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Research Article

Glucono-Delta-Lactone Inhibits Tissue Factor and Thrombin Activity during Human Blood Coagulation

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Abstract:
Many disorders, such as sepsis or trauma, may lead to an upregulation of the coagulation cascade. The resulting procoagulant state may lead to the formation of microvascular thrombi that can disturb organ microcirculation and promote the development of organ dysfunction (2). Glucono-delta-lactone (GDL) is a low toxicity substance that is found naturally in the human body. Previous studies have shown that GDL has an anticoagulant effect in blood (3). This research experiment focuses on testing GDL during procoagulant states of citrated whole blood, specifically during an upregulation of tissue factor and thrombin. This research showed that GDL administration may prove to be useful in prolonging clotting times during these instances, especially early and late in the upregulated coagulation cascade. It is possible that GDL functions by antagonizing a relevant receptor site of thrombin and of tissue factor. However, it is not known at this time by which mechanism GDL employs to oppose the effects of either procoagulants, thrombin or tissue factor.

Keywords: Glucono-delta-lactone (GDL), tissue factor (TF), thrombin, anticoagulant, procoagulants.

INTRODUCTION:

Many disorders, such as sepsis or trauma, may lead to the dysregulation of the coagulation cascade. This could result in an upregulation of clotting factors such as tissue factor or thrombin during the cascade. Furthermore, an upregulation of the coagulation cascade can lead to additional complications if left untreated.

Tissue factor is the initiator of the extrinsic coagulation pathway and is a key regulator of disseminated intravascular coagulation (2). During sepsis or tissue injury in trauma, activation of the extrinsic coagulation pathway upregulates coagulation and simultaneously depresses the inhibitory mechanisms of coagulation and suppresses the fibrinolytic system. The resulting procoagulant state may lead to the formation of microvascular thrombi that can disturb organ microcirculation and promote the development of organ dysfunction (2).

The formation of thrombin is one of the last steps of both the extrinsic and intrinsic coagulation pathways. During sepsis, thrombin is generated while also antagonized by antithrombin, resulting in low blood antithrombin levels in most patients. In addition to consumption, destruction of antithrombin by leukocyte proteases may also occur during sepsis (1). Without antithrombin, thrombin will convert fibrinogen to fibrin which covalently stabilizes the fibrin clot (4). Thrombin itself can exert positive feedback on its own pathway by activating factors V, VIII, and XI (2), increase vascular permeability which leads to tissue damage, and inhibit fibrinolysis via activating thrombin-activatable fibrinolysis inhibitor (TAFI) (4). As a result, thrombin can create a procoagulant state in the patient and lead to organ dysfunction. Currently, activated protein C is the only natural anticoagulant that has demonstrated direct activity in blocking thrombin formation, enhancing fibrinolysis, and diminishing the expression of inflammatory molecules. Glucono-delta-lactone (GDL) is a low toxicity substance that is found naturally in the human body, in many food and cosmetic products, and is on the FDA's "Generally Recognized As Safe" (GRAS) list. Previous studies have shown that GDL has an anticoagulant effect in blood (3). This research experiment focuses on testing GDL during procoagulant states of citrated whole blood, specifically during an upregulation of tissue factor and thrombin

METHODS:

Human citrated whole bloods (CWB) were obtained from University Hospital's clinical labs according to IRB protocol. The bloods were pooled into samples (n=10) of approximately 1.3 mL each. Samples were gently mixed andincubated for 15 minutes at 37^{o}C. Each sample was then divided into four aliquots of 300 uL. Each aliquot was added to cuvettes containing 32 uL of 0.1M $CaCl_2$ (to initiate clotting) and the blood concentrations listed below:

Experiment 1 (n=10)
1. 0.85% saline (Control)
2. 0.09 U/cc thrombin
3. 0.5 mg/mL GDL
4. 0.09 U/cc thrombin + 0.5 mg/mL GDL

Experiment 2 (n=10)
1. 0.85% saline (Control)
2. 0.09 U/cc thrombin
3. 1 mg/mL GDL
4. 0.09 U/cc thrombin + 1 mg/mL GDL

Experiment 3 (n=10)
1. 0.85% saline (Control)
2. 0.09 U/cc thrombin
3. 2 mg/mL GDL
4. 0.09 U/cc thrombin + 2 mg/mL GDL

Experiment 4 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor
3. 0.5 mg/mL GDL
4. 0.045% tissue factor + 0.5 mg/mL GDL

