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Hydroxychavicol, a betel leaf component, inhibits prostate cancer through ROS-driven DNA damage and apoptosis

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ABSTRACT

Dietary phytochemicals are excellent ROS-modulating agents and have been shown to effectively enhance ROS levels beyond toxic threshold in cancer cells to ensure their selective killing while leaving normal cells unscathed. Here we demonstrate that hydroxychavicol (HC), extracted and purified from Piper betel leaves, significantly inhibits growth and proliferation via ROS generation in human prostate cancer, PC-3 cells. HC perturbed cell-cycle kinetics and progression, reduced clonogenicity and mediated cytotoxicity by ROS-induced DNA damage leading to activation of several pro-apoptotic molecules. In addition, HC treatment elicited a novel autophagic response as evidenced by the appearance of acidic vesicular organelles and increased expression of autophagic markers, LC3-IIb and beclin-1. Interestingly, quenching of ROS with tiron, an antioxidant, offered significant protection against HC-induced inhibition of cell growth and down regulation of caspase-3, suggesting the crucial role of ROS in mediating cell death. The collapse of mitochondrial transmembrane potential by HC further revealed the link between ROS generation and induction of caspase-mediated apoptosis in PC-3 cells. Our data showed remarkable inhibition of prostate tumor xenografts by ~72% upon daily oral administration of 150 mg/kg bw HC by quantitative tumor volume measurements and non-invasive real-time bioluminescent imaging. HC was well-tolerated at this dosing level without any observable toxicity. This is the first report to demonstrate the anti-prostate cancer efficacy of HC *in vitro* and *in vivo*, which is perhaps attributable to its selective prooxidant activity to eliminate cancer cells thus providing compelling grounds for future preclinical studies to validate its potential usefulness for prostate cancer management.

Introduction

Polyphenols are major class of phytochemicals known for their disease-fighting and stress-defeating properties. The emerging hypothesis of xenohormesis, a phenomenon by which polyphenols resist stress, combat disease, and confer health benefits (Hooper et al., 2010; Howitz and Sinclair, 2008) has propelled the evaluation of the role of reactive oxygen species (ROS) in dictating health benefits exerted by phenolic phytochemicals. Extensive literature describes the two-pronged approach launched by these phenolic compounds, one that regulates chemopreventive benefits by enhancing the antioxidant defenses, while the other confers chemotherapeutic efficacy due to induction of cellular stress (ROS levels) leading to cell death (Lamming et al., 2004; Surh, 2011; Trachootham et al., 2009a). Several studies suggest that cancer cells display higher reactive oxygen species (ROS) concentrations compared to normal cells (Schumacker, 2006; Szatrowski and Nathan, 1991; Trachootham et al., 2009a,b) and thus an increase in ROS levels can easily tip off the balance in cancer cells due to their higher baseline levels of ROS that result in cell death (Trachootham et al., 2009a).

Inequalities in basal ROS levels in normal and cancer cells can perhaps be ascribed to increased metabolic activity in the latter, which creates a persistent prooxidative condition (Trachootham et al., 2009a). In cancer cells, while moderate or controlled ROS levels lead to survival adaptations that promote their growth and progression, a further increase to higher levels can induce cell death. Excessive levels of ROS disrupt the redox balance maintained in cancer cells by either irreversibly damaging cellular macromolecules including carbohydrates, lipids, proteins and DNA or by interfering with the regulation of redox signaling proteins at transduction or transcriptional levels (Gibellini et al., 2010a).

Abbreviations: ROS, reactive oxygen species; HC, hydroxychavicol; BLE, betel leaf extract; FBS, fetal bovine serum; ATCC, American type cell culture; MTT, tetrazolium bromide solution; DNA, deoxyribonucleic acid; LC3-IIb, light chain 3-IIb; DMSO, dimethylsulfoxide; IC_{50}, inhibitory concentration 50%; LC–MS/MS, liquid chromatography tandem mass spectrometry; PT, permeability transition pore complex; AO, acridine orange; LD_{50}, lethal dose 50%; AVOs, acidic vesicular organelles.

