SHORT COMMUNICATION

Chaetocin antileukemia activity against chronic myelogenous leukemia cells is potentiated by bone marrow stromal factors and overcomes innate imatinib resistance

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Chronic myelogenous leukemia (CML) is maintained by a minor population of leukemic stem cells (LSCs) that exhibit innate resistance to tyrosine kinase inhibitors (TKIs) targeting BCR-ABL. Innate resistance can be induced by secreted bone marrow stromal cytokines and growth factors (BMSFs) that protect CML-LSCs from TKIs, resulting in minimal residual disease. Developing strategies to eradicate innate TKI resistance in LSCs is critical for preventing disease relapse. Cancer cells balance reactive oxygen species (ROS) at higher than normal levels, promoting their proliferation and survival, but also making them susceptible to damage by ROS-generating agents. Bcr-Abl increases cellular ROS levels, which can be reduced with TKI inhibitors, whereas, BMSFs increase ROS levels. We hypothesized that BMSF-mediated increases in ROS would trigger ROS damage in TKI-treated CML-LSCs when exposed to chaetocin, a mycotoxin that imposes oxidative stress by inhibiting thioredoxin reductase-1. Here, we showed that chaetocin suppressed viability and colony formation, and induced apoptosis of the murine hematopoietic cell line TonB210 with and without Bcr-Abl expression, and these effects were potentiated by BMSFs. In contrast, imatinib activities in Bcr-Abl-positive TonB210 cells were inhibited by BMSFs. Further, BMSFs did not inhibit imatinib activities when TonB210 cells expressing Bcr-Abl were cotreated with chaetocin. Chaetocin showed similar activities against LSC-enriched CML cell populations isolated from a murine transplant model of CML blast crisis that were phenotypically negative for lineage markers and positive for Sca-1 and c-Kit (CML-LSK). BMSFs and chaetocin increased ROS in CML-LSK cells and addition of BMSFs and chaetocin resulted in higher levels compared with chaetocin or BMSF treatment alone. Pretreatment of CML-LSKs with the antioxidant *N*-acetylcysteine blocked chaetocin cytotoxicity, even in the presence of BMSFs, demonstrating the importance ROS for chaetocin activities. Chaetocin effects on self-renewal of CML-LSKs were assessed by transplanting CML-LSKs into secondary recipients following *ex vivo* exposure to chaetocin, in the presence or absence of BMSFs. Disease latency in mice transplanted with CML-LSKs following chaetocin treatment more than doubled compared with untreated CML-LSKs or BMSFs-treated CML-LSKs. Mice transplanted with CML-LSKs following chaetocin treatment in the presence of BMSFs had significantly extended survival time compared with mice transplanted with CML-LSKs treated with chaetocin alone. Our findings indicate that chaetocin activity against CML-LSKs is significantly enhanced in the presence of BMSFs and suggest that chaetocin may be effective as a codrug to complement TKIs in CML treatment by disrupting the innate resistance of CML-LSKs through an ROS dependent mechanism.

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INTRODUCTION

Chronic myelogenous leukemia (CML) is maintained by a minor population of leukemic stem cells (LSCs) defined by their ability to transplant disease to a recipient, and that must be eliminated to effect cure.^{1,2} LSCs are homologous to normal hematopoietic stem cells (HSCs), but show enhanced self-renewal capacity, altered expression of various cell surface markers and innate resistance to therapy.^{1–3} Innate resistance can be induced by growth factor and cytokine signaling in the bone marrow microenvironment, which transiently protects a subset of LSCs from therapy, resulting in minimal residual disease.^{3–6} Over time, genetic instability inherent in LSCs, combined with the strong selective pressure of therapy, results in the emergence of permanent, acquired-resistance phenotypes.^{2,6,7}

