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TOLFENAMIC ACID SUPPRESSES CYTOCHROME P450 2E1 EXPRESSION IN MOUSE LIVER

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) play a significant role in the chemoprevention of cancer. We recently showed the chemopreventive response of a NSAID, 2-[(3-chloro-2-methylphenyl)amino]benzoic acid) known as tolfenamic acid (TA) in *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in rats. Pre-clinical studies showed that TA inhibits Specificity protein (Sp) transcription factors and acts as an anti-cancer agent in several cancer models; however the pertinent mechanisms associated with its chemopreventive response in esophageal cancer are not known. Since the bioactivation of carcinogens through cytochrome P450 (CYP) is critical for the induction of cancer, we have studied the effect of TA on critical CYP isozymes in mouse liver samples. Athymic nude mice were treated with vehicle (corn oil) or TA (50mg/kg 3 times/wk) for 4 weeks. Protein extracts (whole cell lysates and microsomal fractions) were prepared from liver tissue and the expression of various CYP isozymes was determined by Western blot analysis. Rat (Sprague-Dawley) livers were harvested and primary hepatocyte cultures were treated with vehicle (DMSO) or TA (50 μ M) and cell viability was assessed at 2 and 5 days post-treatment. TA caused remarkable decrease in the expression of CYP2E1 in both liver lysates and sub-cellular fraction, while its response on other tested isozymes was marginal. TA did not affect the body weight of animals (mice) and viability of rat hepatocytes. These results demonstrate that TA modulates the expression of CYP2E1 which is associated with the bioactivation of carcinogens without causing apparent toxicity. These data suggest that TA-induced inhibition of CYP2E1 attenuates the bioactivation of carcinogens potentially leading to the chemoprevention of NMBA-induced esophageal tumorigenesis in rats.

Keywords

Esophageal cancer; chemoprevention; cytochrome P450; CYP2E1; NSAID; Tolfenamic acid

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INTRODUCTION

Esophageal cancer (EC) is the sixth leading cause of cancer deaths worldwide^{1–2}. Squamous Cell Carcinoma (SCC) and Esophageal Adenocarcinoma (EA) are the major types of EC that constitute 90% of esophageal tumors³. Tobacco-related carcinogens such as nitrosamines are closely associated with this malignancy^{4–6}. Epidemiological evidence suggests that populations exposed to food items containing *N*-nitrosomethylbenzylamine (NMBA) could be at higher risk in developing EC^{4, 7–8}. Animal studies in particular documented strong evidence for NMBA-induced initiation of esophageal SCC in F344 rats and this animal model is frequently used in testing the agents for the chemoprevention of EC^{9–13}. Since, current treatment modalities for EC offer poor survival and limited cure, research focusing on developing strategies for the prevention of EC is gaining momentum. Chemopreventive agents are divided into two major classes ‘blocking agents’ and ‘suppressing agents’¹⁴. While the blocking agents act at the initiation stage of carcinogenesis and prevent damage to cellular DNA, the suppressing agents act at the progression stage of carcinogenesis and modulate cell proliferation, apoptosis, angiogenesis and invasion^{14–15}. Agents such as ellagic acid, diallyl sulfide, theaflavin, curcumin, phenylethyl isothiocyanate (PEITC), phenylbutyl isothiocyanate (PBITC), green tea, black tea, resveratrol, and JTE-522 have been extensively studied for the chemoprevention of EC^{15–22}. Food-based products, freeze-dried vegetables²³, fruit juices^{15, 23–25}, and mixtures of vitamins/minerals were previously tested for their efficacy in the prevention of EC^{26–27}. Other chemopreventive strategies under testing include cell proliferation inhibitors, inhibitors of metabolic activation of pro-carcinogens, and the inducers of protective mechanisms²⁸. Synthetic chemopreventive agents have also been tested, but many of these agents often result in hepatic/renal toxicity and the routine uses of such agents are not warranted due to unpredictable health risks and morbidities.

Several lines of evidence suggest that the use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a decrease in the incidence of several cancers including EC^{29–31}. Long-term use of aspirin and other NSAIDs appear to reduce the risk of cancers that originate in gastrointestinal organs, breast, prostate, lung, and skin³². Furthermore, a meta-analysis showed reduced incidence of EC with the use of NSAIDs. This study reported a strong inverse association between SCC and the use aspirin³³. We have recently tested the chemopreventive response of a NSAID, tolfenamic acid (TA). Tolfenamic acid was extensively studied as an anti-cancer agent in pre-clinical studies using several cancer models^{34–38}. We have evaluated the chemopreventive response of TA using NMBA-induced SCC model. TA treatment after the tumor initiation resulted in significant reduction (~65%) of tumor incidence, multiplicity, and tumor volume³⁹. These findings are very novel and show for the first time that TA can be very effective in lowering tumor formation. These results vividly demonstrated the potential of TA as a possible chemopreventive agent against EC. Although TA is known to target specificity protein transcription factors, c-Met, and other candidates associated with tumorigenesis, the pertinent mechanisms for its chemopreventive response are not clear.

