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Dimeric procyanidins are inhibitors of NF-κB–DNA binding☆

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ABSTRACT

Given the central role of the transcription factor NF-κB in inflammation, molecules that can inhibit NF-κB are being actively investigated. The present work characterize potential interactions between dimeric procyanidins [B-type (B1 and B2) and A-type (A1 and A2)] and NF-κB proteins. B1 and B2, inhibited tumor necrosis factor α (TNFα)- and phorbol 12-myristate 13-acetate (PMA)-induced transactivation of NF-κB-driven genes and the increase of NF-κB–DNA nuclear binding in Jurkat T cells. B1 and B2, added *in vitro* to nuclear fractions, inhibited NF-κB binding to its DNA consensus sequence. B1 and B2 prevented the binding of RelA and p50 recombinant proteins to its DNA consensus sequence. All these effects were not observed with A1 and A2. Putative molecular models for possible interactions of B1, B2, A1 and A2, with NF-κB proteins were constructed, indicating that B-type dimeric procyanidins have higher possibilities of chemical interactions with NF-κB than A-type dimeric procyanidins. The results support the concept that B-type dimeric procyanidins can provide anti-inflammatory benefits due to their ability to reduce NF-κB binding to the DNA.

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1. Introduction

The NF-κB signaling cascade has emerged as a crucial target in the treatment of inflammatory processes, and dietary factors that can influence this pathway are an area of considerable interest [1]. In this regard, plant flavonoids have received particular attention, and it has been speculated that diets rich in flavonoids could have beneficial effects in pathologies with a pro-inflammatory component [2].

The flavan-3-ols catechin and epicatechin are plant secondary metabolites present in high concentrations in certain plants, and foods and beverages based on these plants [3]. In addition to the catechin and epicatechin monomers, many of these plants contain their oligomers (procyanidins), the profile of the oligomers and the concentration of each individual procyanidin varies depending on the plant. For example, while, B-type dimeric procyanidins are found in *Theobroma cacao* (cocoa beans), A-type dimeric procyanidins are present in *Arachis hypogea* L. (peanuts) [4]. Chemically, the monomers of the B-type dimeric procyanidins are linked through a 4β → 6 or 4β → 8 carbon–carbon bond (Fig. 1). In the case of A-type dimeric procyanidins, the monomers are linked by both 4β → 8 carbon–carbon and 2β → O7 ether bonds (Fig. 1).

Transcription factor NF-κB, which can be activated by multiple signals, is a major effector pathway involved in inflammation and certain innate immune responses [5–7]. Excessive activation of the NF-κB pathway can lead to chronic inflammation, which in turn can contribute to the development and progression of a variety of degenerative diseases including tumor development [8]. The Rel/NF-κB family of proteins in human cells includes c-Rel, RelB, RelA (p65), p50/p105 and p52/p100. Generally for the canonical pathway, the phosphorylation by specific IκB kinases (IKK) of two serines (S32 and S36) present in IκBα initiates the activation of NF-κB [9]. Once IκBα is phosphorylated, ubiquitination followed by degradation are required for the complete activation of NF-κB, and its subsequent translocation into the nucleus [9]. In the nucleus, the binding of NF-κB to κB binding sites is a critical event for the regulation of gene expression.

Epidemiological and experimental evidence suggest that the regular consumption of flavan-3-ol-rich foods is associated with a number of positive beneficial health effects [10–13]. In both, *in vitro* and in vivo models, flavan-3-ols and procyanidins have been shown to improve markers of vascular function [14–17], and reduce the risk for inflammation [2,18] and carcinogenesis

☆ The crystal structure of NF-κB is available in the Research Collaboratory for Structural Bioinformatics Protein Databank under PDB # 1VKX.

Abbreviations: PMA, phorbol-12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay; TNFα, tumor necrosis factor alpha.

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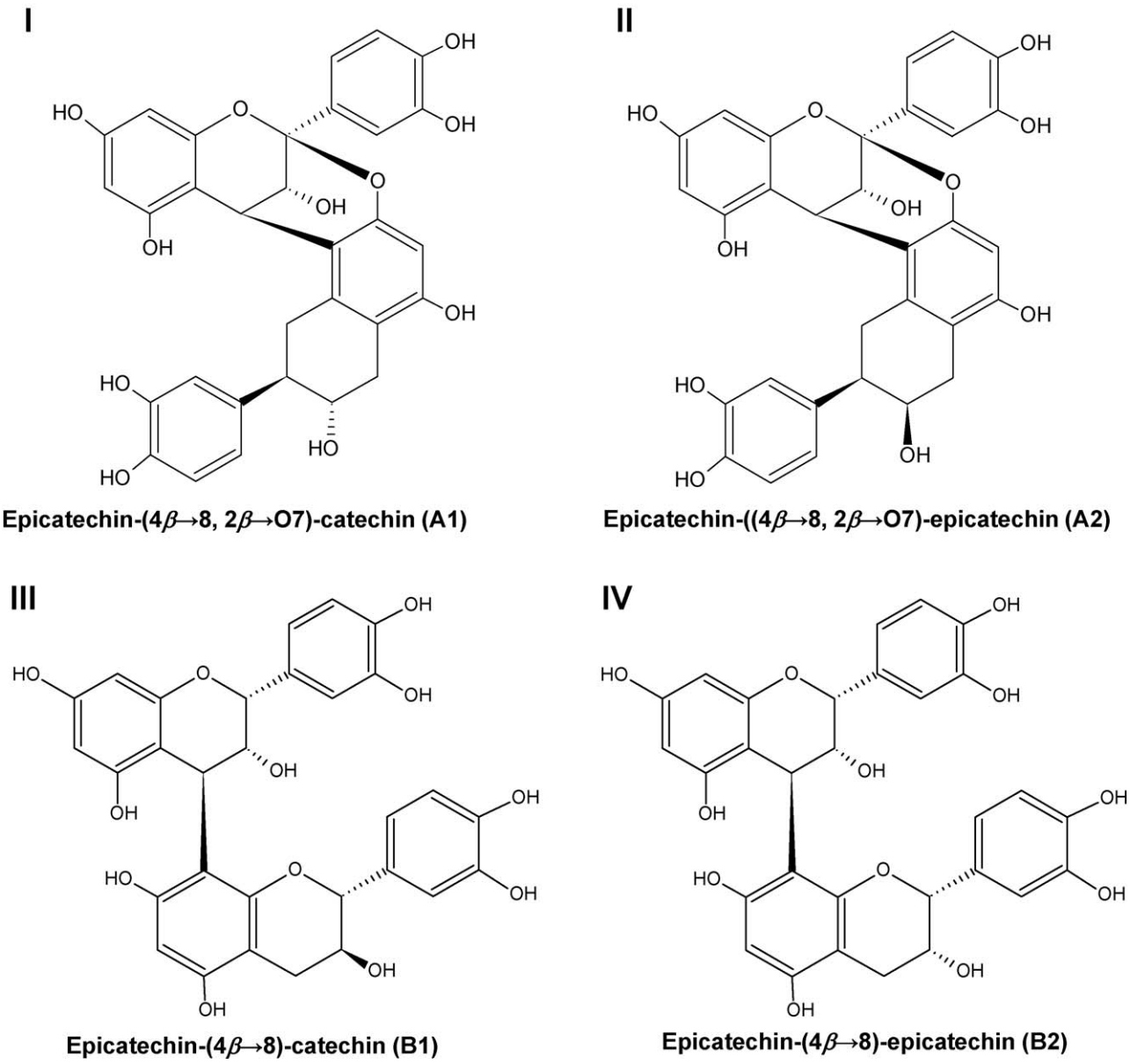

Fig. 1. Chemical structures of dimeric procyanidins. (**I**) Dimer A1, epicatechin-(4β-8,2 β-O7)-catechin; (**II**) dimer A2, epicatechin-(4β-8,2 β-O7)-epicatechin; (**III**) dimer B1, epicatechin-(4β-8)-catechin; and (**IV**) dimer B2, epicatechin-(4β-8)-epicatechin.

