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Gallium Maltolate is a Promising Chemotherapeutic Agent for the Treatment of Hepatocellular Carcinoma

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Abstract. *Background: Hepatocellular carcinoma (HCC) is a particularly lethal cancer with few treatment options. Since gallium is known to accumulate specifically in HCC tumors but not in non-tumor liver, we investigated two gallium compounds, gallium nitrate (GaN) and gallium maltolate (GaM), as potential new agents for treating HCC. Materials and Methods: The anti-proliferative and apoptotic activities of GaN and GaM were assessed in vitro using four HCC cell lines. HCC gene expression data was analyzed to provide a mechanistic rationale for using gallium in the treatment of HCC. Results: Both compounds showed dose-dependent antiproliferative activity in all four HCC cell lines after 6-day drug exposure (IC_{50} values range from 60-250 μM for gallium nitrate and 25-35 μM for gallium maltolate). Gallium maltolate at 30 μM additionally induced apoptosis after 6 days. HCC gene expression data showed significantly elevated expression of the M2 subunit of ribonucleotide reductase, which is a target for the antiproliferative activity of gallium. Conclusion: These data support clinical testing of gallium maltolate, an orally active compound, in the treatment of HCC.*

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the third leading cause of cancer deaths (1). At diagnosis, approximately 10-25% of HCC patients have disease amenable to surgical resection; for the remaining patients, treatment options are very limited (2, 3).

Gallium is a semi-metallic element used in both cancer diagnosis (as radioactive ^{67}Ga for scintigraphic scans) and treatment (4). Gallium nitrate (GaN), as a citrate-buffered formulation for injection (which is approved in the United States to treat hypercalcemia of malignancy), has shown efficacy against several types of cancer, most notably non-Hodgkin's lymphoma and multiple myeloma (4-6). The utility of GaN is limited, however, by renal toxicity, which occurs most often when it is administered by rapid infusion. An orally active compound, gallium maltolate (GaM), is currently in clinical development as a less toxic and more convenient alternative to intravenous GaN (4, 7, 8).

A major mechanism for gallium's antiproliferative activity is its ability to mimic, compete with and substitute for Fe^{3+} in the active site of ribonucleotide reductase (RR), thus inhibiting this enzyme crucial for DNA synthesis (9-11). RRM2 (the M2 subunit of RR) is located in a region (1q:163) of frequent cytogenetic aberration in HCC (12), suggesting it to be a chemotherapeutic target in HCC. Because gallium is known to accumulate in HCC tumors (based on ^{67}Ga scans (13, 14)), and because RR is generally highly overexpressed by HCC cells (determined by analysis of gene expression data, as discussed below), a compelling rationale exists for exploring the potential utility of gallium in treating HCC. The strategy of treating HCC by interfering with cellular iron uptake and metabolism is further supported by the significant observed suppression and regression of human HCC tumor growth in athymic nude mice due to iron deprivation, which had been induced by the administration of deferoxamine (15).

Materials and Methods

Cell lines and materials. The human HCC cell lines Hep3B, HepG2 and SNU475 were purchased from ATCC and cultured according to ATCC recommendations using EMEM (for Hep3B and HepG2) or RPMI (for SNU475) cell culture medium, at 37˚C and 5% CO_2 in a humidified incubator. The Hep 40 human HCC cell line, a gift from Dr. Xin Chen of the University of California, San Francisco, U.S.A., was cultivated under similar conditions in EMEM medium. GaN was purchased from Sigma-Aldrich (St. Louis, MN, USA) and GaM was provided by Titan Pharmaceuticals, Inc. (San Francisco, CA, USA).

Proliferation assay. The antiproliferative activities of GaN and GaM were assessed using Promega's CellTiter 96® AQueous One Solution Cell Proliferation Assay, according to the manufacturer's instructions. Briefly, the cells were seeded at appropriate cell densities in 96-well multititer plates and allowed to incubate at 37˚C overnight before addition of the test compound at the desired

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concentration ranges. The cells were then exposed to the test compound for 6 days before colorimetric assessment of cell viability was done at 490 nm on a plate reader. The assays were performed in triplicate, with n=4 in each experiment.

