Published in final edited form as:

Cancer Chemother Pharmacol. 2015 September ; 76(3): 537–546. doi:10.1007/s00280-015-2828-2.

Characterization of the metabolism of benzaldehyde dimethane sulfonate (NSC 281612, DMS612)

Robert A. Parise^{1,2}, Julie L. Eiseman^{2,3}, Dana M. Clausen^{2,3}, Kimberly P. Kicielski², Pamela A. Hershberger⁴, Merrill J. Egorin^{2,3,5}, and Jan H. Beumer^{1,2,5}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213

²Cancer Therapeutics Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213

³Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

⁴Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263

⁵Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

Abstract

Introduction—Benzaldehyde dimethane sulfonate (BEN, DMS612, NSC281612) is a bifunctional alkylating agent currently in clinical trials. We previously characterized the degradation products of BEN in plasma and blood. The conversion of BEN to its carboxylic acid analogue (BA) in whole blood, but not plasma, suggests that an enzyme in RBCs may be responsible for this conversion. BEN conversion to BA was observed in renal carcinoma cells and appeared to correlate with IC₅₀. To better understand the pharmacology of BEN we aimed to evaluate the metabolism and enzymes potentially responsible for the conversion of BEN to BA.

Methods—Human red blood cells (RBC) were used to characterize kinetics and susceptibility to enzyme specific inhibitors. Recombinant enzymes were used to confirm metabolism of BEN to BA. Analytes were quantitated with established LC-MS/MS methods.

Results—Average apparent V_{max} and K_m were 68 ng/mL•min⁻¹•[10% RBC]⁻¹ and 373 ng/mL, respectively. The conversion of BEN to BA in RBC was not inhibited by carbon monoxide, nitrogen gas or menadione, an inhibitor of aldehyde oxidase. The conversion was inhibited by disulfiram, an inhibitor of ALDH. Of available ALDH isoforms ALDH1A1, ALDH3A1, ALDH2, and ALDH5A1, only ALDH1A1 converted BEN to BA.

Correspondence: Jan H. Beumer, Pharm.D., Ph.D., University of Pittsburgh Cancer Center, Hillman Research Pavilion, Room G27E, 5117 Centre Avenue, Pittsburgh, PA 15213-1863, Tel.: 412-623-3216, Fax.: 412-623-1212, beumerj@gmail.com.

Primary laboratory of origin: University of Pittsburgh Cancer Institute

Disclosures: None

Conclusion—The activating conversion of BEN to BA is not mediated by CYP450 enzymes or aldehyde oxidase but by ALDH1A1. This enzyme, a potential stem cell marker, may be a candidate biomarker for clinical activity of BEN.

Keywords

BEN Metabolism; Alkylators; Degradation; Renal Cell Carcinoma; HPLC; Mass spectrometry; Metabolism; Aldehyde Dehydrogenase; Stability; Cell lines; Cancer

1 Introduction

Approximately 13,000 people die from metastatic renal cell carcinoma (mRCC) in the United States every year [1,2]. Only 10–12% of patients achieve durable complete remission with high dose interleukin-2 therapy [3]. Agents targeting vascular endothelial growth factor and its receptor along with mTOR inhibitors have shown clinical activity [4,5]. However, responses to these agents are generally transient [6], and there is a need for new treatment options for mRCC patients.

Benzaldehyde dimethane sulfonate (BEN, DMS612, NSC281612) is one of a family of dimethane sulfonates that have demonstrated activity in the NCI 60 cell line screen. BEN is proposed to be a bifunctional alkylator with structural similarities to chlorambucil, busulfan and melphalan. However, unlike these compounds, BEN has specific activity against renal carcinoma cells (RCC) in the NCI 60 cell line screen [7]. In addition, BEN has demonstrated antitumor activity in mice with renal carcinoma xenografts, specifically against human A498 and RXF-393 implanted subcutaneously or orthotopically under the renal capsule [Personal communication, Dr. M. Hollingshead, unpublished data [8,9]. The significant *in vitro* and *in vivo* activity against renal carcinoma has led to the ongoing evaluation of BEN in an NCI-sponsored phase I clinical trial (ClinicalTrials.gov Identifier: NCT00923520) [10].

Preliminary experiments showed that BEN undergoes N-dealkylation in liver microsomal fraction by CYP3A, whereas it is oxidized in the cytosolic fraction [11]. Later, we found that in whole blood, but not in plasma, BEN was rapidly converted to its carboxylic acid analogue (BA) [12]. Furthermore, BEN decomposed into at least eleven different products that were structural analogs of either BEN or BA. This led us to hypothesize that the conversion of BEN to BA in whole blood is an enzymatic process [12].

We studied the metabolism of BEN in five different RCC lines and found that the ALDH activity correlated with the amount of BA-OH₂ generated [12]. This further supported for the idea that the conversion of BEN to BA is enzymatic.

In this manuscript we describe the identification of the enzyme responsible for the conversion of BEN to BA, and further characterization of the reaction in biological fluids.

