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Cancer combination therapy with carnosic acid

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Abstract: Carnosic acid (CA) is a natural phenolic diterpene mainly occurring in some species of the Lamiaceae family. Numerous studies described the cytotoxicity of CA towards different types of cancer both *in vitro* and *in vivo*. Particularly, the influence of CA in combination with other drugs, vitamins or natural products through affecting various targets has raised interest. Current experimental *in vivo* data suggested that CA may cooperate with clinically used anticancer drugs promoting their activity against cancer. From this point of view, CA gained importance, because it may alter pharmacodynamic profiles of various agents in the case of their co-administration, and thereby, act in a potentially synergistic manner, which can provide a basis for potential applications of CA in the management of cancer. In the present review, we give an overview of CA as well as CA co-treatment regimens with a special focus on cancer. In this context, the role of CA as an adjuvant treatment alternative is highlighted.

Introduction

Carnosic acid (salvin, CA) is a phenolic diterpene with the chemical formula of $C_{20}H_{28}O_4$ (Fig. 1). CA has been identified in some species of the Lamiaceae family, among which *Rosmarinus officinalis* L. is the most abundant in CA, followed by diverse *Salvia* species (Birtić *et al.*, 2015). CA is mainly known for its antioxidant and antimicrobial properties and has been used in foods, *in vivo* and clinical studies as a chemoprotective, an antioxidant or an anti-inflammatory agent (Birtić *et al.*, 2015). Besides, assorted studies have unraveled the potential of CA against carcinogenesis through diverse actions both *in vitro* and *in vivo* as of yet (Allegra *et al.*, 2020; Moore *et al.*, 2016). To exemplify, anti-angiogenic (López-Jiménez *et al.*, 2013), anti-invasive (Huang *et al.*, 2005), chemosensitive, chemopreventive and chemoadjuvant roles of CA in various cancer types have been demonstrated (Nabekura *et al.*, 2010; Shanmugam *et al.*, 2010).

Numerous proteins or signaling pathways are involved in the emergence of CA-related effects in many cancer types. Many *in vitro* investigations pointed out CA's mechanism in cancer types from different origins. For instance, it exhibited antiproliferative effect on HT-29 colon cells and induced cell cycle arrest. Altered function of detoxifying enzymes and metabolites as well as modified expression of transport and biosynthesis genes were attributed as mechanisms behind its antiproliferative capacity (Valdés *et al.*, 2014). In another example, CA suppressed proliferation of breast cancer cells and improved apoptosis *in vitro* (Yesil-Celiktas *et al.*, 2010; Einbond *et al.*, 2012; Han *et al.*, 2017). Ngo *et al.* (2011) reported numerous preclinical findings on the relevance of CA for cancer prevention, indicating *in vitro* and *in vivo* antitumor effects addressing different molecular targets (Ngo *et al.*, 2011). CA significantly induced tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human renal carcinoma (Caki, ACHN, and A498), human hepatocellular carcinoma (SK-HEP-1), and human breast carcinoma (MDA-MB-231) cells. Besides, it boosted sensitization against TRAIL-linked apoptosis through downregulating c-FLIP and Bcl-2 expression and upregulating ER stress-mediated DR5, Bim, and PUMA expression at the transcriptional level in human carcinoma Caki cells (Jung *et al.*, 2015).

We consider that if CA would ever be used clinically to treat cancer patients, then it would not be used in monotherapy but with all probability as part of combination therapy regimens. Therefore, the question raises, how CA may perform in combination with other drugs. If combined with anticancer drugs, vitamins or natural products, CA indeed exhibited synergistic activity affecting their pharmacodynamic profiles (Fig. 2) (Einbond *et al.*, 2012; Han *et al.*, 2017; Zhang *et al.*, 2019; Ayaz *et al.*, 2019). Therefore, in the present mini-review, we give an overview

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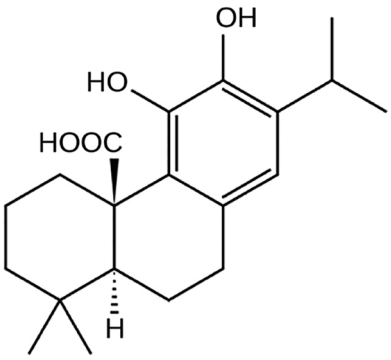

FIGURE 1. Chemical structure of carnosic acid.

