Gallium Maltolate Inhibits Human Cutaneous T-Cell Lymphoma Tumor Development in Mice

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Cutaneous T-cell lymphomas (CTCLs) represent a heterogeneous group of non-Hodgkin's lymphoma characterized by an accumulation of malignant CD4 T cells in the skin. The group IIIa metal salt, gallium nitrate, is known to have antineoplastic activity against B-cell lymphoma in humans, but its activity in CTCLs has not been elaborated in detail. Herein, we examined the antineoplastic efficacy of a gallium compound, gallium maltolate (GaM), *in vitro* and *in vivo* with murine models of CTCLs. GaM inhibited cell growth and induced apoptosis of cultured CTCL cells. In human CTCL xenograft models, peritumoral injection of GaM limited the growth of CTCL cells, shown by fewer tumor formations, smaller tumor sizes, and decreased neovascularization in tumor microenvironment. To identify key signaling pathways that have a role in GaM-mediated reduction of tumor growth, we analyzed inflammatory cytokines, as well as signal transduction pathways in CTCL cells treated by GaM. IFN-γ-induced chemokines and IL-13 were found to be notably increased in GaM-treated CTCL cells. However, immunosuppressive cytokines, such as IL-10, were decreased with GaM treatment. Interestingly, both oxidative stress and p53 pathways were involved in GaM-induced cytotoxicity. These results warrant further investigation of GaM as a therapeutic agent for CTCLs.

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of peripheral, extranodal, non-Hodgkin's lymphomas, resulting in a variety of skin findings, including scaly red patches, plaques, tumors, and erythroderma (Hwang *et al.*, 2008). Patients with tumor stage disease have a poor prognosis with a 5-year survival of less than 50%. The treatment of CTCLs ranges from skin-directed therapies for early disease to more complex chemotherapy-based treatments for later stages (Hwang *et al.*, 2008). Cures are not usually seen, however, and patients require on-going therapy. Hence, there is a great need to continue to develop effective drugs for the treatment of CTCLs.

Mounting evidence indicates that an inflammatory environment is a participatory component of tumor development (Mantovani *et al.*, 2008). In one of our established murine models of CTCLs, C57BL/6 mice injected with MBL2 T-lymphoma cells in the ear can be induced to develop a cutaneous lymphoma in the setting of an inflammatory response generated by a single topical application of 2,4-Dinitro-fluorobenzene (Wu *et al.*, 2011). In this model, tumors do not develop in these animals in the absence of 2,4-Dinitro-fluorobenzene application or when mice are treated with corticosteroids, thus underscoring the importance of the inflammatory microenvironment in this model of CTCL. Moreover, inflammatory macrophages appear to have a critical role, as depletion of macrophages markedly reduces the size of the T-cell tumors (Wu *et al.*, 2014)

Gallium is a group IIIa metal that shares certain chemical similarities with iron (III). These properties enable gallium to bind to the iron transport protein transferrin and to be taken up by cells via transferrin receptor (TfR)-mediated endocytosis of transferrin-gallium (Tf-Ga) complexes (Larson *et al.*, 1980, Harris and Pecoraro, 1983, Chitambar and Zivkovic, 1987). Lymphoma cells are known to express high densities of cell surface TfRs, thus making them selective targets for Tf-Ga (Kvaloy *et al.*, 1984, Chitambar *et al.*, 1989). In contrast to lymphoma cells, normal resting lymphocytes do not express cell surface TfRs and are thus not targeted by Tf-Ga (Chitambar *et al.*, 1989). Tf-Ga interferes with the cellular uptake of transferrin-iron, producing a state of cellular iron deprivation, which can induce apoptosis (Chitambar *et al.*, 1989). Malignant cells have a greater requirement for iron compared with normal cells, rendering them more susceptible to iron deprivation (Richardson *et al.*, 2009). The action of gallium extends beyond interfering with TfR-mediated iron uptake; it inhibits iron-dependent ribonucleotide reductase (deoxyribonucleotide

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Abbreviations: CTCL, cutaneous T-cell lymphoma; GaM, gallium maltolate; GaN, gallium nitrate; PBS, phosphate-buffered saline; Tf-Ga, transferrin-gallium; TfR, transferrin receptor

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synthesis), iron-dependent mitochondrial function, and other processes in the cells, ultimately culminating in cell death (Chitambar, 2012; Chitambar and Antholine, 2013). Gallium also has significant anti-inflammatory and immunosuppressive activities (Chitambar, 2010). The anti-inflammatory action of gallium appears to involve the downregulation of inflammatory T cells and macrophages, as well as possible interference with matrix metalloproteinases. Because many iron compounds are proinflammatory, the ability of gallium to act as a nonfunctional iron mimetic may contribute to its anti-inflammatory activity.

