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An orally active Amazonian plant extract (BIRM) inhibits prostate cancer growth and metastasis

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Abstract Purpose: Poor efficacy of conventional chemotherapeutic drugs against metastatic hormone-refractory prostate cancer (CaP) drives patients to try “alternative medicine”. The antitumor activity of one such agent, “BIRM” (biological immune response modulator; “Simple Ecuadorian Oral Solution: an extract of an Amazonian plant”), was characterized in vitro and in vivo using established CaP cell lines and a tumor model. **Methods:** The cytotoxicity of BIRM in four human and one rat CaP cell line was evaluated using cell proliferation-inhibition and clonogenic survival assays. BIRM-induced apoptosis, alterations in cell cycle phase progression and inhibition of the extracellular matrix-degrading enzyme hyaluronidase were also investigated in these cells. The in vivo efficacy of BIRM was evaluated in rats with subcutaneous tumor implants of Dunning EGFP-MAT LyLu cells. The active species in BIRM were characterized by gel filtration chromatography. **Results:** BIRM inhibited cell proliferation and clonogenic growth of the CaP cells (IC_{50} about 8.0 μ l/ml). It increased cell accumulation in the G_0/G_1 phase by 33.8% and decreased the proportion of cells in S phase

by 54.6%. Apoptotic cell death in BIRM-treated cells was associated with activation of cell death-associated caspases. BIRM inhibited the activity of hyaluronidase, a hyaluronic acid-degrading enzyme, at 1 μ l/ml. Treatment of MAT LyLu tumor-bearing rats with BIRM by oral gavage resulted in a significant decrease in tumor incidence (50%), tumor growth rate (18.6 ± 1.3 days for 1 cc tumor growth in control rats and 25.7 ± 2.6 days in BIRM-treated rats), and only one out of six BIRM-treated rats versus four out of six in the control group developed lung metastasis. Three active ingredients in BIRM with a relative molecular mass (M_r) of ≥ 3500 were identified by ultracentrifugation and gel filtration chromatography and were found to be resistant to proteinase and heat (100°C). **Conclusion:** The plant extract BIRM contains antitumor compounds of $M_r \geq 3500$ with potent antiproliferative activity in vitro and in vivo against prostate cancer cells.

Keywords Natural herbal anticancer products · Prostate cancer · Invasion and metastasis · Chemoprevention · Apoptosis

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Abbreviations *CaP* Prostate cancer/cancer of the prostate · *EGFP* Enhanced green fluorescence protein · *HA* Hyaluronic acid · *HAase* Hyaluronidase · *MTT* Methyl thiozoyl tetrazolium bromide [(3-[4,5-demethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide]

Introduction

Cancer of the prostate (CaP) is the most frequently diagnosed malignant cancer in American men with an estimated 189,000 new cases in 2002 [13]. The majority of CaP-related deaths, estimated to be 30,200 in 2002, are likely the result of failure of all currently available conventional treatments. Besides undergoing conventional therapy, CaP patients often seek treatment by unproven therapeutic approaches [12]. It is estimated that 30–40%

of men with CaP experiment with one or more complementary therapies which include high-dose vitamins and minerals, herbal preparations and supplements of soy, saw palmetto etc. [14]. Moreover, there is a dramatic increase in the number of patients moving towards complementary and alternative medicine and consuming plant extracts from “folklore medicine” [26]. We have come across one such natural herbal medicine “BIRM” (biological immune response modulator; “Simple Ecuadorian Oral Solution: an extract of an Amazonian plant”) formulated by a physician (E.C.-A.), promoted in South America, and based on the local folklore of the Ecuadorian native population. The formulation is dispensed as a natural remedy for a variety of maladies including HIV-1 infection and cancer [1, 3, 4]. Very little systematic information is currently available on BIRM, and no studies have been undertaken to investigate the structure-function correlations in the ingredients of BIRM. Therefore, we decided to evaluate the efficacy and antiproliferative effects of BIRM in a CaP model.

Materials and methods

Test compound

BIRM was a gift from BIRM Inc. (Quito, Ecuador). BIRM is an aqueous extract of dried roots of a plant of the genus *Dulcamara* (family Solanaceae) grown in Ecuador, and marketed as a greenish-brown suspension with a mild bittersweet smell. The inactive ingredients in BIRM comprise 16% solid particles, likely root fibers, and the remainder, a lipid-free liquid. BIRM is prepared by aqueous extraction of dried roots followed by oxidation/reduction of the extract. During this process, the amount of roots and the timing of oxidation/reduction are carefully controlled to minimize batch-to-batch variation. Prior to initiation of this project, the efficacy of BIRM samples from five different batches were selected randomly and tested in two different cell lines (PC-3ML and LNCaP) by the MTT assay to determine the degree of interbatch variation. We found no interbatch variation in the potency of BIRM for induction of cytotoxicity. In the present study, BIRM samples from lot number 011-2000 were used. For all the studies reported here, BIRM clarified by centrifugation at 10,000 g was used.

