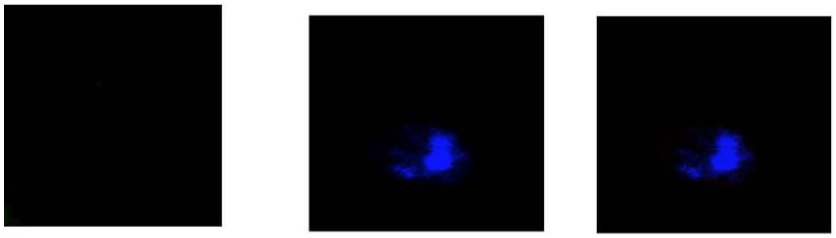
(E) 3,4-epoxy-13-oxo-7*E*,11*Z*,15-cembratriene (**6**) (1000-fold magnification)



(F) 3,4-epoxy-13-oxo-7*E*,11*E*,15-cembratriene (**7**) (1000-fold magnification)



(G) jasplakinolide (**9**) (1000-fold magnification)



(H) background fluorescence (no treatment) (1000-fold magnification)

Fig. 5. (*Continued*).

In our study, the most abundant sources of NF-κB inhibitors were algae, chordates, and bacteria. The algae not only had the highest hit rate in terms of percentage of extracts with strong NF-κB inhibitory potential, they were also the source of extracts with the highest NF-κB inhibitory strength at the single test concentration of 100 μg/mL. Within most phyla, a good correlation between the taxonomy of the source organisms and the NF-κB inhibitory potential of the extracts could be observed. In order to gain further insight into the possible correlations between the taxonomy, metabonomics, and ecological properties of the source organisms and the NF-κB inhibitory potential of the extracts, we evaluated the FA composition of the microalgal samples included in the screening. Algae are known to be rich in FAs [70–72], and their FA composition can, to a certain extent, be used as fingerprints for their taxonomy or for the environmental conditions of their habitat [32,34,70]. Little difference could be seen between the FA composition of the two genera. Amongst the *Chlamydomonas* samples, however, noticeable differences in the FA composition could be observed. While the three tested strains of *Chlamydomonas* are almost indistinguishable in terms of their phylogeny [73], they originate from very different ecosystems. The Antarctic strain CCMP 1619 was the only one to accumulate the ω-3 PUFA linolenic acid (18:3) at a high concentration (12% of the total FA composition). The concentrations of ω-6 PUFA linoleic acid (18:2) and of the ω-9 mono-unsaturated FA eicosenoic acid (20:1) were similar from one species to another. Our results are in accord with Poerschmann et al., who showed that the unsaturation index of FAs in algae and bacteria tends to be higher in colder climates than in warmer climates [70,74]. ω-3 PUFAs, but not ω-6 PUFAs, mono-unsaturated FAs or saturated FAs, have been shown to be potent