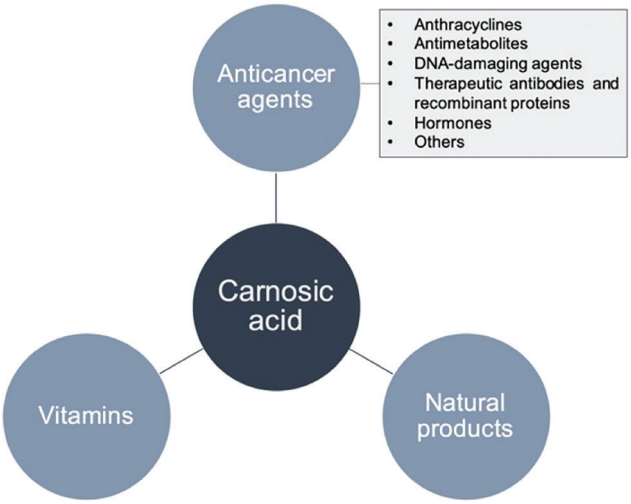

FIGURE 2. Schematic representation of the potential agents with a capability of synergism when combined with carnosic acid.

of the literature deposited in the PubMed database as of May 2021 with a special interest to CA combination therapy. Still, we also mentioned the anticancer potential of CA alone, in order to draw a general concept of the anti-cancer potential of CA and relevant mechanism for a better explanation of CA-associated cytotoxicity in the case of cancer combination therapy.

Anticancer potential and modes of action of carnosic acid monotherapy

The effect of carnosic acid (CA) against various types of cancer has been studied and unraveled in assorted preclinical models, which may provide a basis for forthcoming clinical studies. For instance, recent investigations conducted by El-Huneidi *et al.* (2021) unraveled that CA repressed proliferation and resulted in apoptosis in human gastric cancer cell lines by influencing Akt/mTOR pathway (El-Huneidi *et al.*, 2021). Evaluating the cytotoxicity of CA against a number of known mechanisms of anticancer drug resistance, Mahmoud *et al.* (2020) discovered the capability of CA to induce apoptosis in leukemia cells in addition to the induction of genes as molecular determinants of classical drug resistance (Mahmoud *et al.*, 2020). Likewise, the anticancer potential of CA on chronic myeloid leukemia cells was investigated by Liu *et al.* (2018a), who observed the induction of apoptosis and cell cycle arrest (Liu *et al.*, 2018a).

Jiang *et al.* (2021) observed that CA suppressed cell proliferation, apoptosis, migration, and colony formation of esophageal squamous cell carcinoma in a dose-dependent manner. Cell cycle arrest at the G2/M phase, apoptosis, DNA damage, and MAPK signaling inhibition were detected as associated mechanisms behind its cytotoxicity (Jiang *et al.*, 2021).

In another study conducted by Kar *et al.* (2012), CA inhibited the proliferation of androgen-independent human prostate cancer PC-3 cells via apoptosis through activating serine/threonine protein phosphatase 2A resulting from the AKT/IKK/NF-κB pathway. CA further induced apoptosis in androgen refractory prostate cancer DU145 cells via the activation of caspase-3, enhancement of Bax:Bcl-2 ratio, and cytochrome-c release (Kar *et al.*, 2012).

Lin *et al.* (2018) demonstrated the anticancer potential of CA in melanoma *in vitro* and *in vivo*. CA significantly inhibited the growth of B16F10 melanoma cells and induced cell cycle arrest in addition to its enhancing effect on carmustine- and lomustine-mediated cytotoxicity in the B16F10 tumor model *in vivo* (Lin *et al.*, 2018).

In vivo evidence of the anticancer property of CA further exists in the literature. Petiwala *et al.* (2016) unraveled that CA caused the degradation of androgen receptors and enhanced the expression of endoplasmic reticulum proteins such as CHOP in human prostate cancer cells *in vitro*, which likely contributed to its *in vivo* antiproliferative effect on prostate cancer xenograft tumors, all proving the anticancer property of CA against prostate cancer (Petiwala *et al.*, 2016).

Although the anticancer potential and modes of action of CA have been intensively studied in assorted cancer types, the focus of many studies has been more on the combinatorial application of CA rather than monotherapy.