Cells and tumor lines

Established human CaP cell lines (LNCaP and DU-145) were obtained from the ATCC (Rockville, Md.). A recently established bone metastatic PSA⁺ CaP line (VCaP) was generously provided by Drs. Pienta and Cooper (Karmanos Cancer Center, University of Michigan, Ann Arbor, Mich.) [11, 17, 23]. A metastatic variant of a PC-3 cell line, PC-3ML, was a gift from Dr. M.E. Stearns (Allegheny University Hospitals, Philadelphia, Pa.) [15, 27]. All cultures were maintained in a complete medium containing RPMI-1640 basal medium, 10% fetal bovine serum (Atlanta Biologicals, Atlanta, Ga.), and 10 µg/ml gentamicin. The EGFP-MAT LyLu cell line was generated by stable transfection of Dunning MAT LyLu rat CaP cells with pEGFP-1 plasmid DNA (Clontech, Palo Alto, Calif.) and was maintained in complete medium with 250 nM dexamethasone as described previously [19, 28].

Growth inhibition assay

A ³H-thymidine incorporation assay was performed as described previously [8]. Following incubation in medium containing BIRM or without BIRM, the cells were pulse-labeled with ³H-thymidine

(1 µCi/ml) for 2 h. Incorporation of ³H-thymidine into cellular DNA was stopped by the addition of 10% trichloroacetic acid and the acid-precipitable radioactivity was determined by liquid scintillation counting [8]. Clonogenic survival of CaP cells exposed to BIRM for 24 h was assayed by the colony assay as described previously [8].

Determination of apoptotic activity

BIRM-induced apoptosis was assayed using a cell death ELISA kit (Cell Death ELISA-Plus kit; Roche Molecular Biochemicals, Mannheim, Germany). The assay measured the amount of free nucleosomes in cell lysate resulting from programmed cell death [9]. The relative amount of free nucleosomes present in cell lysates from cultures incubated with BIRM for 4 h or 24 h was estimated according to the supplier's instructions.

Cell cycle analysis

CaP cells (1×10⁵) were cultured in 60-mm culture dishes. After an overnight culture, the cells were treated with 10 or 25 µl/ml of BIRM for 24 h. BIRM-treated and untreated cells were harvested and stained with 50 µg/ml propidium iodide. The amount of propidium iodide bound to DNA was profiled in an EPICS XL flow cytometer as described previously [19]. The fraction of dead cells at the time of harvesting was about 16% as determined by trypan blue exclusion. The majority of these cells were floating, so were discarded at the time of washing. The remaining dead cells were gated out using the forward angle light scatter and side scatter gatings during flow cytometry. About 20,000 propidium iodide-stained cells were analyzed in the flow cytometer from each sample. The MODFIT LT program (Verity Software House, Topsham, Me.) was used for the cell cycle phase analysis [29].

Determination of activation of cell death caspases

Caspase activation in CaP cells treated with BIRM was determined using a kit (Homogeneous Caspases Assay, fluorimetric; Roche) which determined collectively activated caspases nos. 2, 3, 6, 7, 8, 9 and 10. The assay measured the free rhodamine 110 (R110) resulting from the cleavage of a common caspase substrate, DEVD, conjugated with R110. The amount of free R110 was determined fluorimetrically at an excitation wavelength of 499 nm and an emission wavelength of 528 nm, and is expressed as relative fluorescence units (RFU) [25].

HAase assay

We tested whether BIRM affects HAase activity secreted in DU-145 culture-conditioned medium and partially purified HYAL1 using a HAase activity ELISA-like assay [20, 22]. HYAL1-type HAase was partially purified from the urine of patients with high-grade bladder cancer as described previously [20]. The assay was performed in a 96-well microtiter plate coated with HA (200 µg/ml, ICN Biomedicals). Wells were incubated with various concentrations of BIRM or column fractions from a Sephadex G-50 gel-filtration column (see below) in a HAase assay buffer at 37°C for 15 h [20]. Following incubation, HA degraded by HAase was washed off and the HA remaining in the microtiter wells was estimated using a biotinylated bovine nasal cartilage HA-binding protein, and an avidin-biotin detection system (Vector Laboratories, Burlingame, Calif.) [21].

Biochemical characterization of cytotoxic activity in BIRM

To study heat inactivation, BIRM was heated at 100°C for 5 min in a water bath. BIRM was digested with proteinase K (10 U/ml) at

37°C for 18 h. For size fractionation studies, clarified BIRM was loaded into ultrafiltration mini-Centriprep tubes (Millipore, Bedford, Mass.) with membrane barriers with different molecular weight cut-off points (i.e. about 3.5, 10 and 30 kDa). Following three cycles of centrifugation and separation of the low molecular weight fractions, both the filtrate and the retentate were assayed for cytotoxic activity. BIRM solution was also treated with charcoal-dextran (50 mg/ml) at 4°C for 12 h to remove lipids and steroids (if any). Following the various treatments, BIRM was centrifuged and various concentrations of the supernatant were added to PC3-ML cells cultured in 24-well plates (2×10^4 cells/well). BIRM-induced cytotoxicity was estimated using the MTT reduction assay following a 24-h treatment [19].

Gel filtration chromatography

Particle-free BIRM was loaded onto a G-50 Sephadex column (1.5×120 cm) equilibrated with 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl buffer (Tris/NaCl buffer) [20]. The column was eluted in Tris/NaCl buffer at 7 ml/h and 3-ml fractions were collected. Each fraction was assayed for protein (BCA-BioRad), uronate [2], inhibition of cell growth (MTT assay) [19] and inhibition of HAase activity (HAase ELISA-like assay).

