Inhibition of Purified Factor Xa Amidolytic Activity May Not Be Predictive of Inhibition of In Vivo Thrombosis
Implications for Identification of Therapeutically Active Inhibitors

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Objective—In this study we test the hypothesis that blood/plasma-based prothrombinase assays, rather than inhibition of purified factor Xa (fXa), are predictive of in vivo antithrombotic activity.

Methods and Results—Six fXa inhibitors with equivalent nanomolar \( K_i \) were studied in thrombin generation assays using human plasma/blood and endogenous macromolecular substrate. In all assays, benzamidine inhibitors were more potent (100 to 800 nmol/L) than the aminoisoquinolines (5 to 58 \( \mu \)mol/L) or neutral inhibitors (3 to 10 \( \mu \)mol/L). A similar rank order of compound inhibition was also seen in purified prothrombinase assays as well as in a rabbit model of deep vein thrombosis.

Conclusions—Assays using prothrombinase with protein substrates are better predictors of in vivo efficacy than fXa \( K_i \) using amidolytic substrates. (Arterioscler Thromb Vasc Biol. 2003;23:1098-1104.)

Key Words: factor Xa ■ prothrombinase ■ coagulation inhibitors
NaCl, 0.1% PEG-8000, 5 mmol/L CaCl2; pH 7.4). S-2765 (N-α-Z-Arg-Gly-Arg-p Nitroanilide, Diapharma) at 200 μmol/L was added, and substrate hydrolysis was monitored at 405 nm. Data were analyzed by Batch Ki software (BioKin).

**k_{off} Determination**

Inhibitor (200 nmol/L) was preincubated with fXa (100 nmol/L) for 30 minutes. The complex was diluted in 1 mmol/L Spectrozyme FXa (MeO-OC-N-Chg-Gly-Arg-p Nitroanilide, American Diagnostica), and recovery of enzymatic activity was monitored at 405 nm. k_{off} was calculated as in the study by Betz et al.8

**k_{on} Determination**

Equal volumes of FXa (100 nmol/L) and substrate plus inhibitor (400 and 2 μmol/L) were added to each reservoir syringe of a stopped-flow reaction analyzer (Applied Photophysics). Change in absorbance at 405 nm was monitored after the mixing of syringe contents. The stopped-flow traces were analyzed as in the study by Betz et al.8

**Protein Binding**

The extent of rabbit plasma protein binding was determined by the ultra filtration method described in the study by Wright et al.9

**Blood Collection**

Blood from healthy volunteers (a minimum of 3 donors) was drawn and processed to produce a plasma pool as described in the study by Hemker et al.10 Plasma aliquots were stored at −80°C. The platelet-rich plasma (PRP), prepared at the time of the experiment, was the supernatant removed from a 900 g centrifugation of citrated whole blood.

**Thrombin Generation in Reconstituted Human Prothrombinase Complex**

Inhibition assays were carried out as reported.11 FXa was used at a concentration of 1 nmol/L, and after 10 minutes of incubation, the reaction was quenched by addition of soybean trypsin inhibitor (0.5 mg/mL, Sigma).

**Thrombin Generation in Human Plasma**

The protocol was adapted from the method used by Hemker et al.10 Reptilase-treated plasma pool was added to DMSO-solubilized FXa inhibitor in 96-well plates, followed by substrate Pefachrome TG (Pefa-5134, Pentapharm) at a final concentration of 500 μmol/L and CaCl2 at a final concentration of 16.7 mmol/L. Thrombin generation reactions were started by addition of 640 pmol/L TF (Innovin, Dade Behring), and absorbance was monitored at 405 nm at 37°C for 22 minutes.

**Thrombin Generation in Platelet-Rich Plasma**

The assay was modified from that reported by Hemker et al.12 PRP was treated with Pefabloc FG (Pefa-6003, Pentapharm) at 2.3 mg/mL at 37°C for 10 minutes in SigmaCoted (Sigma Aldrich) aggregometer tubes (Chronolog). Additions of 20 nmol/L CaCl2, 2 μmol/L TRAP (a thrombin receptor agonist peptide, Ser[pFPhe]-Har-Leu-Har-Lys-NH2), 500 μmol/L substrate Z-Gly-Gly-Arg AMC (Bachem), and inhibitor in DMSO at various concentrations were added to the tubes. Thrombin generation was triggered by addition of 0.8 pmol/L TF and was monitored for 25 minutes at 37°C using 360 nm excitation and 437 nm emission in a modified luminescence spectrometer (SLM-Aminco).

