Dichamanetin Inhibits Cancer Cell Growth by Affecting ROS-related Signaling Components Through Mitochondrial-mediated Apoptosis

YEONJOONG YONG[1,2,*], SUSAN MATTHEW[1,2,*], JENNIFER WITTWER[1], LI PAN[2], QI SHEN[3], A. DOUGLAS KINGHORN[2], STEVEN M. SWANSON[3] and ESPERANZA J. CARCACHE DE BLANCO[1,2]

Divisions of [1]Pharmacy Practice and Administration, [2]Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Ohio State University, Columbus, OH, U.S.A.; [3]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, U.S.A.

Abstract. *Background/Aim: Dichamanetin is a C-benzylated flavanone isolated as a major secondary metabolite from Piper sarmentosum, a plant used as a spice in Southeast Asia. This study aimed to investigate the path through which dichamanetin exerts its antiproliferative effect. Materials and Methods: The study of several signaling cellular components, namely, reactive oxygen species (ROS) levels, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, mitochondrial membrane potential, DNA binding, poly ADP-ribose polymerase (PARP1) inhibition and proteasome inhibition was performed using an enzyme-linked immunosorbent (ELISA) assay, cell sorting, and western blot. Results: Dichamanetin significantly reduced the cell viability of various types of human cancer cells (HT-29 colon, DU145 prostate, and MDA-MB-231 breast cancer) in a concentration- and time-dependent manner and induced G_1 arrest of the cell cycle. It was also demonstrated that the selective cytotoxic effect of dichamanetin in cancer cells is mediated by the induction of oxidative stress. Conclusion: Our findings suggest that dichamanetin isolated from an edible herb has cancer chemotherapeutic potential.*

*These Authors contributed equally to this study.

Correspondence to: Esperanza J. Carcache de Blanco, Division of Pharmacy Practice and Administration, Division of Medicinal Chemistry and Pharmacognosy, College of Phamacy, The OhioState University, 500 West 12th Avenue, Columbus, OH 43210-1291, U.S.A. Tel: +1 6142477815, Fax: +1 6142921335, e-mail: carcache-de-blan.1@osu.edu

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Plants used as foods that are rich in certain bioactive secondary metabolites have received increased attention over the past several decades as potential agents for the treatment and prevention of several disease states (1). *Piper sarmentosum* Roxb. (Piperaceae), locally known as wild betel, is a glabrous, creeping tropical terrestrial herb popularly consumed as a spice in the cuisine of several Southeast Asian countries (2). There are several literature reports of the anti-amoebic, anti-bacterial, anti-fungal, anti-malarial, anti-neoplastic, anti-protozoal, anti-tuberculosis, hypoglycemic, and insecticidal effects of this species (3-8). In a recent report, our group reported the structures and mitochondrial membrane potential activity of four new *C*-benzylated flavanones, sarmentosumins A-D, five known *C*-benzylflavanones, and several other piperamides (9). Among these compounds, dichamanetin ($C_{29}H_{24}O_6$), a rarely-reported *C*-benzylflavanone, was isolated as a major metabolite (0.9 g; 0.29% yield) from the dried aerial plant parts (311 g) of *P. sarmentosum*. A study of mitochondrial function and induction of nuclear factor kappa-light-chain-enhancer of activated B Cells (NF-κB) are good starting points to investigate the potential anticancer effects of this biologically active compound (10). Mitochondria are the major source of energy in all eukaryotic cells. Mitochondria produce ATP through the process of oxidative phosphorylation and the citric acid cycle. They also regulate calcium homeostasis and modulate apoptosis through release of several cell death-inducing molecules (11, 12). Detection of the mitochondrial permeability transition event provides an early indication of the initiation of cellular apoptosis (13). Hence, mitochondria have been associated with various biological processes involving cell signaling originated by the production of reactive oxygen species (ROS), cellular differentiation, cell death caused by loss of mitochondrial membrane potential (MMP), as well as the control of cell cycle and cell growth related to G_1 phase arrest (14, 15).