Tumor generation and BIRM administration

This experiment was performed according to a protocol approved by the University of Miami Animal Care and Use Committee and as stipulated in the NIH Guide to the Humane Care and Use of Laboratory Animals. A suspension (1×10^5 cells, 0.5 ml) of growing EGFP-MAT LyLu cells was implanted subcutaneously into the dorsal flank of adult (about 250 g) male Copenhagen rats (Harlan Sprague Dawley, Indianapolis, Ind.) under mild anesthesia [19]. The rats were housed in a room under a 12-h light/12-h dark cycle and provided with food and water ad libitum throughout the experiment. Following implantation, the rats were randomly divided into two groups of six animals and gavaged with 1 ml of either distilled water (vehicle control, group 1) or BIRM (group 2) using a 3-inch stainless-steel intubation cannula on day 1 of tumor implantation and then daily for 30 days. Tumor growth was examined by palpating the skin around the site of injection. After the tumors became palpable (about day 5), they were measured three times a week using calipers, and the volumes calculated assuming approximation to an ellipsoid ($\text{length} \times \text{height} \times \text{width} \times 0.524$). Animals were euthanized when the tumor volume was about 10 cc or the tumor became significantly necrotic. At necropsy, lungs were collected and viewed under a Nikon stereomicroscope with a fluorescence attachment (SMZ 1500) to examine the presence of fluorescent metastatic tumor foci.

Statistical analysis

Triplicate samples were assayed in all in vitro experiments. Statistical analysis was performed using parametric and nonparametric Student's *t*-tests.

Results

BIRM inhibits cell proliferation in CaP cells

BIRM inhibited cell proliferation in all the CaP cell lines tested in a dose-dependent manner (Fig. 1). The concentration of BIRM causing 50% growth inhibition

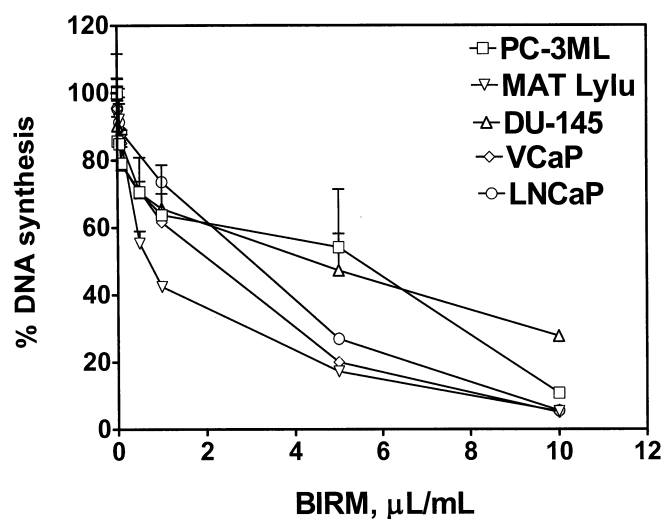


Fig. 1 Cytotoxicity of BIRM against prostate tumor cell lines. CaP cells cultured in growth medium (1×10^4 cells/well, 48-well plates) were exposed to various concentrations of BIRM. Following incubation for 24 h, DNA synthesis was determined by measuring ^3H -thymidine incorporation in the proliferating cells (vertical bars means \pm SEM from three independent assays)

(IC_{50}) was 8 $\mu\text{L/mL}$ (i.e. 0.8% v/v). Furthermore, the inhibitory activity of BIRM was comparable among all CaP cell lines regardless of their androgen sensitivity (androgen-sensitive LNCaP and VCaP cells versus androgen-resistant PC-3ML and DU-145 cells). Similar results were obtained by cell counting and Trypan blue exclusion assays (data not shown). The results presented in Fig. 1 and similar observation from other assays suggested that BIRM-induced inhibition of cell proliferation led to either cell death (cytotoxicity) or arrest of cell proliferation (cytostasis). To distinguish between these mechanisms, we investigated the colony-forming efficiency of CaP cells treated with BIRM. The clonogenic assay revealed a dose-dependent inhibition of colony formation in BIRM-treated CaP cells. Neither cell colonies nor cell clusters were observed in cultures exposed to BIRM at doses of 10 $\mu\text{L/mL}$ and above for 24 h (Fig. 2A). The IC_{50} of BIRM for inhibiting clonogenic survival was also 8 $\mu\text{L/mL}$, the same as the value obtained in the ^3H -thymidine assay (Fig. 2B).

BIRM causes cell cycle arrest in CaP cells

As shown in Table 1, the proportion of cells in G_0/G_1 phase increased significantly from $56.4 \pm 0.9\%$ in control to $75.5 \pm 2.2\%$ in cultures treated with BIRM at 25 $\mu\text{L/mL}$. The increase in the G_0/G_1 phase fraction in the BIRM-treated cells was contrasted with a decrease in the S-phase fraction. The S-phase fraction in BIRM-treated cells was $13.1 \pm 2.9\%$ compared to $28.9 \pm 2.1\%$ in the control. A small decrease of 15–22% in the G_2/M fraction was also observed in BIRM-treated cells.