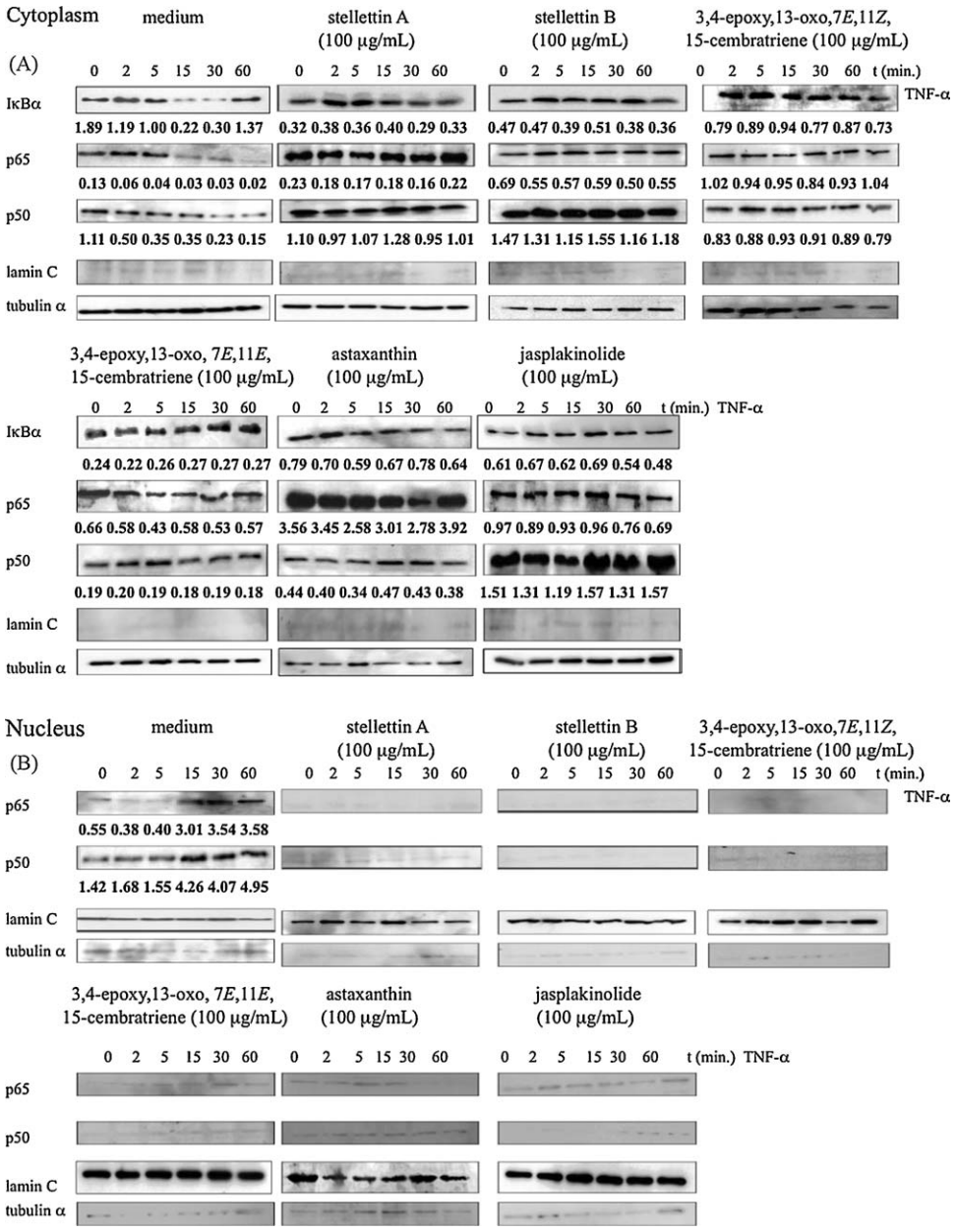


Fig. 6. Effect of stellettin A (**3**), stellettin B (**4**), 3,4-epoxy,13-oxo,7*E*,11*Z*,15 cembratriene (**6**), 3,4-epoxy,13-oxo,7*E*,11*E*,15 cembratriene (**7**), natural astaxanthin (**8**), and jasplakinolide (**9**) on TNF-α-induced IκBα degradation and nuclear translocation of NF-κB in Jurkat leukaemia cells. Jurkat cells (2×10^6 cells/mL) were incubated for 2 h at 37 °C (5% CO_2) with 100 μg/mL of the test compounds. The cells were then treated with 20 ng/mL TNF-α and incubated at 37 °C (5% CO_2) for the indicated times. The cytosolic and nuclear extracts were tested by Western blot analysis for the presence of IκBα, p50, and p65. Equal protein loading and purity of cytosolic/nuclear extracts were evaluated by tubulin-α (cytosolic) and lamin C (nuclear) Western blots. One of two independent experiments is depicted. The level of protein expression (band area × mean band intensity; shown in bold under the individual Western blots) is given as a ratio over the expression of tubulin-α (cytosolic extracts) or of lamin C (nuclear extracts).

NF-κB inhibitors [75–80]. One would hence have expected the CCMP 1619 extract, which has a 12 times higher concentration in ω-3 PUFA than the remaining extracts, to stand out as the strongest NF-κB inhibitor. However, our results disagreed with this hypothesis, as all the microalgal extracts strongly inhibited TNF-α-induced NF-κB activation, without any strong correlation between the bioactivity and the taxonomy of the source organisms or the FA composition of the extracts. A hypothetical explanation which has not been evaluated in our study, for the overall strong NF-κB inhibitory activity of the tested microalgal extracts, could be an ubiquitously high concentration of NF-κB-inhibiting carotenoids. High concentrations of carotenoids, and of astaxanthin in particular, have been previously reported in *Chlamydomonas*, *Chlorococcum*, and other microalgae [50,73,81], and most carotenoids are potent antioxidants [30,82], which renders them capable of interfering with NF-κB activation [83].