[19–22]. While the mechanisms underlying flavan-3-ol/procyanidin-induced inhibition of inflammation have not been defined, the inhibition of NF-κB represents a pathway by which this could occur. In support of this concept, in cell cultures, (−)-epicatechin, (+)-catechin, and certain procyanidins, can modulate the expression of numerous NF-κB-regulated genes involved in inflammation, and carcinogenesis [23–27]. We have shown that (−)-epicatechin, (+)-catechin, and B2, a B-type dimeric procyanidin can inhibit phorbol 12-myristate 13-acetate (PMA)-induced NF-κB activation at multiple levels in the NF-κB pathway in Jurkat T cells [25]. In Hodgkin's lymphoma cells, both (−)-epicatechin and B2 inhibit the constitutive activation of NF-κB [26,28]. Functional evidence supported by a putative molecular model suggests that B2 could interact with NF-κB proteins and prevent the binding of NF-κB to the DNA κB sites [25].

An understanding of the molecular mechanisms supporting a flavanol-NF-κB interaction would be of value in the possible definition of an "anti-inflammatory diet", as well as for the development of pharmacological strategies aimed reducing the risk for chronic inflammation. In the current work we investigated how the interaction of dimeric procyanidins with NF-κB protein components could influence the binding of NF-κB to its DNA consensus sequence. We characterized the relationships between molecular structure, interactions with NF-κB, and biological activity of B-type as compared to A-type dimeric procyanidins. The results suggest that B1 and B2 can interact with the NF-κB proteins p50 and RelA, preventing their binding to the DNA and inhibiting the expression of pro-inflammatory genes In contrast, A-type dimers did not significantly affect NF-κB activity.

2. Materials and methods

2.1. Materials

A1 and A2 dimeric procyanidins isolated from peanut skin, and B1 and B2 dimeric procyanidins isolated from cocoa, were purified [4,29] and supplied by Mars Incorporated (Hackettstown, NJ, USA). Jurkat (T cell leukemia, human) cells were obtained from the

American Type Culture Collection (Manassas, VA). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlbad, CA). The oligonucleotides containing the consensus sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and OCT-1 as well as the reagents for the EMSA, the enzyme assay systems for the determination of luciferase and β-galactosidase activities and the pSV-β-galactosidase control vector were obtained from Promega (Madison, WI). The human-IL-2 ELISA assay and the protease inhibitor cocktail were obtained from Roche Applied Science (Mannheim, Germany). The *Trans*IT-Jurkat Transfection Reagent was purchased from Mirus Bio Corporation (Madison, WI). The PathDetect NF-κB cis reporting system was obtained from Stratagene (La Jolla, CA). Antibodies for RelA and p50 were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant proteins p50 and RelA were purchased from ActiveMotif (Carlsbad, CA). PVDF membranes were obtained from BIO-RAD (Hercules, CA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA). Lactacystin, MG-132 and the 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (NF-κB activation inhibitor) were from EMD (San Diego, CA). The ECL western blotting system was from GE Healthcare (Piscataway, NJ). TNFα, PMA and all the other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO).

2.2. Cell culture and incubation

Jurkat T cells were cultured in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum and antibiotics (10 U/ml penicillin and 10 μg/ml streptomycin). Cells (1×10^6 cells/ml) were preincubated with 2.5–50 μM A1, A2, B1 or B2 for 24 h followed by further incubation without, or with, the addition of either 20 ng/ml TNFα or 100 ng/ml PMA for variable periods of time.

2.3. Determination of IL-2

Cells (1.5×10^6) were preincubated in 1.5 ml medium in the absence, or presence, of 25 μM A1, A2, B1 or B2 for 24 h, added with TNFα or PMA and further incubated for 18 h. IL-2 released to the media was measured after separating the cells by centrifugation at $800 \times g$ for 10 min. IL-2 was measured using the human-IL-2 ELISA assay following the manufacturer's protocols.

2.4. Transfections

Jurkat cells (2.5×10^6 cells) were transfected with the *Trans*IT-Jurkat Transfection Reagent, according to the manufacturer's protocols. As an internal control for transfection efficiency, a vector expressing β-galactosidase (2 μg DNA) was co-transfected with the pNF-κB-Luc plasmid (1 μg DNA). Twenty-four hours after the transfection was initiated, cells were incubated in the absence or presence of 25 μM A1, A2, B1, or B2 for 24 h, added with TNFα or PMA and further incubated for 4 h. Cells were harvested and after lysis, β-galactosidase and luciferase activities were determined following the manufacturer's protocols.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were isolated as previously described [30,31], with minor modifications [32]. For the EMSA, the oligonucleotides containing the consensus sequences for NF-κB or OCT-1 were end labeled with [γ^{-32}P] ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the corresponding labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1× binding buffer [5×: 50 mM Tris–HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl and 0.25 mg/ml poly(dI-dC)]. The possibility that the procyanidins acted in part by inhibiting the binding of the active NF-κB to its DNA consensus sequence was tested. Prior to the addition of the labeled nucleotide, nuclear fractions from cells treated only with TNFα were incubated in the presence of varying concentrations of A1, A2, B1, or B2 (0.1–100 nM) for 30 min. The labeled oligonucleotide was then added and the incubation was done as described above. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel using 0.5× TBE (Tris/borate 45 mM, EDTA 1 mM) as the running buffer. The gels were dried and radioactivity was measured using a Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

2.6. EMSA for p50 and RelA recombinant proteins

Reactions were performed in a medium containing 2 nM constant concentrations of DNA (NF-κB consensus sequence) and 5 nM concentration of recombinant p50 or RelA in the presence of variable concentrations of A1, A2, B1 or B2 (0.01 nM–10 μM) in a 15 μl final volume of binding buffer (50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI-dC), 0.25 mg/ml bovine serum albumin, and 4% (v/v) glycerol). After 30 min incubation at room temperature, samples were loaded onto a 6% (w/v) non-denaturing polyacrylamide gel using 0.5× TBE (45 mM Tris/borate, 1 mM EDTA) as running buffer and run for 3 h at 120 V. Gels were dried and the amount of radioactivity in the bands was determined using a Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

2.7. Molecular modeling

Model building and energy calculations of A1, A2, B1 and B2 were carried out with MacroModel 7.0 and BatchMin 7.0 [33] installed on a Silicon Graphics O2 workstation (R10000, 320 MB RAM, 54 GB hard disk) under the Irix 6.5 operating system. The MM2* force field (the version of Allinger's MM2 force field as implemented in MacroModel) was used. By default, atomic partial charges were calculated from data in the molecular mechanics force field, which also uses distance-dependent dielectric electrostatics, instead of the standard dipole–dipole electrostatics. The conformational searches of A1, A2, B1 and B2 were carried out following an optimized Monte Carlo protocol [34], both in vacuo and in water [35]. The electrostatic cut-off was set to 50 Å. For energy minimization, the conjugate gradient method was used, with a final gradient value of 0.05 kJ $Å^{-1}$ mol^{-1} (0.01 kcal $Å^{-1}$ mol^{-1}) as the criterion for convergence. After 5000 Monte Carlo steps, the resulting set of conformers were fully minimized. Those non-enantiomeric structures lying within a 20 kJ mol^{-1} window above the global minimum were compared. Spatial coordinates of NF-κB in complex with IgκB DNA were taken from the Protein Data Bank (PDB file 1VKX) [36].