2 Materials and Methods

2.1 Chemicals and reagents

4-[bis[2-[(methylsulfonyl)-oxy]ethyl]amino]-2-methyl-benzaldehyde (BEN, NSC 281612), 4-[bis[2-[(methylsulfonyl)-oxy]ethyl]amino]-2-methyl-benzoic acid (BA, NSC 733609), 4-

[bis[2-chloro-ethyl]amino]-2-methyl-benzaldehyde (BEN-Cl₂), 4-[bis[2-chloro-ethyl]amino]-2-methyl-benzoic acid (BA-Cl₂), 4-[bis[2-[(methylsulfonyl)-oxy]ethyl]amino]-benzaldehyde (desmethyl-BEN), and 4-[bis[2-chloro-ethyl]amino]-benzaldehyde (desmethyl-BEN-Cl₂) were obtained from the National Cancer Institute (NCI, Bethesda, MD). 4-[bis[2-hydroxy-ethyl]amino]-2-methyl-benzaldehyde (BEN-(OH)₂) was generated as previously described. [13]. All solvents used for LC-MS/MS were high purity Burdick & Jackson and purchased from Fisher Scientific Co. (Fair Lawn, NJ). Formic acid and DMSO were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Control human blood (citrate anti-coagulated) was obtained from the Central Blood Bank, Pittsburgh, PA and plasma was prepared by centrifugation of whole blood at 2000 × g at room temperature for 20 min. Nitrogen gas for the mass spectrometer was purified with a Parker Balston Nitrogen Generator (Haverhill, MA). Nitrogen gas for the sample evaporation, and nitrogen gas and carbon monoxide gas was purchased from Valley National Gasses, Inc. (Pittsburgh, PA). Reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and disulfiram used for metabolism studies were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Cellgro phosphate buffered saline. (PBS 1x) was purchased from Mediatech (Manassas, VA).

2.2 Analytical Assays

We employed two LC-MS/MS assays: One to quantitate the metabolites generated in the blood metabolism experiments, and a second to quantitate BEN and BA in the purified enzyme experiments. For the blood metabolism experiments we used a previously developed assay to quantitate the analytes in plasma [13,12]. For the purified enzyme experiments we developed an assay to quantitate the analytes in PBS (Assay 2). The LC-MS/MS system consisted of an Agilent (Wilmington, DE, USA) 1100 thermostated autosampler, binary pump, and a Waters (Milford, MA) Quattromicro desktop tandem mass spectrometer (QM system). The liquid chromatography was performed with a gradient mobile phase consisting of A: acetonitrile/0.1% formic acid (v/v) and B: water/0.1% formic acid (v/v). The mobile phase was pumped at a flow rate of 0.3 mL/min and separation was achieved using a Phenomenex (Torrance, CA) Hydro Synergi-RP 4 micron 100 × 2mm column. The gradient mobile phase was as follows: from time zero to until 4.5 min solvent A was maintained at 40% with a flow rate of 0.3 mL/min. At 4.6 min solvent A was increased linearly to 90% and the flow rate was increased to 0.5 mL/min. These conditions were maintained for 2.4 min at which time solvent A was decreased to 40%. The run time was 12 min. During the first two min and the last six min of the LC run the flow leaving the column was diverted to waste. The parameters for the mass spectrometer were as follows: Capillary voltage 4.0 kV, cone voltage 20 V, source temperature and desolvation temperature were 120 °C and 450 °C, respectively. Desolvation gas was 550 L/h and LM1, LM2, HM1 and HM2 were set to 12. The entrance, exit and collision voltages were 1, 23, and 6 respectively. The mass transitions for BEN, BA and desmethyl-BEN were 380>160, 396>300 and 366>270, respectively.

2.3 Standard curves

Sample processing and standard curves for the blood metabolism assays were performed as previously described [13]. The procedure for the preparation of standard curves in PBS was

the same as the procedure for the preparation of the standard curves in plasma except for the following; PBS was used instead of plasma, and only BEN and BA were quantitated from 1 to 1000 ng/mL and desmethyl-BEN was used as the internal standard.

2.4 Determination of the effect of RBC concentration on BEN metabolism

To determine the effect of increasing RBC concentrations on BEN metabolism and to determine an adequate amount of RBCs in further experiments, metabolism was performed with different amounts of RBCs. RBCs were freshly isolated by spinning whole blood and sampling from the bottom of the resulting RBC layer, avoiding the buffy coat. RBCs were reconstituted at the desired concentration in plasma. BEN was added separately to 10 mL of plasma, 0.1% RBCs, 1% RBCs, 10% RBCs to achieve a concentration of 10 µg/mL. The samples were incubated in a shaking water bath at 37 °C. At 0, 5, 10, 15, 30, 45, 60, 120, 180, 240, 360 min, and 24 h, 500 µL aliquots were taken and processed as described above.

2.5 Experiments with carbon monoxide and nitrogen gas

Carbon monoxide (CO) gas and nitrogen (N₂) gas were used to determine whether a heme group or oxygen, respectively was involved in the metabolism of BEN. Either carbon monoxide (CO) or nitrogen gas was passed through a mixture of 10% RBCs in plasma at 37 °C for 5 min in a shaking water bath. The reaction vessels were sealed with a septum. The gasses were continuously passed into the reaction vessel using a stainless steel needle 5 minutes prior and throughout the experiment. A second needle was inserted in the septum to allow the gasses to escape. BEN was added to obtain a concentration of 3 µg/mL and 200 µL aliquots were taken at 0, 60, 120 and 180 min and processed as described above.

