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Suppression of Tumor Necrosis Factor-α-Induced Nuclear Factor κB Activation and Aromatase Activity by Capsaicin and Its Analog Capsazepine

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ABSTRACT Target-specific drugs, including natural products, offer promise for the amelioration of cancer and other human ailments. Capsaicin, the pungent ingredient present in chilies (*Capsicum annuum* L.), and capsazepine, a synthetic analog of capsaicin (collectively referred to as vanilloids), are known to possess a variety of pharmacological and physiological properties. In our continuous effort to discover and characterize cancer chemopreventive agents from natural products, we investigated the effect of vanilloids on nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) activation using stably transfected 293/NFκB-Luc human embryonic kidney cells induced by treatment with tumor necrosis factor-α (TNFα) and on aromatase activity. Capsaicin and capsazepine blocked TNFα-induced NFκB activation in a dose-dependent manner with 50% inhibitory concentration (IC_{50}) values of 0.68 and 4.2 μM, respectively. No significant cytotoxicity was observed at the highest concentrations tested (53.1 μM for capsazepine and 65.5 μM for capsaicin). In addition, these vanilloids inhibited aromatase activity with IC_{50} values of 13.6 and 8.8 μM, respectively. Computer-aided molecular docking studies showed docking scores indicative of good binding affinity of vanilloids with aromatase and NFκB. The highly conserved residues for capsaicin and capsazepine binding with NFκB p50 were Ser299 and Ile278 (H-bond 2.81Å) and with NFκB p100 were Ser6, Arg82, Val86, Arg90 (H-bond 2.89Å), Gly4, and Ser2 (H-bond 2.81Å). The amino acids Trp224, Arg435, and Val373 (H-bond 2.80Å) were found to be important for the binding of capsaicin and capsazepine with aromatase. Based on these findings, aromatase and NFκB are suggested as valid targets for these compounds; additional investigation of chemopreventive or chemotherapeutic potential is required.

KEY WORDS: • ***cancer chemoprevention*** • ***capsaicin*** • ***molecular docking*** • ***nociceptors*** • ***nuclear factor κ-light-chain-enhancer of activated B cells*** • ***red pepper*** • ***tumor necrosis factor-α*** • ***vanilloids***

INTRODUCTION

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the pungent ingredient in a wide variety of red peppers of the genus *Capsicum*. Compounds related to capsaicin, including capsazepine (Fig. 1), collectively referred to as vanilloids, bind specifically to a subclass of sensory neurons called nociceptors that carry the sensation of pain.[1] Prolonged exposure causes nociceptor terminals to become insensitive to capsaicin, as well as to other noxious stimuli.[2] Nociceptor desensitization has led to the use of capsaicin as an analgesic in the treatment of chronic painful disorders.[3] In addition to its neuronal effects, several non-neuronal effects of capsaicin have been reported: induction of apoptosis in transformed cells,[4] stimulation of prostaglandin formation leading to inhibition of gastric lesions,[5] antibacterial activity,[6] inhibition of cardiac excitability,[7] and platelet aggregation.[8] Studies from our laboratory[9–13] and other recent reports[14,15] have described the variety of pharmacological and physiological properties of capsaicin and its related analogs.

Nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) consists of homo- and heterodimeric protein complexes that act as transcription factors. NFκB is found in many animal cell types. It is involved in the regulation of immune response and inflammation, cell lineage development, cell apoptosis, cell cycle progression, and oncogenesis in response to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low-density lipoprotein, and microbial antigens and has been shown to regulate the expression of several genes, including *bcl-2*, *bcl-xl*, *cellular inhibitor of apoptosis protein*, *survivin*, *tumor necrosis factor (TNF) signaling pathway-related regulatory factor*, *cyclooxygenase-2*, *matrix metalloproteinase peptide-9*, and *inducible nitric oxide synthase* and those for cell cycle–regulatory components whose products are involved in tumorigenesis.[16–20] Most carcinogens, pro-inflammatory cytokines such as TNF and interleukin-1, and tumor promoters, including cigarette smoke, phorbol esters, okadaic acid, and H_2O_2, have been shown to activate NFκB, and resulting tumors have misregulated NFκB activity.[21] It is