Cytochrome P450 isozymes (CYPs)⁴⁰ are superfamily of haemoprotein enzymes found on the membrane of endoplasmic reticulum. CYPs are predominantly localized in liver and are responsible for the metabolism of drugs and other xenobiotics. These enzymes acts as catalysts in the metabolism of drugs and are mainly responsible for hydroxylation and hence acting as monooxygenases, dioxygenases and hydrolases⁴¹.

The process of metabolism takes place in two-steps, namely, Phase I (functionalization) and Phase II (conjugation) in the presence of xenobiotic metabolizing enzymes (XMEs). Phase I XMEs, mostly comprising of CYP enzymes (70–80%) induces metabolic activation of

procarcinogens resulting in reactive oxygenated intermediates (ROMs). Phase II XMEs⁴² are responsible for metabolic inactivation of pharmacologically active substances and biotransformation of reactive intermediates to hydrophilic, easily excretable products by conjugating reactions such as, glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. This results in the detoxication of foreign chemicals. CYP-mediated ROMs derived from various foreign chemicals readily binds to DNA and other proteins owing to their highly reactive electrophilic groups. Thereby, functions as carcinogens and mutagens causing tumor promotion and progression.

CYP consists of a large and diverse group of enzymes that catalyze the oxidation of organic substances and are involved in the metabolism of endogenous substrates including xenobiotics. CYP super family includes a number of isozymes which play role in drug metabolism and bioactivation. CYP2E1 is a major catalyst in the bioactivation of several carcinogens and its participation in the metabolic activation of nitrosamines is well known^{4, 43–44}. Due to the active role of CYP2E1 in the bioactivation of potentially carcinogenic compounds, it is linked to the development of carcinogen-induced human cancers^{4, 45–46}. CYP2E1 is expressed predominantly in the liver, and smaller amounts are also found in a number of extrahepatic tissues. In order to understand the role of TA on various CYP isozymes, we have tested the effect of TA on CYP2E1, 1A2, 3A4, and C9 in mouse liver tissue. Interestingly our results showed selective inhibition of these isozymes following TA treatment.

RESULTS

Tolfenamic acid inhibits CYP2E1 expression in mouse liver lysates

Athymic nude mice were treated with vehicle (corn oil) or tolfenamic acid (50 mg/kg, 3 times/wk) for 4 weeks and liver tissue was isolated. The liver tissue was processed to prepare protein extracts (tissue lysates) and the expression of CYP isozymes, 2E1, 1A2, 3A4, and 2C9 was determined by Western blot analysis as described in the Methods section. TA treatment resulted in a remarkable decrease in the expression of 2E1 while other isozymes are not affected (Figure 1). TA caused more than 70% decrease in the expression of 2E1.

Tolfenamic acid inhibits CYP2E1 expression in mouse liver endoplasmic reticulum

CYP2E1 and other CYP isozymes are largely present in the sub-cellular fraction, endoplasmic reticulum (ER). Mouse liver tissue was processed to prepare ER fraction as described in the Methods section and the expression of CYP2E1 and 1A2 was determined by Western blot analysis. Consistent with results from liver lysate, TA caused immense decrease in the expression of 2E1 while its effect on 1A2 was not significant. TA-induced inhibition of CYP2E1 expression was about 60% (Figure 2).

CYP2E1 expression in rat hepatocytes was not affected by 48 h TA treatment

The animal studies demonstrated the response of chronic (long term) treatment (4 weeks) of TA. In order to investigate the acute response of TA on CYP2E1 expression, we have performed an *in vitro* assay using rat hepatocytes. Primary hepatocytes were isolated from Sprague-Dawley rats as described in the Methods section. Cells were seeded in culture plates and treated with vehicle (DMSO) or 50 μ M tolfenamic acid. Cells were collected at 48 h post-treatment and processed to prepare protein extracts (cell lysates). The expression of CYP isozymes 2E1, 1A2, and 2C9 was determined by Western blot analysis. Interestingly TA showed no inhibitory response on CYP isozymes including CYP2E1 (Figure 3). These results demonstrate that acute (short term) treatment is not enough to induce CYP2E1 inhibition in rat hepatocytes.