2.6 Experiments with ALDH and AO inhibitor

Disulfiram and menadione were used to determine the effect of ALDH and aldehyde oxidase (AO) inhibitors on blood metabolism of BEN. Aliquots of 90 µL of 10 mM disulfiram in acetonitrile or 90 µL of 10 mM menadione in DMSO was added to 6 mL of 10% RBCs to obtain a concentration of 150 µM of these compounds.

Additional experiments were conducted with increasing concentrations of menadione of 750 µM and 3 mM. An aliquot of 90 µL of DMSO was added to separate tubes as a solvent control. The resulting mixtures were incubated at 37 °C for 10 min in a shaking water bath before BEN was added to obtain a concentration of 1 µg/mL. Aliquots of 500 µL were taken at 5, 15, 30, 45 and 60 min and processed as described above.

2.7 BEN in lysed blood

To determine the effect of hemolysis on the metabolism of BEN in blood we performed the metabolism of BEN with lysed RBCs. To lyse RBCs a mixture of 10% RBCs in plasma was freeze-thawed 3 times. A volume of 6 mL of both lysed and unlysed RBCs in plasma was incubated separately at 37 °C on a shaking water bath before BEN was added to obtain a final concentration of 10 µg/mL. Aliquots were taken at 5, 60, 120, and 180 min and processed as described above.

We hypothesized the activity of the enzyme in RBCs could be affected by lysis through dilution of cofactors. To determine whether NADH or NADPH would restore the metabolism in lysed RBCs, we evaluated the metabolism in lysed RBC in the absence of presence of either NADH or NADPH. NADH or NADPH was added at a final concentration of 500 μ M to 10% lysed RBCs in plasma and incubated at 37 °C in a shaking water bath. Aliquots of 500 μ L were taken at 5, 30, 60, 90 and 120 min and processed as described as above.

2.8 Determination of BEN blood partitioning

To determine the amount of BEN in whole blood compared to plasma we determined the blood to plasma partitioning ratio. Ten mL of blood (50% RBCs in plasma) was placed into a 15 mL conical tube and placed on ice. Disulfiram was added to obtain a concentration of 1 mM. After 10 minutes, BEN was added to obtain a final concentration of 1000 ng/mL. Five hundred μ L aliquots of blood were taken at 5, 10, 15, 20 and 30 minutes and centrifuged at $12,000 \times g$ for 2 minutes. Two hundred μ L of the resulting plasma was pipetted into micro-centrifuge tubes that contained 10 μ L of 2M H_2SO_4 . The amount of BEN in the plasma samples was determined by LC-MS/MS as described above. The procedure was performed in parallel in 100% plasma to determine the total amount of BEN obtained in plasma without blood. This experiment was performed in triplicate. The blood to plasma concentration partitioning was determined by dividing the concentration of BEN obtained in the 100% plasma experiment divided by the concentration of BEN in the plasma obtained from the BEN added to 50% RBC experiment.

2.9 V_{max} and K_m determination

In order to determine the apparent maximum reaction rate (V_{max}) and the concentration at half-maximum reaction rate (K_m), different concentrations of BEN were added to 10% RBCs in plasma and the amount of BEN remaining was determined at various time points. BEN was added to RBCs that were pre-incubated at 37 °C for five min to achieve a final concentration of 10,000, 5,000, 2,000, 1,000, 800, 600, 400 and 200 ng/mL. Aliquots of 500 μ L were taken at 5, 15, 30, 45, 60, 75, 90, 105 and 120 min and processed as described above. Based on this extensive experiment, more optimal and fewer concentrations could be selected to use in further determinations of apparent V_{max} and K_m .

We determined the apparent V_{max} and K_m in six different lots of human blood. Plasma with 10% RBCs was pre-incubated at 37 °C for five min followed by addition of BEN to achieve final concentrations of 5,000 and 400 ng/mL. 500 μ L aliquots were taken at 5, 15, 30, 45, 60, 75, 90, 105 and 120 min and processed as described above (experiments were performed in triplicate). The resulting concentration versus time data was analyzed with a one compartmental model with saturable elimination using ADAPT5 [14]. In ADAPT we used the equations

$$XP(1) = -V_{max} / (K_m + X(1)/V400) * (X(1)/V400)$$

and

$$XP(2) = -V_{\max} / (K_m + X(2) / V_{5000}) * (X(2) / V_{5000})$$

to determine the K_m and $V_{\max}$ of each experiment.

XP(1)= velocity of 400 ng/mL reaction; $V_{\max}$ = maximum rate of reaction; K_m = concentration at half $V_{\max}$; X(1) = BEN amount in 400 ng/mL incubation; V_{400} = reaction volume of 400 ng/mL incubation; XP(2)= velocity of 5000 ng/mL reaction; X(2)= BEN amount in 5000 ng/mL incubation; V_{5000} = reaction volume of 5000 ng/mL incubation.

To determine if time had an effect on enzyme activity we determined the apparent $V_{\max}$ and K_m of 7 day old blood (stored at 4 °C) compared to freshly obtained blood. Plasma with 10% RBCs was pre-incubated at 37 °C for five min followed by addition of BEN in to achieve final concentrations of 5,000 and 400 ng/mL. Aliquots of 500 μ L were taken at 5, 15, 30, 45, 60, 75, 90, 105 and 120 min and processed as described above.