Treatment of CML patients with imatinib or newer generation Bcr-Abl inhibitors (tyrosine kinase inhibitors, TKIs), such as nilotinib and dasatinib, markedly reduces proliferating BCR-ABL

expressing leukemia cells.^{2–6} However, these inhibitors do not eliminate the CML-LSC population, which shows that inhibiting Bcr-Abl kinase activity alone is insufficient to eradicate the disease, and implicates TKI-insensitive CML-LSCs in relapse.^{3,6,8} *In vitro* studies on human CML cell lines and CD34+ cells isolated from CML patients have shown that bone marrow stromal cell-conditioned media and cytokine cocktails maintain important pro-survival and self-renewal activities in the presence of TKIs, suggesting a role for secreted bone marrow stromal cytokines and growth factors (BMSFs) in innate resistance to BCR-ABL kinase inhibition.^{8–11} Developing therapeutic strategies that can be combined with TKIs to disrupt innate resistance and eradicate TKI-insensitive CML-LSCs is critical for preventing acquired resistance and disease relapse.

The major by-products of cellular metabolism are collectively known as reactive oxygen species (ROS) and include superoxide, hydrogen peroxide and hydroxyl radical.¹² ROS have key roles in

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cell signaling and homeostasis and their effects are concentration dependent.^{12–14} At low and moderate levels, ROS increases cell proliferation and survival through posttranslational modifications of kinases and phosphatases, and by inducing stress-responsive genes and pro-survival signaling pathways.^{12–14} However, at high levels, ROS can damage cellular proteins, lipids and DNA, and induce apoptosis or inflict a catastrophic cellular insult leading to necrosis, even if apoptotic pathways are deregulated.^{12–16} Cancer cells exhibit greater ROS stress than normal cells, and counteract the detrimental effects of high ROS by increasing the production of antioxidant molecules, leading to a tightly controlled balance of ROS and antioxidants at higher than normal levels.^{12,13} Higher ROS levels inherent in cancer cells render them susceptible to cellular redox imbalance and ROS-mediated damage by exogenous ROS-generating agents.^{12,13}

In CML, Bcr-Abl expression increases ROS levels, leading to enhanced cell survival and genomic instability whereas, inhibition of Bcr-Abl activity by imatinib or nilotinib significantly decreases ROS levels.^{7,17–21} It is now established that several cytokines, chemokines and growth factors secreted by bone marrow stromal cells, and implicated in innate TKI resistance, increase ROS levels.^{14,21,22} Thus, we postulated that BMSF-mediated increases in ROS in TKI-treated CML-LSCs might serve as a biochemical basis for triggering ROS-mediated damage with an exogenous ROS-generating agent. Here, we report that chaetocin, a mycotoxin that exhibits anticancer properties and imposes oxidative stress by inhibiting thioredoxin reductase-1,^{16,23,24} significantly enhances the cytotoxic effects of TKIs on BCR-ABL expressing TonB210 cells²⁵ and LSC-enriched CML cell populations isolated from a murine transplant model of CML blast crisis²⁶ that are phenotypically negative for lineage markers and positive for Sca-1 and c-Kit (CML-LSK). Importantly, in contrast to imatinib, the anticancer activity of chaetocin in CML cells is potentiated by BMSFs. Our findings suggest that chaetocin, or other ROS-inducing

compounds, might serve to complement TKIs in the treatment of CML by overcoming innate resistance mediated by BMSFs.

RESULTS AND DISCUSSION

Hematopoietic growth factors and cytokines are implicated in the innate resistance of CML-LSCs to TKIs^{8–11} and are known to increase ROS levels in hematopoietic cells.^{14,21,22} Higher ROS levels in CML-LSCs exposed to BMSFs might render them susceptible to ROS-mediated damage by exogenous ROS-generating agents. Thus, we investigated whether chaetocin, an anticancer mycotoxin capable of imposing oxidative stress,^{16,23,24} might overcome BMSF-mediated innate TKI resistance in CML.

