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Formation of active products of benzaldehyde dimethane sulfonate (NSC 281612, DMS612) in human blood and plasma and their activity against renal cell carcinoma lines

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Abstract

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Conflict of interest None.

Benzaldehyde dimethane sulfonate (BEN, DMS612, NSC281612) is an alkylating agent with activity against renal cell carcinoma and is being evaluated clinically. To support clinical trials, we developed an LC–MS/MS assay to detect and quantitate BEN and its metabolites/decomposition products. We tested the stability and products of BEN and benzoic acid dimethane sulfonate (BA) in plasma, blood and five renal carcinoma cell lines in vitro. Further, we determined the IC₅₀ of BEN, BA and four of their products in these cell lines. Low temperature and pH stabilized the analytes, and utilizing this resulted in an accurate, precise and reproducible assay. The half-lives of BEN and BA added to plasma in vitro were 220 and 5 min, while the half-life of BEN in whole blood was 18 min. The generation and degradation of up to 12 analytes were monitored, and structures confirmed with available authentic standards. The IC₅₀ for BEN was 5- to 500-fold lower than that of any of its products, while the cellular metabolic activity toward BEN correlated with ALDH activity and IC₅₀ values. We detected six of the in vitro products and their respective glucuronides in murine plasma after dosing BEN. The information gained from these experiments will be instrumental in the evaluation of the pharmacology of BEN in ongoing human trials.

Keywords

BEN metabolism; Alkylators; Degradation; Renal cell carcinoma; HPLC; Mass spectrometry; Metabolism; Aldehyde dehydrogenase; Stability; Cell lines; Cancer

Introduction

Approximately 12,000 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 (Fig. 1). However, unlike these compounds, BEN has specific activity against renal carcinoma cells in the NCI 60 cell line screen [7]. BEN causes cell cycle arrest in the G₂–M phases [7] and increases P53 levels, indicating DNA damage, in the renal carcinoma cell line 109 and in the breast cell line MCF-7. In addition, BEN has demonstrated antitumor activity in mice with orthotopic 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 NCI-sponsored phase I clinical trials.

BEN, based on its structure, likely forms an aziridinium intermediate that is attacked by nucleophilic molecules such as DNA, proteins or water. Jumaa et al. reported hydrolysis of BEN in aqueous solution whereby the dimethane sulfonate groups are replaced by hydroxy groups [10]. The hydrolysis of BEN, in vivo and in vitro, may lead to the formation of compounds that are less active as alkylating agents than BEN. In addition to BEN-related analogs, we found benzoic acid (BA) analogs of BEN with their own reactivity.

In order to elucidate the pathways for decomposition and metabolism of BEN and to support ongoing clinical trials of BEN, we developed an LC–MS/MS assay for the detection and quantitation of BEN and metabolites in plasma. Using this assay, we characterized the in

vitro decomposition of BEN in plasma and blood and compared the in vitro BEN products to products that were identified in the plasma from mice that were administered BEN.

We suspect that aldehyde dehydrogenase (ALDH) in red blood cells is the enzyme responsible for the metabolism of BEN to BA and that BA may be the cytotoxic metabolite. Because BEN displays selective activity against renal cell carcinomas, we determined the IC_{50} of BEN and metabolites in five different human renal carcinoma cell lines, and we correlated the metabolism of BEN to its carboxylic acid metabolite BA in renal carcinoma cells with the IC_{50} in these cells. The results of these studies will help to understand the activity of BEN and will aid in elucidating the pharmacology of BEN in the ongoing clinical trial.