2.10 Metabolism using purified ALDH isoforms

To determine whether BEN is a substrate for metabolism by different isoforms of ALDH we performed metabolism studies of BEN with purified human ALDH1A1, 2, 3A1, and 5A1 (PROSPEC, Israel) enzymes. In addition, we performed metabolism studies of BEN with these enzymes in the presence of their respective antibodies, if available, and disulfiram in an effort to inhibit metabolism.

Enzyme incubation mixtures contained 200 μ M Tris-HCl buffer pH 7.4, 200 mM KCl, 4 mM DTT, 5 μ g/mL purified enzyme and 1000 ng/mL BEN. After pre-incubation at 37 °C for 10 min, NAD or NADPH (depending on the cofactor that was required for each isozyme) was added to obtain a concentration of 1 mM. Aliquots of 100 μ L were taken at 0, 10, 20, 30, 45, and 60 min. The aliquots were pipetted into a microcentrifuge tube that contained 5 μ L of 2 M H_2SO_4 . 10 μ L of 1 μ g/mL internal standard and 500 μ L of acetonitrile were added and the samples were briefly vortexed and centrifuged at 12,000 $\times$ g for 5 min. The resulting supernatant was transferred to 12 $\times$ 75 mm glass tubes and blown down under a stream of nitrogen at 37 °C. The samples were reconstituted in 100 μ L of 20:80 acetonitrile/ H_2O with 5% 2 M H_2SO_4 (v/v/v) and 10 μ L was injected into the LC-MS/MS system.

2.11 Immunoprecipitation of ALDH1A1 in RBCs

To determine if ALDH was responsible for the metabolism of BEN, we performed immunoprecipitation of ALDH1A1 enzyme and then performed BEN metabolism in RBCs. One hundred μ L of 50 % lysed RBCs in plasma were added to 900 μ L of immunoprecipitation buffer which consisted of 5 mM EDTA, 0.02% sodium azide and 18 μ L of 50x protease cocktail inhibitor (BD baculogold). The sample was briefly vortexed and centrifuged for 10 min at 12,000 $\times$ g at 4 °C. Either 5 μ L of ALDH1A1 purified MaxPab rabbit polyclonal antibody (D01P; Abnova, Taipei, Taiwan) or IgG polyclonal rabbit control antibody (Abcam, Cambridge, MA) was added. To a third control sample nothing was added. The samples were placed on a rotary agitator at 4 °C. After 24 h, 100 μ L of protein A

sepharose B conjugate was added to each tube and the sample was placed on a rotary agitator for 4 h at 4 °C. The sample was then centrifuged at $2,000 \times g$ for 5 min and the resulting supernatant was used for BEN metabolism.

The samples were placed in a shaking heated water bath at 37 °C. After 10 min, BEN was added to each sample to achieve a concentration of 1000 ng/mL and NADH was added to achieve a concentration of 3 mM. Aliquots of 100 μ L were taken at 0, 15, 30 and 60 min and added to microcentrifuge tubes that contained 5 μ L 2 M H_2SO_4 . 10 μ L of 1 μ g/mL internal standard and 500 μ L of acetonitrile were added and the samples were briefly vortexed and centrifuged at $12,000 \times g$ for 5 min. The resulting supernatant was transferred to 12 $\times$ 75 mm glass tubes and blown down under a stream of nitrogen at 37 °C. The samples were reconstituted in 100 μ L of 20:80 acetonitrile/ H_2O with 5% 2 M H_2SO_4 (v/v/v) and 10 μ L was injected into the LC-MS/MS system.

2.12 Western blot

To determine if RBCs had ALDH1A1 we performed Western blot analysis as previously described.[15] One hundred μ L of 50 % lysed RBCs in plasma was added to 890 μ L of cell lysate buffer containing 200 mM Tris-HCl, pH 7.4, 100 μ M 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM EGTA, and 1% Triton X-100. Protein concentration was determined using the BCA Protein Assay kit (Pierce, Invitrogen, Grand Island, NY, USA), and 10, 20, 40 and 80 μ g protein were subjected to SDS-PAGE electrophoresis (4–15% SDS-polyacrylamide, Bio-Rad) followed by electrotransfer to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline and then probed with primary antibodies ALDH1A1 purified MaxPab rabbit polyclonal antibody (D01P; Abnova, Taipei, Taiwan) overnight at 4 °C. Blots were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Bio-Rad, Hercules, CA, USA). The enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA) was used to facilitate detection of protein bands.

3 Results

3.1 The effect of RBCs on BEN metabolism

The rate of disappearance of BEN increased as the percentage of RBCs was increased. After one hour, the reaction that contained no RBCs had 99% of BEN remaining whereas the reaction with 10% RBCs had only 54% BEN remaining. After 6 hours the reaction with no RBCs had 59% remaining while the reaction with 10% RBCs had only 5% BEN remaining, see Figure 1A. Another measure of metabolism is the formation of BA-(OH)₂ or lack of metabolism is the formation of BEN-(OH)₂. The 10% RBC reaction produced the most BA-(OH)₂ and the least amount of BEN-(OH)₂, see Figure 1B and C.

