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Are glycyrrhizin derivatives specific inhibitors of HMGB1/DNA interactions

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ABSTRACT

Previous work in our laboratory investigated the ability of ammonium glycyrrhizinate (GLA) and two related compounds (carbenoxolone and glycyrrhetic acid) to inhibit the DNA-binding properties HMGB1 in an in vitro screening system based on a quantitative capillary electrophoresis mobility shift assay (CEMSA). Our results demonstrated that GLA and GAK inhibited the DNA-binding of HMGB1 at concentrations above the critical micelle concentration. Thus, the inhibitory effects were observed only under conditions where the triterpenes formed aggregates and not when the compounds were in solution. Literature evidence suggests that compounds capable of forming aggregates inhibit or activate unrelated proteins including chymotrypsin, β -lactamase, β -galactosidase and alkaline phosphatase. The present work investigates the possible promiscuous nature of the inhibition of HMGB1/DNA interactions observed for GLA derivatives. The model proteins used in the study were glutathione S-transferase (GST) and alkaline phosphatase. Our results indicate that the GLA derivatives tested do not inhibit enzyme activity in the enzymes tested. On the contrary, GLA activated GST while GAK activated alkaline phosphatase. Taken together with our previous results, the present data suggest that GLA and GAK could be utilized in the inhibition of macromolecular interactions with minimal effects on cellular enzymes.

Keywords: GLA derivatives, aggregates, promiscuous inhibition, glutathione S-transferase, alkaline phosphatase

INTRODUCTION

High mobility group box-1 (HMGB1) is a ubiquitous nuclear non-histone protein, with high electrophoretic mobility. Originally identified as an architectural transcription factor, HMGB1 plays a pivotal role in transcriptional regulation [1]. Currently it is a focus of intensive studies [2-6] because of its critical role in regulation of multiple intracellular and extracellular processes.

Modulation of biological activity of HMGB1 can affect many intracellular processes; therefore, this protein is a potential target for therapeutic intervention. Anti-HMGB1 agents that have been reported include polyclonal neutralizing antibodies, nicotinic agonists, stearyl lysophosphatidyl choline, ethyl pyruvate, serine protease inhibitors (nafamostat mesilate), steroidal pigments, and ethacrynic acid [7]. Preliminary reports suggest that small molecule inhibitors of HMGB1 are important experimental tools which may find clinical applications [8].

The lack of high affinity leads (including a specific radioligand) and an appropriate assay for screening protein/DNA interactions make the development of small molecule inhibitors of HMGB1/DNA interactions especially challenging. A variety of approaches have been used to investigate the interactions of HMGB1 with DNA and to identify possible inhibitors of the interaction. Gel shift assay demonstrated the ability of glycyrrhizic acid (GLA) to inhibit HMGB1/single stranded DNA (ssDNA) complex formation [9]. Bianchi and co-authors have reported the direct binding of GLA and a derivative (carbenoxolone) to HMGB1 using nuclear magnetic resonance (NMR) and fluorescence studies [10,11].

Our interest in studying HMGB1/DNA interactions and in the identification of small molecule inhibitors led to the development of a capillary electrophoresis mobility shift assay (CEMSA) to study HMGB1/double stranded DNA (dsDNA) interactions in the presence and absence of GLA derivatives [8]. Glycyrrhizin (GLA), carbenoxolone disodium (CGA) and potassium glycyrrhetinate (GAK) were tested for the ability to inhibit the binding of HMGB1 to double-stranded DNA. Data obtained for GLA derivatives in the CEMSA were not characteristic of competitive inhibitors and suggested that the inhibition of DNA-HMGB1 binding may be dependent on the ability of GLA derivatives to form micelles or supramolecular aggregates.

The ability of aggregate-forming compounds to inhibit or activate unrelated protein targets is well known and has been referred to as promiscuous inhibition or activation [12, 13]. In particular, the promiscuous inhibition or activation effects were demonstrated for polyanionic compounds or compounds capable of forming aggregates in model enzymes including chymotrypsin, β -lactamase, and alkaline phosphatase. The existing literature reports suggest that the polyanionic properties of GLA [14] and the micelle forming abilities of GLA and derivatives [15] could result in non-specific modes of interaction with proteins.

