Title: Carnosol: A promising anti-cancer and anti-inflammatory agent

Short Title: Carnosol for cancer

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1. Introduction

The identification and characterization of the anti-cancer properties of natural products, especially in the area of cancer chemoprevention, has received significant interest over the years[1-4]. A class of compounds known as diterpenes, is receiving increasing attention for a variety of health promoting properties such as anti-microbial [5], anti-inflammatory [6], neuroprotective[7], anti-oxidant [8], and anti-cancer properties[9]. One agent in particular is carnosol which has been reported to have broad anti-cancer properties in several cell line models including prostate, breast, leukemia as well as others. These studies have begun to identify the pro-apoptotic properties and targeting of multiple deregulated pathways with carnosol, however, a comprehensive evaluation of carnosol as an anti-cancer agent is lacking. This mini-review presents the current knowledge of carnosol as it applies towards several cancers including prostate, breast, skin, leukemia, and colon cancer.

2. Mediterranean Diet and Lifestyle

The Mediterranean diet has received significant attention for its cardiovascular and metabolic health promoting properties[10]. This diet is best understood by the food patterns in Crete, Greece and the southern part of Italy which has high intakes of fresh fruit and vegetables, olive oil, unrefined cereals, and low consumption of meat products. Attention is now being given to how the Mediterranean diet impacts other disease states such as cancer where several population based studies are suggestive of a reduced risk of breast, colon, stomach, and prostate cancers with this type of diet and lifestyle[11-16]. The majority of these studies focus on the consumption of various meats, fruits, vegetables and olive oil.

What is less understood is the impact of Mediterranean herbs such as rosemary, sage, basil, oregano, and others that are often incorporated into various foods and oils. Each of these herbs have a variety of phytochemicals such as diterpenes including carnosol and carnosic acid. For
this reason we are focusing on understanding the current knowledge of carnosol as an anti-cancer agent.

A case-control study in patients diagnosed with lung cancer evaluated the relationship between components of the Mediterranean diet and lung cancer[15]. Eligible participants were living in the Lazio region, which includes the city of Rome, were between the ages of 35 and 90 years of age. Control participants were matched by gender (1:1 for males and 1:2 for females) and age. As a part of the questionnaire participants were evaluated for consumption of herbs including parsley, rosemary, basil, and sage. All of four of these herbs trended towards a decreased overall risk with the most significant overall risk reduction observed with sage [OR = 0.43, 95% CI = 0.29-0.65]. Parsley was also associated with a reduced overall risk [OR = 0.31, 95% CI = 0.11-0.84] while a trend towards a reduced risk was observed with rosemary [OR = 0.66, 95% CI = 0.37-1.15] and basil [OR = 0.63, 95% CI = 0.31-1.30]. These results at a minimum are suggestive that the phytochemicals isolated from these culinary herbs should be investigated for their health promoting properties.

3. Carnosol: Sources, Chemistry, and Synthesis

Mediterranean herbs including rosemary and sage have been used for culinary purposes and for their medicinal properties for millennia. Carnosol was first isolated from sage (Salvia carnosa) in 1942 and the chemical structure was first established in 1964 by Brieskorn et al[17]. Rosemary and sage have been known to contain a variety of polyphenols such as carnosol, carnosic acid, rosmarnol, rosmarinic acid as well as others[18]. It has been estimated that approximately 5% of the dry weight of rosemary leaves contains carnosol and carnosic acid, however, this fraction is estimated to account for > 90% of the antioxidant activity[19].
Carnosol is an ortho-diphenolic diterpene with an abietane carbon skeleton (Figure 1) with hydroxyl groups at positions C-11 and C-12 and a lactone moiety across the B ring[20]. Carnosol is the product of oxidative degradation of carnosic acid (Figure 2)[21]. The most popular and expensive way to procure a highly purified form of carnosol is through extraction and purification from natural sources such as rosemary. Recently, a semi-synthetic process has been described using pisiferic acid (Figure 2) extracted from Sawara (Chamaecyparis pisifera) leaves, a cypress tree native to Japan, to synthesize carnosic acid and then to carnosol[22]. Carnosic acid was prepared through oxidation of pisiferic acid using three different methods: a) mCBPO, b) CAMCBP in CH$_2$CL$_2$, or c) IBX in CHCl$_2$-CH$_3$OH. The third semi-synthetic reaction developed had an overall yield of 72% while the first two had considerably lower yields as low as 10%. Carnosol was prepared from carnosic acid in the presence of silver oxide in CH$_2$CL$_2$ with a purified yield of 67%. Alternatively, carnosic acid in the presence of methanol for 1 week at room temperature will oxidize carnosic acid to carnosol [23,24].