3.2 Effects of carbon monoxide and nitrogen gas on BEN metabolism

We performed incubations of BEN in 10% RBCs in the presence of either carbon monoxide or nitrogen gas to determine if a heme group or oxygen was involved in the metabolism of BEN. The metabolism of BEN was not appreciably inhibited by the presence of these gasses

as the rate of disappearance of BEN appeared a little greater in the presence of the gasses. After approximately 60 min, the concentration of BEN was halved in all incubations (data not shown).

3.3 Effects of disulfiram and menadione on BEN metabolism

To determine whether the metabolism of BEN in RBCs was due to ALDH or AO we performed incubations of BEN with 10% RBCs in plasma in the presence of the ALDH inhibitor disulfiram or the AO inhibitor menadione. The incubation performed with the ALDH inhibitor disulfiram resulted in an almost complete inhibition of BEN metabolism, whereas the AO inhibitor menadione appeared to have only a partial inhibitory effect on the metabolism of BEN. The amounts of BEN remaining in the control, disulfiram and menadione treated samples were 0.5%, 92% and 18%, respectively (Figure 2A).

Since menadione caused a partial inhibition of BEN metabolism, we performed additional experiments with increasing concentrations of menadione, in order to increase the inhibition of BEN. These experiments showed that vehicle control DMSO alone (1.5% DMSO v:v) caused the same extent of inhibition of BEN in RBCs as 3 mM menadione at the same concentration of DMSO (Figure 2B).

3.4 BEN in lysed blood

We performed experiments in lysed RBCs in order to determine whether a complete cell structure is required for the metabolism of BEN, or whether lysing the cells resulted in dilution of cofactors. RBCs that had been lysed before metabolism did not metabolize BEN. Adding NADH to the lysed RBCs incubation, however, restored the ability of lysed RBCs to metabolize BEN. Adding NADPH partially restored the ability of lysed RBCs to metabolize BEN (Figure 3).

3.5 BEN blood to plasma concentration ratio

We determined the blood to plasma concentration ratio of BEN by measuring the amount of BEN added to 50% RBCs in plasma and added to plasma as described in the methods section. The average plasma to whole blood partition ratio was found to be 1.25 ± 0.08 . The amount of BEN detected in the blood remained nearly constant throughout the experiment (data not shown), indicating that the equilibrium between RBCs and plasma was complete by 5 minutes.

3.6 Determination of $V_{\max}$ and K_m

We determined the metabolism of BEN at different concentrations in 10% RBCs. We found that a starting concentration of 5000 ng/mL captured both the saturable and linear components of BEN metabolism. In addition, starting concentrations below 400 ng/mL appeared to display linear metabolism, see Figure 4.

Next, we evaluated six different lots of blood for the metabolism of BEN. Based on the exploratory data we choose to perform the metabolism of BEN using 5000 and 400 ng/mL to best capture the apparent $V_{\max}$ and K_m of BEN in blood. The apparent K_m ranged from

196.3 to 799.6 ng/mL and the apparent $V_{\max}$ ranged from 65.4 to 113.2 ng/mL⁻¹•min⁻¹•[10% RBC]⁻¹, see Table 1 and.

3.7 Metabolism using purified ALDH isoforms

To determine if ALDH is responsible for the metabolism of BEN we assessed the metabolism of BEN with the commercially available isoforms of ALDH. These included ALDH1A1, 2, 3A1 and 5A1. Activity of the purified ALDH isoforms was confirmed with appropriate substrates and co-factors, see. ALDH1A1 was the only isoform that showed the ability to metabolize BEN. In three independent experiments the rate of disappearance of BEN was 0.172, 0.224 and 0.154 ng/min/μg of enzyme, see Figure 5 for a representative experiment. Disulfiram did not inhibit the reaction of ALDH1A1. This is likely due to the fact that disulfiram is inactivated by the dithiothreitol present in the reaction mixture, thereby preventing it from inhibiting ALDH. Furthermore, the addition of 100x of the ALDH1A1 and IgG antibodies did not inhibit the metabolism of purified ALDH1A1 (data not shown).

3.8 Immunoprecipitation of RBCs

We performed immunoprecipitation of RBCs with antibodies for ALDH1A1. The purpose was to deplete ALDH1A1 enzyme, preventing BEN metabolism and thereby implicate it in the metabolism of BEN by RBCs. Despite our best efforts, however, the control RBC experiment with no antibodies added would not metabolize BEN after being subjected to the immunoprecipitation procedure. In an attempt to preserve metabolism of BEN with RBCs, we varied the amount of protease inhibitors and concentration of the different buffer used, without success (data not shown).

3.9 Western blot

To determine whether RBCs have ALDH1A1 and to test whether the ALDH1A1 antibody used for the immunoprecipitation experiment would bind to the enzyme we performed a western blot assay. The western blot showed a band in the 55 kDa region, which is the size of ALDH1A1 enzyme (55 kDa) (Figure 6).

4 Discussion

BEN is an alkylating agent currently being investigated in phase I clinical trials. BEN has structural similarities to both busulfan and melphalan. As with related alkylators, we have previously shown that BEN undergoes hydrolysis in blood and plasma [12]. In whole blood, but not in plasma, BEN is rapidly converted to its carboxylic acid analogue BA, suggesting conversion by an enzyme present in red blood cells. In plasma, BEN and BA decompose according to a parallel pathway to a number of hydroxylated and chlorinated analogues [12]. The *in vitro* half-life of BA in plasma is very short at ~5 min compared to that of BEN at 22 min. Previously reported data suggests that BA is more reactive than BEN, and BA represents the effector of DNA alkylation [12]. In the current study, we show that the activation of BEN to BA is mediated by ALDH1A1.

