Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-κB and AP-1

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Aims: Kolaviron, a bioflavonoid isolated from the seeds of Garcinia kola has been reported to possess anti-inflammatory, antioxidant, antigenotoxic and hepatoprotective activities in model systems via multiple biochemical mechanisms. The present study investigated the possible molecular mechanisms underlying the hepatoprotective effects of kolaviron.

Main methods: Biomarkers of hepatic oxidative injury, histological and immunohistochemical techniques were used. In addition, the protein expression levels of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) were evaluated by western blotting while DNA-binding activities of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) were determined by electrophoretic mobility shift assay.

Key findings: Kolaviron administered orally at doses of 100 and 200 mg/kg for 7 days significantly lowered the activities of serum transaminases and γ-glutamyl tranferase induced by single intraperitoneal administration of dimethyl nitrosamine (DMN) (20 mg/kg) and preserved the integrity of the hepatocytes. Also, kolaviron at both doses reduced the DMN induced elevated hepatic levels of malondialdehyde and reversed DMN mediated decrease in hepatic glutathione. The hepatoprotective effect of kolaviron was compared to that of curcumin, an established hepatoprotective agent. Kolaviron inhibited the DMN induced expression of COX-2 and iNOS. Immunohistochemical staining of rat liver verified the inhibitory effect of kolaviron on DMN-induced hepatic COX-2 expression. Furthermore, kolaviron abrogated DMN induced binding activity of NF-κB as well as AP-1.

Significance: The ability of kolaviron to inhibit COX-2 and iNOS expression through down regulation of NF-κB and AP-1 DNA binding activities could be a mechanism for the hepatoprotective properties of kolaviron.

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Introduction

Dimethyl nitrosamine (DMN) is a representative chemical of a family of N-nitroso compounds and has been found in processed meats and industrial products. It is a potent hepatotoxin, carcinogen and mutagen (George et al., 2001). DMN exerts carcinogenic effects and induces hepatic necrosis through metabolic activation by CYP2E1 (Guengerich et al., 1991) in experimental animals. The formation of reactive oxygen species (ROS) like H₂O₂, superoxide anion (O₂⁻) and hydroxyl radicals (OH·) has been demonstrated during the metabolism of nitrosamines resulting in oxidative stress, which may be one of the key factors in the induction of pathological conditions such as hepatocellular necrosis, carcinogenicity, neoplastic changes, and tumor formation (Nakae et al., 1997; Pradeep et al., 2007; Wills et al., 2006). Teufelhoefer et al. (2005) also demonstrated that metabolism of the nitrosamine by CYP2E1 in mouse liver stimulated Kupffer cells leading to generation of superoxide and other ROS capable of damaging liver cells.

Cyclooxygenase-2 is an enzyme involved in inflammatory processes and a rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid. Inappropriate up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues (Mohan and Epstein, 2003). COX-2 has been further implicated in tumorigenesis by increased susceptibility of COX-2 over-expressing transgenic mice (Muller-Decker et al., 2002) and relative resistance of COX-2 knockout animals to spontaneous or experimentally-induced carcinogenesis (Tiano et al., 2002).

Like other early-response gene products, COX-2 can be induced rapidly and transiently by proinflammatory mediators, endotoxins as well as carcinogens (Kim et al., 2005). Studies have demonstrated carcinogen-induced expression of COX-2 by activating nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (Chun et al., 2003, 2004; Kim et al., 2005). Therefore targeted inhibition of COX-2 and modulation of abnormal up-regulation of NF-κB and AP-1 have now been recognized as the molecular basis of chemoprevention by structurally diverse dietary phytochemicals. A number of dietary phytochemicals have been shown to inhibit COX-2...
and transcription factors (NF-κB and AP-1) controlling this gene in both in vivo and in vitro models of carcinogenesis (Chun et al., 2004; Surh, 2003). Inducible nitric oxide synthase (iNOS) is another inducible enzyme that causes the overproduction of nitric oxide during inflammation and tumor development (Chun et al., 2007). Nitric oxide has been implicated in initiation, promotion stage of neoplastic transformation (Mordan et al., 1993) and in tumor progression by regulating angiogenesis (Chin et al., 1997). Therefore, suppression of the induction and activity of COX-2 and/or iNOS has been considered a new paradigm in cancer chemoprevention in several organs (Chung et al., 2007).