We first characterized chaetocin activity using TonB210, a murine hematopoietic cell line with doxycycline-inducible BCR-ABL expression.²⁵ In the absence of exogenous IL-3, TonB210 cells are dependent on doxycycline-induced BCR-ABL expression for cell survival and proliferation. TonB210 permitted us to compare chaetocin effects on cell viability in the presence or absence of BCR-ABL expression and in response to BMSFs obtained by harvesting conditioned media from cultures of the murine bone marrow fibroblast cell line M2-10B4.²⁷ In TonB210 cells expressing BCR-ABL, the half maximal inhibitory concentration (IC₅₀) of imatinib was 1.5 μ M (Figure 1a), whereas in the absence of BCR-ABL expression, imatinib did not affect viability (Figure 1b). In contrast, chaetocin caused significant cytotoxicity in both BCR-ABL expressing TonB210 cells and TonB210 cells lacking BCR-ABL expression with an IC₅₀ value of 175 nM (Figures 1c and d). Effects of chaetocin on innate TKI resistance were then investigated by assessing viability, apoptosis induction and colony formation in imatinib treated and untreated TonB210 cells, in response to BMSFs. As expected, imatinib showed no effect on the growth of TonB210 cells lacking BCR-ABL expression in the presence or absence of BMSFs (Figures 2a, c and e). Consistent with previous reports implicating BMSFs in innate TKI resistance in CML, bone

Figure 1. Chaetocin antileukemia activity in TonB210 model CML cells. TonB210 cells with doxycycline-induced BCR-ABL expression (**a** and **c**) and noninduced BCR-ABL (**b** and **d**) were treated with the indicated concentrations of imatinib or chaetocin and incubated at 37 °C for 48 h. Viability was measured using trypan blue exclusion and is presented as the percentage of viable cells. Error bars represent s.d. from three independent experiments.

Figure 2. Chaetocin antileukemia activity is potentiated by BMSFs and overcomes BMSFs-mediated imatinib resistance in TonB210 cells. (**a** and **b**) The effect of chaetocin, imatinib and/or BMSFs on the viability of TonB210 cells. TonB210 cells without BCR-ABL induction (**a**) or with doxycycline-induced expression of BCR-ABL (**b**) were pretreated with BMSFs for 4 h and then imatinib and/or chaetocin was added at the indicated concentrations. Viability was measured using trypan blue exclusion 48 h after imatinib and/or chaetocin addition and is presented as the percentage of viable cells. (**c** and **d**) The effect of chaetocin, imatinib and/or BMSFs on the induction of apoptosis in TonB210 cells. TonB210 cells without BCR-ABL induction (**c**) or with doxycycline-induced expression of BCR-ABL (**d**) were treated with imatinib, chaetocin and BMSFs as in (**a** and **b**). Cells undergoing apoptosis were detected using annexin V FITC and propidium iodide stain following 48 h of drug exposure. The frequency of apoptosis is expressed as the percentage of annexin V positive cells. (**e** and **f**) The effect of imatinib, chaetocin and/or BMSFs on TonB210 colony formation. TonB210 cells without BCR-ABL induction or with doxycycline-induced expression of BCR-ABL were pre-exposed to BMSF, treated with indicated concentrations of imatinib and/or chaetocin for 48 h, and then plated in methylcellulose at a density of 1000 cells per 150 μl. Colonies were counted after 7 days. Statistical analyses: unpaired *t*-test (*n* = 3), **P* < 0.05, ***P* < 0.005 and ****P* < 0.0005. Error bars represent s.d. from three independent experiments.

marrow stromal cell-conditioned media protected BCR-ABL expressing TonB210 cells from imatinib-mediated growth suppression. These effects were most pronounced on cell viability and colony formation (Figures 2b, d and f). In contrast to imatinib, chaetocin significantly suppressed the growth of both BCR-ABL-expressing TonB210 cells and TonB210 cells lacking BCR-ABL expression and these effects were potentiated by BMSFs (Figures 2a–f). The combination of chaetocin and imatinib almost completely eliminated viable BCR-ABL expressing cells (Figures 2b, d and f).