2.8. Statistical analysis

One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe, were performed using Statview 5.0.1 (Brainpower Inc., Calabazas CA). A p value <0.05 was considered statistically significant. Values are shown as means ± SEM.

3. Results

3.1. B-type dimeric procyanidins inhibit TNFα- and PMA-induced activation of NF-κB-driven genes in Jurkat T cells

The effects of dimeric procyanidins on NF-κB-driven transactivating activity were evaluated measuring the expression of

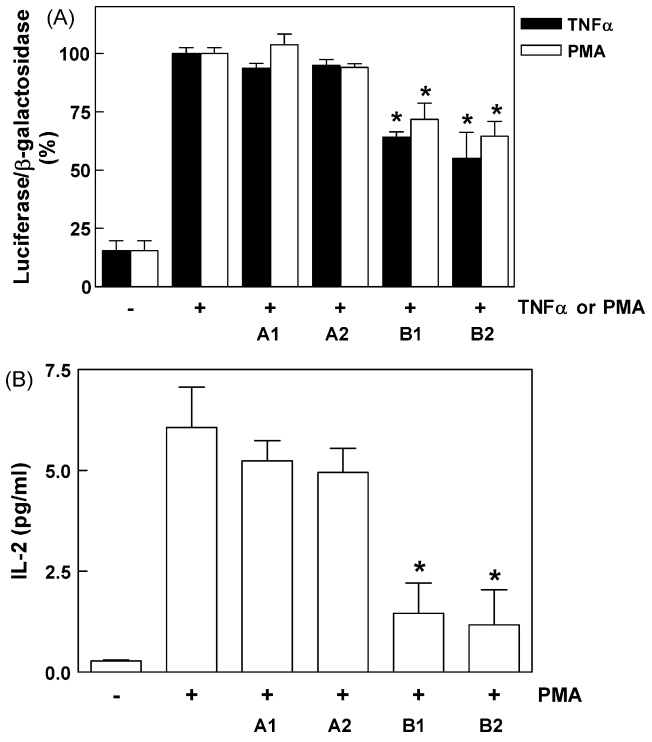

Fig. 2. Effects of dimeric procyanidins in NF-κB-driven transactivating activity in Jurkat cells. (A) Cells were co-transfected with a pNF-κB-Luc plasmid and a β-galactosidase plasmid. After 24 h of initiating the transfection, cells were preincubated in the absence, or presence, of 50 μM A1, A2, B1 or B2 for 24 h, followed by 4 h incubation without (−), or with (+), 20 ng/ml TNFα or 100 ng/ml PMA. Values are expressed as the ratio luciferase/β-galactosidase activity. *Significantly different compared to the TNFα or PMA control group ($p < 0.05$, one way ANOVA test). (B) Cells were preincubated for 24 h in the absence or presence of 50 μM A1, A2, B1 or B2, followed by 18 h incubation without (−), or with (+), 100 ng/ml PMA. IL-2 release to the media was measured by ELISA following the manufacturer's protocol. Results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to the PMA control group ($p < 0.05$, one way ANOVA test).

reporter and endogenous genes. For the reporter gene assay, Jurkat cells were co-transfected with a vector expressing a pNF-κB-Luc plasmid and β-galactosidase (as a control for transfection efficiency). After 24 h of preincubation in the presence of 50 μM A1, A2, B1 or B2, cells were treated with TNFα or PMA for a further 4 h period. Luciferase activity, corrected for β-galactosidase activity, was approximately 34–52% lower in the cells preincubated with B1 and B2, than in cells treated with TNFα or PMA alone (Fig. 2A). A1 and A2 did not affect TNFα- or PMA-mediated increases of luciferase activity. The expression of the NF-κB-regulated gene, IL-2, was evaluated by measuring the release of IL-2 to the media using an ELISA assay. IL-2 production by non-stimulated cells was below the level of detection; PMA treatment resulted in a significant stimulation of IL-2 production. PMA-induced IL-2 release to the media was decreased by 80 and 85% in the cells pretreated with 50 μM B1 and 50 μM B2, respectively, compared to that in cells treated with PMA alone (Fig. 2B). PMA-stimulated IL-2 production was not influenced by 50 μM A1 or A2.

3.2. B-type, but not A-type, dimeric procyanidins inhibit TNFα- and PMA-induced NF-κB nuclear binding activity in Jurkat T cells

NF-κB–DNA binding was measured by EMSA in nuclear extracts isolated from Jurkat cells incubated without, or with, 5–50 μM A1, A2, B1 or B2 for 24 h, and subsequently treated with either 20 ng/ml TNFα or 100 ng/ml PMA (Fig. 3). As evaluated by EMSA supershift assay, the specificity of the NF-κB–DNA binding was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB or OCT-1. In addition, among the members of the Rel/NF-κB family of proteins, RelA and p50 are the main components of the active NF-κB in Jurkat cells (data not shown). Fig. 3A depicts the time (15–240 min)-dependent increase of NF-κB–DNA binding activity in nuclear fractions from cells treated without, or with, either 20 ng/ml TNFα or 100 ng/ml PMA. The maximum levels of NF-κB–DNA binding were observed after 60 min of treatment with both stimuli, and this time point was used for the subsequent experiments (Fig. 3A). A concentration-dependent (5–50 μM) inhibition of NF-κB–DNA binding activity was observed for the B1 and B2 procyanidins (Fig. 3B). The lowest NF-κB–DNA binding was observed at concentrations of 50 μM B1 and 50 μM B2 (86–102 and 90–105% reductions, respectively), compared to that observed in control cells treated with only TNFα or PMA (Fig. 3C). No changes in NF-κB–DNA binding activity were observed in the cells preincubated with 50 μM A1 or A2, compared to cells treated with TNFα or PMA alone (Fig. 4A and B). Subsequently, the inhibitory capacity of B1 and B2 was compared to that of other well known NF-κB inhibitors. B1 and B2 had similar inhibitory effect as the NF-κB inhibitors lactacystin, MG-132, and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline in preventing TNFα- and PMA-induced increase in NF-κB–DNA binding activity (Fig. 4C).

3.3. B1 and B2, but not A1 nor A2, prevent in vitro NF-κB binding to its DNA consensus sequence in nuclear extracts

We next tested the capacity of the dimeric procyanidins to exert a direct inhibition of the binding of the active NF-κB to DNA κB-sites. For this purpose, nuclear fractions were isolated from cells treated with, or without TNFα for 1 h. Aliquots of this sample, containing 4 μg of protein, were added 30 min prior to the binding reaction with different concentrations of A1, A2, B1, B2 (0.1–100 nM) (Fig. 5A). The addition of B1 or B2 reduced (60 and 80%, at 10 nM concentration; and 83 and 90%, at 100 nM concentration, respectively) the binding of the active NF-κB to DNA. The binding of the active NF-κB to its consensus sequence was not affected by 100 nM A1 or A2 (Fig. 5B). As a control, we evaluated the effects of A1, A2, B1 and B2 on the DNA binding of the unrelated transcription factor OCT-1. As shown in Fig. 5A, none of the dimers affected its binding.