Garcinia kola Heckel (Guttiferae) is a largely cultivated tree and highly valued in west and central Africa for its edible nuts. The seed, commonly known, as ‘bitter kola’ is eaten by many and it is culturally acceptable in Nigeria. Extractives of the plant have been employed in the African herbal medicine for the treatment of ailments such as laryngitis, liver diseases, cough and hoarseness of voice (Iwu, 1982; Farombi, 2003).

Kolaviron is a fraction of the defatted ethanol extract, containing Garcinia biflavonoids GB1, GB2 and kolaflavanone. Several studies have demonstrated the protective effects of kolaviron against hepatotoxicity and oxidative stress induced by 2-acetylaminoflourene (Farombi et al., 2000b), carbontetrachloride (Farombi, 2000), aflatoxin B1 (Farombi et al., 2005), potassium bromate-induced nephrotoxicity (Farombi et al., 2002) and dibutylphthalate-induced testicular oxidative stress (Farombi et al., 2007) in animal model. Furthermore, kolaviron has been shown to exhibit potent antioxidant and metal chelating activities in vitro (Farombi and Nwaokefor, 2005) as well as inhibition of carcinogen-induced genotoxicity in human liver-derived HepG2 cells (Nwankwo et al., 2000). However the molecular mechanisms underlying the hepatoprotective and other chemopreventive/chemoprotective effects of kolaviron remain largely unknown. In the present study, we report that kolaviron inhibits DMN-induced liver injury in rats by suppressing COX-2 and iNOS expression via NF-κB and AP-1.

Materials and methods

DMN was purchased from Sigma Chemical Co. (St Louis, MO, USA). Rabbit polyclonal COX-2 antibodies were products of Cayman Chemical Co. (Ann Arbor, MI, USA). Anti-rabbit, anti-goat and antimouse horseradish peroxidase-conjugated secondary antibodies were products of Zymed Laboratories (San Francisco, CA, USA). ARE oligonucleotide was obtained from Bionsis (Seoul, Korea). Enhanced chemiluminescence (ECL) detection kit and [γ-32P] ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents used were in the purest form available commercially.

Extraction of kolaviron

Kolaviron was isolated according to published procedure (Iwu, 1985). Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60 °C) 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 ethylacetate (6×300 ml). The concentrated ethylacetate yielded a golden yellow solid termed kolaviron which has been shown to consist of Garcinia biflavonoid GB-1 (3′,4′,4″,5,5″,7,7″-heptahydroxy-3,8″-biflavonone), GB-2 (3′,4″,4‴,5,5″,7,7″-octa-hydroxy-3,8″-biflavonone), and kolaflavanone (3′,4″,4‴,5,5″,7,7″-octahydroxy-4‴-methoxy-3,8″-biflavonone). Kolaviron was identified by direct comparison of the 1H nuclear magnetic resonance (NMR), 13C NMR and electron ionization (EI)-mass spectral results with previously published data (Iwu, 1985).

Animal treatment

Male albino rats Wistar strain were purchased from Charles River (Tokyo, Japan). They were housed in a temperature-controlled (25 °C) room with alternating 12-h light/12-h dark cycles. All animals were acclimatized for 7 days before experiment, fed standard pellet chow and given fresh water ad libitum. The experimental protocols were approved by the Animal Care and Use Committee of the Seoul National University. Thirty (30) male rats were divided into five groups. Control rats received the vehicle only. Rats in group 3 and 4 were administered 100 and 200 mg/kg kolaviron respectively for 7 consecutive days (Farombi et al., 2005, 2004). Rats in groups 2, 3, 4 and 5 received a single intraperitoneal dose of DMN (20 mg/kg) 48 h before sacrifice. The protective effect of kolaviron on DMN-induced hepatotoxicity was compared to that of curcumin (200 mg/kg) which has been confirmed to have hepatoprotective effects (Park et al., 2000). At the end of each experiment, rats were sacrificed by cervical dislocation. Blood was collected by heart puncture for serum isolation. Liver was excised, rinsed in physiological saline and stored at ~80 °C until use for western blot analysis and nuclear extract preparation.

Measurement of serum enzyme activities

Serum was prepared from the whole blood by centrifugation at 3000 g for 10 min at 4 °C. Serum aspartate amino transferase (AST), alanine amino transferase (ALT) and γ-glutamyl transferase (γ-GT) activities were determined using commercially available kits.