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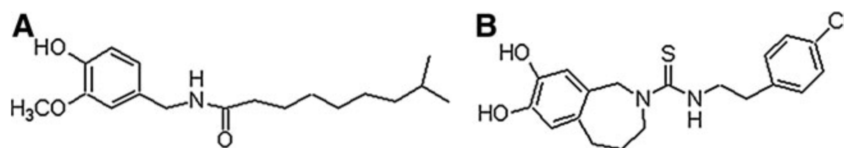

FIG. 1. Chemical structure of (**A**) capsaicin and (**B**) capsazepine.

now well established that aberrant regulation of NFκB and the signaling pathways that control its activity are involved in cancer development and progression, as well as in drug resistance, especially during chemotherapy and radiotherapy.[22] Blocking NFκB can cause tumor cells to stop proliferating, go to apoptosis, or to become more sensitive to the action of antitumor agents.[23,24] Thus, agents that can suppress NFκB activation have the potential to suppress carcinogenesis or tumorigenesis.

Aromatase, a key cytochrome P450 enzyme, catalyzes the rate-limiting aromatization step in the conversion of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone).[25–27] Thus, aromatase inhibitors decrease bioavailable estrogen. Aromatase inhibitors have been shown to have considerable antitumor activity in most estrogen receptor–positive breast cancers,[28] and they have been recognized as potential chemopreventive agents with proven efficacy in animal models. Non-steroidal aromatase inhibitors are already in clinical use for the treatment of breast cancer.[29] Such compounds ultimately reduce estradiol receptor stimulation and reduce formation of genotoxic estrogen metabolites.[30] The aromatase complex is associated with the endoplasmic reticulum and consists of a specific cytochrome P450 heme protein (CYP19) and the flavoprotein NADPH cytochrome P450 reductase.[31] In our assay, purified CYP19 hydrolyzes dibenzylfluorescein, and aromatase activity was quantified by measuring the fluorescent intensity of the resultant fluorescein.

Because capsaicin and its related analogs have been reported to show diverse biological activity, we have targeted NFκB and aromatase activity to determine the chemopreventive potential of capsaicin and capsazepine in an effort to understand the mechanism of action of vanilloids. Our preliminary observation of the non-neuronal activity of capsaicin led us to investigate other vanilloids for further biological testing. This report describes NFκB and aromatase inhibitory activity by vanilloids. The effect of vanilloids on NFκB and aromatase activity has been compared with that of *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK) and naringenin, known inhibitors of NFκB and aromatase, respectively. In addition, molecular docking studies of NFκB and aromatase in complex with capsaicin and capsazepine are described. These studies provide insight into the mechanism of NFκB and aromatase inhibition by capsaicin and related compounds.

MATERIALS AND METHODS

Chemicals

Capsaicin, capsazepine, dimethyl sulfoxide, sulforhodamine B, NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $MgCl_2$, and dibenzylfluorescein were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, antibiotic-antimycotic, hygromycin B, and minimal essential medium sodium pyruvate were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Reporter lysis buffer and the luciferase assay system were purchased from Promega (Madison, WI, USA). TNFα was purchased from Calbiochem (Gibbstown, NJ, USA).

NFκB luciferase assay

The NFκB inhibition assay was conducted using luciferase expression as previously described.[32,33] Human embryonic kidney cells (293/NFκB-Luc) were used to monitor alteration of the NFκB pathway. This cell line is stably transfected with a luciferase reporter construct regulated by the NFκB response element. Transcription factor can bind to the response element when stimulated by TNFα, allowing transcription of the luciferase gene. Luciferase reacts with substrate, emitting light that was detected using a luminometer. Cells were seeded (10^5 cells/mL) on sterile white-walled 96-well plates and grown to approximately 80% confluence by incubating for 48 hours. Capsaicin and capsazepine were tested at a concentration of 20 μg/mL followed by 1:3 serial dilutions for dose-dependent studies. After treatment with test compounds for 10 minutes, cells were incubated for an additional 6 hours with or without TNFα (10 ng/mL). The cells were gently washed twice with phosphate-buffered saline and kept at −80°C with cell lysis buffer (Promega). The luciferase assay was performed using the Luc assay system from Promega following the manufacturer's instructions. Luciferase activity was monitored using a LUMIstar Galaxy luminometer (BMG, Offenburg, Germany), dose–response curves were constructed, and results were expressed as values for the concentration required to inhibit TNFα-activated NFκB activity by 50% (IC_{50}).

