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Assessment of the anti-tumor potential of Bithionol *in vivo* using a xenograft model of ovarian cancer

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Abstract

Objective—In regards to the concept of ‘drug repurposing’, we focused on pharmaceutical grade Bithionol (BT) as a therapeutic agent against ovarian cancer. Our recent *in-vitro* study provides preclinical data suggesting a potential therapeutic role for BT against recurrent ovarian cancer. BT was shown to cause cell death via caspases-mediated apoptosis. The present preliminary study further explores the anti-tumor potential of pharmaceutical grade BT in an *in vivo* xenograft model of human ovarian cancer.

Methods—Nude Foxn1^{nu} mice bearing SKOV-3 human ovarian tumor xenografts were treated with titrated doses of BT and therapeutic efficacy of pharmaceutical BT was determined using bioluminescence imaging. BT induced changes in cell proliferation and apoptosis were evaluated by Ki-67 immunochemical staining and TUNEL assay. The effect of BT on autotaxin (ATX) levels in serum, ascitic fluid and tumor tissue was assessed by colorimetric and western blot techniques.

Results—BT treatment did not exhibit anti-tumor potential nor enhanced survival time, at any of the doses tested. No apparent signs of toxicity were observed with any of the doses tested. Immunohistological analysis of tumor sections did not demonstrate a significant decrease in cellular proliferation (Ki-67 assay). An increase in apoptosis (via TUNEL assay) was observed in all BT-treated mice, compared to vehicle-treated mice.

Conclusion—Although BT did not show significant antitumor activity in the present study, the ability of BT to induce apoptosis makes it still a promising therapeutic agent. Further confirmatory and optimization studies are essential to enhance the therapeutic effects of BT.

Keywords

Bithionol; ovarian cancer; xenografts; apoptosis; cellular proliferation; autotaxin

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INTRODUCTION

Most epithelial ovarian cancer (EOC) patients are diagnosed at an advanced stage when disease has spread beyond the ovary. The majority of women diagnosed with advanced ovarian cancer have a low overall survival [1]. Although most ovarian cancer patients (70–80%) initially respond to cytoreductive surgery and adjuvant paclitaxel and platinum-based chemotherapy, the long-term prognosis is generally poor, with recurrence and development of drug-resistant disease [2, 3]. Therefore, it is essential to explore new agents that can act via novel mechanism(s) of action to improve the overall survival for ovarian cancer patients. New drug discovery demands enormous cost and time. An alternative approach is ‘drug repurposing’ wherein clinically approved drugs for one indication are re-explored for new applications. The advantage of ‘drug repurposing’ is cost and time effectiveness as absorption, distribution, metabolism, excretion (ADME) and basic toxicity are already well established and can be immediately taken to Phase II/III clinical trials. Keeping this concept in mind, we focused on pharmaceutical grade Bithionol [2, 2′-Sulfanediylbis (4, 6-dichlorophenol)] (BT), a clinically approved anti-parasitic drug as an anti-ovarian cancer drug. BT is not commercially available in the U.S., but pharmaceutical grade BT capsules were obtained from the Centers for Disease Control and Prevention (CDC) with their approval. The pharmaceutical grade BT has received Food and Drug Administration approval as a second-line orally administered medication for the treatment of helminthic infection and has been safely dosed in humans [4].

In our recent *in vitro* study, we demonstrated the ability of BT (chemical grade from Sigma) to exert cytotoxic effects on a panel of ovarian cancer cell lines regardless of their cisplatin sensitivities [5]. BT was shown to induce cell death via apoptosis and the mechanism(s) of action was shown to be via cell-cycle regulation, reactive oxygen species (ROS) generation, NF-κB inhibition and autotaxin (ATX) inhibition [5]. To assess whether our *in vitro* findings would extend to an *in vivo* model, the present study explored the anti-tumor potential of pharmaceutical grade BT using a xenograft model of human ovarian cancer in nude mice. As studies regarding stability, dissolution, best formulation, vehicle and route of delivery were already established; the major focus of the current study was determination of the optimal dose required for inhibiting the growth of implanted human EOC in nude mice and the effective treatment duration.