In order to investigate the possible promiscuous nature of the inhibition of HMGB1/DNA interactions observed for GLA derivatives, the compounds were evaluated using two unrelated proteins, glutathione S-transferase and alkaline phosphatase. Data collected in these enzyme assays are reported herein and the results discussed in the context of promiscuous inhibition/activation by these compounds.

MATERIALS AND METHODS

Glycyrrhizic acid ammonium salt, GLA, carbenoxolone disodium (CGA), 18 β -glycyrrhetic acid, 3-(N-morpholino) propane sulfonic acid (MOPS), 4-nitrophenyl phosphate disodium salt, triethylamine were purchased from Sigma Aldrich (St. Louis, MO, USA). Alkaline phosphatase from bovine intestine (27 U/ μ l) was from (Sigma, St. Louis, MO). Glycyrrhetic acid potassium

salt (GAK) was prepared from 18 β -glycyrrhetic acid (an aglycone of GLA). GST assay was carried out using GST detection kit (GE healthcare Biosciences Corp, NJ, USA).

Cloning, Expression, and Purification of GST. Recombinant glutathione S-transferase (GST) from *Schistosoma japonicum* was expressed in *E. coli* BL21 strain transformed with pGST-2T expression vector (GE Healthcare), per manufacturer's instructions. Briefly, after promoter induction with isopropyl- β -D-thiogalactoside, cells were harvested, lysed, and GST expression was monitored by GST detection module (GE Healthcare). GST protein was purified using Glutathione Sepharose 4B. GST-containing fractions were collected, combined and dialyzed against buffer F (100 mM HEPES pH 7.9; 100 mM NaCl; 0.5 mM EDTA; 5% glycerol; 5 mM DTT), and concentrated using Centricon-10 cartridge (Millipore, Billerica, MA). Glycerol was added to the protein solution to the final concentration 20%; protein was stored at (-20°C).

Enzyme assays

Compounds were tested for their ability to interact with Glutathione S-transferase (GST) and Alkaline phosphatase. Assays were performed in 50mM MOPS/triethylamine-50mM KCl buffer pH 7.5. Stocks of test compounds were prepared at 10 mM in deionized water. All reactions were monitored spectrophotometrically using UV1201 (Shimadzu, Japan). The assays were done in triplicate.

For both the assays mixture of different concentrations of test compounds and enzymes (1 μ L) in MOPS/triethylamine buffer were incubated for 15min in ice followed by 30 min centrifugation at 15,000xg to precipitate aggregates from solution [16]. The supernatant was transferred to the quartz microcuvette (1 ml). The reaction was initiated by addition of suitable substrate.

GST assay: The assay was carried out by manufacturer's instructions using the GST detecting module (GE Healthcare). Reaction mixture (1 ml) contained the variable concentrations of test compound (100 μ L), MOPS/triethylamine buffer, reduced glutathione, 100mM 1-chloro-2,4-Dinitrobenzene (CDNB) in ethanol (10 μ L) and GST enzyme (1 μ L). The reaction progress was monitored for 5 min at 340 nm.

Alkaline phosphatase assay: Reaction mixture (1 ml) contained the variable concentrations of the test compound (100 μ L), MOPS/triethylamine buffer, 2.5mM p-nitrophenyl phosphate (250 μ L) and alkaline phosphatase (1 μ L). The hydrolysis was monitored for 5 min at 405 nm.

Statistical Analysis of the significance of the data was carried out using Tukey-Kramer HSD.

RESULTS

When tested as inhibitors of HMGB-1/DNA interactions in the CEMSA, GLA and derivatives (especially GAK) did not display typical concentration dependent inhibition but rather an all or none response (Fig. 1). These observations may be related to the ability of GLA derivatives to form aggregates. Therefore, the micelle/aggregate forming abilities of GLA, CGA and GAK were investigated using fluorometric and dynamic light scattering studies (DLS) [8].