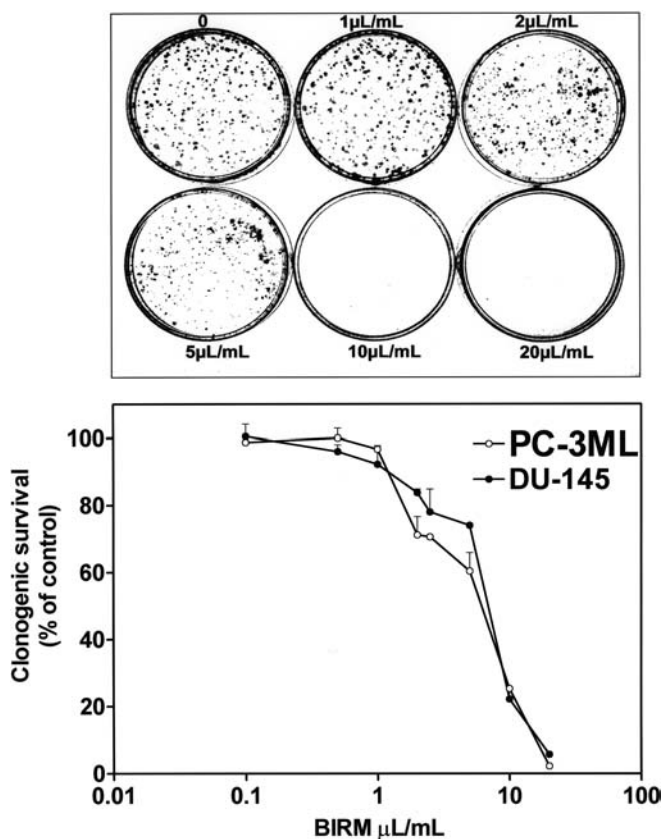


Fig. 2a, b Effect of BIRM on the growth of CaP cells. Cells cultured at low density in 60-mm culture dishes were exposed to BIRM for 24 h. Surviving cells at the end of incubation were allowed to form adherent cell colonies during the next 7–10 days. Cell colonies stained with 0.1% crystal violet and colonies containing > 50 cells were counted manually using a hand-held electronic counter in a blinded fashion. **a** Colonies of surviving PC3-ML cells exposed to BIRM for 24 h. **b** Clonogenic survival of CaP cells cultured with BIRM. The results are presented as means \pm SEM from three independent experiments

BIRM induces apoptosis in CaP cells

We did not observe a significant difference in the levels of free nucleosomes in BIRM-treated cells during 4 h of treatment, but after 24 h of treatment the intracellular levels of free nucleosomes showed a two- to threefold dose-dependent increase (Fig. 3).

Table 1 Flow cytometric cell cycle fractionation analysis of PC-3ML cells treated with BIRM for 24 h. The distributions of cells in the various cell cycle phases were calculated from the DNA content of propidium iodide-labeled cells using the MODFIT program.

Treatment	G ₀ /G ₁ phase		S phase		G ₂ /M phase	
		Difference (%) ^a		Difference (%) ^a		Difference (%) ^a
Control	56.37 \pm 0.9		28.9 \pm 2.1		14.6 \pm 1.2	
BIRM (10 µl/ml)	67.93 \pm 2.1	+ 20.5	19.5 \pm 2.4	–32.5	12.5 \pm 0.28	–15
BIRM (25 µl/ml)	75.46 \pm 2.2	+ 33.8	13.1 \pm 2.9	–54.6	11.39 \pm 0.29	–22

^aCalculated as [(% of cells in respective phase of untreated samples – % of cells in respective phase in BIRM-treated samples) / (% of cells in respective phase in untreated control)] \times 100

Activation of cell death-associated caspases

As shown in Fig. 4, the activities of one or more of caspases 2, 3, 7, 8, 9 and 10 were increased significantly in BIRM-treated cells as compared to the activities in control cells. We initially detected a time-dependent increase in combined caspase activity, beginning at 4 h of exposure to BIRM and peaking at 18 h. The dose-dependent increase in caspase activities showed a 50% increase in cells treated at 5 µl/ml BIRM over the activity in control cells following incubation for 18 h or longer (Fig. 4).

BIRM inhibits tumor growth and metastasis

As shown in Fig. 5, following tumor implantation, oral administration of BIRM (4 ml/kg body weight) to rats resulted in slow tumor growth. While the tumor incidence was 100% in the control group, only four out of six BIRM-treated animals (67%) developed tumors. The tumor growth rate estimated using non-linear regression analysis of tumor volumes over time for each animal confirmed a decreased growth rate in BIRM-treated animals. The time taken for tumors to reach 1 cc was 18.6 ± 1.3 days in control animals and 25.7 ± 2.6 days in BIRM-treated animals (mean \pm SE from four animals). A 38% delay in tumor growth was observed in BIRM-treated animals compared with control animals. The difference in the growth rate between control and BIRM-treated animals was statistically significant (unpaired *t*-test: $P = 0.03$, $t = 2.773$, $df = 6$, 95% CI 0.835–13.36). Fluorescence imaging of the lungs at necropsy revealed that only one out of six BIRM-treated animals had metastatic lung foci, whereas five out of six control animals had tumor metastasis to the lungs. Furthermore, the tumor foci in the lungs of the BIRM-treated animal were significantly smaller than those in control animals (Fig. 6). These results indicate that ingredients in BIRM either delay or block spontaneous lung metastasis.