In the present study, we established an ovarian cancer xenograft model by intraperitoneal injection of SKOV3-luc-D3 human EOC cells into **Nude Foxn1^{nu} mice**. Following tumor establishment, mice were treated with varying doses of pharmaceutical grade BT. As most ovarian cancer patients are diagnosed at an advanced stage when the disease has spread beyond the ovary [6], we purposefully delayed BT treatment to mimic the human disease at detection stage. BT treatment was started at 5 weeks post-tumor inoculation to ensure that the majority of mice would show steady and stable tumor burden. Mice that reached predetermined humane or experimental end points were sacrificed as per the Laboratory Animal Care and Use Committee (LACUC) guidelines. Kaplan-Meier survival curves were generated. Following sacrifice, blood and ascitic fluid were collected for ATX measurements. In addition, tumor implants were removed and examined by haematoxylin

and eosin (H&E) staining and immune-histochemistry (IHC) for assessment of cell proliferation rate (Ki67 expression), apoptosis (terminal deoxynucleotide transferase-mediated dUTP nick end labelling - TUNEL) and ATX status (as a marker of tumor cell motility, survival, angiogenesis, proliferation).

MATERIALS AND METHODS

Cell lines and Chemicals

SKOV3-luc-D3 cell lines (derived from human ovarian carcinoma cells) purchased from Caliper Life Sciences (Hopkinton, MA, USA) were used to induce tumor growth. The luciferase reporter, luc-D3 tagged to SKOV-3 cells facilitates non-invasive monitoring of tumor progression and response to BT treatment via luciferase expression. The cells were tested for absence of mycoplasma before injecting into mice. Pharmaceutical grade BT in the form of capsules was obtained from the CDC Drug Service, Atlanta, GA. The content of the capsule was suspended in injection grade water (Gibco Cat # A12873-02) to prepare BT suspensions for administration. Primary antibodies, e.g., Ki67, cleaved caspase-7 and cPARP were purchased from Cell Signaling Technologies (Danvers, MA). ATX antibody was purchased from Santa Cruz Biotechnology Inc., (USA). TumorTACS™ In Situ Apoptosis Detection Kit for TUNEL assay was purchased from Trevigen (Gaithersburg, MD).

Animal care

The animal study was approved by the LACUC of Southern Illinois University School of Medicine, Springfield, IL, USA. Animals were 4–6 week old female heterozygous HSD: Athymic Nude Foxn1^{nu} mice purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in static micro-isolators (up to five mice per cage) and kept under standard conditions: 22 ± 2 °C, 45 ± 10% relative humidity, and 12 h light/12 h dark cycle each day, with free access to sterile food and water. All mice were held in specific pathogen free conditions in an ABL-2 barrier facility. Mice underwent a period of 2 weeks of observation and acclimatization before tumor inoculation.

Mice Xenograft study

Fifty, 4–6 week-old HSD: Athymic Nude Foxn1^{nu} mice, weighing between 22–25grams, were inoculated IP with 5.0×10⁶ SKOV3-luc-D3 cells in Dulbecco's phosphate buffered saline (DPBS). Following injection, the animals were imaged twice a week using a non-invasive, *in vivo* imaging system (IVIS, Xenogen, Caliper Life Sciences, CA, USA) to ensure implantation and to determine treatment start date. Regions of interest (ROIs) were established for individual tumors, data was analysed using the Living Image® Software v. 3.0 (Xenogen, Caliper Life Sciences, CA, USA) and luminescent signal for those tumors were measured. Mice that showed a marked increase in luminescence signal over the basal value (day 1 of SKOV3-Luc-D3 inoculation) were considered as tumor bearing. Only mice that showed steady and stable increase in tumor intensity were considered for BT treatment. A time period of 5 weeks was needed for the majority of the mice to show well established tumor. Mice with similar signal intensities were randomly distributed into 5 groups of 8 animals each (4 treatment groups and 1 control group). As BT capsules are for oral

administration, we administered capsular contents as aqueous suspension via oral gavage. Each treated group received a different oral dose of BT (30, 60, 120 or 240 mg/kg BW). Each dose was divided into three doses and administered on day 1, 3 and 5 weekly. The control group received vehicle (injection grade water) orally on same schedule. The treatment continued until animals reached standard signs of pain and distress or our pre-determined experimental end points (80–100% reduction in tumor). Body weight, expressed in grams, was used to monitor for systemic toxicity of BT. Mice that reached pre-determined end points (either humane or experimental) were sacrificed per LACUC guidelines. Mice were euthanized in a special euthanasia chamber using carbon dioxide (CO₂). Kaplan-Meier survival curves were generated for the time period starting from the first day of BT treatment until the day of the event of interest (either humane or experimental end points). Following sacrifice, blood, ascitic fluid and tumor tissues were collected. Blood was collected by cardiac puncture in the presence of an anticoagulant (heparin) for ATX measurements. Tumor implants were excised and examined by H&E staining and IHC. Expression of Ki-67 was used to assess cell proliferation rate, TUNEL assay was used to assess apoptosis and change in ATX levels were compared between the control and treated groups.