Animal growth is not affected by Tolfenamic acid

NSAIDs are known to cause some side-effects including gastro-intestinal damage. Such side-effects may potentially lead to loss of body weight. In order to document the effect of TA on mouse body weight, we have continuously monitored the changes in the body weight of animals (mice) during the experimental period (4 weeks post-treatment) in this experiment. Our results showed that animals tolerated the treatment and did not lose body weight until the termination of the experiment after 4 weeks (Figure 4A).

Tolfenamic acid is not toxic to rat hepatocytes

TA was tested for possible anti-proliferative activity in rat hepatocytes. Primary hepatocytes were isolated from adult male Sprague-Dawley rats as described in the Methods section. Hepatocytes were seeded in culture plates. Cells were treated with vehicle (DMSO) or 50 μ M tolfenamic acid and cell viability was assayed at 2 and 5 days post-treatment as described in methods. TA treatment did not result in cell death and there was no change in the cell viability among control and TA treated samples (Figure 4B) even at 5 days post-treatment.

DISCUSSION

NSAIDs have been widely tested in cancer therapy^{47–50}, and cancer chemoprevention. Some of the mechanisms by which many NSAIDs act as anti-cancer agents are already known. NSAIDs inhibit cancer cell growth through modulation of cell cycle genes, inducing apoptosis and promoting anti-angiogenic activities^{47, 51}. NSAIDs are expected to reduce chronic inflammation, which is a driving force behind the progression of a number of cancers and other diseases. NSAID are known to inhibit the production of cyclooxygenases (COX), and the disruption of this pathway slows the growth of abnormal cells and facilitates the normal process of programmed cell death, or apoptosis. Hence, the anti-cancer activity of NSAIDs was primarily linked to COX-dependent response³¹. The profile of NSAID-induced responses in cancer therapy is well established, recent studies are focused on the development of NSAIDs as a new class of mechanism-based drugs for the prevention of several cancers. Recently, we have shown that a NSAID, tolfenamic acid inhibits esophageal tumorigenesis in NMBA-induced SCC animal model (F344 rats)³⁹; however the indubitable mechanisms associated with such chemopreventive response of this NSAID is not known.

CYP super family consists of numerous isozymes classified 21 families. Among these CYP 1, 2 and 3 account for 70% of total hepatic CYPs content and are responsible for 94% of drugs metabolism in liver⁵². CYPs play a key role in cancer formation and treatment by catalyzing the metabolic activation of various precarcinogens and participate in the inactivation and activation of anticancer drugs. These CYP enzymes are most directly related to the development of cancer as a result of the environment and share substrate specificity. We have chosen a few candidates (CYP2E1, 1A2, 3A4 and 2C9) for screening in this investigation. CYP2E1 accounts for 7% of total CYP content in liver. It participates in the metabolic activation of many low molecular mass procarcinogens (acetaminophen)⁵³ and non-drug environmental toxicants (benzene, ethyl bromide, alcohol, etc.) leading to the development of certain chemically-mediated cancers⁵⁴, while CYP1A2 accounts for 13% of total hepatic content of isoenzymes and is responsible for the *N*²-hydroxylation of arylamine and heterocyclic amine carcinogens^{55–56}. CYP1A2 activity in conjunction with higher *N*-acetyltransferase (Phase II enzyme) activity has been associated with a high risk for colorectal cancer in individuals consuming well-done meat. CYP3A4 is one of the major enzymes for drug metabolism in adults, both, making up to nearly 30% of the total CYP enzymes expressed in the human liver⁵⁷ and it is responsible for oxidation of xenobiotics and drugs and a variety of compounds (eg., steroids, fatty acids, pesticides, and

carcinogens)⁵⁸. CYP2C9 is the most abundant enzyme in the CYP2C subfamily that accounts for about 20% of the total CYP enzymes in human liver. Inhibition of this isoform results in several clinically important drug interactions^{59,60}.