BIRM inhibits the activity of HYAL1-type HAase

Investigation of inhibition of matrix metalloproteinase activity by BIRM using a ³H-labeled collagen-degradation assay [18] showed no changes in matrix

Values are mean \pm SE percentages from three independent experiments. Similar results were obtained from the cell cycle fractionation analysis of LNCaP cells exposed to BIRM

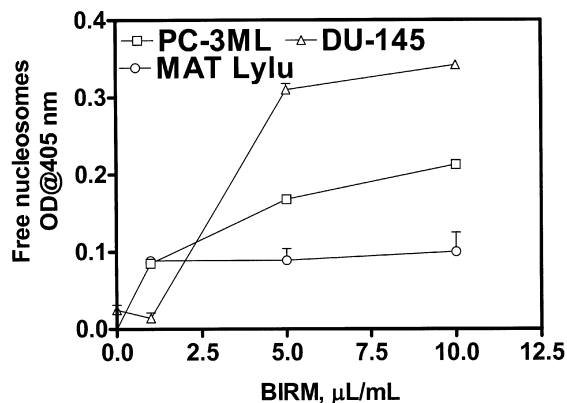


Fig. 3 BIRM kills tumor cells by induction of apoptosis. PC-3ML, DU-145 and Mat LyLu CaP cells cultured in growth medium (1×10^4 cells/well; 48-well plates) with or without BIRM at various concentrations for 24 h were analyzed for apoptotic activity using the Cell Death ELISA Plus assay kit, which allowed measurement of soluble nucleosomes by spectrophotometry. The data presented as means \pm SEM from three independent experiments

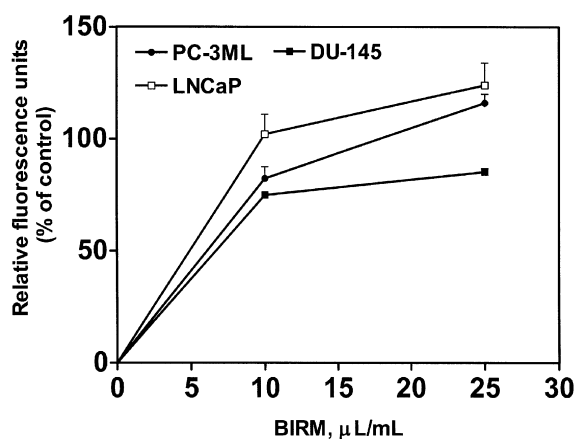


Fig. 4 BIRM induced increases in caspase activity. CaP cells were treated with BIRM for 4 to 24 h and the total activities of cell death-associated caspases were measured using a kit that employed a rhodamine110-conjugated peptide substrate common to all cell death-related caspases. The results are presented as means \pm SEM from three independent experiments in each of which cell lysates incubated with BIRM for 24 h were used for caspase activity determination. Caspase activity in untreated control cultures was detectable, but was typically ten times less than the activity detected in the positive controls provided with the assay kit

metalloproteinase activity following BIRM treatment (data not shown). We next examined whether BIRM could inhibit the activity of HYAL1-type HAase. We have previously shown that HYAL1 is the major HAase expressed in cancers of the prostate and bladder [21, 22]. Furthermore, invasive tumor cells express high levels of HYAL1 [5, 21, 22]. As shown in Fig. 7, BIRM potently inhibited HAase activity. BIRM inhibited the HAase activity present in the culture-conditioned medium of DU-145 cells (a good source of HYAL-1 [21]) and the activity of partially purified HYAL1, in a dose-dependent manner (IC_{50} 0.25 $\mu\text{L/mL}$).

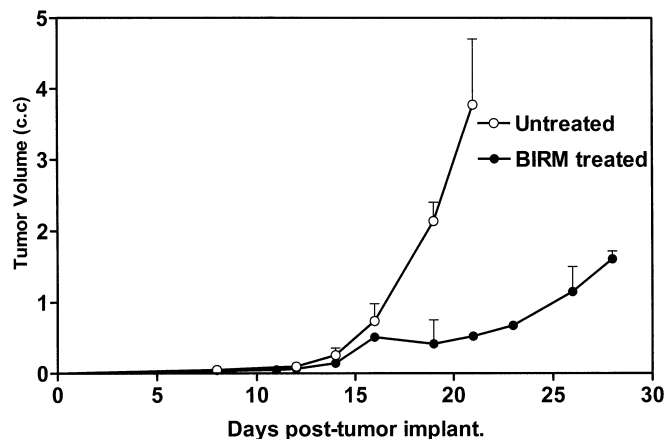


Fig. 5 Effect of daily dosing of BIRM on growth and metastasis of Dunning MAT LyLu tumors in rats. Tumor growth in rats with subcutaneous tumor implant with EGFP-MAT LyLu cells (1×10^5 cells/animal). The data are presented as means \pm SEM of each treatment group over time. Data from the control group include measurements from six tumor bearing rats, whereas the BIRM-treated group had only four animals; the other two animals in this group did not develop tumors

Characterization of active ingredients in BIRM solution

BIRM retained its full cytotoxic activity after boiling for 5 min or digestion with proteinase K, indicating that reactive species present in BIRM most likely are heat-stable proteinase-resistant compounds. Similarly, charcoal-dextran extraction also did not result in any loss in the cytotoxic activity associated with BIRM, suggesting that the active species present in BIRM are not lipid-soluble compounds such as alkaloids or steroids. No loss in cytotoxic activity was found upon ultrafiltration through a 3.5-kDa membrane barrier. However, a 40% loss in activity was observed after ultrafiltration through 10-kDa and 30-kDa membranes (Fig. 8). These results indicate that BIRM contains at least two species with different molecular mass, i.e. one with a molecular mass between 3500 and 10,000 and a second with a molecular mass of $\geq 30,000$, with cytotoxic activity against CaP cells. The possibility of the growth-inhibitory activity being associated with carbohydrate derivatives was investigated using the Bitter and Muir modified carbazole assay to measure glycosaminoglycans and proteoglycans containing D-glucuronic acid (i.e. uronate) [23]. The results showed that BIRM is rich in uronate-containing carbohydrates (19.5 mg/mL). Gel filtration chromatography on a Sephadex G-50 column also showed two active fractions (fraction nos. 28 and 42, Fig. 9).