3.4. B1 and B2, but not A1 or A2, inhibit the in vitro binding of the p50 and RelA recombinant proteins to its DNA consensus sequence

To further define the capacity of B1 and B2 to prevent NF-κB binding to the DNA, we tested if these B-type dimeric procyanidins inhibit the binding of the recombinant proteins p50 or RelA to their consensus sequence. First, the experimental system was characterized by studying the specific binding of p50 and RelA to the consensus oligonucleotide. For this purpose, a control sample was incubated in the presence of antibodies for p50 or RelA, and the supershift of the band was assessed (Fig. 6A). In addition, to determine the specificity of the p50– or RelA–DNA complex, aliquots of p50 (left panel) or RelA (right panel) recombinant proteins were incubated in the presence of increasing amounts (0.01–500 nM) of unlabeled oligonucleotide containing the consensus sequence for NF-κB prior to the binding assay (Fig. 6B). B1 and B2 caused a concentration-dependent inhibition of both p50 and RelA binding to the DNA (Fig. 6C). At 100 nM, B1 and B2 caused a 29–38%, and 38–47%, inhibition of either p50 or RelA binding to its consensus sequence. A1 and A2 did not inhibit the binding of p50 and RelA recombinant proteins to the DNA (Fig. 6C).

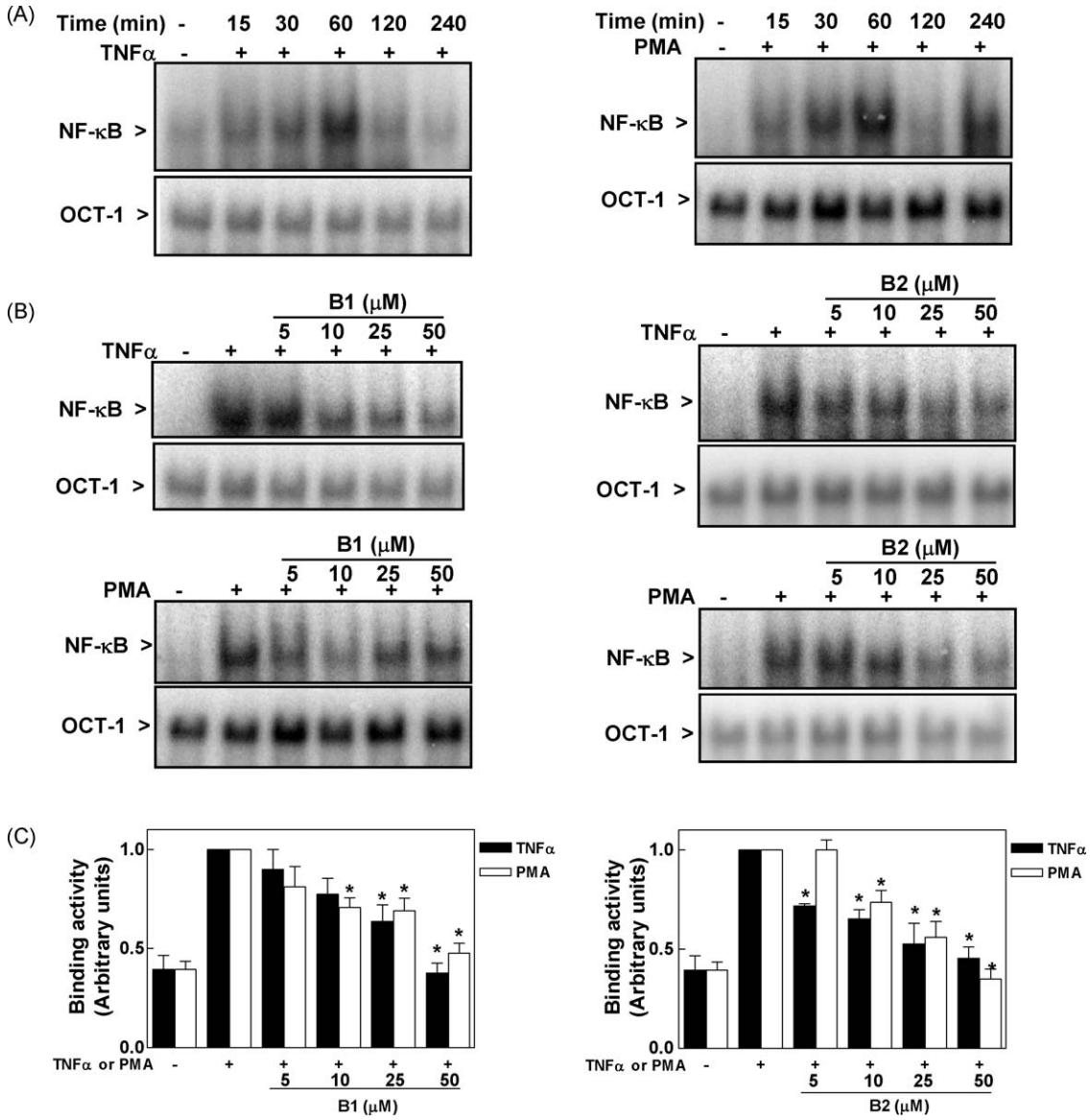

Fig. 3. B-type procyanidins inhibit TNFα- and PMA-induced increases in NF-κB–DNA binding in Jurkat cells. (A) EMSA of nuclear fractions isolated from cells incubated for different periods of time (15–240 min) without (−) or with (+) 20 ng/ml TNFα (left panel) or 100 ng/ml PMA (right panel). (B) EMSA of nuclear fractions isolated after 24 h of preincubating cells in the absence, or in the presence of 5–50 μM B1 or B2, and a further 1 h incubation without (−) or with (+) 20 ng/ml TNFα or 100 ng/ml PMA. (C) Bands corresponding to the experiments described in B were quantitated and results are shown as means ± SEM of 3 independent experiments. *Significantly different compared to the TNFα or PMA group ($p < 0.01$, one way ANOVA test).

3.5. Molecular modeling for NF-κB–dimeric procyanidins interactions

Given the above findings, molecular models of how the different dimeric procyanidins might interact with NF-κB proteins were constructed using MacroModel 7.0 [33]. The procedure used was the following: (i) first, a conformational search was carried out with an optimized Monte Carlo procedure [34]; this produced a very similar ensemble of conformers where a common structure for each dimeric procyanidin predominate (see below); followed by (ii) fitting the minimum energy conformer found (for each dimeric procyanidin) at the previous step onto the base-specific binding residues (Arg 54 and Arg 56 of p50) of NF-κB (PDB entry 1VKX) was achieved by manual docking.

Molecular models of A1, A2, B1 and B2 were initially constructed, as previously described [25], using the model-building facility implemented in MacroModel. Briefly, coordinates of these structures were later used as input for BatchMin, the calculation module of this program. The minimum energy conformers for B1 and B2 represent a folded structure where ring B′ stacks onto ring A orienting the hydroxyl groups toward the same edge of the molecule. The above was not observed for A1 and A2, where ring B′ and A are not stacked (Fig. 7).