In order to evaluate the underlying mechanisms of TA-induced chemoprevention of SCC, we have treated athymic nude mice with TA and investigated the changes in the above mentioned CYP isozymes. Our results showed selective response on various CYP isozymes. We have examined the expression of 2E1, 1A2, 3A4, and 2C9 expression in mouse liver following four weeks of treatment and found that tolfenamic acid inhibits CYP2E1 expression both in liver lysates (Figure 1) and ER fraction (Figure 2). This inhibition was about 60–70% while other isozymes were un-altered or minimally affected. This data suggest the differential response of TA in modulating CYP family, and in this case specifically inhibiting CYP2E1. It is not clear if the response of TA is due to chronic (4 weeks) treatment or an early event. So we conducted an *in vitro* experiment using rat hepatocytes. When hepatocytes were treated with 50 μ M of TA for 48 h, there was no discernible change observed in all CYP isozymes tested (Figure 3). These results support the idea that chronic treatment of TA is required to modulate the expression of CYP2E1 in animal models.

CYP2E1 is a major catalyst in the bioactivation of several carcinogens and participates in the metabolic activation of nitrosamines including NMBA^{44, 61}. Bioactivation of carcinogens through CYP enzyme system is critical especially in chemical or dietary related carcinogenesis. Several CYP isozymes are known to be involved in the drug metabolism and biotransformation mechanisms. It is known that CYP2E1 participates in carcinogen activation and promotes lipid peroxidation by the generation of activated oxygen species during turnover in the absence and presence of substrates^{62–63}. Since CYP2E1 is critical in the bioactivation of pre-carcinogens to transform into ultimate carcinogens, the changes in the expression of this isozyme could affect the efficacy of carcinogens in causing cancer. In order to understand the role of TA on various CYP isozymes we have tested the effect of TA on CYP2E1, 1A2, 3A4 and C9 in liver tissue of mice treated with TA. Interestingly our results showed a selective inhibition of these isozymes following TA treatment. As presented in Figure 5A, tolfenamic acid inhibits the expression of CYP isozymes involved in the bioactivation of carcinogens and potentially impeding carcinogenesis. The mechanism of NMBA-induced tumorigenesis in rat esophagus is shown in Figure 5B. Initially, CYP enzymes cause the hydroxylation of methylene carbon forming α -hydroxynitrosamine which spontaneously disintegrates into two chemical moieties, methyldiazohydroxide and benaldehyde. Methyldiazohydroxide methylates guanine residues at N⁷ and O⁶ positions and form DNA adducts. O⁶- methylguanine adducts are poorly repaired, become carcinogenic (Figure 5B) and ultimately leading to tumorigenesis.

Liver is the major site for CYP metabolism. CYP content is very high in liver and esophageal microsomes expresses only 7% of hepatic microsomal CYP⁶⁴. Apparently, CYP2E1 is expressed predominantly in the liver; however smaller amounts are found in some extra-hepatic tissues. Several studies collectively indicated that CYP2E1 is a major catalyst in the oxidation of benzene, styrene, carbon tetrachloride, vinyl chloride and other hazardous chemicals which are known to cause cancer^{44, 65–66}. Metabolic activation of pre-carcinogens is an important step in carcinogenesis. For example, ethanol decomposes to induce genotoxic effect and oxidative stress thereby leading to the generation of carcinogenic etheno-DNA adducts 1,N(6)-ethenodeoxyadenosine and 3,N(4)-ethenodeoxycytidine. Induction of liver CYP2E1 is significantly correlated with the generation of these carcinogenic DNA adducts⁶⁷. As the role of CYP2E1 is very evident in the bioactivation of pre-carcinogens to ultimate carcinogens, suppressing the activity of this isozyme may be a plausible mechanism of chemopreventive action. Studies also reported the

association between CYP2E1 genetic polymorphism and the cancer incidence among human population in certain parts of the world⁶⁸. A recent meta-analysis involving 11 case controlled studies with 1088 cases and 2238 controls, demonstrated that CYP2E1 polymorphism was linked to the decreased risk of developing EC among Asian populations⁶⁹.

In order to test the toxic response of TA, we have monitored the changes in the body weights of mice over 4 weeks (Figure 4A). We also tested the effect of TA on the cell death of rat hepatocytes. We did not observe cell death even at 5 days post-treatment (Figure 4B). Since primary cultures are difficult to maintain long term, we conclude that TA has minimal effect on causing cell death in rat hepatocytes. These results confirm that TA-induced inhibition of CYP2E1 in mouse and rat hepatocytes occurs without causing apparent toxicity. The development of CYP2E1 inhibitors is under testing for the therapeutic application and to understand the mechanism of CYP2E1-mediated toxicity. Curcumin is shown to reduce esophageal cancer in F344 rats potentially affecting the initiation and progression phases following exposure to NMBA. CYP2E1 inhibitors such as disufiram, pyrazole, 4-methylpyrazole, and chlormethiazole causes some toxic responses, hence there is a need for developing compounds that effectively diminishes CYP2E1 and are also innocuous for a long term use. In this context TA may be a promising candidate for such an application.