Induction of NF-κB is another major approach in studying cellular response because it is very sensitive to internal stimuli such as cytokines and free radicals, and external stimuli such as ultraviolet irradiation, bacterial or viral antigens (16). In the present study, the effects of dichamanetin on the anti-proliferative potential using several human cancer cells *in vitro* through the loss of MMP, the production of ROS, the regulation of NF-κB and inhibitor of nuclear factor kappa-B kinase subunit alpha (IκK-α), the change of cell distribution in cell cycle and cell cytotoxicity were investigated.

Materials and Methods

Extraction and isolation of dichamanetin. Dichamanetin was isolated and purified from *P. sarmentosum* according to the procedure previously reported (9). Briefly, the dried aerial plant parts were extracted with methanol and partitioned with various organic solvents followed by bioguided fractionation. The bioactive chloroform soluble fraction was subsequently subjected to chromatography over silica gel using a dichloromethane/acetone gradient to yield pure dichamanetin (Figure 1). This compound was identified by its spectroscopic data and in comparison with literature values (9). All reagents used were of analytical and biological grade.

Cell culture. Cancer cells (HT-29 colon, DU-145 prostate, and MDA-MB-231 breast) were purchased from the American type culture collection (ATCC, Manassas, VA, USA and cultured in RPMI-1640 and Dulbecco's modified Eagele's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 10% antibiotic-antimycotic (Gibco, Rockville, MD, USA) at 37˚C with humidified air and 5% CO_2. Cells of three to 10 passages in actively growing conditions were used for the experiments conducted.

JC-1 MMP assay. HT-29 colon cancer cells were cultured in 10 cm dishes at a density of 5×10^5 cells/ml at 37˚C in a 5% CO_2 incubator. After 24 h, cells were treated with or without dichamanetin and incubated for another 24 h. Then, 100 μl of JC-1 staining solution (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl- benzimidazolylcarbocyanine) per milliliter of culture medium were then added to each dish and allowed to incubate for 30 min. Stained cells were harvested and immediately analyzed using a Becton-Dickinson FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA). Mitochondria containing red JC-1 aggregates in healthy cells were detected in a FL2 channel using an excitation of 530 nm and an emission at 580 nm. Green JC-1 monomers in apoptotic cells were detected in a FITC channel (FL1) using excitation and emission wavelength at 485 and 530 nm, respectively.

XTT assay. The XTT assay is a colorimetric assay based on the cleavage of the yellow tetrazolium salt (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; XTT) to an orange formazan dye by metabolically active cells. Exponentially growing cells were seeded in 96-well plates (HT-29: 12,000 cells; DU-145: 8,000 cells; MDA-MB-231: 12,000 cells), incubated at 37˚C with 5% CO_2, and 24 h later treated with different concentrations of dichamanetin (0.2 to 100 μM), or ellipticine, as a positive control, or 1% ethanol as solvent control. After 72 h of treatment, cell viability was measured using XTT (final concentration of 0.06 μM) and an electron-coupling reagent phenazine methosulfate (PMD). Cells were treated with test compounds in a dose-dependent manner for a 24 h period. Each sample was tested in triplicate and was repeated in two separate experiments.

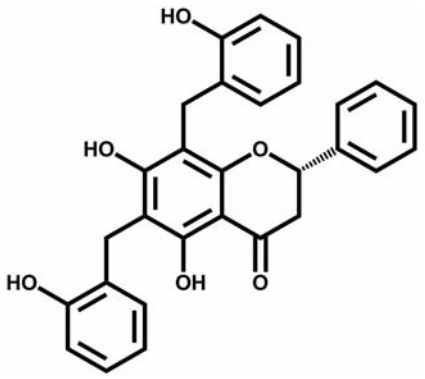

Figure 1. *Structure of dichamanetin*.

