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Inhibitory effect of iron withdrawal by chelation on the growth of human and murine mammary carcinoma and fibrosarcoma cells

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ABSTRACT

Since iron uptake is essential for cell growth, rapidly dividing cancer cells are sensitive to iron depletion. To explore the effect of iron withdrawal on cancer cell growth, mouse and human mammary carcinoma cells (4T1 and MDA-MB-468, respectively) and mouse and human fibrosarcoma cells (L929 and HT1080, respectively) were cultured in the absence or presence of DIBI, a novel iron-chelating polymer containing hydroxypyridinone iron-ligand functionality. Cell growth was measured by a colorimetric assay for cell metabolic activity. DIBI-treated 4T1, MDA-MB-468, L929 and HT1080 cells, as well as their normal counterparts, showed a dose- and time-dependent reduction in growth that was selective for human cancer cells and mouse fibrosarcoma cells. The inhibitory effect of DIBI on fibrosarcoma and mammary carcinoma cell growth was reversed by addition of exogenous iron in the form of iron (III) citrate, confirming the iron selectivity of DIBI and that its inhibitory activity was iron-related. Fibrosarcoma and mammary carcinoma cell growth inhibition by DIBI was associated with S-phase cell cycle arrest and low to moderate levels of cell death by apoptosis. Consistent with apoptosis induction following DIBI-mediated iron withdrawal, fibrosarcoma and mammary carcinoma cells exhibited mitochondrial membrane permeabilization. A comparison of DIBI to other iron chelators showed that DIBI was superior to deferiprone and similar to or better than deferoxamine for inhibition of fibrosarcoma and mammary carcinoma cell growth. These findings suggest that iron withdrawal from the tumor microenvironment with a selective and potent iron chelator such as DIBI may prevent or inhibit tumor progression.

1. Introduction

There is abundant evidence that elevated iron levels in the body result in an increased cancer risk and that cancer cells have an increased requirement for iron (Marques et al., 2014; Toyokuni, 2009; Torti and Torti, 2013a, 2013b). For example, epidemiological studies show that a high dietary intake of iron from red meat is associated with an elevated risk of breast cancer in women (Ferrucci et al., 2009; Kallianpur et al., 2008). A link between iron intake and breast cancer is supported by a report of increased incidence of 1-methyl-1-nitrosourea-induced mammary carcinomas in rats on a diet modified to be high in iron (Thompson et al., 1991). In another study, subcutaneous injections of iron (II) sulfate promoted the development of mammary tumors in rats given dimethylbenz[a]anthracene (Diwan et al., 1997). Although iron is required for the growth of all cells, the demand for iron is higher in cancer cells because of their rapid rate of proliferation (Heath et al., 2013). Thus, high levels of dietary iron enhance the growth of spontaneous mammary tumors in mice while transplanted colon adenocarcinoma, hepatoma, and mammary carcinoma cells grow slower in iron-deficient mice (Hann et al., 1988, 1991). In addition, iron homeostatic mechanisms are frequently disrupted in multiple myeloma and breast cancer cells (Gu et al., 2015; Yang et al., 2001; Zhang et al., 2014). It follows that depriving cancer cells of iron results in cytotoxicity (Hoke et al., 2005; Jiang et al., 2002; Macková et al., 2012).

Iron chelators as anti-cancer agents that may be able to synergize with other cancer therapies show potential to reduce tumor burden (Buss et al., 2003; Kalinowski and Richardson, 2005; Pogribny et al., 2013). However, iron chelators (deferasirox, deferoxamine, and deferiprone) that are currently available for clinical use have limitations such as adverse side effects, short biological half-life, and high cost (Neufeld, 2010). DIBI is a novel highly selective iron chelator that is a member of a family of soluble copolymers with selectable molecular weights and defined contents of the modified hydroxypyridinone iron chelator, 3-hydroxy-1-(β-methacrylamidoethyl)-2-methyl-4(1 H)-

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pyridinone (MAHMP) (Holbein and Mira de Orduña, 2010). Hydroxypyridinones have the potential to yield new specialized iron chelators through chemical functionalization and modification, and present with low toxicities and good biocompatibility (Santos et al., 2012; Zhou et al., 2012). Unlike deferoxamine, which is a siderophore for some bacteria (Neupane and Kim, 2009), DIBI and deferiprone are synthetic hydroxypyridinone-class chelators. DIBI is predicted to bind iron in a molar ratio of greater than one and more avidly than deferiprone or MAHMP (coordination number greater than 2), given its multiple integral MAHMP residues, each of which can presumably coordinate iron intra-cooperatively, i.e., proximal MAHMP residues in DIBI coordinating iron.

In this study, we investigated the effect of iron chelation with DIBI on the growth of human and murine mammary carcinomas and fibrosarcomas, as well as the normal counterparts of these malignant cells. We also compared the efficacy of DIBI with deferiprone, deferoxamine, and MAHMP in our tumor model systems. Our findings suggest that iron withdrawal by highly selective iron chelators such as DIBI warrants further investigation as a possible treatment for solid tumors.