Developing new therapeutic strategies that can be combined with TKIs to disrupt innate resistance is critical for eliminating TKI-insensitive CML-LSCs implicated in minimal residual disease and disease relapse. Thus, we compared effects of chaetocin as a single agent, and in combination with imatinib, on the viability, apoptosis induction and colony forming capacity of CML-LSCs in response to BMSFs. Doses of chaetocin and imatinib used in these studies were determined by performing individual chaetocin and imatinib dose response studies using CML-LSCs and assaying for viability at 48 h (Figure 3). We observed that the cytotoxicity of

Figure 3. Chaetocin possesses antileukemia activity against murine BCR-ABL+, NUP98/HoxA9+ model CML-LSKs. (**a** and **b**) CML-LSKs obtained from the BCR-ABL+, NUP98/HoxA9+ murine model of CML were treated with indicated concentrations of imatinib or chaetocin and incubated at 37 °C for 48 h. Viability was measured using trypan blue exclusion and is presented as the percentage of viable cells. Error bars represent s.d. from three independent experiments.

chaetocin increased in the presence of BMSFs and that combination treatment overcame BMSF-mediated imatinib resistance (Figure 4a). In agreement, the addition of BMSFs reduced the induction of apoptosis by imatinib, but potentiated apoptosis in chaetocin-treated cells (Figure 4b). Moreover, combination treatment with imatinib and chaetocin induced apoptosis more effectively than either drug alone, which was not inhibited by BMSFs (Figure 4b). Colony formation by CML-LSKs was inhibited by both imatinib and chaetocin (Figure 4c). Noticeably, the presence of BMSFs conferred almost complete protection from imatinib cytotoxicity, whereas a complete loss of colony formation was observed in cells exposed to chaetocin and BMSFs (Figure 4c). The combination of imatinib and chaetocin further reduced colony formation compared with treatment with imatinib or chaetocin alone, and no colonies were observed when CML-LSKs were treated with both agents in the presence of BMSFs (Figure 4c).

Cancer cells balance ROS and antioxidants at higher levels than normal cells, which promotes their proliferation and survival, but also makes them susceptible to damage by exogenous ROS-generating agents.^{12,13} BCR-ABL expression increases intracellular ROS levels,¹⁷ whereas TKI inhibition of Bcr-Abl reduces ROS.^{18,20,21} Furthermore, BMSFs, which are implicated in innate TKI resistance, can increase ROS levels.^{14,21,22} These studies implicate higher ROS levels in the growth and survival of CML cells, and suggest a potential therapeutic role for ROS-generating agents. As the imposition of oxidative stress is believed to be central to chaetocin's anticancer activity,^{16,23,24} we investigated effects of chaetocin on ROS levels in CML-LSKs in response to BMSFs. Both

BMSFs and chaetocin increased ROS in CML-LSKs (Figure 4d). Addition of BMSFs and chaetocin resulted in significantly higher levels of ROS in CML-LSKs compared with chaetocin or BMSFs alone (Figure 4d). Pretreatment of CML-LSKs with the antioxidant *N*-acetyl-cysteine blocked chaetocin cytotoxicity, even at high doses of chaetocin (500 nM) and in the presence of BMSFs (Figure 4e). These findings strongly suggested that chaetocin had activity against CML-LSKs, and its potentiation by BMSFs, was mediated through increased ROS. To further support this, we showed that the activity of phenethyl isothiocyanate, an ROS-inducing antileukemia agent,²⁸ was also enhanced by BMSFs and that phenethyl isothiocyanate activity in the presence and absence of BMSFs was blocked by *N*-acetyl-cysteine (Figure 4f).

