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Original Contribution

(6)-Gingerol-induced myeloid leukemia cell death is initiated by reactive oxygen species and activation of miR-27b expression

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ABSTRACT

The natural polyphenolic alkanone (6)-gingerol (6G) has established anti-inflammatory and antitumoral properties. However, its precise mechanism of action in myeloid leukemia cells is unclear. In this study, we investigated the effects of 6G on myeloid leukemia cells in vitro and in vivo. The results of this study showed that 6G inhibited proliferation of myeloid leukemia cell lines and primary myeloid leukemia cells while sparing the normal peripheral blood mononuclear cells, in a concentration- and time-dependent manner. Mechanistic studies using U937 and K562 cell lines revealed that 6G treatment induced reactive oxygen species (ROS) generation by inhibiting mitochondrial respiratory complex I (MRC I), which in turn increased the expression of the oxidative stress response-associated microRNA miR-27b and DNA damage. Elevated miR-27b expression inhibited PPARγ, with subsequent inhibition of the inflammatory cytokine gene expression associated with the oncogenic NF-κB pathway, whereas the increased DNA damage led to G2/M cell cycle arrest. The 6G-induced effects were abolished in the presence of anti-miR-27b or the ROS scavenger *N*-acetylcysteine. In addition, the results of the in vivo xenograft experiments in mice indicated that 6G treatment inhibited tumor cell proliferation and induced apoptosis, in agreement with the in vitro studies. Our data provide new evidence that 6G-induced myeloid leukemia cell death is initiated by reactive oxygen species and mediated through an increase in miR-27b expression and DNA damage. The dual induction of increased miR-27b expression and DNA damage-associated cell cycle arrest by 6G may have implications for myeloid leukemia treatment.

Myeloid leukemia is characterized by an impaired hematopoietic process with rapid proliferation of undifferentiated and immature blood cells of the myeloid lineage [1]. Chemotherapeutic treatment strategies for both acute and chronic myeloid leukemia are effective at the early stages of the disease. However, major limitations of standard chemotherapy in the clinical setting are side effects, such as cardiac and renal toxicity, severe myelosuppression, and development of chemoresistance, leading to poor survival outcomes [2–5]. Therefore it is imperative to look for novel therapeutic agents with lesser side effects urgently to address the underlying causes of poor treatment outcomes associated with conventional therapeutics. In search of novel yet nontoxic chemotherapeutic agents, attention has been focused on natural agents in recent times [6–11].

Many natural polyphenolic compounds have been known to restrict cancer cell proliferation through distinct mechanisms [12]. These compounds are preferred either as single agents or as adjuvants for chemotherapy owing to their immense antioxidative potential, lesser side effects, and ease of metabolism [12,13]. (6)-Gingerol (1-(4′-hydroxy-3′-methoxyphenyl)-5-hydroxy-3-decanone; 6G)[2], is a polyphenolic alkanone present in the major pungent extracts of ginger (*Zingiber officinale* Roscoe, Zingiberaceae) with established antitumorigenic and proapoptotic activities [14]. Its potent anti-tumor activity has been reported in a variety of

Abbreviations: 6G, (6)-gingerol; iNOS, inducible nitric oxide synthase; NF-κB , nuclear factor κB; miRNA, microRNA; MRC, mitochondrial respiratory complex; ETC, electron transport chain; PBMC, peripheral blood mononuclear cell; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; NHE-1, sodium/hydrogen exchanger 1; ROS, reactive oxygen species; PARP, poly(ADP ribose) polymerase; PPARγ, peroxisome proliferator-activated receptor γ; miR-27b, microRNA 27b; PI, propidium iodide; NAC, *N*-acetylcysteine; DMSO, dimethyl sulfoxide; PCNA, proliferating cell nuclear antigen; IL, interleukin; TUNEL, terminal deoxynucleotidyl transferase enzyme-mediated dUTP nick-end labeling

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cancer types, including breast, pancreatic, gastric, colon, and colorectal cancers, and certain hematological malignancies [14–23]. Apart from cell culture models its antitumorigenic effects have also been studied in in vivo animal models [24,25]. The potential mechanism of its action is presumably through the inhibition of inducible nitric oxide synthase (iNOS), suppression of IκBα phosphorylation, nuclear factor κB (NF-κB) nuclear translocation, release of cytochrome *c*, caspase activation, and increase in apoptotic protease-activating factor-1, oxidative stress, and DNA damage leading to apoptosis induction [20–25].

Despite the promising anti-cancer activity of 6G, its limited clinical application is attributed to its low bioavailability and efficacy at relatively high concentrations in vitro [14–23,26–28]. Although ginger and its constituents such as 6G at doses up to 2.0 g daily have shown very low levels of toxicity and high levels of tolerability in both animal and human studies [26], it is rapidly metabolized into glucuronides and sulfates [28]. 6G has a terminal half-life ranging from 7.23 to 8.5 min in rat plasma [27], whereas its elimination half-life varies between 75 and 120 min at the 2.0-g dose in human plasma [28]. Despite these pharmacokinetic limitations, 6G has emerged as a modulator of key oncogenic signaling pathways in a variety of cancer cells [27]. Therefore, cancer-specific preclinical studies are essentially required to validate the usefulness of 6G as a chemotherapeutic or chemopreventive agent [29]. Its development as a chemotherapeutic agent holds promise because of its nontoxic and inexpensive nature and ease of availability and metabolism [14]. Therefore, characterization of the cancer-specific molecular mechanism of 6G action along with identification of its molecular and cellular targets could be an essential way forward in the phytochemical-derived drug discovery process [29,30].

