Protective Effects of Macelignan on Cisplatin-Induced Hepatotoxicity Is Associated with JNK Activation

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Cisplatin is one of the most effective antineoplastic drugs, but it has undesirable side effects such as hepatoxicity at high doses. This study investigated the protective effect of macelignan, isolated from Myristica fragrans HOUTT. (nutmeg), against cisplatin-induced hepatotoxicity and the possible mechanisms involved in these effects in mice. Pretreatment with macelignan for 4 d significantly prevented the increased serum enzymatic activities of alanine and aspartate aminotransferases in a dose-dependent manner. The results also showed that the protective effects of macelignan on cisplatin-induced hepatotoxicity may be associated with the mitogen-activated protein kinase (MAPK) signaling pathway. Cisplatin-induced phosphorylation of c-Jun N-terminal kinase 1/2 (JNK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) was abrogated by pretreatment with macelignan, however, that of p38 was not significantly affected. It was also found that macelignan attenuated the expression of phosphorylated c-Jun in cisplatin-treated mice. Accordingly, it is suggested that the hepatoprotective effects of macelignan could be related to activation of the MAPK signaling pathway, especially JNK and c-Jun, its substrate. The present findings suggest that co-treatment of cisplatin with macelignan may provide more advantage than cisplatin treatment alone in cancer therapy.

Key words macelignan; cisplatin; hepatotoxicity; mitogen-activated protein kinase; c-Jun N-terminal kinase; c-Jun

In the clinical field, the most important form of toxic hepatic injury is caused by therapeutic agents. Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug from the market, and it is also responsible for more than 50% of cases of acute liver failure in the United States. More than 600 drugs have been associated with hepatotoxicity, and there are now a number of reports that reactive metabolites formed from drugs such as acetaminophen, tamoxifen, diclofenac, and troglitazone are known to cause hepatotoxicity.21

Cisplatin (cis-diaminedichloroplatinum(II)) is one of the most effective antineoplastic drugs, and it is particularly used for the treatment of ovarian, testicular and head and neck cancers.3,4 In spite of its chemotherapeutic activity, various adverse effects, including renal dysfunction, nausea and vomiting, myelosuppression, ototoxicity, and nephrotoxicity, associated with its clinical use are well known.5 Among them, nephrotoxicity is frequent and a major limitation to the use of this drug. Several mechanisms, including oxidative stress, inflammation, genotoxic damage, and cell cycle arrest, have been examined in cisplatin-induced nephrotoxicity.6–8 However, cisplatin-induced liver toxicity and the mechanisms have been little studied, except for some clinical reports.9–11

Myristica fragrans HOUTT. (Myristicaceae) is a perennial herb native to Indonesia and cultivated in South Africa, the Molucca Islands, India and other tropical areas. Its fruits, commonly known as mace (outer husk) or nutmeg (inner seed or kernel), has been used traditionally for spice and also possesses carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, anxiogenic, anti-diarrheal and anti-inflammatory activities.12–14 Macelignan (Fig. 1) isolated from M. fragrans has been reported to have antioxidant activity and cause alteration in hepatic enzyme activities,15–17 however, the protective effect of macelignan against hepatotoxicity has not yet been examined.

The objective of the current study was to examine the protective effects of macelignan on cisplatin-induced hepatotoxicity. For this purpose, we evaluated the levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Several studies have reported that cisplatin activity is related to the cascades of mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinases (JNKs), also referred to as stress-activated protein kinases (SAPKs), extracellular signal-regulated kinases (ERKs), and p38 kinases.18,19 Accordingly, we examined whether the protective activity of macelignan against cisplatin-induced hepatotoxicity is associated with the mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS

Isolation of Macelignan

Macelignan (Fig. 1) was isolated from Myristica fragrans HOUTT. (Myristicaceae) as described previously.20 Dried seed kernels (100 g) of Myristica fragrans were ground and extracted twice with 75% aqueous methanol (400 ml, v/v) for 24 h at room temperature. The methanol extract was concentrated, frozen, lyophilized (7 g) and further fractionated successively with ethyl acetate, n-butanol and water. Each fraction was evaporated and dried

