Appendix
Supplemental Digital Content1
Rosemary: Overview of Health Benefits
Keith Singletary PhD

Methods of literature search

The PubMed literature database was consulted for this general overview. Search terms included *Rosmarinus officinalis* L., rosemary extract, carnosol, carnosic acid, rosmarinic acid and 1,8-cineole. Full reports and English abstracts of foreign-language articles from peer-reviewed journals were primary sources of information. The quality of some studies' methodologies varied, particularly in regard to adequately describing the composition of test samples. Nonetheless, these were included in this discussion so that the variety of information can be evaluated and important issues for future research can be identified. Commercial and governmental reports were also supplementary sources. Cell culture and especially animal studies were selected for inclusion along with human studies where available.

Bioavailability of rosemary and individual constituents

The bioavailability of major bioactive diterpenoids in a rosemary ethanol extract containing 39% w/w carnosic acid (CA) was reported in rats. In an acute study (1) lean rats were fed diets supplemented with rosemary extract (0.5%) for 15 days, followed by a single bolus (100 mg, i.g.) of the extract. This corresponded to 577 mg/kg* (*unless otherwise indicated mg/kg refers to mg sample/kg body weight) of the rosemary extract or 230 CA/kg. Tissue and blood samples were taken at intervals from 25 to 800 min. CA was rapidly absorbed into bloodstream with a T_{max} of 25 min. Maximum plasma concentrations of CA, CA-glucuronide and carnosol were 26.5, 59.9 and 18.1 μM,

respectively. Lean and obese Zucker rats also were fed a diet supplemented with this extract (0.5% w/w) for 64 days (1). In this chronic study carnosol-glucuronide and CA-glucuronide were present in the plasma of lean rats at 9.2 and 10.5 μM, respectively, and a hydroxyl rosmariquinone metabolite was detected at 195.6 μM. Free CA was not detected and carnosol was present at 3.5-6.0 μM. Of interest, CA and CA-12-methyl ether were detected in the brain at levels of 1.9 and 4.0 μg/g tissue, respectively. Moreover, numerous glucuronide conjugates of rosemary constituents were detected in small intestine contents. It is noteworthy that no significant differences in the metabolic profiles between lean and obese rats were observed. In two other studies the bioavailability of CA, when administered as a single compound (65-90 mg/kg), has been reported in rats to be 40-65%, depending on the method of administration. Following acute oral dosing, CA was found to be circulating in its free form at a concentration of 42-106 μM (2,3). Additionally, for mice dosed orally with CA (3 mg/25g mouse), CA was detected in the brain within 1 hr, suggesting that CA is rapidly absorbed and subsequently able to penetrate the blood-brain barrier. The absorption efficiency and bioavailability of oral rosmarinic acid (RA) in rats was measured after a single dose of 50 mg/kg (4). The maximum level of free and conjugated forms of RA in the plasma was 4.63 μM after 30 min, indicating that oral RA is rapidly absorbed. RA is known to be metabolized by host enzymes and gut microflora to such intermediates as caffeic acid and ferulic acid (4-12). Percutaneous dosing of RA can substantially increase its bioavailability (13), suggesting that this route may be promising for therapeutic applications of this compound. The absorption of 1,8-cineole in possums was determined following consumption in a diet (1-4% w/w) for 3 days (14). The blood levels of cineole

did not exceed 51.8 μM regardless of the dietary amount. It should be noted that the presence of other dietary factors, as well as type and gender of animal used may cause variability in bioavailability findings (1).

Neurological actions

Neuroprotective effects

Individual constituents

Rosmarinic acid

Several *in vitro* investigations provide evidence for a general neuroprotective action of RA. For example, RA (14-56 μM) protected human dopaminergic neuronal cells against H_2O_2-induced apoptosis, an effect in part due to induction of the antioxidant enzyme heme oxygenase-1 (15). In three *in vitro* models of neuronal cell death RA (0.1-100 μM) significantly protected cells from neurotoxic insult in part by preventing oxidative stress and intracellular Ca^{++} overload, as well as by increasing the expression of peroxisome proliferator-activated receptor (PPAR)-γ, a change known to lower the expression of pro-inflammatory genes (16). RA also was reported to modulate neuroprotective heat shock proteins when added to cultures (15 μM) of rat PC12 cells (17).

Some *in vitro* actions of RA relate specifically to neurodegenerative conditions. For example, RA pretreatment (10 and 100 μM) of MES23.5 dopaminergic cell cultures protected them from 6-hydroxydopamine (6-OHDA)-induced neurotoxicity (18). This is noteworthy since one neuropathological feature of Parkinson's disease (PD) is the loss of dopaminergic neurons in the *substantia nigra pars compacta* region of the brain with the consequent decrease in dopamine in the striatum. Another *in vitro* experiment evaluated