**Thrombin Generation in Whole Blood**

FXa inhibitor was added to blood within 2 minutes of blood collection. The blood was stirred on a Multi-magnet stir plate (Laboratory Line Instruments) at room temperature. Aliquots withdrawn at specific time points were treated with a quench solution.14 Enzyme immunoassay for the determination of human thrombin-antithrombin III complex (TAT) was done according to manufacturer’s instructions (Enzygnost TAT micro, Dade Behring). Quantitation of fibrinopeptide A (FPA) was by LC/MS/MS.

**Rabbit Deep Vein Thrombosis Model**

The experimental protocol has been described.15 Briefly, weight of a thrombus formed on cotton threads introduced in the abdominal vena cava of an anesthetized rabbit was measured with and without administration of inhibitor. In this study, a modification of the published protocol was used in that the thrombogenic device was introduced simultaneously with the initiation of drug administration rather than 30 minutes before drug infusion. Blood samples obtained during the experiment were monitored for inhibitor plasma concentration and coagulation parameters. Prothrombin times (PTs) were evaluated using Thromboplastin C Plus (Dade) on an automated coagulation timer (ACL 3000, Instrumentation Laboratory). Quantitation of plasma concentration of inhibitors was carried out by LC/MS/MS. Except where noted, 6 animals per group were used for each inhibitor tested and each group had its own control set of animals (n=6). Our protocol was approved by the Institutional Animal Care and Use Committee at Millennium Pharmaceutical Inc and was in compliance with United States Drug Association federal guidelines (Guide for the Care and Use of Laboratory Animals).

**Results**

**Inhibition of Purified Human FXa**

A series of FXa inhibitors from different chemical classes have been designed to improve their pharmacokinetic properties. The FXa inhibitory activity of the inhibitors is shown in Table 1. The compounds represent 3 chemical classes of inhibitors (P1 benzamidines, aminoisoquinolines and neutrals), and the FXa Ks are similar. FXa specificity is demonstrated by lack of inhibition against thrombin. Additionally, all compounds have similar values for k_{on} and k_{off}. Extent of rabbit plasma protein binding is similar for the benzamidines and higher for the aminoisoquinolines and neutrals.
properties. We have selected 6 of these inhibitors for our comparative study.\textsuperscript{16,17,18} C2092-28 and C1942-36 belong to the P1 benzamidine series, C2072-24 and C1924-81 are in the class of P1 aminoisoquinolines, and C2112-42 and C2104-75 contain neutral moieties in both the P1 and P4 position. As shown in Figure 1, all of the chosen compounds were capable of inhibition of fXa at low nanomolar concentrations and, when assayed in the same concentration range, did not cross-react with the related serine protease thrombin. Representative inhibitors from the 3 classes (C2092-28, C1942-36, C1924-81, and C2104-75) were also evaluated for inhibitory activity against amidolytic substrate cleavage by fVIIa/TF and by fIXa. For each of the 4 compounds assayed to a concentration of 1 \( \mu \text{mol/L} \), no inhibitory activity was detected against either fVIIa or fIXa (data not shown). Stopped-flow measurements of \( k_{\text{on}} \) and \( k_{\text{off}} \) showed that the compounds share similar modes of inhibition against tripeptide substrate cleavage by solution phase fXa. Compared with previously reported transition state analog peptide inhibitors of fXa, all 6 compounds would be classified as reversible inhibitors.\textsuperscript{3,8} Because our in vivo and some of our in vitro evaluations involve the presence of blood components, we measured the extent of plasma protein binding by these agents and found protein binding for the 2 benzamidines was significantly lower than that for the neutral and aminoisoquinoline inhibitors (Figure 1). To investigate the effect of variable protein binding capacity on the potency of compounds, \( K_i \) determinations for fXa inhibition were carried out in buffer A containing 1 mg/mL BSA. The observed \( K_i \) for C2104-75 (a 96% protein-bound inhibitor) as well as those for C2092-28 and C1942-36 (80% to 83% protein-bound inhibitor) did not change in the presence of added protein in the assay media (data not shown).

Figure 2. Tissue factor–mediated thrombin generation in human plasma. Each of the 4 panels depicts a dose-response curve for inhibition of the rate of thrombin generation (dA/dt) over the time course of experiment. Each inhibitor was independently tested 4 to 6 times. Representative curves are shown here. The range of concentration evaluated for each inhibitor is as follows: C1942-36 (8 concentrations between 0 and 20 \( \mu \text{mol/L} \), bolded at 10 \( \mu \text{mol/L} \)); C2092-28 (8 concentrations between 0 and 20 \( \mu \text{mol/L} \), bolded at 10 \( \mu \text{mol/L} \)); C2104-75 (8 concentrations between 0 and 50 \( \mu \text{mol/L} \), bolded at 10 \( \mu \text{mol/L} \)); and C1924-81 (8 concentrations between 0 and 100 \( \mu \text{mol/L} \), bolded at 10 \( \mu \text{mol/L} \)). The thrombin lag 2X extension is calculated by determining the concentration of inhibitor required to extend the time of peak thrombin generation by 2-fold.