Fig. 1. Chemical Structure of Macelignan

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under reduced pressure (ethyl acetate fraction 4.2 g, butanol fraction 0.7 g, water fraction 2.1 g). Further separation was performed using silica gel column chromatography (70—230 mesh, Merck & Co., Whitehouse Station, NJ, U.S.A.) by eluting with n-hexane : ethyl acetate solution (10 : 1, v/v), and 10 ml volumes of eluant were collected in test tubes. The collected tubes were divided into six fractions (Fr. I—Fr. VI) following silica TLC (thin layer chromatography; 60 F254, Merck & Co., Whitehouse Station, NJ, U.S.A.). Fr. III, providing considerable inhibitory activity against S. mutans, was further separated through a silica gel column using n-hexane : ethyl acetate (20 : 1, v/v), yielding Fr. III-B (0.52 g). Fr. III-B was eluted with 80% methanol using Rp-18 column chromatography (LiChroprep, 25—40 μm, Merck & Co., Whitehouse Station, NJ, U.S.A.), and Fr. III-B-2 (0.5 g) was finally obtained as a single compound.

Chemicals Cisplatin (cis-diaminedichloroplatinum [II]), curcumin, and corn oil were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Antibodies used in this experiment were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), and ECL solution was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

Animals and Treatment Male ICR mice weighing 25±5 g (5 weeks old) were purchased from Samtako Bio Korea (Seoul, Korea). The animals were allowed free access to normal standard chow diet and tap water, and maintained in controlled conditions of 25±2 °C, 55±5% relative humidity with a 12 h dark/light cycle. The protocol for mouse maintenance was approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, according to the Guide for Animal Experiments edited by the Korean Academy Medical Association. Mice were kept in this environment for 1 week before an experiment. Macelignan in corn oil (25 or 50 mg/kg per day) was administered orally for four consecutive days. Three hours after the final treatment with macelignan, a single dose of cisplatin (45 mg/kg, in corn oil and PBS) was used as vehicle control, and the positive group animals were given cisplatin with curcumin (50 mg/kg). Sixteen hours after administrating cisplatin, all animals were killed with CO2 anesthesia, and blood and liver samples were collected.

Determination of Serum Biochemical Parameters Blood samples were kept at room temperature for 1 h, and serum was prepared by centrifugation at 4000 rpm for 15 min and stored at −70 °C until analysis. The levels of serum ALT and AST were measured using an automatic photometer (PRIME, BPC BioSed S.r.l., Italy).

Histopathological Examination Portions of the liver were fixed in 10% formalin and then embedded in paraffin. Five micrometer thick microtome sections were prepared from each liver sample and stained with hematoxylin–eosin. The sections were examined for the pathological findings of hepatotoxicity such as centrilobular necrosis and lymphocyte infiltration.

Western Blot Analysis Powdered liver tissues under liquid nitrogen were homogenized with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 0.25% deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF)) and incubated on ice for 20 min. The mixture was then centrifuged, and the supernatant was used to determine the concentration of protein. Fifty micrograms of total cellular protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding was blocked overnight with 5% skim milk in Tris-buffered saline Tween-20 (TBST) (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) at 4 °C, and then blots were incubated with 1:1000 diluted primary antibody for 2 h. After washing with TBST, the membranes were probed with 1:2000 secondary antibody for 1 h and washed with TBST three times. Immunoblots were visualized using a chemiluminescent reagent. The densities of the bands were measured using an RFLPscan version 2.1 software program (Scanalytics Inc., Fairfax, VA, U.S.A.).

Statistics All experiments used 6—8 animals per treatment group, and the results are presented as the mean±standard deviation (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffé’s test (SPSS 12.0). The criterion for statistical significance is expressed as *p<0.05, **p<0.01.