***In vivo* bioluminescence imaging**

In vivo bioluminescence imaging (BLI) was performed as described previously [7, 8] using IVIS Imaging system equipped with a highly sensitive cooled CCD camera. To measure tumor growth in terms of luciferase activity, 150 mg/kg BW of d-luciferin (sodium salt) in sterile 0.9% sodium chloride was injected into the mouse peritoneal cavity 10 min. prior to imaging. Mice were anesthetized using an isoflurane gas anesthesia system. ROIs were established for individual tumors, data analyzed using the Living Image® Software v.3.0 and luminescent signal for those tumors were measured. The signal intensities from manually derived ROIs were obtained and expressed as photon flux (photons/s).

Tumor immunohistochemical and histologic analysis

Tumor tissue from four mice (in each group) was processed for histological analysis and to assess the extent of apoptosis and cell proliferation (Ki67). Tissue sections, stored at –80°C, were fixed with cold fixative (acetone) for 10 min. prior to immunostaining. To assess morphological changes, tissue sections were subjected to routine H&E staining. Cell proliferation was assessed by Ki-67 staining using anti-mouse Ki-67 antibody (1:400 dilution) and detected using anti-mouse HRP-DAB Cell and Tissue Staining Kit from R&D Systems, according to the manufacturer's instructions. Briefly, acetone fixed sections were treated with 2% hydrogen peroxide to inactivate endogenous peroxidase and nonspecific binding was blocked by treatment with a blocking reagent. The primary antibody was applied to each section for 1 hr. at 37°C and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was applied at a dilution of 1:100 for 1 hr. at room temperature. Immunoreactivity was subsequently detected using a 3, 3'-diaminobenzidine system (DAB). Ki-67-positive cells (brown staining) were scored by visual examination of 10 randomly selected microscopic fields.

For assessing TUNEL-positive cells, TumorTACS *in Situ* Apoptosis Detection Kit was used. Histologic analysis of DNA fragmentation was used to identify apoptotic cells in sections of

the xenograft tumors. During apoptosis, the chromosomal DNA is cleaved by endonucleases to generate DNA fragments with free 3'-hydroxyl residues. In situ, enzyme terminal deoxynucleotidyl transferase (TdT) adds biotinylated nucleotides at the 3' ends of cleaved DNA fragments. The incorporation of biotinylated nucleotides allows chromosomal DNA fragmentation to be visualized by binding streptavidin-HRP followed by reaction with 3, 3'-diaminobenzidine (DAB) to generate a dark brown precipitate. When viewed under a standard light microscope, apoptotic cells are clearly distinguished by the dark brown staining.

Evaluation of BT Toxicity

For mice treated with BT, gross measures such as weight loss, distress signs, behavior changes, body condition and diarrhea, in particular, were investigated for potential signs of BT related toxicity. A regular record of body condition scoring was maintained. To assess body condition, we used the 'body condition scoring' system as described by Ullman-Cullere and Foltz [9].

Western blot assays

Western blotting was carried out to analyze expression of ATX and effectors of apoptosis, such as cleaved caspase-7 and cleaved PARP, using specific antibodies. After resection, tumor tissue was immediately stored at -80°C until analysis. Tumor tissues from two mice (in each group) were pooled and assessed. The study was repeated with 3 sets of such pooled tissues for each group. The tissue was homogenized on ice in RIPA buffer containing protease inhibitors, clarified and subjected to a BCA protein assay and analysed by western blotting. The relative levels of protein expression were estimated using the Odyssey imaging software. For tumor tissue, individual proteins were normalized to β -actin loading controls. The ratio of protein to β -actin was normalized to 1 in the control group. For ascites, the amount of protein in each sample was estimated by BCA assay and equal amount of protein was loaded from each sample. For assessment of equal protein loading, the gels were stained with coomassie blue stain. Post transfer loading was assessed by Ponceau S staining of the immunoblot. ATX expression in the control was considered as 1, against which other groups were compared.

Autotaxin (ATX) Assay

ATX activity from plasma and ascites was assessed using colorimetric and western blotting techniques. The phosphodiesterase activity of ATX was measured colorimetrically as described previously [5, 10]. Briefly, 20 μ L of plasma/ascites was incubated with 80 μ L substrate containing p-nitrophenylphosphonate (pNppp) at a final concentration of 5 mM. The reaction product was measured by reading the absorbance at 410 nm. ATX levels of the vehicle-treated control group were considered as 100% against which BT-treated cells were compared. The data was expressed as %ATX vs. control. Ascites and plasma from four mice (in each group) were assessed for ATX activity. Data are shown means $\pm$ SD of four mice from each group. The assay was repeated twice for each mouse. ATX protein from tumor tissue and ascites was assessed by western blotting as described above.