4. Anti-oxidant Activity of Carnosol

The health promoting properties of rosemary and sage have been attributed to the antioxidant activity of polyphenols present in these extracts[25-28]. Reactive oxygen species and depletion of antioxidant enzymes have been suggested to promote a variety of biological responses including neurodegenerative, inflammatory conditions, cardiovascular disease, and carcinogenesis of various tissues [29]. Rosemary extracts were prepared and shown by the DPPH (2,2-diphenyl-1-pycrilhydrazil hydrate) radical scavenging activity assay to have a radical scavenging activity up to 95.1% [25] with approximately 90% of the antioxidant activity attributed to the diterpenes carnosol and carnosic acid [19]. Carnosol has been shown to inhibit Cu$^{2+}$ induced LDL oxidation and lipid free radicals in mouse liver microsomes[30] and are good scavengers of peroxyl radicals (CCl$_3$O$_2$) [19]. The antioxidant response element is believed to
be activated through the catechol-hydroxyl groups of carnosol and is converted to a carnosol quinone[8]. This quinonederivative is the main anti-oxidation product of carnosol essentially voiding it of any antioxidant activity [31]and under appropriate conditions the antioxidant activity can be recovered[32].

The glutathione-S-transferase (GST) family of phase II detoxification enzymes catalyze the reaction of glutathione with electrophiles and have been a target of interest for cancer [33]. Carnosol by intraperitoneal administration has been shown to enhance the in vivo activity of GST and quinonereductase in the liver of the female rat [34]. Carnosol (100-400 mg/kg) increased GST activity by 1.6 to 1.9 fold increase.

5. Anti-inflammatory Activity of Carnosol
Deregulated inflammatory signaling including excess nitric oxide (NO) produced by NO synthase (iNOS) occurs during inflammation and the multi-step process of carcinogenesis which has led to the search for agents that decrease inflammatory signaling pathways. Raw 264.7 cells treated with carnosol reduced LPS stimulated NO production with an IC<sub>50</sub> of 9.4 uM[35]. This led to an inhibition of the NF-κB, p38 and p44/42 mitogen activated protein kinase (MAPK). In another study, carnosol was shown to activate the peroxisome proliferator-activated receptor gamma. Carnosol has also been shown to reduce the pro-inflammatory leukotrienes in intact polymorphonuclear leukocytes (PMNL), inhibit 5-lipoxygenase, antagonize the intracellular ca2+ mobilization, and inhibit the secretion of leukocyte elastase[6]. In addition, carnosol blocks protein kinase C signaling and inhibits the binding of AP-1 to the COX-2 promoter which should be noted is fundamentally different than the synthetic COX-2 inhibitors (e.g. celecoxib) that function as direct inhibitors of Cox-2[36].
6. Anti-Cancer properties of Carnosol