Assessment of apoptotic cell morphology. Cells were seeded in chamber slides at 5,000 to 20,000 cells per chamber (depending on the growth characteristics of each cell line); GaM was added 24 h later at 10 μM or 30 μM. After 6-day drug exposure, the cells were fixed using absolute methanol at –20˚C for 10 min, then stained with hematoxylin and eosin. Cell morphology was observed with a light microscope at 20x magnification.

Immunoblotting. Cells were exposed to the desired concentrations of GaM for appropriate periods; whole cell lysates were then prepared and total protein quantified using the Bradford Assay (Bio-Rad, Hercules, CA, USA). Typically, 20 μg of protein was electrophoresed on pre-cast gels (Bio-Rad) and transferred onto PVDF membranes (Bio-Rad). Non-specific sites were blocked by incubation with 5% non-fat milk in TBS-1% Tween solution for 1 h at room temperature. The proteins were then detected with specific antibodies against CD71 (transferrin receptor 1) (Zymed Laboratories, San Francisco, CA, USA) or PARP (BD-PharMingen, San Diego, CA, USA) at recommended dilutions, followed by appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and the SuperSignal West Dura Extended Duration ECL kit from Pierce (Rockford, IL, USA).

Gene expression data analysis. Gene expression data on 75 liver tumor and 72 non-tumor liver tissues (16) were retrieved from the Stanford Microarray Database and analyzed by the web-based microarray data analysis program GABRIEL (Genetic Analysis By Rules Incorporating Expert Logic; http://gabriel.stanford.edu). GABRIEL is a rule-based computer program designed to apply domain-specific and procedural knowledge systematically for the analysis and interpretation of data from DNA microarrays (17). We retrieved data for 4841 clones based on the following selection criteria: all non-flagged spots with >1.5-fold intensity over local background in either channel, 75% good data, and genes whose $\log_2$ of Red/Green normalized ratio (mean) was greater than 3-fold for at least four arrays. To obtain biologically meaningful data, we rescaled the data set such that the relative tumor (T) *versus* non-tumor (N) expression ratio ($\log_2$(T/N)) was calculated by $\log_2$(T/U) – $\log_2$(N/U), where U is the common reference consisting of a pool of mRNA from several cell lines (16). If an HCC sample did not have a corresponding non-tumor sample, the global mean of the non-tumor gene expression ratios was used.

We used the GABRIEL t-score pattern based rule to identify genes that were significantly differentially expressed between tumor and non-tumor liver tissues. The t-score is a measure of variability (average/standard deviation), and thus provides an indication of the consistency of the expression values across all samples. A t-score threshold of 2 was used. Proband-based analysis was used to search for genes having a linear correlation with tumor stage.

Results and Discussion

Antiproliferative effects of gallium nitrate and gallium maltolate in HCC. The antiproliferative activity of GaN was observed in all four HCC cell lines tested following 6 days of drug exposure, with IC_{50} values ranging from 60 to 250 μM (Figure 1A). These values are consistent with the IC_{50} values of GaN in other human cancer cell lines (*e.g.*, lymphoma, cervical cancer, breast cancer) (18-20). Using the same four human HCC cell lines, we observed antiproliferative effects of the orally active compound GaM, with IC_{50} values of 25-35 μM after 6 days of drug exposure (Figure 1B). Thus, both GaN and GaM exert antiproliferative activity against human HCC cell lines, with the latter showing greater potency. Little antiproliferative activity was seen for either GaN or GaM following 3 days of drug exposure (data not shown). This result is consistent with gallium not being acutely cytotoxic, but rather acting to prevent DNA synthesis and cell division, since the doubling-times for the HCC cell lines studied were 3 to 5 days.

