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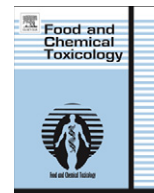

Methylated chrysin, a dimethoxy flavone, partially suppresses the development of liver preneoplastic lesions induced by *N*-Nitrosodiethylamine in rats

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ABSTRACT

The modifying effect of chemically modified chrysin on formation of preneoplastic foci induced by *N*-nitrosodiethylamine (DEN) was investigated in male rats. Male Wistar rats were administered three intraperitoneal injections of DEN (200 mg/kg bodyweight) interspersed by 2 weeks with or without an oral dose of dimethoxy flavone (DMF 100 mg/kg bodyweight), 2 weeks after DEN initiation. The number of GST-Pi positive foci and proliferating cell nuclear antigen (PCNA)-positive cells were significantly suppressed by the administration of DMF. Real-time RT-PCR analysis revealed that DMF treatment increased mRNA expression levels of apoptotic proteins *p53* and *fas*, cell cycle regulatory proteins *chek 2*, *cdkn1a, rad 50,* anti-inflammatory protein *pparg* whereas the mRNA expression levels of b*cl-2* and p*rdx-2* were decreased compared to mRNA levels in DEN-treated group. Therefore, we propose that DMF partially suppresses the formation of preneoplastic lesions in rats following DEN exposure by regulating anti-inflammatory and apoptosis-promoting events and restoring the cellular redox balance altered by DEN.
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1. Introduction

Dimethoxy flavone (DMF), a naturally occurring flavone contained in pepper leaves can be manufactured from chrysin by chemical modification (Ahmad et al., 1997; Zheng et al., 2003). DMF has more bioavailability and greater metabolic stability than chrysin (Walle, 2007). Although chrysin is not toxic and hence is being consumed by humans, studies on the role of DMF *in vivo* are lacking (Walle, 2009). Pushpavalli et al. (2010) have recently reported that chrysin renders widespread hepatoprotective properties in the galactosamine induced liver injury model. However, the mechanism of the chemopreventive effect of DMF is still unknown.

Diethylnitrosamine is a potent carcinogenic *N*-nitroso compound widely accepted for induction of preneoplastic lesions and hepatic tumors in rats. Initiation by DEN involves perturbations of nuclear enzymes involved in DNA repair or replication (Aiub et al., 2004). DEN is present in tobacco products, cosmetics and as a contaminant of drinking water (Reh and Fajen, 1996; Lubick, 2006; Zhou et al., 2007).

DEN-induced lesions as well as tumors in rodents show marked biochemical, histological and molecular similarity to the progression of hepatocellular carcinoma (HCC) in humans (Feo et al., 2000). In India, liver cancer is the third most common form of cancer in men (Indian Council of Medical Research, 2001). Increasing incidence, morbidity and mortality from liver cancer is currently driving the quest for identification of novel compounds that can combat this deadly disease. Chemoprevention using dietary non-nutrients offers a credible option for cancer treatment since they are likely to influence a plethora of subcellular events leading to suppression of cell proliferation, modulation of oncogenic pathways, inhibition of inflammation and induction of apoptosis (Surh, 2003; Sunny, 2005). We recently reported that another bioflavonoid morin is capable of suppressing rat hepatocellular carcinoma through co-ordinated modulation of expression of Akt and inflammation mediators like Cox-2 during DEN-induced liver cancer. (Sivaramakrishnan and Niranjali, 2009).

Therefore, the current study was planned to investigate the chemopreventive role of DMF and quantify its effect on molecular events in the experimentally validated preneoplastic lesion model. (Mandal et al., 2008).

Abbreviations: bcl 2, B Cell CLL/lymphoma–2; *cdkn1a*, Cyclin dependent kinase inhibitor 1A; *chek 2*, Cell-cycle checkpoint kinase2 homolog (S.pombe); DEN, N-diethylnitrosamine; DMF, dimethyl flavone; *fas*, apoptosis stimulating fragment; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; GST-Pi, glutathione-S-transferase Pi form; *p53*, Tumor protein 53; PCNA, proliferating cell nuclear antigen; *pparg*, Peroxisome proliferator-activated recptor gamma; *prdx 2*, peroxiredoxin-2; *rad 50*, RAD50 homolog (S.cerevisiae); RT-PCR, reverse transcriptase-polymerase chain reaction.