RA's impact on a histopathological marker of PD, the aggregation of fibrillar proteins, such as α-synuclein, within filamentous inclusions called Lewy bodies in nigral dopaminergic neurons. RA (10 and 50 μM) was able to inhibit the formation of new fibrils and to accelerate the destabilization of preformed fibrils in solutions of α-synuclein (19). Of interest is the report that RA was selected as a lead compound for PD drug development based on computational analysis of its docking affinity with the dopamine D3 receptor and its lack of toxicity. This finding is important, since one therapeutic approach for PD is direct stimulation of dopamine receptors (20). There is also *in vitro* evidence for a benefit of RA toward Alzheimer's disease (AD). In PC12 cell cultures it was reported that RA treatment (0.1 – 100 μM) afforded protection from amyloid-β peptide (Aβ)-induced neurotoxicity, in part by inhibiting Aβ-associated reactive oxygen species formation, lipid peroxidation, neuronal apoptosis, and tau protein hyperphosphorylation (21). The authors suggested, in light of RA's low toxicity, that it be considered as a potential agent in therapeutic strategies for AD. Additionally, RA was shown by NMR analysis, to promote disaggregation of $A\beta_{1-42}$ peptides, the oligomers of which are believed to be the most toxic Aβ species *in vivo* (22). Overall, RA demonstrated strong anti-Aβ-aggregation activity *in vitro*. RA was identified as a promising nonpeptidyl ligand mimicking peptidyl inhibitors of caspase-3 in *in silico* molecular docking analyses evaluating potential candidates for therapy of multineurodegenerative disorders (23).

In vivo confirmation of RA's action toward degenerative diseases is more limited. For example, brains of rats were microinjected with 6-OHDA, and for 21 days thereafter rats were administered RA (20 mg/kg, i.g.). RA protected against several 6-

OHDA-induced changes, including dopamine depletion in the striatum, decreased tyrosine hydroxylase-positive neurons in the *substantia nigra,* and increased iron-containing cells of the *substantia nigra* (24). RA also counteracted 6-OHDA-induced downregulation of the Bcl-2/Bax ratio. Taken together, these findings indicate that RA has substantial neurorescue activity in this model. In an AD model of transgenic mice (Tg2576) animals were fed diets supplemented with RA (0.5%) for 9 months. RA intake was associated with significantly lower Aβ plaque burden in the brain (25). Additional biochemical analyses suggested that RA also inhibited the Aβ aggregation pathway known to progress from Aβ monomers to oligomers, and, ultimately, to Aβ deposition. Thus, RA appears to be, as the authors suggested, an attractive candidate for AD therapy or prevention strategies. It is not known what concentration of RA occurs in the brain following long-term oral administration, although it has been reported that RA was found in the brain of rats administered (i.p.) the Lamiaceae herb *Plectranthus barbatus* (20). In an animal model of amyotrophic lateral sclerosis (ALS), human SOD1 G93A transgenic mice were administered RA (0.13 mg/kg, i.p.) twice per week for 13 weeks. RA significantly extended survival of the mice, improved motor function, and suppressed body weight loss, compared to controls. It also attenuated the degeneration of spinal cord motor neurons (26). Similar results were obtained when animals were dosed with an undefined rosemary extract. A mouse kindling model was used to monitor the effects of RA (1,2 or 4 mg, i.p.) for 16 days prior to pentylenotetrazole (PTZ)-induced seizures (27). Compared to controls, the 4 mg RA increased latency and decreased percentage of seizures but only at day 4 post-PTZ. Only the 4 mg dose reduced DNA damage in the cortex.

Carnosic acid

As assessed *in vitro* there are several means by which carnosic acid (CA) can be neuroprotective. One mechanism is by enhancing the expression of nerve growth factor (NGF) and stimulating neurite outgrowth. For example, exposure to CA of human astrocytes (2-50 μM), PC12 cells (5-20 μM), and human glioblastoma cells (5-50 μM) showed that it was neurotrophic and induced neuronal differentiation, in part by activation of the Nrf2-p62/ZIP pathway (28-31). In one report (28) CA was much stronger in promoting NGF synthesis than its derivative carnosol. CA also may suppress oxidative stress and inflammation in nerves as another mechanism for neuroprotection. CA at concentrations of 1-10 μM acted variably in neuronal cell cultures by inhibiting NO production and iNO synthase induction, increasing glutathione levels, and protecting against reactive oxygen species (ROS) (29-32). With regard to PD, CA (0.1-1.0 μM) prevented 6-OHDA-induced death of human dopaminergic neuronal cells, and carnosol (1-10 μM) protected nigral dopaminergic cells from rotenone-induced toxicity *in vitro* (33,34). In relation to AD, in cultures of human neuronal cells, exposure to CA (30 μM and 50 μM) suppressed $A\beta_{1-42}$ peptide production (35,36) and attenuated the subsequent apoptosis induced by amyloid β (37). In another study, membranes rich in muscarinic acetylcholine receptor were prepared using human brain tissue from AD cases. Carnosol (0.8 μM) was able to prevent oxidation damage-induced inactivation of this receptor, a significant finding, since decreased cholinergic transmission is a key feature of AD (38).