Fluorescence activated cell sorting (FACS) analysis. Cells (HT-29 and MDA-MB-231) were seeded into 10 cm^3 dishes and 24 h later treated with different concentrations of dichamanetin or the control solvent. After 24 h of incubation, cells were trypsinized, pelleted by centrifugation, washed with PBS and fixed in ice-cold 70% ethanol. DNA was stained with 10 μg/ml propidium iodide (Invitrogen) in a reaction solution containing 1 mM of ethylene diamine tetra-acetate and 100 μg/ml of RNase A (Sigma, St. Louis, MO, USA). Fluorescence emitted from the propidium iodide–DNA complex was quantified using a Becton-Dickinson FACS Calibur flow cytometer (BD Bioscience).

Poly ADP-ribose polymerase (PARP-1) assay. PARP-1 chemiluminescent assay kit was purchased from BPS Bioscience (San Diego, CA, USA) and the functional activity of human PARP-1was carried out according to the protocol supplied by the manufacturer. The assay for PARP activity employs biotinylated NAD^+ as a substrate for PARP to incorporate biotinyl-poly (ADP-ribose) into the PAR chain on an immobilized histone substrate. PARP-1 biotinylated substrate was incubated with dichamanetin at different concentrations and an assay buffer that containing the PARP-1 enzyme (1). Finally the plate was treated with streptavidin horseradish peroxidase (HRP) followed by addition of the HRP substrate to produce chemiluminescence that was then measured using the chemiluminescence reader FLUOstar Optima (BMG Labtechnologies GmbH Inc., Durham, NC, USA).

DNA binding assay. A DNA intercalation assay was set up using the method described by Cain *et al*. (18) and Saiple *et al*. (19). In brief, 10-fold dilutions of dichamanetin in DMSO were placed onto a 96-well black microtiter plate in 10 μl aliquots. Then, 190 μl of a mixture of ethidium bromide (EtBr) and a DNA substrate in reaction buffer was added to each well. EtBr fluorescence was monitored at excitation and emission wavelengths of 545/595 nm and 355/590 nm, respectively. The final concentrations of the reagents used in this assay were 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM

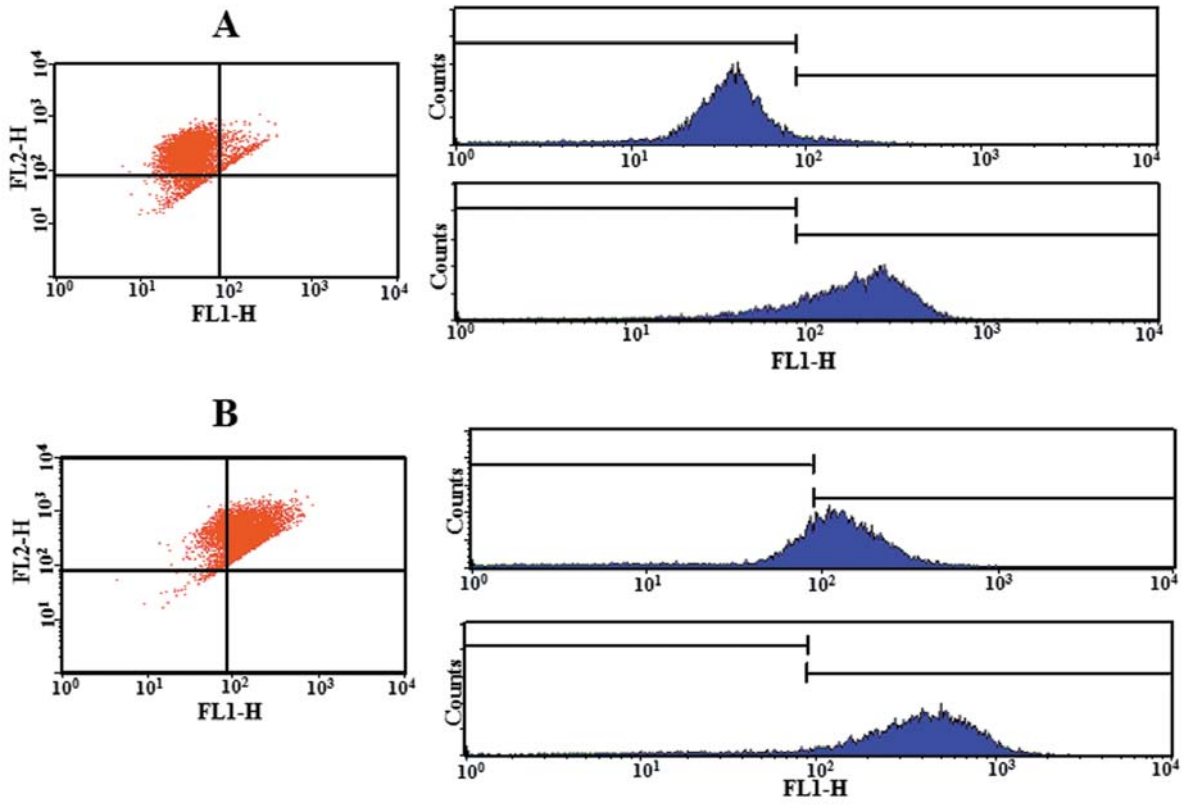

Figure 2. *Dichamanetin induces the loss of mitochondrial membrane potential in HT-29 cells. Cells were exposed to dichamanetin (100 μM) or vehicle for 24 h. The cells were then stained with JC-1 (0.38 μM, 30 min, 37˚C), washed with PBS and analyzed by flow cytometry. A: Untreated cultured cells. B: Cells exposed to dichamanetin.*

EDTA, 0.05% Brij-35, 100 pM-100 μM inhibitor, 1 μM EtBr, and 133 nM DNA substrate to maintain a ratio of 1:2 EtBr to DNA base pairs. The percentage fluorescence was calculated relative to a negative control (DMSO only). The assay is based on a ligand displacement-type approach, which makes use of the increased fluorescence of intercalated EtBr /DNA over that of free EtBr. Fluorescence decreases when EtBr is displaced by a competing intercalator. The results were obtained from triplicate samples in two separate experiments.

Measurement of ROS. Colon cancer HT-29 cells (10,000 cells/well) were plated in a 96-well plate and cultured for 24 h prior to treatment. Cells were then treated with vehicle (0.5% DMSO) and dichamanetin (0.2 to 100 μM). After 5 h of incubation, a solution of H_2O_2 was added to induce oxidative stress and cells were incubated for 30 min (20). A 5-μM aliquot of dichlorodihydrofluorescein diacetate (Sigma) in PBS was then added, and the plates were incubated at 37˚C for 15-30 min. Fluorescence was measured at an emission wavelength of 485 nm and excitation at wavelength 530 nm. All treatments were performed in triplicate and are representative of at least two different experiments.

NF-κB inhibition assay. An enzyme-linked immunosorbent (ELISA)-based NF-κB inhibitory chemiluminescent assay was performed as described previously (21, 22). Briefly, HeLa cells (ATCC) were treated with various concentrations of the test compounds, or the positive control, or solvent control, and their nuclei were extracted using the NE-PER kit (Pierce Biotechnology, Rockford, IL, USA). The specific binding ability of activated p65 subunits of NF-κB in the nucleus was detected. Rocaglamide (Enzo Life Sciences International, Ann Arbor, MI, USA) was used as a positive control [inhibitory concentration (IC_{50}) value of 0.07 μM]. The chemiluminescent activity was measured using a plate reader (FLUOstar Optima; BMG Labtechnologies GmbH, Inc.). Treatments were performed in triplicate and calculations included data for at least two different experiments.