6.1 Carnosol and Prostate Cancer

In our laboratory, we are currently investigating the potential role of carnosol for the prevention and treatment of prostate cancer (PCa). Earlier we provided evidence that carnosol promotes $G_2$ cell cycle arrest in PC3 cells decreasing cell viability[37]. The anti-cancer properties of carnosol were associated with a potential to modulate multiple signaling pathways such as the cell cycle related proteins, PI3K/AKT, and apoptotic related proteins [38]. To understand the effects of carnosol in PC3 cells we performed an antibody array that identified 36 proteins down-regulated at least 50% and 24 proteins that were up-regulated at least 200% including a target within the 5'-AMP-activated protein kinase (AMPK) protein subunit. AMPK has been shown to regulate the growth and survival of cancer cells. We observed a dose dependent increase in the phosphorylated forms of AMPK-α (Thr$^{172}$) which has been shown to be upstream of the mammalian target of rapamycin (mTOR). We were also able to show a decrease in the phosphorylation of mTOR (Ser$^{2448}$) and related downstream targets.

Recently, we have shown that carnosol has a unique property where it functions as a dual disruptor of both androgen and estrogen receptor α in PCa[9]. Using a TR-FRET assay we were able to show that carnosol is an antagonist at both the AR and ERα with no agonist properties. To our knowledge this is a unique property as several synthetic agents including fulvestrant, toremifene, and tamoxifen have been investigated as dual disruptors, however, through dose escalation it is not uncommon for these synthetic agents to display agonist properties at the AR[39-42]. Carnosol displayed minimal effects on non-tumorigenic prostate epithelial cells when treated with increasing concentrations and was similar in effect with what was observed with flutamide and tamoxifen. Interestingly, we observed an increase in protein expression of androgen receptor with tamoxifen and flutamide which was reversible with co-
treatment with carnosol. In a xenograft study, athymic nude mice were implanted with 22Rv1 cells and treated with carnosol orally five days weekly. At the conclusion of the study mice treated with carnosol had a significant suppression in tumor growth by 36% \((P = 0.028)\) and circulating PSA by 26% \((P = 0.0042)\).

6.2 Carnosol and Breast Cancer

Carnosol and carnosic acid were investigated for their antimicrobial and anti-cancer properties in breast cancer \([43]\). Both carnosol and carnosic acid were shown to have cytotoxic activity against MCF-7 cells with an \(\text{IC}_{50}\) of 82 and 96 µM. We have observed similar results with carnosol decreasing the cell viability of MCF-7 cells \([9]\), AU565, and MDA-MB-231 (unpublished results). Further studies are needed to determine if carnosol preferentially targets ER+ breast cancer cells versus ER- breast cancer cells.

Carnosol was evaluated to determine the ability of carnosol to inhibit the formation of DMBA-DNA adduct formation and DMBA-induced mammary tumorigenesis in the female rat\([44]\). Intraperitoneal administration of rosemary and carnosol were shown to significantly inhibit mammary adduct formation by 44% and 40%, respectively. At week 20 post DMBA treatment carnosol (100 and 200 mg/kg) administration resulted in a significant inhibition of tumor formation was observed with decreases of 33% \((P < 0.001)\) and 30% \((p < 0.005)\), respectively. The role of carnosol in preventing DMBA-induced mammary tumorigenesis may be partially explained by carnosol inducing detoxification enzymes including GST and quinonereductase which carnosol has been shown to modulate in other studies\([34,45]\). Another consideration is the microsomal metabolism of endogenous estrogens. CD-1 mice were administered rosemary (2%) in their diet led to an increase in the oxidation and glucuronidation of estradiol and estrone inhibiting their uterotropic action \([46]\).
Using mammary epithelial cells carnosol was shown to block the increased binding of AP-1 to the COX-2 promoter [36]. In addition, carnosol was shown to inhibit the activation of PKC, ERK1/2, p38, and c-jun NH2-terminal kinase mitogen activated protein kinase. Overexpression of c-juninhibited the suppressive effects of carnosol.