Statistical Analysis

All data are reported as means $\pm$ SD and statistical significance was defined as $p < 0.05$. The Kaplan–Meier method and the log-rank (Mantel-Cox) test were used to compare survival between groups using the Statistical analysis SPSS 17.0 software (SPSS Inc., Chicago, IL).

RESULTS

Effect of BT on tumor growth

To investigate whether pharmaceutical grade BT can inhibit the growth of implanted human EOC xenografts in a nude mice model, animals were inoculated with SKOV3-luc-D3 cells via the IP route as described in Materials and Methods. Representative bioluminescent images (dorsal views) of control and BT-treated mice are shown in Figure 1. Mice that showed detectable tumor with bioluminescence (BLI) values above 1.0×10^8 photons/sec (approx. 5 weeks post-tumor inoculation) were only included in the BT treatment study (Figure 2, B–F). Each group received different dosage of pharmaceutical grade BT, as an aqueous suspension, via oral gavage. The control group received only vehicle (injection grade water). Tumor growth was assessed by bioluminescent imaging. The mean tumor growth curves representing each treatment group are shown in Figure 2. As shown in Figure 2A, the mean tumor intensity in the control group increased proportionately with time. Similar increase in tumor intensity was observed with BT-treated groups as well. Our data demonstrated that pharmaceutical grade BT did not show antitumor potential at any of the doses tested.

Effect of BT on Survival

Kaplan-Meier survival curves were generated for the time period starting from day 1 of BT treatment until the day of the event of interest (either humane or experimental end points). The log-rank (Mantel-Cox) test was used to compare survival curves between groups. As shown in Figure 3A, BT treatment did not significantly affect survival ($p = 0.3677$, Log-rank). Compared to vehicle-treated mice, the 30 mg/kg BW BT-treated group showed a relatively higher overall survival, yet not statistically significant. The median survival (post-BT treatment) for the 30 mg/kg BW group was 45 days whereas the vehicle-treated control group survived 35 days. The median survival time for the 60, 120 and 240 mg/kg BW groups were 42, 39 and 32 days, respectively.

BT Toxicity

To assess general health, mice were regularly monitored for appearance, activity (natural vs. provoked) and body condition. For mice treated with BT, gross measures such as weight loss, clinical signs of illness, behaviour changes and diarrhoea, in particular, were investigated for potential signs of BT-related toxicities/side effects. A regular record of body condition was maintained. None of the mice treated at any BT dose showed signs of morbidity. Body weights were not affected by BT treatment (Figure 3B). None of the mice showed abdominal distension until just before euthanasia. No difference was observed between vehicle-treated and BT-treated mice in any of the clinical signs of illness.

Effect of BT on tumor histology

To investigate the effect of BT on the histology of EOC xenografts, tumor tissue was processed for morphological studies (H&E staining). SKOV-3 cells are a well characterized platinum-resistant phenotype of serous ovarian cancer [11]. As shown in Figure 4, most of the tumors exhibit poorly differentiated serous cystadenocarcinomas with variable nuclear sizes. Tumor implants from vehicle controls were composed primarily of ovarian epithelial cancer cells with cobblestone-like characteristics and were relatively uniform epithelial cells with irregular and hyperchromatic nuclei. Similar morphology was seen with mice treated with higher doses of BT, e.g., 120 mg/kg BW and 240 mg/kg BW. In contrast, mice treated with BT at 30 mg/kg BW showed a slightly increased proportion of non-cellular stromal components with pleomorphic and hyperchromatic nuclei.

Inhibition of cell proliferation in vivo

We performed Ki-67 immunohistochemistry to investigate effect of BT on cell proliferation. Ki-67 was used as a surrogate marker of tumor aggressiveness, with dark brown nuclear staining regarded as positivity for Ki-67 (Figure 5). Based on the scoring of 10 randomly selected microscopic fields, the extent of proliferation was expressed as proliferative index (percentage of Ki-67-positive cells). Data was expressed as mean $\pm$ SD of four mice from each group. As shown in Figure 5 (graph), the proliferation index was significantly higher in the vehicle-treated tumors compared to the 30 mg/kg BW BT group (51.33 ± 6.11 % vs. 34.33 ± 3.21 , respectively, $P < 0.05$, student 't' test). Our results suggest that the delayed tumor growth observed at 30 mg/kg BW dose initially may be caused by a reduction in the proliferative index of tumors based on Ki-67 staining. However, no significant difference was observed between control and all other treatment doses. The proliferative index was 51.33 ± 6.11 % (untreated) vs. 41.67 ± 4.51 (60mg/kg BW), 49.01 ± 12.29 (120mg/kg BW), and 52.67 ± 8.33 (240 mg/kg BW, $p > 0.05$, student 't' test).

