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ORIGINAL CONTRIBUTION

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Non-pungent capsaicinoids from sweet pepper

Synthesis and evaluation of the chemopreventive and anticancer potential

Summary *Background* Capsiate, the non-pungent ester isoster of capsaicin, and its dihydroderivative are the major capsaicinoids of sweet peppers. The remarkable difference between the sensory properties of capsaicin *vs* capsiate is solely due to the way the vanillyl and the acyl moieties of this basic structural motif are linked, via an amide bond in capsaicin-type compounds and via an ester bond in capsiate-type compounds. *Aim of the study* Since capsaicin induces apoptosis in tumoral cells by a vanilloid receptor type 1(VR1)-independent pathway, we examined the effects of capsiates derived from sweet peppers in the ROS generation and induction of apoptosis in tumoral cells and if these are mediated independently from VR1. *Methods* We have developed an expeditious synthesis of capsiates based on the esterification of vanillol with the Mitsunobu protocol. Capsiate-induction of apoptosis, generation of reactive oxygen species and disruption of the mitochondria transmembrane potential in tumoral cell lines were measured by flow cytometry. Chemopreventive activity was studied in a two-stage mouse skin carcinogenesis assay. *Results* Capsiates induce apoptosis that was preceded by an increase in the production of reactive oxygen species and by a subsequent loss of mitochondria transmembrane potential ($\Delta\psi_m$). These properties were retained in simplified synthetic analogues of natural capsiates, one of which (*nor*-dihydrocapsiate) showed powerful chemopreventive activity. *Conclusions* These results suggest that capsiates and related synthetic analogues target a variety of pathways involved in cancer development and inflammation, and have considerable potential for dietary health benefits as well as for pharmaceutical development.

Key words capsiate – VR1 – ROS – apoptosis and cancer

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Abbreviations

CPT	*nor*-dihydrocapsiate
DBMA	dimethylbenz[*a*]anthracene
CoQ	coenzyme Q-*10*
CPS	capsaicin (*E*-8-methyl-*N*-vanillyl-6-nonenamide)
Eth	ethidium
HE	dihydroethidine
PI	propidium iodide
PM	plasma membrane
PMOR	plasma membrane NADH-oxidoreductase
PMA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species
VR1	vanilloid receptor 1
$\Delta\Psi_m$	mitochondrial transmembrane potential

Introduction

The hot taste of chili pepper (*Capsicum annuum* L.) has generated an intense research activity aimed at the characterization and exploitation of the pungent compounds responsible for the sensory properties of this spice. The active principle, capsaicin (*E*-8-methyl-*N*-

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vanillyl-6-nonenamide, CPS, *1*), was first isolated in 1876, and an enormous body of literature has been accumulated on its chemistry and pharmacology [1]. The outgrowth of this attention was the discovery and cloning of a cell surface receptor (VR1) that binds CPS in a specific way, and a structurally heterogeneous group of pungent compounds named vanilloids [2]. VR1 belongs to the family of putative store-operated calcium channels, and is expressed mainly in peripheral pain-sensing neurons. The generation of *knock out* mice for the VR1 gene supported a correlation between the pungency of CPS and the activation of this receptor "in vivo" [3, 4], while several investigations have highlighted the therapeutic value of vanilloids to treat painful disorders such as peripheral neuropathies and rheumatoid arthritis [5]. Considerable amounts of CPS-like compounds have also been reported in sweet pepper (*Capsicum annuum* L.) [6], but the lack of pungency of these CPS analogs has long made them unattractive targets for research. Recent studies have shown that hot pepper and sweet pepper contain similar compounds, whose structural hallmark is the presence of a vanillyl core bound to a branched fatty acid [7]. The remarkable difference between the sensory properties of these plants is solely due to the way the vanillyl and the acyl moieties of this basic structural motif are linked, via an amide bond in hot pepper (capsaicin (*1*)-type compounds) and via an ester bond in sweet pepper (capsiate (*3a*)-type compounds or capsiates). Despite the growing interests in secondary metabolites from edible plants, and the presence of sizeable amounts of capsiates in sweet pepper, no biological property apart from the lack of pungency has been reported for these compounds.

