Targeting Chronic Myelogenous Leukemia with Imatinib and Glycyrrhizic Acid Combination Therapy

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ABSTRACT

An estimated 8,990 new cases of chronic myelogenous leukemia (CML) were diagnosed in 2019. The prevalence of CML has been on the rise since the discovery of tyrosine kinase inhibitors (TKI) in 2001. Imatinib (IMT), the first TKI approved for clinical use, is the gold standard for CML treatment, although rising resistance often requires patients to switch TKI therapy at least once. Glycyrrhizic acid (GA) is a versatile drug due to its numerous therapeutic properties. Anti-tumor properties of GA indicate its use as a chemotherapeutic agent, and previous data from our lab has found apoptosis-inducing effects of GA in CML cell lines. In the current study we examined the efficacy of an IMT+GA combination therapy on chronic myelogenous leukemia cell lines. Cell proliferation and viability post-treatment were determined using Trypan Blue exclusion assay and MTT assay. Induction of apoptosis post-treatment was examined using Annexin V-FITC assay and Western blot analysis. Proliferation and viability of CML cell lines was negatively correlated with IMT+GA cotreatment in a dose-dependent manner. The expected IMT-induced apoptosis of CML was further exacerbated when GA was added to treatment at a concentration of 2.0 mM or greater. At these concentrations of GA in combination with IMT, enhanced PARP cleavage compared to control was found. Together we show that a chemotherapy consisting of imatinib and glycyrrhizic acid may be a novel method of treating chronic myeloid leukemia. Future studies will attempt to elucidate the mechanism of action of GA therapy by focusing on modulation of hyaluronidase activity and sumoylation levels post-treatment.

METHODS

Cell culture

- K562 cells were cultured for 48-hour intervals with varying concentrations of IMT alone, GA alone, or an IMT-GA combination.
- Imatinib-resistant K562 (RK562) cells were maintained in 1 uM IMT prior to treatment (described above).

Assays

- Viability: Equal dilution of cell solution and 0.4% trypan blue dye solution and analysis using a TC20™ Automated Cell Counter.
- Proliferation: MTT colorimetric assay to determine proliferation based on metabolic activity and formazan crystal formation.
- Apoptosis: Annexin V-FITC assay using annexin V-FITC and propidium iodide fluorescent tags and flow cytometry analysis to determine stage of cellular apoptosis. Western Blot analysis of PARP cleavage to further confirm drug-induced apoptosis.
- Gene Expression: RT-qPCR to examine post-treatment expression patterns of genes associated with HA (hyaluronic acid) synthesis, HA cleavage, SUMO (small ubiquitin-like modifier), and SUMO regulation. The ΔΔCT method was used for analysis.

ACKNOWLEDGEMENTS

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RESULTS

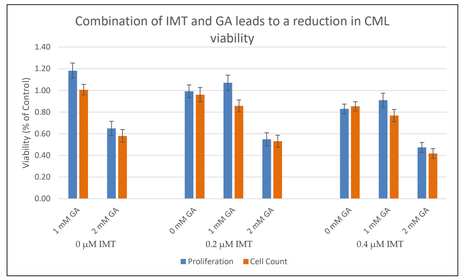
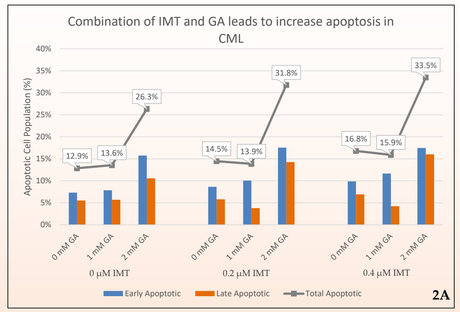


Figure 1. MTT cell proliferation data and cell count data of K562 cell line following 48-hour incubation with IMT, GA, or IMT+GA at varying concentrations. Data analyzed as a fraction of the respective control (untreated) value.