Methods

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 733 609), 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 Developmental Therapeutics Program, National Cancer Institute (NCI, Bethesda, MD). Based on early results (see below), the benzoic acid analog of BEN (BA) and its products were included in further studies. All solvents used for LC-MS/MS were high-purity Burdick & Jackson and purchased from Fisher Scientific Co. (Fair Lawn, NJ). Formic acid was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Control human blood was obtained from the Central Blood Bank, Pittsburgh, PA, and plasma was prepared by centrifugation of whole blood at $2,000 \times g$ at room temperature for 20 min. 4-[bis[2-hydroxy-ethyl]amino]-2-methyl-benzaldehyde (BEN-(OH)₂) was generated by heating 13.6 mg BEN in approximately 4 mL of 0.05 N aqueous NaOH for 48 h at 50 °C. The progression of the reaction was monitored by mass spectrometry. 4-[bis[2-hydroxy-ethyl]amino]-2-methyl-benzoic acid (BA-(OH)₂) was generated by heating 13.2 mg BA in a solution of 0.03 N NaOH in 7 mL of acetonitrile/water (1:1, v/v) for 48 h at 50 °C. The progression of the reaction was assessed by mass spectrometry, and the conversion was 100 % in both cases. The final solutions were neutralized with hydrochloric acid and acidified with formic acid for a final concentration of 1 mg/mL of the respective product.

Qualitative determination of metabolites

In order to determine the structures of the products generated by the in vitro decomposition experiments and by mice administered BEN, we established an LC-MS/MS system using both MS scan mode and enhanced product ion scan mode. The LC-MS/MS system consisted of an Agilent (Wilmington, DE, USA) 1200 SL thermostated autosampler, binary pump, DAD, thermal column compartment and an ABI SCIEX (San Jose, CA, USA) 4000Q hybrid linear ion trap tandem mass spectrometer. 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 0.2 mL/min, and separation was achieved using a Phenomenex (Torrance, CA) Hydro Synergi-RP 4 micron 100×2 mm column. The gradient mobile phase was as follows: At time zero, solvent A was 15 % and was increased linearly to 40 % over 20 min. The percentage of solvent A was increased linearly to 80 % at 21 min, and the flow rate was increased to 0.4 mL/min. This was maintained for 2 min at which time solvent A was decreased linearly to 15 % at 24 min. This was maintained for 6 min, for a total run time of 30 min. During the

first 2 min and the last 6 min of the run, the flow leaving the column was diverted to waste. The parameters of the mass spectrometer were as follows: curtain gas 20, IS voltage 5,500 V, probe temperature 400 °C, GS1 40, GS2 30, declustering potential 50 V, collision energy 30 V, collision energy spread 20 V and scan rate 1,000 amu/s.

Quantitative assay

This LC–MS/MS assay was modified to quantify the metabolites generated in the decomposition and mouse metabolism experiments. The instrumentation, mobile phase and column were the same as the qualitative method described above. Mass spectrometric parameters were optimized for all analytes available. For MRM transitions, see Table S 1. The parameters of the mass spectrometer for the quantitation method were as follows: curtain gas 20, IS voltage 5,500 V, probe temperature 400 °C, GS1 40, GS2 30, declustering potential 50 V and collision energy 12 V with a dwell time of 50 ms on each MRM transition.

Standard curve—Stock solutions of BEN, BEN-Cl₂BA, BA-Cl₂BEN-(OH)₂ and BA-(OH)₂ were prepared separately at 1.0 mg/ mL in acetonitrile containing 0.1 % formic acid. A mixture was made containing all six analytes at a concentration of 0.1 mg/mL in acetonitrile with 0.1 % formic acid. All stock solutions were stored at –80 °C. On assay days, this solution was diluted with acetonitrile to obtain the lower calibration working solutions of 0.01 and 0.001 mg/mL. These calibration working solutions were diluted in plasma with 2 M H₂SO₄ (5 %, v/v) to produce 200- µL aliquots of the following concentrations: 1, 3, 10, 30, 100, 300, 500, 750 and 1,000 ng/mL. Aliquots of 10 µL of a mixture of desmethyl-BEN and desmethyl-BEN-Cl₂ (1 µg/mL each in acetonitrile) were added as internal standards. The analyte-to- internal standard ratios (response) were calculated by dividing the area of the compound peak by the area of the respective internal standard peak. Standard curves were constructed by plotting the analyte-to-internal standard ratio versus the nominal concentration and fitting by linear regression with weighting by 1/ year². The deviations of back-calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, reflected the assay performance.