Immunoblot analysis. HeLa cells (ATCC) were seeded in 10-cm^3 dishes and 24 h later treated with different concentrations of dichamanetin or the solvent control. Following incubation (8 h), whole-cell protein lysates were prepared using PhosphoSafe lysis buffer (Novagen, Madison, WI, USA) and the protein concentrations were measured using the BCA Protein Assay kit (Pierce Biotechnology). Cell lysates containing 30 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, probed with antibodies and detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). Anti-NF-κB (p65), anti-Ικκα, anti-β-actin, and anti-

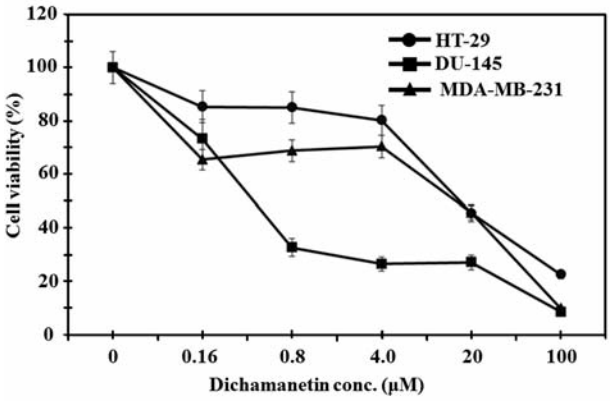

Figure 3. *Concentration-dependent cytotoxicity of dichamanetin against human cell lines HT-29 (17.7 μM), DU145 (60.0 μM) and MDA-MB-231 (8.7 μM). Ellipticine served as positive control [inhibitory concentration (IC_{50}) 13.8, 16.7 and 10.6 μM in HT-29, DU145 and MDA-MB-231, respectively].*

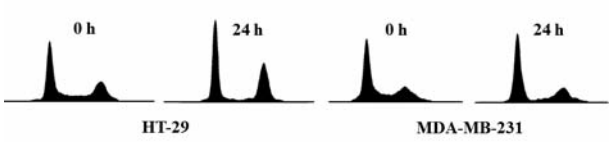

Figure 4. *Effect of dichamanetin on cell cycle progression. HT-29 colon cancer cells and MDA-MB-231 breast cancer cells were treated with dichamanetin (50 μM) for 24 h and analyzed using CELLQuest for propidium iodide-stained DNA content.*

Table I. *Inhibition of poly ADP ribose polymerase (PARP-1) enzyme after treatment with dichamanetin.*

Dichamanetin (μM)	0.17	0.85	4.27	21.35	107.73
PARP-1 (%)	12.8	18.2	32.0	96.2	99.0

rabbit antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis. IC_{50} values per each cell line were calculated using the curve-Fit Simple Equation, Table Curve 2Dv4 (System Software Inc., San Jose, CA, USA). Data were expressed as means±SEM of three determinations and the functions of fitting in experimental values for the IC_{50} values were chosen to be greater than 0.95 (R^2) and less than 10 (standard error of estimate), respectively.