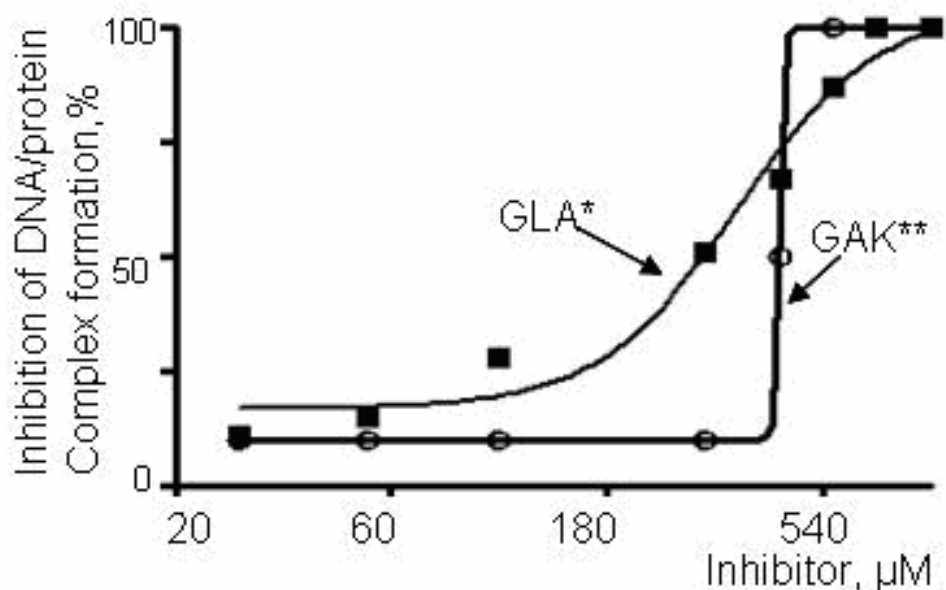


Fig. 1 Inhibition of DNA-HMGB1 complex formation with GLA and GAK as a function of increasing concentration of inhibitors under different conditions: GLA *buffer A (20 mM Tris-HCl pH 8.0; 5mM MgCl₂), or in GAK** buffer B (50mM MOPS-triethylamine, pH 7.5; 50mM KCl). (Source: *Der Pharmacia Lettre* 2, 432)

The results of these studies suggested that the compounds formed aggregates of varying size depending on the aqueous environment. Hence, GLA formed micelles in water (critical micelle concentration found to be above 0.3μM) while GAK formed aggregates in the MOPS/triethylamine buffer (pH 7.5) used in the CEMSA (critical aggregation concentration found to be above 0.3μM) (Fig. 2A, 2B). These data suggest that the inhibition of HMGB-1/DNA interactions reported previously may be due to promiscuous inhibition.

