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Comparison of 2, 2-Bis (bromomethyl)-1, 3-propanediol induced genotoxicity in UROtsa cells and primary rat hepatocytes: relevance of metabolism and oxidative stress

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Abstract

2, 2-Bis (bromomethyl)-1, 3-propanediol (BMP) is a brominated flame retardant used in urethane foams and polyester resins. In a two year dietary study, BMP caused neoplastic lesions at multiple sites including the urinary bladder of both rats and mice. However, liver was not a target tissue. We previously reported that BMP elicited oxidative DNA damage in a human uroepithelial cell line (UROtsa). The present *in vitro* study investigated the susceptibility of target (UROtsa cells) and non-target cells (primary rat hepatocytes) to BMP-induced genotoxicity. In contrast to hepatocytes, BMP exhibited greater genotoxic potential in UROtsa cells as evidenced by the concentration dependent increase in DNA strand breaks and DNA binding. Total content of intracellular GSH quantified in UROtsa cells (2.7 ± 1.0 nmol/mg protein) was 4 fold lower than that in hepatocytes (10.7 ± 0.3 nmol/mg protein). HPLC analysis indicated BMP was not metabolized and/or consumed in UROtsa cells at any of the concentrations tested (10–250 μ M) but was extensively converted to a mono-glucuronide in hepatocytes. These results demonstrate that a target cell line such as UROtsa cells are more susceptible to BMP-induced DNA damage when compared to non-target cells. This increased susceptibility may relate to the deficiency of antioxidant and/or metabolic capabilities in UROtsa cells.

Keywords

2, 2-Bis (bromomethyl)-1, 3-propanediol; UROtsa cell; primary rat hepatocyte, cell susceptibility; metabolism; oxidative stress

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

1. Introduction

2, 2-Bis (bromomethyl)-1, 3-propanediol (BMP) is a brominated flame retardant widely used in the manufacture of urethane foams, polyester resins and molded products. It has been classified as a high-production-volume chemical by the U.S. Environmental Protection Agency (EPA) since 1990, indicating that annual production or import exceeded 1 million pounds (EPA, 2006). The high production volume of BMP has resulted in environmental contamination as it has been identified in dust particles and wastewater (EPA, 1983). EPA's report refers to it as a compound that is anticipated to last in water for long periods of time (EPA, 1983). The estimated half life of BMP in ground water is over 100 years (Ezra et al., 2006; Ezra et al., 2010). Due to its persistence in the environment, humans may be exposed to BMP by multiple routes such as inhalation, oral and dermal contact (NTP, 1996).

The National Toxicology Program (NTP) reported that in a two year dietary feeding study BMP exposure caused neoplastic lesions at multiple sites in both genders of rats and mice (NTP, 1996). Some of the affected tissues were in direct contact to BMP, such as oral cavity and gastrointestinal tract. This suggested that the parent compound was most likely the causative agent. However, neoplasms observed at other sites (kidney, urinary bladder, lung, mammary gland etc.) required systemic exposure to the absorbed chemical and/or its metabolites. Interestingly, liver was not a target tissue for BMP in either species. As the principal organ in biotransformation processes of exogenous substances, the liver would be expected to metabolize BMP and be exposed to its metabolites. Results of previous *in vitro* studies in our laboratory have shown that liver preparations extensively convert BMP to a water-soluble mono-glucuronide conjugate. No other metabolites were detected (Rad et al., 2010). When BMP was administered to F344 rats, either as a single dose (IV or PO) or repeated daily doses for 10 days (PO), the only metabolite detected in plasma and urine was BMP-glucuronide (Hoehle et al., 2009).

Since glucuronidation is the key metabolic process that governs the clearance of BMP from the body, its role in BMP-induced genotoxicity was investigated in the studies reported here. They build upon our observation that BMP induces strand breaks, as assessed by the comet assay, in human bladder epithelial cells (UROtsa; considered a target cell population) (Kong et al., 2011). Specifically, the *in vitro* studies compare the genotoxic potential (induction of DNA strand breaks and covalent binding to DNA) of BMP in UROtsa cells (target cells) and primary rat hepatocytes (non-target cells) and relate these outcomes to the glucuronidation capacity of these two cell types. In addition, as BMP-induced oxidative stress plays a key role in BMP-associated DNA damage (Kong et al., 2011), the basal level of intracellular GSH content and the effect of BMP on GSH levels were assessed in both the target cells and non-target cells.