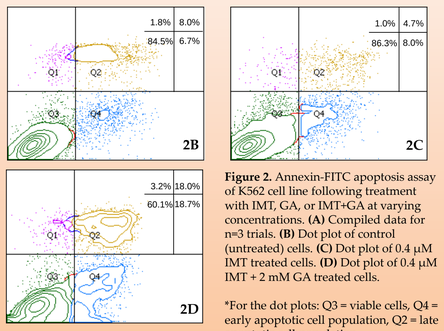
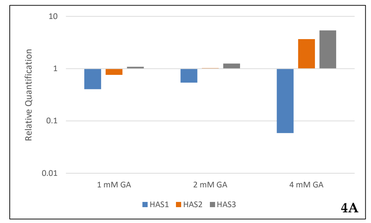
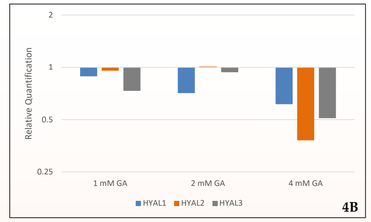
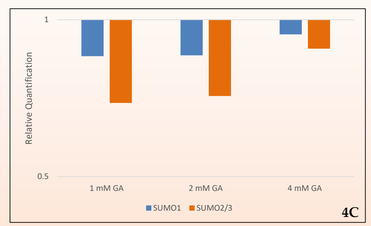


Figure 2. Annexin-FITC apoptosis assay of K562 cell line following treatment with IMT, GA, or IMT+GA at varying concentrations. **(A)** Compiled data for n=3 trials. **(B)** Dot plot of control (untreated) cells. **(C)** Dot plot of 0.4 μM IMT treated cells. **(D)** Dot plot of 0.4 μM IMT + 2 mM GA treated cells.

*For the dot plots: Q3 = viable cells, Q4 = early apoptotic cell population, Q2 = late apoptotic cell population







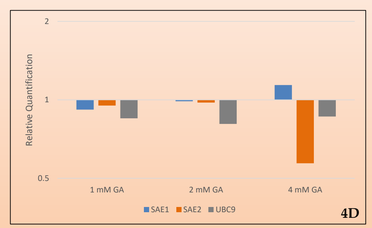


Figure 4. RT-qPCR analysis of K562 gene expression following incubation with varying concentrations of GA. Relative quantification was determined using HPRT, B2M, and 18S as housekeeping genes. **(A)** HA synthases (HAS1, HAS2, HAS3). **(B)** Hyaluronidases (HYAL1, HYAL2, HYAL3). **(C)** SUMO proteins (SUMO1, SUMO2/3). **(D)** SUMO regulatory enzymes (SAE1, SAE2, UBC9).

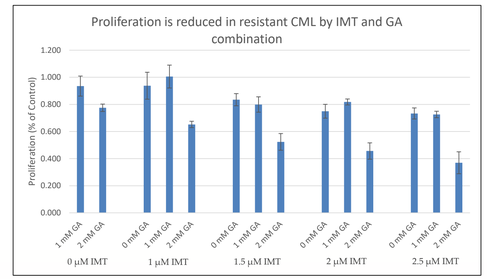
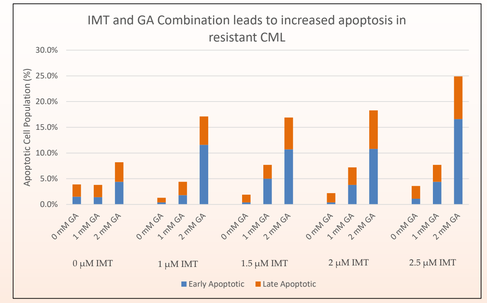


Figure 5. MTT cell proliferation data of RK562 cell line following 48-hour incubation with IMT, GA, or IMT+GA at varying concentrations. Data was normalized to the control (untreated) group.



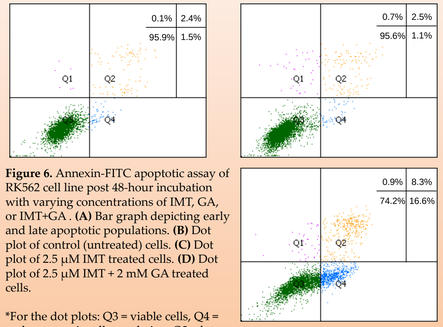


Figure 6. Annexin-FITC apoptotic assay of RK562 cell line post 48-hour incubation with varying concentrations of IMT, GA, or IMT+GA . **(A)** Bar graph depicting early and late apoptotic populations. **(B)** Dot plot of control (untreated) cells. **(C)** Dot plot of 2.5 μM IMT treated cells. **(D)** Dot plot of 2.5 μM IMT + 2 mM GA treated cells.