CPS, and possibly other vanilloids as well, is endowed with a pleiotropic pattern of biological activities, some of which are mediated by the activation of cellular targets different from VR1. Thus, CPS can inhibit the plasma membrane NADH oxidase [8, 9] and induce apoptosis in tumoral cells and in activated T cells in a VR1-independent way [10–12]. Analogues of CPS, devoid of VR1 activity but retaining the ability of the natural product to induce apoptosis in cancer cells, have potentially far-reaching therapeutic applications. We present evidence that capsiates lack significant vanilloid activity, but can nevertheless exert pro-oxidant activity and induce apoptosis in cancer cells. These properties were retained in the simplified analogue *nor*-dihydrocapsiate (*3c)*, which could strongly prevent tumorogenesis in athymic mice.

Materials and methods

Syntheses

General

Column chromatography: Merck Silica Gel. IR: Shimadzu DR 8001 spectrophotometer. NMR: Bruker AM 200 (200 MHz and 50 MHz for ^{1}H and ^{13}C, respectively). For ^{1}H NMR, $CDCl_3$ was the solvent, with $CHCl_3$ at $\delta = 7.26$ as the reference. For ^{13}C NMR, $CDCl_3$ was the solvent, with $CDCl_3$ at $\delta = 77.0$ as the reference. CH_2Cl_2 was dried by distillation from CaH_2, and THF by distillation from Na/benzophenone. Na_2SO_4 was used to dry solutions before the evaporation. Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates, which were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. (Z)-8-Methyl-6-nonenoic acid, its *E*-isomer and its hydrogenation product were prepared according to literature procedures [13].

Synthesis of natural (3a, 3b) and synthetic (3c, 3d) capsiates. Synthesis of *nor*-dihydrocapsiate (3c) as exemplificative

To a cooled (0 °C) solution of vanillyl alcohol (*2*) (2 g, 13 mmol) in dry THF (50 mL), nonanoic acid (2.05 g, 13 mmol, 1 mol equiv) triphenylphosphine (3.4 g, 1.3 mmol, 1 mol equiv) and diethylazodicarboxylate (DEAD, 2.333 g, 1.3 mmol, 1 mol equiv) were added. The cooling bath was removed, and the solution was allowed to warm to room temperature over 2 h. The solvent was then removed, and the residue was purified by CC (hexane-EtOAc 9:1 as eluant) to give 2.675 g (70 %) *3d* as a colorless oil. IR (KBr) ν_{max} cm^{-1} 3584, 1732, 1518, 1277, 1159, 1122, 1035; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.66 (3H, br m), 5.03 (2H, s), 3.91 (3H, s), 2.33 (2H, t, $J = 7.8$ Hz), 1.83 (12H, br m), 0.88 (3H, t, $J = 0.88$ Hz); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 173.7 (s), 146.3 (s), 145.5 (s), 127.8 (s), 121.8 (d), 114.2 (d), 111.1 (d), 66.7 (t), 55.7 (q), 34.2 (t), 31.6 (t), 29.0 (t), 24.8 (t), 22.5 (t), 13.9 (q). HRMS *m/z* 294,1840 (calcd for $C_{17}H_{26}O_4$, 294,1831).

Z-Capsiate (*3b*): colorless oil. IR (KBr) ν_{max} cm^{-1} 3453, 1732, 1614, 1518, 1464, 1277, 1159, 970; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.88 (3H, br m), 5.19 (2H, br s), 5.03 (2H, s), 3.90 (3H, s), 2.57 (1H, m), 2.35 (2H, t, $J = 7.8$ Hz), 2.07 (2H, m), 1.66 (2H, m), 1.38 (2H, m), 0.94 (6H, d, $J = 0.88$ Hz); HRMS *m/z* 306,1822 (calcd for $C_{18}H_{26}O_4$, 306,1831).

Biological assays

Cell lines and reagents

Jurkat cells (TIB-152, ATCC, Rockville, MD, USA) were maintained in exponential growth in RPMI-1640 medium (Bio-Whittaker, Verviers, Belgium), and human embryonic kidney-derived 293T cells (CRL-1573, ATCC) in DMEM (Bio-Whittaker). The culture media were supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine and the antibiotics penicillin and streptomycin (Gibco, Paisley, Scotland). All other reagents not cited above or later were from Sigma Chemical Co. (St. Louis, MO, USA).