NF-κB specifically recognizes κB DNA elements with a consensus sequence of 5′-GGGRNYYYCC-3′ (where R is an unspecified purine; Y is an unspecified pyrimidine; and N is any nucleotide). In particular for p50, the specific binding to DNA bases involves several critical aminoacid residues including Arg 54 and Arg 56. Carbons of these arginines form hydrogen bonds with nitrogen and oxygen groups of guanine$_{-4}$ and guanine$_{-3}$ of the NF-κB consensus sequence. Based on this, we explored the possibility that dimers could directly interfere with the binding between the mentioned arginine residues of p50, and the guanines of the κB–DNA consensus sequence. Fig. 8 depicts the superimposition of the base-specific guanines of the κB–DNA consensus sequence and the different A-type and B-type dimeric procyanidins, and their interaction through hydrogen bonds with the pairs of arginine

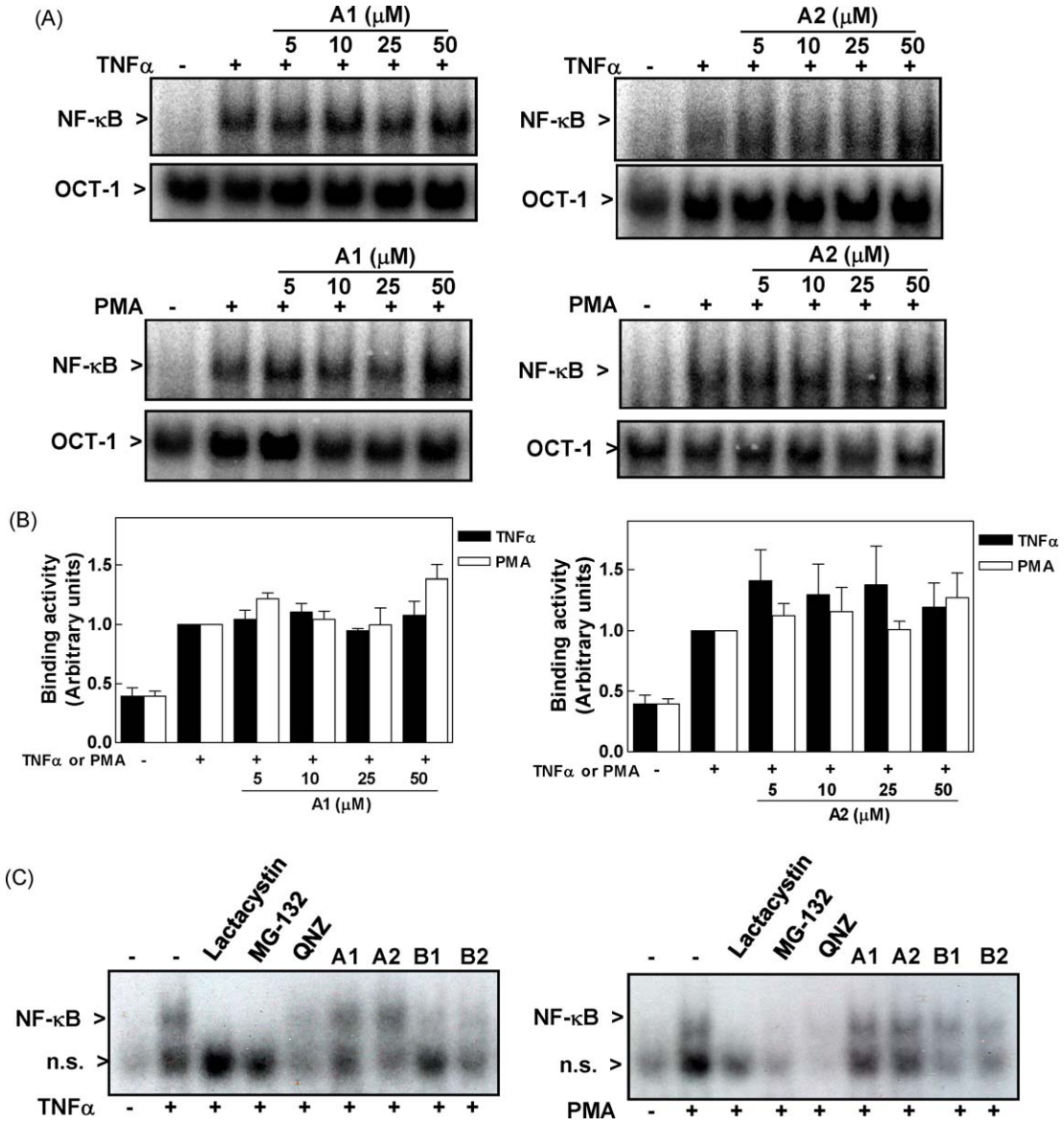

Fig. 4. A-type procyanidins do not inhibit TNFα- and PMA-induced increase in NF-κB–DNA binding in Jurkat cells. (A) Nuclear fractions were isolated after 24 h of preincubating Jurkat cells in the absence, or in the presence of 5–50 μM A1 or A2 and a further 1 h incubation without (−) or with (+) 20 ng/ml TNFα or 100 ng/ml PMA. EMSA of nuclear fractions incubated with different concentrations of A1 or A2. (B) Bands were quantitated and results are shown as means ± SEM of 3 independent experiments. (C) EMSA of nuclear fractions from cells preincubated for 1 h with different NF-κB inhibitors [10 μM lactacystin, 1 μM MG-132, 1 μM 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ)] or for 24 h with 50 μM A1, A2, B1 or B2, and a further 1 h incubation with TNFα (left panel) or PMA (right panel). n.s.: non-specific. One EMSA out of two independent experiments is shown.

residues present in the DNA binding region of p50. For each dimeric procyanidin analyzed, the ensemble of structures was scrutinized to search for common conformational motifs. The criterion for similarity was considered shape, as well as the spatial distribution and kind of charged/polar atoms. In particular, features such as ring stacking and hydrogen bond donor/acceptor ability at the edges of the planar aromatic systems were taken into special regard. Overall, B1 and B2, but not A1 or A2, behave as a reasonable mimic of the guanine pairs.

4. Discussion

The present work was aimed to study the interactions between the dimers B1, B2, A1 and A2 and the NF-κB proteins. The results indicate that dimers B1 and B2, can interact with NF-κB proteins. These interactions with B1 and B2 can lead to act inhibition of NF-κB binding to the DNA, and a subsequent reduction in the transactivation of NF-κB-driven genes. The occurrence and specificity of the B-type dimer interactions is supported by the findings that in each of the three systems that were tested (NF-κB activation in whole cells, NF-κB binding to the DNA in cell nuclear fractions, and RelA and p50 recombinant protein binding to the DNA), B1 and B2, but not A1 and A2, inhibited the binding of NF-κB to DNA and the expression of NF-κB-regulated genes. The theoretical three dimensional molecular models of the B-type dimers support the idea that these compounds can interact with NF-κB, while an interaction with the A-type dimers would not be predicted.