Determination of lipid peroxidation

Lipid peroxidation was assessed in terms of malondialdehyde (MDA) formation in the rat liver 10,000 g supernatant fraction. The measurement of thiobarbituric acid reacting substances (TBARS) was
performed as described previously (Aeschbach et al., 1994). MDA was quantitated by using $\Sigma = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Buege and Aust, 1978).

**Determination of reduced glutathione (GSH)**

GSH levels were measured using the commercially available colorimetric assay kit BIOXYTECH GSH-400 (OXIS Research, Portland, OR, USA). Briefly 50 μl of R1 solution (solution of chromogenic reagent in HCl) was added to the 700 μl of the 10,000 g liver supernatant fraction followed by gentle vortex-mixing. Following the addition of 50 μl of R2 solution (30% NaOH), the mixtures were incubated at 25±3 °C for 10 min. After centrifugation, the absorbance of the clear supernatant was read at 400 nm.

**Western blot analysis**

Livers were homogenized in ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM DTT, 1 mM Na$_2$VO$_4$ and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14,800 g for 30 min, and aliquots of supernatant containing 30 μg protein were boiled in sodium dodecylsulfate (SDS) sample buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane, and the blots were blocked with 5% fat-free dry milk-PBST buffer (phosphate-buffered saline containing 0.1% Tween 20) for 1 h at room temperature and then washed in PBST buffer. The membranes were incubated for 4 h at room temperature with 1:1000 dilution of primary antibodies for COX-2. Blots were washed three times with PBST at 10 min intervals followed by incubation with 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (rabbit) for 1 h and again washed in PBST three times. The transferred proteins were visualized with an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech, Inc) according to the manufacturer’s instructions.

**Histological studies and immunohistochemical staining**

Liver specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. After deparaffinization and dehydroxylation, the paraffin blocks were stained with hematoxylin and eosin for microscopic examination. For immunohistochemical analysis, the enzymatic activity of endogenous peroxidases in the liver section was first blocked with 3% hydrogen peroxide, followed by incubation with rabbit polyclonal antirat COX-2 antibody (Cayman Chemical CO., Ann Arbor, MI, USA) at room temperature for 40 min. The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally counterstaining was performed using Mayer’s hematoxylin.

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**Fig. 2.** Effects of kolaviron and curcumin on DMN-induced histological changes in the livers of rats. (A) Liver from a vehicle treated rat, (B) DMN-treated rat liver, (C) liver from the rat treated with kolaviron (100 mg/kg) plus DMN, (D) liver from a rat treated with kolaviron (200 mg/kg) and DMN, (E) liver from a rat treated with curcumin (200 mg/kg) and DMN. Liver sections were stained with hematoxylin and eosin. ×400.
Preparation of nuclear extracts from rat liver

The nuclear extract from rat liver was prepared as described previously (Chun et al., 2003). Briefly, liver samples were homogenized in 1 ml of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14,000 g. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed once with 500 μl of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol] and centrifuged for 5 min at 14,800 g. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of protein concentrations.

Electrophoretic mobility shift assay (EMSA)

EMSA for determining the rat ARE-binding activity was performed using a DNA–protein binding detection kit (Gibco BRL, Grand Island, NY), according to the manufacturer’s protocol. Briefly, the ARE oligonucleotide probe was labeled with [γ-32P]ATP by using T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in a total volume of 25 μl of 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 10 μg of nuclear extracts and 100,000 cpm of [γ-32P]ATP-end labeled oligonucleotide. A 100-fold excess of unlabeled oligonucleotide (competitor) was added where necessary. After 50-min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturating polyacrylamide gel at 150 V for 2 h. Finally, the gel was dried and exposed to an X-ray film.

Statistics

The data were analyzed using one-way ANOVA followed by Dunnett’s test. A P value less than 0.05 was considered statistically significant. All the statistics were carried out in SAS (The SAS System for windows, v8; SAS Institute Inc., Cary, NC, USA).