RESULTS

Effect of Macelignan on Cisplatin-Induced Hepatotoxicity The protective effect of macelignan on cisplatin-induced hepatotoxicity was examined by quantifying the levels of serum ALT and AST. In cisplatin-treated mice, serum levels of ALT and AST were increased to 494% and 333% compared to the normal group (Fig. 2). However, pretreatment with macelignan was protective, as indicated by significant reduction of ALT and AST activities, and showed higher activity than that with curcumin (50 mg/kg). In contrast, there were no significant differences in the activities of ALT and AST in mice treated with macelignan (50 mg/kg) alone when compared with control. This indicates that macelignan itself does not have a hepatotoxic effect.

Effect of Macelignan on Hepatic Morphological Changes of Cisplatin-Induced Hepatotoxicity Histological effects were also examined in this study. In contrast to cisplatin-untreated control mice (Fig. 3a) and macelignan only treated mice (Fig. 3b), cisplatin-treated mice showed severe centrilobular necrosis and lymphocyte infiltration (Fig. 3c). Pretreatment with macelignan strongly prevented the development of severe hepatic lesions induced by cisplatin, with marked reductions in necrosis (Fig. 3d). These results were consistent with those of serum hepatotoxicity indices shown in Fig. 2.

Effect of Macelignan on Cisplatin-Mediated Activation of MAP Kinases and Transcription Factors To investigate whether the protective effect of macelignan is related to MAP kinase signaling, protein samples obtained from mouse liver were subjected to Western blot analysis. As shown in Fig. 4, although the unphosphorylated form of JNK1/2 was not changed by cisplatin treatment, its administration led to an increase in the level of phosphorylated JNK1/2. However, pretreatment with macelignan before cisplatin injection significantly suppressed JNK1/2 activation, and this activity of macelignan was almost comparable to that of curcumin at the
same dose (50 mg/kg). Cisplatin also induced expression of phosphorylated ERK1/2. These effects were slightly attenuated by macelignan, but the inhibitory activity was not apparent compared to that in JNKs. The protein levels of unphosphorylated and phosphorylated p38 were not affected by either cisplatin or a combination treatment of cisplatin with macelignan. Moreover, phosphorylated JNK1/2, ERK1/2 and p38 were not affected when treated with macelignan alone (data not shown).

Based on the above data, the protein expression levels of AP-1 subunit, c-Jun and c-Fos were examined. The results showed that expression of phosphorylated c-Jun was increased in cisplatin-treated mice, although unphosphorylated c-Jun and c-Fos production were not changed by administration of cisplatin or macelignan (Fig. 4). However, increased phosphorylated c-Jun expression was highly attenuated by treatment with macelignan. In addition, macelignan presented more efficacious activity than curcumin at the same dose.

DISCUSSION

Clinical evidence of cisplatin-induced hepatic injury has been demonstrated by elevated activities of serum amino-transferase including ALT and AST. The rise in levels of serum ALT and AST has been attributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm, and are released into the circulation after hepatic damage. Therefore, the protective effect of macelignan on cisplatin-induced hepatotoxicity was examined by quantifying the levels of ALT and AST. As shown in Fig. 2, pretreatment with macelignan was protective, as indicated by the significant reductions in ALT and AST activities. Histopathological analysis of liver sections indicated a moderate centrilobular necrosis and lymphocytic infiltration in mice treated with macelignan plus cisplatin compared with those treated with cisplatin alone (Fig. 3).

The cytotoxic action of cisplatin is mediated by its interaction with DNA to form DNA adducts, which activate several signal transduction pathways including MAPK and culminate in the activation of apoptosis. The MAPK signaling pathways play important roles in regulating gene expression, cell proliferation, cell motility, and cell survival and death in eukaryotic cells. Three distinct groups of MAPKs have been identified in mammalian cells: c-Jun N-terminal kinase (JNKs), extracellular-regulated kinase (ERKs), and p38. A major role for MAPKs is to regulate the activities of transcription factor and transmit extracellular signals to the nucleus, which induce target gene expression. Numerous mammalian transcription factors and their coregulatory proteins are known as targets of the MAPK cascades.