Determination of mitochondrial transmembrane potential and ROS generation

To study the mitochondrial transmembrane potential ($\Delta\psi_m$) and the superoxide anion generation (ROS), cells were incubated (10^6/ml) in phosphate-buffered saline (PBS) with $DiOC_6(3)$ (green fluorescence) (20 nM) (Molecular Probes, Eugene, OR, USA) and dihydroethidine (HE) (red fluorescent after oxidation) (2 μM) (Sigma) for 20 min at 37 °C, followed by analysis on an Epics XL Analyzer (Coulter, Hialeah, FL, USA). When indicated, the percentage of dead and living cells was determined by incubating an aliquot of the cells with 10 μg/ml of propidium iodide (PI) for 2 min at room temperature. Cells permeable to PI (dead cells) appeared as red fluorescent by flow cytometry.

Determination of nuclear DNA loss and cell cycle analysis

The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70 %, for 24 h at 4 °C). Then, the cells were washed twice with PBS and subjected to RNA digestion (RNAse-A, 50 U/ml) and PI (20 μg/ml) staining in PBS for 1 h at room temperature, and analyzed by cytofluorimetry. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows the determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).

Transient transfections and determination of cell death through the VR1

293T cells (10^5/ml) were transiently co-transfected with the pEGFP-C1 plasmid encoding a brighter fluorescent variant of the *Green Fluorescent Protein* (Clontech, Palo Alto, CA, USA), and with either the plasmid encoding the complete rat cDNA VR1 [2] or the empty vector, pcDNA3 (Invitrogen, Breda, The Netherlands). The transfections were made by using the lipofectamine reagent (Life Technologies, Madrid, Spain) according to the manufacturer's recommendations. Forty-eight h after transfection the cells were stimulated with the various compounds for 6 h, the detached cells removed (dead cells) and the percentage of adherent green fluorescent cells (living cells) measured by flow cytometry. All the experiments were repeated at least three times.

Two-stage skin carcinogenesis test

Two groups of 6 female CD-1 mice for control or 7 mice for CPT treatment (4 to 5 weeks old) were initiated by a single topical application of 200 μmol of DBMA in 0.2 ml of acetone in the shaved dorsal region. One week later, the mice were treated with 0.2 ml of vehicle (acetone) or capsiate (200 μmol in 0.2 ml acetone) one hour before the application of 2.5 μg of PMA in 0.2 ml acetone, twice weekly for 18 weeks. Animals were weighed weekly, and the number of visible tumors was counted once every week. The mean volume of skin papillomas was calculated as an ellipsoid. Statistical analyses were performed by Fisher's and *t*-tests.

Results

The capacity of different vanilloid analogues to induce apoptosis in human transformed cell lines was investigated. The diagram of the synthesis and the compounds used in this study are portrayed in Fig. 1; capsaicin, CPS (*1*); capsiate (*3a*); *Z*-capsiate (*3d*); dihydrocapsiate (*3b*); *nor*-dihydrocapsiate (*3c*).

Induction of apoptosis by CPS and capsiates in Jurkat cells

It has been suggested that a mitochondrial $\Delta\psi$ breakdown and a later ROS generation are invariant features of early apoptosis [14, 15]. Thus, we studied the role of the mitochondria ($\Delta\psi_m$) and ROS generation in the apoptotic pathway in Jurkat T cells treated with 100 μM of the following compounds: capsaicin (*1*), dihydrocapsiate (*3b*), *nor*-dihydrocapsiate (*3c*), capsiate (*3a*) and *Z*-capsiate (*3d*). Compounds *3a* and *3b* are natural capsiates, while the remaining compounds are synthetic. The cells were treated for 6 h, and ROS generation and $\Delta\psi_m$ dissipation were detected by double staining experiments, using HE (non-fluorescent), which is oxidized to ethidium (Eth, red fluorescent) via ROS and $DiOC_6(3)$ (green fluorescent), a cationic probe that accumulates in mitochondria as a function of their membrane potential. In these experiments, untreated cells were used to establish control values, considering these cells to have a high $\Delta\psi_m$ ($DiOC_6(3)^{high}$) and low levels of intracellular ROS ($HE \rightarrow Eth^{low}$) (Fig. 2A). As expected, CPS treatment of Jurkat cells leads to an increase in the percentage of