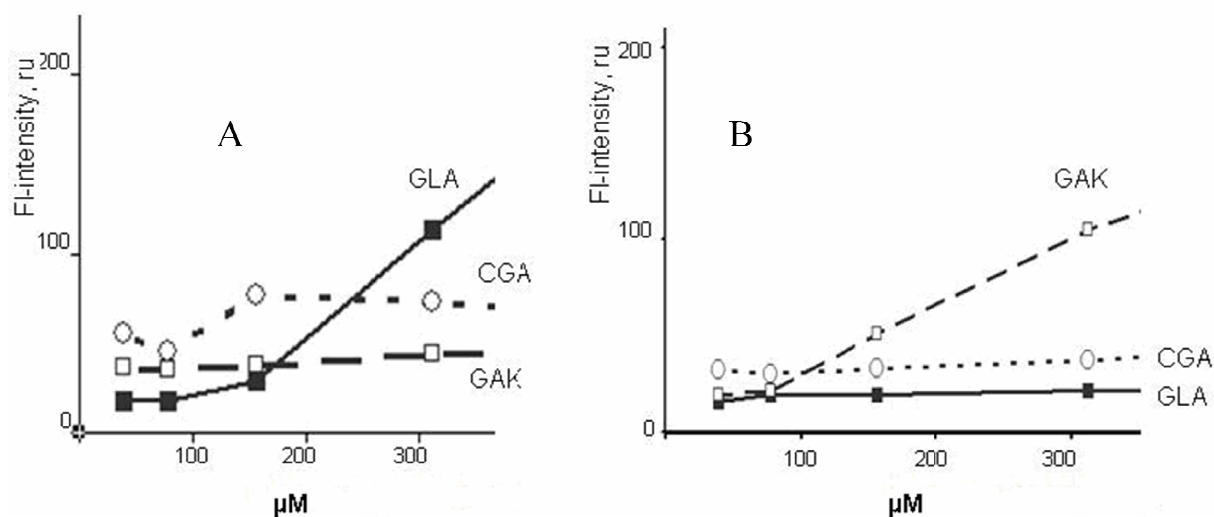
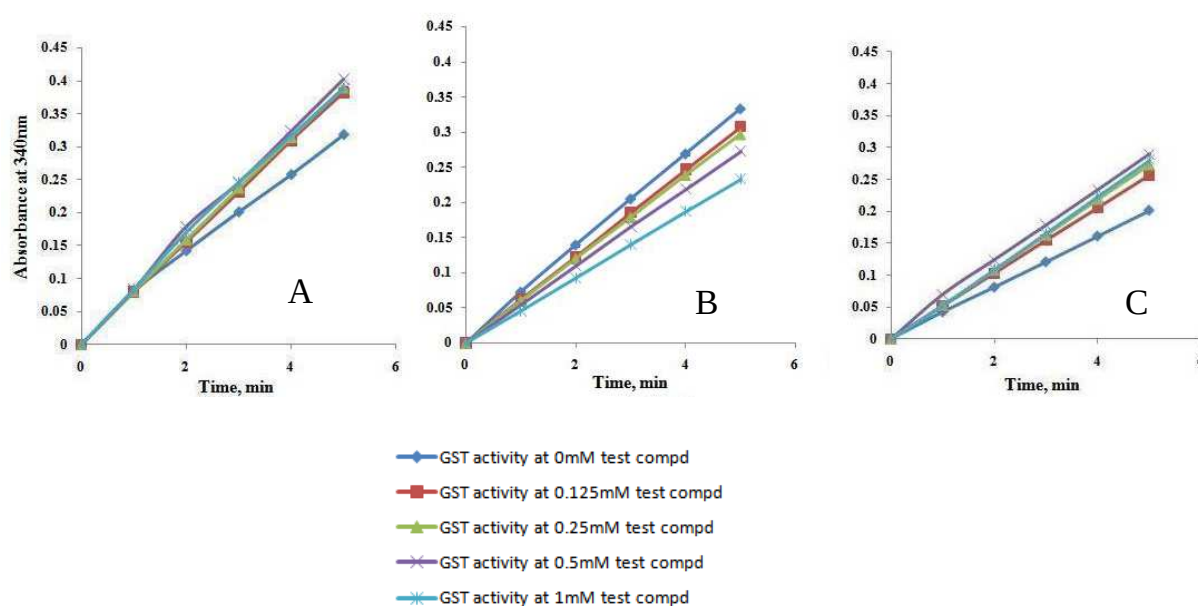


Fig. 2 CMC evaluation of three GLA derivatives (GLA, CGA and GAK) by fluorescent method with DPH as a probe, in water (A) and buffer B (MOPS-triethylamine buffer, pH 7.5; (D). (Source: *Der Pharmacia Lettre* 2, 432)

To investigate the possible promiscuous nature of the inhibition by GLA derivatives, GLA, CGA and GAK were tested for their ability to inhibit the activity of two unrelated enzymes; Glutathione S-Transferase (GST) and Alkaline phosphatase.