Regarding *in vivo* evidence for neuroprotection by CA, in one study CA was provided orally to mice (3 mg/25g mouse) and within 1 hr was measured at significant levels in the brain, an indication that CA was able to penetrate the blood-brain barrier

(32). In a follow-up study diets supplemented with 0.03% CA were fed to mice for 1 week, which resulted in induction of the brain phase 2 enzymes HO-1 and γ-glutamyl cysteine ligase, and an increase in total GSH and total GSSG levels. The authors concluded that orally-administered CA reaches the brain, activates potentially neuroprotective pathways, and is converted at some point *in vivo* to an electrophilic (quinone) form (32). Oral dosing of rats with CA (20 mg/kg; 3 weeks) prior to lesioning of right striatum of rats by 6-OHDA treatment reversed the reduction in striatal GST pi associated with the lesioning (39). In a follow-up study these authors found, compared to controls, that oral pretreatment with CA of rats lesioned with 6-OHDA exhibited improved behavior performance, reduced brain peroxidation, increased expression of antioxidant enzymes in the brain, and counteraction of 6-OHDA-induced apoptosis in the striatum and substantia nigra (40). The authors suggested that CA was a candidate for prevention of Parkinson's disease neurodegeneration. Another study evaluated mice given CA in the diet (0.05% wt/wt) prior to and during subacute exposure to KCN for 8-12 days (41). Compared to controls, CA reduced neurotoxicity in the cortex, hippocampus and striatum of KCN-treated mice and improved neurobehavioral outcomes. The authors recommended that CA be considered for therapy of cyanide-induced brain injury. In an animal model of controlled cortical impact (CCI) traumatic brain injury (TBI), mice were administered CA (i.p. at 0.3, 10.0 or 3.0 mg/kg) at 15 minutes post-brain injury (42). Compared to controls, CA dosing resulted in a dose-dependent decrease in cortical lipid peroxidation and cytoskeletal breakdown markers. CA also preserved mitochondrial respiratory function that was usually compromised by CCI-induced TBI. In 2 rat studies intranasal (i.n.) CA was administered for 4 days (2-4

mg/kg), either with chitosan or as part of a nanoparticle formulation. Compared to controls, CA in both formulations significantly elevated levels of brain hippocampal NGF and brain-derived neurotrophic factors. The authors suggested that i.n. delivery of CA may be a promising therapeutic strategy, although acute effects of CA on the olfactory mucosa need to be evaluated (43,44). In an animal model of ALS, CA administered for 133 days (0.13 mg/kg, i.p., twice weekly) improved motor function of human SOD1 G93A transgenic mice (26). However, CA did not lessen the body weight loss accompanying disease progression and only marginally improved survival of the mice.

Antinociceptive actions

Rosemary essential oil

Rosemary essential oil administration for pain alleviation was evaluated in several *in vivo* models. Oral dosing of rodents (45) with various amounts of oil significantly suppressed carrageenan-induced paw edema (250-500 mg/kg), and chemically-induced nociception (70-250 mg/kg), compared to controls, but did not affect heat-induced nociception (125-500 mg/kg). The oil did not cause acute toxicity at doses up to 3000 mg/kg, p.o. This essential oil was reported to be composed of myrcene (25%), 1,8-cineole (20%), 1-O-menthen-8-ol (6%), 2-ethyl-4,5-dimethylphenol (7%), and geranyl acetate (5%). The authors suggested, in light of the oil's significant activity against chemical stimuli but not against thermal stimuli, that it likely inhibits peripheral rather than central pain mechanisms. In a second study rosemary oil (100-600 mg/kg, i.p.) significantly suppressed dysfunction in the "pain-induced functional impairment in the rat" (PIFIR) model (46). Constituents in this oil included α-pinene (14%), camphene (11%), β-pinene (12%), myrcene (3%), 1,8-cineole (9%), and α-phellandrene (8%). In

this study, the antinociceptive response from the oil was blocked by concomitant administration of naloxone and WAY100635, which suggested that, in part, 5-hydroxytryptamine1a receptors and endogenous opioids were involved in the oil's antinociceptive activity. The analgesic effect of rosemary oil was evaluated in mice (20 mg/kg, p.o.) for 7 days prior to testing in a heat-induced pain model (47). Compared to controls, rosemary significantly increased the latency time of animal response to the thermal stimulus. The major constituents of this oil were 1,8-cineole, camphor and α-pinene. Differences in dosing protocols and compositions of oils among these studies make it difficult to identify the specific bioactive chemical(s) mediating these effects.

Rosemary extracts

An undefined ethanol extract was tested orally in two mouse models of nociception and one rat model of arthritic pain (PIFIR model). This extract significantly inhibited chemically-induced pain (10-300 mg/kg, p.o.) and inflammation-associated pain (30-300 mg/kg, p.o.), compared to controls. It also produced a dose-dependent (30-3000 mg/kg, p.o.) antinociceptive response in the PIFIR model (48). The constituents responsible for these effects were not determined. In another study in mice, three different extracts of rosemary were evaluated in the chemically-induced abdominal constriction model. When tested at 100 mg/kg (i.p.) the ethanol and ethyl acetate fractions produced greater antinociceptive responses than a hexane extract (49). Subsequent fractionation of the ethyl acetate and ethanol extracts identified three triterpenes as major contributors to the antinociceptive actions.