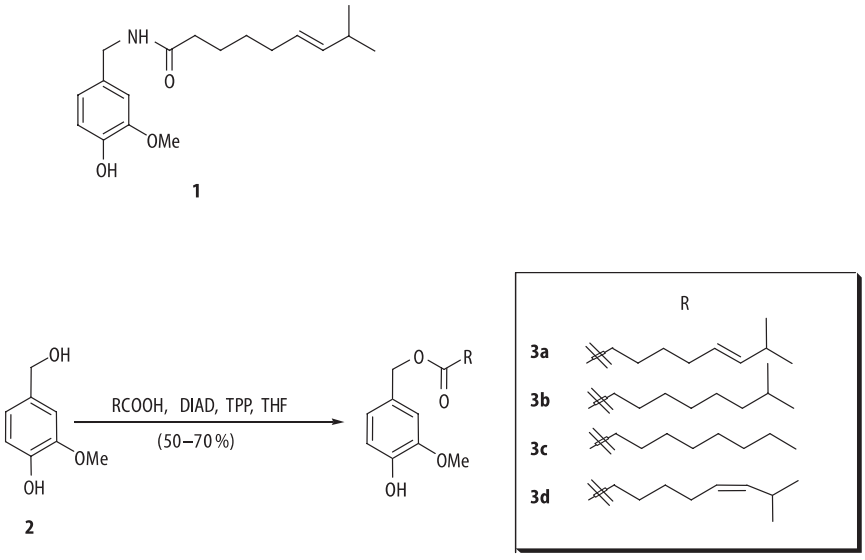

Fig. 1 Chemical structure of capsaicin (*1*) and schematic synthesis of natural (*3a, 3b*) and synthetic (*3c, 3d*) capsiates

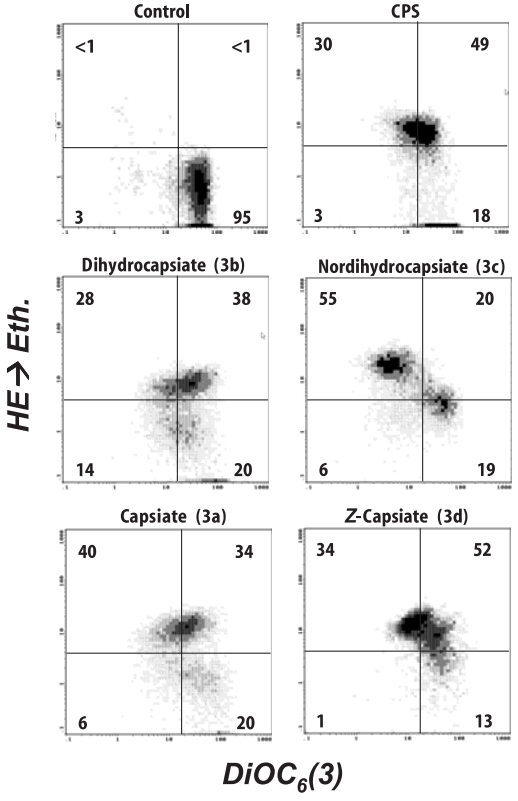

Fig. 2 *Reactive oxygen generation by capsaicinoids in Jurkat cells.* Jurkat cells were treated with the indicated compounds at 100 µM. After a 6-h treatment, cells were collected and the simultaneous $\Delta\psi_m$ disruption and ROS generation detected by flow cytofluorimetry. The numbers in the corners represent the percentage of cells obtained in biparametric histograms delimited by four compartments, namely, i) bottom-"right" that correspond to normal cells that have $\Delta\psi_m^{high}$ indicated by $DiOC_6(3)^{high}$ staining and low levels of ROS indicated by $(HE \rightarrow Eth)^{low}$; ii) $\Delta\psi_m^{low}/DiOC_6(3)^{low}$ and $(HE \rightarrow Eth)^{low}$ (bottom-"left"); iii) ROS generating cells $(HE \rightarrow Eth)^{high}$ with $\Delta\psi_m^{high}/DiOC_6(3)^{high}$ (top right), and iiii) ROS generating cells $(HE \rightarrow Eth)^{high}$ with $\Delta\psi_m^{low}/DiOC_6(3)^{low}$ (preapoptotic cells, top left compartment). Results are representative of five independent experiments