Induction of Apoptosis in vivo

The extent of apoptosis in tumor tissue was assessed by DNA fragmentation (TUNEL) and expression of apoptotic effectors such as cleaved caspase-7 and cleaved PARP. Apoptotic cells with condensed and irregularly shaped nuclei stain positively for TUNEL (dark brown). Based on the scoring of 10 randomly selected microscopic fields, the apoptotic index (the percentage of brown stained apoptotic cells) was calculated for each group. Data was expressed as mean $\pm$ SD of four mice from each group. The TUNEL assay showed an increase in apoptotic-positive cells in all the BT-treated groups compared to the vehicle-treated group (Figure 6). The apoptotic index increased from 8 ± 1 % in vehicle-treated (controls) to 41 ± 5.29 % in the 30 mg/kg BW group (Figure 6 – bar graph). The apoptotic index was 28.33 ± 4.51 , 28.67 ± 5.03 and 29.33 ± 2.08 % in the 60 mg/kg, 120 mg/kg and 240 mg/kg BW groups, respectively, which was statistically significant compared to the vehicle-treated control group (8 ± 1 %, $P < 0.01$ for all groups). The observation of apoptosis in BT-treated tumor tissue was also confirmed by expression of cleaved caspase-7 and cleaved PARP using western blotting. The cleavage of PARP and procaspase 7 are well documented indicators of apoptosis [12, 13]. As shown in Figure 7A, enhanced expression of cleaved caspase-7 and cleaved PARP was observed in BT-treated tumors compared to

vehicle-treated. Very weak expression was observed in vehicle-treated tumor tissue. Our results demonstrate apoptosis inducing potential of BT.

Effect of BT treatment on ATX inhibition

ATX activity was estimated in plasma, ascites and tumor tissue using colorimetric and western blotting techniques. All methods showed similar trends. Ascites and plasma from four mice (in each group) were assessed for ATX activity by colorimetric method. Data was shown as means $\pm$ SD of four mice from each group. ATX levels in the vehicle-treated control group were considered 100% against which all treated groups were compared. As shown in Figure 7B, statistically significant differences in ATX levels were not observed between BT treated and untreated groups, in plasma and ascites. High intra-group variations in ATX levels were observed in plasma and ascites.

ATX expression in ascites was also assessed by western blotting. For ascites, ATX expression in the BT-treated groups was normalized to ATX expression in the control (which was considered equal to 1). As shown in Figure 7C & Figure 7D (densitometry analysis), no significant difference was observed between BT-treated and untreated mice.

In the case of tumor tissue, ATX expression was assessed by western blotting (Figure 7A). The ratio of protein to actin was normalized to 1 in the control group. As shown in Figure 7D, the relative level of ATX expression, as assessed by densitometry, was not statistically different between treatment groups and vehicle. Due to high intra-group variability of ATX levels in plasma and ascites, it is difficult to make meaningful conclusions regarding the effect of BT on ATX, and further studies using a greater number of animals will be needed to elucidate the role of ATX.

DISCUSSION

Major hurdles associated with the treatment of ovarian cancer include chemoresistance and toxicity of standard drugs [14, 15]. It is essential to develop novel strategies to overcome resistance and to improve the long term survival of ovarian cancer patients. 'Drug repurposing' is a cost-effective alternative to new drug discovery. In this study, we focused on pharmaceutical grade Bithionol (2, 2'-Sulfanediylbis (4, 6-dichlorophenol), BT, a clinically approved anti-parasitic drug re-purposed as a potential anti-ovarian cancer drug. BT was shown to be an effective anti-cancer agent in preclinical models representing breast cancer, melanoma, ovarian and cervical cancer [5, 16–18]. In our recent *in vitro* study, we demonstrated the ability of BT (chemical grade from sigma) to exert cytotoxic effects on a panel of ovarian cancer cell lines regardless of their cisplatin sensitivities. BT was shown to induce cell death via apoptosis and the mechanism(s) of action were shown to be via cell cycle regulation, ROS generation, NF-kB inhibition and autotaxin inhibition [5]. In view of these *in vitro* observations, the major objective of the present study was to assess whether BT (pharmaceutical grade) is capable of inhibiting the growth of implanted human EOC xenografts in a nude mouse model and to understand its mechanisms(s) of action *in vivo*.

In the present study, we established an ovarian cancer xenograft model by IP injection of SKOV3-luc-D3 human EOC cells into nude mice. Tumor growth and treatment response

were studied using bioluminescence imaging (BLI). SKOV-3 cell lines were selected as they are known to produce aggressive intraperitoneal disease with carcinomatosis and ascites that resemble human ovarian cancer in many aspects. Furthermore, our *in-vitro* data demonstrated susceptibility of SKOV-3 cells to cytotoxic effects of BT. BT was shown to cause cell death via caspases mediated apoptosis in SKOV-3 cells and the mechanism(s) of action was shown to be via cell cycle arrest, ROS generation and inhibition of ATX [5]. Our *in vitro* data support significance of SKOV-3 xenografts as an experimental model of EOC apoptosis and offers an attractive paradigm to study drugs mechanisms of action.

