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

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Abstract – Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a major constituent of rhubarb. Although it has been claimed to have a wild 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 (1,3,8trihydroxy-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 Polygonam 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, vander Hoeven JC, 1984) Lack of emodin genotoxicity was also evident in a mouse micronucleus assay (Mengs U *et*

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al., 1997). 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, benzo(a)pyrene (BaP) and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, USA). The S9 fraction was purchased from Moltox® 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 benzo(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 μl of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5h at 4°C and then for 20min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 μg/ml). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Nottingham, UK).

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 cytochalasin 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-cRNAs 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

absence of S9 was 149.67±17.10 and the MF of bacteria exposed to 10 μg/plate 2-aminofluorene in the presence of S9 was 238.00±20.52. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in revertant. The MF of solvent control bacteria were 24.33±0.58 in the absence of S9 and 24.00±3.00 in the presence of S9. The MF of emodin (3.3, 10, 33.3, 66.6, 100 μg/plate) treated bacteria were 30.00±4.58, 35.67±2.08, 31.33±3.51, 30.00±6.56, 17.67±4.04 in the absence of S9, 29.33±9.24, 24.00±5.29, 27.00±1.73, 21.67± 3.06, 21.33±5.69 in the presence of S9, respectively. Emodin treatments significantly increased revertant numbers in TA98 with or without S9. In TA1535 strain, the MF of 1.5 μg/plate sodium azide treated bacteria in the absence of S9 was 329.00± 39.89 and the MF of bacteria exposed to 10 μg/plate 2-aminofluorene in the presence of S9 was 280.67±64.73. The positive control chemicals, sodium azide and 2-aminofluorene showed large increases in revertant numbers. The MF of solvent control bacteria were 16.67±1.53 in the absence of S9 and 19.00±2.00 in the presence of S9. The MF of emodin (3.3, 10, 33.3, 66.6, 100 μg/plate) treated bacteria were 71.67±11.68, 78.67±2.31, 71.00±7.00, 58.67±15.18, 75.67± 4.16 in the absence of S9, 141.67±27.02, 231.00±21.00, 244.67 ±71.67, 275.67±83.91, 382.33±34.96 in the presence of S9, respectively. Emodin treatments significantly increased revertant numbers in TA1535 with S9.

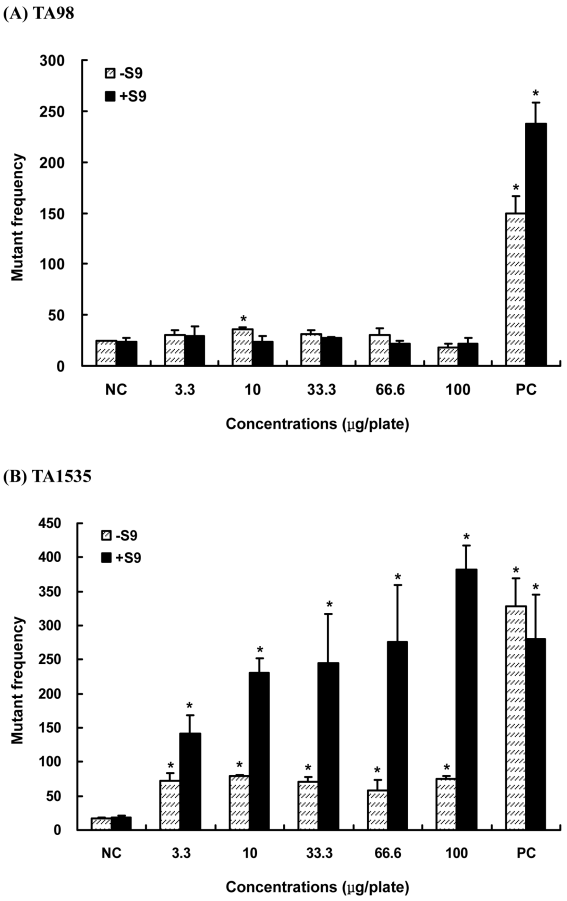

Fig. 1. The mutagenicity of Emodin tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using Salmonella *typhimurium strains* TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. PC: positive control (-S9: 1.0 μg/palte 2-nitrofluorene 1.5 μg/plate sodium azide, +S9: 10 μg/plate 2-aminofluorene)