$(HE \rightarrow Eth^{high})$ cells that reflects ROS hypergeneration (49 %) which was followed by a loss in the mitochondria transmembrane potential (30 % of $DiOC_6(3)^{low}$ cells). Similar results were obtained with all natural and synthetic capsiates tested. In all the cases, $\Delta\psi_m$ dissipation was preceded by an increase in intracellular ROS as detected in kinetic experiments (data not shown). Nevertheless, in Jurkat cells treated with dihydrocapsiate (*3b*) a significant percentage (14 %) of cells with $DiOC_6(3)^{low}/HE \rightarrow Eth^{low}$ was detected. All capsiates tested were biologically active, and further experiments were carried out with the simplified analogue *nor*-dihydrocapsiate (*3c*). Next, we analyzed apoptosis by measuring the percentage of hypodyploid cells and, as shown in Fig. 3A, both compounds induced apoptosis in Jurkat cells measured as the percentage of cells that undergo chromatinolysis. The apoptosis induced by these capsaicinoids was found to be dose-dependent, with *nor*-dihydrocapsiate being more potent than CPS in this biological activity (IC50 70 µM and 128 µM for *nor*-dihydrocapsiate and CPS, respectively). Taken together, these results strongly suggest that capsiate induces apoptosis in Jurkat cells through mechanisms similar to those described for CPS [10, 16].

Nor-dihydrocapsiate does not activate the vanilloid receptor type 1

CPS can induce cell death in 293T cells transfected with the rat VR1. This cell death occurs rapidly and with concentrations of CPS incapable of inducing apoptosis in the absence of VR1 [2, 11]. To evaluate whether capsiate binds and activates the VR1, we co-transfected the cells either with an expression plasmid containing the entire VR1 cDNA or with the empty vector (pcDNA3), and

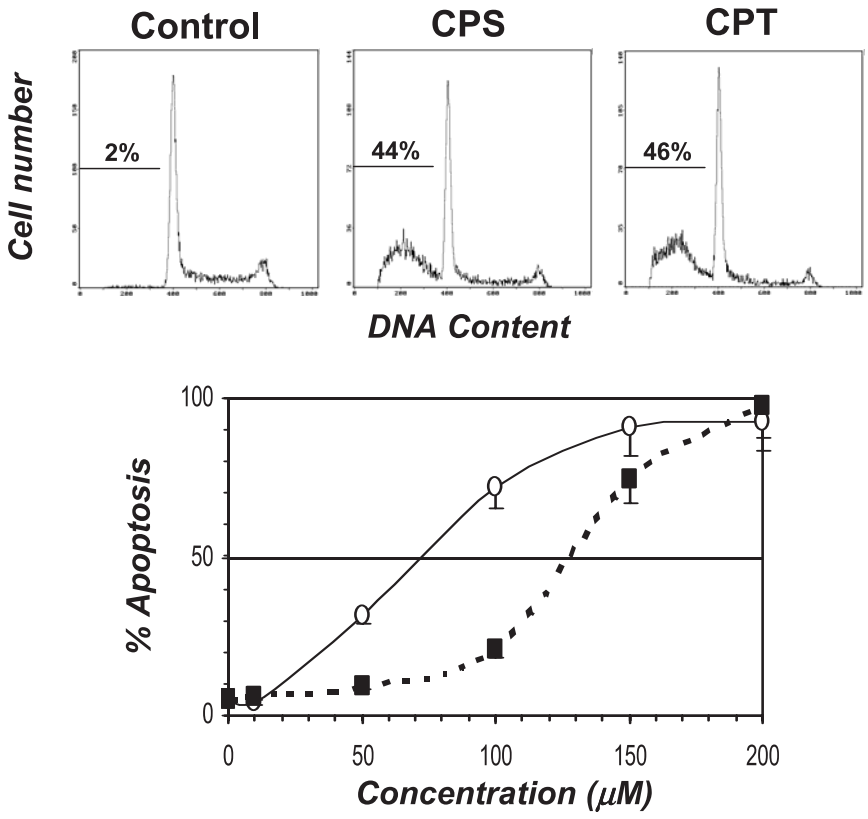

Fig. 3 *Induction of apoptosis by CPS and CPT in Jurkat cells. Detection of cells with fragmented DNA*. Jurkat cells were treated with increasing concentrations of CPS (filled squares) and capsiate (open circles) for 18 h and the percentage of apoptotic cells determined by cell cycle analysis (hypodiploidy). Values are means ± SE of three independent experiments