2. Materials and methods

2.1. Cell lines

MDA-MB-468 human mammary carcinoma cells were kindly provided by Dr. P. Lee (Dalhousie University, Halifax, NS). 4T1 mouse mammary carcinoma cells and HT1080 human fibrosarcoma cells were provided by Dr. D. Waisman (Dalhousie University). Dr. R. Garduno (Dalhousie University) generously provided the L929 mouse fibrosarcoma cells. Human mammary epithelial cells and human dermal fibroblasts were purchased from Lonza Inc. (Walkersville, MD). HC11 mouse mammary epithelial cells were kindly provided by Dr. Hyo-Sung Ro (Dalhousie University). 3T3 mouse fibroblasts were purchased from ATCC (Manassas, VA). All cancer cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich Canada, Oakville, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine (Invitrogen, Burlington, ON), hereafter referred to as complete DMEM (cDMEM). HMECs and human fibroblasts were grown in Clonetics® mammary epithelial cell growth medium (MEGM) and fibroblast growth medium (FGM-2), respectively (Lonza Inc.). HC11 cells were grown in DMEM containing 5% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. 3T3 fibroblasts were grown in cDMEM. Cancer cells were cultured at 37 °C in a humidified 10% CO_2 incubator and were regularly passaged using TrypLE (Invitrogen) to maintain optimal growth conditions. Normal cells were cultured at 37 °C in a humidified 5% CO_2 incubator and were passaged no more than 6 times.

2.2. Reagents

DIBI and MAHMP were provided by Chelation Partners Inc. (Halifax, NS). The DIBI preparation used in this study had an average MW of 29 kDa and a MAHMP content of 21% (*w*/*w*). A stock solution of DIBI was prepared in cDMEM and stored at 4 °C until use. Dimethyl sulfoxide (DMSO), deferoxamine, deferiprone, iron (III) citrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich Canada.

2.3. MTT assay

Cell growth was assessed by the colorimetric MTT assay (Mosmann, 1983). The effect of DIBI on cell number was examined with the MTT assay at time-points of 24, 48 and 72 h. Cells were harvested, counted, and plated in triplicate or quadruplicate into 96-well flat-bottom plates at 1.2–5 × 10^3 cells/well (depending on the cell line) and allowed to adhere for 24 h. Cells were then treated with cDMEM alone or the desired concentrations of DIBI, MAHMP, deferoxamine, deferiprone, and/or iron (III) citrate. Cells were cultured for 24, 48 or 72 h and 2 h before the end of culture MTT solution was added to each well at a final concentration of 0.5 mg/ml. Cell-free supernatant was discarded and formazan crystals were solubilized in 0.1 ml of DMSO. Absorbance was then measured at 570 nm on an ASYS Expert 96 plate-reader (Montreal Biotech Inc., Kirkland, QC). Absorbance in each treatment group was compared to the vehicle control to determine percent reduction in mitochondrial succinate dehydrogenase activity.

2.4. Oregon green 488 cell proliferation assay

MDA-MB-468, 4T1, HT1080 and L929 cells were harvested, counted, and plated at 5 × 10^4 cells/well into 6-well plates and allowed to adhere for 24 h culture, then the medium was discarded and cell monolayers were washed with PBS. Serum-free DMEM containing 1.25 μM Cell Trace™ Oregon Green®488 carboxylic acid diacetate (Invitrogen) was then added to all wells. After 1 h, cells were washed 3 times with warm cDMEM. A fourth wash of cDMEM was left on the cells for 3 h at 37 °C. At this point, an aliquot of non-proliferative cells was harvested as a baseline control, fixed in 1% paraformaldehyde and stored at 4 °C until the end of the experiment. All other cells were treated with cDMEM alone or the desired concentration of DIBI and cultured for 72 h. Cells were then harvested and mean channel fluorescence (MCF) was determined by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Mississauga, ON) and Becton Dickinson CellQuest™ software (version 3.3). Data shown represent cell proliferation relative to the cDMEM control where relative cell division = $(MCF_{sample} / MCF_{cDMEM\ control}) \times 100$.

2.5. Cell cycle analysis

Cells were synchronized by serum starvation for 24 h, then harvested, counted, and plated at 2.5 × 10^4 cells/well (4T1) or 5 × 10^4 cells/well (MDA-MB-468, L929, HT1080) into 6-well plates. Cells were allowed to adhere for 24 h and then treated with either cDMEM alone or the desired concentrations of DIBI for 48 h. Cells were harvested, washed and resuspended in cold PBS, followed by the addition of cold 70% ethanol and incubation at −20 °C for at least 24 h. Cells were then washed with PBS and resuspended in PBS containing 0.1% Triton X-100, 0.2 mg/ml of RNase A and 0.02 mg/ml of PI. Following staining at room temperature for 30 min, cells were analyzed by flow cytometry. ModFit software (Verity Software House, Topsham, ME) was used to determine the percent of cells in each phase of the cell cycle.

2.6. Apoptosis/necrosis assay

Cells were harvested, counted, and plated at 2.5 × 10^4 cells/well (4T1) or 3 × 10^4 cells/well (MDA-MB-468, L929, HT1080) into 6-well plates and allowed to adhere for 24 h. Cells were then treated with cDMEM alone or the desired concentrations of DIBI and cultured for 48 h. Cells in suspension and adherent cells were then harvested, washed once with PBS and resuspended in flow cytometry buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$) containing 2% Annexin-V-FLUOS (*v*/*v*; Roche Diagnostics, Laval, QC) and 1 μg/ml of PI. After staining for 15 min at room temperature cells were analyzed by flow cytometry.

2.7. Mitochondrial membrane permeabilization assay

Cells were harvested, counted, and plated into 6-well plates at 2.5–3 × 10^4 cells/well (depending on the cell line) and allowed to adhere for 24 h. Cells were cultured in the absence or presence of the