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2. Materials and methods

2.1. Chemicals

Chrysin (C80105) and N-diethylnitrosamine (DEN; 73861) were purchased from Sigma Chemical Company (St.Louis, MO, USA) with purities of >97 and >99%, respectively. Dimethoxy flavone was synthesised chemically from chrysin following the method of Zheng et al. (2003). All other reagents were commercially available and of analytical grade.

2.2. Animals and experimental design

Animals received humane care in accordance with the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India and prior permission was sought from the institutional animal ethics committee (IAEC No: 01/089/09). Six-week old male Wistar rats were purchased from the Kings Institute of Preventive Medicine (Guindy, Chennai) and maintained in an air-conditioned room with a 12 h light/dark cycle (room temperature, 22 ± 3 °C; relative humidity, 65 ± 11%), and given free access to a pellet diet (Amrut Laboratory Animal Feed, Bangalore, INDIA; containing protein 22.06%, oil 4.28%, fibre 3.02%, ash 7.8%, sand (silica) 1.37% w/w) and tap water. After a one-week acclimatization period, a short-term liver carcinogenesis bioassay to study the modifying effect of DMF on neoplastic lesion formation induced by DEN was performed by the following procedure. Sixty rats were divided into 4 experimental groups. Fifteen rats designated as Group A, were fed basal diet and served as blank control, 15 rats designated as Group B, were fed diet along with oral injections of 100 mg DMF/kg bodyweight, three times weekly, starting at week-9 served as a drug control. A preliminary dosage fixation study with varying concentrations of DMF showed 100 mg/kg to be the optimum oral dose with no long-term toxicity (data not shown). Fifteen rats, designated as group C received an intraperitoneal injection of 200 mg DEN/kg body weight, three times during the total experimental period interspersed by a fortnight at week-7, -9 and -11 and served as control to induce preneoplastic lesions. Group D rats received DEN similar to group C rats, beginning from week-7 and were co-administered DMF starting from week-9 so that treatment commences after induction of putative preneoplastic liver foci. At the end of the week-13, rats were euthanized by exsanguinations under ether anesthesia, and the livers were excised and weighed. Sliced liver samples were fixed in 10% phosphate-buffered formalin for histopathology and immunohistochemistry. Remaining pieces of the livers were frozen in dry ice and stored at −80 °C until further analysis.

2.3. Histology and immunohistochemistry

After sacrifice, resected liver slices from the neoplastic nodules formed in the centrilobular region were fixed and dehydrated in graded ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H.E.) for histological examinations. Immunohistochemical staining of PCNA and immunofluorescence staining of GST-Pi were performed using the following procedure: the deparaffinised liver sections were treated with 0.3% H_2O_2 in methanol for 30 min, and then incubated with a solution of normal goat serum and rabbit anti-GST-Pi antibody (1:300 dilution; #3369, Cell signaling Technology, Beverly, MA, USA or mouse anti-PCNA antibody (1:200 dilution; MS-106-P, Neomarkers, USA) overnight at 4 °C. This was followed by washing in phosphate-buffered saline for 5 min, after which the slides were incubated with goat anti-mouse secondary antibody IgG-HRP (1:400 dilution) for PCNA and with goat anti-rabbit secondary antibody IgG-FITC (1:400 dilution) for GST-Pi at room temperature for 45 min. The slides were then developed with 3,3′-diaminobenzidine as the chromogen, followed by light counterstaining with hematoxylin, dehydration and mounting with DPX for PCNA or detection by immunofluorescence with the green filter for GST-Pi. The numbers and areas of GST-Pi positive cells (>0.2 mm diameter) were designated as preneoplastic foci. A majority of PCNA-positive labelled nuclei that were nested in foci-like areas to the total area of the liver sections were quantified using Scion Image (Scion Corp, Frederick, MD, USA). Each section was examined at magnification (20×) and the ratio of GST-Pi and PCNA-positive area to hepatic tissue area was calculated. The result was regarded as the mean of five different fields on each section.