with an equimolar concentration of the plasmid pEGFP-C1. Forty-eight hours after transfection, the cells were stimulated with either CPS or *nor*-dihydrocapsiate (5 μM) for 6 h and the percentage of green fluorescent cells was detected by flow cytometry. We found that this cell type is transiently transfected with a high efficiency, and Fig. 4 shows that about 60 % of the cells express the green fluorescent protein. The percentage of positive cells did not change after the addition of CPS to pcDNA3 co-transfected cells (59 %), while a clear reduction in this percentage was observed in cells co-transfected with the plasmid pcDNA3-VR1 (32 %). Interestingly, *nor*-dihydrocapsiate could not affect the percentage of green fluorescent cells, indicating that cell death and detachment from the culture plate did not occur with this compound. These results strongly suggest that *nor*-dihydrocapsiate does not bind and activate VR1, data that correlate with the general lack of pungency of capsiates [7].

Chemopreventive activity of nor-dihydrocapsiate

The biological activity of *nor*-dihydrocapsiate was investigated *in vivo* in the two-stage mouse skin carcinogenesis assay using dimethylbenz[*a*]anthracene (DBMA) as an initiator and PMA as a promoter. During a 119-day study, 100 % of incidence was detected in PMA-treated CD-1 mice and the number of skin papillomas increased progressively with an average of 29.6 tumors per animal at the end of this period. Treatment of mice with *nor*-dihydrocapsiate 1 h before the PMA application not only reduced the tumor incidence to 50 % (15.2 tumors per animal) at the doses tested, but also caused a significant delay in the appearance of tumors (Fig. 5A). Interestingly, at day 119 of the study the mean tumor volume was also greatly reduced in the group of mice treated with *nor*-dihydrocapsiate (Fig. 5B).

Discussion

Eukaryotic cells continuously produce ROS as side products of redox reactions. Generation of ROS is mainly under mitochondrial control, and involves the production of hydrogen peroxide, hydroxyl radicals, and superoxide anions. Production of ROS by aerobic organisms is a "double-edged sword" since these species are required for signal transduction in pathways controlling cell growth, but can also damage structures essential for cell integrity and homeostasis [reviewed in 17]. The plasma membrane of certain types of cells contains a further electron transport chain, apparently essential to control cell growth and differentiation. This

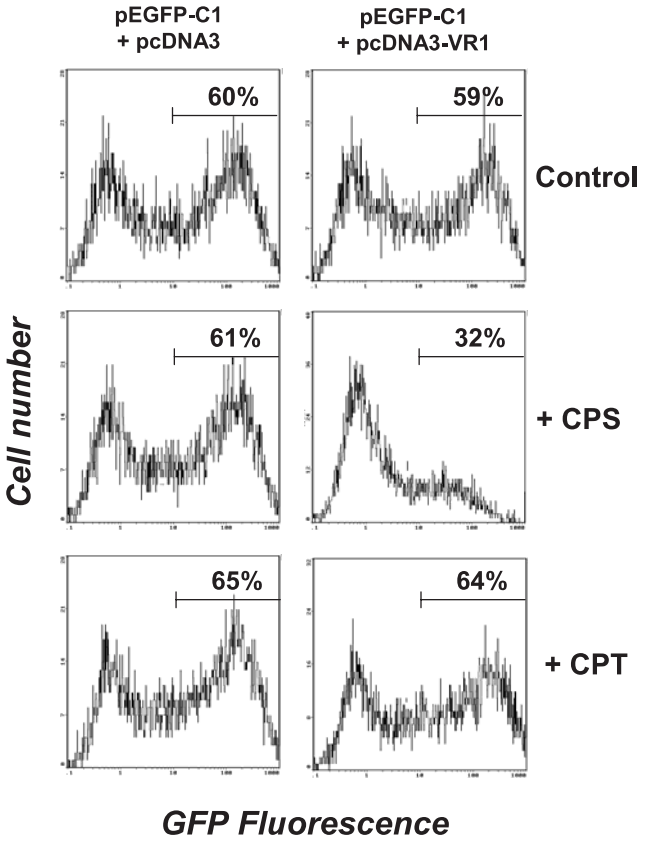

Fig. 4 *CPS but not CPT induces cell death in VR1-transfected cells.* The cells were co-transfected with the plasmid pEGFP-C1 and with either pcDNA-3 or pcDNA3-VR1 plasmids. After 48 h, the cells were stimulated with either CPS or CPT (5 μM) for 6 h and the remaining adherent living cells were analyzed for green fluorescent protein expression by cytometry. Results are representative of five different experiments

