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Kolaviron Modulate Aberrant Crypt Multiplicity and Xenobiotic Metabolizing Enzymes During 1,2-Dimethylhydrazine-Induced Colon Carcinogenesis in Rats

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Abstract: Cancer of the colon is one of the most common cancers in developed countries and its chemoprevention is of great interest throughout the world. We have investigated the efficacy of kolaviron on xenobiotic-metabolizing enzymes against 1,2 dimethyl hydrazine (DMH)-induced colon carcinogenesis. Male albino Wistar rats were randomly divided into four groups. Group 1 served as control, animals have access to rodent feeds for 8 weeks plus 1mM EDTA-saline injection subcutaneous (s.c) once a week for 4 weeks. Group 2 rats served as kolaviron (KV) control received 100 mg/kg body weight of kolaviron per oral (p.o.) every day. Group 3 served as carcinogen control, received pellet diet and 30 mg/kg bodyweight of 1,2-dimethylhydrazine (DMH) dissolved in 1mM EDTA-saline subcutaneous injection once a week for 4 weeks to induce colon carcinogenesis. Group 4 rats received 30 mg/kg body weight DMH dissolved in 1mM EDTA-saline subcutaneous injection and kolaviron 100 mg/kg body weight. DMH exposure showed elevated activities of phase I enzymes and reduced activities of phase II enzymes in the liver and colon of colon carcinogenesis bearing rats. Kolaviron supplementation significantly reversed these effects. These findings suggest that kolaviron can significantly modulate the xenobiotic-metabolizing enzymes during DMH induced colon carcinogenesis in rats.
Keywords: colon cancer, kolaviron, DMH, xenobiotic enzymes, carcinogenesis.

INTRODUCTION

Colon cancer is a serious health problem worldwide. Highest intake of alcohol, smoking, high fat diets and lack of physical activity are considered as an etiological factor for the development of colon cancer. It has three distinct stages initiation, promotion and progression stages [1].

The metabolism of 1,2-dimethylhydrazine (DMH) in the liver predominates over organ-specific metabolism resulting in the transport of metabolites to the colon giving rise to DNA alkylation in the colonocytes. It is believed that alkylation of specific sites in DNA leads to events which may result in tumour initiation [2]. The metabolic activation of DMH follows (i) hydroxylation of DMH to methylazoxymethanol, which occurs in the liver and in the colonic mucosa via a cytochrome P450-dependent pathway and (ii) oxidation of methylazoxymethanol to methylazoxyformaldehyde, which is catalyzed by the microsomal/cytosolic enzymes of the liver and colon. The unstable compound, methylazomethanol, readily yields methyldiazonium ion, which can have effect on the enzymatic and non-enzymatic processes in the liver and colon [3]. Since liver is the major organ responsible for DMH metabolism, assay of the hepatic and colonic phase I and II xenobiotic metabolizing enzymes, could provide additional information regarding the efficacy of kolaviron on DMH-induced colon carcinogenesis. Carcinogens are activated by phase I enzymes. This can be prevented by inhibition of these enzymes phase I or by induction of phase II enzymes, leading to detoxification and accelerated excretion of carcinogens [4].

Bitter kola (Garcinia kola) belongs to the family of plants called Guttiferae and the genus Garcinia. Kolaviron (KV) is an extract from the seeds of Garcinia kola, containing a complex mixture of bioflavonoids and polyphenols [5].

Many studies have confirmed the antioxidative, anti-lipid peroxidation, chemoprevention properties of kolaviron and anti-inflammatory effects of kolaviron in chemically-induced toxicity in animal models of diseases and in cell culture [6-9].

In the present study, the effect of kolaviron on colon carcinogenesis initiated with DMH was investigated in male Wistar rats by determining xenobiotic-metabolizing enzymes.

MATERIALS AND METHODS

Chemicals

DMH was purchased from Sigma Chemical Co. (USA). All other chemicals and reagents used were of analytical grade.

Extraction of Kolaviron

Garcinia kola seeds purchased from a local market in Yenagoa, Nigeria, were certified at the Department of Botany, Niger Delta University, Nigeria. Seeds were sliced, pulverized with an electric blender and dried at 40ºC in a drying oven. Powdered seeds were extracted with light petroleum ether (boiling point 40–60ºC) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethyl acetate (6 x 300 mL). The concentrated ethyl acetate yielded kolaviron [10] as a golden yellow solid.