The etiology of EC clearly identifies the significant role of tobacco as a risk factor along with others such as excessive alcohol consumption, exposure to organic solvents, nitrosamines, benzopyrenes etc., and intake of hot beverages. Although the association of the above agents in causing EC is well established, the risk appears to be impacted by (healthy) life styles and dietary habits (taking natural antioxidants and other dietary chemopreventive agents)⁷⁰. Limitations in conventional therapy and rapid invasiveness of certain malignancies highly impact mortality rates in cancer patients. Hence there is an urgent need for developing intensive approaches for cancer prevention. Several agents have been tested for the chemoprevention of cancers; however the difficulties in understanding precise mechanisms of these candidates, and assessing the response due to long-term use still poses major setbacks in this field of research. In this context here we introduce tolfenamic acid, a NSAID with limited side-effects for potential implications in the chemoprevention of EC. The results presented in this investigation shows strong response of TA in modulating the expression of CYP2E1 consequently changing the course of carcinogenesis. These results are interesting and in fact promising. Since CYP family is involved in the metabolism of drugs as well as toxins, if TA shows a global response inhibiting many isozymes, it might also exhibit interactions with concomitantly administered drugs. If the actions of TA is selectively on the isozymes that are primarily participating in metabolic activation of carcinogens, tolfenamic acid could serve as an ideal candidate for testing in the chemoprevention of human cancers. However, it would require extensive investigations to exclude its involvement with all other relevant drug metabolizing CYP isozymes. Experiments to further evaluate the effect of TA on the activity of CYP2E1 in liver of mouse and rat and the expression/activity of other important isozymes in liver and other organs are under investigation.

MATERIALS AND METHODS

Cell culture

Primary hepatocytes were isolated from adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN). Hepatocytes were isolated by using collagen perfusion as described previously^{71–72}. Briefly, liver was perfused first with Krebs's buffer (121 mM NaCl, 25 mM NaHCO₃, 4.8 mM KCl, 2 mM MgSO₄, 1.2mM KH₂PO₄) and then with type

II collagenase (Worthington Biochemical Corp., Lakewood, NJ) in Kreb's buffer. After liver perfusion, hepatocytes were collected, pelleted and washed twice with RPMI1640 (Life Technologies, Grand Island, NY). Hepatocytes were re-suspended in RPMI1640 supplemented with 1 μ M dexamethasone (Sigma, St. Louis, MO), 100 nM insulin (Sigma-Aldrich Corp, St. Louis, MO), 5% FBS (Life Technologies, Grand Island, NY), 1% penicillin streptomycin (Sigma-Aldrich Corp, St. Louis, MO). Cells were seeded on a 60 mm collagen coated dish (BD Biosciences, San Jose, CA) with the density of 3 $\times$ 10⁶ cells per plate. After 4h, unattached cells were removed and fresh media was added followed by overnight incubation.

Animal Treatment/Tissue collection

Female athymic nude mice (6–8 week old) were purchased from National Cancer Institute (Bethesda, MD) and housed at University of Central Florida (UCF) animal facility. After acclimatization, mice were treated with vehicle (corn oil) or 50 mg/kg tolifenamic acid 3 times/wk for 4 weeks. Animal body weights were measured and euthanized. Liver from control and treated groups were isolated and snap frozen in liquid nitrogen. All the procedures were approved by the Institutional Animal Care and Use Committees of MD Anderson Cancer Center Orlando and UCF.

Preparation of hepatic endoplasmic reticulum (ER)

ER was prepared from either mouse liver tissue or isolated rat primary hepatocytes using the same methodology as described previously^{73–74}. In brief, samples were washed with phosphate buffered-saline (PBS) and re-suspended in buffer A (0.25 M sucrose in 10 mM Hepes, pH 7.2) containing 0.5 mM EDTA and protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN). Samples were then homogenized in a Parr bomb using 1100 psi for 40 minutes followed by the isolation of ER^{73–76}. The ER was purified using a sucrose step density gradient^{73–76}. The purity of ER was confirmed by immunoblotting for ER marker protein.