Sample preparation—Ten microliter of a mixture of 1 µg/mL desmethyl-BEN and 1 µg/mL desmethyl-BEN-Cl₂ in acetonitrile (internal standards) and 1 mL of acetonitrile were added to 200 µL of standard or sample. Samples were vortexed for 1 min at a setting of 10 on a Vortex Genie-2 (Model G-560 Scientific Industries, Bohemia, NY) and centrifuged at 12,000 × g at room temperature for 5 min. The resulting supernatants were transferred to 12 mm × 75 mm borosilicate glass tubes and evaporated to dryness under a gentle stream of nitrogen at 37 °C. Dried residues were reconstituted in 100 µL of 20:80:0.05 acetonitrile/ water/2 M H₂SO₄ (v/v/v), and 10 µL was injected into the LC–MS/MS system.

Autosampler stability—Stability of the compounds in the autosampler was determined by 48 repeat injections of 500 ng/mL of all six analytes extracted from plasma. Samples were reconstituted with acetonitrile/water (20:80), with and without either 5 % 2 M H₂SO₄ or 0.1 % formic acid. The autosampler temperature was 4 °C. The amount of decomposition was assessed by the absolute peak area over time.

Plasma stability of BEN and BA—BEN or BA was added to plasma at a concentration of 10 µg/mL (*n* = 3) and incubated under the following conditions: 37, 4 or 37 °C with the addition of 5 % (v/v) of 2 M H₂SO₄ to the plasma. A 200-µL aliquot was removed at the following time points: 0, 5, 15, 30, 60, 120, 180 and 240 min. Samples were immediately frozen at –80 °C until analyzed by LC–MS/MS.

Incubation of BEN and products in human blood

We evaluated the stability of BEN, BEN-Cl₂ and BEN-(OH)₂ in blood ($n = 3$). Each of the compounds was added separately to 10 mL of human blood to achieve a concentration of 10 µg/mL. Samples were incubated in a shaking water bath at 37 °C for 0, 5, 10, 15, 30, 45, 60, 120, 180, 240, 360 min, and 24 and 48 h. At these times, a 500-µL aliquot of blood was removed and centrifuged at $12,000 \times g$ for 2 min at 4 °C. An aliquot of 200 µL of plasma was removed and added to 10 µL 2 M H₂SO₄. Samples were analyzed as described.

Incubation of BEN, BA and products in human plasma

To test whether the decomposition of BEN and BA in blood was in any way related to the presence of red blood cells, we repeated the blood experiment in plasma at 37 °C out for 96 h. At each time point, a plasma aliquot of 200 µL was removed and added to 10 µL 2 M H₂SO₄. In addition, BEN-Cl₂ and BA-Cl₂ were also incubated to assess their products. Samples were analyzed as described.

BA degradation in phosphate-buffered saline

To confirm that the observed degradation of BA in plasma was chemical, and independent of endogenous plasma components, we compared the decomposition of 10 µg/mL BA in plasma to the decomposition in PBS with and without 50 mg/mL human albumin. Triplicate aliquots of 200 µL were taken at 5, 10, 15, 20, 25 and 30 min. The aliquots were added to 10 µL of 2 M H₂SO₄. Samples were analyzed as described.

Half-life of analytes

To determine the half-lives of the analytes in blood and plasma, we analyzed the data from the experiments with PK Solutions 2.0 (Summit Research Services, Montrose, CO; www.summitPK.com). In order to determine statistical parameters, half-lives and relative rates in the protein binding experiment were determined by analysis with ADAPT5 [11] based on a one-compartmental model.

Protein binding

In order to determine the protein binding of BA, we modified a previously published approach, assuming that only the free fraction of an analyte can react [12].

Cell lines

Human renal carcinoma cell lines, ACHN, 786-0, A498, SN12K1 and CAKI-1, were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute and National Institutes of Health (Bethesda, MD). Human non-small-cell lung adenocarcinoma A549 cells for ALDH-positive control were obtained from Dr. Pam Hershberger, University of Pittsburgh Cancer Institute (UPCI). Human colorectal carcinoma HCT116 cells used for ALDH-negative control were obtained from Dr. Su-Shu Pan UPCI.