2. Material and methods

2.1 Chemicals

Radioactive ($[^{14}\text{C}]$ -labeled) BMP (Lot No. 10426-17-34) in absolute ethanol (1 mCi/ml) with a reported specific activity of 65.1 mCi/mmol (247 $\mu\text{Ci}/\text{mg}$) was received from Midwest Research Institute (Kansas City, MO) and stored at 4°C. The radiochemical purity of BMP was reported to be 97.3%, which was confirmed by HPLC-radiometric analysis. This radiochemical purity was monitored routinely over the course of the studies. Non-radiolabeled BMP (Lot No. 04119MD) was obtained from Sigma-Aldrich (St. Louis, MO) as a crystalline solid and stored at room temperature. Stated chemical purity was given as 98%. Non-radiolabeled BMP was dissolved in and serially diluted with 100% EtOH for cell culture dosing. $[^{14}\text{C}]$ -BMP dosing solutions were prepared by dissolving the appropriate amount of non-radiolabeled and radiolabeled compound in EtOH.

Hydrogen peroxide was purchased from JT Baker (Phillipsburg, NJ) and was diluted with sterilized distilled water (dH₂O) before use. Dulbecco's Modified Eagle Medium (DMEM), Liver Digest Medium, Williams Media E (WME), Hank's balanced salt solution (HBSS), penicillin-streptomycin, trypsin-EDTA, L-glutamine and trypan blue were acquired from Gibco Invitrogen Corporation (Carlsbad, CA) and fetal bovine serum (FBS) from Atlanta Biologicals (Lawrenceville, GA). Flo-Scint III and Pico-Fluor 40 scintillation cocktail solutions were received from PerkinElmer (Torrance, CA). Other chemicals and general reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise, and used without further purification.

2.2 Biological materials

2.2.1 Animals—For preparation of hepatocytes, male SD rats, 10–12 weeks of age (250–325 g), were obtained from Harlan Laboratory, Inc. (Indianapolis, IN). They were housed in the University of Arizona Animal Care Facility (UAC) which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Upon receipt, the animals were taken to a designated animal room where they were acclimated for 5–7 days in polyethylene cages (two animals per cage) before being used in the experiments. The room temperature was maintained between 20–23 °C and the relative humidity between 40–60%. A light/dark cycle was maintained at 12 hour intervals. Biannual testing for coliform and nitrates was performed by the UAC Diagnostics Laboratory Medical Technologist. During the acclimation period, rats were fed standard commercial diets (Harlan Teklad 4% rodent diet, Harlan, Indianapolis, IN) and were provided food and water for *ad libitum* consumption.

2.2.2 UROtsa cell culture—UROtsa cells were generously provided by Drs. Donald and Maryann Sens (University of North Dakota, Grand Forks, ND). This cell line was generated from the normal human bladder epithelium and immortalized with the SV-40 large T antigen (Rossi et al., 2001). Cell culture conditions were as previously described by (Kong et al., 2011; Wnek et al., 2011).

2.2.3 Rat hepatocytes preparation—Primary hepatocytes from male SD rats were prepared in-house via a two stage perfusion method as described by (Pritchett et al., 2002). Only those cell preparations with >80% viability (as determined by trypan blue exclusion) were used for primary culture. For the 1 h incubation with BMP, freshly isolated hepatocytes were diluted with WME to achieve a concentration of 5×10^5 cells/ml. For 24 h exposures, isolated hepatocytes were subjected to low speed centrifugation ($50 \times g$ for 10 min), harvested and re-suspended in a plating medium containing 10% FBS, 10 µg/ml insulin, 100 U/ml penicillin and 10 µg/ml streptomycin in DMEM. The hepatocytes were then plated in the 6-well cell culture plates at a concentration of 5×10^5 cells/ml. Following the plating procedure the cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C for 15 h. The culture media was then replaced with fresh plating medium containing BMP and the cells were incubated as described above for 24 h.

2.3 Single cell gel electrophoresis assay

The single cell gel electrophoresis assay (comet assay), was applied to assess DNA strand breaks induced by BMP in the cells. The assay was performed as previously reported by (Kong et al., 2011). Briefly, UROtsa cells (5×10^5 cells/ml) and freshly isolated, suspended hepatocytes (5×10^5 cells/ml) were incubated with BMP (10–100 µM) at 37 °C for 1 h. All control and treated cultures received the same final concentration of vehicle (0.5% EtOH). Hydrogen peroxide (50–500 µM) was used as positive control. The experiment was repeated at least three times independently.