Results

When colon cancer (HT-29) cells were treated with 100 μM dichamanetin for 24 h and compared to controls (untreated) after incubation, a change in MMP was detected. Flow cytometric analysis of MMP in HT-29 treated-cells was performed using JC-1 fluorescence (0.38 μM, 30 min). As shown in Figure 2A, JC-1 fluorescence was evident in the left side, which suggested that HT-29 cells in the normal state were located in the JC-1 inactive quadrant (FL1) and active quadrant (FL2). Untreated control cells exhibited a normal high membrane potential. In healthy cells, JC-1 forms J-aggregates and these complexes emit an intense red fluorescence at 560/595 nm (excitation/emission). Dichamanetin-treated cells, on the other hand, exhibited only green fluorescence at 485/535 nm (excitation/emission) because they were in an apoptotic state and JC-1 remained in the monomeric form. Dichamanetin clearly induced the loss of MMP in HT-29 cells. In order to confirm the cytotoxic effect of dichamanetin on different cancer cells, three different cancer cell types (colon, prostate and breast) were tested. Dichamanetin cytotoxic potential was four-times more potent in MDA-MB-231 breast cancer cells (Figure 3) than in DU-145 prostate cancer cells and two times more potent than in HT-29 colon cancer cells. The cytotoxic effect of dichamanetin on HT-29 was shown by only 28% over that of the control. The IC_{50} value for HT-29 colon cells was one third that of DU-145 prostate cancer cells. Hence, dichamanetin exhibited the most potent cytotoxic effect on the triple-negative MDA-MB-231 breast cancer cells. The effects of the loss of MMP observed with JC-1 correlate with the IC_{50} values obtained when using the XTT assay. The effect of dichamanetin on cell-cycle progression was determined by flow cytometry using propidium iodide-stained HT-29 colon cancer cells. As shown in Figure 4, treatment with dichamanetin (50 μM) for 24 h led to G_1 phase arrest cells (16% increase over that of the control). Treated cells were only slightly arrested in the G_2/M phase. The G_1 phase of treated MDA-MB-231 breast cancer cells was increased by 26.2% when compared with the non-treated cells. Dichamanetin significantly inhibited PARP-1 enzyme in a concentration-dependent manner when using HT-29 cells (Table I). PARP-1 was completely inhibited by dichamanetin in the concentration range of 20-100 μM. As a result of dichamanetin treatment, IKK-α was also inhibited by dichamanetin in a concentration-dependent manner. In order to obtain information on dichamanetin as a potential DNA-intercalating agent, an assay based on DNA binding affinity was performed following Cain's method of ligand displacement (18). In this test of competitive displacement, dichamanetin at 100 μM showed 50% displacement by EtBr at 545/595 nm and 355/590 nm. EtBr-alone was used as control. In Figure 6, NF-κB p65 is shown to be inhibited by dichamanetin in dose-dependent concentrations from 0.16 to 100 μM. Figure 5 shows the induced effects of dichamanetin on ROS expression, known to correlate with activation of the

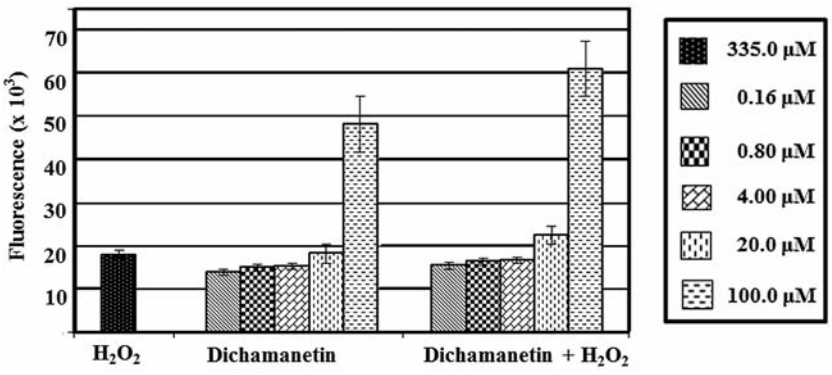

Figure 5. *Effect of dichamanetin on reactive oxygen species (ROS) induction, in the absence and presence of H_2O_2. H_2O_2 was used as control (335 µM).*

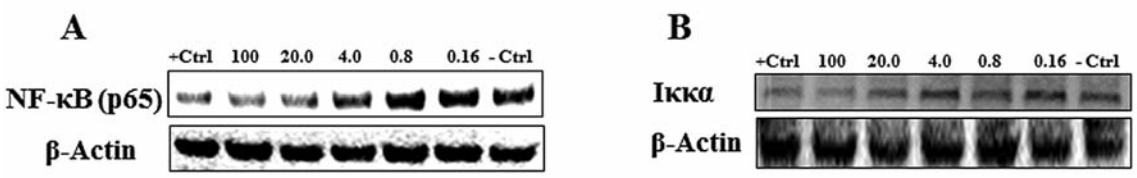

Figure 6. *Nuclear factor kappaB (NF-κB) inhibitory effects exhibited by dichamanetin. Inhibition of NF-κB (p65) (A) and Ικκα (B). Cells were treated with dichamanetin at 0.16 to 100 µM (0.08 to 50 µg/ml). β-Actin was used as a control for equal loading.*

mitochondrial pathway for apoptosis. ROS as an oxidative signal was dramatically induced by more than three-fold after treatment of dichamanetin (100 µM) in the presence of H_2O_2.