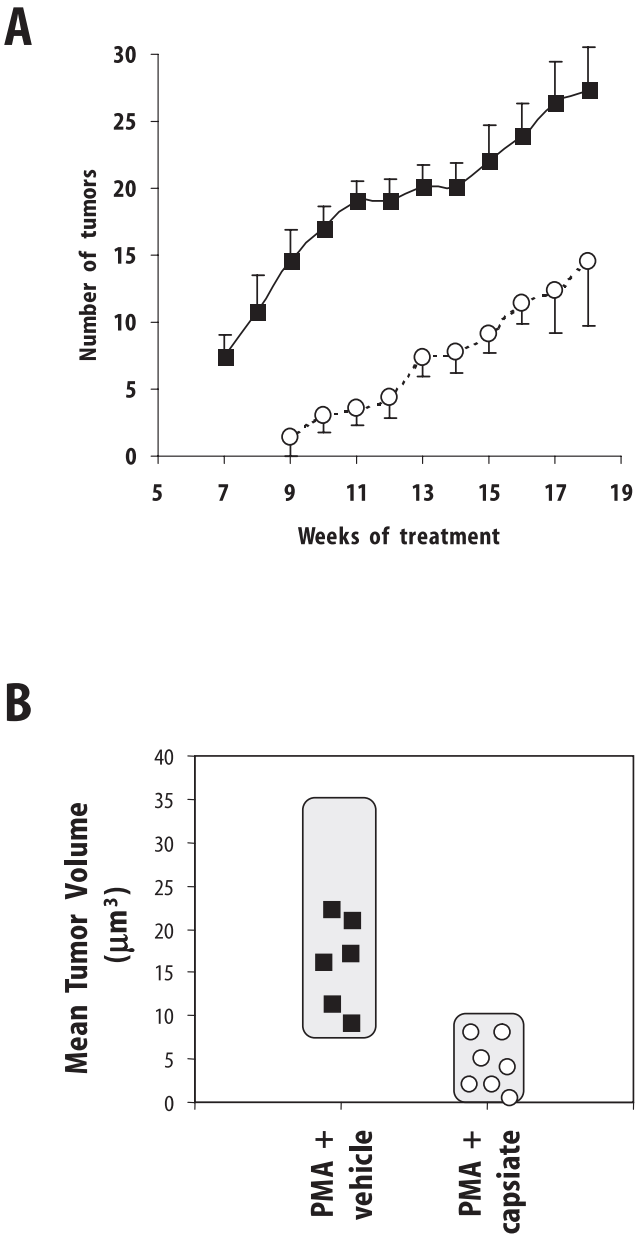

Fig. 5 *Chemopreventive effect of capsiate on skin carcinogenesis.* **A** Number of papilloma skin tumors appeared in mice treated with PMA as carcinogen, alone (filled squares) or with CPT (open circles). Results are mean ± SE of 6 mice (7 mice in the CPT-treated group), $p \leq 0.005$ for all points. **B** Mean of skin tumor volume in each tumor-bearing mouse at the end of the experiment (119 days). Shared areas represent the SE distribution of each overall group, $p \leq 0.02$