Synergistic or additive cytotoxic effects in combination with anticancer drugs

Anthracyclines

Additive cytotoxic properties have been described for CA-anthracycline combinations. A remarkably reduced number of leukemia cells and a higher percentage of apoptotic cells were observed in a mouse model of acute myeloid leukemia (AML) upon co-administration of CA and doxorubicin compared to mice treated with doxorubicin alone. Thus, the potential of CA as a promising adjuvant anti-cancer drug was emphasized (Wang *et al.*, 2015). Another study investigated daunorubicin accumulation in the presence of various rosemary phytochemicals including CA in a P-glycoprotein (P-gp)-overexpressing multidrug-resistant human KB-C2 cervical carcinoma cell line. CA enhanced the cellular accumulation of daunorubicin in a concentration-dependent manner through inhibiting P-gp-mediated efflux of daunorubicin, improving the efficacy of chemotherapy regimens (Nabekura *et al.*, 2010; Khan *et al.*, 2015). Similarly, CA alleviated the 50% inhibition concentration (IC_{50}) of doxorubicin by enhancing its cellular concentration and, thereby, increased the sensitivity of K562/AO2 cells through downregulation of MDR1 and inhibition of P-gp-mediated drug efflux (Yu *et al.*, 2008).

Antimetabolites

Cytarabine or cytosine arabinoside (1-β-D-arabinofuranosylcytosine) is a pyrimidine nucleoside analog substantially used for myeloid or lymphoid leukemias as well as Hodgkin or Non-Hodgkin lymphomas (di Francia *et al.*, 2021). Doxercalciferol (1-D2)

in combination with CA boosted the therapeutic activity of cytarabine in patient-derived AML blasts and triggered apoptotic signals in cells sustaining from DNA injury upon cytarabine treatment. This was caused by activation of the vitamin D-upregulated protein TXNIP (Wang *et al.*, 2019). Likewise, cytarabine-induced cell death in HL-60 and U937 AML cells was remarkably enhanced by the addition of 1α-hydroxyvitamin D2 and CA to cytarabine in comparison to cytarabine monotherapy. The enhanced cell death was induced by apoptosis and necrosis and caused by increased DNA injury with higher levels of the DNA damage response activated marker Chk1 (Wang *et al.*, 2016). Cytarabine in combination with 1-D2 and CA exhibited selective and enhanced cell death in malignant AML blasts, which was linked to the activation of the monocytic differentiation program as well as the elevated expression levels of the vitamin D receptor. Besides, caspase-dependent apoptosis with enhanced expression of the apoptosis modulator Bim was displayed as the background mechanism in association with this combination regimen (Harrison *et al.*, 2016).

DNA-damaging agents

A number of studies have proved the accelerative effect of CA in combination with DNA-damaging agents against cancer. If combined with cisplatin, CA exhibited greater anti-growth and pro-apoptotic effects by discouraging myeloid-derived suppressor cells in Lewis lung cancer xenografts with fewer side effects than those of cisplatin (Liu *et al.*, 2018b). Shao *et al.* (2019) established the cellular mechanisms of CA in combination with the alkylating agent temozolomide (TMZ). The cytotoxicity of TMZ was enhanced in glioma cells in addition to the increment in TMZ-associated suppression of colony formation and cell migration as well as TMZ-associated cell cycle arrest and cellular apoptosis. Furthermore, autophagy was induced upon their co-administration. Cyclin B1 inhibition and activation of poly (ADP-ribose) polymerase and caspase-3 were attributed as mechanisms underlying TMZ-induced cell cycle arrest and cellular apoptosis, while TMZ-induced cellular autophagy was encouraged by CA by the inhibition of p-AKT, the downregulation of p62, and the transition of LC3-I to LC3-II (Shao *et al.*, 2019).

Therapeutic antibodies and recombinant proteins

The humanized monoclonal antibody trastuzumab cooperatively inhibited migration of oncoprotein ERBB2-positive breast cancer cells and induced cell cycle arrest in G0/G1 if combined with CA. Furthermore, CA reversibly improved trastuzumab-associated inhibition of cell survival. These effects were linked to the downregulation of ERBB2, the upregulation of both CDKN1A/p21 WAF1 and CDKN1B/p27 KIP1, and the modulation of the PI3K/AKT/mTOR signaling pathway. Besides, CA partially enhanced sensitivity towards trastuzumab in SKBR-3-trastuzumab-resistant cells (D'Alesio *et al.*, 2017).

Hormones

CA induced apoptosis in breast cancer cells *in vitro* by the caspase-3 signaling pathway/TRAIL activation if administered with the selective estrogen receptor modulator tamoxifen. Besides, the cooperation of CA and tamoxifen displayed a better inhibition of breast cancer growth in a mouse xenograft model *in vivo* in comparison to the treatments of CA or tamoxifen alone (Han *et al.*, 2017). In combination with the corticosteroid dexamethasone, CA exerted better cytotoxicity levels than that of CA alone towards pulmonary adenocarcinoma (Coyne and Narayanan, 2019).