Induction of apoptosis by gallium maltolate. Previous research showed that iron deprivation, due to exposure to either deferoxamine or 12.5 - 100 μM GaN, induced apoptosis in human leukemic CCRF-CEM cells (21). Similarly, GaN at 50 - 100 μM induced apoptosis in human peripheral blood mononuclear cells (22), while GaN at 500 μM or Ga-transferrin at 50 μM induced apoptosis in MCF-7 human breast cancer cells (23). In all of these cases, introducing excess iron with the gallium prevented apoptosis. The ability of gallium compounds to promote apoptosis was also recently demonstrated by Joseph *et al.* (24), who showed that GaN induces apoptosis in mantle cell lymphoma cells *via* induction of Bax, generation of reactive oxygen species and down-regulation of cyclin D1.

We found that treatment of HCC cells with GaM (30 μM, 6-day drug exposure) produced cellular morphology indicative of apoptosis (Figure 2A). Additionally, we observed different extents of cleavage of the enzyme poly(ADP-ribose) polymerase (PARP) in HCC cell lines Hep 3B, Hep 40 and SNU475 following 6-day exposure to 30 μM GaM (Figure 2B). HepG2 cells appear to lack PARP, suggesting that apoptosis may be mediated by other pathways in this cell line. PARP is involved in DNA damage repair, and its expression is triggered by DNA-strand breaks (25). In cells undergoing apoptosis, PARP is cleaved from a full-length 116 kDa peptide into 89 kDa and 24 kDa polypeptides by caspase-3 during the degradation of cellular DNA, thus preventing DNA damage repair.

Dose-dependent regulation of transferrin receptor expression by gallium maltolate. The preferential uptake of gallium by tumor cells is, in most cases, due to overexpression of the transferrin receptor by tumor cells (26). Gallium, particularly following oral administration of gallium maltolate, binds to serum transferrin (7), and gallium-transferrin is taken up via transferrin receptors on tumor cells, though non-transferrin-mediated uptake may also occur (27).

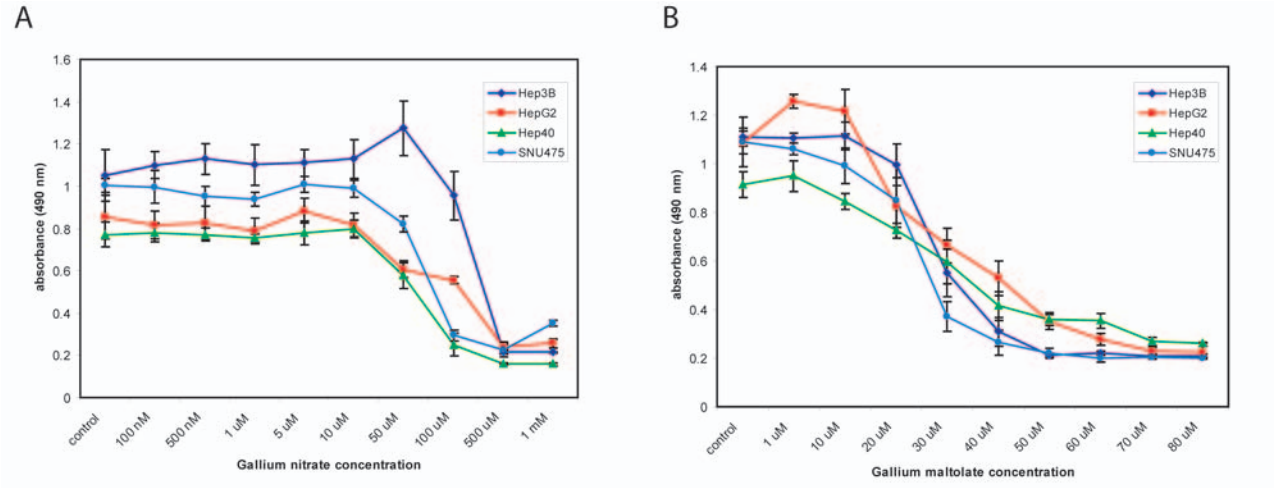

Figure 1. *The effects of (A) gallium nitrate or (B) gallium maltolate on proliferation of four human HCC cell lines following six days of drug exposure.*