PMOR system has been found in animal, plant and yeast cells [18] and has been suggested to play a role in the maintenance of the cellular redox state. The constituents of the PMOR system have long remained elusive, but a plasma membrane NADH-oxidase (NOX) was eventually characterized as a 32 kDa ectoenzyme located on the outer surface of the plasma membrane [8, 19]. This NADH oxidase activity is inhibited by the vanilloid CPS, and may be an important target for anticancer drugs, since the upregulation of the PMOR system could be a mechanism by which cancer cells tolerate the high levels of ROS associated to their increased metabolic rate. Since cancer cells also have a low superoxide-dismutase activity, they are especially sensitive to drugs which interfere with the regulation of enzymes regulating the levels of intracellular ROS, which in turn leads to free-radical damage to mitochondrial membranes, release of cytochrome c, and apoptosis [20]. The apoptotic properties of known inhibitors of the NADH-oxidase activity like CPS cogently validate this point [8, 12]. The chemopreventive and antitumoral profile of CPS is marred by its irritancy and vanilloid activity. Here, we present evidence that capsiates, a class of non-pungent analogues of CPS contained in sweet pepper, can inhibit the PMOR system in tumoral cells to a greater extent than CPS and without activating the vanilloid receptor.

Cell-cycle analysis in Jurkat cells and measurements of ROS generation and disruption of $\Delta\psi_m$ by flow cytometry showed that natural (*3a*, *3b*) and synthetic (*3c*,

3d) capsiates are able to alter the redox state of the cells and to induce apoptosis. Capsiates were unable to induce apoptosis in normal T cells at the concentrations used to kill tumoral cells, a property shared with CPS [10, 21]. It is possible that interference of capsiate with the CoQ binding site of the PMOR system seemingly redirects the electron flow in the complex, generating an excess of ROS and a pro-oxidative environment. This, in turn, might induce oxidation of thiol groups in the mitochondrial permeability transition pores (PT) [22], allowing their opening, the free distribution of solutes at both sides of the inner mitochondrial membrane, and the eventual activation of the apoptotic-executor caspase-3. Alternatively, capsiates might induce mitochondrial depolarization by direct inhibition of complex I of the respiratory chain.

No significant differences in apoptotic potency was observed between the natural capsiates and their synthetic analogues, showing that a discrete branching and unsaturation have little effect on activity, at least if the length of the linear chain is maintained. On account of its easier availability, *nor*-dihydrocapsiate (*3c)*, a synthetic analogue almost twice as potent as CPS to induce apoptosis, was selected for further "in vivo" experiments. Using cells transfected with the cloned rat VR1, we could not detect any significant functional vanilloid activity for *3c*, while binding experiments with ^{3}H-RTX did not show any activity in binding experiments either (A. Szallasi, personal communication with G. A.). The presence of a hydrogen bonding donor at the benzylic position is an important requirement for the vanilloid activity of benzyl type (C6-C1-X-COR) capsaicinoids [23, 24]. Thus, the observation that capsiates lack pungency and VR1 functional activity is not surprising, although their inverted analogues (compounds of the C6-C1CO-X-R type) retain considerable pungency and vanilloid activity [23, 24].

The antitumoral activity of CPS has been documented "in vivo" not only in tumor prevention, but also when injected directly on the tumor [25]. Nevertheless, this type of vanilloids may not be suitable for systemic use, as they can also target the vanilloid receptor VR1 and can have important side effects [26]. Systemic administration of capsiates was well tolerated in animals [21]. This observation and the interesting profile of in vitro activity led us to investigate the chemopreventive activity of capsiates *in vivo*. The synthetic analogue *nor*-dihydrocapsiate (*3c*) was employed because of its easier availability in sizeable amounts compared to the natural products. A two-stage mouse skin carcinogenesis assay using dimethylbenz[*a*]anthracene (DBMA) as an initiator and PMA as a promoter showed powerful chemopreventive activity, reducing tumor incidence to 50 %, causing a significant delay in the appearance of tumors, and greatly reducing the mean tumor volume in the treated mice group. Nonetheless, the chemopreventive activity of CPT, in addition to the effect on the PMOR system, may also be a consequence of its capacity to inhibit the transcription factor NF-κB in response to either PMA or proinflammatory cytokines [21].

Chemoprevention of cancer by ingestion of chemicals is thought to be a major way to reduce the risk of carcinogenesis, and dietary natural products may be of special relevance because of their long record of safe human consumption. The research on secondary metabolites from edible plants has long been limited to compounds that are detected by our senses and are relevant in the study of flavor and taste. The discovery of the remarkable properties of capsiates exemplifies the rich pharmacological potential of dietary secondary metabolites which go undetected by our senses, and suggests that, while the nutritional aspects of food have been well characterized, their pharmacological properties are still largely unexplored and unexploited.

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