Other drugs

CA reversed P-gp-linked multi-drug resistance and sensitized KB-C2 cells to vinblastine (Nabekura *et al.*, 2010). CA and arsenic trioxide co-treatment possessed a greater rate of apoptosis in a mice model of AML, which was attributed to the modulated expression levels of cleaved caspase-3, PTEN, p27 gene mRNA, and proteins compared with CA or arsenic trioxide monotherapy. Besides, the longest survival time was observed in mice receiving CA and arsenic trioxide combination therapy, indicating the synergistic potency against leukemia (Li *et al.*, 2016). Together with arsenic trioxide, CA promoted the cytotoxic activity of arsenic trioxide and caused G1 arrest and apoptosis in HL-60 leukemia cells by modulation of PTEN/AKT signaling pathway and displaying synergistic effects (Wang *et al.*, 2012b). CA activated nuclear transcription factor E2-related factor 2 (NRF2) in AML, alleviated the cytotoxic effects of arsenic trioxide through stimulation of the glutathione biosynthetic pathway and, thereby, accelerated arsenic excretion from the cells (Nishimoto *et al.*, 2016). CA stimulated the expression of NAD(P)H quinone oxidoreductase (NQO1) and markedly augmented the cytotoxicity of β-lapachone (β-lap) in all of the tested melanoma cell lines through stabilization of NRF2. This can be taken as a hint that CA enhances the clinical response to NQO1-linked antitumor drugs (Arakawa *et al.*, 2018).

Synergistic or additive cytotoxic effects in combination with vitamins

CA combined with the physiologically active form of vitamin D3, 1,25-dihydroxyvitamin D3 (1,25D), potentiated differentiation of HL-60 myeloid leukemia cells and exerted anticancer effects by attenuating the cellular reactive oxygen species content and advancing glutathione levels (Danilenko *et al.*, 2003; Danilenko and Studzinski, 2004). The potentiated pro-differentiation effect of 1,25D was associated with an activation of the JNK pathway as well as increased expression of early growth response-1 (EGR-1) and c-FOS (Wang *et al.*, 2005b). Similarly, 1,25D and CA combination treatment activated the NRF2/antioxidant response element pathway and acted cooperatively to differentiate myeloid leukemia cells (Bobilev *et al.*, 2011).

CA and CA-rich ethanolic extracts of rosemary leaves markedly augmented the *in vitro* differentiating and antiproliferative effects of 1,25D and its low-calcemic analog, 1,25-dihydroxy-16-ene-5,6-trans-cholecalciferol (Ro25-4020) in WEHI-3BD- murine myelomonocytic leukemia cells by cell cycle arrest in the G0/G1 phase and pronounced cell growth inhibition. Furthermore, the combined administration of rosemary extract and Ro25-4020 strongly exhibited cooperative antitumor effects without causing hypercalcemia (Sharabani *et al.*, 2006). In another study, 1,25D in combination with CA

and the p38 MAPK inhibitor SB202190 boosted the differentiation of OCI-AML3 (p53 wild type) and HL60 (p53 null) AML cells by enhancing the inhibitory phosphorylation levels of MEK-1. On the other hand, this combination treatment decreased the levels of activated ERK1/2 (Thompson *et al.*, 2010). 1α,25-Dihydroxyvitamin D_2, and its analogs PRI-1916 and PRI-1917 cooperated with CA to induce cell differentiation and G1/S cell cycle inhibition (Nachliely *et al.*, 2016a). Likewise, the *in vitro* differentiation of HL-60 and freshly obtained leukemic cells induced by 1,25D analogs was enhanced by 1,25D-CA co-treatment *ex vivo* through the JNK pathway (Wang *et al.*, 2005a). Hematopoietic progenitor kinase 1 (HPK1) holds a dual role as (1) positive regulator of 1,25D-linked differentiation and cell cycle arrest of AML cells and as (2) mediator of vitamin D resistance. A combination of CA and a selective inhibitor of the α and β isoforms of p38 MAPK (SB202910) increased the sensitization of 40AF cells to 1,25D through increasing HPK1 protein levels if simultaneously added to 1,25D (Chen-Deutsch and Studzinski, 2012). In another study, an analog of 1,25D, 1-D2, exhibited antitumor activity with significantly reduced calcemic effects compared with those of 1,25D. In comparison to 1-D2 monotherapy, the combination of CA and 1-D2 enhanced monocytic differentiation of HL-60 and U937 cells and induced cell cycle arrest, which was attributed to the modulated expression of microRNA181a (Duggal *et al.*, 2012). If administered with the novel double-point modified analogs of 1,25-dihydroxyvitamin D2 (PRI-5201 and PRI-5202), CA augmented the antileukemic effects with upregulated vitamin D receptor protein levels, which was probably linked to increased activation of differentiation-associated vitamin D response elements (Nachliely *et al.*, 2016b). Administration of CA together with 1,25D and a kinase inhibitor SB202190 jointly enhanced the intensity of differentiation through upregulating activated JNK1p46 and the transcription factors modulated by the JNK pathway, c-JUN, JUNB, ATF2 as well as C/EBP β (Chen-Deutsch *et al.*, 2009).