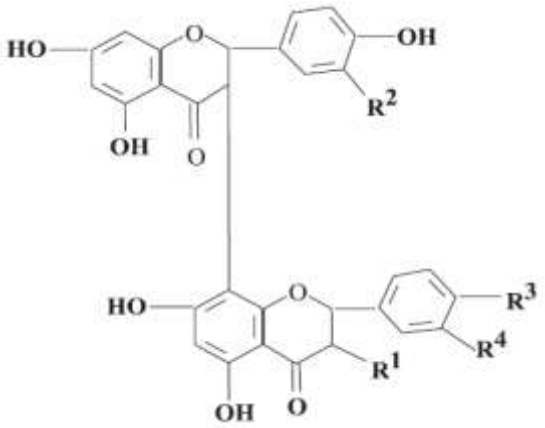

Fig-1: Structure of kolaviron

	R_1	R_2	R_3	R_4
Garcinia Biflavonoid 1	OH	H	OH	H
Garcinia Biflavonoid 2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Animals

Three to four-weeks-old, male albino rats (100–130 g) of Wistar strain were obtained from Central Animal House of Niger Delta University, Bayelsa State, Nigeria. Animals were quarantined for 1 week and allocated randomly to experimental and control groups. Animals were maintained as per the principles and guidelines [25]. The animals were housed thirteen per cage in a specific pathogen-free animal room under controlled conditions of a 12 h light/12 h dark cycle, with temperature of 22±1 °C and relative humidity of 50±10% till the end of 8 weeks of the experimental procedure.

Induction of Colon Carcinogenesis

DMH was dissolved in 1 mM EDTA-saline just prior to use and the pH adjusted to 6.5 with 1 mM NaOH to ensure the stability of the carcinogen. The rats were given subcutaneous injections of DMH for 4 consecutive weeks at a dose of 30 mg/kg body weight [11].

Experimental Design

Group 1 animals received 1mM EDTA-saline injection subcutaneous (s.c) once a week for 4 weeks. Group 2 rats served as kolaviron (KV) control received 100 mg/kg body weight of kolaviron per oral (p.o.) every day. Group 3 served as carcinogen control, received 30 mg/kg body weight of 1,2-dimethylhydrazine (DMH) subcutaneous injection once a week for 4 weeks to induce colon carcinogenesis. Group 4 rats received DMH injection and kolaviron 100 mg/kg body weight. At the end of 56 days (8 weeks) rats were sacrifice by cervical dislocation after an overnight fasting.

Determination of Aberrant Crypt Foci

The detached colons of eight rats were washed thoroughly with 0.9% NaCl, opened longitudinally from caecum to anus and fixed flat between two pieces of filter paper. Microscopic slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. After 24 h in buffered formalin, the colon was stained with 0.2%

methylene blue as described by Bird and Good [25]. It was then placed mucosal side up, on a microscopic slide and observed under a light microscope. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from laminae to basal surface of cells, and the easily discernible per cryptal zone. Crypt multiplicity was determined as the number of crypts in each focus, and was categorized as containing 1, 2, 3, 4 or more aberrant crypts/focus. For topographical assessment of the colon mucosal ACF was counted using a light microscope.

Preparation of liver and colon cytosolic and microsomal fractions

The excised liver and colon tissues were homogenized in 0.1 M phosphate buffer pH 7.4, centrifuged at 9000×*g* for 20 min and the supernatant was collected, to this 0.2 vol. of 0.1 M $CaCl_2$ in 0.25 M sucrose were added and the samples were kept on ice centrifuged at 27,000×*g* for 20 min, and the clear cytosolic fractions were used for phase II enzymes. Microsomal pellets were washed twice by suspending in 7ml of 10 mM Tris–HCl (pH 7.4); 0.25M sucrose and centrifuged at 9000×*g* for 20 min, to obtain microsomal fractions, for phase I enzymes.

Determination of protein

Microsomal and cytosolic proteins were determined using the method of Lowry *et al*. [12].

Assay of cytochrome P4502E1 activity

Both the standard, liver/colon and 0.2M Potassium phosphate buffer, pH 6.8 are incubated for 3min at 37ºC in a shaking water bath according to the method of [22], but 10mM NADPH is only added to the samples to initiate the transformation of 1mM p-nitro phenol to 4-nitrocatechol, but not the standard. After 10 min incubation at 37ºC, the reaction is terminated by the addition of 0.5ml ice-cold perchloric acid 0.6N. This lowers the pH, the colour disappears and the proteins in the microsomes denature. The protein is precipitated by centrifugation at 3000rpm for 10min. 1ml of the supernatant is then added to 0.1ml of 10M NaOH in a cuvette. The absorbance is read immediately at 536nm since the colour fades with time. Concentration of 4-nitrocatechol formed is read from the standard curve. Cytochrome P4502E1 is expressed as millimoles p-nitro catechol liberated per minute per milligram protein.