In this work, we administered BT capsular contents as aqueous suspension via oral gavage. We tested a wide range of BT doses from 30mg/kg to 240mg/kg BW. The dose range selected was based on our *in vitro* BT cytotoxicity data and also on the published data regarding toxicity, metabolism, clearance kinetics and oral LD₅₀ of BT in animals and humans. Furthermore, we took into account a previous published study which showed growth inhibition of breast cancer xenografts tumors when treated with BT at 60 mg/kg BW [16]. Our *in vitro* results demonstrated the cytotoxic effects of BT towards all the ovarian cancer cell lines tested. SKOV-3-luc-D3 cell lines purchased from Caliper Life Sciences have shown BT IC₅₀ values of 51±6 μM and 31±4 μM at 48 and 72 hrs post BT treatment respectively. Although pharmacokinetics varies from one species to another, several published studies reported that oral administration of 50 mg/kg BT divided in three alternate daily doses for 5 days will maintain serum levels of BT in the range of 140 to 550 μM in rabbits, dogs and humans [16, 17, 19–22].

With regard to treatment duration, we followed similar treatment regimen usually prescribed for humans when treating parasitic infections. In humans, the therapeutic doses are given orally on alternate days, usually in 2 or 3 divided doses. In the present study, each treated group received a different dose of BT orally (30, 60, 120 or 240 mg/kg BW). Each dose was administered (in three divided doses) on day 1, 3 and 5 of each week. In our study, the treatment continued until animals reached humane (signs of pain and distress) or experimental end points (80–100% reduction in tumor). The treatment duration was also selected based on the reports of lack of BT accumulation in many species (humans, dogs, rabbits). In humans, accumulation of BT was not observed even after ten oral doses of 50mg/kg administered on alternate days [16, 20–22]. It may be essential to maintain a certain concentration of the drug to prevent repopulation of tumor cells. In fact, tumor repopulation between cycles of chemotherapy likely has a negative effect on clinical outcome in ovarian cancer patients [23, 24]. In view of these facts, we treated the mice continuously with BT. In absence of toxicity, the treatment continued until the specified end point had been reached (either humane or experimental).

In the present study, pharma grade BT did not exhibit significant anti-tumor potential at any of the doses tested. Ling Zhai et al. reported anti-tumor activity of BT as a single drug in a human ovarian cancer xenograft mouse model [25]. In another study growth inhibition of breast cancer xenograft tumors was observed when treated with BT-cyclodextrin at 60 mg/kg BW [16]. The reasons for lack of therapeutic effect of BT in our study may be due to (i) the use of pharma grade BT, (ii) administration as an aqueous suspension and/or (iii) disease stage at which treatment started. In the present study, the contents of the BT capsules were

administered as a homogenous aqueous suspension via oral gavage. Although we do not have the pharmacokinetic data to interpret our results, the generally observed poor water solubility and subsequent poor absorption of BT may explain our findings. The aqueous solubility is a major indicator for the solubility in intestinal fluids and its potential contribution to bioavailability issues. In addition to solubility, the stage of the disease at which treatment started may also influence the outcome. To mimic human disease at detection stage, we delayed the treatment until 5 weeks post-tumor inoculation in order to have strong tumor signal. A future study, with early BT intervention, could help us draw a bigger picture of the potential of BT as an anti-tumor drug as well as its mechanism of action.

In our previous *in vitro* study, BT (chemical grade) induced cell death via apoptosis, and by affecting cell cycle regulation, ROS generation, NF- κ B inhibition and ATX inhibition [5]. We investigated whether pharmaceutical grade BT can induce similar effects in *in vivo* as well. In the present study, we investigated the effect of BT treatments on cellular proliferation, apoptosis and ATX levels in an IP xenograft model of human ovarian cancer. As observed *in vitro*, a significant decrease in cellular proliferation (Ki-67 staining) was observed in mice treated with BT (30 mg/kg BW) vs. vehicle. This correlated well with the delayed tumor growth observed in animals treated with the same BT dose. However, at higher BT doses, significant inhibition of cellular proliferation was not observed.