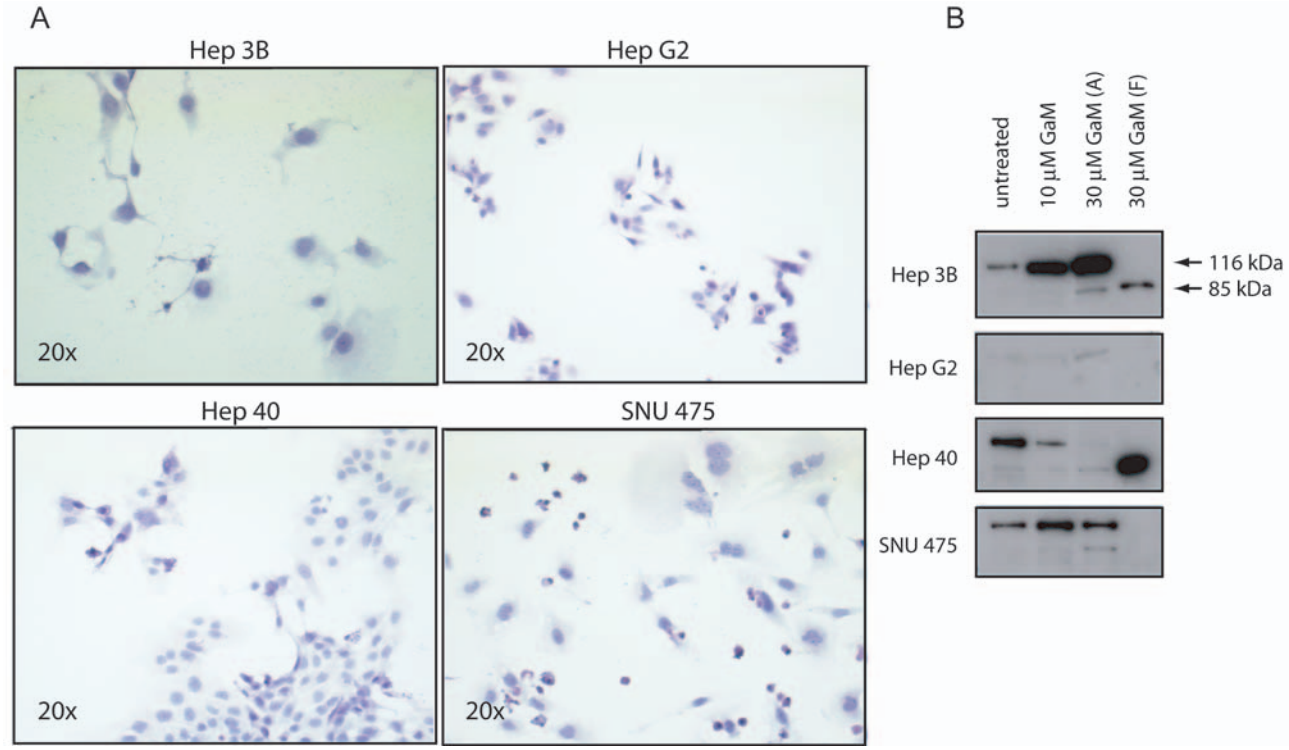

Figure 2. *(A) Changes in cell morphology indicative of apoptosis following exposure to 30 µM gallium maltolate (GaM) for six days. (B) PARP cleavage following exposure to 30 µM GaM for 6 days in three HCC cell lines (HepG2 has undetectable levels of PARP). The attached (A) and floating (F) cells were collected and assessed separately.*