2.4 Cell-based BMP metabolism assay

Hepatocytes (5×10^5 cells/ml) in suspension or as plated were incubated at 37 °C with [^{14}C]-BMP (10–250 μM) for 1 h (shaking incubator) or 24 h (cell culture incubator), respectively. UROtsa cells were seeded in 6-well plate at 5×10^5 cells/well and maintained for 48 h until chemical exposure. UROtsa cells were exposed to [^{14}C]-BMP (10–250 μM) alone, [^{14}C]-BMP (10–250 μM) with UDPGA (1 mM) or [^{14}C]-BMP (10–250 μM) with NADPH (1 mM) for 1 or 24 h in a cell culture incubator. For these incubations, the specific activity of [^{14}C]-BMP ranged from 2.1 to 50 mCi/mmol (100 $\mu\text{Ci/ml}$). At the end of incubation, aliquots (200 μl) of the medium were mixed with ice cold methanol (1:1) and centrifuged at $10,000 \times g$ for 10 min. The supernatants were subjected to HPLC analysis. The incubations were conducted in triplicate three independent times (i.e. hepatocytes derived from three different rats).

To test whether UROtsa cells possess β -glucuronidase activity, cells were incubated with the conditioned medium containing [^{14}C]-BMP-glucuronide (10 μM , biosynthesized by microsomal incubation) or [^{14}C]-BMP (10 μM) for 24 h at 37 °C. Aliquots (200 μl) of the medium were then mixed with ice cold methanol (1:1) and centrifuged at $10,000 \times g$ for 10 min. The supernatant was analyzed by HPLC. Incubation of β -glucuronidase (5000 U/ml) with the [^{14}C]-BMP-glucuronide in DMEM for 24 h at 37 °C was performed at the same time to validate the assay.

2.5 Measurement of DNA binding

Following incubation with [^{14}C]-BMP for 1 h or 24 h, the cell pellets or attached cells were washed with fresh cold PBS three times and lysed by DNA digestion buffer (50 mM Tris PH=7.6, 1 mM EDTA and 1% SDS). The cell lysates were incubated with Proteinase K (1 mg/ml) and RNase (1 mg/ml) for 1 h at 55 °C. DNA was extracted by the standard phenol/chloroform method and precipitated by ethanol/acetate as described by (Kirby and Cook, 1967). The amount and purity of extracted DNA was quantified from the absorbance at 260nm (NanoDrop 2000C Spectrophotometer, Thermo Fisher Scientific). The A_{260}/A_{280} and A_{260}/A_{230} ratios (A_{260}/A_{280} 1.8 and A_{260}/A_{230} 2.3) indicated that this phenol/chloroform extraction yielded high quality/highly purified DNA. The total radioactivity per sample was assessed by LSC using Beckman LS 3801 Liquid Scintillation Counter (Brea, CA). The radioactivity measured in the DNA extracted from the non-radiolabeled chemical exposed cells was used to correct for the background. The results are expressed as pmol [^{14}C]-BMP equivalents/mg DNA. The experiments were repeated three times in triplicate.

2.6 HPLC analysis

A method for reversed phase HPLC-radiometric analysis of [^{14}C]-BMP and its metabolites was developed for qualitative and quantitative analysis of [^{14}C] radiolabeled chemical entities. The details have been reported by (Hoehle et al., 2009). In this study, 10 to 50 μl of samples were injected onto a 250×4.6 mm i.d., 5 μm reversed phase Luna C18 column coupled with a 4.0×3.0 mm i.d. Security Guard C18 guard cartridge (Phenomenex, Torrance, CA). Data were acquired and analyzed using ChemStation for LC 3D (Revision: B.01.01, Agilent Technologies) and WinFlow software (Version 1.5; LabLogic, Sheffield, UK). The limit of detection (LOD) was 0.72 $\mu\text{g/ml}$. The limit of quantification (LOQ) is 2.15 $\mu\text{g/ml}$ based on the signal-to-noise of five-to-one as the minimal signal determination.