Experiment 5 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor
3. 1 mg/mL GDL
4. 0.045% tissue factor + 1 mg/mL GDL

Experiment 6 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor
3. 2 mg/mL GDL
4. 0.045% tissue factor + 2 mg/mL GDL

Experiment 7 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor
3. 0.09 U/cc thrombin
4. 0.045% tissue factor + 0.09 U/cc thrombin

Experiment 8 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor + 0.09 U/cc thrombin
3. 0.5 mg/mL GDL
4. 0.045% tissue factor + 0.09 U/cc thrombin + 0.5 mg/mL GDL

Experiment 9 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor + 0.09 U/cc thrombin
3. 1 mg/mL GDL
4. 0.045% tissue factor + 0.09 U/cc thrombin + 1 mg/mL GDL

Experiment 10 (n=10)
1. 0.85% saline (Control)
2. 0.045% tissue factor + 0.09 U/cc thrombin
3. 2 mg/mL GDL
4. 0.045% tissue factor + 0.09 U/cc thrombin + 2 mg/mL GDL

Experiments 8, 9, and 10 were repeated using plasma (acellular).

The samples were analyzed using the Sonoclot Coagulation Analyzer (Sienco, Wheat Ridge, CO, USA), a mini-viscometer that is sensitive to fibrin polymer formation and fibrinolysis (5). This instrument is currently FDA approved for evaluating human blood coagulation. Samples were evaluated for clotting time, the time required for liquid blood to become a thicker gel.

Clotting times were compared using a statistical program to perform paired t-tests and repeated measure analysis of variance (ANOVA). Significance was defined as test values with $p<0.05$.

RESULTS:

Experiments 1, 2, 3 – GDL's effect on thrombin during blood coagulation

Thrombin administered at doses of 0.09 U/cc final concentration significantly shortened the clotting times of all test groups.

The procoagulant effect of thrombin was reduced in blood samples treated with GDL. Two-tailed paired t-tests indicated a significant increase in clotting time between samples treated with thrombin versus thrombin with 0.5 mg/mL GDL ($p<0.0001$) (Figure A), 1 mg/mL GDL ($p<0.0001$) (Figure B), and 2 mg/mL GDL ($p<0.0001$) (Figure C), respectively.

Figure A: Clotting times of 0.09 U/cc thrombin and 0.5 mg/mL GDL treated whole bloods

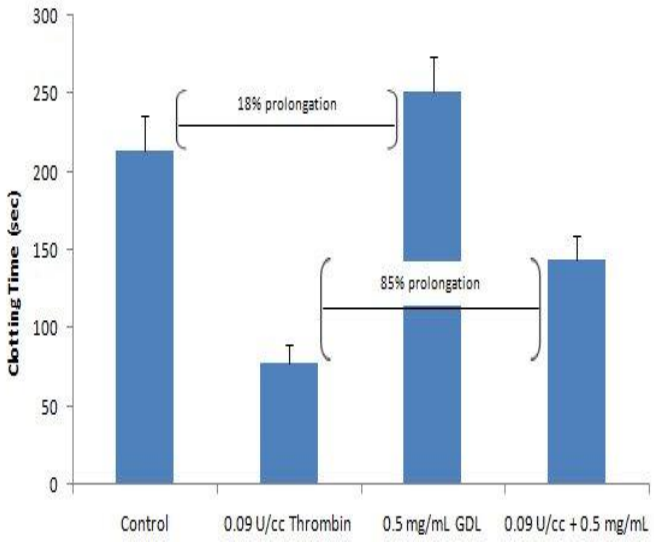


Figure B: Clotting times of 0.09 U/cc thrombin and 1 mg/mL GDL treated whole bloods