MTT assay

Human renal carcinoma cells were grown in RPMI 1640 complete medium containing 10 % FBS, 10 units/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5 % CO₂ at 95 % humidity. On day 1, SN12K1, CAKI-1 and A498 cells were plated in triplicate at 2500 cells/well in 96-well plates, and ACHN and 786-0 cells were plated in triplicate in 96-well plates at 1500 cells/well. After 24 h, BEN, BEN-Cl₂, BEN-(OH)₂, BA, BA-Cl₂ or BA-(OH)₂ was added to wells separately such that the final concentrations ranged between 1 and 30,000 ng/mL in medium containing less than 0.1 % acetonitrile. The MTT assay was performed

following the manufacturer's instructions using a TAC3 MTT cell proliferation assay (Trevigen, Gaithersburg, MD) and a Dynex NRX Revelation microplate reader (Dynex Technologies, Chantilly, VA). As a control, acetonitrile 0.1 % in medium was used. The results were expressed as percentage inhibition relative to control. The IC₅₀ values of BEN and products for each cell line were calculated from triplicate experiments using the Hill equation and the computer program ADAPT 5.

BEN metabolism in renal carcinoma cells

SN12K1, ACHN, CAKI-1, 786-0 or A498 cells (3×10^5 cells/well) in logarithmic growth were plated into 6-well culture plates in 2 mL of medium. After 24 h, BEN was added at a final concentration of 1,000 ng/mL. Aliquots of medium (100 μ L) were taken at 0, 2, 4, 6 and 24 h, placed into a microcentrifuge tube containing 5 μ L 2 M H₂SO₄ and stored at -80°C until analysis using calibration curves in control medium.

Aldehyde dehydrogenase (ALDH) activity in renal carcinoma cells

SN12K1, A498, human colorectal HCT116 and human non-small-cell lung cancer A549 cells were grown as described. Cells were harvested by scraping, washed with PBS and spun at $900 \times g$. Cells (1×10^8) were homogenized in 100 mM Tris-HCl buffer (pH 8.0) with a Dounce homogenizer on ice. The resulting homogenate was centrifuged at $9,000 \times g$ for 15 min at 4°C . The $9000 \times g$ supernatant (300 μ L) was added to 1.5 mL of 100 mM Tris-HCl buffer pH 8.0, with 3 mM β -nicotinamide adenine dinucleotide (NAD), 4 mM acetaldehyde, 100 mM potassium chloride and 10 mM 2-mercaptoethanol. As a control, $9,000 \times g$ supernatants were added to the sample without addition of acetaldehyde which served as the blank in the reaction. Absorbance at 340 nm was recorded for 5 min in a Beckman Coulter DU 640 spectrophotometer (Indianapolis, IN, USA) maintained at 37°C . The rate of absorbance increase at 340 nm per minute was used to quantitate the reduction of NAD to NADH, which stoichiometrically corresponds to the conversion of acetaldehyde to acetic acid. The activity is reported as the number of units per 1×10^7 cells. The experiment was performed thrice for each cell line.

In vivo products of BEN

In order to determine whether the products observed in vitro would be generated in vivo, we administered BEN to mice. Mice were obtained and handled in accordance with the Guide for the Care and Use of Laboratory Animals as previously described [13]. Adult female CD2F1 mice were administered a 20 mg/kg i.v. bolus of BEN (2 mg/mL in 50 % hydroxyl-propyl- β -cyclodextrin in saline) or 50 % hydroxyl-propyl- β -cyclodextrin vehicle at 0.01 mL/g body weight. Mice were euthanized 30 min post-dose. Blood was removed by cardiac puncture and placed on ice. Plasma was obtained by centrifuging blood for 2 min at $14,000 \times g$ at 4°C . A 200- μ L aliquot was added to 10 μ L 2 M H₂SO₄ and frozen at -80°C until analysis as described.