Individual rosemary constituents

1,8-cineole and rosmarinic acid

Two *in vivo* investigations reported antinociceptive activity of 1,8-cineole, a component of rosemary essential oil. When tested orally in rodents (100-400 mg/kg), 1,8-cineole was effective for reducing pain in three models of nociception (50). Similarly, in another study, 1,8-cineole (0.3 mg/kg, i.p.) and another essential oil component β-pinene (0.3 mg/kg, i.p.) significantly suppressed pain-induced responses in two rodent models (51). In this latter report naloxone failed to reverse the effect of 1,8-cineole, suggesting a non-opioid-like mechanism of action. RA administration to rats (5 and 10 mg/kg, i.p.) had no beneficial effect on the tail-flick test for nociception (52), compared to controls. In contrast, oral administration of RA (100 and 150 mg/kg) just prior to initiation of heat- and chemically-induced pain tests significantly reduced the induction of pain. The authors suggested that RA possesses both central and peripheral antinociceptive activities (53).

Carnosol

Oral treatment of mice with carnosol (10 mg/kg) reduced nociceptive behavior in two models of chemically-induced pain, compared to controls, but did not have a benefit in a chemically-induced model of mechanical allodynia (54).

Hesperidin

The flavonoid hesperidin was identified as an active compound in an ethanol extract of rosemary that was tested in the PIFIR assay. When examined individually hesperidin (100-1778 mg/kg, p.o.) demonstrated antinociceptive activity, although it was 5.5 times less potent than the original extract, suggesting that other compounds in the extract were involved in the pain-diminishing action of rosemary (55). Although

hesperidin's mechanism of action was not fully characterized, the response partly involved participation of the TRPV1 receptor.

Triterpenes

Three triterpenes were isolated from an ethyl acetate extract of rosemary and tested in a chemically-induced constriction model in mice. Compared to controls, significant, dose-dependent antinociceptive responses were observed for administration (i.p.) of micromeric (ED_{50} = 1.1 mg/kg), oleanolic (ED_{50} = 2.1 mg/kg), and ursolic (ED_{50} = 1.6 mg/kg) acids (76).

Cognition benefits

Rosemary extracts

In a scopolamine-treated rat model the effects of administration of an ethanol extract of rosemary (200 mg/kg, p.o.) for 28 days on behavioral and cognitive responses was determined (56). Treatment with rosemary extract improved long-term memory, compared to controls. Moreover, the extract stimulated butyrylcholinesterase activity in the hippocampus and frontal cortex. The major compounds in the extract were 5.5% RA, 2.5% CA, 1.3% carnosol, and 13% hydroxycinnamic derivatives. In another study using 9-month old rats, rosemary extract (containing 40% carnosic acid) was fed to animals (i.g., 50,100, 200 mg/kg/day) for 12 weeks (57). Compared to controls, rats given 100 mg/kg of the extract showed better spatial memory retrieval scores, and there was increased activity of SOD, GPx and CAT in the hippocampus of the rats.

Individual rosemary constituents

Rosmarinic acid

In two animal studies RA improved cognitive performance. Subchronic administration of mice with RA (2 mg/kg, p.o.) for periods of 2-3 weeks enhanced spatial memory in the Morris water maze test (58). Furthermore, in brain samples from these RA-treated mice, prolyl oligopeptidase (POP), an enzyme involved in modifying neuropeptides, was inhibited in a non-competitive manner (IC_{50} = 63.7 μM). It was suggested that inhibition of POP could enhance cognitive function by increasing brain concentrations of neuropeptides such as arginine-vasopressin, substance P and oxytocin. In a separate experiment involving an AD animal model, mice receiving RA (0.05-4 mg/kg, i.p.) for 9 days after $A\beta_{25-35}$ dosing (i.c.v.) evidenced less memory impairment caused by either $A\beta_{25-35}$ or peroxynitrite treatment (59). Finally, RA administration (16 and 32 mg/kg/day. p.o.) for 7 days improved learning and memory not only in control rats (no scopolamine) receiving RA but also reversed learning and memory deficits in rats dosed with scopolamine and receiving (60).

Anti-depressant-like effects

Rosemary essential oil

Rosemary essential oil suspensions (5,10,20%) were administered (0.1 ml/100g, i.p.) to rats 1 hr prior to measuring behavioral changes in the forced swim test (FST). Rosemary injection was associated with a significant antidepressant-like effect for the 5% and 10% suspensions but not the 20%, compared to controls. The composition of the oil was not reported and a mechanism for any effect was not determined (61). Similarly, the essential oil given orally to mice (0.1-100 mg/kg) produced significant antidepressant-like effects in two behavioral tests (62). In this study, the oil was reported to contain mainly 1,8-cineole (45%) and camphor (22%).