It is well known that damaged apoptotic pathways are hallmarks of cancer cells and an important cause of resistance to cytotoxic agents [26]. The ability to induce apoptosis is a critical factor for any effective treatment against cancer [27]. Our previous *in vitro* study revealed the inhibitory effect of BT (chemical grade) on ovarian cancer cell growth via induction of caspase-3/7 activity, DNA fragmentation and loss of mitochondrial potential. The present study confirms the pro-apoptotic role of BT (pharmaceutical grade) *in vivo* as shown by increased expression of apoptotic markers, cleaved caspase-7 and cleaved PARP and by increased nuclear fragmentation (TUNEL Assay). A significant increase in apoptosis was observed in all BT-treated groups compared to vehicle. It is unclear why we observed minimal tumor inhibition despite enhanced apoptosis. The ability of BT to induce apoptosis is encouraging.

It is understood that one of the mechanisms by which BT inhibits tumor growth is by inhibition of ATX enzyme activity or secretion. ATX is known to increase the aggressiveness and invasiveness of transformed cells, and directly correlates with tumor stage and grade in several human malignancies, including ovarian cancer [28, 29]. Tumors with increased ATX expression include breast cancer [30], teratocarcinoma [31], neuroblastoma [32], glioblastoma [33], lung carcinoma [34], thyroid carcinoma [35] and ovarian cancer [28, 29, 36]. Currently there are no small molecule inhibitors of ATX on the market or in the clinic. It is essential to identify putative ATX inhibitors that reduce tumor metastasis and tumor growth, both *in vitro* and *in vivo*. In this context, further exploration of BT as an autotaxin inhibitor is highly desirable. Previously, BT was shown to inhibit solid tumor growth in several preclinical cancer models by targeting ATX [5, 17, 37]. In our recent *in vitro* study we also demonstrated inhibition of ATX in BT (chemical grade)-treated ovarian cancer cell lines [5]. Ling Zhai et al., proposed inhibition of ATX enzyme activity and ATX secretion as

one of the mechanisms by which BT inhibits tumor growth in a human ovarian cancer xenograft mouse model [25]. Given the significance of ATX in ovarian cancer [38–41], we studied the effect of BT on ATX *in vivo* using an EOC IP xenograft model. In this present study, no significant difference in ATX levels were observed in tumor tissue, ascites and plasma from BT-treated mice vs untreated. Due to the high variability observed within each group, it is difficult to make any meaningful conclusions regarding the effect of BT on ATX. Further work using a greater sample size will be necessary to confirm the role of ATX.

Although our study did not demonstrate the anti-tumor potential of pharmaceutical grade BT *in vivo*, the ability of BT to induce apoptosis still makes it a promising therapeutic agent. Additionally, lack of toxicity at any of the tested doses and delayed tumor growth observed at lower doses are important features shown in our present study. These characteristics are worth exploring further in future optimization studies. In order to improve bioavailability and anti-tumor response, our future studies would also focus on pharmacokinetic data using different BT formulations (chemical *versus* pharmaceutical grade), different vehicles, different routes of delivery and wide dose ranges. If not effective as single agent, it can also be tested in combination with other standard chemo drugs for its ability to sensitize the cells to chemotherapy. However, this is the first preliminary *in vivo* anti-tumor efficacy study, using pharmaceutical grade BT.

Conclusions

Keeping in view the concept of ‘drug repurposing’, we evaluated the anti-tumor potential of pharmaceutical grade BT using ovarian cancer xenograft model. No significant antitumor activity was observed at any of the doses tested. However, the ability of BT to induce apoptosis makes it still a promising therapeutic agent. Further confirmatory and optimization studies are essential to enhance the therapeutic effects of BT.

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ABBREVIATIONS

BT	Bithionol
ATX	Autotaxin
IC₅₀	Half maximal inhibitory concentration
IP	intra peritoneal
ROS	Reactive oxygen species
NF-κB	Nuclear factor kappa B

EOC	Epithelial ovarian cancer
H&E	Hematoxylin & Eosin
TUNEL	Terminal deoxynucleotide transferase-mediated dUTP nick end labelling

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Figure 1. *In vivo* bioluminescent images (dorsal) of tumor in mice treated with BT at different doses
Representative bioluminescent images (dorsal views) of tumor growth in mice treated with different doses of BT (30 mg/kg BW, 60 mg/kg BW, 120 mg/kg BW, and 240 mg/kg BW). Control mice were treated with vehicle (gavage administration of injection grade water). The images represent tumor growth in the same mice at different days post BT treatment. The color scale bar, from blue (least intense) to red (most intense) indicates photon count per sec. Regions of interest (ROIs) were established for tumors and data was analysed using Living Image® Software v.3.0 (Xenogen, Caliper Life Sciences, CA, USA) and luminescent signal for these tumors were measured.