Aromatase assay

Aromatase activity was assayed as previously reported[29] with the necessary modifications to perform the test in 384-well plates. The test substance (3.5 μL at 20 μg/mL) was preincubated with 30 μL of NADPH regenerating system (2.6 m*M* $NADP^+$, 7.6 m*M* glucose 6-phosphate, 0.8 U/mL glucose 6-phosphate dehydrogenase, 13.9 m*M* $MgCl_2$, and 1 mg/mL albumin in 50 m*M* potassium phosphate buffer, pH 7.4) for 10 minutes. The enzyme and substrate mixture (33 μL of 1 μ*M* enzyme [165 μU of CYP19, BD Biosciences, San Jose, CA, USA], 0.4 μ*M* dibenzylfluorescein, and 4 mg/mL albumin in 50 m*M* potassium phosphate, pH 7.4) was added, and the plate was incubated for 30 minutes at 37°C before quenching with 25 μL of 2 *N* NaOH. After termination

of the reaction and shaking for 5 minutes, the plate was further incubated for 2 hours at 37°C. This enhances the ratio of signal to background. Fluorescence was measured using a BioTek (Winooski, VT, USA) Synergy 2 plate reader at 485 nm (excitation) and 530 nm (emission). IC_{50} values and dose–response curves were based on three independent experiments performed in duplicate using five concentrations of tested substance.

Cytotoxicity assay

LNCaP (androgen-sensitive human prostate carcinoma, ATCC number CRL-1740), MCF7 (human mammary gland adenocarcinoma, ATCC number HTB-22), and Hepa 1c1c7 (murine hepatoma, ATCC number CRL-2026) cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA). LU-1 (lung cancer carcinoma) was provided by the Department of Surgical Oncology, University of Illinois Chicago, Chicago, IL, USA. Evaluation of cytotoxic potential was performed by the method of Skehan *et al.*[34] In brief, using 96-well plates, cells were seeded at a density of 5.0×10^4 cells/mL and treated with concentrations of each compound ranging from 0 to 20 μg/mL (10 μL of solution in 10% dimethyl sulfoxide). A day 0 control plate was incubated for 30 minutes at 37°C; all other plates were incubated for 72 hours. After incubation, cells were fixed by addition of 50 μL of cold 50% trichloroacetic acid, incubated at 4°C for 30 minutes, washed with tap water, and air-dried. Cells were then stained with 100 μL of 0.4% sulforhodamine B in 1% acetic acid for 30 minutes at 23°C. After washing with 1% acetic acid (four times) to remove excess dye and air-drying, bound dye was solubilized by addition of 200 μL of 10 m*M* Tris base (pH 10). Finally, the plates were shaken for 5 minutes, and absorption at 515 nm was determined with a BioTek ELx800 plate reader. All determinations were performed in triplicate, and the IC_{50} values of active samples were assessed by linear regression analyses of dose–response data.

In silico studies

In order to gain a better understanding of the interactions of capsaicin and capsazepine with NFκB and aromatase, docking studies were carried out. The three-dimensional structures of capsaicin (CID_1548943), capsazepine (CID_2733484), naringenin (CID_932), letrozole (CID_3902), and TPCK (CID_9824) were retrieved through the PubChem database at the National Center for Biotechnology Information web server (pubchem.ncbi.nlm.nih.gov). X-ray crystallographic three-dimensional structures of NFκB (PDB ID 1BFS for NFκB p-50; PDB ID 2D96 for NFκB p-100) and aromatase (PDB ID 3EQM) were retrieved from the Brookhaven Protein DataBank (www.pdb.org).

Docking was carried out using GOLD (Genetic Optimization for Ligand Docking) software (version 3.2; Cambridge Crystallographic Data Centre, Cambridge, United Kingdom), which uses the Genetic Algorithm (GA). This method allows for partial flexibility of the protein and full flexibility of the ligand (gold.ccdc.cam.ac.uk). All water molecules and heteroatoms were removed from the targets to evaluate the scoring functions. The default parameters to run the software were as follows: population size, 100; selection-pressure, 1.1; number of operations, 10,000; number of islands, 1; niche size, 2; and operator weights for migrate of 0, mutate of 100, and crossover of 100 were applied. The active site was defined within 15 Å, and the ligand-binding interactions were analyzed using scoring functions (*i.e.*, Goldscore [GS]). For each of the lead–target interactions, independent GA runs were performed on a set of two groups with a population size of 100 individuals. When the top three solutions attained root mean square deviation (RMSD) values within 1.5 Å, GA docking was terminated. The RMSD values for the docking calculations are based on the RMSD matrix of the ranked solutions. We observed that the best-ranked solutions were always among the first 10 GA runs, and the conformation of molecules based on the best fitness score was further analyzed.