Rosemary extracts

In a recent study, mice drank water containing rosemary tea (2% w/v infusion) for 4 weeks prior to testing for behavioral changes (63). Consumption of rosemary tea significantly improved anxiety/fear responses and depression-like behavior, compared to controls, and produced a significant decrease in activities of cerebral cholinesterase isoforms. In another report, mice were orally administered different doses of rosemary extracts prepared from hexane (0.1-10 mg/kg), ethanol (0.1-100 mg/kg) and ethyl acetate (0.1-1.0 mg/kg) prior to two behavioral assays. Significant antidepressant-like effects were observed (62), compared to controls. The effects of all fractions were similar to that produced by the antidepressant drug fluoxetine. Analysis of the fractions indicated that the hexane extract contained carnosol and rosmarinic acid, while the ethyl acetate fraction contained predominantly ursolic and betulinic acids and carnosol. The ethanol fraction contained mostly ursolic acid and secondarily betulinic acid. In another mouse study ethanol extracts produced antidepressant activity in the FST (100 mg/kg, p.o.) and tail suspension (10-100 mg/kg, p.o.) behavioral tests. This action appeared to be due to the extract's interaction with the monoaminergic system (64,65). The composition of this extract was not reported. Likewise, in a separate report, an ethanolic extract of rosemary administered (50-100 mg/kg, p.o.) for 7 days to mice significantly reduced immobility times in the tail suspension test, compared to controls. This extract contained 1.3% CA and 4% RA. Furthermore, brain analyses of these mice showed that the antidepressant-like effect was associated with down-regulation of mitogen-activated protein kinase (MKP-1) gene expression, upregulation of tyrosine hydroxylase and pyruvate carboxylase genes (involved in dopamine synthesis and glutamate-glutamine conversion).

At the highest dose, a significant increase in brain cortex concentrations of 5 neurotransmitters was detected (61). The authors suggested that this extract exhibited the ability to enhance dopaminergic, seratonergic and cholinergic functions in the mouse brain, and speculated that regular dietary intake of rosemary may have important health benefits in light of its good oral bioavailability and few side effects. Multiple actions of a rosemary ethanol extract were tested in a unique olfactory bulbectomized (OB) animal model of depression, which is characterized by behavioral, neurochemical and neuroendocrinological alterations mimicking human depression. This extract was reported to contain 16% ursolic acid, 10% carnosol and 6% each of betulinic, oleanolic and rosmarinic acids. Oral administration of the extract (10-300 mg/kg) for 14 days to mice reversed OB-induced depression-associated hyperactivity and abolished anhedonic-like behavior, but did not counteract learning deficits cause by OB. The 10 mg/kg dose also reversed the decline in serum glucose and the increase in hippocampal acetylcholinesterase activity usually accompanying OB surgery (66). Collectively, these various animal studies testing rosemary extracts suggest that multiple constituents of rosemary are likely contributing to these antidepressant-like effects. Furthermore, these diverse investigations used oral dosing protocols over a range of doses, which suggests that dietary examination of these antidepressant-like actions is clearly warranted.

Individual constituents

Rosmarinic acid

Several laboratories have evaluated neurobehavioral actions of RA. RA was identified as an antidepressive substance in extracts from the leaves of *Perilla frutescens* Briton var. *acuta* Kudo (67). When administered orally (35.7-150 mg/kg) or i.p. (1-4

mg/kg) RA produced a dose-dependent U-shaped response in duration of immobilization in the FST in mice. Similarly, administration of RA (0.25-4 mg/kg, i.p.) demonstrated a dose-dependent U-shaped response in freezing behavior in mice exposed to conditioned fear stress (68). The authors speculated that the lack of effect of RA at the higher (2-4 mg/kg) doses may be due to its conversion to a metabolite that interferes with the effect of RA. It was suggested that RA may thus be able to inhibit emotional disturbances accompanying stress. In another study using the FST, RA (2 mg/kg, i.p.) significantly reduced the duration of immobility in mice without affecting spontaneous motor function. Analyses of brain samples showed that RA did not affect the uptake of monoamines to synaptosomes or mitochondrial monoamine oxidase activity (69). In a rat study RA administration (2 and 4 mg/kg, i.p.) produced anxiolytic-like activity without altering locomotor functions or causing DNA damage in brain tissue (70). Extended administration of RA (1-4 mg/kg, i.p.) to mice for 7 and 14 days resulted in a dose-dependent decrease in duration of immobility in the FST (71). Of interest, RA treatment of these mice for up to 4 days produced a significant dose-dependent increase in cell proliferation in the dentate gyrus of the hippocampus, which, in part, may have contributed to RA's antidepressant-like actions. This is relevant since disruption of neurogenesis in this brain region is believed to be involved in stress-associated depression. In a rat model evaluating post-traumatic stress disorder (PTSD)-like symptoms, RA was given for 14 days (5 and 100 mg/kg/day, i.g.) prior to testing by the enhanced single prolonged stress (ESPS) paradigm (72). Compared to controls, rats administered the 10 mg/kg RA showed less PTSD-like symptoms induced by traumatic stress and exhibited a restoration of hippocampal proliferation.

Carnosol and ursolic and betulinic acids

Antidepressant-like effects of ursolic acid (0.01-0.1 mg/kg, p.o.), carnosol (0.01-0.1 mg/kg, p.o.) and betulinic acid (10 mg/kg, p.o.) all isolated from rosemary have been reported in several mouse studies. The effect of ursolic acid appeared to involve the dopaminergic system (62,73).