Discussion

A systematic investigation of promising plant products has led to the discovery and development of antineoplastic agents with unique modes of action and striking efficacy (e.g. paclitaxel, vinblastine, etoposide, etc) [7]. In

Fig. 6A, B EGFP-MAT LyLu rat prostate tumors metastatic to lungs. The figure shows rat lungs with fluorescent tumor foci (arrows). **A** Control animals ($\times 40$); **B** a typical tumor metastatic to the lung in a BIRM-treated animal ($\times 20$). Tumor foci in the lungs of BIRM-treated animals were typically ten times smaller or absent

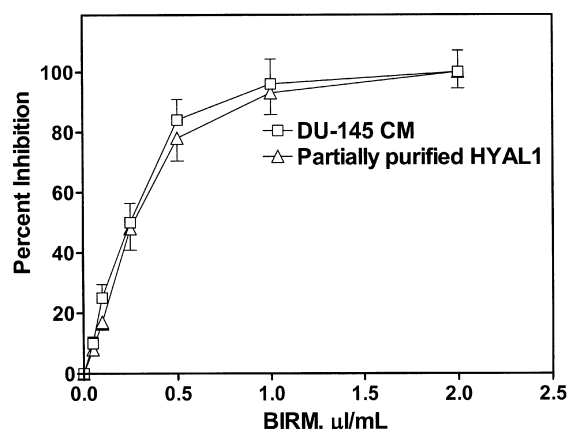
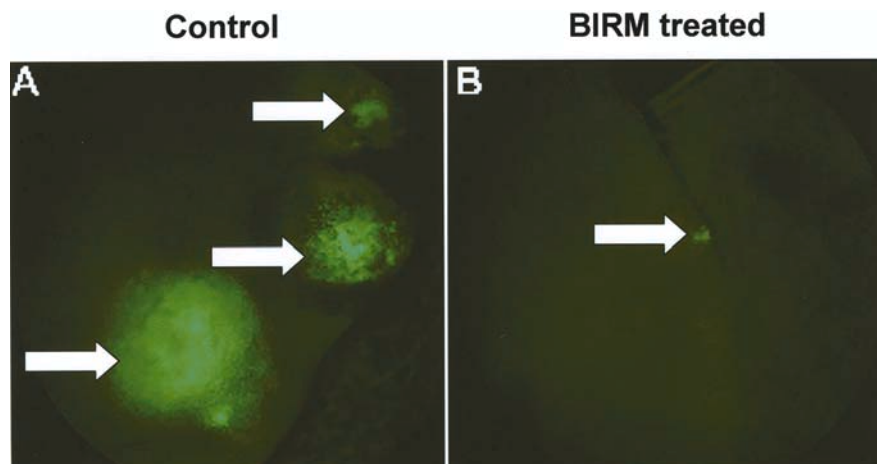


Fig. 7 BIRM inhibits tumor-derived HAase. Effect of BIRM on the HAase activity present in DU-145 cell-conditioned medium and partially purified preparation of HYAL1 was carried out using an HAase ELISA-like assay as described in Materials and methods. The data shown are from a typical experiment. Similar results were obtained in three other experiments using LNCaP cells (data not shown)

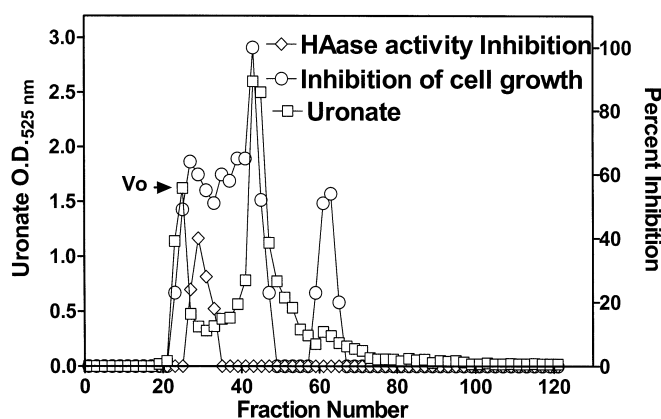


Fig. 9 Fractionation of antineoplastic ingredients present in BIRM by gel filtration chromatography. Clarified BIRM (5 ml) was fractionated on a Sephadex G-50 column. The column fractions were assayed for protein ($A_{280\text{ nm}}$), uronate concentration (Bitter and Muir assay), HAase activity (HAase activity ELISA) and cytotoxicity (MTT assay). A single protein peak was detected in fraction no. 20, which had neither cytotoxic activity nor HAase-inhibitory activity

