Isolation and characterization of an anticancer catechol compound from *Semecarpus anacardium*

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**A B S T R A C T**

Ethnopharmacological relevance: The fruits and seeds of *Semecarpus anacardium* are used widely for the treatment of human cancers and other diseases in the Ayurvedic and Sidda systems of medicine in India.

**Aim of the study:** The principal aim of this investigation was to isolate and characterize the anticancer compound from the kernel of *Semecarpus anacardium* nut.

**Materials and methods:** The bioactivity-tailored isolation and detailed chemical characterization were used to identify the active compound. Cytotoxicity, apoptosis, cell cycle arrest as well as synergism between the identified anticancer compound and doxorubicin in human tumor cell lines were analyzed.

**Results:** GC/MS, IR, proton NMR, carbon NMR and collisionally induced dissociation (CID) spectra analysis showed that the isolated active compound is (Z)-pentadecadienyl) catechol (SA-3C). SA-3C is cytotoxic to tumor cell lines with IC50 values lower than doxorubicin and even multidrug resistant tumor cell lines were equally sensitive to SA-3C. SA-3C induced apoptosis in human leukemia cell lines in a dose-dependent manner and showed synergistic cytotoxicity with doxorubicin. The cell cycle arrest induced by SA-3C at S- and G2/M-phases correlated with inhibition of checkpoint kinases.

**Conclusion:** SA-3C isolated from the kernel of *Semecarpus anacardium* can be developed as an important anticancer agent for single agent and/or multigent cancer therapy.

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**1. Introduction**

In the cancer drug discovery program, a paradigm based on ethnobotanical and ethnopharmacological data would be more economical and beneficial for identifying potential anticancer molecules than mass screening of plant species. In this regard, the fruits of *Semecarpus anacardium*, a tropical tree growing wild in the Indian subcontinent, are used extensively for the treatment of human cancers in the Ayurvedic medicine. The nut milk extract of *Semecarpus anacardium* is one of the ingredients in a Sidda medicine (India) preparation called “Kalpaamruthaa” that is reported to have antioxidant, analgesic, antipyretic and ulcerogenic properties (Arulkumaran et al., 2006; Mythilypriya et al., 2007; Veena et al., 2007), The nut extract exhibits antitumor activity due to the suppression of hypoxic and angiogenic factors (hypoxia inducible factor-1 alpha, vascular endothelial growth factor, and inducible nitric oxide synthase) [Mathivadhani et al., 2007a]. *Semecarpus anacardium* nut milk extract also inhibits pro-inflammatory cytokine production in rheumatoid arthritis patients (Singh et al., 2006) and maintains the glutathione redox status by restoring the associated enzymes against oxidative stress in experimental mammalian carcinoma (Mathivadhani et al., 2006). The oil of *Semecarpus anacardium* nut is reported to have cytotoxic effects against acute myeloblastic leukemia (HL-60), chronic myelogenous leukemia (K-562), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa) cell lines (Chakraborthy et al., 2004). The shell of *Semecarpus anacardium* has been subjected to extensive investigations and a number of compounds including anacardic acid, semicarpol, bilalowan, monolefin I, dilefin II, bilalawan-A, bilalawan-B and biflavonoids such as biflavone A1, A2, B and C, tetrahydroamentoflavone, tetrahydrobustaflavone, jeediflavanone,
semicarpuflavanone, and gulluflavanone have been isolated and characterized either from the nut shell or from the vesicant oil (Murthy, 1983a,b,c). But the nut kernel has not been investigated thoroughly for the anticancer activity, in spite of the reports of anticancer activity of fruits by several investigators (Gothoskar and Ranadive, 1971; Chitnis et al., 1980; Indap et al., 1983; Pathak et al., 1983; Vaishnav et al., 1983). Gil et al. (1995) isolated a phenolic glucoside from the kernel, which lacked any anticancer activity. In the present investigation, we have isolated and characterized one of the active principles in the kernel of Semecarpus anacardium nut that is selectively cytotoxic to human tumor cell lines.