The ability of NADH supplementation to restore the ability of lysed RBCs to metabolize BEN suggested an enzymatic reaction requiring NADH as a co-factor. The inability of carbon monoxide to inhibit metabolism indicated that hemeproteins are not responsible for the metabolism of BEN, while the inability of nitrogen to abolish metabolism suggests oxygen is not required. Menadione did not abolish BEN metabolism, while disulfiram did abolish BEN metabolism, suggesting ALDH activity was responsible for BEN metabolism to BA.

Using commercially available purified ALDH isozymes, we evaluated the ability of ALDH1A1, 3A1, 2 and 5A1 to metabolize BEN into BA. Only ALDH1A1 could be shown to metabolize BEN into BA. We also performed the metabolism of BEN with purified ALDH1A1 in the presence of 1A1 antibody, which was successful in binding the enzyme as evidenced by the Western blot experiments of RBCs. The ALDH1A1 antibody was not able to inhibit the reaction. This result may be explained by the antibody binding to an ALDH1A1 epitope without hindering ALDH1A1 metabolic activity. Disulfiram did not slow the metabolism, which may be due to disulfiram inactivation by dithiothreitol, a reducing agent required for to keep ALDH in a reduced state.

ALDHs are NAD(P)⁺ dependent enzymes that metabolize both aromatic and aliphatic aldehydes into carboxylic acids [16]. While this process generally detoxifies aldehydes, the metabolism of BEN to BA represents an increase in potency [12]. Although we evaluated four different ALDH isozymes for their ability to metabolize BEN to BA, there are 19 different known human isoforms of ALDH, and we cannot exclude the possibility that ALDH isozymes other than ALDH1A1 are capable of this conversion.

The apparent $V_{\max}$ and K_m of BEN were determined in 6 different lots of blood to evaluate biological central tendency and variability. BEN displayed saturable kinetics. Although there was variability, all lots of blood displayed K_m values within an order of magnitude of the exposures observed in mice (16 ng/mL, after 20 mg/kg BEN equivalent to 60 mg/m² in humans), or in humans (14.5 ng/mL after 9 mg/m²) [13,10]. Therefore we would expect linear kinetics of BEN in humans. The inter-subject variability for $V_{\max}$ and K_m was 22%CV and 41%CV, respectively. This is not surprising as the inter-individual biological variability for the quantity of certain enzymes can be as much as 73 percent and the inter-individual activity of e.g. CYP3A4 can range from 200 to 300 percent [17,18]. In addition, there are two known polymorphism of ALDH1A1, ALDH1A1*2 and ALDH1A1*3 [19]. These polymorphisms occur in 7% percent of the population and are responsible for reduced enzymatic activity [20]. Reduced ALDH activity may also be caused by comedication, including disulfiram (AntabuseTM), an irreversible inhibitor of certain isoforms of ALDH, prescribed to treat chronic alcoholism [21], and furazolidone, used in the treatment of *Helicobacter pylori* [22,23]. The impact of ALDH1A1 polymorphisms and inhibitory comedication on the pharmacokinetics and pharmacodynamics of BEN remains to be investigated.

In conclusion, BEN has shown promising *in vitro* and *in vivo* activity and is currently being studied in a phase I clinical trial with documented clinical responses (ClinicalTrials.gov Identifier: NCT00923520) [10]. In this paper we demonstrate that ALDH1A1 likely is the

enzyme that is responsible for the conversion of BEN to its active metabolite BA. Cancers that are known to overexpress ALDH1A1 may be a target for BEN. It is noteworthy to mention that certain tumor stem cells have been found to over express ALDH1A1 and that BEN may be an ideal candidate for these cells as well [24,25]. Future patients enrolled on trials of BEN should have their tumors analyzed for ALDH1A1 expression as a potential predictive marker of response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

5 Funding Information

This work was supported by the National Cancer Institute [contract N01-CM-52202] and [grants U01-CA099168, UM1CA186690, P30-CA47904].

This research was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute.