A previous study has demonstrated that MAPK members can be activated following exposure of tumor cells to cisplatin. It has been well documented that curcumin has a hepatoprotective effect and is an inhibitor of JNK in hepatocytes. In this study, cisplatin induced expression of phosphorylated JNK1/2 and ERK1/2 in mice liver, however, pretreatment with macelignan before cisplatin injection suppressed JNK and ERK activation more than curcumin (Fig. 4).

Cisplatin-induced kinase activation and its inhibition by macelignan were apparent in JNK1/2 signaling. JNK and ERK activation in cisplatin-treated cells has been reported, however, their roles are still controversial. Most studies have shown that activation of JNK is associated with induction of cell death. In contrast, other studies have suggested that JNK signaling plays a role in enhanced cell survival of cis...
platin-treated cells. JNK activation associated with cell fate is dependent on the type of stimuli and circumstances, but it seems clear that JNK is activated in cells treated with anti-neoplastic agents, such as cisplatin, vincristin or adriamycin, although its role on apoptosis is not well established. Like JNK, ERK is also activated by cisplatin in a variety of cell lines. According to previous studies, ERK activation is associated with either inducing apoptosis or antagonizing apoptosis of cisplatin-treated cells. Both effects of survival and death signaling mediated through MAPK are possible, and this phenomenon could reflect differences in cell context or the extent of DNA damage.

It is well known that MAPK signaling pathways regulate expression of the AP-1 family members c-Jun and c-Fos. AP-1 transcription factors could be involved in both the induction and prevention of apoptosis. c-Jun is a central component of all AP-1 complexes, which act often as transcriptional activators. MAPKs such as JNK, ERK5, and p38 can regulate the expression levels of genes encoding these transcription factors by controlling the c-jun promoter depending on the stimulus. c-Jun is targeted by the JNK pathway in response to genotoxic stress and pro-inflammatory cytokines, and phosphorylation of c-Jun enhances its transcriptional activity. c-Jun is necessary for the induction of apoptosis in cells exposed to genotoxic stress or UV radiation. On the other hand, some previous studies have showed that c-Jun is rapidly activated in response to mitogens, indicating that c-Jun probably has a function in the control of cell proliferation. Balance between the pro-apoptotic and anti-apoptotic target gene expression determines whether the final outcome will be cell survival or cell death. This balance may be dependent on the type and duration of stimulus, as well as on the activation of other transcription factors.

It is reported that persistent activation of JNK can be involved in cisplatin-induced apoptosis, and c-Jun, the main substrate of JNK is necessary for induction of apoptosis in response to cisplatin. In this study, the administration of cisplatin led to the activation of JNKs, and its induction was significantly attenuated by macelignan treatment. Pretreatment with macelignan also inhibited c-Jun activation in cisplatin-treated mice. These results indicate that macelignan attenuates activation of JNK and c-Jun caused by cisplatin.
which prevents hepatocyte apoptosis.

Like c-Jun, the activity of c-Fos, another component of AP-1, can be regulated by JNK signaling pathways. Induction of c-Fos is important for AP-1 activity because Jun/Fos heterodimers are more stable than Jun/Jun homodimers. However, in this study, the protein expression of c-Fos was not changed in cisplatin-treated mice liver. Considering other reports about AP-1 activity, several mechanisms could be involved in regulating AP-1 activity. The investigation of a more exact mechanism about JNK signaling and its substrates affected by treating cisplatin and macelignan is needed.

In conclusion, macelignan reduced cisplatin-induced hepatotoxicity in mice, and its protective activity might be related to the JNK signaling pathway. Phosphorylated JNKs and c-Jun, its substrate, were strongly induced in cisplatin-treated mice. Considering other reports about AP-1 activity, several mechanisms could be involved in regulating AP-1 activity. The investigation of a more exact mechanism about JNK signaling and its substrates affected by treating cisplatin and macelignan is needed.

REFERENCES