*For the dot plots: Q3 = viable cells, Q4 = early apoptotic cell population, Q2 = late apoptotic cell population

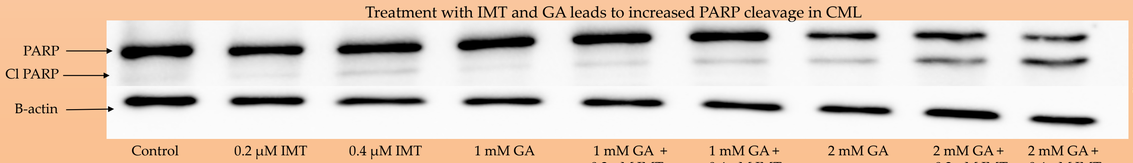


Figure 3. Western Blot image of K562 cell line following 48-hour incubation with IMT, GA, or IMT-GA treatment and subsequent protein isolation. PARP cleavage was used to indicate apoptosis. B-actin was used as a loading control.

DISCUSSION

In the current study, we demonstrate that the combination of IMT and GA treatments is an effective therapy against CML. Viability and proliferation of K562 cells was reduced following treatment with GA and IMT in a dose-dependent manner, with the most significant reduction being seen in 0.4 μM IMT + 2 mM GA (Fig. 1). Furthermore, treatment with 0.4 μM IMT + 2 mM GA led to a 2-fold increase and a 1.3-fold increase in the total drug-induced apoptotic cell population from 0.4 μM IMT alone and 2 mM GA alone, respectively (Fig. 2). Lastly, PARP cleavage was analyzed via Western Blot to confirm apoptosis. Cleavage of PARP by caspases is one of the final steps of the programmed cell death pathway. Taken together, this indicates the potential for a combination therapy to be more effective than either IMT or GA alone.

Since the development of IMT and other TKIs, CML patients now have near-typical life expectancies. Due to this, patients have a greater chance of resistant CML developing and roughly 1-in-4 patient will have to switch TKIs at least once. This study found that when IMT-resistant K562 (RK562) cells were cultured in 1 uM or 1.5 μM IMT, there was no significant decrease in proliferation compared to the control (untreated) group. A significant decrease was seen at 1.5 μM IMT + 2 mM GA and all greater combinations. When using 1 μM IMT as the control group, since RK562 were maintained in 1 μM IMT, a significant decrease was seen at 1.5 μM, 2 μM, and 2.5 μM only in the presence of 2 mM GA (Fig. 5). Furthermore, we found that the addition of 2 mM GA led to a 2- to 13-fold increase in the total apoptotic cell population from the IMT alone treatments. The greatest increase in total apoptotic cells was seen at 1 μM IMT + 2 M GA (13-fold change from IMT alone), while the greatest total apoptotic population was found after incubation with 2.5 μM IMT + 2 mM GA (Fig. 6). These findings point to the possibility of GA's use in treating resistant CML in combination with a patient's current TKI therapy.

This study also began to examine the mechanism of action of GA in the treatment of CML via RT-qPCR analysis. Since mounting research has found that HA plays an important role in cancer biology, we chose to examine the genes coding for HA synthases (HAS1, 2, 3) and the genes coding for hyaluronidases (HYAL1, 2,3). HYALs are responsible for the cleavage of HA into smaller fragments. GA has been shown to act as a putative HYAL inhibitor. We further examined gene expression for SUMO proteins (SUMO1, 2/3) and the SUMO machinery (SAE1, SAE2, UBC9), due to evidence that GA inhibits SUMOylation and the role SUMO plays in cancer biology. We found that HAS1 was significant downregulated following treatment with 1 mM and 4 mM GA, yet 4 mM GA treatment led to significant upregulation of HAS2 and HAS3 (Fig. 4A). While all HYALs were downregulated following treatment with GA, the only significant downregulation was seen in HYAL2 and HYAL3 following treatment 4 mM GA (Fig. 4B). There was so significant increase or decrease of SUMO protein or SUMO machinery expression following GA treatment (Fig. 4C and 4D). Future studies will need to further investigate the effect of GA treatment on HA production and modulation, and on SUMO regulation. RT-qPCR analysis will further look into gene expression of the target genes. Western Blot analysis will examine protein levels following GA treatment. Immunoprecipitation assays will examine protein-protein interaction following GA treatment. Finally, HYAL enzymatic activity assays will examine the effect of GA treatment on enzyme activity.