Cell viability assay

Cell viability was measured using CellTiter Glo kit (Promega, Madison, WI) according to instructions provided by the supplier. Briefly, cells (hepatocytes) were plated in 96-well white walled clear bottom plate (Lonza, Basel, Switzerland) and treated with vehicle (DMSO) or 50 μ M TA. At 2 and 5 days post-treatment, 100 μ l of assay reagent was added to each well, mixed and the plate was incubated in dark for 15 min. Luminescence values were obtained measuring the plate using Optima plate reader. All treatments were performed at least in triplicate.

Western immunoblot

Protein extracts were prepared from control (DMSO) and TA-treated hepatocytes or the liver tissue harvested from mice and the expression of CYP isozymes, 2E1, 1A2, A3 and C9 was determined through Western immunoblot. Hepatocytes were cultured in 100 mm dish and treated with vehicle (DMSO) or 50 μ M tolifenamic acid for 48 h. Cells were collected, washed once with PBS and re-suspended in cell lysis buffer containing protease inhibitor. Liver tissue was briefly homogenized in the cell lysis buffer with protease inhibitor. The samples were used to determine the protein expression by Western immunoblot as described before and the respective bands were scanned and quantitated using Kodak imaging system software^{34,35,39}.

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Figure 1. Tolfenamic acid inhibits CYP2E1 expression in liver lysates

Mice were treated with 50 mg/kg 3 times/wk for 4 weeks. Liver tissues were processed to prepare tissue lysates and the expression of various CYP isozymes 2E1, 1A2, 3A4 and 2C9 was determined by Western blot analysis. β -actin was used as loading control. The Western blot results were quantitated by densitometric analysis of bands using Kodak imaging system. Bars (arbitrary units-normalized over β -actin) in Figure A represent the mean $\pm$ S.D. of 3–4 samples. Representative gels for each isozyme was presented in the Figure (1B). Con: Control; TA: Tolfenamic Acid

Figure 2. Tolfenamic acid inhibits CYP2E1 expression in liver microsomes

Mice were treated with 50 mg/kg 3 times/wk for 4 weeks. Liver tissues were processed to prepare microsomal fraction (endoplasmic reticulum). The expression of CYP2E1 and 1A2 was determined by Western blot analysis. The bands of the immunoblots for CYP2E1 were quantitated by densitometric analysis using Kodak imaging system. Calreticulin was used as loading control as well as for confirming the purity of the ER fraction. Bars (arbitrary units-normalized over β -actin) in the figure represent the mean $\pm$ S.D. of 3–4 samples.

Representative gels were shown in the insert.

Con: Control; TA: Tolfenamic Acid

Figure 3. Expression of CYP isozymes in hepatocytes was not affected by 48 h TA treatment
Hepatocytes were cultured from Sprague-Dawley rat livers as described in the Methods and treated with 50 μ M tolfenamic acid for 48 h. Cells were harvested, protein extracts were made and the expression of CYP isozymes, 2E1, 1A2 and 2C9 was determined by Western blot analysis. β -actin was used as loading control. Representative gels were shown in the figure.

Con: Control; TA: Tolfenamic Acid

Figure 4. Effect of tolfenamic acid on mice body weights

(A) Athymic nude mice were treated with vehicle (Control) or TA (50 mg/kg 3 times/wk for 4 weeks). The changes in the body weight of Control and TA groups were continuously monitored. Data shown is the mean $\pm$ S.D. of body weight of control and TA group animals (n=5–6) at the termination of the experiment. (B) Rat hepatocytes were treated with vehicle (DMSO) or 50 μ M tolfenamic acid. Cell viability was measured at 2 and 5 days post-treatment using Cell Titr Glo (Promega) kit. Data represent mean $\pm$ S.D. of 3 independent determinations.

Con: Control; TA: Tolfenamic Acid

Figure 5. Proposed role of tolfenamic acid in the metabolic activation of NMBA

(A) Cytochrome P450 enzymes play critical role in carcinogenesis by participating in the bioactivation of carcinogens. Tolfenamic acid inhibits the expression of critical CYP isozymes thereby changing the course of carcinogenesis. (B) NMBA. Hydroxylation of methylene carbon by CYP enzymes produces α -hydroxynitrosamine which spontaneously splits into methyldiazohydroxide and benzaldehyde. Methyldiazohydroxide forms methylcarbonium ion that methylates guanine residues at N⁷ and O⁶ and form N⁷-methylguanine and O⁶-methyl guanine. O⁶-methyl guanine adducts are poorly repaired and eventually leading to carcinogenesis. When tolfenamic acid is given to the animals, it modulates the CYP enzyme system and potentially inhibits the bioactivation of NMBA and disrupts carcinogenesis.