Antiinflammatory actions

Extracts of rosemary

Ethanol extracts of rosemary suppressed inflammation in three rodent skin models. For example, an undefined ethanol extract applied topically (10-1000 $\mu g/cm^2$) to mice significantly reduced phorbol-12-myristate-13-acetate (PMA)-induced ear edema (74), which was accompanied by a markedly lower number of leukocytes at the site of injection, compared to controls. The extract also was found to inhibit nitric oxide (NO) synthesis and NF-kB activation. Similarly, an undefined rosemary ethanol extract signficantly inhibited skin lesions in an atopic dermatitis mouse model (75). Another ethanol extract (containing carnosol, carnosic acid and rosmarinic acid at 0.57, 1.98 and 0.43 mg/g dried leaves, respectively), when co-injected (intradermally, 1mg) with *Propionibacterium acnes* into mouse ears signficantly reduced *P. acnes*-induced swelling and granulatomatous response, compared to controls (76). An aqueous extract of rosemary (100, 200 or 400 mg/kg, i.g.) was administered to rats 1 hr prior to injection of carrageenan in subcutaneous tissue of the dorsal region (77). Oral treatment with this extract led to a dose-dependent decrease in neutrophil migration, compared to controls, and SOD, PGE2, IL-6 and TNF-α levels were reduced in the inflamed exudate.

Individual constituents

Carnosic acid

CA applied to PMA-treated ears of mice significantly suppressed inflammation (EC_{50} = 10.2 μg/cm^2), reduced leukocyte infiltration, decreased expression of TNF-α and IL-1β and inhibited COX-2 expression (74). In the mouse paw edema model, carnosol (10 mg/kg, p.o.) significantly blocked formalin-induced inflammation, compared to controls (54).

Rosmarinic acid

RA stimulated anti-inflammatory activity in different animal models. For example, in diabetic ischemia-reperfused (I/R) rats RA dosing (50 mg/kg, i.v.) provided long-term improvements in neuronal functional recovery following cerebral I/R by blocking inflammatory responses (78). RA administration (5-10 mg/kg, i.p.) also suppressed leukocyte migration to the pleural cavity of carrageenan-treated rats, compared to controls (52). In another report RA suppressed inflammation in two organs. Treatment of mice with RA inhibited LPS-induced acute lung injury (5-20 mg/kg, i.p.) and LPS-induced liver injury (135 mg/kg, p.o.). An inhibition of TNF-α and inflammatory cytokine production was associated with the beneficial effects of RA in the lung model but not the liver injury model. The liver protection by RA appeared to be due to scavenging of superoxide or peroxynitrite species (79,80). In a rat liver I/R model RA administered at 25 mg/kg (i.v.) 30 min prior to induction of ischemia led to significant reduction in serum levels of transaminases and lactate dehydrogenase, compared to controls (81). In the same report RA dosing 5 min prior to induction of thermal injury caused a significant reduction in organ dysfunction markers in liver, kidney and lung. When administered to mice injected with the inflammatory cytokine high mobility group

box-1 (HMGB1) RA treatment (0.7-1.4 μg/ mouse, i.v.) significantly lowered HMBG1-induced leukocyte migration and permeability in peritoneal cavities, compared to controls. RA inhibited skin inflammation in 12-tetradecanolyphorbol-13-acetate (TPA)-treated mouse skin (0.25-1.35 mg cutaneous RA/mouse) and suppressed 2,4-dinitrofluorobenezene-induced atopic dermatitis in Nc/Nga mice (10-50 mg/kg, i.p.) (82-84). Mechanisms for these various protective effects of RA included scavenging of reactive oxygen radicals, inhibiting COX-2 induction, reducing chemokine and eicosanoid synthesis, modulating NF-kB and metalloproteinase 1, and inhibiting cell adhesion. In another model, mice made septic by a cecal ligation and puncture procedure, showed significantly improved survival rates when dosed with RA twice (1.4 μg/dose, i.v.), compared to controls (85). In light of this the authors suggested that RA is a candidate for inclusion in treatment strategies for severe vascular inflammatory diseases, such as sepsis or septic shock. RA also exhibited protective effects *in vivo* against arthritis symptomology. For example, compared to controls, daily administration of RA (50 mg/kg, i.p.) for 14 days significantly suppressed synovitis in a mouse model of collagen-induced arthritis (86). Moreover, fewer COX-2 expressing cells were observed in synovial tissues of the RA-treated mice. In one experiment with horses, addition of a high rosmarinic acid mint sample (*Menthus spicata*) for 24 days to feed preparations (1.9g RA/day, p.o.; ~3.6 mg/kg) prior to injection of LPS into the intercarpal joint, resulted in reduced PGE_2 and glycosaminoglycan in synovial fluid as well as lower levels of plasma total white blood cells, segmented neutrophils and leukocytes (87). In a Korean study peripheral blood mononuclear cells were isolated from 28 patients with rheumatoid arthritis. When cells were incubated with RA (50 μM) for 48 hr, compared to

controls, RA induced preferential apoptosis of activated T cells and effector T cells by a mitochondrial pathway (88). Some have suggested that rosemary may be beneficial in the treatment of destructive inflammatory joint diseases such as rheumatoid arthritis and recommended that the effect of RA on pathogenesis of human rheumatoid arthritis be further characterized (89,90).