2.7 Glutathione assay

Total glutathione (GSH) content of UROtsa cells and hepatocytes was measured by a GSH assay kit from Cayman Chemical (Ann Arbor, MI). The assay was performed according to the instructions included in the kit. Briefly, UROtsa cells and freshly isolated hepatocytes

were incubated with or without BMP (25–500 μ M) for 1 h. In some incubations, potassium bromate (KBrO_3 , 2 mM) or N-acetyl-L-cysteine (NAC, 2 mM) were incubated with cells for 1 h as positive controls. Following collection by centrifugation ($1000 \times g$ at 4°C), cell pellets were sonicated in cold MES buffer (50 mM 2-(N-morpholino)ethanesulfonic acid containing 1mM EDTA, pH 6~7). Cell lysates were centrifuged at $10,000 \times g$ for 15 min at 4°C and supernatant was collected. After estimation of total protein concentration by the Bradford assay (Bradford, 1976), an aliquot of supernatant was deproteinized by adding an equal volume of 10% metaphosphoric acid followed by centrifugation at $3000 \times g$ for 15 min. Next, 1 ml of supernatant was collected and mixed with 50 μ l of triethanolamine (4 M). 50 μ l of each sample was added to each well of a 96-well plate that contained 150 μ l of the freshly prepared assay cocktail (containing glutathione reductase, cofactors and DTNB). Following 25 min incubation in the dark, absorbance in the wells was measured at 412 nm using a micro-plate reader (BioTek, Winooski, VT). The concentration of GSH in a test sample was determined by calculating from the regression curve generated from several standards of GSH. Results were expressed as nmol/mg protein. Each assay was done in triplicate and repeated three times.

2.8 Data analysis

All data are presented as means $\pm$ SEM. Data sets were subjected to unpaired t-test or one way ANOVA followed by Dunnett's post-hoc analysis (GraphPad Prism 5, GraphPad software, Inc). $P < 0.05$ was accepted as the level of significance.

3. Results

3.1 Cell susceptibility to BMP-induced genotoxicity: DNA strand breaks and DNA binding

When incubated with freshly isolated rat hepatocytes for 1 h, BMP (25–100 μ M) did not increase the percent of DNA in the comet tail ($T_{\%DNA}$) at any of the tested concentrations compared to vehicle control (Fig 1A). The positive control, H_2O_2 , caused DNA strand breaks in hepatocytes as the $T_{\%DNA}$ was significantly increased at concentrations of 100 and 500 μ M. In UROtsa cells, incubation with BMP (10–100 μ M) for 1 h resulted in a concentration dependent increase in the $T_{\%DNA}$. UROtsa cells were also more sensitive to H_2O_2 than hepatocytes when the $T_{\%DNA}$ was compared at the 50 μ M, a concentration used for both cell types (Fig 1B).

To assess the covalent DNA binding in these two cell populations, UROtsa cells and rat hepatocytes (suspended or plated) were incubated with [^{14}C]-BMP (10–250 μ M) for 1 and 24 h, respectively. Following 1 h of incubation, the amount of [^{14}C]-BMP equivalents bound to DNA was similar for hepatocytes and UROtsa cells (Fig 2A). It was in the range of 5–98 pmol/mg DNA for UROtsa cells and 3–77 pmol/mg DNA for hepatocytes. However, after 24 h of incubation with [^{14}C]-BMP, the extent of binding of [^{14}C]-BMP equivalents to DNA was clearly different. At this time point, the extent of binding measured in rat hepatocytes (13–111 pmol/mg DNA) was significantly lower than that measured in UROtsa cells (48–1041 pmol/mg DNA) at each concentration of [^{14}C]-BMP (Fig 2B).

3.2 Glucuronidation of BMP by primary rat hepatocytes and UROtsa cells

The data presented in Fig 3 and Fig 4 show rat hepatocytes converted BMP to BMP-glucuronide with no evidence of other metabolites being formed. The extent of glucuronidation by rat hepatocytes was dependent on the substrate ([^{14}C]-BMP) concentration and duration of exposure. The data presented in Fig 5 show the concentration dependent, stoichiometric conversion of BMP to BMP-glucuronide by primary rat hepatocytes at 1 h and 24 h. [^{14}C]-BMP was extensively metabolized at low substrate concentrations (10–50 μ M). As the substrate concentration increased, the extent of