Our Western blotting results showed that all four studied HCC cell lines expressed readily detectable levels of CD71 (transferrin receptor 1), with Hep 40 expressing it at higher levels than the others (Figure 3A). Following 6-day exposure to 10 µM or 30 µM GaM, the protein level of CD71 demonstrated a dose-dependent increase in all four HCC cell lines (Figure 3B). The increase of CD71 expression following drug exposure may result from a feedback regulatory attempt to increase iron uptake to overcome competition by gallium. This increased expression of CD71, however, sets up a self-destructive loop as it promotes further gallium uptake, which ultimately inhibits cell division, leading to cell death.

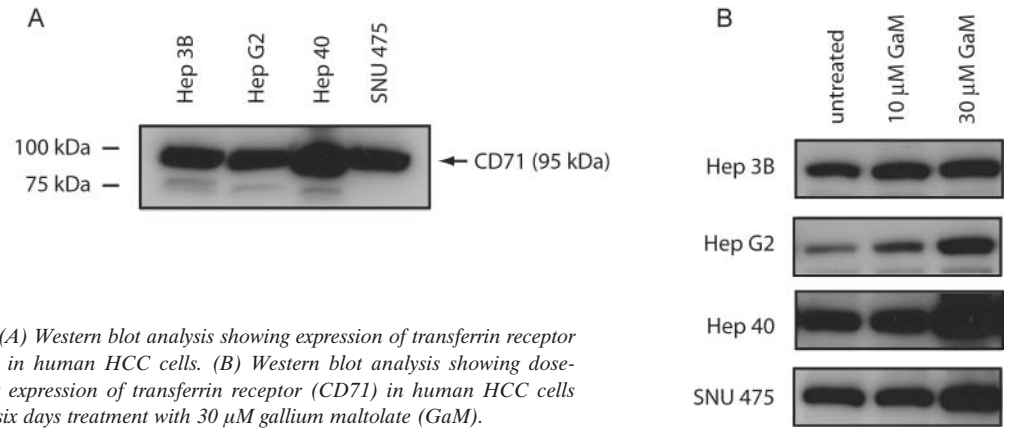

Figure 3. *(A) Western blot analysis showing expression of transferrin receptor 1 (CD71) in human HCC cells. (B) Western blot analysis showing dose-dependent expression of transferrin receptor (CD71) in human HCC cells following six days treatment with 30 µM gallium maltolate (GaM).*