2. Materials and methods

2.1. Bioactivity-tailored isolation and characterization

Semecarpus anacardium kernel (450 g) was purchased from Garry and Sun, Reno, NV. A voucher specimen was supplied by the vendor and it was confirmed with the Florida International University Herbarium specimen (Miami, FL). The kernels were washed in hexane, air-dried, ground to coarse powder and stirred with hot methanol (1.5 L) for 24 h with occasional boiling. The methanolic extract was concentrated in a rot evaporator under reduced pressure at 50 °C and the dense oily mass obtained was dissolved in 25 ml ethyl acetate and filtered. The filtrate was again concentrated by rot evaporation under reduced pressure at 50 °C and the residue was chromatographed on a silica gel column for initial clean up and fractionation. The fractions having cytotoxic activity (MTT assay) were collected, pooled together and evaporated off the solvent under reduced pressure to obtain an oily fraction. It was saponified with sodium hydroxide and the non-saponified fraction was extracted into ethyl acetate, neutralized the excess alkali, washed, dried and the solvent evaporated off in a rot evaporator under reduced pressure at 50 °C to obtain an oily product. Analysis of the product on GC/MS showed two main peaks with mass number 316 and 318, and the fragmentation pattern of the olefinic linkages are established by semipreparative Thin Layer Chromatography on Silica Gel (RP-18 EMD Chemicals, San Diego, CA) and elution with acetonitrile–water (95:5). The pure fractions were pooled together and the solvent evaporated under vacuum yielding an oily product. The cytotoxicity assay was performed with individual drug (SA-3C or doxorubicin) as well as combination of both drugs. In the case of combination protocol, drug concentrations were escalated keeping a constant ratio between the two drugs so as to conform to CalcuSyn software (Biosoft, Ferguson, MO). The fraction of surviving cells under each concentration was used for analysis of synergism between SA compound and doxorubicin by CalcuSyn software (Chou and Talalay, 1983; Ramachandran et al., 2004).

2.2. Cell lines

Human leukemia (CEM and CEM/VLB), breast carcinoma (MCF-7), colon carcinoma (SW620 and SW620/Ad300), transformed human mammary epithelial cell line (MCF-10A) and peripheral blood mononuclear cells (PBMMN) were used for the investigation. CEM/VLB and SW620/Ad300 are multidrug resistant cell lines derived from CEM and SW620 cell lines, respectively. Human leukemia, colon carcinoma and PBMMNs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO2 incubator maintained at 37 °C. MCF-7 and MCF-10A cells were grown in DMEM medium supplemented with 10% FBS and antibiotics.

2.3. Cytotoxicity studies

Cells were plated at a density of 10,000 cells/well in 96-well plates with RPMI/DMEM medium and treated with isolated drug at varying doses for 48 h at 37 °C in a 5% CO2 incubator. Cell Proliferation assay kit 1 (MTT) from Roche Molecular Biochemicals (Indianapolis, IN) was used for cytotoxicity assays and the manufacturer’s protocol was followed. The percentage of surviving cells over untreated control cells was calculated and plotted against drug concentrations. The IC50 value for individual cell lines was derived from the dose–response curve (Ramachandran et al., 2006).

2.4. Synergistic effect of SA-3C with doxorubicin

To determine the synergism, cytotoxicity assay was performed with individual drug (SA-3C or doxorubicin) as well as combination of both drugs. In the case of combination protocol, drug concentrations were escalated keeping a constant ratio between the two drugs so as to conform to CalcuSyn software (Biosoft, Ferguson, MO). The fraction of surviving cells under each concentration was used for analysis of synergism between SA compound and doxorubicin by CalcuSyn software (Chou and Talalay, 1983; Ramachandran et al., 2004).

2.5. Apoptosis

Apoptosis assay was performed using the Annexin-V-EGFP Apoptosis Detection kit (MBL International Corporation, Woburn, MA) according to the manufacturer’s protocol. Tumor cells (0.2 × 106) were treated with different doses of SA-3C (0, 0.2, 0.5 and 1 µg/ml) for 48 h at 37 °C and washed with PBS twice. The cells were stained with 5 µl of Annexin V-Fluorescein (Annexin V-FITC) and 5 µl of propidium iodide (PI) in 500 µl of binding buffer before flow cytometric analysis in a Coulter Elite cytometer with FL1 (FITC) and FL2 (PI) detectors (Ramachandran et al., 2006).