Recent studies have shown that natural agents, including isoflavone, curcumin, indole-3-carbinol, 3,3′-diindolylmethane, (−)-epigallocatechin-3-gallate, resveratrol, etc., exert their anticancer activities through changes in a group of endogenous small noncoding RNAs of 19–25 nucleotides (~22 nt) in length, known as microRNAs (miRNAs). MiRNAs regulate gene expression by binding to the 3′ untranslated region (3′-UTR) of target mRNA, resulting in either mRNA degradation or inhibition of translation [31]. Natural agents have been reported to either inhibit miRNAs associated with the oncogenic signaling pathways or activate expression of miRNAs associated with the cell death pathways, leading to the inhibition of cancer cell proliferation, induction of apoptosis, reversal of epithelial–mesenchymal transition, or enhancement of efficacy of conventional cancer therapeutics [10,32]. However, there is no information available concerning the regulation of miRNA expression by 6G.

Therefore in this study, as an initial step in the assessment of 6G as a chemotherapeutic agent against myeloid leukemia, we investigated the effects of 6G on myeloid leukemia cell lines, primary leukemia cells, and mouse xenografts and further examined the cell death mechanism. Our results unveil a novel mechanism of action of 6G involving ROS generation through the inhibition of mitochondrial respiratory complex I (MRC I) and ROS-associated increase in miR-27b expression and DNA damage, leading to G2/M cell cycle arrest and apoptosis in myeloid leukemia cells. These preclinical studies suggest that 6G could serve as a promising agent for myeloid leukemia treatment.

Material and methods

Reagents

6G was extracted and purified from the rhizomes of ginger (*Z. officinale*) at the medicinal process chemistry division of the CSIR–Central Drug Research Institute, India, as per a standardized procedure (CDRI Plant Code 4735) [33]. The purified compound was dissolved in dimethyl sulfoxide (DMSO) to a final stock concentration of 10 mM. The stock solution was aliquotted and stored at −20 °C until further use. Primary antibodies for cleaved PARP (No. 9541), β-actin (No. 4970), cyclin B1 (No. 4138), Cdk1 (No. 9112), Cdc25B (No. 9525), Cdc25C (No. 4688), PPARγ (No. 2430), Bak (No. 6947), caspase-3 (No. 9661) (Cell Signaling Technologies, Boston, MA, USA); Bcl2 (No. 610538), Bad (No. 610391), Bax (No. 610982), XIAP (No. 610716), BclXL (No. 610746) (BD Biosciences, San Jose, CA, USA); and PCNA (No. sc-25280) and p-H2AX (No. sc-101696) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in this study. The secondary antibodies used in the experiments were from Chemicon (Temecula, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture

Chronic myeloid leukemia (CML) cell lines (K562, LAMA-84, JURL-MK1) and acute myeloid leukemia (AML) cell lines (U937, HL-60, NB4) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), both from Gibco (Carlsbad, CA, USA), along with 1% penicillin and streptomycin from Sigma in a humidified incubator at 37 °C with 5% CO_2. Peripheral blood samples were obtained from normal healthy donors and myeloid leukemia patients of various clinically defined stages at the CSM Medical University (Lucknow, India), after written informed consent in compliance with the Declaration of Helsinki 2002. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Hypaque density gradient (1.0 g/ml) centrifugation. Subsequently, the isolated cells (10^6/ml) were cultured in complete RPMI 1640 medium supplemented with 10% FBS and subjected to 6G treatment for various time periods. Transfections were carried out in these cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At the end of the treatment period the samples either were processed for flow cytometry-based cell viability analysis or were resuspended in Trizol reagent or cell lysis buffer and processed for RT-PCR or Western blotting analysis, respectively.

Cell viability assay

The cell viability assay was performed using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's instructions.

Apoptosis and TUNEL assays

6G-induced apoptosis was measured by using the Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instructions. Cells were seeded in six-well culture plates at a density of 1×10^6 cells per well and were treated with 6G for 24, 48, and 72 h. At the respective time points cells were harvested and washed twice with phosphate-buffered saline (PBS). Subsequently the cell pellet was resuspended in 1× binding buffer containing annexin V–FITC and propidium iodide and incubated for 10 min in the dark. Both control and treated samples were analyzed for live, necrotic, and early and late apoptotic cells using a FACSCalibur (BD Biosciences) flow cytometer. The TUNEL assay was done using a flow cytometry-based kit (Roche, Mannheim, Germany) as per the manufacturer's instructions.

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