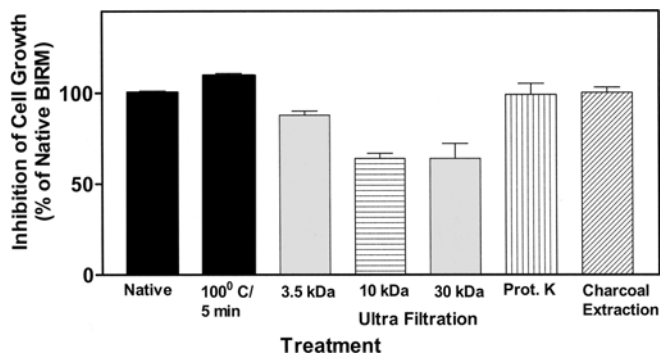


Fig. 8 Cytotoxicity of modified BIRM as assayed by MTT assay and compared with unmodified BIRM. BIRM solution was treated with proteinase K, heated for 5 min in a bath of boiling water or subjected to ultrafiltration as described in Materials and methods. Following treatment, untreated and treated BIRM solutions were tested for cytotoxic activity in PC3-ML cells by MTT assay, as described in Materials and methods (vertical bars means \pm SEM from four independent assays)

this report, we present evidence of the antineoplastic activity of a plant-derived nutritional supplement, BIRM. The medicinal value of BIRM is believed to be associated only with the Amazonian variety of the plant *Dulcamara*, where the micronutrients present in the soil of the Upper Amazon basin promote the synthesis of medicinal compounds in the plant. BIRM inhibited cell proliferation and clonogenic survival (Figs. 1 and 2) and caused apoptotic cell death via the caspase activation pathway (Figs. 3 and 4). In vivo studies on the growth and metastasis of Dunning MAT LyLu tumors suggested that oral dosing with BIRM resulted in lower tumor incidence, slower tumor growth and reduced spontaneous metastasis to the lungs (Figs. 5 and 6). A preliminary biochemical characterization and size-exclusion chromatography suggested that there were at least four active species present in BIRM, three with cytotoxic activity and one with HAase-inhibitory activity. We have not yet determined whether the three

cytotoxic species present in BIRM have the same chemical composition but different polymer length. Nonetheless, all four active ingredients were heat-stable and unlikely to be proteins or lipid-soluble compounds.

Inhibition of tumor growth in the rat CaP model following the oral administration of BIRM clearly suggests that the active ingredient(s) of BIRM are absorbed in the gastrointestinal tract. The reduction in tumor incidence (33%) and the number of tumor foci in the lungs (>80%) in BIRM-treated animals suggest that BIRM may exert both antiproliferative and antimetastatic activities. It is estimated that 20–40% of patients initially diagnosed with local CaP have either locally advanced disease (stage C) or metastatic disease (stage D) [10, 16], and the cure of metastatic disease still remains a challenge. Our observation that CaP cell cultures treated with BIRM showed a significant reduction in cell proliferation and undergo apoptosis (Figs. 1, 2, and 3) indicates that the active ingredients present in BIRM have potential for use in controlling advanced hormone-refractory prostate cancer. We investigated whether the BIRM-induced cytotoxic effect was due to inhibition of mitotic spindle separation. This would lead to mitotic inhibition and arrest of cells in the G₂/M phase. Contrary to our expectation, incubation with BIRM arrested CaP cells in G₀/G₁, which was compensated for by a significant decrease (i.e. –56%) in the proportion of cells in S phase and a modest decrease in G₂/M, indicating lack of mitotic arrest or cytokinesis (Table 1). This finding is novel in the sense that most plant-derived compounds used in cancer therapy interfere with tubulin polymerization (e.g. vinblastine and vincristine) or depolymerization (paclitaxel), or inhibit topoisomerase I activity (e.g. irinotecan, topotecan, 9-aminocamptothecin and 9-nitrocamptothecin) [6] or topoisomerase II activity, leading to cell cycle arrest in the G₂/M phase (e.g. paclitaxel, etoposide and teniposide) [24].

Apoptotic cell death may be one of the mechanisms involved in BIRM-induced cytotoxicity. BIRM increased the apoptosis in three CaP cell lines (Fig. 3). Furthermore, induction of apoptosis in BIRM-treated CaP cells was coincident with activation of cell-death caspases (Fig. 4). In addition to its cytotoxic effects, BIRM appeared to be a potent inhibitor of metastasis. Although, the mechanism by which it may inhibit metastasis is unknown at present, our results suggests that BIRM is a potent inhibitor of HAase, a class of matrix-degrading enzymes whose levels have been shown to correlate with CaP progression [20, 22].

The recommended minimum dose of BIRM for human consumption is 4 ml/day (as indicated on the bottle label), a significantly lower dose than that used in the current study. We based the dosage to rats on the observed efficacy in vitro. We found no observable systemic toxicity in rats at a dose of 4 ml/kg. Given its effect on tumor growth and metastasis and no systemic toxicity, inclusion of BIRM as an adjuvant to standard therapy has potential to reduce/halt disease progression.

In summary, our study demonstrated that BIRM shows cytotoxic activity against both androgen-dependent and androgen-independent CaP cells in vitro. More importantly, it reduced tumor incidence, delayed tumor growth and caused a significant reduction in metastasis in an experimental model of late-stage CaP. Furthermore, no systemic toxicity was seen following continuous administration of BIRM in an in vivo rat model. These useful properties of BIRM indicate that further investigation of its mechanism of action and clinical trials involving its use in advanced CaP are warranted.