We like to thank Dr. Joseph M. Covey at the Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, for his intellectual input. We would like to thank Dr. Merrill Egorin for his help and guidance on conducting the experimentation that was required for this manuscript.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60 (5):277–300. [PubMed: 20610543]
2. Cohen HT, McGovern FJ. Renal-cell carcinoma. *N Engl J Med.* 2005; 353 (23):2477–2490.
3. Facchini G, Perri F, Caraglia M, Pisano C, Striano S, Marra L, Fiore F, Aprea P, Pignata S, Iaffaioli RV. New treatment approaches in renal cell carcinoma. *Anticancer Drugs.* 2009; 20 (10):893–900. [PubMed: 19752718]
4. Rini BI, Atkins MB. Resistance to targeted therapy in renal-cell carcinoma. *Lancet Oncol.* 2009; 10 (10):992–1000. [PubMed: 19796751]
5. Rini BI. Metastatic renal cell carcinoma: many treatment options, one patient. *J Clin Oncol.* 2009; 27 (19):3225–3234. [PubMed: 19470934]
6. Reeves DJ, Liu CY. Treatment of metastatic renal cell carcinoma. *Cancer Chemother Pharmacol.* 2009; 64 (1):11–25. [PubMed: 19343348]
7. Mertins SD, Myers TG, Holbeck SL, Medina-Perez W, Wang E, Kohlhausen G, Pommier Y, Bates SE. In vitro evaluation of dimethane sulfonate analogues with potential alkylating activity and selective renal cell carcinoma cytotoxicity. *Mol Cancer Ther.* 2004; 3 (7):849–860.
8. Mertins, S. Treating Renal Cancer Using a 4-[Bis[2-[(methylsulfonyl)oxy]ethyl]amino]-2-methyl-benzaldehyde. Patent Application Publication; United States: 2011.
9. Carter, J. In vivo efficacy of an aldehyde degradation product of dimethane sulfonate (NSC 281612) in an orthotopic RXF-393 human renal tumor model. *Proceedings of the American Association of Cancer Research*; 2005; p. 322-323.
10. Appleman LJ, Balasubramaniam S, Parise RA, Bryla C, Redon CE, Nakamura AJ, Bonner WM, Wright JJ, Piekarz R, Kohler DR, Jiang Y, Belani CP, Eiseman J, Chu E, Beumer JH, Bates SE. A Phase I Study of DMS612, a Novel Bifunctional Alkylating Agent. *Clinical cancer research: an official journal of the American Association for Cancer Research.* 2015; 21 (4):721–729. 10.1158/1078-0432.CCR-14-1333 [PubMed: 25467180]

11. Kicieliński KP, Parise RA, Holleran JL, Eiseman JL, Covey JM, Egorin MJ. In vitro metabolism & decomposition of benzaldehyde dimethane sulfonate (NSC 281612) (BEN). *Proceedings of the American Association for Cancer Research*. 2006; 47:#544.
12. Parise RA, Anyang BN, Eiseman JL, Egorin MJ, Covey JM, Beumer JH. Formation of active products of benzaldehyde dimethane sulfonate (NSC 281612, DMS612) in human blood and plasma and their activity against renal cell carcinoma lines. *Cancer chemotherapy and pharmacology*. 2013; 71 (1):73–83.10.1007/s00280-012-1980-1 [PubMed: 23053264]
13. Parise RA, Beumer JH, Clausen DM, Rigatti LH, Ziegler JA, Gasparetto M, Smith CA, Eiseman JL. Effects of the aldehyde dehydrogenase inhibitor disulfiram on the plasma pharmacokinetics, metabolism, and toxicity of benzaldehyde dimethane sulfonate (NSC281612, DMS612, BEN) in mice. *Cancer chemotherapy and pharmacology*. 2013; 72 (6):1195–1204.10.1007/s00280-013-2296-5 [PubMed: 24061865]
14. D'Argenio, DZSA.; Wang, X. *Biomedical Simulations Resource*. Los Angeles: 2009. ADAPT 5 User's Guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software.
15. Lavalley CR, Bravo-Altamirano K, Giridhar KV, Chen J, Sharlow E, Lazo JS, Wipf P, Wang QJ. Novel protein kinase D inhibitors cause potent arrest in prostate cancer cell growth and motility. *BMC chemical biology*. 2010; 10:5.10.1186/1472-6769-10-5 [PubMed: 20444281]
16. Marchitti SA, Bocker C, Stagos D, Vasilou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin Drug Metab Toxicol*. 2008; 4 (6):697–720. [PubMed: 18611112]
17. Sebastian-Gambaro MA, Liron-Hernandez FJ, Fuentes-Arderiu X. Intra- and inter-individual biological variability data bank. *European journal of clinical chemistry and clinical biochemistry: journal of the Forum of European Clinical Chemistry Societies*. 1997; 35 (11):845–852.
18. Chen M, Nafziger AN, Bertino JS Jr. Drug-metabolizing enzyme inhibition by ketoconazole does not reduce interindividual variability of CYP3A activity as measured by oral midazolam. *Drug metabolism and disposition: the biological fate of chemicals*. 2006; 34 (12):2079–2082.10.1124/dmd.106.011742 [PubMed: 16997909]
19. Liu J, Zhou Z, Hodgkinson CA, Yuan Q, Shen PH, Mulligan CJ, Wang A, Gray RR, Roy A, Virkkunen M, Goldman D, Enoch MA. Haplotype-based study of the association of alcohol-metabolizing genes with alcohol dependence in four independent populations. *Alcoholism, clinical and experimental research*. 2011; 35 (2):304–316.10.1111/j.1530-0277.2010.01346.x
20. Spence JP, Liang T, Eriksson CJ, Taylor RE, Wall TL, Ehlers CL, Carr LG. Evaluation of aldehyde dehydrogenase 1 promoter polymorphisms identified in human populations. *Alcoholism, clinical and experimental research*. 2003; 27 (9):1389–1394.10.1097/01.ALC.0000087086.50089.59
21. Petersen EN. The pharmacology and toxicology of disulfiram and its metabolites. *Acta psychiatrica Scandinavica Supplementum*. 1992; 369:7–13. [PubMed: 1471556]
22. Karamanakos PN, Pappas P, Boumba VA, Thomas C, Malamas M, Vougiouklakis T, Marselos M. Pharmaceutical agents known to produce disulfiram-like reaction: effects on hepatic ethanol metabolism and brain monoamines. *International journal of toxicology*. 2007; 26 (5):423–432.10.1080/10915810701583010 [PubMed: 17963129]
23. Morgner A, Labenz J, Miehle S. Effective regimens for the treatment of *Helicobacter pylori* infection. *Expert opinion on investigational drugs*. 2006; 15 (9):995–1016.10.1517/13543784.15.9.995 [PubMed: 16916269]
24. Visus C, Wang Y, Lozano-Leon A, Ferris RL, Silver S, Szczepanski MJ, Brand RE, Ferrone CR, Whiteside TL, Ferrone S, DeLeo AB, Wang X. Targeting ALDH(bright) human carcinoma-initiating cells with ALDH1A1-specific CD8(+) T cells. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2011; 17 (19):6174–6184.10.1158/1078-0432.CCR-11-1111 [PubMed: 21856769]
25. Su Y, Qiu Q, Zhang X, Jiang Z, Leng Q, Liu Z, Stass SA, Jiang F. Aldehyde dehydrogenase 1 A1-positive cell population is enriched in tumor-initiating cells and associated with progression of bladder cancer. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2010; 19 (2):327–337.10.1158/1055-9965.EPI-09-0865

