Genetic Toxicity Test of Emodin by Ames, Micronucleus, Comet Assays and Microarray Analysis Showing Differential Result

Seo Y. GO, Kyung J. Kwon, Sue N. Park, and Yhun Y. SHEEN*

College of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, Korea
Department of Toxicological Researches, National Institute of Toxicological Research, Korea Food and Drug Administration, 5 Nokbeon-dong, Eunpyeong-gu, Seoul 122-704, Korea

(Received 19 June 2007; Accepted 21 August 2007)

Abstract – Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a major constituent of rhubarb. Although it has been claimed to have a wide spectrum of therapeutic value, its side effects, especially in human kidney cells have not been well characterized. In this study, we have carried out in vitro genetic toxicity test of emodin and microarray analysis of differentially expressed genes in response to emodin. The result of Ames test showed mutations with emodin treatment in base substitution strain TA1535 both with and without exogenous metabolic activation. Likewise, emodin showed mutations in frame shift TA98 both with and without exogenous metabolic activation. The result of COMET assay in L5178Y cells with emodin treatment showed DNA damage both with and without exogenous metabolic activation. Emodin did not increase micronuclei in CHO cells both with and without exogenous metabolic activation. 150 Genes were selected as differentially expressed genes in response to emodin by microarray analysis and these genes would be candidate biomarkers of genetic toxic action of emodin.

Keywords □ Emodin, Ames test, COMET assay, MN assay, Microarray, S9 fraction

INTRODUCTION

Emodin is one of the oldest and best known Chinese herbal medicines (Da Huang) (Maclean and Townsend, 1999; Hoffman, 2003). Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone present in the roots and barks of numerous plants and an active ingredient of Chinese herbs including Rheum officinale and Polygonum cuspidatum (Kuo et al., 2001; Sato et al., 2000). It is also found in the roots, leaves, and bark of senna and aloe (Merck, 1998; Nature’s Field, 1999). Preparations of all of these plants have been used in herbal laxatives (National Toxicology Program, 2003). Emodin is also found in the wild mushroom Dermocybe sanguinea (von Wright et al., 1992), and various fungi (Wehner et al., 1979). Emodin has been shown to possess biological activities of anticancer (Yeh et al., 1988), antivirus (Barnard et al., 1992), inhibition on NADH oxidase, xanthine oxidase, succinate oxidase, as well as vasorelaxation (Lin et al., 1996). Recent studies have suggested that in the HER-2/neu-overexpressing breast cancer cell emodin may act as a tyrosine kinase inhibitor and in T-lymphocytes as a strong suppressing factor on proliferation (Kumar et al., 1998; Kuo et al., 1997, 2001; Lee, 2001a,b; Zhang et al., 1999a,b). In addition, emodin has been reported to induce apoptosis in human lung squamous cell carcinoma (Lee, 2001a,b; Zhang and Hung, 1996; Zhang et al., 1998). Despite of its therapeutic value, potential side effects of emodin have been revealed through experimental studies. In 2001, the National Toxicology Program (NTP) reported that exposure of rats to emodin resulted in an increase in incidence of renal tubule hyaline droplets and severities of renal tubule pigmentation in both male and female animals (National Toxicology Program, 2001).

Emodin isolated from different sources was reported to be mutagenic in Salmonella typhimurium strains TA97, TA98, TA100, TA102, and TA1537 with or without metabolic activation (Wehner FC et al., 1979; Krivobok S et al., 1992). However, there are also reports showing no evidence of mutagenicity for emodin. In mammalian test systems using V79 Chinese hamster cells, no genotoxicity of emodin was found either with or without metabolic activation (Bruggeman IM, van der Hoeven JC, 1984) Lack of emodin genotoxicity was also evident in a mouse micronucleus assay (Mengs U et

*Corresponding author
Tel: +82-2-3277-3028, Fax: +82-2-3277-2851
E-mail: yysheen@ewha.ac.kr
Thus far, no further study has not been carried out to find out the underlying mechanism of genetic toxic action of emodin. In this study, we have tested emodin using Ames test, in vitro micronuclei assay in CHO cells, single cell gel/comet assay in L5178Y cells, microarray analysis of gene expression profiles in L5178Y cells in order to find out biomarker gene candidates in response to genetic toxicity of emodin.

**MATERIALS AND METHODS**

**Materials**

Emodin, 2-aminofluorene, 2-nitrofluorene, sodium azide, methanesulfonic acid methyl ester, benz(a)pyrene (BaP) and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, USA). The S9 fraction was purchased from Moltex® S9 (Canbiotech, U.S.A).

**Ames test**

The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using *Salmonella typhimurium* strains TA98 and TA1535. The tester strains were cultured overnight in nutrient broth medium at 37°C. To the 0.1 ml of bacterial suspension, 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 ml of S9 was added and then 0.1 ml of emodin (3.3, 10, 33.3, 66.6, 100 µg/plate) or positive control chemicals such as 2-aminofluorene, 2-nitrofluorene and sodium azide were added and incubated for 20 min at 37°C. After incubation, 2.0 ml of top agar was added to mix and the mixture was poured onto a minimal glucose agar plate. 48 Hours after the incubation at 37°C, the numbers of revertant colonies were counted (Kasamatsu et al, 2005).

**Comet assay**

Comet assay was carried out according to Singh et al. (Singh et al., 1988) with slight modification. L5178Y mouse lymphoma cells were seeded in 12 well plates and were exposed to 7.5, 15, 30 µg/ml emodin for 2 h. For the positive controls, cells were exposed to 150 µM methyl methanesulfonate (MMS) in the absence of S9, 50 µM benz(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 µl of cell suspension were mixed and the mixture was poured onto a minimal glucose agar plate. 48 Hours after the incubation at 37°C, the numbers of revertant colonies were counted (Kasamatsu et al, 2005).

**In vitro cytokinesis block micronucleus assay**

The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders et al., 2003). CHO-K1 cells were grown in 24-well plates and treated with emodin (3.75, 7.5, 15 µg/ml) or cyclophosphamide (2.5, 5, 15 µg/ml) for 4 h with or without S9. After the treatment, cells were washed with PBS and further incubated for 20 h in the medium containing 3 µg/ml cytchalasin B. Cells were harvested and spread on glass slide, and fixed with 100% methanol for 5 min and stained with 0.24 mM acridine orange in 6.7 mM phosphate buffer (pH 6.8) for 3 min. Micronuclei were scored under the fluorescence microscope at 1000 magnification.

**Microarray**

The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate. After 2 h of treatment with emodin (30 µg/ml), cells were resuspended in media without emodin and cultured for 20 h. Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by a RNeasy mini kit (QIAGEN, Hilden, Germany). Total RNA (1 µg) was amplified using the Affymetrix one-cycle cDNA synthesis protocol. For each array, 15 µg of amplified biotin-cRNA was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, USA) for 16 h at 45°C in a rotating hybridization oven. Slides were stained with streptavidin/phycoerythrin and washed for antibody amplification. Arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037).

**RESULTS**

**Emodin induced gene mutations in both TA98 and TA1535 strains.**

The mutant frequency (MF) was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to different concentrations of emodin (Fig. 1). In TA98 strain, the MF of 1.0 µg/plate 2-nitrofluorene treated bacteria in the