GS performs a force field–based scoring function and is made up of four components: (1) protein–ligand hydrogen bond energy (external H-bond); (2) protein–ligand van der Waals energy (external vdw); (3) ligand internal van der Waals energy (internal vdw); and (4) ligand intramolecular hydrogen bond energy (internal H-bond). The external vdw score is multiplied by a factor of 1.375 when the total fitness score is computed. This is an empirical correction to encourage protein–ligand hydrophobic contact. The fitness function has been optimized for the prediction of ligand binding positions: $GS = S(hb_ext) + S(vdw_ext) + S(hb_int) + S(vdw_int)$, where $S(hb_ext)$ is the protein–ligand hydrogen bond score, $S(vdw_ext)$ is the protein–ligand van der Waals score, $S(hb_int)$ is the score from intramolecular hydrogen bonds in the ligand, and $S(vdw_int)$ is the score from intramolecular strain in the ligand.

Both capsaicin and capsazepine, along with the positive controls (TPCK and naringenin), were analyzed using docking studies, and the IC_{50} values of each are described in detail in Results.

Estimation of binding affinity

Binding affinity (K_i) values were calculated by using Autodock version 4.0 (autodock.scripps.edu). Polar hydrogen atoms were added to the macromolecules, and all rotatable bonds present in the ligand were set as rotatable bond for experiments. A grid box was created for macromolecule (dimensions of the grid box was $40 \times 40 \times 40$ with grid spacing of 0.381). A GA was used to run Autodock. Population sizes of 150 and 10 million energy were used for the 100 times search, with $40 \times 40 \times 40$ dimensions of grid box size and 0.381 Å grid spacing around the catalytic triad. The best conformation was selected from the lowest docking energy identified among the different pockets of the macromolecule for each ligand, with not more than 1.5 Å RMSD. The formula for calculation is as follows: $K_i = \exp(\Delta G/RT)$ (http://autodock.scripps.edu/faqs-help/faq/how-autodock-4-converts-binding-energy-kcal-mol-into-ki).

Statistical analysis

All the determinations were performed in duplicate, and data are mean ± SD values of three independent analyses. IC_{50} values were calculated by using Table Curve 2D Windows version 4.07 (SPSS Inc., Chicago, IL, USA). Results were subjected to analysis of variance, and differences between means at the $P < .05$ level were considered significant compared with the control in Student's *t* test.

RESULTS

We report here aromatase inhibition and suppression of TNFα-induced NFκB activation determined with stably transfected 293/NFκB-Luc human embryonic kidney cells by vanilloids. Capsaicin and capsazepine both demonstrated significant inhibition of NFκB activation, with IC_{50} values of 0.68 μM and 4.2 μM, respectively. TPCK was adopted as the positive control for the NFκB assay ($IC_{50} = 5\ \mu M$) (Table 1). Both compounds were also found to inhibit aromatase: capsazepine with an IC_{50} of 8.8 μM and capsaicin with an IC_{50} of 13.6 μM. This inhibition is comparable to that of the positive control, naringenin ($IC_{50} = 1\ \mu M$). At the highest concentration tested (53.1 μM for capsazepine and 65.5 μM for capsaicin), the vanilloids did not affect cell viability.

In silico docking results of capsaicin and capsazepine indicated strong binding compared with TPCK and naringenin (Table 1 and Fig. 2) and are in keeping with the *in vitro* biological activity.

The important amino acids participating in binding pocket formation for NFκB were Phe295, Lys334 (H-bond 3.03Å), Gln279, Phe298 (H-bond 2.84Å), Asp297, Gly296 (NFκB p50), Leu74, Gly72, Ala71, Asp69, Leu70 (H-bond 3.0Å), Gly91, Ser11, Gly1, and Ser3 (NFκB p100). The unique and highly conserved residues in capsaicin and capsazepine for NFκB p50 were Ser299 and Ile278 (H-bond 2.81Å) and for NFκB p100 were Ser6, Arg82, Val86, Arg90 (H-bond 2.89Å), Gly4, and Ser2 (H-bond 2.81Å). Specific residues interacting with TPCK were Ile280, Thr313, Lys315, Pro314, Gly293, and Arg281 for NFκB p50, whereas no unique amino acids were found with NFκB p100. Four of these residues (Thr313, Lys315, Glu293, and Arg281) are hydrophilic in nature, which could reduce binding affinity.