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Cancer cells can succumb to death due to these high ROS levels via several mechanisms including apoptosis, necrosis, autophagy, mitotic catastrophe, and even due to the loss of drug-resistance adaptation (Surh, 2003). Intrinsically driven apoptosis due to excess ROS is mediated directly through the mitochondria and involves the opening of permeability transition (PT) pore complex followed by the release of cytochrome c into the cytosol, which triggers the caspase cascade culminating in cell death (Gibellini et al., 2010b). Thus, drugs that act as prooxidants and tip over the ROS balance in cancer cells by promoting the leakage of free radicals from the mitochondria may prove to be valuable anticancer therapeutics. Although plant phenolics have been long known to have antioxidant functions, their role in enhancing ROS levels is emerging.

Chemically, polyphenols are redox moieties and this property confers on them the unique dual ability to quench as well as generate ROS (Decker, 1997; Sakihama et al., 2002). The chemopreventive efficacy of certain polyphenols lies in their antioxidant properties and their ability to scavenge ROS thereby reducing oxidative stress. Such species at low concentration and in normal cells would act as chemopreventive agents (Bouayed and Bohn, 2010; Surh, 2011). Other species of polyphenols can act as prooxidants and generate ROS. The ROS surge induces DNA damage and eventually apoptosis thereby exerting a potential chemotherapeutic action (Martin and Barrett, 2002; Trachootham et al., 2009a). Such species are effective at higher concentrations in cells with higher levels of oxidative stress (Azam et al., 2004). Literature suggests that polyphenols with catechol and/or pyrogallol groups can exhibit prooxidant properties, either by reducing iron (III) or copper (II) ions while chelation or by the reaction of ortho-hydroxyphenoxy radical, produced from their oxidation, with other free-radical species to oxidize ortho-quinones and O_2^- (Mira et al., 2002). Thus, the unique properties that include relative abundance in nature, lack of selectivity-related side effects, and patient compliance, which potentially underscore their anticancer benefits, have kindled a lot of interest in the recent times to further investigate the phenolic phytochemicals.

We have recently reported the bioactive constituents of *Piper betel* leaf extract (BLE), and its significant antiproliferative activity in *in vitro* and *in vivo* prostate cancer models (Paranjpe et al., 2013). Our study concluded that the phenolic compound, hydroxychavicol (HC) is the most abundant phytochemical in betel leaves that majorly contributed to the antiproliferative efficacy of BLE. Although HC is present at ~26%, vital factors like environment, cultivation, harvest period, extraction procedures etc., may greatly influence its relative abundance (Quideau et al., 2011; Sasidharan et al., 2011). To identify the optimal concentration at which HC imparts maximum anticancer efficacy and further evaluate its pharmacological significance, it is essential to isolate and purify HC from betel leaves.

Literature reports suggest that HC is effective in impeding cell cycle progression of prostate cancer and oral KB carcinoma cells (Chang et al., 2002, 2007). Also, HC has been shown to impart anti-mutagenicity (Amonkar et al., 1986; Bhide et al., 1991a,b; Kumar et al., 2010), antiulcerogenic (Bhattacharya et al., 2005; Sarkar et al., 2008), and antioxidative (Bhattacharya et al., 2005; Chen et al., 2000) properties specifically against cancer cells. There have also been speculations regarding its stress-inducing properties (Chang et al., 2002; Chen et al., 2000). However, there is a dearth of data emphasizing the *in vivo* efficacy of HC and its prooxidant nature in prostate cancer cells, which could potentially lead to its development as a single-agent chemotherapeutic agent or in an adjuvant setting. Here we report the prooxidant property of HC obtained from betel leaves, as well as its anticancer mechanisms in in vitro and *in vivo* prostate cancer models emphasizing the HC-induced ROS effects on various pathways.