Discussion

Loss of MMP is an early event and a main indicator of process of programmed cell death. Dichamanetin triggered HT-29 cells, leading to a loss of MMP. Cells in an apoptotic state are scheduled to die. Our results suggest that dichamanetin could be a useful lead compound in the development of new anticancer agents from edible natural sources for the treatment of cancer, particularly of triple-negative breast cancer. These data advocate for an analysis of the effects of dichamanetin on the cell cycle of treated cancer cells. Moreover, the XTT assay has been used to monitor the amount of NADPH in living cells through its reduction to a yellow formazan dye. Therefore, it can also be used to assess the imbalance of DNA single-strand break repair by indirectly measuring PARP-1 activation through the depletion of intracellular NADPH. Triple-negative cells have been reported to be highly sensitive to PARP-1 inhibition and the cytotoxic data correlated with the fact that the triple-negative MDA-MB-231 breast cancer cells had lower IC_{50} values in the XTT assay. Thus, analysis of the levels of PARP inhibition would provide more insight into the potential of dichamanetin for the treatment of triple-negative breast cancer. In the cell cycle, each checkpoint control is very important in the process of maintaining cell homeostasis. Many diseases, including cancer, are related to cell-cycle dysregulation. Among checkpoints in the cell cycle, the G_1 checkpoint determines if cells divide or remain in a state of G_0 phase because the G_1 phase is involved in metabolic processes that participate in cell division (23). For this reason, cell arrest at the G_1 checkpoint is very useful in the inhibition of continuous cancer cell proliferation. These data demonstrate that dichamanetin induces G_1 phase arrest in HT-29 and MDA-MB-231 cells. G_1 arrest is well-known to be an outcome of DNA damage, which is related to the induction of p53. This is a tumor suppressor protein that regulates the cell cycle and is crucial in cancer development. Therefore, G_1 phase arrest by dichamanetin is of importance for its possible development as a new potential anticancer agent for the treatment of triple-negative breast and colon cancer. Having confirmed the effect of dichamanetin on the G_1 phase of the cell cycle in breast and colon cells, it may be suggested that the loss of MMP and the cytotoxicity observed in the XTT assay might be correlated to DNA damage, as well as with the cell oxidative process. Thus, both PARP-1 and ROS evaluations were performed on this compound. PARP-1 is an abundant and ubiquitous nuclear enzyme that catalyzes the NAD (+)-dependent addition of

ADP-ribose polymers on a variety of nuclear proteins. Members of the PARP family of proteins are involved in DNA repair and apoptosis regulation. PARP-1 is especially important for repairing single-strand breaks and plays a critical role in cancer (24). Due to its significance in cancer cell proliferation, inhibitors of PARP-1 are well investigated (18, 25). In this study, the results of dichamanetin treatment suggest the importance of further studying its effects on PARP-1. Due to the nature of the PARP assay used to test dichamanetin, DNA-intercalating agents can give positive results. There are various DNA mono-intercalating agents with potent, broad-spectrum experimental antitumor activity (26). The planar-cationic structure of dichamanetin leads to the suggestion that it might act as a DNA intercalator due to an ability to slot between base pairs and DNA. If dichamanetin has a DNA-intercalating characteristic, it could have potential use in chemotherapy. Examples of anticancer drugs with DNA-intercalating properties include doxorubicin (anthracycline antibiotic) and actinomycin D (polypeptide antibiotics). Thus, we investigated the dichamanetin DNA-intercalating site of action with a displacement assay using EtBr. The discovery of a new type of compound able to intercalate DNA is very significant in the search for new anticancer drugs and the effect of dichamanetin on PARP-1 activity seems to be, in part, directly correlated to its DNA-intercalating properties.