Emodin induced DNA damage in L5178Y cells

The Olive Tail Moment (OTM) was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lymphoma cells exposed to different concentrations of emodin (7.5- 30 μg/ml) for 2 h (Fig. 2). The OTM of MMS-treated cells (150 μM, positive control in the absence of S9) was 67.98±15.21 and the OTM of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 46.75±9.76. The OTM of control cells was 1.86±1.68 in the absence of S9 and 3.13±2.44 in the presence of S9. Cells were exposed to 7.5, 15, 30 μg/ml emodin for 2 h. OTMs

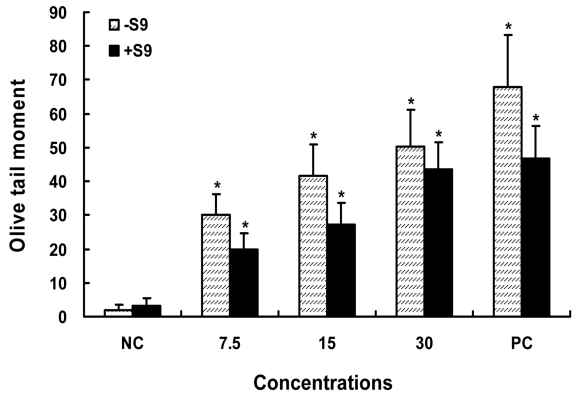

Fig. 2. Olive tail moments by Emodin in L5178Y mouse lymphoma cells. Olive tail moments were measured using comet assay according to Singh et al. (Singh et al., 1988) with slight modification as described in methods. Olive tail moments of L5178Y mouse lymphoma cells exposed to 7.5, 15, 30 μg/ml Emodin for 2h. Negative control was medium. Positive controls were MMS (150 μM) in the absence of S9 and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. NC: negative control. PC: positive control, Data are means ± S.D. (n=15)

induced by emodin were 30.26±6.07, 41.78±9.14, 50.14±10.95 in the absence of S9 and 19.95±4.85, 27.29±6.36, 43.60±7.95 in the presence of S9, respectively. It thus caused a significant increase in DNA damage in comparison to the solvent control and the lowest effective concentration was 7.5 μg/ml.

Emodin induced micronuclei in CHO-K1 cells.

CHO-K1 cells cultured in 24-well plate using RPMI medium and treated with cyclophosphamide (CPA) in the presence of S9. As expected, numbers of micronuclei of cells treated with 2.5, 5, 15 μg/ml CPA were 11.67±4.16, 8.33±2.08, 12.67±3.79 in the absence of S9 and 25.67±4.04, 51.00±5.29, 83.00±5.57 in the presence of S9. The MF of solvent control such as H_2O, DMSO were 7.33±0.58, 7.00±2.00 in the absence of S9 and 9.00±2.00, 9.33±1.53 in the presence of S9. Numbers of micronuclei of 3.75, 7.5, 15 μg/ml emodin treated cells were 5.33±0.58, 8.33±2.31, 10.00±3.61 in the absence of S9 and 10.67±1.15, 12.67±3.21, 7.00±4.36 in the presence of S9, respectively. Increase in the numbers of micronuclei with emodin treatment was statistically significant and concentration-dependent (Fig. 3).

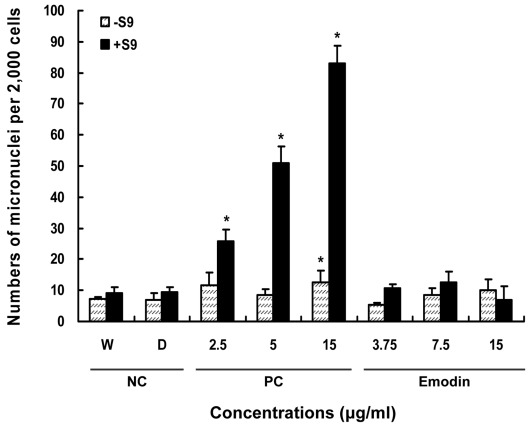

Fig. 3. Micronucleus formation by Emodin in CHO-K1 cells. The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders et al., 2003) as described in methods. 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. Cells were stained with 0.24 mM acridine orange and micronuclei were scored under the fluorescence microscope at 1000 magnification. Data are means ± S.D. n=3 *: Statistically different from concurrent control at $p<0.05$. NC: negative control. PC: positive control (cyclophosphmide 2.5, 5 and 10 μg/ml).