glucuronide formation (expressed as a percentage of the administered BMP) decreased. Correspondingly, the percentage of parent BMP remaining in the incubation increased. When BMP was incubated with UROtsa cells, no BMP glucuronide was formed and no other metabolites were detected under any conditions that were tested. These conditions included the addition to the incubation of 1 mM UDPGA (a co-factor for glucuronidation) or NADPH (a co-factor for CYP450 mediated oxidative metabolism). Essentially, all of the radioactivity obtained after incubation of [^{14}C]-BMP with UROtsa cells eluted from the HPLC column as a single peak at 13.5 min, which is the retention time of [^{14}C]-BMP. The column recovery was >95% in all the samples.

The minor peaks that eluted at 3–5 min, which accounted for < 4% of total radioactivity applied to the column, were present in all analyzed samples in the current study and in previous studies (Hoehle et al., 2009; Rad et al., 2010). These peaks did not change with substrate concentration or incubation time. They seem to be the degradation products of a low level ^{14}C -contaminant present in [^{14}C]-BMP.

3.3 Measurement of the basal level of intracellular GSH

The basal level of GSH, a major intracellular antioxidant molecule, was compared in UROtsa cells and hepatocytes. The total content of intracellular GSH in UROtsa cells (2.7 ± 1.0 nmol/mg protein) was 4 fold lower than that in rat hepatocytes (10.7 ± 0.3 nmol/mg protein) (** $P < 0.01$).

3.4 Depletion of GSH by BMP in primary rat hepatocytes and UROtsa cells

Incubations of hepatocytes for 1 h with BMP (100 and 500 μM) significantly decreased the intracellular concentration of GSH. This depletion of GSH was not observed in UROtsa cells incubated with the same concentrations of BMP for 1 h. Potassium bromate (KBrO_3 , 2 mM), the positive control used in the assay, significantly depleted intracellular GSH in both cell types. Administration of NAC (2 mM) as an exogenous source of thiols and a GSH precursor significantly increased the intracellular GSH in UROtsa cells (Fig 6A and B).

4. Discussion

The present studies were conducted to investigate the susceptibility of two different cell types to BMP-induced genotoxicity and to determine potential mechanisms to explain the observed differences. First, the genotoxicity of BMP, with respect to its induction of DNA strand breaks and its degree of covalent binding to DNA, was evaluated in target cells (UROtsa) and non-target cells (rat hepatocytes). Second, the formation of BMP-glucuronide, the predominant and only identified metabolite of BMP, was compared in these two cell types. Finally, the total intracellular GSH content in these two cell populations, as a marker of antioxidant activity, and the ability of BMP to alter GSH were assessed. The results indicated the target cell line is more susceptible to BMP-induced genotoxicity when compared to non-target cells. This increased susceptibility may relate to the deficiency of antioxidant and/or metabolic capabilities in the target cells.

In the current study BMP significantly increased the extent of DNA strand breaks in UROtsa cells compared to vehicle control following 1 h of exposure. However, this DNA damaging effect of BMP was not observed in rat hepatocytes under the same conditions applied to UROtsa cells. In addition, the covalent binding of BMP to DNA was significantly lower in rat hepatocytes than that in UROtsa cells following 24 h of exposure. One important reason for this difference is rat hepatocytes can convert BMP to a less DNA reactive metabolite, an ether glucuronide. BMP was extensively metabolized to BMP-glucuronide in rat hepatocytes at low concentrations (10–50 μM). At BMP concentrations of 125 μM or higher, the extent

of glucuronidation decreased when expressed as a percentage of BMP concentration. Importantly, there was no evidence of other metabolites being formed at all conditions (concentration and incubation time). These results are consistent with previous *in vivo* and *in vitro* studies conducted in this species (rat). Incomplete conjugation of BMP and persistence of free parent BMP at high concentrations is most likely due to the concentration dependent saturation of UDP-glucuronosyltransferases (Court et al., 2001). Similarly, *in vivo* results obtained in rats indicated a saturation of the BMP glucuronidation capacity at higher doses (Hoehle et al., 2009). Unlike rat hepatocytes, UROtsa cells lack the ability to biotransform BMP. HPLC analysis indicated BMP was not metabolized and/or consumed in incubations with UROtsa cells (up to 24 h of incubation) at any of the concentrations tested (10–250 μ M). Addition of metabolic co-factors (UDPGA and NADPH) to the culture media also did not result in the formation of a BMP-glucuronide or other metabolites. In addition, results of a preliminary study (unpublished data) with BMP-glucuronide, which was biosynthesized by and isolated from microsomal incubations, demonstrated the glucuronide metabolite bound less to DNA and produced less DNA strand breaks than BMP. Taken together, these data support a detoxification role of glucuronidation in BMP associated genotoxicity.