Results

Method development and performance

Chromatographic method—Our goal was to provide adequate separation for all six analytes and all the other possible products while minimizing the run time. In order to achieve this, we assessed three different columns: a Phenomenex Luna C18(2) 150×2 mm, Phenomenex Synergi 4 μ Polar-RP 100×2 mm and a Phenomenex Synergi 4 μ Hydro-RP 100×2 mm. In addition, the percentage of acetonitrile and water was varied over time. We found that the Synergi Hydro-RP and the gradient described above resulted in adequate separation with the shortest run time at 30 min. As expected, the BA analogs eluted before

their respective BEN analogs, and modifications of the side chains resulted in the OH–OSO₂CH₃–Cl elution order. We found that using two internal standards, desmethyl-BEN for the early eluting compounds and desmethyl-BEN-Cl₂ for later eluting peaks, resulted in a more precise and reproducible assay. A representative chromatogram is shown in Figure S1.

Autosampler stability—Analytes, neat or extracted from plasma, decomposed in the autosampler after reconstitution in acetonitrile or water. We found that 5 % (v/v) of 2 M H₂SO₄ slowed down decomposition sufficiently to allow quantitation. The percentage loss after 48 sequential injections over 24 h in the presence of acid was BEN 4.6 %; BEN-Cl₂ 6.3 %; BEN-(OH)₂ 9.3 %; BA 5.2 %; BA-Cl₂ 2.2 %; and BA-(OH)₂ 4.0 %.

Plasma stability—After 4 h at 4 °C in plasma, BEN and BA had not degraded (100 and 102 % remaining), respectively, relative to time 0 h. At 37 °C, the amount of BEN was 92 %, whereas <1 % of BA remained. At 37 °C with 2 M H₂SO₄ (5 % v/v), the degradation of BEN and BA in plasma was slowed, and the amounts of BEN and BA remaining after 4 h were 105 and 91 %, respectively (see Fig. 2).

Plasma calibration curve and lower limit of quantitation (LLOQ)—The assay proved to be linear and had regression coefficients > 0.989 for each of the three standard curves for each analyte. The lower limit of quantitation (LLOQ) was 1 ng/mL for BEN, BEN-Cl₂BA, BA-Cl₂ and BA-OH₂ and 10 ng/mL for BEN-OH₂ at a signal-to-noise ratio >10. The assay was accurate and precise with CV% <15 % at all concentrations. The mean and standard deviations of the back-calculated values in the standard curves of each analyte are displayed in Table S2.

Incubation of BEN and products in human blood

In whole blood at 37 °C, BEN had an overall apparent half-life of 18 min. However, the rate of disappearance appeared to increase when the concentration of BEN dropped below approximately 1 µg/mL. BEN decomposed into at least eleven different products that were structural analogs of either BEN or BA. In addition, the BA analogs had peak areas much larger than the BEN analogs. These compounds were identified by LC–MS/MS, and their structures are depicted in Fig. 1. For both BEN and BA, we observed and structurally elucidated products in which one or both of the methane sulfonate leaving groups were replaced by either a chlorine (from plasma chloride ions) or hydroxyl (from plasma water) moiety, resulting in a total of 12 analytes, including parent compounds. We quantitated the absolute amount (where standards were available, Fig. 3a) formed or the peak area/IS area ratio (Fig. 3b).

In addition, BEN-Cl₂BA-Cl₂ and BEN-(OH)₂ were also incubated separately, and their conversion to respective products was determined. When BEN-Cl₂ was placed into blood, BA-Cl₂ and BA-(OH)₂ were detected. The addition of either BA-Cl₂ or BEN-(OH)₂ to blood resulted in the detection of only BA-(OH)₂.

Incubation of BEN, BA and products in human plasma

To test whether the decomposition of BEN and BA was related to the presence of red blood cells, we repeated the blood experiment in plasma. Incubated in plasma at 37 °C, BEN generated only BEN analogs (Fig. 4a) and BEN had an apparent half-life of 220 min. Incubation of BA only generated BA analogs (Fig. 4b), and BA had an apparent half-life of 5 min. Plasma incubation of BEN-Cl₂ or BA-Cl₂ generated the respective Cl–OH and (OH)₂ analogs (data not shown).