The high hit rate observed for chordate-derived extracts is novel and highly promising, as the only natural products isolated from this phylum and reported as NF-κB inhibitors to date is the quinone 3-demethylubiquinone Q_2 from the ascidian *Aplidium glabrum* [84].

At the end of the large-scale screening, five extracts from the IAS Marine Collection (University of the South Pacific, Suva, Fiji) were selected to pursue an in-depth investigation of the chemical characteristics and of the mechanisms of action of the natural products responsible for the NF-κB inhibitory activity observed in the crude extracts. Together, the extracts yielded eight compounds with diverse chemical structures which inhibited TNF-α-induced

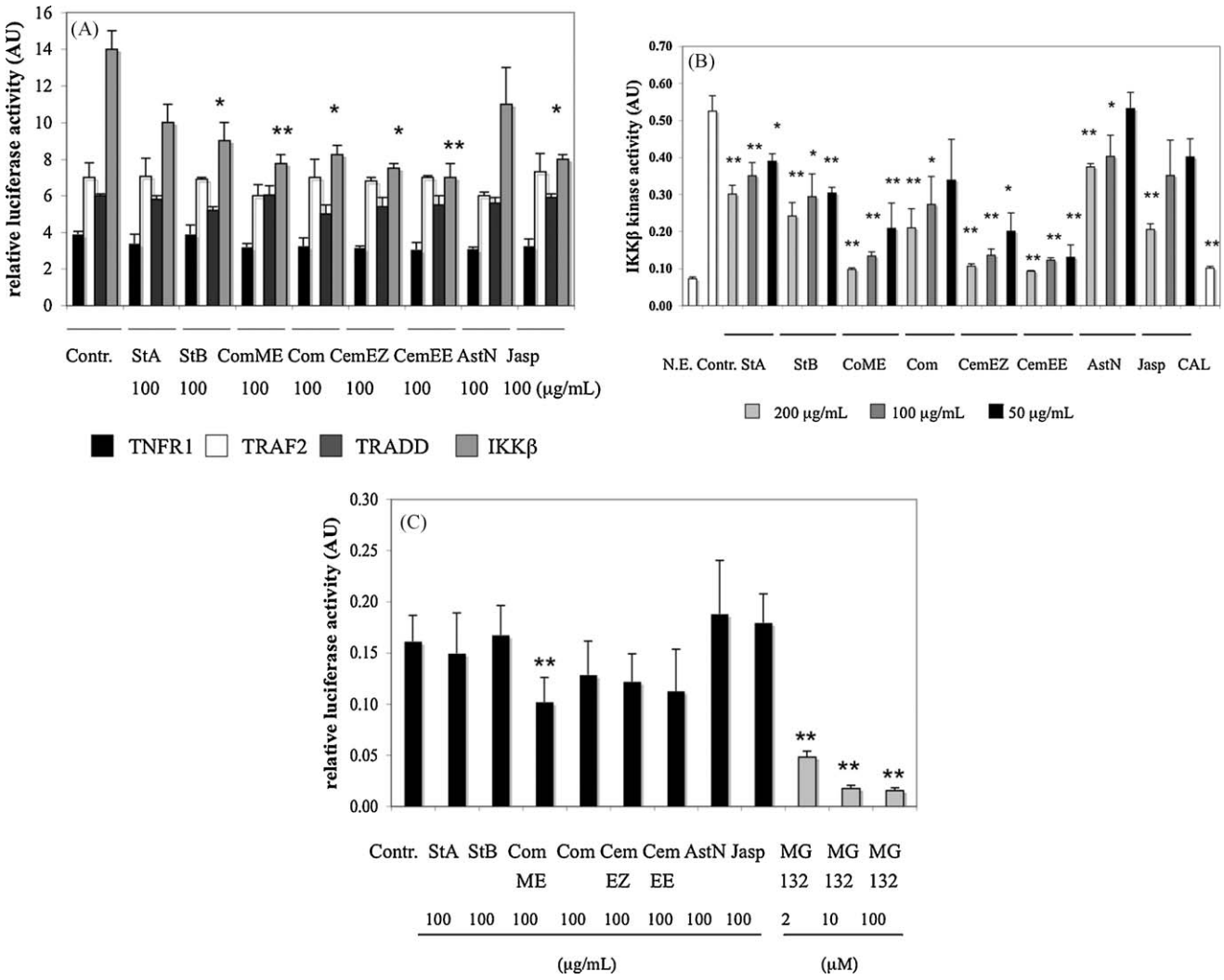


Fig. 7. Effects of stellettin A (StA), stellettin B (StB), 6-methoxy-comaparvin-5-methyl ether (ComME), 6-methoxy-comaparvin (Com), 3,4-epoxy,13-oxo,7*E*,11*Z*,15-cembratriene (CemEZ), 3,4-epoxy,13-oxo,7*E*,11*E*,15-cembratriene (Cem7EE), natural astaxanthin (AstN), or jasplakinolide (Jasp) on NF-κB activation induced by the over-expression of TNFR1, TRAF2, TRADD, or IKKβ in K562 cells (A), on the kinase activity of IKKβ (B), or on the proteolytic activity of the 26S proteasome in K562 cells (C) at the indicated test concentrations. In (A), the results are given as a ratio of the luminescence measured for the firefly luciferase divided by the luminescence measured for the *Renilla* luciferase, and the negative control (Contr.) refers to the transcription activity of NF-κB in absence of any test substance. In (B), (N.E.) refers to a negative control in absence of IKKβ, while (Contr.) refers to a negative control in presence of IKKβ, but in absence of any test compound. Calbio IV® (CAL) (1 μM) was used as a positive control. In (C), the results are shown as a ratio of the firefly luciferase activity measured in the proteasome assay over the firefly luciferase activity measured in a viability assay run in parallel with the proteasome assay. The negative control refers to the proteolytic activity of the 26S proteasome in absence of any test compound. MG132 (Z-Leu-Leu-Leu-CHO) (2–100 μM) was used as a positive control. All the results are shown as mean ± SD of at least two individual measurements. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, compared to the negative control.