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References

1. BIRM Carbohydrate of low molecular weight ECA10-142 controls AIDS (1994) Tenth International Conference on AIDS (Yokohama, Japan). Abstracts, vol 2 (abstract no. 0291)
2. Bitter T, Muir H (1966) Mucopolysaccharides of whole human spleens in generalized amyloidosis. *J Clin Invest* 45:963–975
3. Cevallos EA (1994) Binational experience in the treatment of AIDS with a low molecular weight natural carbohydrate (ECA-10-142), as a stimulant of the immune system. Tenth International Conference on AIDS (Yokohama, Japan). Abstracts, vol 1 (abstract no. 0294)
4. Cevallos EA (1996) “BIRM: La estrategia terapeutica del futuro”. Abstract of Congreso Mundial de SIDA en Vancouver
5. Delpech B, Girard N, Bertrand P (1997) Hyaluronan: fundamental principles and applications in cancer. *J Intern Med* 242:41–48
6. Dennis RAM, Adriana BN, Rocha DA, Gilberto S (2000) Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compound. *Oncologist* 5:185–198
7. Donehower RC, Rowinsky EK (1993) Anticancer drugs derived from plants. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Principles and practice of oncology, 4th edn. Lippincott, New York
8. Dudak SD, Lopez A, Block NL, Lokeshwar BL (1996) Enhancement of radiation response of prostatic carcinoma by lonidamine. *Anticancer Res* 16:3665–3671
9. Eisel D, Fertig G, Fischer B, Manzow S, Schmelig K (eds) (2000) Guide to cell proliferation and apoptosis methods, 2nd edn (technical manual). Roche Applied Science, Mannheim, Germany
10. Harris K, Reese DM (2001) Treatment options in hormone-refractory prostate cancer: current and future approaches. *Drugs* 61:2177–2192
11. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP (1983) LNCaP model of human prostatic carcinoma. *Cancer Res* 43:1809–1818
12. Jacobson JS, Chetty AP (2001) Complementary and alternative medicine in prostate cancer. *Curr Oncol Rep* 3:448–452
13. Jamel A, Thomas A, Murray T, Thun M (2002) Cancer statistics 2000. *CA Cancer J Clin* 52:23–47
14. Jones HA, Metz JM, Devine P, Hahn SM, Whittington R (2002) Rates of unconventional medical therapy use in patients with prostate cancer: standard history versus directed questions. *Urology* 59:272–276
15. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 17:16–23

16. Kojima M, Troncoso P, Babaian RJ (1995) Use of prostate-specific antigen and tumor volume in predicting needle biopsy grading error. *Urology* 45:807–812
17. Korenchuk S, Lehr JE, Mclean L, Lee YG, Whitney S, Vessella R, Lin DL, Pienta KJ (2001) VCaP, a cell-based model system of human prostate cancer. *In Vivo* 15:163–168
18. Lokeshwar BL, Selzer MG, Block NL, Gunja-Smith Z (1993) Secretion of matrix metalloproteinases and their inhibitors (tissue inhibitor of metalloproteinases) by human prostate in explant cultures: reduced tissue inhibitor of metalloproteinase secretion by malignant tissues. *Cancer Res* 53:4493–4498
19. Lokeshwar BL, Selzer MG, Zhu BQ, Block NL, Golub LM (2002) Inhibition of cell proliferation, invasion, tumor growth and metastasis by an oral non-antimicrobial tetracycline analog (COL-3) in a metastatic prostate cancer model. *Int J Cancer* 98:297–309
20. Lokeshwar VB, Lokeshwar BL, Pham HT, Block NL (1996) Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. *Cancer Res* 56:651–657
21. Lokeshwar VB, Young MJ, Goudarzi G, Iida N, Yudin AI, Cherr GN, Selzer MG (1999) Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1. *Cancer Res* 59:4464–4470
22. Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, Nadji M, Lokeshwar BL (2001) Stromal and epithelial expression of tumor markers hyaluronic acid and hyaluronidase in prostate cancer. *J Biol Chem* 276:11922–11932
23. Mickey DD, Stone KR, Wunderli H, Mickey H, Paulson DF (1980) Characterization of a human prostate adenocarcinoma cell line (DU 145) as a monolayer culture and as a solid tumor in athymic mice. *Prog Clin Biol Res* 37:67–84
24. Pienta KJ, Naik HN, Jeffrey EL (1996) Effect of estramustine, etoposide and taxol on prostate cancer cell growth in vitro and in vivo. *Urology* 48:164–170
25. Roche Applied Science (2002) Homogeneous caspases assay, fluorimetric (pack insert/product instruction). Roche Applied Science, Mannheim Germany
26. Smith M, Mills EJ (2001) Select complementary/alternative therapies for prostate cancer: the benefits and risks. *Cancer Pract* 9:253–255
27. Wang M, Stearns ME (1991) Isolation and characterization of PC-3 human prostatic sublines, which preferentially metastasize to select organs in S.C.I.D. mice. *Differentiation* 48:115–125
28. Wenger AS, Mickey DD, Hall M, Silverman LM, Mickey GH, Fried A (1984) In vitro characterization of MAT LyLu: a Dunning rat prostate adenocarcinoma tumor subline. *J Urol* 131:1232–1236
29. Yamamura Y, Rodriguez N, Schwartz A, Eylar E, Bagwell B, Yano N (1995) A new flow cytometric method for quantitative assessment of lymphocyte mitogenic potentials. *Cell Mol Biol (Noisy-le-grand)* 41:121–132