The important amino acids participating in binding pocket formation for aromatase were Phe134, Arg115 (H-bond 2.78Å), Ile133, Trp224, Arg376, Met374, Leu477, Ile395, Val373, Ser479, Leu396, Leu372, Val370, Val369, Pro429 (H-bond 2.1Å), Phe430, Gly431 (H-bond 2.69Å), Gly436, Cys437, and Asp371. Trp224, Arg435, and Val373 (H-bond 2.80Å) were found to be unique for both capsaicin and capsazepine, whereas no unique amino acids were found with naringenin.

For comparison, in addition to naringenin, we performed docking experiments using the well-known pharmacologic inhibitor letrozol. The GS for the interaction was 50.37. Trp224 was found as a unique and common amino acid among the capsazepine, capsaicin, and letrozole binding pockets. The important amino acids participating in binding pocket formation for aromatase have been discussed, including a few uncommon amino acids like Thr310, Gly439, Phe148, Met127, Phe221, Glu302, Ala306, Asp309, Met311, and Ala306. These extra amino acids may be the reason for the high GS for letrozole compared with naringenin, capsazepine, and capsaicin.

For analyzing viability and biological activity of the target molecule, about 11 structural properties were calculated through the project leader module of Fujitsu Computer Systems (Westwood, MA, USA) Scigress Explorer™ version 7.7 (*e.g.*, conformation minimum energy [in kcal/mol], dipole moment [in debye], electron affinity [in eV], dielectric energy [in kcal/mol], steric energy [in kcal/mol], total energy [in Hartree units], heat of formation [in kcal/mol], highest occupied molecular orbital energy [in eV], lowest unoccupied molecular orbital energy [in eV], ionization potential [in eV], polarizability, etc.) (Table 2). Drug-like properties of the compounds were analyzed using Lipinski's Rule of Five: the molecule should have not more than five hydrogen bond donors (OH and NH groups), not more than 10 hydrogen bond acceptors (notably N and O), a molecular weight under 500, and a partition coefficient log *P* of < 5.[35]

We observed that Autodock results showing low binding energy have low K_i values as well. To address this, we used Autodock version 4.0 for the estimation of K_i. GOLD is a well-known docking software, but it has limitations in

TABLE 1. INHIBITION OF AROMATASE ACTIVITY AND NUCLEAR FACTOR κB ACTIVATION BY CAPSAICIN AND CAPSAZEPINE

	Aromatase		*NFκB*	
	IC_{50} (μM)	*Docking score (GS)*	*IC_{50} (μM)*	*Docking score (GS)*
Capsaicin	13.6 ± 0.14	46.14	0.68 ± 0.03	37.31 (p50), 38.59 (p100)
Capsazepine	8.8 ± 0.33	51.25	4.2 ± 0.08	31.67 (p50), 36.77 (p100)
Naringenin	1.0 ± 0.18	45.64	ND	ND
TPCK	ND	ND	5.1 ± 2.1	35.71 (p50), 34.63 (p100)

Data are mean ± SD 50% inhibitory concentration (IC_{50}) values (in μM). Naringenin and *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK) were used as positive controls for the aromatase and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) assays, respectively. Docking scores (Goldscore [GS]) of vanilloids and positive controls with aromatase, NFκB p50, and NFκB p100 receptors were calculated.

ND, not determined.