Oxidative stress plays a well-known role in cellular change and oxygen-derived species, such as superoxide radicals, hydrogen peroxide, singlet oxygen and hydroxyl radicals, which are well-known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases, including cancer. Effects of ROS on cancer cells are well-documented and include not only roles in cancer cell apoptosis and cell damage but also in the induction of host defense genes (27-29). Evaluation of ROS levels using human cells is, therefore, of great importance. The results from the XTT assay suggested that NADPH intracellular levels are affected by dichamanetin. In turn, NADPH oxidase is directly correlated with intracellular ROS levels. Thus, the data support further analysis of the effects of dichamanetin on ROS levels and on the oxidative pathway of the cells. Hence, it seems that dichamanetin induced ROS, causing a loss of MMP and in turn inducing cell apoptosis.

There are five different types of NF-κB proteins including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. These transcription factors are associated with a number of diseases such as cancer, chronic inflammation, asthma, neurodegenerative diseases, and heart diseases (30). NF-κB is considered to be an instigating factor unleashing inflammatory responses in chronic disease conditions such as cancer. The NF-κB pathway is generally thought to be a primary oxidative stress-response pathway, as nearly all pathways leading to NF-κB activation are blocked by a variety of antioxidants. Since ROS were induced by dichamanetin in the ROS assay, it was considered of value to look into the effects of dichamanetin on the NF-κB pathway. Additionally, activation of NF-κB has been associated with stimuli from different sources, such as cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens. Moreover, inhibition of p65 is used as an indicator to check the cellular response in cell proliferation because RelA (p65) contains potent transactivation domains. The inhibiton of IκK-α induction in turn inhibits the function of IκB kinase complex, which plays a major role in regulating NF-κB. Degradation or inhibition of IκB frees NF-κB to migrate to the nucleus where it can act as a transcription factor. The degradation of IκB proteins signals activation of the NF-κB on cells. Therefore, inhibition of IκB is a useful tool to inhibit NF-κB activation *via* IκB. In this experiment, it was confirmed that IκK-α expression was reduced remarkably in the presence of dichamanetin at 20 and 100 μM. The inhibition of NF-κB and IκK-α by dichamanetin suggests it may be of use as a potential inhibitor of cell growth.

The overall effect of cancer cell inhibition by dichamanetin was predominantly related to oxidative pathway components. Accordingly, this investigation has provided information regarding the effects of dichamanetin on ROS signaling components. The cytotoxic effects of dichamanetin were also reflected by IC_{50} values (8.68 and 17.09 μM) for triple-negative breast and colon cancer cells, respectively. Ellipticine (used as positive control) and dichamanetin had similar IC_{50} values; ellipticine functions as a small molecule DNA intercalator with anti-neoplastic effects. The major characteristics of dichamanetin also include cytotoxicity, significant DNA intercalation, ROS induction, and MMP. It also exerts inhibitory effects an IκK-α, NF-κB (p65) and PARP-1 in a concentration-dependent manner. Dichamanetin significantly reduced the viability of various types of cancer cells in a dose- and time-dependent manner. When it comes to the process of cell-cycle arrest, lead compounds should have the potential to arrest cells at specific phases such as G_1 or G_2/M phase in the cell cycle. Dichamanetin was shown to arrest cells at the G_1 and G_2/M phases in the present study. Furthermore, dichamanetin induced loss of MMP as an evidence of cellular apoptosis.

Thus, the results of this study suggest that dichamanetin could be developed to block cancer cell proliferation, particularly for triple-negative breast cancer and colon cancer.

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