GST activity in presence of GLA derivatives

GLA, CGA and GAK were tested for their effect on the enzymatic activity of GST at concentration ranges used in our previous studies (ranging from 0.125 mM to 1mM). Figure 3, Graph A illustrates a significant increase in GST activity at all concentrations of GLA, while CGA (Figure 3; Graph B) showed no significant effect at any of the concentrations tested. An apparent increase in GST activity produced by GAK at all concentrations tested was found to be non-significant based on the Tukey Kramer HSD (Fig. 3; Graph C).

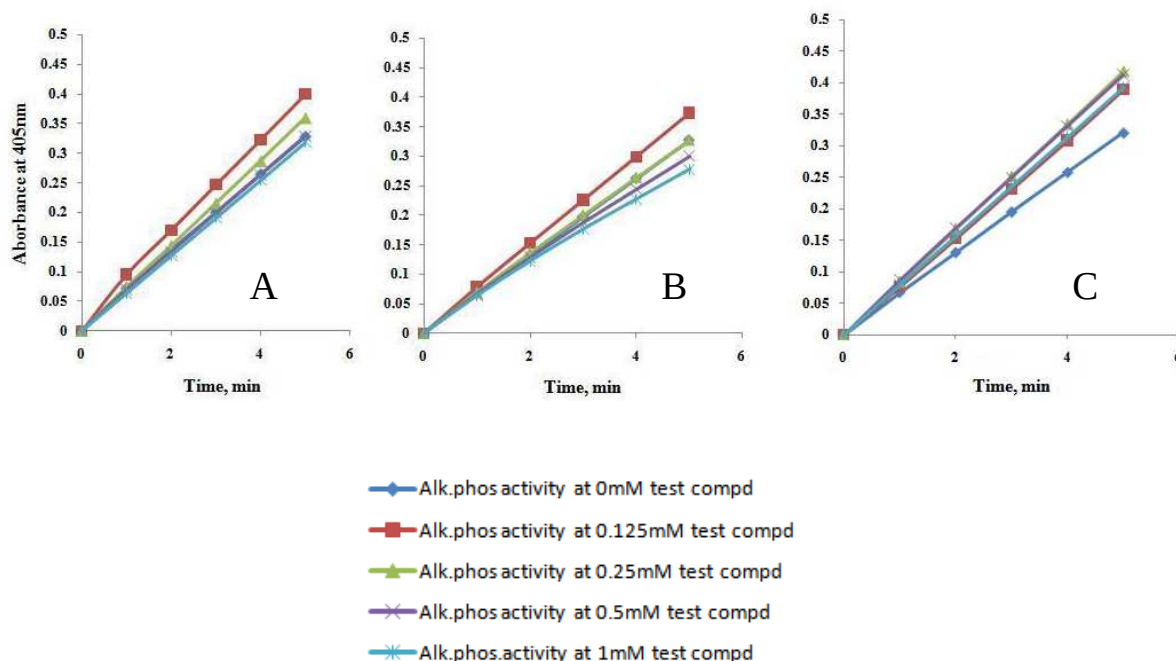


A: Glutathione S-Transferase activity in presence of GLA
B: Glutathione S-Transferase activity in presence of CGA
C: Glutathione S-Transferase activity in presence of GAK

Fig. 3 Glutathione S-transferase (GST) activity in the presence of GLA, CGA & GAK (MOPS/triethylamine buffer pH 7.5). GST assay points have an average error of $\pm 3\%$.

Alkaline phosphatase activity in presence of GLA derivatives

GLA, CGA and GAK were tested for their effect on the enzymatic activity of alkaline phosphatase at concentration ranges used in our previous studies (ranging from 0.125 mM to 1mM). The addition of GAK (Figure 4; Graph C) resulted in a significant activation of alkaline phosphatase at all the concentrations tested. In contrast, GLA (Figure. 4; Graph A) and CGA (Figure. 4; Graph B) did not show any statistically significant effect on enzyme activity.



A: Alkaline phosphatase activity in presence of GLA
B: Alkaline phosphatase activity in presence of CGA
C: Alkaline phosphatase activity in presence of GAK

Fig. 4 Alkaline phosphatase activity in presence of GLA, CGA & GAK (MOPS /triethylamine buffer pH 7.5). The Alkaline phosphatase points have an average error of $\pm 3\%$.