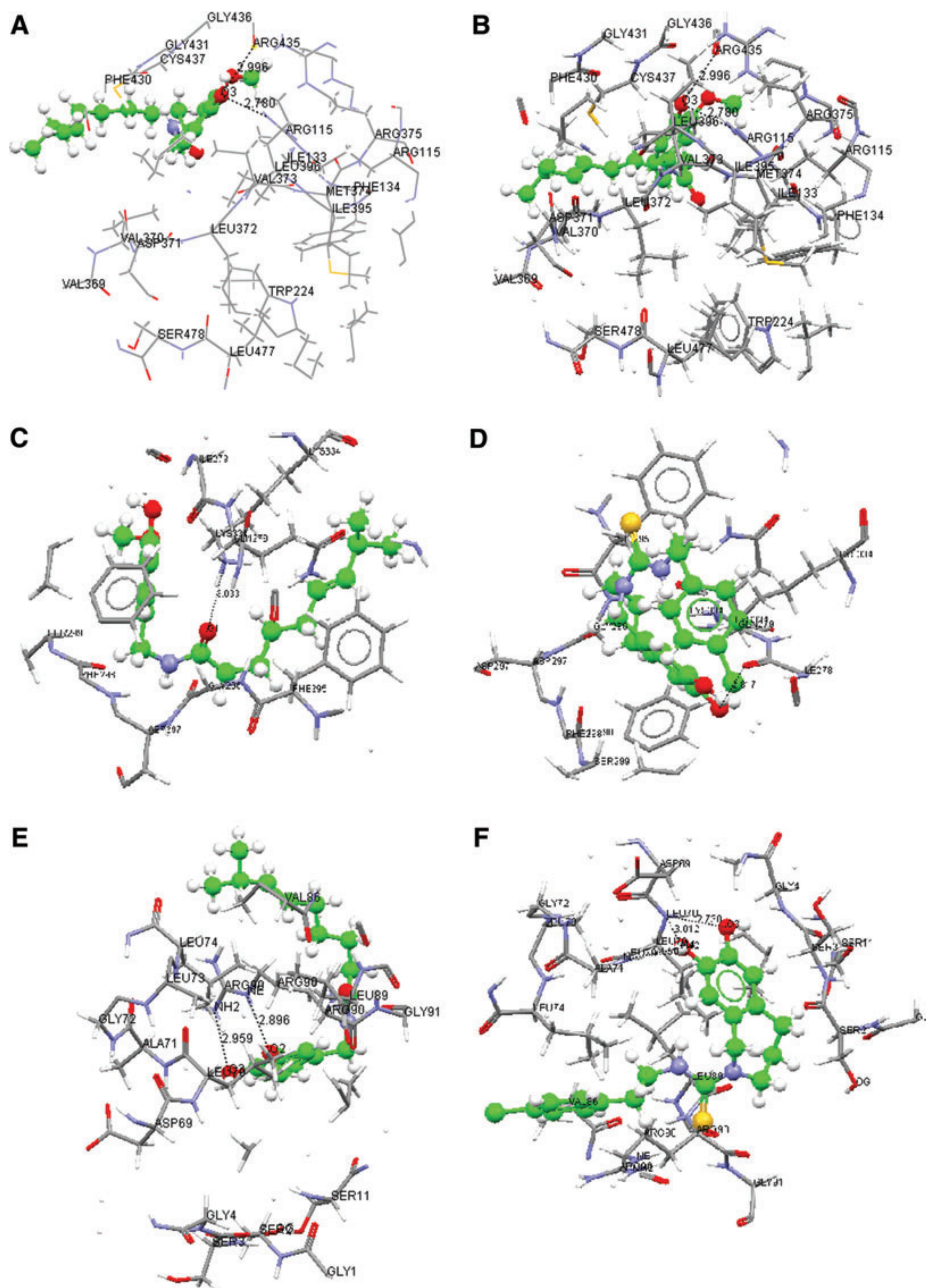

FIG. 2. Binding domains of aromatase, NF*κ*B p50, and NF*κ*B p100 receptors: (**A**) capsaicin docked on aromatase, (**B**) capsazepine on aromatase, (**C**) capsaicin on NF*κ*B p50, (**D**) capsazepine on NF*κ*B p50, (**E**) capsaicin on NF*κ*B p100, and (**F**) capsazepine on NF*κ*B p100. Color images available online at www.liebertonline.com/jmf

calculating K_i. On the basis of the estimated K_i value, we have categorized our compounds in descending order (*i.e.*, for aromatase, capsazepine > naringenin > capsaicin; for NF*κ*B p100, TPCK > caspazepine > capsaicin; and for NF*κ*B p50, TPCK > capsazepine > capsaicin) (Table 3).