Assay of microsomal NADH-cytochrome P450 reductase

NADH-cytochrome P450 reductase activity was assayed by the method of Mihara and Sato [23]. The reaction mixture contained 0.1M potassium phosphate buffer (pH 7.5), 0.1mM NADH, 1mM potassium ferricyanide and microsomal preparation in a final volume of 1 ml. The reaction was started at 25 ^{0}C by the addition of NADH, and the rate of reduction of potassium ferricyanide by NADH was measured at 420 nm. The enzyme activity was calculated using the extinction coefficient of 1.02mM^{-1} cm^{-1}. One unit of enzyme activity is defined as that causing the reduction of one micro mole of ferricyanide per minute or the oxidation of NADH.

Assay of microsomal NADPH-cytochrome b_5 reductase

NADPH-cytochrome b5 reductase was assayed by the method reported by Omura and Takesue [24] by measuring the rate of oxidation of NADPH at 340 nm. The reaction mixture contained 0.3M potassium phosphate buffer (pH 7.5), 0.1mM NADPH, 0.2mM potassium ferricyanide and the microsomal preparation in a final volume of 1ml. The reaction was started at 25 ^{0}C with the addition of NADPH. The enzyme activity was calculated using the extinction coefficient of 6.33mM^{-1}cm^{-1}. One unit of enzyme activity is defined as that causing the oxidation of one mole of NADPH per minute.

Assay of DT-diaphorase (DTD)

DTD activity in cytosoles was measured spectrophotometrically with NADPH as the electron donor and 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor at 620 nm [13]. The reaction mixture consisted of 25 mM Tris. HCl buffer (pH 7.4), 4 mM DCPIP, 6 mM NADPH and an appropriate concentration of cytosolic enzyme in a final volume of 1.0 ml. The reaction was started at 25 ^{0}C by addition of NADPH. The enzyme activity was calculated using the extinction coefficient of 21 mM^{-1}cm^{-1} for difference in absorbance at 620 nm. One unit of enzyme activity is defined as the amount of enzyme causing the reduction of 1.0 µmole of DCPIP per minute under the conditions described above.

Assay of cytosolic GST

Glutathione S-transferase was assayed by the method of Habig *et al*. [14]. The reaction mixture contained 100mM phosphate buffer (pH 6.5), 30mM 1-choloro 2, 4-dinitrobenzoic acid (CDNB) and 30mM reduced glutathione. The reaction was started by the addition of cytosolic sample and the absorbance measured at 340 nm. The specific activity of glutathione S-transferase is expressed as mmoles of GSH-CDNB conjugate formed/min/mg protein using the extinction coefficient 9.6mM^{-1} cm^{-1}.

RESULTS

Modulation of kolaviron on liver and colon phase I and phase II enzymes of the control and experimental rats.

	Control	KV	DMH	DMH + KV
P4502E1liver	7.35 ± 0.11[a]	6.71± 0.51[a]	12.49±0.19[b]	8.11±0.13[c]
P4502E1colon	2.19 ± 0.09[a]	1.90 ± 0.33[a]	4.72±0.71[b]	2.61±0.67[c]
P450 R liver	22.13±0.87[a]	19.81±0.40[a]	45.91±0.29[b]	24.11±0.72[c]
P450 R colon	6.36±0.22[a]	7.21±0.18[a]	17.46±0.37[b]	8.19±0.18[c]
P450b_5R liver	13.56±0.76[a]	12.97±0.57[a]	28.11±0.91[b]	12.59±0.27[c]
P450b_5R colon	4.76±0.89[a]	5.09±0.48[a]	10.82±0.12[b]	4.98±0.35[c]
DTD liver	5.06±0.61[a]	5.24±0.73[a]	2.08±0.38[b]	4.92±0.30[c]
DTD colon	3.34±0.20[a]	3.41±0.50[a]	1.11±0.52[b]	3.24±0.75[c]
GST liver	6.48±0.10[a]	7.01±0.37[a]	2.49±0.12[b]	6.79±0.67[c]
GST colon	3.68±0.49[a]	3.98±0.29[a]	1.48±0.67[b]	4.01±0.25[c]

Data are mean±SD of five rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $p < 0.05$

GST in millimoles of CDNB–GSH conjugate formed per minute per milligram protein

DTD is expressed in micromoles of 2,6-DCPIP reduced per minute per milligram protein

NADH Cytochrome P450 Reductase µmoles of NADH oxidised/mg protein/min

Cytochrome P450 b5 Reductase µmoles of NADPH oxidised/mg protein/min

CytochromeP4502E1 millimoles p-nitrocatechol /mg protein/min

Modulation of kolaviron on DMH induce Aberrant Crypt Foci in control and experimental rats