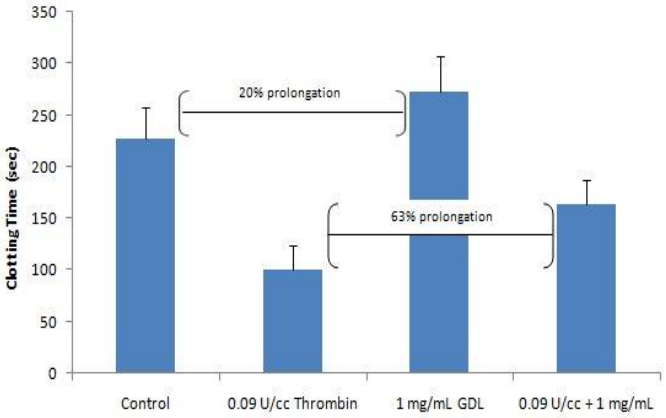
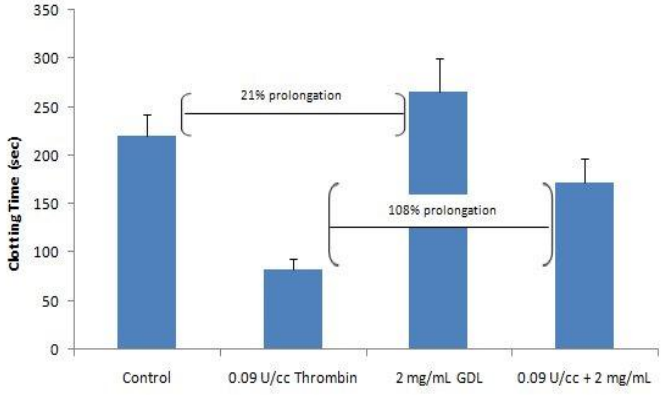


Figure C: Clotting times of 0.09 U/cc thrombin and 2 mg/mL GDL treated whole bloods



Experiments 4, 5, 6 – GDL's effect on tissue factor during blood coagulation

Tissue factor administered at doses of 0.045% final concentration significantly shortened the clotting times of all test groups.

The procoagulant effect of tissue factor was reduced in blood samples treated with GDL. Two-tailed paired t-tests indicated a significant increase in clotting time between samples treated with tissue factor versus tissue factor with 0.5 mg/mL GDL ($p<0.0001$) (Figure D), 1 mg/mL GDL ($p<0.0001$) (Figure E), and 2 mg/mL GDL ($p=0.0002$) (Figure F), respectively.

Figure D: Clotting times of 0.045% tissue factor and 0.5 mg/mL GDL treated whole bloods

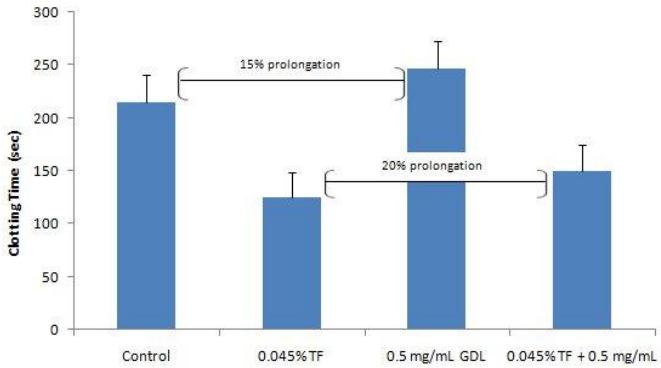


Figure E: Clotting times of 0.045% tissue factor and 1 mg/mL GDL treated whole bloods

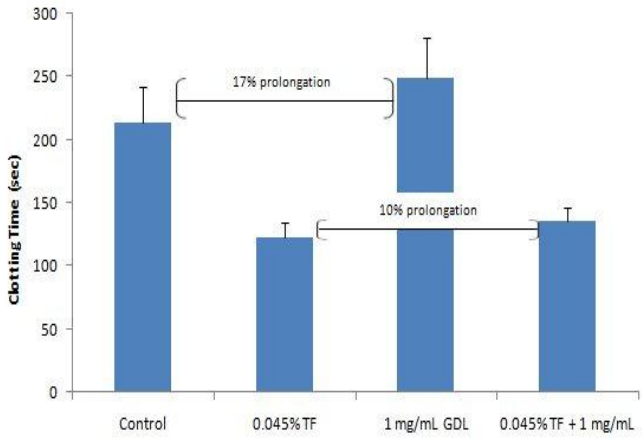
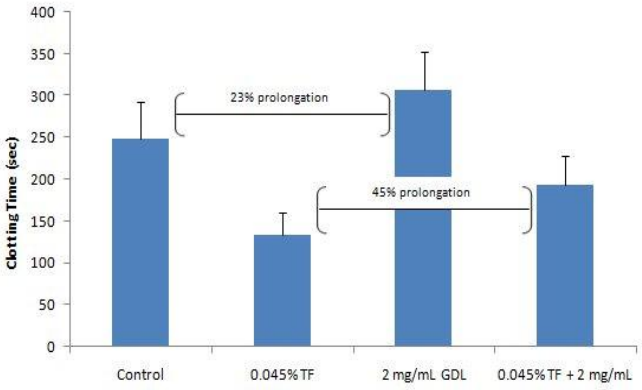