Results

The protective effect of kolaviron on DMN-induced liver injury was evaluated by determining the levels of AST, ALT and γ-GT. As shown in Fig. 1, kolaviron administered at a dose of 100 and 200 mg/kg significantly lowered the activities of AST, ALT and γ-GT that were released into serum as a consequence of DMN-induced hepatic damage. The preventive effect of kolaviron on DMN-induced hepatotoxicity was
dose-dependent and at a dose of 200 mg/kg the effect was comparable to that of curcumin. Histological examination of liver samples also corroborated the above findings. Thus, liver specimens from rats treated with DMN revealed massive and severe hepatocyte necrosis at the centrilobular zone (Fig. 2B) compared with the control liver (Fig. 2A). In both kolaviron and curcumin-pretreated rats, the integrity of the hepatocytes was relatively well preserved (Fig. 2C-E).

Enhancement of oxidative stress has been reported to be implicated in DMN-induced fibrosis and possibly hepatocarcinogenesis in rats (Vendemiale et al., 2001). In agreement with this notion, DMN caused a decrease in intracellular GSH levels (Fig. 3A) with concomitant increases in MDA production (Fig. 3B). The effects of DMN on both parameters were abrogated by kolaviron and curcumin.

The protein expression levels of COX-2 and iNOS were evaluated by western blotting. As shown in Fig. 4A and B, both proteins were highly induced following DMN administration. However, oral administration of kolaviron (100 and 200 mg/kg) for 7 days prior to DMN treatment significantly decreased the levels of COX-2 and iNOS proteins. The inhibition of DMN-stimulated COX-2 and iNOS expression by kolaviron at a dose of 200 mg/kg was better than the reference compound curcumin. Immunohistochemistry demonstrated increased COX-2 expression in the DMN-treated rats. Also immunohistochemical analysis of sections of rat liver treated with DMN verified the inhibitory effect of kolaviron on DMN-induced hepatic COX-2 expression (Fig. 5). As shown in Fig. 5D and E, pretreatment with kolaviron significantly inhibited the number of epidermal COX-2 positive cells in comparison with DMN treatment alone. Since DMN is known to be bioactivated by CYP 2E1, we thought it necessary to examine whether kolaviron can modulate the metabolism of DMN by interfering with this enzyme. Kolaviron did not change the expression of CYP 2E1 compared with control (data not shown).

In response to oxidative stimulus and carcinogenic insult, the up regulation of COX-2 expression requires the activation of several transcription factors including NF-κB and AP-1 (Lee et al., 2007). Also it has been demonstrated that NF-κB is involved in the regulation of iNOS and COX-2 expression (Surh et al., 2001). In order to evaluate whether the preventive effect of kolaviron on DMN-induced liver injury was related to its ability to regulate the DNA-binding activities of transcription factors, an EMSA was performed using the probe of either NF-κB or AP-1. As shown in Fig. 6, the administration of DMN increased the binding of NF-κB and AP-1. However, pretreatment with either kolaviron (100 mg/kg or 200 mg/kg) or curcumin prevented the DMN-induced binding activity of NF-κB and AP-1 (Fig. 6).

**Discussion**

Although diverse mechanisms have been presented to account for the chemopreventive effects of dietary phytochemicals (Lee et al., 2007; Surh, 2003), attention has recently been focused on signaling molecules mediating in inflammation and cancer. Because of a causal link between inflammation and cancer (Clevers, 2004), targeted blockade of intracellular signaling pathways mediating inflammatory response is now considered a road map for developing molecular target-based chemopreventive agents (Surh et al., 2005). Therefore modulation of cellular signaling network involved in induction and activity of COX-2 and/or iNOS has been considered a new paradigm for preventing carcinogenesis (Chung et al., 2007).

**Fig. 5.** Immunohistochemistry of COX-2 in the liver of kolaviron and curcumin-treated rats. Treated rat livers were used for immunohistochemical analysis of COX-2, using rabbit polyclonal anti-rat COX-2 antibody as a primary antibody. Immunohistochemical staining was carried out as described in Materials and methods. Positively stained COX-2 staining yielded a brown-colored product. Vehicle treated control rat, X 100 (A); DMN treated, X 100 (B); DMN treated, X 400 (C); kolaviron (100 mg/kg) plus DMN treated, X 100 (D); kolaviron (200 mg/kg) plus DMN treated, X 100 (E); curcumin (200 mg/kg) plus DMN treated, X 100 (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Several experimental studies have demonstrated the antioxidant, antihepatotoxic and antigenotoxic properties of kolaviron in various experimental models (Farombi et al., 2000a; Nwankwo et al., 2000). However the underlying molecular mechanisms of hepatoprotection of kolaviron remain to be elucidated. In the present study, we report that kolaviron inhibits COX-2 and iNOS expression in rat liver through down regulation of NF-κB and AP-1 in a DMN-induced hepatotoxic model.