1,8-cineole

1,8-cineole (or eucalyptol) was tested for an impact on airway inflammation in guinea pigs. Animals were given an ovalbumin challenge, then treated acutely by inhalation with 1,8-cineole (aerosolized 1mg/ml solution) or saline (controls), and after 24 hr bronchoalveolar lavage fluid (BALF) and tracheal tissue were obtained. Compared to controls, the BALF of 1,8-cineole-treated animals evidenced significantly fewer leukocytes and lower levels of the pro-inflammatory cytokines TNF-α and IL-1β. Moreover, 1,8-cineole impaired the development of airway hyperresponsiveness in tracheal rings isolated from the animals (91). In a related study, the same authors reported that 1,8-cineole was a tracheal myorelaxant which acted preferentially on electromechanical coupling (92), a result consistent with other animal studies (93). As reported in three studies from the same group, the anti-inflammatory actions of 1,8-cineole may depend on the route of administration. When administered via s.c. injection (20 μl/paw), 1,8-cineole induced inflammatory edema in rodent paws (94,95). However, when dosed systemically (100-400 mg/kg, p.o.) this phytochemical significantly reduced carrageenan-induced paw edema (96). Moreover, compared to controls, instillation of 1,8-cineole intrarectally in rats (200-400 mg/kg), prior to TNBS-induced colitis,

significantly attenuated the extensive inflammation and ulceration associated with the chemically-induced colonic damage (97).

Triterpenes

Topical administration of the rosemary triterpenes ursolic, oleanolic and micromeric acids (25-200 μg/cm^2) significantly reduced edema in mice with croton oil-induced ear dermatitis (98).

Alleviation of metabolic disorders (obesity and diabetes)

Rosemary essential oil

Inconsistent effects of rosemary oil on blood glucose levels in animals have been reported. Rosemary essential oil administered topically (25 μl, twice/day for 3 days) to alloxan diabetic mice produced a significant suppression of blood glucose levels up to 12 days after dosing. An opposite effect was reported in normal rabbits (99). Specifically, normal rabbits were given an intraperitoneal glucose tolerance test along with administration of rosemary essential oil (25 mg/kg, i.m.). A time-dependent 55% increase in plasma glucose levels was observed within 2 hr, compared to controls. Furthermore, in those rabbits administered the rosemary oil after the glucose dose, there was a significant 30% decrease in serum insulin within 30 min (100). No effect of the oil on fasting glucose levels was observed. Additionally, in alloxan diabetic rabbits, similar treatment with the essential oil increased fasting glucose levels by 17% above that observed in untreated animals. The mechanisms for this hyperglycemic effect of the essential oil on glucose and insulin levels in these rabbit models were not determined. The reasons for this disparity between the mouse and rabbit studies are not known but may be due to differences in the experimental models and protocols used, and to the

composition of samples of rosemary tested. In both investigations the composition of the test materials was not reported.

Individual constituents

Carnosic acid

In several animal studies CA demonstrated potential anti-obesity actions by modulating lipid absorption and disposition. Specifically, CA administration (5-20 mg/kg, p.o.) for 14 days significantly inhibited serum triglyceride elevation in mice fed a high-fat diet, compared to controls. Furthermore, at the highest dose, CA reduced body weight gain and the accrual of epididymal fat weight (101). Of interest, carnosol and oleanolic acid, other phytochemicals in rosemary, had no effect on these end points at a dose of 200 mg/kg, p.o. In a study using ob/ob mice, supplementing diets with 0.05% CA (w/w) for 5 weeks resulted in significant weight loss, a decrease in visceral adiposity, lower serum triglyceride (TG) levels and a substantial suppression of liver TG content, compared to controls (102). Food intake was not affected. Additionally, CA improved glucose tolerance, although mice in the CA-supplemented group had a 50% higher level of fasting glucose than the control mice. The reason for this elevated fasting glucose was not determined. It was suggested by the authors that CA is a novel agent that could have potential use for obesity-related disorders (103). In a follow-up investigation, electron microscopic examination of livers from the ob/ob mice fed this CA-supplemented diet detected less lipid accumulation in hepatocytes, compared to controls. PPAR-γ protein levels were significantly decreased in liver nuclear fractions from the treated mice, whereas the expression of lipogenic genes SREBP-1 and fatty acid synthase was unchanged, compared to controls. Additionally, compared to controls, CA feeding

increased the expression of p-MAPK, pEGFR and pAMKα in livers of treated mice and was also associated with reduced serum levels of at least 6 inflammatory cytokines (104). In a similar manner mice fed a high-fat diet supplemented with 0.01% and 0.02% w/w CA for 12 weeks exhibited decreased hepatic steatosis, compared to controls (105). This change was associated with reduced *de novo* lipogenesis and increased fatty acid β-oxidation as measured by analyzing the expression of multiple hepatic fatty acid-related genes. Moreover, blood glucose levels following the intraperitoneal glucose tolerance test were significantly lower in CA-high-fat-fed mice, compared to high-fat fed controls, and blood lipid profiles also were improved. In a recent study by these authors hepatic expression of liver fatty acid binding protein, stearoyl-CoA desaturase 1 and fatty acid synthase (genes involved in lipogenesis and lipolysis) were significantly decreased in ob/ob mice fed diets supplementated with CA, compared to controls (106). Additionally, hepatic genes involved in fatty acid oxidation and regulation of energy balance were upregulated. Of note CA decreased mRNA expression of regulator of calcineurin 2-3 in the hypothalamus, which is a regulator of food intake and weight gain. The consequences of these gene expression changes need further characterization.