Chaetocin effects on CML-LSK self-renewal *in vivo* were assessed using *ex vivo* secondary transplantation assays. CML-LSKs were purified from primary CML mice and transplanted into secondary recipients following *in vitro* exposure to chaetocin with or without added BMSFs. Consistent with its strong effect on *in vitro* colony formation, *ex vivo* chaetocin exposure more than doubled the disease latency period in comparison with transplantation with untreated CML-LSKs or CML-LSKs exposed to BMSFs (Figure 4g). Furthermore, chaetocin treatment in the presence of BMSFs significantly extended survival time compared with chaetocin treatment alone (Figure 4g). This showed that chaetocin had activity against leukemia stem cells, and this activity was significantly enhanced in the presence of BMSFs.

It is becoming clear that TKI resistance can be mediated by multiple cytokines and growth factors and that this phenomenon extends to a variety of cancers, including those of nonhematological origin, such as melanoma, breast cancer and nonsmall-cell lung cancer.^{29–32} Recent reports have described strategies for overcoming TKI resistance mediated by BMSFs centered on inhibiting various components of the JAK/STAT signaling axis.^{10,11,29,30} Our data show that innate TKI resistance can be overcome by chaetocin through alternate pathways, primarily centered on ROS. Thus, chaetocin may serve as a broadly applicable codrug for overcoming innate TKI resistance in a variety of hematological and nonhematological cancers.

MATERIALS AND METHODS

Tissue culture and drugs

All tissue cultures were performed at 37 °C and 5% CO₂ in RPMI or IMDM supplemented with 10% fetal bovine serum (Invitrogen/Gibco, Burlington, ON, Canada). TonB210 cells²⁵ were cultured in either 10 ng/ml IL-3 (R&D Systems, Minneapolis, MN, USA) or 2 μg/ml doxycycline. BMSFs were obtained by harvesting media conditioned by M2-10B4 cells⁹ after 5–7 days of culture and used at a 50% ratio in subsequent culture assays. Chaetocin and imatinib (Sigma Aldrich, St Louis, MO, USA) were dissolved in DMSO and diluted in phosphate-buffered saline. Phenethyl isothiocyanate (Sigma Aldrich) was diluted in DMSO immediately before use. The final concentration of DMSO did not exceed 0.1%.

Growth assays

Cell viability was determined using trypan blue staining reagent (Invitrogen) and counting viable cells by light microscopy with a hemocytometer. Apoptotic cells were assayed by flow cytometry using the Annexin-V PE Apoptosis Detection Kit I (BD Pharmingen, Mississauga, ON, Canada) according to the manufacturer's instructions. Colony formation was measured using MethoCult GF M3434 methylcellulose (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Cells were plated in triplicate at a density of 1000 cells per 150 μl and incubated at 37 °C with 5% CO₂ for 7–10 days and the resulting colonies were counted using light microscopy.