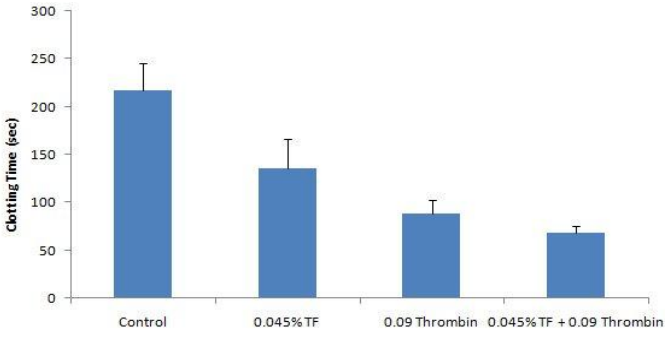
Figure F: Clotting times of 0.045% tissue factor and 2 mg/mL GDL treated whole bloods



Experiment 7 – Synergistic effect of tissue factor with thrombin

The procoagulant effects of tissue factor and thrombin were increased in blood samples containing both substances. Two-tailed paired t-tests indicated a significant decrease in clotting time between samples treated with tissue factor versus tissue factor and thrombin ($p<0.0001$) and samples treated with thrombin versus tissue factor and thrombin ($p=0.0011$) (Figure G).

Figure G: Clotting times of 0.045% tissue factor and 0.09 U/cc thrombin treated whole bloods



Experiments 8, 9, 10 – GDL's effect on tissue factor with thrombin during blood coagulation

Tissue factor administered at doses of 0.045% final concentration mixed with thrombin administered at doses of 0.09 U/cc final concentration significantly shortened the clotting times of all test groups.

The procoagulant effect of tissue factor with thrombin was reduced in blood samples treated with GDL.

Two-tailed paired t-tests indicated a significant increase in clotting time between samples treated with tissue factor and thrombin versus tissue factor, thrombin and 0.5 mg/mL GDL ($p<0.0001$) (Figure H), 1 mg/mL GDL ($p<0.0001$) (Figure I), and 2 mg/mL GDL ($p<0.0001$) (Figure J), respectively.

Figure H: Clotting times of 0.045% tissue factor, 0.09 U/cc thrombin, and 0.5 mg/mL GDL treated whole bloods.

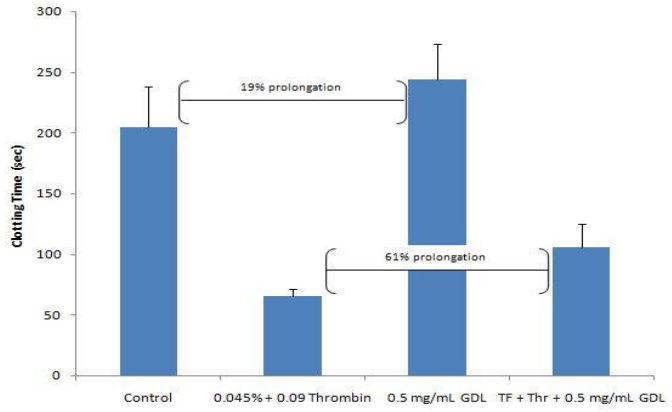


Figure I: Clotting times of 0.045% tissue factor, 0.09 U/cc thrombin, and 1 mg/mL GDL treated whole bloods