DISCUSSION

The study of natural products can reveal interesting biology and generate leads pertaining to specific cellular targets, activities, and therapeutic manipulations. Capsicum is

Table 2. Quantitative Data and Physicochemical Properties of Capsaicin, Capsazepine, Naringenin, and TPCK

Molecule	*Conformation minimum energy (kcal/mol)*	*Dipole moment (debye)*	*Electron affinity (eV)*	*Dielectric energy (kcal/mol)*	*Steric energy (kcal/mol)*	*Total energy (Hartree)*	*Heat of formation (kcal/mol)*	*HOMO energy (eV)*	*Ionization potential (eV)*	*LUMO energy (eV)*	*Polarizability*
Capsaicin	−22.498	2.123	−0.020	−0.424	−23.128	−165.864	−124.702	−9.123	9.118	0.026	35.355
Capsazepine	−17.838	4.209	0.517	−0.937	−17.860	−184.890	−27.060	−8.439	8.440	−0.513	40.057
Naringenin	−16.833	4.171	0.607	−0.568	−16.795	−153.053	−158.860	−9.271	9.272	−0.610	29.335
TPCK	2.028	4.612	0.828	−0.816	2.108	−174.804	−67.797	−9.559	9.560	−0.827	35.102

HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

extensively used as a natural food additive for flavoring, seasoning, coloring, and antiseptic properties. Its use is especially popular in hot climates where consumption may be as high as 2.5 g/day.[36] The ubiquitous human consumption of foods containing capsaicin and other vanilloids suggests that these agents may also be important as dietary antioxidants and/or anti-inflammatory mediators.[37]

Recently, disruption of mitochondrial transmembrane potential, bioenergetics, and redox state, apoptosis by activation of peroxisome proliferator-activated receptor-γ, generation of reactive oxygen species, and antioxidant activity of a group of vanilloid compounds were described, as well as structure–activity relationships.[38] It is interesting that capsaicin has been reported to inhibit NADH oxidase activity in transformed cells[39,40] and in rat plasma membranes.[41–44] The inhibitory effect of capsaicin causes reactive oxygen species generation and oxidative stress in transformed cells. However, in erythrocytes, capsaicin acts as an antioxidant. Several studies show that capsaicin protects against lipid peroxidation, plasma membrane redox system function, -SH group oxidation, and protein carbonyl formations in erythrocytes.[10,12,13,38]

Among inhibitors of NFκB, salicylates, aspirin,[45] apigenin, and curcumin[46,47] were reported to suppress activation by preventing the degradation of the NFκB inhibitor (IκB), and therefore NFκB was retained in the cytosol. The serine protease inhibitor TPCK can block NFκB activation completely. TPCK inhibits the processing of NFκB subunits as well as IκB degradation.[48] It has also been demonstrated that TPCK inhibits DNA replication by G_0–G_1 growth arrest and thus promotes apoptosis by other cytotoxic stimuli.[49] The present study provides evidence that vanilloids can act as inhibitors of NFκB and aromatase. The inhibitory actions of vanilloids do not appear to result from nonspecific cellular toxicity but rather are specific for NFκB-dependent gene transcription. In other studies, it has been demonstrated that capsaicin inhibits phorbol ester–induced activation of NFκB and activator protein-1 by blocking the signal transducer and activator of transcription 3 signaling pathway.[50–52] In addition, we have found that capsazepine, a vanilloid receptor antagonist, also inhibits TNFα-induced NFκB activation and aromatase activity. Our findings on capsazepine are in agreement with a previous report on inhibition of nitric oxide and inducible nitric oxide synthase protein synthesis induced by lipopolysaccharide in RAW 264.7 macrophages; this inhibition occurred via suppression of mRNA expression through inactivation of NFκB[53] and inhibition of IκB-α degradation in lipopolysaccharide-stimulated peritoneal macrophages[54] by capsaicin. However, to our knowledge, this is the first report to demonstrate inhibition of aromatase activity by vanilloids.

