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Anti-leukemic effects of gallic acid on human leukemia K562 cells: Downregulation of COX-2, inhibition of BCR/ABL kinase and NF-κB inactivation

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ABSTRACT

Gallic acid (GA) induces apoptosis in various cancer cell lines. In this study, we investigated the apoptotic activity induced by GA on chronic myeloid leukemia (CML) cell line-K562 and the underlying mechanism. GA reduced the viability of K562 cells in a dose and time dependent manner. GA led to G_0/G_1 phase arrest in K562 cells by promoting p21 and p27 and inhibiting the levels of cyclin D and cyclin E. Further studies indicated apoptosis with impaired mitochondrial function as a result of deranged Bcl-2/Bax ratio, leakage of cytochrome *c* and PARP cleavage along with DNA fragmentation and by up-regulating the expression of caspase-3. GA also activated the protein expressions of fatty acid synthase ligand and caspase-8. GA is more effective in imatinib resistant-K562 (IR-K562) cells (IC_{50} 4 μM) than on K562 cells (IC_{50} 33 μM). GA inhibited cyclooxygenase-2 (COX-2) in K562 as well as IR-K562 cells appears to be COX-2 involved in the suppression of growth. Interestingly, GA also inhibited BCR/ABL tyrosine kinase and NF-κB. In conclusion, GA induced apoptosis in K562 cells involves death receptor and mitochondrial-mediated pathways by inhibiting BCR/ABL kinase, NF-κB activity and COX-2.

1. Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is widely used as an antioxidant in the food industry and was shown to exhibit variety of pharmacological and biological activities, including anti-cancer (Inoue et al., 1995; Kaur et al., 2009; Yeh et al., 2011). Inhibition of ribonucleotide reductase and cyclooxygenases (COXs) *in vitro* in human HL-60 promyelocytic leukemia cells was reported (Madlener et al., 2007). GA, isolated from the leaf extract of *Pistacia weinmannifolia*, was shown to significantly inhibit the growth of K562 cells with an IC_{50} value less than 5 μg/ml (Hou et al., 2000). Recent pre-clinical studies clearly demonstrated the chemopreventive effect of GA on dimethyl hydrazine (DMH) induced colon carcinogenesis in animal models (Giftson et al., 2010).

COXs are the key enzymes that catalyze the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. In most tissues, COX-1 is expressed constitutively, whereas COX-2 is induced by growth factors, cytokines, and carcinogens. Thus, increased COX-2 expression appears to be involved in the development of cancer by promoting cell division and inhibiting apoptosis (Tang et al., 2002; Rizzo, 2011). The inhibition of COX-2 activity by traditional NSAIDs blocks these activities and thus may account for their anti-carcinogenic activity (Evans and Kargman, 2004; Khan et al., 2011). Celecoxib, a COX-2-selective inhibitor, exerted antitumor effects in a wide variety of cancers (Entezari et al., 2011; Subhashini et al., 2005). It also showed synergistic antitumor effects when combined with gemcitaine or 5-fluorouracil in patients with advanced pancreatic cancer (Milella et al., 2004). Subhashini et al. (2005) showed that celecoxib exerts antileukemic effects in K562 cells by cell cycle arrest, caspase-3 activation and down-regulation of COX-2 expression. These effects of celecoxib were shown to be synergistic with hydroxyurea or imatinib (Zhang et al., 2006). The mechanism underlying the antitumor activity of COX-2 inhibitors is thought to involve inhibition of COX-2 enzyme activity, but it is unclear whether COX-2 inhibition is required to induce apoptosis. It has been reported that the anti-proliferative effects of celecoxib were similar for both hematopoietic and epithelial cancer cell lines, despite the fact that the COX-2 expression was negative in most of the hematopoietic cell lines, including K562 cells (Waskewich et al., 2002). Giles et al. (2002),

Abbreviations: C-PC, C-phycocyanin; CA, chebulagic acid; GA, gallic acid; TPL, Triphala; IR-K562, imatinib resistant-K562; DRI, dose-reduction index; CI, combination-index; PARP, poly ADP ribose polymerase; APAF, apoptotic protease activating factor.

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on the other hand, reported COX-2 expression in K562 cells and Arunasree et al. (2008) reported COX-2 over expression in imatinib resistant-K562 (IR-K562) cells.

The BCR/ABL tyrosine kinase in the cytosol activates various intracellular signaling pathways including NF-κB, which regulates extracellular stimuli (Goldman and Melo, 2003; Wong and Witte, 2004). Though imatinib mesylate (Gleevec; 2-phenyl amino pyrimidine) is a specific inhibitor of BCR/ABL tyrosine kinase, the outcome of its therapy in the accelerated and blastic phases of chronic myelogenous leukemia (CML) is unacceptably poor (Savage and Antman, 2002). Also resistance develops to gleevec therapy, warranting the need for the search of novel molecules against CML. A novel multi- BCR/ABL and Src family tyrosine kinase inhibitor, dasatinib was developed as oral drug for CML patients with imatinib resistance. Latest reports suggest the association of dasatinib with side effects at higher dosage. Hence, development of effective BCR/ABL tyrosine kinase inhibitors with fewer side effects is of great therapeutic significance.