Different flavanols and procyanidins have been reported to inhibit transcription factor NF-κB and the expression of NF-κB-regulated genes at multiple steps of the NF-κB activation pathway [23–27,37] Green tea (−)-epigallocatechin-3-gallate inhibits NF-κB in CD4+ T cells, through the blockage of the 20S/26S proteasome complex, followed by the cytosolic accumulation of IκBα [38]. In

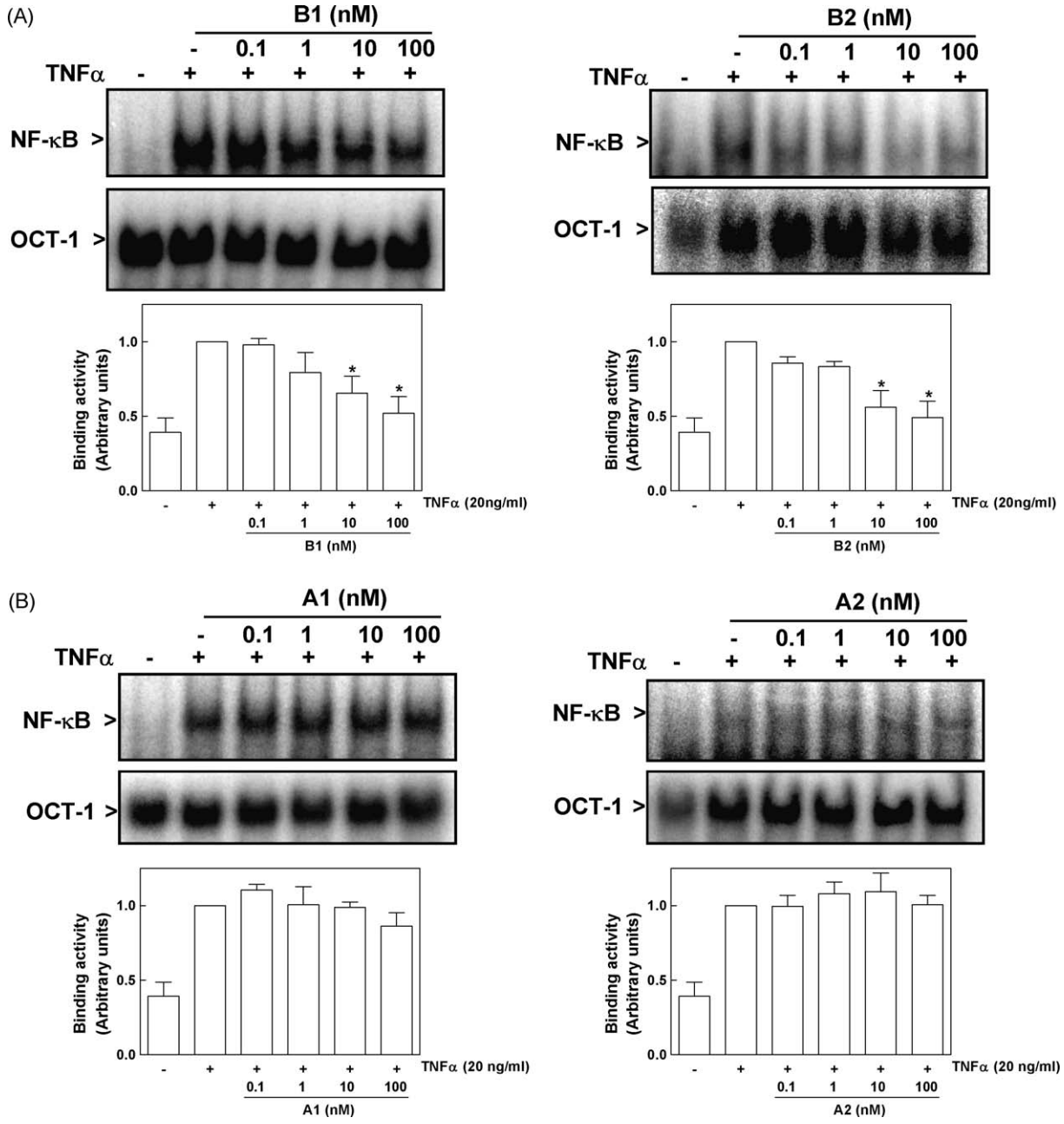

Fig. 5. B1 and B2, but not A1 nor A2, inhibit *in vitro* NF-κB binding to its DNA consensus sequence in nuclear extracts. (A) NF-κB binding activity was measured by EMSA in nuclear fractions isolated from Jurkat cells incubated for 1 h without (−) or with (+) 20 ng/ml TNFα. Nuclear fractions were incubated with 0.1–100 nM B1 or B2 (A), or A1 or A2 (B) for 30 min prior to the EMSA assay. After quantitation results are shown as means ± SEM of 3 independent experiments. *Significantly different compared to the TNFα group ($p < 0.05$, one way ANOVA test).

addition, (−)-epigallocatechin-3-gallate also inhibits UVB-induced NF-κB activation in human epidermal keratinocytes [39]. In different cell types, diverse procyanidins have been shown to have the ability to modulate the *in vitro* expression of IL-2, IL-1β, and IL-4 [27,40,41]. Flavan-3-ols, and the procyanidin dimers, B1 and B2, can inhibit the interferon γ-induced expression of NF-κB-dependent genes, the secretion of TNFα, and the expression of a NF-κB-driven luciferase reporter gene in RAW 264.7 macrophages [27]. We have reported that (−)-epicatechin and B2 can inhibit NF-κB constitutive activation in Hodgkin's lymphoma cells. In these cells, treatment with (−)-epicatechin and B2 caused a decreased expression of NF-κB-regulated proteins including (cytokines IL-6, RANTES and TNFα), and proteins involved in the modulation of apoptotic cell death (Bcl-xL, Bcl-2, XIAP, cFLIP) [24,26]. In agreement with the above, in the current study we observed that cells pretreated with B1 or B2 showed a reduced NF-κB-dependent gene expression compared to cells treated with TNFα or PMA alone. The observed decreased in IL-2 expression cannot univocally be associated to NF-κB inhibition given that other transcription factors, e.g. AP-1, CREB, and NFAT, regulate IL-2 expression. However the inhibition of NF-κB-dependent expression of a NF-κB reporter gene, backs up the differential inhibition by the tested dimers on NF-κB-dependent transactivation.

The findings that flavanol-related chemical structures differentially inhibit NF-κB activation, prompts the need to define not only the relevance of the chemical structure but also of the conformation of the procyanidins being studied. Based on the mentioned observations, and considering the lower NF-κB nuclear binding activity found in the cells pretreated with B1 and B2, but not A1 or A2, we speculated that the B-type dimeric procyanidins