Table 3. Binding Energy and Constant Inhibition of Tested Compounds

Receptor/ligand	*Estimated binding energy for Aromatase*	*NFκB (p50)*	*NFκB (p100)*	*Estimated K_i (μM)*
Naringenin	−7.22	ND	ND	4.87031×10^{-6}
Capsaicin	−6.42	ND	ND	1.88883×10^{-5}
Capsazepine	−7.95	ND	ND	1.41392×10^{-6}
Letrozole	−6.41	ND	ND	1.92110×10^{-5}
Capsaicin	ND	ND	−5.22	1.44260×10^{-4}
Capsazepine	ND	ND	−6.33	2.19996×10^{-5}
TPCK	ND	ND	−6.49	1.67759×10^{-5}
Capsaicin	ND	−5.13	ND	1.68023×10^{-4}
Capsazepine	ND	−6.02	ND	3.71973×10^{-5}
TPCK	ND	−6.48	ND	1.70626×10^{-5}

K_i, inhibition constant; ND, not determined.

To substantiate these responses, we performed docking experiments using capsaicin and capsazepine (ligands) with NFκB and aromatase (receptors). All the targets showed an acceptable minimum docking score. Docking with NFκB showed higher binding affinities for capsaicin and capsazepine compared with TPCK, possibly because of interaction with the hydrophobic residues Ile278, Leu73, and Val86. Interaction with Trp224 and Val373 may be responsible for the higher binding affinity of capsaicin and capsazepine with aromatase.

Results were analyzed to find common and unique amino acids. Amino acids common in binding pocket formation for test compounds as well as control compounds as in the case of NFκB were Phe295, Lys334 (H-bond 3.03Å), Gln279, Phe298 (H-bond 2.84Å), Asp297, and Gly296 for NFκB p50 and Leu74, Gly72, Ala71, Asp69, Leu70 (H-bond 3.0Å), Gly91, Ser11, Gly1, and Ser3 for NFκB p100. Other than these common amino acids, there were a few unique amino acids that were present in binding pockets of capsaicin and capsazepine for NFκB p50 (Ser299 and Ile278 [H-bond

Table 4. Evaluation of Capsaicin, Capsazepine, Naringenin, and TPCK for Lipinski's Rule

Molecule	Molecular weight < 500	Log P < 5	Group count for H-bond donors (< 5)			Atom count for H-bond acceptors (< 10)		Lipinski's Rule of 5 violations
			Amine	Secondary amine	Hydroxyl	Nitrogen	Oxygen	
Capsaicin	305.416	3.530	0	1	1	1	3	0.000
Capsazepine	376.900	5.699*	0	1	2	2	2	1.000
Naringenin	272.257	1.987	0	0	3	0	5	0.000
TPCK	351.847	4.183	0	1	0	1	3	0.000

*Violates Lipinski's Rule by 1 (Log $P < 5$).

2.81Å]) and NFκB p100 (Ser6, Arg82, Val86, Arg90 [H-bond 2.89Å], Gly4, and Ser2 [H-bond 2.81Å]). The important amino acids participating in binding pocket formation for aromatase were Phe134, Arg115 (H-bond 2.78Å), Ile133, Trp224, Arg376, Met374, Leu477, Ile395, Val373, Ser479, Leu396, Leu372, Val370, Val369, Pro429 (H-bond 2.1Å), Phe430, Gly431 (H-bond 2.69Å), Gly436, Cys437, and Asp371. Trp224, Arg435, and Val373 (H-bond 2.80Å) were found to be unique for both capsaicin and capsazepine, whereas no unique amino acids were found with naringenin. In the case of aromatase, the IC_{50} of naringenin is low compared with those of capsaicin and capsazepine. This may be due to differences in chemical configurations or bioavailability. Naringenin is a flavonoid, whereas capsaicin/capsazepine are vanilloids.

Lipinski's ''Rule of 5'' test for drug-likeness indicates that capsaicin is a viable drug candidate. For capsazepine, the log *P* (octanol/water partition coefficient) value was greater than 5, which could indicate low intestinal absorption (Table 4). The analysis of NFκB and aromatase using the X-ray structure as an alignment template led to the identification of important amino acids involved in the ligand-binding interactions. Hence, vanilloids, including capsaicin, could be used as a pharmacophore model for designing new cancer chemopreventive agents.

Based on these encouraging responses and molecular interaction studies, we suggest that the suppression of NFκB activation and aromatase activity by vanilloids could play a chemopreventive role in carcinogenesis. Further investigations into the actions of vanilloids, especially those related to the NFκB signaling pathway, aromatase inhibition, and *in vivo* models, could provide new insights into the medicinal value of these molecules.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist. All the authors have substantially participated in the investigation, data analysis, and the preparation of the manuscript and accept full responsibility for its content.

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