BA degradation in phosphate-buffered saline

BA was added to human plasma, PBS and 5 % human albumin in PBS. The rate of BA disappearance was greatest in PBS with an apparent half-life of 4 min compared to 5 min in plasma and 8 min in PBS with albumin (Figure S2). Under the assumption that only unbound BA can degrade, the plasma protein binding of BA was determined to be 15 % (3.7–26.7 % 95 % CI) and the binding of BA to 5 % albumin was determined to be 49 % (36.4–61.4 % 95 % CI). BEN is 74–85 % protein bound in human plasma [14].

Growth inhibitory potential of BEN and products

The growth inhibitory potential, as expressed by the IC_{50} of BEN, BA, BEN- CL_2 BA- CL_2 BEN-(OH) $_2$ and BA-(OH) $_2$ was determined in human renal carcinoma cell lines. Although BEN was chemically stable, the IC_{50} values of BEN in these cell lines were 1.5- to 500-fold lower than those of the other analytes tested (Table 1). The IC_{50} values of BEN-(OH) $_2$ and BA-(OH) $_2$ could not be determined in the concentration range tested. The growth inhibitory potential for BEN in the cell lines was A498 > CAKI-1 > ACHN > 786-0 > SN12K1.

BEN metabolism in human renal carcinoma cells

BEN underwent either metabolism or chemical conversion to both BEN-(OH) $_2$ and BA-(OH) $_2$ in all cell lines. In contrast, BEN was converted to only BEN-(OH) $_2$ in control, cell-free medium (Fig. 5). The rate of disappearance of BEN was greatest in A498 cells followed by 786-0, ACHN, CAKI-1 and lastly SN12K1 cells (ratio of A498/SN12K1 of 35).

Aldehyde dehydrogenase activity in cell lines

To explain the different fate of BEN incubated with different cell lines, we measured ALDH activity in human renal carcinoma A498 cells and SN12K1 cells, human lung tumor A549 cells and human colorectal HCT116 cells. These cell lines were chosen because the A498 cells generated the most BA-(OH) $_2$ and the SN12K1 cells generated the least amount of BA-(OH) $_2$ after incubation of BEN. As a positive and negative control we used the A459 and HCT116 cells which have been shown to have high and low levels of ALDH activity, respectively, in a screen of NCI 60 cell lines for ALDH activity [15]. The A498 cell line had an aldehyde dehydrogenase activity 38 times greater than the SN12K1 cell line, 6.25 (± 0.17) units/ 10^7 cells versus 0.163 (± 0.102) units/ 10^7 cells, respectively. The A549 cells, used as a positive control, had an activity of 26.2 (± 0.3) units/ 10^7 cells, while HCT116 displayed no ALDH activity.

BEN products in plasma from a mouse dosed with BEN

We detected 12 different BA-derived products in plasma of a mouse dosed with BEN, 6 of which were glucuronides. Each of the BA products generated had a corresponding glucuronide. The non-glucuronide products were identical to the BA products generated in human blood and plasma.

Discussion

BEN is an alkylating agent currently being investigated in phase I clinical trials, with structural similarities to busulfan and melphalan. BEN is reported to undergo hydrolysis in aqueous environment [10]. This led us to hypothesize that BEN likely undergoes hydrolysis in plasma and blood. The hydrolysis of BEN was expected to decrease the alkylating activity. In order to better understand the pharmacology of BEN and its products, we aimed to characterize the generation and growth inhibitory potential of BEN products.

First, we developed an assay that would allow us to quantitate BEN and its presumed products. The degradation of the analytes could be slowed by lowering temperature and pH. BEN and its analogs were found to be more stable than BA and its respective analogs.

In whole blood, but not in plasma, BEN was rapidly converted to its carboxylic acid analog BA, suggesting conversion by an enzyme present in red blood cells. BA rapidly decomposed in plasma with a half-life of approximately 5 min. The rate of BA degradation in plasma was slightly slower than in PBS, which is not surprising since only free BA can react. However, the rate of degradation in plasma was greater than in PBS with human albumin at physiological concentrations. This may be due to the chemical environment of plasma. Plasma may have other components that enable BA to react faster than in PBS with albumin. Under the assumption that only free BA is subject to degradation, we can conclude that BA is likely approximately 15 % protein bound and that the decomposition of BA in plasma is most likely chemical in nature, not enzymatic.