Synergistic or additive cytotoxic effects in combination with natural products

Many studies confirmed the combinatorial use of CA with natural products as exemplified below.

Co-treatment of CA and the flavonoid fisetin exerted cytotoxicity towards lung cancer *in vitro* and *in vivo*. The combination therapy of CA and fisetin suppressed lung cancer growth even more in comparison to CA and fisetin monotherapy through caspase-3-dependent apoptosis (Shi *et al.*, 2017). If combined with curcumin, CA exhibited potentialized growth inhibitory effects compared to CA alone in MDA-MB-468 triple-negative breast cancer cells, indicating varying synergy rates ranging from slight to strong based on their concentration through inhibiting Na-K-ATPase activity (Einbond *et al.*, 2012). Likewise, curcumin-CA co-treatment each combined at low, non-cytotoxic doses, synergistically induced apoptosis through disrupting cellular Ca^{2+} homeostasis in AML cells but not in non-neoplastic hematopoietic cells, proving its cancer-selective action. Even more, co-administration of curcumin and CA significantly alleviated disease progression in mice suffering from AML, suggesting a model for a new Ca^{2+}-targeted pharmacological approach (Pesakhov *et al.*, 2016). Another study by Pesakhov *et al.* (2010) described the synergistic antiproliferative effect and apoptosis induction by curcumin-CA combination treatment in HL-60 and KG-1a human AML cells at non-cytotoxic concentrations of each agent. The caspases-8, -9, and -3 and the proapoptotic protein Bid were activated in curcumin-CA-induced apoptosis (Pesakhov *et al.*, 2010). If rats with bleomycin-induced lung fibrosis were treated with a combination of rosemary leaf extracts (RLE) abundant in CA or RLE abundant in rosmarinic acid following industrial elimination of essential oils, an enhanced antifibrotic effect was observed compared with RLE abundant in CA or RLE abundant in rosmarinic acid monotherapy (Bahri *et al.*, 2021).

Discussion

The use of CA in food, *in vivo*, and clinical studies has been sufficiently documented so far. The function of CA towards assorted conditions was diverse ranging from chemoprotective to anti-inflammatory (Birtić *et al.*, 2015; Gomez-Garcia *et al.*, 2013; Park and Mun, 2013). Despite increased utilization of CA in food, nutritional health, and cosmetic industries, it may exhibit a side effect profile. For instance, high-dose CA was proved to be relatively safe due to the fact that short-term oral administration exhibited a low toxicity profile in different organs of rats (Wang *et al.*, 2012a). Evaluated its potential harm in primary human hepatocytes and microsomes, a dose-dependent enhancement in hepatotoxicity and increased CYP3A enzyme activity in comparison to rifampicin were observed (Dickmann *et al.*, 2012). On the other hand, Lin *et al.* (2018) unraveled that CA alleviated the values of aspartate aminotransferase and alanine aminotransferase *in vivo*, pointing out the safety of CA as a likely chemotherapeutic agent. Still, to stay on the safe side, CA needs a safety assessment prior to its planned use.

Nature is a unique source of new chemical entities and provides selective and effective drug leads with fewer side effects. For instance, 83% of approved drugs between 1981 to 2004 in the field of anticancer drugs were natural products, natural product derivatives, and natural product mimics (Newman and Cragg, 2016). In this context, natural compounds hold vital importance with improved pharmacological features in terms of the discovery of new drug leads and the design of prospective (semi)synthetic derivatives. CA certainly deserves further preclinical validation to move to the clinics, specifically in the field of oncology.

Conclusion and Perspectives

Numerous investigations demonstrated the beneficial effects of co-treatment regimens of CA together with clinically established anticancer drugs, vitamins, hormones, or natural compounds. Preclinical studies pointed to the synergistic (or at least additive) potency of combined applications of CA as its probable future applications. Therefore, CA may represent a useful alternative (1) to improve cytotoxic treatment with classical anticancer drugs, (2) for differentiation therapy with vitamin D derivatives, (3) for phytotherapy, and (4) for

reducing side effects of classical anticancer drugs. Clinical trials are warranted confirming its potential use as an adjuvant and efficient agent in cancer therapy.

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