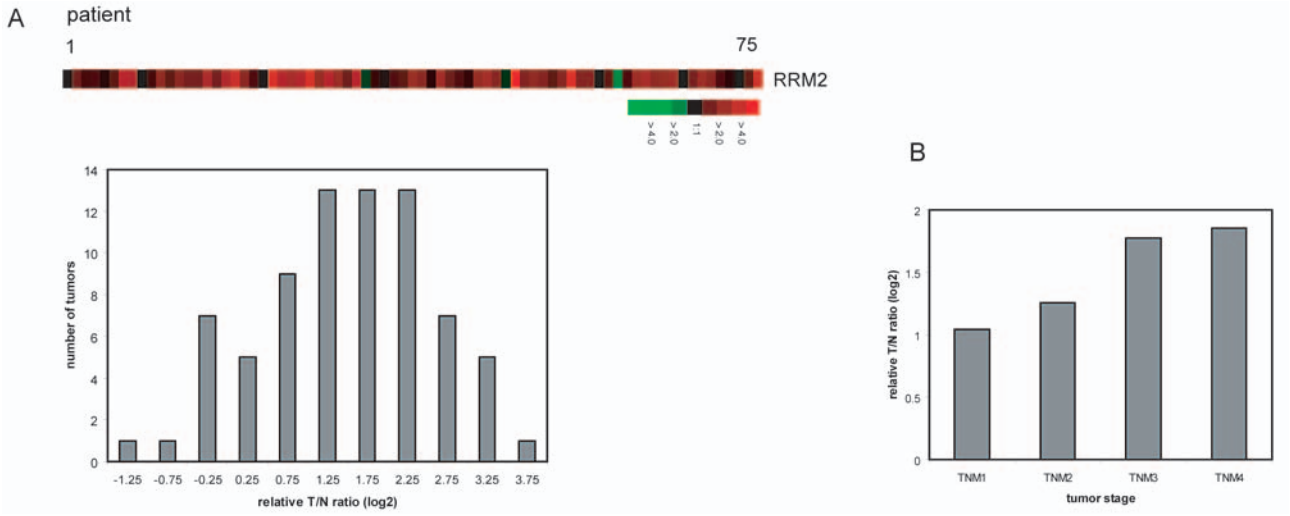

Figure 4. *(A) The relative expression of RRM2 in HCC tumor compared to non-tumor liver tissue in 75 patients (57 of whom had matched HCC and non-tumor liver samples). The relative abundance of RRM2 transcript of each patient is represented by the color scale at the bottom right corner (red indicates overexpression, black indicates equal expression, whereas green indicates underexpression relative to the mean expression level of RRM2 across all samples). Grey represents missing data. The distribution of the 75 patients over the range of T/N expression ratios for RRM2 is also depicted as a histogram. (B) RRM2 expression as a function of HCC stage in the same 75 tumor samples.*

Overexpression of RRM2 in HCC. Gallium is known to compete with and substitute for Fe^{3+} in RRM2 (9-11). We surveyed the HCC gene expression data from our earlier study to determine whether RRM2 is a viable target for HCC. We found 1616 genes from the 75 HCC patients to be over-expressed in HCC tumors relative to normal liver tissue, based on their having a t-score >2 (with a false-positive rate of 0.007 and a false-negative rate of 0.013). Of particular interest, RRM2 (t-score=8.51) was among the top 2% of genes with a t-score >2, suggesting that it was consistently (65 out of 75 tumor samples) and significantly differentially up-regulated in tumor *versus* non-tumor liver (Figure 4A).

Proband-based analysis was used to search for genes that have a linear positive correlation with progressive tumor stage (according to the TNM system). At a correlation threshold of 0.6, 864 genes satisfied this rule (false-positive rate=0, false-negative rate=0.031). RRM2 had a correlation of 0.843, indicating a strong positive correlation with tumor stage (Figure 4B). Not only was RRM2 over-expressed in all stages of HCC compared to non-tumor liver, but its increased expression with progressive tumor stage implied that it is a good chemotherapeutic target, particularly for advanced stages of HCC (when most patients are diagnosed).

Conclusion

Gallium maltolate appears to be a promising chemotherapeutic agent for the treatment of HCC, due to: (a) the ability of gallium to preferentially accumulate in liver tumors; (b) the enhanced expression by HCC tumors of RRM2, which is

targeted by gallium; (c) the *in vitro* antiproliferative and pro-apoptotic effects of GaN and GaM in HCC cell lines; (d) the clinical antitumor effect of gallium (as intravenous GaN) in other cancers; and (e) the clinical safety and convenience of GaM. The ability of HCC tumors to accumulate gallium, while surrounding normal liver tissue and other tissues do not (13, 14), could result in highly preferential antiproliferative targeting, with normal tissues being spared. The avidity of even distant HCC metastases for gallium (28) raises the possibility of the first potential treatment for metastatic HCC. Given the resistance of HCC to currently available chemotherapy, and the urgent need for new HCC therapies, a clinical trial to assess the efficacy of GaM in HCC patients appears warranted. Further research also appears desirable to more extensively study gallium's mechanisms of action in HCC, and to assess whether a combination of GaM and other chemotherapeutic agents (including other inhibitors of RR or DNA synthesis) could bring about a synergistic antitumor effect against HCC.

Acknowledgements

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