2.6. Cell cycle analysis

Following drug exposure for 48 h, cells were centrifuged and resuspended in propidium iodide–hypotonic citrate solution for 1 h before flow cytometrical analysis of cell cycle distribution (Ramachandran et al., 2003).

2.7. Checkpoint kinase inhibition assay

To analyze the role of Cdc25C in the SA-3C-induced cell cycle phase-arrest, checkpoint kinase activity was measured using the Cyclex Checkpoint Kinase Assay Inhibitor Screening kit-1 (Cyclex Co., Ltd., Japan). Tumor cells were incubated with varying doses of SA-3C for 48 h at 37 °C in a 5% CO2 incubator and collected by centrifugation for 10 min at 1000 rpm. Total proteins were extracted using the TNE lysis buffer (10 mM Tris pH 8.0, 1% NP-40, 0.15 M NaCl, 1 mM EDTA) containing protease inhibitor (1 tablet for 10 ml of solution). About 100 µg total protein was used for checkpoint kinase estimation by ELISA. The protocol supplied by the Cyclex Co. (Japan) was used for the analysis and the percentage of inhibition.
was calculated based on the kinase activity of untreated control cells.

2.8. Statistical analysis

Microsoft Excel program was used for statistical analysis and preparation of graphs. Mean and standard deviation values were calculated from three independent experiments. Analysis of variance was used to compare the treatment means.

3. Results

3.1. Structure

The molecular ion peak at m/z 316, in the mass spectra of the compound (m/z 123, 100), 136, 163, 189 and 316 [M+H]⁺ 315, [M+H]⁺ 317) corresponded to that of a pentadecylidienylcatechol and the base peak at m/z 123 confirmed the beta cleavage to the benzene ring. The IR spectra (3436 (b), 3008, 2957, 2927, 2854, 1622, 1594, 1505, 1476, 1276, 777, 732 cm⁻¹) showed characteristic absorptions for a 1,2,3 tri-substituted benzene ring (out of plane bending deformations at 777 and 732 cm⁻¹) and hydroxyl group (broad absorption at 3436 cm⁻¹) in addition to the olefinic linkages. The proton NMR spectra showed peaks for the terminal methyl (δ 0.90 t 7 Hz 3H), homobenzylic (1.63 q 7 Hz 2H), allylic (2.08 m 4H), benzyl (2.62 t 8 Hz 2H), diallylic (2.80 t 6 Hz 2H), phenolic (5.30 s 2H), olefinic (5.39 m 4H), the apparent singlet for 1,2,3 substituted benzene (6.73 m 3H) and the multiplet at δ 1.54 (10 H) corresponded to the protons attached to other carbon atoms in the lipid chain.

The carbon NMR spectra confirmed the assignment for the protons as evidenced by the peaks: terminal methyl (δ 13.79), diallylic (25.66), allylic (27.23 and 29.24), benzyl and homobenzylic (29.75), olefinic (128.02, 128.19, 129.95, 130.16) and aromatic (112.91, 120.13, 122.12, 129.38, 141.9, 143.94 for C6, C4, C5, C3, C2, C1, respectively). The HETCOR spectra clearly demonstrated that the peak at 29.75 is a two carbon signal from the cross-peak of it C1, respectively). The HETCOR spectra clearly demonstrated that the peak at 29.75 is a two carbon signal from the cross-peak of it C1, respectively).

3.2. Anticancer effects of SA-3C

We have analyzed the anticancer effect of SA-3C in drug sensitive and resistant leukemia, breast cancer and colon cancer cell lines and compared with normal peripheral mononuclear cells using MTT assay kit (Fig. 2). SA-3C is cytotoxic to cancer cells line including the pre-malignant human mammary epithelial cell line MCF-10A whereas cytotoxicity was much lower in PBMs (<30% at the highest concentration). SA-3C IC₅₀ values ranged from 0.10 to 5.9 μg/ml (0.32–18.70 μM) among different cancer cell lines. SA-3C was highly cytotoxic to leukemia cells, and both drug sensitive (CEM) as well as multidrug resistant (CEM/VLB) cell lines were equally sensitive to the compound (IC₅₀ = 0.10 μg/ml). Pre-malignant breast epithelial cell line (MCF-10A) has a little higher IC₅₀ value (0.82 μg/ml) than the breast cancer cell line (MCF-7, 0.6 μg/ml). The drug sensitive (SW620) and resistant colon carcinoma cell lines (SW620/Ad300) have similar SA-3C IC₅₀ values (5.9 μg/ml).