In plasma, BEN and BA decomposed according to a parallel pathway to a number of hydroxylated and chlorinated analogs, as depicted in Fig. 1. The relatively fast rate of production of BA products relative to the generation of BEN products is likely indicative of the relative reactivities of the acid analogs relative to the aldehyde analogs. We hypothesize that the unbound electron pair on the aniline nitrogen is the initiator of an S_N2 attack on the β -carbon, generating the reactive aziridinium intermediate. Mesomeric withdrawal of the aniline unbound electron pair by the para-aldehyde moiety, or occupation of the electron pair by a proton under acidic conditions would be expected to decrease the generation of the aziridinium intermediate and thereby decrease the reactivity. The abundance of BA analogs and lack of BEN analogs after incubation of BEN in whole blood can be attributed to the relative rates of the competing pathways of BEN conversion. The conversion of BEN to BA is much more rapid than conversion of BEN to downstream aldehyde analogs. In addition, any aldehyde products formed may also be converted to their respective acid counterparts by the same enzymatic pathway that converts BEN to BA, as was shown for the conversion of BEN-(OH)₂ to BA-(OH)₂ in whole blood.

Intravenous administration of BEN to a mouse confirmed the presence of the BA-related analytes and their glucuronides, but no BEN 30 min after dosing. The detection of glucuronides of BA analogs without hydroxyethyl moieties suggests that glucuronidation takes place on the carboxylic acid functionality. More complete assessment of the pharmacokinetics and metabolism of BEN in mice is currently being conducted.

Although ten of the 12 entities observed in plasma have the potential to alkylate DNA (all except the dihydroxy metabolites), and BEN-Cl₂ has been shown to be cytotoxic in the NCI 60-cell line screen, the BEN products that are generated in plasma and blood had IC₅₀ values that were severalfold higher than those of BEN in a panel of human renal carcinoma cells. During incubation with human renal carcinoma cells, BEN undergoes chemical conversion to BEN-(OH)₂ or metabolism to BA and subsequent conversion to BA-(OH)₂ in all cell lines tested. A498 cells displayed the greatest metabolism of BEN to BA as shown by the amount of BA-(OH)₂ generated; in fact, no BEN-(OH)₂ could be detected. In contrast, SN12K1 cells generated very little BA-(OH)₂. The ratio of BEN disappearance for A498/SN12K1 was 35-fold. It is noteworthy that the A498 cell line was the most sensitive to BEN demonstrated by the lowest IC₅₀ value. To test whether ALDH may be responsible for the metabolic conversion of BEN to BA, we measured the ALDH activity in A498 and SN12K1 cells. We found that A498 cells had ALDH activity ~40- fold greater than SN12K1 cells, paralleling the BA-(OH)₂ generating activity and conversely the metabolism of BEN to BA. These results are in line with the previously reported ALDH activities [15]. These data support the notion that intracellular ALDH is responsible for the metabolism of BEN to

BA in these cells and that this intracellular conversion mediates its cytotoxic effects. ALDH may also be responsible for the observed metabolism of BEN to BA in whole blood as red blood cells contain ALDH [16].

We hypothesize that the A498 cell line is able to most efficiently metabolize BEN to its more reactive metabolite BA by ALDH inside the cell, which then rapidly alkylates cellular constituents resulting in cell damage. The high IC_{50} values of BA added to medium suggest that BA reacts with other nucleophiles in the medium before it is able to reach its intracellular targets. BEN- Cl_2 and BA- Cl_2 had intermediary IC_{50} s. This suggests that BEN- Cl_2 gets into the cells and is locally converted to BA- Cl_2 which reacts with cellular targets. However, because BA- Cl_2 is not as reactive as BA, a fair amount likely diffuses out of the cell without exerting cytotoxic effects. Conversely, when BA- Cl_2 was added to the cell incubation, the relative stability of BA- Cl_2 allowed enough of it to diffuse into the cells to cause intracellular damage. Taken together, these data suggest that BEN is a pro-drug that requires intracellular activation to the highly reactive BA, which likely exerts rapid and localized damage via alkylation inside the cells.