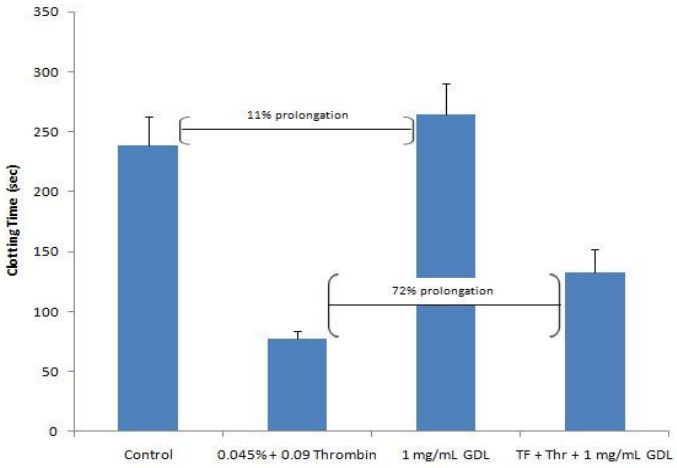
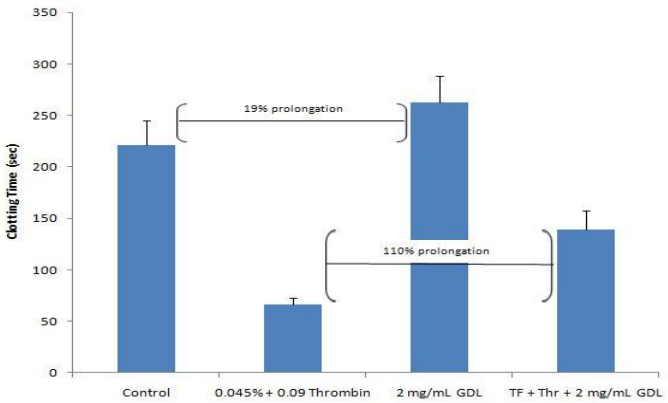


Figure J: Clotting times of 0.045% tissue factor, 0.09 U/cc thrombi 2 mg/mL GDL treated whole bloods



Experiments 8, 9, 10 in plasma – GDL's effect on tissue factor with thrombin in plasma

Tissue factor administered at doses of 0.045% final concentration mixed with thrombin administered at doses of 0.09 U/cc final concentration significantly shortened the clotting times of all test groups.

GDL only significantly prolonged the clotting time of plasma control samples when administered at doses of 2 mg/mL final concentration.

The procoagulant effect of tissue factor with thrombin was reduced in plasma samples treated with GDL. Two-tailed paired t-tests indicated a significant increase in clotting time between samples treated with tissue factor and thrombin versus tissue factor, thrombin and 0.5 mg/mL GDL ($p<0.0001$) (Figure K), 1 mg/mL GDL ($p<0.0001$) (Figure L), and 2 mg/mL GDL ($p<0.0001$) (Figure M), respectively.

Figure K: Clotting times of 0.045% tissue factor, 0.09 U/cc thrombin, and 0.5 mg/mL GDL treated plasma

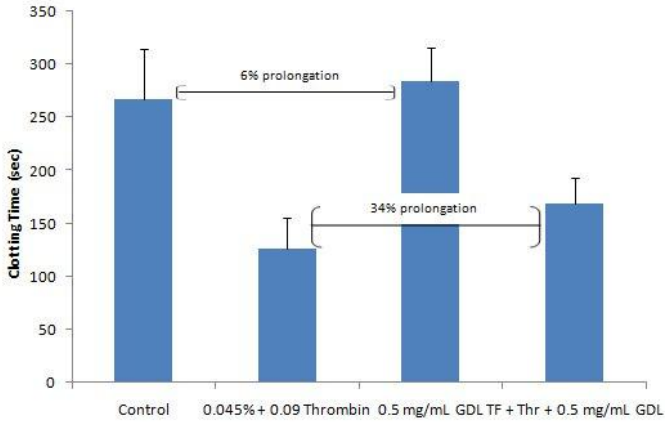


Figure L: Clotting times of 0.045% tissue factor, 0.09U/cc thrombin, and 1 mg/ml GDL treated plasma

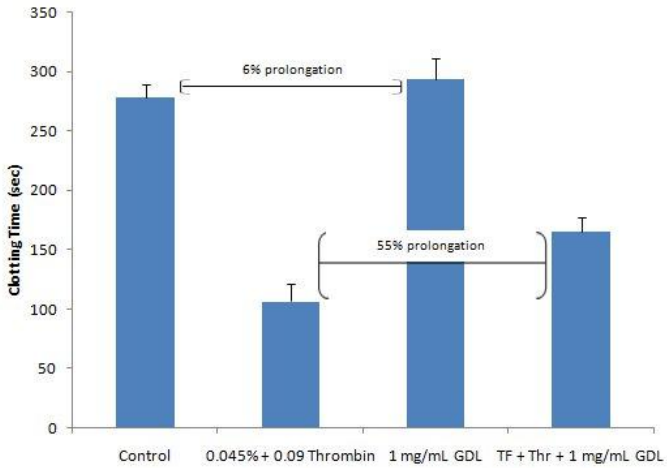
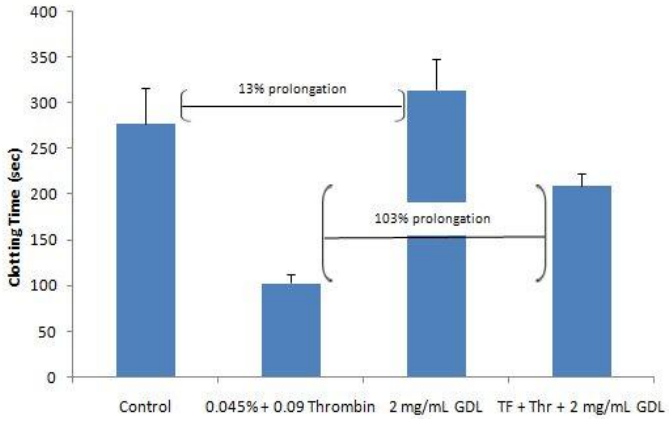