NF-κB activation in a concentration-dependent manner, with MIC values as low as 141 μM. All the bioactive compounds except asthaxanthin (**8**) were shown to directly interfere with the binding of NF-κB to DNA. Upstream of the nuclear translocation of NF-κB, IKKβ was shown to be the major molecular target for most of the tested compounds. The naphtopyrones 6-methoxy-comaparvin-5-methyl ether (**1**) and 6-methoxy-comaparvin (**2**) isolated from the echinoderm *C. parvicirrus* and the isomalabaricanes stellettin A (**3**) and stellettin B (**4**) isolated from the sponge *R. globostellata* possess lactone rings with α,β-unsaturated carbonyl groups which may be responsible for the latter's interferences with IKKβ and with the binding of NF-κB to DNA as described previously for other NF-κB-inhibiting natural products [21]. 6-Methoxy-comaparvin-5-methyl ether (**1**) was the only compound shown to strongly inhibit the proteolytic activity of the 26S proteasome at the test concentration of 100 μg/mL. The carotenoid astaxanthin (**8**) isolated from the gorgonian *Subergorgia* sp., as well as its synthetic counterpart, was the only NF-κB-inhibiting compound included in our study for which no molecular target could be pinpointed. Most likely, the mechanism of action of the NF-κB inhibitor astaxanthin (**8**) is through its strong antioxidant potential, although the latter hypothesis was not tested in the present study. Astaxanthin is, indeed, a very potent antioxidant and free-radical scavenger [30,82,85], and oxidative stress is known to be implicated in the activation of NF-κB [83,86,87].

We conclude that marine algae, bacteria and invertebrates are a highly promising and hitherto largely unrecognized source of NF-κB inhibitors. Furthermore, NF-κB-inhibiting marine natural products target a wide range of molecules along the NF-κB activation pathway. Hence, marine natural products can be viewed as a treasure-case for anticancer, anti-inflammatory, and other NF-κB-related drug discovery. Unfortunately, marine drug discovery, especially when based on marine invertebrate-derived natural products, has been dogged since its early days by the difficulty to supply sufficient biomaterial for drug development [20]. Our results, which show that the cultured algae and bacteria are

amongst the richest sources of NF-κB inhibitors, provide some hope that this hurdle will soon become surmountable, as large amounts of active biomass of these organisms can be obtained through culture and fermentation. Further extracts identified in our large-scale screening assay as sources of NF-κB inhibitors are currently being investigated in order to determine the chemical and biological properties of the natural products responsible for the NF-κB inhibitory activity observed in the crude extracts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.05.009.

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