Clinical trials have shown gallium nitrate (GaN), a first generation gallium compound, to have significant antineoplastic activity in B-cell non-Hodgkin's lymphoma (Straus, 2003). However, drug resistance to GaN may be encountered, thus limiting its anti-tumor efficacy in some patients. Our recent studies have shown that gallium maltolate (GaM), a next generation of gallium compound, displays greater cytotoxicity compared with GaN against a panel of lymphoma cell lines, including lymphoma cells resistant to growth inhibition by GaN (Chitambar *et al.*, 2007). In contrast to GaN, which is a simple metal salt, GaM consists of three maltolate ligands bidentately bound to a central gallium atom in a propeller-like arrangement (Chitambar *et al.*, 2007). Gallium's anti-inflammatory properties may be relevant to CTCLs, as our studies have shown that CTCL develops in a background of inflammatory changes in the skin. Beyond a single case report of a marked response to GaN in a patient with peripheral T-cell lymphoma refractory to conventional therapy (Huang *et al.*, 2005), there are limited data and research on the efficacy of gallium compounds in CTCLs. In the present study, we examined the antineoplastic efficacy of GaM *in vitro* and *in vivo* in preclinical murine models of CTCLs and investigated the molecular mechanisms that mediate the anti-tumor activity of this metallocompound.

RESULTS

Cytotoxic activity of GaM in CTCL cells

Hut78 and HH are both CTCL cell lines that were established from human CTCL patients. Because TfR-mediated endocytosis of Tf-Ga is the initial step in gallium-mediated cell death, we first examined whether TfR was present on the surface of Hut78 and HH cells. Using anti-CD71 (TfR) antibody, we detected the homogeneous expression of TfR on both cell lines by flow cytometry (Supplementary Figure S1 online), indicating that these cells may be selectively targeted by TfR-Ga through TfR-ligand engagement. Next, we exposed both cultured cell lines to GaM at increasing concentrations (Figure 1a). Both CTCL cell lines exhibited dose-dependent cytotoxicity with GaM. Of note, a slight proliferative effect of GaM was seen after day 1 of treatment at 100 μM, but complete killing of Hut78 cells was seen after 3 days of incubation. The ability of gallium to mimic iron, an essential element for cell growth, may account for the initial proliferative effects of low-dose GaM. Although HH cells were also sensitive to GaM exposure, they required a higher concentration of GaM and a longer incubation period for complete killing (Figure 1a). In the flow cytometry–based apoptosis assay, Hut78 cells showed marked increases of annexin V or 7-AAD positivity after 1 day exposure to GaM at 100 μM (47.5% in GaM vs. 10.9% in phosphate-buffered saline (PBS control; Figure 1b). Interestingly, we noticed that GaM displayed only slight toxicity against HaCat keratinocyte cells (Supplementary Figure S2 online), suggesting that GaM may have selective toxicity toward the two malignant T-cell lines.

A Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) was utilized to measure oxygen consumption in CTCL cell lines. Incubation of HuT78 cells with increasing concentrations of GaM over 8 hours resulted in a dose-dependent decrease in the cellular oxygen consumption rate before measurable changes in cell death could be detected (Figure 1c), suggesting that an early event in gallium-induced tumor cell death involves inhibition of mitochondrial respiratory function, and thus cellular metabolism.

Inhibition of tumor growth by GaM in CTCL mouse models

In order to determine whether GaM can block tumor growth *in vivo*, we established xenograft CTCL mouse models through the subcutaneous (SC) implantation of CTCL cells, either Hut78 or HH cells, in the flank skin of Nod-Scid IL-2 γ-chain knockout (NSG) mice. Tumors were apparent after 2 weeks with most growth occurring during the third and fourth weeks. To observe the treatment effect by GaM, we injected GaM at the same site with a daily dosage of 400 μg per mouse for 5 days 1 day after tumor cell inoculation. Four weeks after implantation, mice were killed and examined (Figure 2a). Solid tumors were found in SC area in the mice in PBS-treated group, whereas tumors were rarely found in GaM-treated mice (Figure 2b). Significant decreases in tumor formation were revealed in both Hut78 and HH cell line experiments (Supplementary Figure S3 online, Hut78, $P = 0.0021$, HH, $P = 0.0061$).

In addition, we sought to determine the anti-tumor effect of GaM on established CTCL tumors. Therefore, instead of on day 2, we started GaM treatment on day 8 or 15 after tumor implantation as indicated (Figure 2a). Mice were killed 4 weeks after implantation. With treatment starting at day 8, tumor growth was greatly inhibited as confirmed by hematoxylin and eosin staining (Figure 2c). Even with treatment starting at day 15, the GaM-treated tumors showed a significant reduction in size compared with PBS treatment ($P = 0.0415$), although tumor growth was only partially inhibited (Figure 2d and e). Thus, even in established tumors, GaM treatment can effectively inhibit growth of CTCL tumors in NSG mice (Figure 2d and e).

Decreased tumor vascularization with GaM treatment in tumor-bearing mice

To understand the impact of GaM treatment on the tumor environment, we first assessed tumor vascularity in Hut78 tumor tissues isolated from PBS or GaM-treated tumor-bearing mice. Immunofluorescent staining showed a significant decrease in CD31, a recognized marker for vascular endothelial cells, in the tumors from GaM-treated mice (Supplementary Figure S4a and b online). Although positive signals highlighting both small vessels and individual cells were frequently observed amidst condensed tumor area in the PBS control