6.3 Carnosol and Skin Cancer

A methanol extract of rosemary was evaluated for its inhibition on tumor initiation and promotion in mouse skin[23]. Several constituents were characterize by HPLC in the rosemary extract and found to contain ursolic acid (16.5-19.2%), carnosol (3.8-4.6%) and carnosic acid (0.1-0.5%). The rosemary extract was found to inhibit the covalent binding of benzo(a)pyrene to skin epidermis DNA. In mice treated with B(a)P and tetradecanoylphorbol-13-acetate (TPA) mice treated with rosemary (1.2 or 3.6 mg) five minutes prior to application of B(a)P led to a reduction in tumors by 54 or 64%, respectively. Topical application of rosemary was also found to decrease TPA induced ornithine decarboxylase (ODC) activity, TPA induced inflammation, arachadonic acid-induced inflammation, TPA induced hyperplasia, and TPA induced tumor promotion. In mice initiated with DMBA followed by TPA for promotion rosemary, (0.4, 1.2, or 3.6 mg) was found to decrease the number of skin tumors by 40, 68 or 99%, respectively. The topical application of carnosol also inhibited TPA induced ear inflammation, ODC activity, and tumor promotion. Carnosol at 1, 3, or 10 µM inhibited the number of skin tumors per mouse by 38, 63, or 78%, respectively.

The migration and invasion of B16/F10 mouse melanoma cells was shown to be inhibited by carnosol [47]. Carnosol treatment resulted in the decrease of MMP-9 mRNA and protein expression with an IC$_{50}$ value for MMP-9 mRNA at 5 µM suggesting regulation at the transcriptional level. Several upstream regulators of MMP-9 including AKT, p38, and JNK and
to a lesser extent Erk1/2 phosphorylation activities were modulated by carnosol. The activity of NF-kB was also inhibited by carnosol (10 µM).

6.4 Carnosol and Leukemia
Carnosol was shown to induce apoptosis by disrupting the mitochondrial membrane potential in three acute leukemia cell lines which included SEM, RS4:11, and MV4:11[48]. At 18 µM carnosol did not induce cell death of peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers while the same dose in cell lines resulted in apoptosis. Apoptosis and alterations in mitochondrial membrane potential resulted from carnosol treatment. There is evidence to suggest that carnosol targets the anti-apoptotic members of the Bcl-2 family of proteins. A reduction in Bcl-2 protein expression ranged from 33 to 53% in the three cell lines. Several phytochemicals have been reported to have sensitizing properties when used in combination with chemotherapies in a variety of cancer cell lines. Interestingly, co-treatments of carnosol and AraC, or methotrexate, or vincristine resulted in a delay in chemotherapy induced DNA fragmentation[49]. Further studies are needed to understand how co-treatment of carnosol with chemotherapeutic agents delays DNA fragmentation.

6.5 Carnosol and Colon Cancer
Using a model for colon tumorigenesis dietary administration of carnosol was observed to decrease intestinal multiplicity by 46% in the C57BL/6J/Min/+ (Min/+ ) mouse[50]. Mice were administered a diet with or without carnosol (0.1%). Carnosol was shown to restore E-cadherin and β-catenin to the enterocyte membranes producing a phenotype similar to the APC+/+ wild-type (WT) littermate. Inherited mutations in the APC tumor suppressor gene result in the generation of familial adenomatous polyposis coli with somatic mutations in >80% of sporadic colon cancers [51]. These results suggest that carnosol prevents Apc-associated tumorigenesis in a mouse model, however, further studies are needed.
7. Carnosol Safety and Toxicity

In the Ames Salmonella tester strain TA102 carnosol was found to have significant antioxidant activity with anti-mutagenic activity similar to ascorbic acid [52]. Several studies have suggested that carnosol is protective against environmental toxins in experimental animal models of hepatotoxicity [53-55], bronchial cells [56], and may assist in inducing phase II detoxification enzymes [57]. In the micronucleus test for mutagenesis carnosol was found to be more effective than L-ascorbic acid for gamma-ray radioprotection capacity both before and after radiation exposure [58].