In the present study, the efficacy of GA on the proliferation of imatinib sensitive K562 and imatinib resistant-K562 (IR-K562) cells and the mechanisms involved were investigated.

2. Materials and methods

2.1. Cell lines and reagents

The human chronic myeloid leukemia cell line, K562, MDA-MB-231 (breast carcinoma), and COLO-205 (colon cancer) were obtained from National Center for Cell Science (NCCS), Pune, India. IR-K562 cells were developed as described by Arunasree et al. (Arunasree et al., 2008). RPMI 1640, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Penicillin, Gentamycin and Streptomycin were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc., Grand Island, NY). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Poly-L-lysine, glutaraldehyde, DEAE-cellulose, DAPI (4,6-diamidino-2-phenylindole), Proteinase K, RNase A, Propidium iodide (PI), Phenyl methyl sulfonyl fluoride (PMSF), Leupeptin, Aprotinin, Pepstatin A, Trypsin, Tween-20, Triton X-100, Ponceau S, Igepal CA-630 were from Sigma Chemical Company (St. Louis, USA). Mouse monoclonal antibodies against Bcl-2, PARP (Poly ADP-ribose polymerase), Bax and anti-Tyr were from Upstate Biotechnology (Charlottesville, VA, USA) cytochrome *c* was obtained from Chemicon (Temecula, CA, USA), Cyclin D, Cyclin E, Caspase-3, Caspase-8 and FASL antibodies were a kind gift from Dr. Bindu Aramati and Enzyme immunoassay kit for the measurement of NF-κB was purchased from Invitrogen Inc., (CA, USA). IL-1β (R & D Systems, USA) was a kind gift from CCMB, Hyderabad, India. All other chemicals and reagents were purchased from local companies and are of molecular biology grade. Imatinib was a gift from Natco Pharma Ltd., India. GA was extracted and purified from the fruits of *T. bellerica* and characterized by LC-MS, NMR, IR and crystal structure data (Reddy et al., 2010).

2.2. Cell culture and treatment

Cell lines used in this study except K-562 were maintained in monolayer in tissue culture Petri dishes. Cells were maintained in RPMI-1640 medium supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were sub-cultured twice each week and the exponentially growing cells were used in all treatments. GA dissolved in DMSO was used in the treatments. Before the treatment with test compound cells were washed with PBS and fresh medium was added. At the time of treatment, working solutions were diluted accordingly in RPMI. Final concentrations of GA ranged from 0.01 to 50 μM. The drugs were added to the cells, 12 h after the sub-culture. The final concentration of the vehicle (DMSO) never exceeded 0.1%.

2.3. Cell proliferation assay

Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann (Mosmann, 1983). The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The dehydrogenases using NADH or NADPH as co-enzyme can convert the yellow form of the MTT salt to insoluble, purple formazan crystals (Liu et al., 1997). Formazan solution is read spectrophotometrically after the crystals are dissolved in organic solvent (DMSO). Cells (5×10^3 cells/well) were incubated in 96-well plates in the presence or absence of GA for 24 h in a final volume of 100 μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the absorbance was measured at 570 nm on microtiter plate reader (μ Quant Bio-tek Instruments, Inc.). Each concentration was tested in three different experiments run in four replicates. The viable cells were counted by the trypan blue exclusion assay with a hemocytometer. Further to know whether the effects observed are brought about by the parent compound or by a consecutive product formed by either spontaneous chemical decomposition or metabolic conversions such as oxidation, esterification or monomethylation, we have checked the metabolism of GA in mice plasma using reverse phase-HPLC (shown in Supplementary data).

2.4. Analysis of interactions

The level of interaction between GA and imatinib was assessed by combination-index (CI) methods (Chou and Talalay, 1983; Chou et al., 1993). The CI method is a quantitative representation of pharmacological interaction between two drugs. Briefly, variable ratios of drug concentrations were used in several different combinations for the treatment of imatinib sensitive and imatinib resistant-K562 cells for 24 h. Cell growth inhibition was determined using the MTT assay, as previously described. The anti proliferative data obtained were analyzed using mutually exclusive equations to determine the CI (Mosmann, 1983; Liu et al., 1997). CI value of 1 indicates an additive effect, whereas a CI < 1 or>1 indicates synergism or antagonism, respectively. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate). The CI values were calculated at *x*% cell growth inhibition, as:

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

where, $(Dx)_1$ and $(Dx)_2$ are the doses of D_1 (drug #1, for example, the GA) and D_2 (drug #2, for example, the imatinib) alone that gives *x*% inhibition, whereas $(D)_1$ and $(D)_2$ are the doses of D_1 and D_2 in combination that also inhibits *x*% (*i.e.* isoeffective). The $(Dx)_1$ and $(Dx)_2$ can be readily calculated from the Median-effect equation of Chou (Chou and Talalay, 1983; Chou et al., 1993).

The dose-reduction index (DRI) defines the extent (folds) of dose reduction possible in a combination, for a given degree of effect, compared with the dose of each drug alone: $(DRI)_1 = (Dx)_1/(D)_1$ and $(DRI)_2 = (Dx)_2/(D)_2$. The relationship between DRI and CI is, therefore, expressed as: $CI = (Dx)_1/(D)_1 + (Dx)_2/(D)_2 = 1/(DRI)_1 + 1/(DRI)_2$.