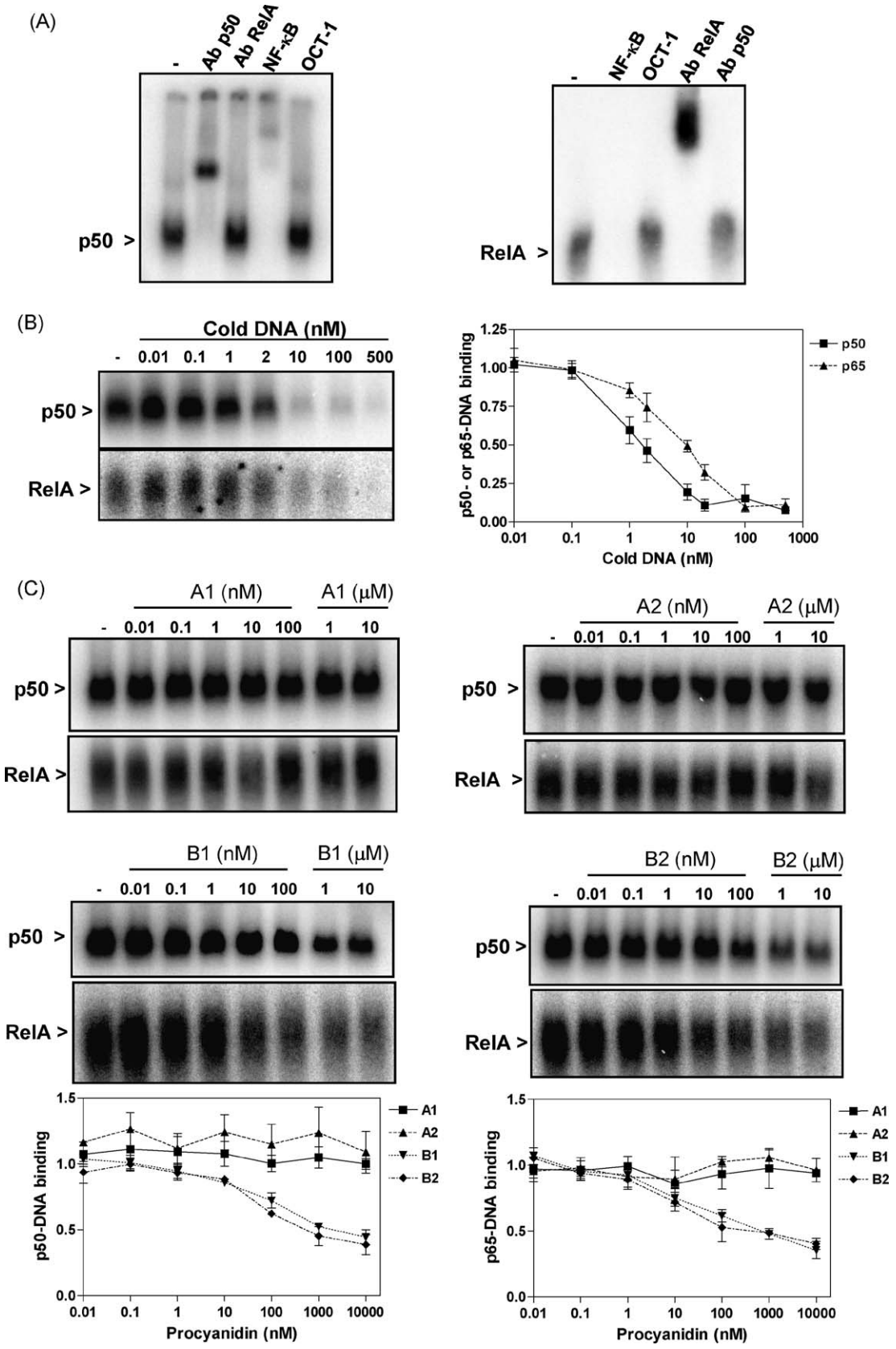

Fig. 6. B1 and B2, but not A1 nor A2, prevent *in vitro* recombinant proteins p50 and RelA binding to its DNA consensus sequence. (A) To determine the specificity of the p50– or RelA–DNA complex, an aliquot of either p50 (left panel) or RelA (right panel) recombinant proteins was incubated in the presence of a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB (NF-κB) or OCT-1 (OCT-1) prior to the binding assay. To determine the binding of p50 and RelA to its consensus sequence, control samples were incubated in the presence of antibodies for p50 (Ab p50) or RelA (Ab RelA), 30 min prior to the binding assay. (B) The dependence of p50 and RelA binding to DNA with DNA concentration was measured by EMSA. p50 or RelA samples were incubated with 0.01–500 nM unlabeled specific NF-κB oligonucleotide for 30 min prior to the EMSA assay. (C) p50 or RelA samples were incubated with 2 nM DNA and 0.01–10,000 nM A1, A2, B1 or B2. The binding of p50 or RelA to DNA was measured by EMSA assay. Results are shown as mean ± SEM of 3 independent experiments.

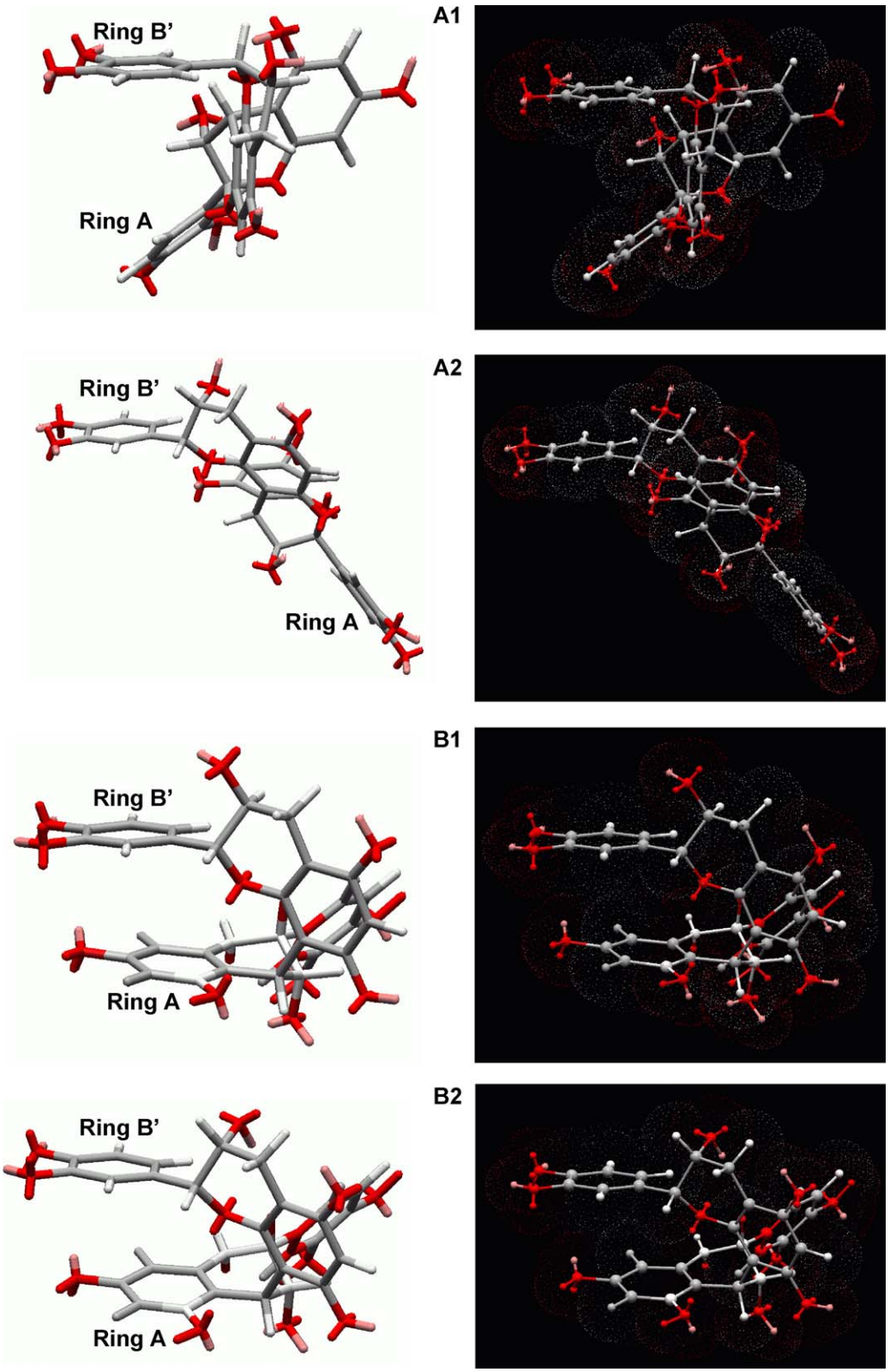

Fig. 7. Minimum energy conformer for A1, A2, B1 and B2. Left panel: polytube conformational representation of the global minimum energy conformer of A1, A2, B1 or B2, respectively, found after the optimized Monte Carlo search, as implemented in MacroModel (see Section 2.7). Note that the minimum energy conformers for B1 and B2 represent a folded structure where ring B′ stacks onto ring A orienting the hydroxyl groups toward the same edge of the molecule. Right panel: polytube conformational representation of the global minimum energy conformer of A1, A2, B1 or B2, showing van der Waals forces to depict the observed stacking in B1 and B2. Carbon atoms are represented in gray, oxygen atoms in red and hydrogen atoms in pink. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