Figure M: Clotting times of 0.045% tissue factor, 0.09 U/cc thrombin, and 2 mg/mL GDL treated plasma



Discussion

The initiation of blood coagulation by tissue factor will lead to the formation of thrombin during the coagulation cascade. Thrombin (Table 1) and tissue factor (Table 2) significantly shortened the clotting time of whole blood when added, respectively. Tissue factor upregulates coagulation by initiating the extrinsic coagulation pathway. As a result, the introduction of tissue factor to citrated whole blood increased clot formation and significantly decreased clotting time. The conversion of prothrombin to thrombin is one of the final steps of the coagulation pathway. The addition of exogenous thrombin also upregulated coagulation and resulted in a significantly shortened clotting time. In reality, tissue factor and thrombin are likely to be present simultaneously in the blood during procoagulant states such as in sepsis or trauma. In this experiment, tissue factor with thrombin significantly decreased clotting time compared to adding either substance alone (Figure G). This indicates that both substances can contribute to shortening clotting time without one masking the effects of the other.

GDL was clearly capable of increasing the clotting time in blood samples treated with thrombin (Figures A, B, C) and tissue factor (Figures D, E, F), respectively. At the tested concentrations, GDL significantly increased the clotting times of both sample types. This suggests that GDL is effective during procoagulant states that involve an upregulation of tissue factor or thrombin.

GDL significantly increased the clotting time in samples with tissue factor and thrombin mixed together (Figures H, I, J). This implicates that GDL can antagonize different parts of the coagulation cascade simultaneously. In practice, anticoagulants may not always be administered to patients at the commencement of the coagulation cascade but instead may be administered when coagulation is already in progress. This research experiment showed that GDL administration may prove to be useful in prolonging clotting times during these instances, especially late in the coagulation cascade when prothrombin is converted to thrombin.

Furthermore, each concentration of GDL tested prolonged the clotting times in blood samples treated with thrombin more than that treated with tissue factor (Figures A, B, C versus Figures D, E, F, respectively). The addition of GDL to blood samples treated with both thrombin and tissue factor showed a prolongation increase similar to that found when GDL was added to blood samples treated with thrombin alone (Figures A, B, C versus Figures H, I, J, respectively). Thrombin enhances adhesion between tumor cells, platelets, endothelial cells, and the extracellular matrix by mobilizing adhesion molecules to the cell surface (4). It is possible that GDL functions by antagonizing a receptor site of thrombin more effectively than a receptor site of tissue factor. However, it is not known at this time exactly what method GDL uses to oppose the effects of either thrombin or tissue factor.

When experiments 8, 9, and 10 were repeated using plasma, GDL was able to significantly increase the clotting time in samples with tissue factor and thrombin mixed together (Figures K, L, and M). However, GDL was only able to significantly increase the clotting time of plasma control samples when administered at 2 mg/mL final concentration (Table 5). This gives some insight into GDL's mechanism of action since there are no red blood cells present in plasma. It may be possible that GDL can bind to tissue factor and thrombin, effectively inhibiting either from reaching its respective receptor. Again, it may also be possible that GDL functions by antagonizing a receptor site.

Tissue factor is the initiator of the blood clotting cascade. GDL inhibits the starting of this critical physiological event .In addition, GDL mitigates the detrimental procoagulant effect of thrombin. This agent reacts with fibrinogen in the latter stages of clotting, to generate fibrin, the undesirable clot. That GDL reveals these important properties may help in the production of more effective anticoagulant products.

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