3.3. Synergism between SA-3C and doxorubicin

The results of combination effect of SA-3C and doxorubicin are presented in Fig. 3. The median-effect plots and isobologram on the cytotoxicity of SA-3C and doxorubicin combination in CEM and CEM/VLB cell lines clearly indicated a synergistic effect between these two agents in leukemia cell lines. The combination index values of SA-3C and doxorubicin are all less than 1 at IC₅₀ (CEM = 0.356; CEM/VLB = 0.649), IC₇₅ (CEM = 0.190; CEM/VLB = 0.403) and IC₉₀ (CEM = 0.121; CEM/VLB = 0.301) levels which indicated a strong synergistic effect of cytotoxicity between doxorubicin and SA-3C in human leukemia cell lines.

3.4. Induction of apoptosis

Fig. 4 shows the flow cytometric analysis of apoptosis using Annexin-V-EGFP kit. SA-3C compound induced significant level of apoptosis even at lower doses of up to 1 μg/ml (p<0.01). The population in quartet B2 is apoptotic cells (%) and the population in B4 is necrotic cells. The percentage of apoptotic cells increased with increasing concentrations of SA-3C in both drug sensitive and resistant leukemia cell lines. SA-3C induced comparatively higher percentage of apoptosis in CEM cell line than CEM/VLB. SA-3C at 1 μg/ml concentration induced 30.5% and 24.1%
Fig. 3. Analysis of cytotoxicity data using CalcuSyn program to determine the combination effect of SA-3C and doxorubicin. Median–effect plots and isobolograms of SA-3C and doxorubicin in CEM and CEM/VLB cell lines are presented. The median–effect plot is based on logarithmic form of Chou and Talalay’s (1983) equations. ED50, ED75 and ED90 represent IC50, IC75 and IC90 levels based on surviving cells (% of control).

Fig. 4. Flow cytometric analysis of SA-3C-induced apoptosis in CEM and CEM/VLB cell lines using Annexin-V-EGFP apoptosis detection kit. The cells in quadroon B1 and B3 are non-apoptotic cells, B2 are apoptotic cells and B4 are necrotic cells. A dose-dependent induction of apoptotic cells (%) was noticed with SA-3C treatment.
3.5. Cell cycle

SA-3C induced cell cycle blockage in a dose-dependent manner (Fig. 5). The percentage of subG0/G1 cells increased with increasing concentrations of SA-3C with 0.4% at untreated group to 10.8% at 1 µg/ml in CEM. G0/G1 population decreased with increasing concentrations of SA-3C (45.9% at 0 µg/ml to 34.9% at 1 µg/ml in CEM). Similarly G2/M population showed an increase with escalating concentrations of SA-3C (16.6% at 0 µg/ml to 22.5% at 1 µg/ml). In CEM/VLB cell line, subG0/G1 population increased from 0.2% at 0 µg/ml to 7.9% at 1 µg/ml SA-3C.

3.6. Checkpoint kinase inhibition

The inhibition of checkpoint kinases [Checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2) and Cdc25C-associated kinase 1 (C-TAK1)] by anticancer agents is indicative of the effect of these agents on the G1/S transition and G2/M progression. Three different Cdc25 family members exist in humans with Cdc25A regulating the G1/S transition, whereas Cdc25B and Cdc25C are involved in G2/M progression. Checkpoint kinases are involved in the phosphorylation of Cdc25 family members. ELISA results presented in Fig. 6 show significant inhibition of checkpoint kinase with SA-3C treatment, and almost complete inhibition at 0.5 µg/ml in both CEM and CEM/VLB cell lines (p < 0.01). A dose-dependent inhibition was also evident in both cell lines.