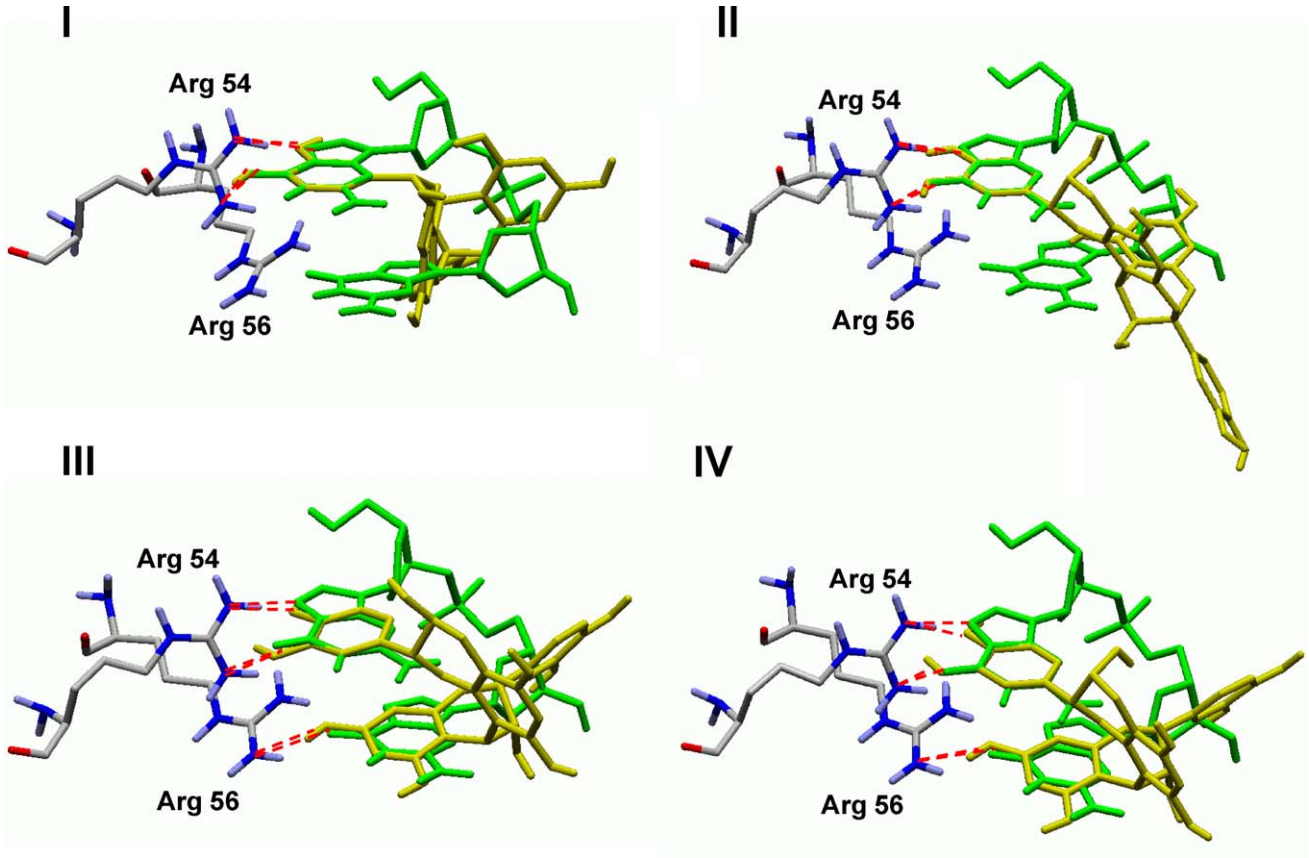

Fig. 8. Superimposition between A1, A2, B1 or B2 and guanine pairs. Superimposition of the minimum energy conformer of A1 (I), A2 (II), B1 (III) or B2 (IV) (in yellow) upon the guanine pairs (in green) taken from the double dodecamer in the complex 1VKX [36]. Putative hydrogen bonding interactions (in red) mediated by Arg residues of p50 subunit (top) are shown. Superimposition was forced upon three atoms in the top guanine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

have a special capacity to interact with the active NF-κB. Supporting such idea, the addition of B1 or B2 to nuclear fractions isolated from TNFα-stimulated cells resulted in a concentration-dependent inhibition of NF-κB binding to its consensus sequence. Under similar experimental conditions, dimeric procyanidins A1 and A2 did not affect the binding of NF-κB, indicating a selective effect of B1 and B2 on the binding of NF-κB to its DNA consensus sequence. Furthermore, we observed that B1 and B2 inhibited the binding of p50 and RelA recombinant proteins to the DNA. The similar results obtained in the three different experimental conditions; whole cells, nuclear extracts, and recombinant proteins p50 and RelA, support the occurrence of a stereo-specific interaction between B1 or B2 and the NF-κB proteins.

The pioneering work by Natarajan et al. implied the importance of phenolic conformation by demonstrating that rotationally constrained variants of caffeic acid phenethyl ester have less ability to inhibit DNA binding of NF-κB than the parent molecule [42]. To better define the stereo-chemical possibilities for the interaction between dimeric procyanidins and NF-κB proteins we constructed molecular models. As previously showed, the minimum energy conformers for B1 and B2 represent a folded structure where ring B′ stacks onto ring A orienting the hydroxyl groups toward the same edge of the molecule. However, the above was not observed for A1 and A2, where ring B′ and A are not stacked. To note, stacked rings B′ and A of B1 and B2 lie very close to the positions occupied by the two guanine rings in the NF-κB consensus sequence. Moreover, the polar atoms of B1 and B2 are favorably placed to give rise to a similar hydrogen bonding pattern to that observed in the complex. In contrast, rings B′ and A from A1 and A2 dimeric procyanidins are not stacked possibly due to the extra covalent bridge present between the two monomers. This extra covalent bridge (2β → O7 ether bond) generates a more rigid molecule that impairs the formation of a stacked molecule. We suggest that this difference contributes to the differential inhibitory effects of these dimeric procyanidins, with respect to their ability to reduce the binding of NF-κB to its consensus sequence.

From a physiological point of view, the question if relevant concentrations of dimers or other procyanidins can be reached by dietary sources remains open. Results in this paper were obtained using dimer concentrations that exceed the expected plasma concentrations of dimers, even with the ingestion of very high flavanol meals [43–45]. However, it is important to note that micromolar concentrations of the dimers could be achieved in certain organs, e.g. the gastrointestinal tract. Thus it is reasonable to speculate that the gut immune system can be influenced by certain dietary procyanidins. This may have important implications for conditions such as inflammatory bowel disease.

In summary, results presented in the current paper show that the dimeric procyanidins B1 and B2, but not the dimeric procyanidins A1 or A2, can inhibit the binding of active NF-κB to κB sites. This is supported by the results obtained in the three independent systems that were tested, i.e. NF-κB activation in whole cells, NF-κB binding to the DNA in cell nuclear fractions, and RelA and p50 recombinant protein binding to the DNA. The relevance of B-type dimeric procyanidins as dietary or pharmacological anti-inflammatory agents needs to be confirmed by further in vivo studies.

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