Materials and methods

Cell culture, chemicals and reagents. Piper betel leaves were purchased from the local farmer's market in Atlanta, GA. Dichloromethane (DCM), and methanol (MeOH) were obtained from Fisher Scientific (Pittsburgh, PA). The silica used for classical chromatography was from EMD Biosciences (Billerica, MA). Thin-layer chromatography (TLC) plates were from EMD chemicals (Billerica, MA). Androgen-independent prostate cancer cells, PC-3, DU145, C4-2 and 22Rv1 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% penicillin/streptomycin. The normal prostate epithelial, RWPE-1 cells purchased from American Type Culture Collection (ATCC, Manassas, VA) were cultured in Keratinocyte-SFM medium kit (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Luciferase-expressing PC-3 cells (PC3-luc) were from PerkinElmer (Hopkinton, MA) and were maintained in MEM medium with 10% FBS, Hyclone, (Pittsburgh, PA). All the cell lines were made sure to be devoid of mycoplasma contamination using Universal Mycoplasma Detection Kit from ATCC (ATCC, Cat#30-1012K, Manassas, VA). The MTT dye (thiazolyl blue tetrazolium bromide, 98% TLC), acridine orange (AO), 2′,7′-dichlorofluorescein diacetate (DCFDA), chloroquine, 3-methyladenine (3-MA), dimethyl sulfoxide (DMSO), Hoechst stain and β-actin antibody were from Sigma (St. Louis, MO). Dihydroethidium (DHE), 4,5-dihydroxy-1, 3-benzenedisulfonic acid disodium salt monohydrate (tiron), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), apocynin, rotenone and cyclosporin A were from Fisher Scientific. The concentrations of the above reagents used in the study were: 100 μM HC, 25 μg/ml of AO, 5 μM DHE, 25 μM DCFDA, 2.5 μg/ml of JC-1, 0.5 mM 3-MA; 1 mM tiron, 100 nM rotenone, 10 μM apocynin and 5 μM cyclosporin A. Primary antibodies for beclin-1, light chain 3 (LC3IIb), cleaved caspase-3, cleaved PARP, γ-H2AX, and cytochrome c were from Cell Signaling (Beverly, MA). MitoTracker Red, Alexa 488- or 555-conjugated secondary antibodies were from Life Technologies (Grand Island, NY). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. Hydroxychavicol (HC) was extracted from betel leaves and was characterized for >99% purity.

Isolation of HC from betel leaves. Freshly chopped Piper betel leaves were submerged in deionized water and extraction was carried out in a boiling apparatus for 3 h followed by collection of the supernatant by filtration for three consecutive days. The pooled supernatant was then concentrated to 1/12th of the original volume under reduced pressure at a temperature of 50 °C. This concentrated aqueous extract was further extracted 6 times in a separating funnel with 250 ml of DCM each time, followed by vacuum filtration through celite bed. The resultant clear DCM fraction was then concentrated under reduced pressure. The residue was then subjected to silica gel column chromatography (100–200 mesh) where the elution was initiated with a total of 1400 ml of DCM followed by 800 ml of 1% MeOH in DCM. Fractions of 100 ml each were collected and subjected to TLC in DCM:MeOH (19:1). The fractions 4–22 were found to contain pure HC and thus were pooled and concentrated under vacuum. HC was further characterized by spectral analysis for both quantitation and >99% purity via HPLC using similar method as described earlier (Paranjpe et al., 2013).

Antiproliferative MTT assay. Androgen-independent human prostate cancer cells, PC-3, DU145, C4-2 and 22Rv1, were seeded in 96 well plates at a density of 3500 cells per plate. After 24 h of incubation, the medium was aspirated and replaced by media dosed with HC at concentrations of 1, 10, 25, 50, 75, 100 and 250 μM. A primary stock was prepared by dissolving HC in DMSO at a concentration of 25 mM from which a secondary concentration of 250 μM was prepared in media. Further dilutions were made using the secondary stock. A total volume of 100 μl was added to each well. After 48 h, the drug-containing medium was replaced with 100 μl of medium containing MTT (tetrazolium bromide solution in PBS (5 mg/ml)). The yellow thiazolyl groups of MTT are reduced to purple tetrazolium crystals by viable cells, which were