In vitro evaluations of CA provide supportive evidence for CA's antiadipogenic actions. In HepG2 cells CA treatment (10 μM) lowered lipid accumulation and inhibited PPARγ activity through modulation of EGFR/MAPK signaling (104). In two studies CA treatment of preadipocyte cell cultures inhibited differentiation (IC_{50} = 0.86 μM) (107,108), and inhibited pancreatic lipase activity (IC_{50} = 36 μM) (153). The anti-adipogenic effect of CA was exerted through interfering with mitotic clonal expansion, altering the ratio of different C/EBPβ forms, and blocking PPARγ and C/EBPα

expression (107). The effect of CA on PPARγ may be dose-dependent and cell/tissue specific, since others have reported activation of PPARγ by CA (109,110), an aspect of CA's actions that needs to be more carefully examined *in vivo* (111). Additional mechanisms for CA effects in adipocytes include decreasing TNF-α-mediated inflammation and insulin resistance (112) and attenuation of fatty acid desaturase activity (113).

Rosmarinic acid

Several reports provide *in vivo* evidence that RA can alleviate complications of diabetes, although its effects on glucose regulation are inconsistent. For example, STZ-induced diabetic rats were administered RA (7.5-15 mg/kg, i.g.) for 16 weeks. Although RA treatment had no effect on STZ-induced glycemia, compared to controls, it did lessen diabetic neuropathy (DN). Specifically, urinary albumin levels were decreased, kidney pathology was ameliorated and serum levels of cystatin C (a measure of impaired kidney function) and connective tissue growth factor (a fibrosis mediator of DN-associated signaling) were decreased, compared to controls (114). Similarly, painful diabetic neuropathy was ameliorated for STZ-induced rats administered RA (10 and 30 mg/kg/day, p.o.) for 8 weeks (115). In another experiment with STZ-induced diabetic rats, RA dosing (50 mg/kg, i.g. for 10 weeks) was followed by excision of aortas. RA treatment did not affect blood glucose levels or blood pressure of STZ-treated rats, compared to controls. Yet, examination of the aorta samples indicated that RA treatment did counteract some diabetes-associated problems, such as impaired endothelium-dependent relaxation and damage to endothelial monolayer structures. RA also prevented the upregulation of the proinflammatory cytokines IL-1β and TNF-α in diabetic rat aorta,

and decreased endothelin converting enzyme-1 (ECE-1) and the expression of ET_A and ET_B receptors (116). The authors suggested that RA decreased the adverse effects of preproendothelin-1 (ET-1) on the vasculature which contributed to improvement of endothelial function and stimulation of remodeling. For STZ-induced diabetic rats exposed to ischemia and reperfusion, administration of RA for 14 days (50 mg/kg, i.v.) alleviated cerebral injury and attenuated blood-brain barrier breakdown, compared to controls. These effects likely involved modulation of high mobility group box-1 (HMGB1) and NF-kB signaling pathways (78). However, RA dosing had no effect on diabetic hyperglycemia in this study. In contrast, it was observed by others that dosing of STZ-induced rats with RA (577 μg/ml in drinking water) for 4 days decreased fed-state plasma glucose levels in animals fed a high-carbohydrate (HC) diet, compared to HC-fed controls (117). This effect in part was due to RA-associated modulation of the trafficking of the Na^+/glucose cotransporter-1 to the brush border membrane. Brain complications of diabetes were studied in STZ-induced diabetic rats provided RA (10 mg/kg/day, i.g.) for 21 days prior to isolation of cerebral cortex, hippocampus, striatum and cerebellum regions of the brain (118). Compared to controls, RA treatment prevented the diabetes-induced increase in lipid peroxidation and the increase in acetylcholinesterase activities in the hippocampus, cortex and striatum regions. The authors suggested that RA is a candidate compound for therapy of patients with hyperglycemia-induced cholinergic disorders. Regarding insulin status, an intriguing study reported the effect of RA administration (200 mg/kg, i.p.) for 8 days on implantation of islet allografts in STZ-induced diabetic mice. RA synergized with the monocolonal antibody anti-CD154 in prolonging survival of the transplanted islets (119). Long-term allografts (>150 days) that

received the combined therapy exhibited larger islet clusters and contained more insulin- and glucagon-positive cells compared to anti-CD154 allografts alone. The action of RA was associated with fewer apoptotic cells, possibly due to RA's inhibition of reactive nitrogen and oxygen species. This report is relevant to type-1 diabetes, since studies show that pancreatic islet transplantation can correct abnormal glucose metabolism, although the accompanying prolonged immunosuppressant therapy has substantial side effects. Another study found that RA treatment (100 mg/kg/day, i.g.) for 30 days protected pancreatic tissue from oxidative stress-induced glucolipotoxicity in STZ-induced diabetic rats fed a high-fat diet, compared to controls (120). Specifically, RA dosing was associated with increased insulin sensitivity, reduced blood glucose, HbA1C, and inflammatory mediator levels, reduced levels of lipid peroxidation products, and improved status of multiple antioxidant markers. Furthermore, RA significantly improved degenerative diabetes-induced changes in pancreatic β-cells and improved their structural and functional integrity.

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