4. Discussion

One of the herbal preparations commonly used in the Indian Ayurvedic medicine against human cancers contain Amoora rohituka stem bark, Semecarpus anacardium fruits and Glycyrrhiza glabra roots (Ramachandran et al., 2003, 2006; Rabi et al., 2003). Semecarpus anacardium is a well-studied medicinal plant and investigators have described anticancer properties of its nut milk extract (Mathivadhani et al., 2006, 2007a,b). Similarly, anti-inflammatory and anti-arthritic effects have been described for the nut milk extract (Ramprasath et al., 2004, 2005, 2006a,b; Singh et al., 2006). We have isolated the anticancer principle, 3-[8’(Z),11’(Z)-pentadecadienyl]catechol (SA-3C) from the kernels of Semecarpus anacardium nuts and have analyzed for the first time, the mechanism of cell death induced by the agent in tumor cells. Chemically, non-isoprenoid phenolic lipids such as SA-3C are derivatives of mono- and dihydroxy phenols (catechol, resorcinol and hydroquinone) and they are strongly amphiphilic in nature because of hydroxy benzene ring and the hydrophobic aliphatic side chain. Alkyl resorcinols (resorcinolic lipids) are naturally occurring agents...
that mediate DNA cleavage by sequence-selective strand scission, and the DNA cleavage efficiency is proportional to the length of the side chain (Scannell et al., 1988). Resorcinolic lipid from Ginkgo biloba (5-n-pentadec-8-enyl resorcinol) had been reported to exhibit strong antitumor effects against S180 tumors in mice while those from Lysimachia japonica were reported to exhibit anti-cancer activity against KB cell cultures as well as B–16, PC-13, L-S178-Y, P388 and HEp-2 cell lines. The most active compounds were 5-n-tridecyl resorcinols (Arisawa et al., 1989; Itokawa et al., 1989). It is also reported that the most active homologues of these lipids have 11–15 carbon atoms in the aliphatic side chain and introduction of double bond in the side chain increased the cytotoxicity activity fourfold. Further, the length and degree of unsaturation of the side chain in 5-alk(en)yl resorcinols are reported to induce permeability of biological membranes (Scannell et al., 1988).

The IC50 values of SA-3C are quite comparable to or better than that of agents like doxorubicin. Doxorubicin IC50 values are 0.13 and 13.24 μg/ml for CEM and CEM/VLB cell lines, respectively (Ramachandran et al., 2003). SA-3C is also cytotoxic to other types of tumor cell lines including colon carcinoma and breast carcinoma. Interestingly, SA-3C has almost identical sensitivity in both sensitive and multidrug resistant cell lines. This may indicate that SA-3C may not be a substrate for multidrug resistance (MDR)-associated proteins like P-glycoprotein and the compound may not be effluxed out of the MDR cells (Ramachandran and Melnick, 1999; Higgins, 2007). Also co-incubation of SA-3C with doxorubicin failed to enhance the cellular doxorubicin accumulation in multidrug resistant CEM/VLB cells in the present investigation (data not shown). This agent also induced apoptosis of cancer cells significantly, which appeared to be the major mechanism of cell death. A dose-dependent induction of apoptosis was also evident in both CEM and CEM/VLB cell lines. Cell cycle analysis revealed that SA-3C caused blockage of cells at S-phase and G2/M phases. The decrease in the percentage of G0/G1 population was also noticed concurrently. SA-3C also inhibited the checkpoint kinases in a dose-dependent manner. Three members of checkpoint kinases, checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2) and Cdc25C-associated kinase 1 (C-TAK1) are involved in the phosphorylation of Cdc25C and cell checkpoint abrogation has been proposed as one of the ways to sensitize cancer cells to DNA-damaging agents (Xiao et al., 2006). Furthermore, P53 is the major player in a checkpoint that arrests cells at the G1/S boundary while Chk1 is critical for G2/M and S checkpoints that prevent cell cycle progression after replication defects. Therefore, SA-3C-induced DNA damage may be contributing to the inhibition of checkpoint kinases resulting the cell cycle arrest at S and G2/M phases (Zhou and Sausville, 2003; Luo and Leventon, 2005).

When the tumor cell lines were treated with SA-3C and doxorubicin simultaneously, synergistic cytotoxic effect was observed. Since both these agents induced cell cycle block and apoptosis such outcome is quite plausible. The combination index values were less than 1 indicating strong synergism between these two agents in leukemia cell lines (Chou and Talalay, 1983; Ramachandran et al., 2004). These results indicate that the combinations of SA-3C and doxorubicin are more advantageous than single agents and therefore, SA-3C can be developed as an adjuvant chemotherapeutic agent against cancer.

References