Treatments	Number of Aberrant crypt in a foci containing				
	1	2	3	4	Total aberrant crypt
DMH	14±2[b]	10±3[b]	8±2[b]	7±1[b]	39±3[b]
DMH + KV	8±2[c]	6±3[c]	4±2[c]	1±2[c]	19±2[c]

Data are means±SD of 8 rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $p < 0.05$

DISCUSSION

We chose to study the active bioflavonoid of Garcinia kola in a colon cancer model due to previous observations seen in which certain populations had decreased incidence of colon cancers, and these same populations have been identified with lower risk of colon cancer. It was postulated that a dietary fiber and phytochemicals have effect accounted for lowered colon cancer. Hence, in the present study, we investigated the chemo preventive effect of kolaviron, a naturally occurring bioflavonoid, on DMH induced colon carcinogenesis in Westar rats.

The earliest microscopic recognizable biomarkers of colorectal carcinogenesis are the ACF. These are considered to be the useful biomarkers to assess the chemo preventive potential of natural products against colon carcinogenesis [15]. In this study, the inhibitory effects of kolaviron on the occurrence of ACF were observed during colorectal carcinogenesis.

Significant reduction in the occurrence of ACF in DMH treated rats supplemented with kolaviron denotes that it has remarkable potential in inhibiting the occurrence of pre-neoplastic changes. This result is in line with the work of Venkatachalam *et al*. [16] who also show the inhibitory properties of rosmarinic acid against ACF formation.

Xenobiotic detoxification is controlled by the liver as the main organ and to a lesser extent other organs like the colon. The detoxification reaction, in microsomes involves cytochrome (cyt) P450 system, electron flows from NADPH or NADH through a flavoprotein cyt P450 Reductase or cyt b5 Reductase to different cyt P450 and cyt b5. Cyt P4502E1 has an important role in oxidative activity, controlling the excretion of most xenobiotic compounds. [17]. The sequential biotransformation of the procarcinogen DMH to azomethane, azoxymethane and MAM are catalysed by cytP4502E1 enzyme system. In the present study, the DMH alone-treated rats showed increased activities of the phase I enzymes, NADH cyt P450 Reductase and cyt P450 b5 reductase and cytP4502E1 due to the metabolism of DMH in the liver microsomes and colonic epithelial cells to yield reactive genotoxic intermediates. These intermediates covalently bind to DNA to form DNA adducts and initiate the carcinogenic process [18]. Supplementation with kolaviron to DMH-treated rats suppressed the activities of the phase I enzymes in the liver and colonic mucosa. In this context, biflavonoids are known to play a crucial role in modulating the carcinogen-metabolizing system such as inhibiting the NADH cyt P450 Reductase and cyt P450 b5 reductase and cytP4502E1 [26].

Generally, DT Diaphorase detection in liver and colon was induced by kolaviron is a means for determining the potency of many anticarcinogenic substances. DTD protects liver and colon from the toxicity of quinines and their metabolic precursors (benzene, aromatic hydrocarbon, hydroquinone, etc.) [16]. The metabolism of quinines to hydroquinone, annihilating semiquine radicals and subsequent oxygen-radical production, is assisted by DTD. In the present study results shows that DMH treated rats in liver and colon DTD activity decreased significantly as compared with the control values, but chemoprevention with kolaviron alone and in the DMH challenged rats shows increased activity of DTD depicting the modulatory role of kolaviron. These results are similar with the works of Venkatachalam *et al*. [16] and Ozen and Korkmaz [19].

GST, which plays an important role in conjugating the metabolites resulting from the action of cytochrome P450, with endogenous ligands (reduced glutathione) favoring their elimination from the body of organisms, is a critical detoxification enzyme [20]. It has already been reported that glutathione S-transferase induces protection against various cytotoxic, mutagenic and carcinogenic chemicals [21]. In the present study results of liver and colon GST activity decreased significantly in DMH treated rats as compared with the control values, but chemoprevention with kolaviron alone and in the DMH challenged rats shows increased activity of GST depicting the modulatory role of kolaviron. These results are in tune with the works of Eboh *et al*. [9] and Ozen and Korkmaz [19] that also show the chemopreventive properties of kolaviron and Urtica dioica.

In conclusion kolaviron in the present study shows modulatory activities of xenobiotic metabolizing enzymes during DMH induction of colon carcinogenesis; this could be part of the explanation why kolaviron is claimed in many literatures to possess chemopreventive properties.

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