Figure 1.

A) The disappearance of BEN with different percentages of RBCs. B) The production of BA-(OH)₂ when BEN is added to different concentrations of RBCs. C) The production of BEN-(OH)₂ when BEN is added to different concentrations of RBCs. 0% RBCs (■), 0.1% RBCs (□), 1% RBCs (▲), and 10% RBCs (△).

Figure 2.

BEN added to 10% RBCs with and without enzyme inhibitors. A) BEN added to 10% RBCs (◇); BEN added to 10% RBCs in the presence of menadione (■); and BEN added to 10% RBCs in the presence of disulfiram (▲). B) BEN added to 10% RBCs with and without different amounts of menadione. BEN alone (■); BEN + 1.5% DMSO (□); BEN added after 150 μ M menadione (▲); BEN added after 750 μ M menadione (○); and BEN added after 3 mM menadione (●).

Figure 3.

BEN added to lysed and non-lysed RBCs. 10% RBCs (◇); 10% lysed RBCs (■); 10% lysed RBCs + NADH (▲); and 10% lysed RBCs + NADPH (◆).

Figure 4.

BEN metabolism with 10% RBCs. 200 ng/mL (■), 400 ng/mL (□), 600 ng/mL (▲), 800 ng/mL (△), 1000 ng/mL (●), 2000 ng/mL (○), 5000 ng/mL (◆), and 10000 ng/mL (◇).

Figure 5. Metabolism of BEN in the absence (□) and presence (■) of purified human ALDH1A. A) Disappearance of BEN, and B) generation of BA. Each point is the average of three measurements.

Figure 6.

Western blot of ALDH1A1 from human RBCs. Lanes from left: lane 1 – ladder (49.9kDa), lanes 2 and 3 - blank, lanes 4 and 5 - 80 μ g protein, 6 and 7 - 40 μ g protein, 8 and 9 - 20 μ g protein, lanes 10 and 11 - 10 μ g protein, and lane 12 ladder (49.9 kDa).

Table 1**K_m, V_{max}, and Cl of BEN in six different lots of human blood**

Lot 1		SE (CV%)	Confidence interval (95%)
Km	196.3	8.399	[161.7 , 230.9]
Vmax	65.44	4.288	[59.54 , 71.34]
Cl	0.333	5.585	[0.2942 , 0.3725]
Lot 2		SE (CV%)	Confidence interval (95%)
Km	319.5	4.457	[289.6 , 349.4]
Vmax	87.42	1.125	[85.35 , 89.49]
Cl	0.2736	3.643	[0.2527 , 0.2946]
Lot 3		SE (CV%)	Confidence interval (95%)
Km	256.2	5.414	[227.1 , 285.4]
Vmax	72.27	2.236	[68.88 , 75.67]
Cl	0.282	3.978	[0.2585 , 0.3056]
Lot 4		SE (CV%)	Confidence interval (95%)
Km	609.8	10.04	[483.5 , 736.2]
Vmax	113.4	2.865	[106.6 , 120.1]
Cl	0.1859	7.653	[0.1565 , 0.2152]
Lot 5		SE (CV%)	Confidence interval (95%)
Km	481.8	7.136	[410.8 , 552.8]
Vmax	76.5	2.063	[73.24 , 79.76]
Cl	0.1588	5.253	[0.1416 , 0.1760]
Lot 6		SE (CV%)	Confidence interval (95%)
Km	371.2	7.729	[312.0 , 430.5]
Vmax	68.16	1.811	[65.61 , 70.71]
Cl	0.1836	6.142	[0.1603 , 0.2068]
Lot 4 +7 days		SE (CV%)	Confidence interval (95%)
Km	699.6	7.276	[594.5 , 804.7]
Vmax	100.2	2.416	[95.17 , 105.2]
Cl	0.1432	5.145	[0.1280 , 0.1584]
		Mean	CV%
Km	419.2	44.3	
Vmax	83.3	21.5	
Cl	0.22	32.3	

Units: Km (ng/mL), Vmax (ng/mL⁻¹•min⁻¹•[10% RBC]⁻¹), Cl (mL/min).