Figure 2. Evaluation of BT treatment on growth of implanted human ovarian cancer xenografts by bioluminescence imaging (BLI)

Following tumor formation, mice were treated with different doses of BT (30 mg/kg BW, 60 mg/kg BW, 120 mg/kg BW and 240 mg/kg BW). Control mice were treated with vehicle (gavage administration of injection grade water). Tumor growth was assessed by BLI using the IVIS imaging system. The tumor intensity was expressed as photons/sec. (A) Effect of BT treatment on mean tumor growth of each group at different days post BT treatment. Data represent Mean $\pm$ SEM. (B – F) Individual tumor growth curves of each mice (each group) Vs days post treatment. Each curve represents each mouse.

Figure 3. Assessment of BT treatment on survival rates and body weight

(A) Kaplan–Meier survival curves. Mice treated with vehicle (gavage administration of injection grade water) were used as control. Kaplan–Meier survival curves were generated from the day 1 of BT treatment until the day of an event of interest (either humane or experimental end points). The log-rank (Mantel–Cox) test was used to compare survival curves between groups. No statistically significant difference was observed in survival upon BT treatment ($p = 0.3677$, Log-rank). (B) Effect of BT on body weight (gms). Mice treated with different doses of BT were regularly monitored for body weight. No significant changes in body weight were observed in mice treated at any BT dose

Figure 4. Histological analysis of tumor tissue sections in BT-treated and untreated mice
Tumor sections from BT-treated and untreated mice were stained with hematoxylin & eosin staining. The magnified image (X200) was shown in inset. Tumor tissue from four mice (in each group) was processed. Duplicate tissue slides were prepared for each mouse. The figure shows representative images for each group. The tumors were composed primarily of ovarian epithelial cancer cells with cobblestone-like characteristics and were relatively uniform epithelial cells with irregular nuclei and hyperchromatic nuclei. Mice treated with BT at 30mg/kg BW showed an increased proportion of non-cellular stromal components with pleomorphic and hyperchromatic nuclei.

Figure 5. Effect of BT on expression of Ki-67 protein in tumor sections of BT-treated and untreated mice

Ki-67 was used as a surrogate marker of cell proliferation. Dark brown stained cells represent Ki-67 positive cells. The magnified image (X200) was shown in inset. Tumor tissue from four mice (in each group) was processed for assessment of cellular proliferation. Duplicate tissue slides were prepared for each mouse. **Graph:** The figure demonstrates correlation between cellular proliferation and tumor growth. The extent of proliferation was expressed as proliferative index calculated as the percentage of Ki-67-positive cells (brown stained). Proliferation index was averaged from scoring of 10 randomly selected microscopic fields from both slides. Data was expressed as mean $\pm$ SD, * $p < 0.05$, as compared to vehicle-treated control, Student 't' test. Proliferation index was significantly higher in the vehicle-treated tumors compared to the 30 mg/kg BW BT group ($P < 0.05$).

Figure 6A

Figure 6B

Figure 6. Effect of BT on cell apoptosis in tumor sections of BT-treated and untreated mice

The extent of apoptosis in tumor tissue was assessed by TUNEL assay. Apoptotic cells with condensed and irregularly shaped nuclei stain positively for TUNEL (dark brown). The magnified image (X200) was shown in inset. Tumor tissue from four mice (in each group) was processed for evaluation of apoptosis. Duplicate tissue slides were prepared for each mouse. **Bar Graph:** The apoptotic index (the percentage of brown stained apoptotic cells) was calculated for each group. Apoptotic index was averaged from scoring of 10 randomly selected microscopic fields from both slides. Data was expressed as mean $\pm$ SD, * $p < 0.05$, compared to vehicle-treated controls. The TUNEL assay showed an increase in apoptotic-positive cells in the all the BT-treated groups compared with the vehicle-treated group ($P < 0.01$ for all, student 't' test).

Figure 7. Effect of BT on expression of ATX protein and apoptosis effectors in xenograft tumors of nude mice

(A) ATX activity from plasma and ascites was assessed using a colorimetric technique. ATX levels in the vehicle-treated control group were considered as 100%. The data was expressed as %ATX vs. control. Data are shown as means $\pm$ SD of at least four samples from each group. (B) ATX protein from ascites was assessed by western blotting. (C) Expression of ATX protein, cleaved caspase-7 and cleaved PARP from tumor tissue were assessed by western blotting. (D) Quantification of ATX protein from ascites and tumor tissue by densitometry. ATX expression was assessed by western blotting. The relative levels of protein expression were estimated using the Odyssey imaging software. In tumor tissue, the ratio of protein to β -actin was normalized to 1 in the control group. The relative level of ATX expression showing relatively reduced expression in the 30 mg/kg BW group compared to control; however, this was not statistically significant. In ascites, the ATX expression in the control was considered as 1 against which other groups were compared.