Figure 4. Chaetocin activity is mediated through ROS and reduces leukemic engraftment. **(a)** The effect of chaetocin, imatinib and/or BMSFs on the viability of CML-LSCs obtained from the BCR-ABL+, NUP98/HoxA9+ murine model of CML. Cells were pretreated with BMSFs for 4 h and then imatinib and/or chaetocin was added at the indicated concentrations. Viability was measured using trypan blue exclusion 48 h after drug addition and is presented as the percentage of viable cells. **(b)** The effect of chaetocin, imatinib and/or BMSFs on the induction of apoptosis in CML-LSCs. Cells were treated with imatinib, chaetocin and BMSFs as in panel A. Cells undergoing apoptosis were detected using annexin V FITC and propidium iodide stain following 48 h of drug exposure. The frequency of apoptosis is expressed as the percentage of annexin V positive cells. **(c)** The effect of imatinib, chaetocin and/or BMSFs on the colony formation of CML-LSCs. Cells were pre-exposed to BMSF for 4 h, treated with imatinib and/or chaetocin for 48 h and plated in methylcellulose at a density of 1000 cells per 150 μl. Colonies were counted after 7 days. **(d)** Reactive Oxygen Species (ROS) levels in chaetocin-treated cells. CML-LSCs were treated with BMSFs for 4 h and then chaetocin was added at 500 nM. 4 hours after the addition of chaetocin, CellROX Orange was added to achieve 5 μM final concentration. Fluorescence intensity ($n = 5$) is displayed in arbitrary units. **(e)** The effect of the antioxidant *N*-acetyl-L-cysteine (NAC) on chaetocin activity. CML-LSCs were pretreated with NAC for 30 min and then treated with BMSFs for 4 h. Following this, chaetocin was added and viability measured 24 h later by trypan staining. **(f)** The effect of NAC and BMSF on phenethyl isothiocyanate (PEITC) activity. CML-LSCs were pretreated with NAC for 30 min and then treated with BMSFs for 4 h. Following this, PEITC was added and viability measured 24 h later by trypan staining. **(g)** The effect of chaetocin and BMSFs on leukemia engraftment. CML-LSCs were pre-exposed to BMSF for 4 h, treated with 250 nM chaetocin for 24 h and then injected intravenously into sublethally irradiated mice. Animals were monitored and euthanized when disease symptoms were observed. Five mice were used per treatment condition. $P < 0.05$ chaetocin vs chaetocin/BMSF. Statistical analyses: unpaired t -test ($n = 3$), * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$. Error bars represent s.d. from three independent experiments.

ROS assay

ROS were detected in CML-LSCs by CellROX Orange (Invitrogen Life Technologies, Burlington, ON, Canada) using flow cytometry. Briefly, cells were treated with BMSFs for 4 h before the addition of chaetocin and then cultured for an additional 4 h. CellROX orange was added directly to the culture media and cultures incubated 100 min at 37 °C with 5% CO₂. Cells were then washed twice with PBS and analyzed by flow cytometry.

Murine CML model and isolation of CML-LSCs

CML-LSCs were obtained for *in vitro* and *ex vivo* secondary transplantation assays using an established BCR-ABL-Nup98/HoxA9 CML model of blast crisis.²⁶ HSCs were purified from the bone marrow of C57BL6/J mice (Charles River Laboratories, St-Constant, QC, Canada) using standard approaches. Mice were euthanized and single cell suspensions of bone marrow were obtained by flushing both tibia with PBS using a 26-gauge needle. Lineage-positive (LIN+) cells were depleted with a Lineage Cell Depletion Kit (Miltenyi Biotec, San Diego, CA, USA) and purified HSCs (LIN[−], CD117+ and Sca-1+ cells) were obtained from the LIN[−] fraction by fluorescence-activated cell sorting using a MoFlo Astrios instrument (Beckman Coulter, Mississauga, ON, Canada). Purified HSCs were transduced with BCR-ABL-GFP and Nup98/HoxA9-YFP by plating in culture dishes coated with retronectin (Takara Bio, Mountain View, CA, USA) and culturing for 3 days in IMDM supplemented with SCF, Flt3, IL6 and IL3 (R&D Systems) with replacement of 50% of culture media with viral supernatant every 12 h starting on day 2. CML was induced by intravenous injection of 2–10 × 10⁴ transduced HSCs into sublethally irradiated (6 Gy) C57BL recipient mice. Mice were closely monitored and those exhibiting morbid features associated with leukemic engraftment (for example, cachexia, decreased movement and poor grooming) were euthanized and tissues immediately harvested for subsequent studies. CML-LSCs were obtained by harvesting leukemia cells from the bone marrow or spleens of primary mice with leukemic engraftment and fluorescence-activated cell sorting purifying GFP+/YFP+ LSCs (Sca-1+, CD117low and LIN[−] cells). All animal experiments were performed in accordance with Canadian Council on Animal Care guidelines and with the approval of the University of Saskatchewan Committee on Animal Care and Supply. For *ex vivo* secondary transplantation assays, mice were sublethally irradiated 24 h before intravenous injection of CML-LSCs cultured *in vitro* with chaetocin and/or BMSFs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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