An interesting observation is that the degree of covalent binding of [14 C]-BMP equivalents to cellular DNA was similar in both cell types at 1 h but clearly different at 24 h. This difference observed at 24 h may reflect a lag time necessary for the induction of UGTs in hepatocytes. Induction of UGT activity would not be a factor for UROtsa cells because they do not glucuronidate BMP. Another explain for this difference at 24 h is that hepatocytes, as compare to UROtsa cells, may repair DNA adducts more efficiently.

Total content of intracellular GSH was quantified in the two cell populations based on previous results that suggested oxidative stress contributed to the DNA lesions in UROtsa cells following acute exposure of BMP. Results showed total GSH in UROtsa cells was 4 fold lower than that in hepatocytes suggesting UROtsa cells may have inadequate antioxidant defense mechanisms. Unexpectedly, a significant depletion of GSH was measured in hepatocytes but not in UROtsa cells following 1 h exposure of BMP (100 and 500 μ M). This loss of GSH in primary hepatocytes may represent the ability of these cells to counteract oxidative damage due to the high basal level of GSH. It needs to be noted that the UROtsa cell line is an immortalized cell line which contains inactivated p53 as a result of SV40 transformation. Several studies have shown that multidrug resistance proteins (MRPs), the major efflux transporters for GSH and its conjugates, were induced by transfection of inactivated p53 (Iida et al., 2001; Lin-Lee et al., 2001; Sullivan et al., 2000). Therefore, the low basal level of GSH in UROtsa cells may be partially explained by their increased ability to export GSH. It is unclear why GSH is not depleted in UROtsa cells after BMP exposure. This probably relates to the method used to measure GSH. The concentration of GSH in UROtsa cells is at or just above the limit of detection, making it difficult to demonstrate that BMP depletes GSH in these cells. The depletion of GSH in hepatocytes by high concentrations of BMP (100 and 500 μ M) explains the absence of DNA strand breaks in hepatocellular DNA. When glucuronidation of BMP is nearing saturation, the high levels of GSH are able to detoxify BMP-associated reactive oxygen species that damage DNA. Thus, hepatocytes, possess two detoxification mechanisms, effective conjugation and antioxidant activity, both of which are deficient in UROtsa cells.

The results of a preliminary *in vivo* microarray analysis provide additional evidences that BMP exposure disrupts the redox cycling and antioxidant enzyme system in target cells. A number of antioxidant genes in bladders of B6C3F1 mice were highly up-regulated after 6 h of BMP dosing (300 mg/kg, PO) (unpublished results). The up-regulated genes include heme oxygenase (decycling) 1 (Hmox1, 46-fold), glutathione S-transferase (Gsta1 and

Gsta2, 8-fold), glutathione synthetase (Gss, 5-fold), glutathione –cysteine ligase (Gclm, 5-fold), glutathione peroxidase 2 (Gpx2, 5-fold), thioredoxin reductase (Txnrd1, 5-fold), metallothionein 2 (Mt2, 5-fold) and glutathione reductase 1 (Gsr1, 4-fold). As many of these genes are downstream targets regulated by transcription factor Nrf2, these microarray data are consistent with our previous finding that Nrf2 was induced by BMP in UROtsa cells (Kong et al., 2011). It is likely that these downstream genes of Nrf2 are also induced in UROtsa cells following BMP exposure. Future studies should validate these gene expressions, especially genes related to GSH regulation (Gst, Gss, Gpx, Gclm, Gsr) and BMP metabolism (UDP-glucuronosyltransferase, Ugt), in both target cells and non-target cells.