DISCUSSION

A CEMSA was developed to study HMGB1/dsDNA interactions and then used to screen potential inhibitors of HMGB1 binding. The inhibition observed for GLA derivatives was not characteristic of competitive inhibition (Fig. 2) and led to further experimentation [8] evaluating the surfactant properties of GLA and its derivatives. Fluorometric determinations and dynamic light scattering studies (DLS) revealed that GLA formed micelles in water (critical micelle concentration above $300\mu\text{M}$) while GAK formed aggregates (critical micelle/aggregation concentration above $300\mu\text{M}$) in the MOPS/triethylamine buffer (pH 7.5) used in the CEMSA. These observations led to the hypothesis that inhibition was observed only when assay conditions favored the formation of micelles or aggregates. In order to test this hypothesis GLA, CGA and GAK were tested against two unrelated enzymes, glutathione S-transferase, and alkaline phosphatase. Glutathione S-transferase has a molecular mass in the same range as HMGB1 (25-30 kDa) while alkaline phosphatase (molecular mass 160 kDa) had been used as a model enzyme to evaluate promiscuous properties of the tested compounds. MOPS/triethylamine buffer (pH 7.5) was used for the assays in order to simulate the conditions of CEMSA. While enzymatic assays involving promiscuous inhibitors or activators typically use detergents (e.g., Triton X-100 at 0.1% v/v) and supplemental proteins (e.g., bovine serum

albumin, BSA), we did not include these components, to simulate the assay conditions of CEMSA.

Several studies reported that aggregating compounds exhibit diverse effects (activation, inhibition or no effect) across enzyme classes [13]. In our study, we detected the activation of GST by GLA (Figure 3A). This observation was unexpected since GLA did not form micelles/aggregates in MOPS/triethylamine buffer. There are no reports in the literature on the effect of GLA on GST activity in solution. However, GLA has been reported to activate GST in cell based assays [17]. CGA (Figure 2, Graph B) has not been shown to form aggregates under the test conditions used and did not show any effect on GST activity. In spite of the fact that GAK has been shown to form aggregates in the MOPS buffer, the compounds produced no significant change in enzyme activity at the concentrations tested (Figure 1; Graph C). The effects of GAK on alkaline phosphatase activity were in sharp contrast to the effect observed for GST. GAK showed significant activation of alkaline phosphatase. GLA and CGA did not show any effect on the activity of alkaline phosphatase.

CONCLUSION

Earlier studies involving the DNA binding protein HMGB1 indicated that certain GLA derivatives inhibited protein–DNA interactions at concentrations approximating the critical micelle concentration. These observations were interpreted to suggest that the observed inhibition was due to macromolecular interaction of aggregates with the target protein (HMGB1). Using the same compounds at identical concentrations, the ability of GLA derivatives to affect the activity of glutathione S-transferase (GST) and alkaline phosphatase was evaluated. Under conditions where aggregate formation was favored the GLA derivatives were found to have no inhibitory effects on the enzymes tested. Hence, the ability of these agents to form aggregates does not result in enzyme inhibition. In contrast, GLA was found to activate GST enzymatic activity while the aggregate-forming GAK activated alkaline phosphatase but had no effect on GST. Taken together, these data suggest that the GLA derivatives tested herein may affect enzyme activity in a non-specific manner due to their detergent properties, but that aggregation alone is not sufficient to explain the effect of the compounds on the proteins studied. Based on these preliminary studies, promiscuous inhibition by these GLA derivatives does not appear to explain the inhibitory action observed on HMGB1-DNA interactions. Hence, GLA and GAK may find utility in the inhibition of macromolecular interactions with minimal effects on cellular enzymes. Experiments involving these compounds and additional model enzymes (e.g., chymotrypsin, β -lactamase, and β -galactosidase) may help to better understand their range of effects (inhibition, no effect, or increase in activity) on enzyme/protein activity.

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