Several animal studies have suggested that daily oral administration of carnosol is well tolerated. In our study, we observed that oral administration of carnosol at 30 mg/kg was well tolerated when administered five days weekly over a 28 day period as evidenced by daily body weight measurements which did not vary between carnosol or vehicle treated mice [9]. In another study, Sprague-Dawley rats were administered an AIN-76A diet with up to 1% carnosol for two weeks with no observable effects on body weight [44]. In a separate experiment with the same group carnosol at 200 mg/kg administered intraperitoneally for five consecutive days was well tolerated as evidenced by measuring liver weight. C57BL/6J/Min/+ (Min/+) Mouse were administered carnosol up to 0.1% in their daily diet was well tolerated over a ten week period [50].

As an alternative approach, future studies should consider evaluating highly characterized rosemary extracts that are standardized to carnosol. This strategy has been employed for studying EGCG (epigallocatechin-3-gallate) in clinical trials through the use of a highly characterized green tea extract standardized to EGCG and other green tea polyphenols [1].
This approach has been recognized by the FDA as seen in their guidance titled, “Guidance for Industry – Botanical Drug Products” and by botanical products receiving investigational new drug (IND) status such as Polyphenon E. The benefit of using a highly characterized extract would be cost to procure carnosol would be cheaper because less purification would be required. A second benefit could be that additional constituents that are found in rosemary would have the opportunity to work synergistically with carnosol. Further studies are needed to evaluate highly characterized rosemary extracts standardized to carnosol. Recently, the European Union has approved a rosemary extract standardized to carnosol for its application towards food preservation and has been adopted into the EU food additive legislation [59]. This extract was well tolerated in short and long term toxicity studies. In a 13 week oral study in male and female rats the NOAEL (no observable adverse event level) of different rosemary extracts was between 180 to 400 mg/kg/bw/day which was equivalent to 20-60 mg/kg /day of carnosol and carnosic acid per day. The adult mean intake of this extract was estimated to be between 500 and 1500 mg of carnosol and carnosic acid per day. Further studies are needed to determine the anti-cancer properties of these rosemary extracts in addition to evaluating isolated constituents from rosemary.

8. Concluding Remarks

The bioavailability and metabolism of carnosol in either animals or humans has not been investigated. The bioavailability of carnosic acid, which shares structural similarities to carnosol are summarized briefly [60]. The $C_{\text{max}}$ of carnosic acid was 128 µM (42.52 mg/L) when administered intragastrically at 90 mg/kg and the absolute bioavailability of carnosic acid was 65.09%. These results are promising suggesting that diterpenes are well absorbed, however, further studies are needed to determine if carnosol has similar pharmacokinetic parameters to carnosic acid. Pharmacokinetic studies of carnosol will be critical in determining the potential use of carnosol for application as an anti-inflammatory and anti-cancer agent. Several pre-
clinical studies have suggested that carnosol selectively targets tumorigenic cell as opposed to non-tumorigenic cells and is safe and tolerable in animals. Further studies are needed to understand molecular interactions of carnosol with deregulated pathways associated with inflammation and cancer. To understand the full potential of carnosol as a chemopreventive or chemotherapeutic agent more mechanistic studies are needed.
Conflict of Interest Statement

None declared

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Legends:

Figure 1. The chemical properties of carnosol.

Figure 2. Semi-synthesis of carnosol from pisiferic acid. Pisiferic acid represents the starting material that has been used for semi-synthetic process of carnosic acid. Carnosic acid is oxidized to form carnosol. This synthesis has been adapted from Tada et al [22].
References


Chemical Properties of Carnosol

Synonyms: 1,3,4,9,10,10aS-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-isopropyl-2H-9S,4aR-(epoxymethano)phenanthren-12-one

CAS Number: 5957-80-2

Formula weight: 330.4

Molecular Formula: C_{20}H_{26}O_{4}

Figure 1. Johnson JJ.
Figure 2. Johnson JJ.