Another mechanism by which BMP may cause genotoxicity is formation of DNA adducts. The data presented here most likely represent a variety of [¹⁴C]-BMP adducts in the DNA of UROtsa cells. These results are important in that they demonstrate the capacity of BMP to form DNA adducts in target cells. Quantitative comparison of DNA binding and DNA strand breaks suggests that formation of adducts may be a more efficient genotoxic pathway. Our data showed the level of DNA strand breaks is roughly increased by an order of magnitude after 1 h of exposure of UROtsa to BMP (100 μM). Several studies have shown that the level of strand breaks in normal somatic mammalian cells is below 0.5 breaks per million nucleotides (Billen, 1990; Vilenchik and Knudson, 2000, 2003). Thus, the level of BMP-induced strand breaks in UROtsa cells is around 5 breaks per million nucleotides. Under the same conditions, the amount of bound BMP is around 70 pmol per mg DNA which corresponds to more than 20 adducts per million nucleotides. The frequency is almost 10-times larger at 24 h. In order to better characterize the biological relevance of this DNA binding, future studies should focus on the identification of the actual BMP-DNA adducts. It is known that some DNA adducts possess more mutagenic potential than others (Wyatt and Pittman, 2006). For example, O⁶-guanine and O⁴-thymine adducts have greater mutagenic potential than N⁷-guanine adducts, which are reported to have limited mutagenic potential (Saffhill et al., 1985).

In summary, the results of the studies presented here demonstrate that metabolic activity and intracellular antioxidant capacity are two key factors modulating cell susceptibility to BMP-induced genotoxicity in vitro. Increased susceptibility in the target cells used here is related to the deficiency of antioxidant and/or metabolic capabilities in these cells. Besides these, other factors such as DNA repair capacity may also contribute to the cell susceptibility and deserve further investigation.

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Highlights

- Comparing to hepatocytes, BMP exhibits greater genotoxic potential in UROtsa cells.
- BMP is not metabolized and/or consumed in UROtsa cells.
- Hepatocytes extensively convert BMP to a mono-glucuronide.
- UROtsa cells have significantly lower level of intracellular GSH than hepatocytes.
- Metabolic and antioxidant capacities modulate cell susceptibility to BMP.

B

Figure 1.

DNA strand breaks detected in A) rat hepatocytes and B) UROtsa cells following incubation with various concentrations of BMP for 1 h. T%DNA represents % DNA in the comet tail. Because of the similarity of the data, results obtained in UROtsa cells were combined with those of previous experiments. Data are shown as mean $\pm$ SEM, N=3–7. * P <0.05, ** P <0.01, *** P <0.001 versus vehicle control.

B

Figure 2.

Covalent binding of [^{14}C]-BMP equivalents to DNA measured in rat hepatocytes and UROtsa cells following incubation with [^{14}C]-BMP (10–250 μM) for (A) 1 h and (B) 24 h. Data are shown as mean $\pm$ SEM, $N=3-4$. * $P<0.05$, ** $P<0.01$ versus corresponding concentration applied to rat hepatocytes.

Figure 3. Representative HPLC-radio chromatograms of BMP glucuronide formed after incubation of BMP (10–250 μ M) with (A) primary rat hepatocytes (suspension, 1×10^6 cells) or (B) cultured UROtsa cells (roughly 1.2×10^6 cells) for 1 h. UDPGA (1 mM) and NADPH (1 mM) were added in some of the UROtsa cell incubations. All incubations were performed three independent times with identical results.

Figure 4.

Representative HPLC-radio chromatograms of BMP glucuronide formed after incubation of BMP (10–250 μ M) with (A) cultured rat hepatocytes or (B) cultured UROtsa cells for 24 h. UDPGA (1 mM) and NADPH (1 mM) were added in some of the UROtsa cell incubations. Incubations were performed three times with identical results.

Figure 5. Stoichiometric conversion of various concentration of BMP (10–250 μ M) into BMP-glucuronide by primary hepatocytes from male SD rats. (A) 1 h incubation of hepatocytes suspension (1×10^6 cells) with BMP (B) 24 h incubation of plated hepatocytes (roughly 5×10^5 cells) with BMP. Results are expressed as % of the administered BMP. Data are shown as mean $\pm$ SEM, N=3.

A

B

Figure 6.

Total GSH content measured in (A) rat hepatocytes and (B) UROtsa cells after 1 h exposure of BMP (25–500 μM), KBrO₃ (2 mM) or NAC (2 mM). Results are expressed as % of GSH level in vehicle control treated cells. Data are shown as mean ± SEM, N=3. *P<0.05, **P<0.01 versus vehicle control.