The apparent reduction in DMN-mediated increase in the activities of serum enzymes and lipid peroxidation by kolaviron in the present study is in agreement with previous observations on the hepatoprotective ability of this natural compound on several hepatotoxicants (Farombi et al., 2005; Farombi et al., 2000a).

Various hepatotoxic compounds such as carbon tetrachloride have been demonstrated to activate the well defined eukaryotic transcription factors such as NF-κB and AP-1 (Kim et al., 2002). NF-κB plays a central role in general inflammation as well as tumorigenesis (Surh et al., 2001). Rapid phosphorylation of IκBα and its subsequent degradation following exposure of cells to external stimuli such as carcinogens, inflammatory cytokines and reactive oxygen species lead to increased nuclear translocation and DNA binding of NF-κB. Our results show that DMN increased DNA-binding activity of NF-κB. However, pretreatment of rats with kolaviron abrogated the DNA binding activity of NF-κB. Curcumin, a reference compound used in this study also attenuated the effect of DMN on these transcription factors. The physiological function of curcumin has been closely associated with its ability to inhibit the activation of transcription factors such as NF-κB (Nanji et al., 2003).

COX-2 and iNOS are well known proinflammatory genes and their expressions have been shown to be regulated by NF-κB and AP-1 (Nanji et al., 2003). The present study revealed that kolaviron and curcumin abolished the expression of these proteins in DMN-treated rat liver. The results therefore suggest that kolaviron may be important not only in alleviating liver inflammation but also for the prevention of liver cancer. Apart from NF-κB, AP-1 regulates the transcription of a vast variety of genes some of which are involved in neoplastic transformation and tumor promotion (Lee et al., 2007; Young et al., 2003). It has been shown that AP-1 regulates partly the expression of COX-2 in mouse skin stimulated with TPA (Chun et al., 2004). Considering therefore the importance of AP-1 in toxicity and neoplasm, the inhibition of DMN-mediated DNA binding of this transcription factor and expression of its components by kolaviron partly explains the molecular basis of the hepatoprotective effect of kolaviron in drug-induced hepatotoxicity and possibly hepatocarcinogenesis.

CYP 2E1 mediates the biotransformation of DMN to reactive species (Frei et al., 2001; Guengerich et al., 1991). Therefore, one cannot rule out the possibility that kolaviron can attenuate DMN-induced hepatic injury by inhibiting this enzyme. However, we found that kolaviron did not influence the constitutive expression of CYP 2E1, compared with the control (data not shown). In our previous study, it was also observed that kolaviron did not affect the activity of aniline hydroxylase, a CYP 2E1 representative enzyme (Farombi et al., 2000a). Based on these findings, it is likely that protection of DMN-induced hepatotoxicity by kolaviron is associated with its inhibition of proinflammatory enzymes, such as COX-2 and iNOS rather than suppression of the metabolic activation of DMN.

**Fig. 6.** Inhibitory effects of kolaviron and curcumin on DMN-induced activation of NF-κB (A) and AP-1 (B) in rat liver. Lane 1, free probe; lane 2, control; lane 3, DMN alone; lane 4, kolaviron (100 mg/kg) plus DMN; lane 5, kolaviron (200 mg/kg) plus DMN; lane 6, curcumin (200 mg/kg) plus DMN; lane 7, competitor. *P<0.001 compared with lane 2; **P<0.001 compared with lane 3.
Conclusion

Taken together, the ability of kolaviron to inhibit COX-2 and iNOS expression through down regulation of NF-κB and AP-1 DNA binding activities could be a mechanism to explain the hepatoprotective effect of kolaviron on drug-induced hepatotoxicity. The edible G. kola nut from which kolaviron is obtained plays a prominent role in the social customs of people in the west Africa and used traditionally in the treatment of liver disorders. In view of the present study, kolaviron merits further consideration as an edible phytochemical with potential therapeutic application in the chemoprevention of liver cancer.

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