Microarray analysis of differentially expressed genes with emodin treatment in L5178Y cells

Differentially expressed genes from L5178Y cells treated with emodin (30 μg/ml) were analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays. 2816 genes were emodin specifically regulated and their fold of change were greater than Log 2. Among them 462 genes were selected after the Welch's T-test and Volcano plot analysis (Fig. 4). Figure 5 showed the results of clustering analysis of emodin regulated genes. Table I showed genes which expressed were

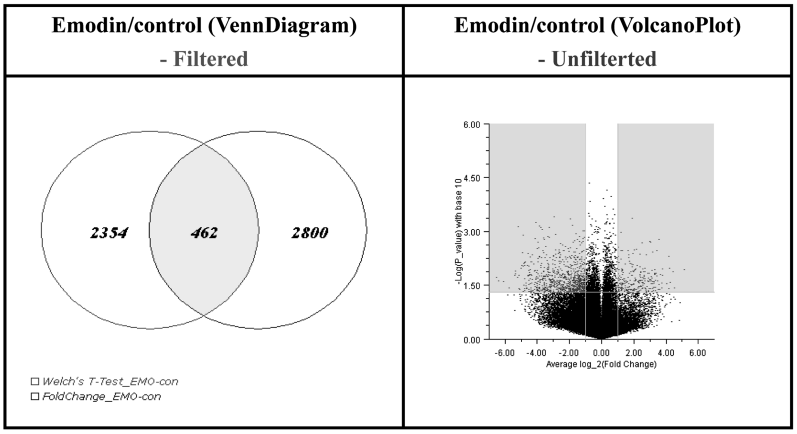

Fig. 4. Numbers of Emodin regulated genes in L5178Y cells. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with Emodin (30 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). Profiles were analyzed by VennDiagram (A) and Volcano Plot (B).

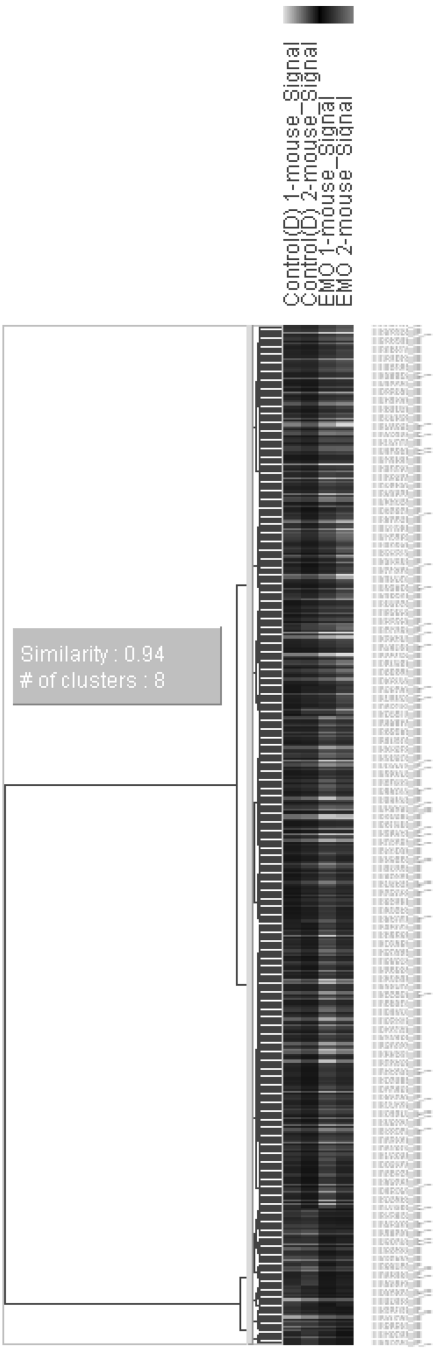

Fig. 5. Results of hierarchical clustering of genes regulated by Emodin. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with Emodin (30 μg/ml), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). The data were analyzed by hierarchical clustering, and green represents down regulation of the transcripts; black, no change; red, up regulation of the transcript.

increased with emodin treatment. If these genes expression would be related to genetic toxicity of emodin, it would need further study.