2.4. Real-time RT-PCR analysis

The expression of the genes listed in Table 1 was quantified using quantitative real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) analysis. The genes listed in Table 1 were selected based on the result of our previous studies in which genes of specific tumor suppressors and inflammatory mediators were significantly increased in rats treated with DEN. Briefly, the total RNA from six rats per treatment group was extracted using TRIzol reagent (Sigma Chemical Company, USA), according to the manufacturer's instructions. The total RNA was reverse transcribed using ThermoScript reverse transcriptase (SuperScript III First-Strand Synthesis System; Invitrogen). All the reactions were performed using SYBR Green I chemistry (Invitrogen, USA) and were carried out under the following conditions using Strategene M3000 Sequence Detection System (Stratagene, USA) with the following protocol: incubation at 50 °C for 2 min followed by 95 °C for 10 min, then 40 cycles for 15 s at 95 and 60 °C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in triplet repeat, and PCR reactions without the addition of template cDNA were used as blank controls. The forward and reverse primers listed in Table 1 were designed using the NCBI-primer BLAST public database. The relative differences in gene expression were calculated using the cycle time (Ct) values that were first normalised with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the endogenous control in the same sample, and then relative control Ct value following the 2- ΔΔct method as previously described (Livak and Schmittgen, 2001). The data represents the average fold changes with standard deviation.

2.5. Statistical analysis

All data were expressed as means with their standard deviation. Multi-groups (DEN-treated [group C] and DMF-treated DEN group [group D]) were used to test the homogeneity of variance between the groups using Tukey's test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Toxicological parameters and histopathological findings in liver

Treatment with DMF only did not affect clinical symptoms, food consumption or water intake (Table 2). Mean body weight in DMF-treated DEN-induced rats (group D) was significantly higher than in the DEN-induced rats (group C) from week 11 to week 13 (Fig 1 and Table 2). Both absolute and relative liver weights significantly increased ($p < 0.01$ and $p < 0.001$ respectively), whereas body weight decreased in the DEN-induced control group (group C) compared with the DEN-induced DMF treatment group (group D, Table 2). However, a slight increase in the absolute liver weight was seen in DEN-induced DMF treated groups (group D) when compared to blank control rats (group A) resulting in a small but significant ($p < 0.05$) increase in relative liver weight in group D rats. This perhaps points to the partial protection rendered by DMF against the DEN-induced putative lesions.

Histologically, DEN exposure led to diffuse hepatocellular hypertrophy with formation of hepatic foci containing mainly eosinophilic or basophilic hepatocytes (Fig 2).

Table 1
Sequence of primers used for real-time RT-PCR analysis.

Accession No.	Gene description	Gene symbol	Forward	Reverse
NM_030889.3	Tumor protein 53	*p53*	TTGGACCCTGGCACCTACAATG	GCAGACAGGCTTTGCAGAATGG
NM_053677.1	Cell-cycle checkpoint kinase2 homolog (S.pombe)	*Chek 2*	AGTTGCACGACCTCTCACAG	TATCCCTCCCGAACCAGTAG
NM_022246.1	RAD50 homolog (S.cerevisiae)	*Rad 50*	GCAACTTCCAGCTTCTGGTC	CCTGCCATACACAGTTCCC**T**
NM_080782.3	Cyclin dependent kinase inhibitor 1A	*Cdkn 1a*	CTGGTGATGTCCGACCTGTT	GGGCTCAGGTAGATCTTGGG
NM_139194.2	Apoptosis stimulating fragment	*Fas*	ACCTGGTGACCCTGAATCTG	TGATACCAGCACTGGAGCAG
NM_016993	B Cell CLL / lymphoma - 2	*Bcl-2*	AAGACACCCCTTCATCCAAG	TCAGGCTGGAAGGAGAAGAT
NM_017169	Peroxiredoxin-2	*Prdx-2*	AGGACTTCCGAAAGCTAGGC	TTGACTGTGATCTGGCGAAG
NM_013124.3	Peroxisome proliferator-activated recptor gamma	*Pparg*	GGAGATCCTCCTGTTGACCC	GTGGAGCAGAAATGCTGGAG
NM_017008.3	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	TCAAGAAGGTGGTGAAGCAG	AGGTGGAAGAATGGGAGTTG

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