In summary, we developed an analytical assay that will prove instrumental in defining the preclinical and clinical pharmacology of BEN in ongoing clinical trials. We have identified a number of products and metabolites of BEN and characterized their relative growth inhibitory properties. In addition, we have shown that ALDH may be responsible for the metabolism of BEN to BA and that BEN is more active in cell lines that express high levels of ALDH. The potential importance of this finding lies in the reported overexpression of ALDH in stem cells of many tumor types, making these stem cells potential targets for BEN therapy, existing polymorphisms in ALDH and potential drug–drug interactions with inhibitors of ALDH such as disulfiram and metronidazole [17–20]. More complete in vitro and in vivo studies are underway to define BEN pharmacokinetics and metabolism, and the relative contribution of BEN and metabolites to plasma exposure of alkylating species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Chemical structures of busulfan, melphalan and chlorambucil and proposed metabolic scheme for BEN in blood. BEN analogs can also be converted to their corresponding BA analog. In plasma there is no conversion of BEN to BA. BEN is converted only to BEN analogs, and BA is converted to BA analogs, respectively

Fig. 2.
Stability of BEN (**a**) and BA (**b**) in plasma at room temperature (open circle), 4 °C (*filled square*) and at room temperature with 5 % (v/v) 2 M sulfuric acid (*open triangle*)

Fig. 3.

The disappearance of BEN in whole blood at 37 °C over time. **a:** BEN (*filled square*), BEN-Cl₂ (*filled diamond*), BEN-(OH)₂ (*filled triangle*), BA (*open square*), BA-Cl₂ (*open diamond*) and BA-(OH)₂ (*open triangle*). **b** BEN-Cl (*filled square*), BEN-OH (*filled diamond*), BEN-Cl-OH (*filled triangle*), BA-Cl (*open square*), BA-OH (*open diamond*) and BA-Cl-OH (*open triangle*). Analytes in graph A were quantitated using reference compounds. The analytes in graph B were measured by dividing the area of the analyte by the area of the internal standard

Fig. 4. BEN (a) and BA (b) decomposition in plasma at 37 °C and formation of structural analogs. **a:** BEN (filled square), BEN-Cl₂ (filled circle), BEN-(OH)₂ (filled triangle), BEN-Cl (open square), BEN-Cl-OH (open triangle) and BEN-OH (open circle). (b): BA (filled square), BA-Cl₂ (filled circle), BA-(OH)₂ (filled triangle), BA-Cl (open square), BA-Cl-OH (open triangle) and BA-OH (open circle). For the analytes in which no reference compound was available, the ng/mL BEN equivalents were calculated by using the analyte-to-internal standard ratio back-calculated with the equation of the BEN standard curve

Fig. 5. Metabolism of BEN (a) and formation of BA (b), BEN-(OH)₂ (c) and BA-(OH)₂ (d) in intact human renal carcinoma cells. SN12K1 (open circle), ACHN (open square), CAKI-1 (open triangle), 786-0 (filled triangle), A498 (filled square), blank media (filled circle)

Table 1Growth inhibition (IC₅₀) for BEN and related analogs in human renal carcinoma cell lines

Compound/cell	786-0	ACHN	SN12K1	CAKI-1	A498
BEN	0.267 (0.097)	0.263 (0.047)	0.790 (0.105)	0.202 (0.133)	0.0463 (0.0119)
BEN-Cl ₂	6.88 (2.77)	6.13 (2.06)	10.4 (1.2)	1.30 (0.65)	0.0677 (0.0072)
BEN-(OH) ₂	>10	>10	>10	>10	>10
BA	9.23 (5.53)	18.9 (8.9)	19.7 (5.6)	15.8 (8.1)	23.5 (9.6)
BA-Cl ₂	4.74 (0.70)	5.72 (0.51)	9.35 (2.76)	6.60 (1.35)	6.57 (3.49)
BA-(OH) ₂	>10	>10	>10	>10	>10

IC₅₀ expressed as µg/mL, mean (SD) of triplicate experiments