DISCUSSION

Human exposure to emodin is predominantly oral via ingestion of herbal remedies or wild mushrooms. Estimated maximum human exposure to emodin, based on the emodin content in the constituents of herbal formulations, is 3 mg/kg/day for a 70 kg person. Emodin is biotransformed by the microsomal cytochrome P450 enzymes into hydroxyemodins, some of which are direct mutagens to the test strains and could, therefore, explain the basis for mutagenic nature of emodin (Masuda T and Ueno Y., 1984; Masuda T *et al*, 1985 ; Murakami H *et al.*, 1987; Mueller SO and Lutz WK, 1998). Alternatively, 2-hydroxyemodin, one of the metabolic products of emodin, in turn can produce active oxygen and can induce DNA strand breaks suggesting a possible role of active oxygen in the process of mutagenesis, even though there is a report against the involvement of oxygen. (Kodama M *et al.*, 1987; Bosch R *et al*., 1987) Another conceivable mechanism could be the non-covalent binding of emodin to DNA leading to the inhibition of the catalytic activity of topoisomerase II, at least in part, contributing to emodin-induced genotoxicity and mutagenicity (Mueller *et al*, 1998; Mueller and Stopper, 1999; Muller *et al*., 1996)

To obtain an in depth knowledge regarding its predicted mutagenicity and to verify its historical claims of potential benefits, 2-year genetic toxicology and carcinogenesis studies of emodin were conducted by National Toxicological Program (NTP) of National Cancer Institute (NCI), USA (NTP, 2001). The results showed no evidence of carcinogenic activity for emodin in male F344/N rats and female B6C3F mice and equivocal evidence of carcinogenic activity in female 344/N rats and male B6C3F mice (NTP, 2001). Therefore, assessment of the genotoxicity profile of emodin in light of other data from animal metabolism and rodent carcinogenicity studies do not support concerns that senna laxative components pose a genotoxic risk to humans when consumed under prescribed use conditions (Brusick D and Mengs, 1997). There are reports about antimutagenicity of emodin in Salmonella typhimurium TA98. The crude extracts (containing 3.4 mg of emodin, 2.1 mg of chrysophanol, and 1.8 mg of rhein in 10 g of dry matter) as well as emodin induced a dose-dependent decrease in the mutagenicity of benzo[a]pyrene (B[a]P), 2-amino-3-methylimidazo[4,

Table I. Results of gene ontology analysis.

Accession No	Gene symol	Gene description	Fold change
NM_144906	Sgip1	SH3-domain GRB2-like (endophilin) interacting protein 1	16.91
NM_172966	Sh3rf2	SH3 domain containing ring finger 2	9.56
NM_009397	Tnfaip3	tumor necrosis factor, alpha-induced protein 3	8.89
NM_009465	Axl	AXL receptor tyrosine kinase	6.08
NM_010930	Nov	nephroblastoma overexpressed gene	4.65
NM_011078	Phf2	PHD finger protein 2	3.98
NM_015764	Greb1	gene regulated by estrogen in breast cancer protein	3.88
NM_173738	BC027344	cDNA sequence BC027344	3.77
XM_140497	Zfp508	zinc finger protein 508	3.44
NM_181853	Trim66	tripartite motif-containing 66	3.12

5-f]quinoline (IQ) and 3-amino-1-methyl-5H-pyrido[4,3b] indole (Trp-P-2), and 1-Nitropyrene. On the whole, the mutagenicity of emodin may depend on (i) its activation by microsomal enzymes, (ii) the cell system in which these studies are carried out. It seems when used alone, emodin exhibits mild mutagenicity at least in microbes. Most procarcinogens require metabolic activation to its active form by cellular detoxifying enzymes. Emodin has been shown to inhibit cytochrome P4501A1, thus counteracting the effects of mutagen. 48 Among a panel of 10 anthraquinones, emodin emerged as the most active inhibitor for cytochrome P4501A1 (Mengs U *et al.,* 1997). The reason for the varied effects as shown above by emodin is not clear. Overall, there is no consensus regarding the presence of mutagenicity for emodin by the several in vitro/ vivo assays reported.

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