Tissue Factor–Mediated Thrombin Generation in Human Plasma

We decided to use a human plasma-based system to compare the relative potency of fXa inhibitors during cleavage of the physiological substrate prothrombin. The time course of thrombin generation was measured by following the kinetics of cleavage of a specific paranitroanilide substrate. The derivative of the optical density measurement is proportional to the sum of activities derived from free thrombin and thrombin-\( \alpha \text{2} \) macroglobulin complex. Figure 2 shows the concentration-dependent extension of lag of TF-mediated thrombin generation. The bolded rates of thrombin generation correspond to approximately equivalent amount (10 to 12.5 \( \mu \text{mol/L} \)) of each of the fXa inhibitors. The concentration required for 2-fold extension of time required for peak thrombin generation can be used for a relative ranking of the 6 compounds. As shown in Table 1, the superior potency observed for C1942-36 and C2092-28 translates into much lower effective inhibitory concentrations (400 and 800 \( \text{nmol/L} \), respectively) than those for C2104-75 and C1924-81 (5 and 47 \( \mu \text{mol/L} \), respectively). In general, the potency of the P1 benzamidines was at least 7-fold superior to the other 2 classes of compounds.

Thrombin Generation in Platelet-Rich Plasma

The interactions between activating/aggregating platelets and components of the coagulation cascade are of importance during thrombotic events. Because an active site-directed fXa inhibitor should be capable of inhibition of prothrombinase complexes on the surface of activated platelets, we decided to use thrombin generation in PRP to rank the inhibitors from different classes. Two P1 benzamidines, 1 P1 aminoisoquinoline, and 1 P1 neutral inhibitor were used for this purpose. The continuous determination of thrombin generation and the first derivative (rate of generation) for selected inhibitor
concentrations are shown in Figure 3. All 4 compounds tested were inhibitory in a dose-responsive manner but with greatly differing potencies. The 2 P1 benzamidines show a 50% decrease of fluorescence signal from the averaged control at approximately 100 nmol/L. The P1 neutral inhibitor (C2104-75) can achieve comparable inhibition but at a 5 times higher concentration. The P1 aminoisoquinoline fared the worst and needed greater than 50 times the amount for half-maximal inhibition. The fluorescence titration data were used to calculate the amount of inhibitor required for half-maximal inhibition of area under the curve (Table 1).

**Thrombin Generation in Whole Blood**

To investigate inhibitory capacity of the inhibitors in the presence of all the components of whole blood, we monitored 2 markers of thrombin generation in freshly drawn, nonanticoagulated blood. Unlike the 2 previously described systems, the initiation of clotting was not carried out by addition of exogenous TF but by activation of platelets by gentle magnetic stirring. This experimental protocol produces micromolar amounts of FPA, which was subsequently degraded during the 60-minute observation period (Figure 4). TAT, the other marker of thrombin generation, attained peak levels after a slightly longer lag period and remained relatively stable during sampling. Figure 4 shows a representative experiment comparing 2 P1 benzamidines, 1 P1 neutral and 1 P1 aminoisoquinoline. To achieve comparable potency of inhibition, the agents had to be evaluated at varying doses with C1942-36 and C2092-28 tested between the concentrations of 200 nmol/L and 1 μmol/L and C2104-75 tested at 10 μmol/L. However C1924-81 did not attain similar inhibitory activity at the highest dose tested (20 μmol/L) (Figure 4 and Table 1).

### Table 1. Correlation of In Vitro and In Vivo Parameters

<table>
<thead>
<tr>
<th>Compound</th>
<th>FXa Ki, nmol/L</th>
<th>Thrombin Lag 2X Extension, nmol/L</th>
<th>Thrombin Generation in PRP IC50, nmol/L</th>
<th>Whole Blood Thrombin Generation</th>
<th>In Vivo Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2092–28</td>
<td>1.8</td>
<td>800</td>
<td>214</td>
<td>Inhibitory at 500 nmol/L</td>
<td>Inhibitory at 0.8 μmol/L*</td>
</tr>
<tr>
<td>C1942–36</td>
<td>1.1</td>
<td>400</td>
<td>106</td>
<td>Inhibitory at 200 nmol/L</td>
<td>Inhibitory at 0.2 μmol/L*</td>
</tr>
<tr>
<td>C2072–24</td>
<td>1.3</td>
<td>5000</td>
<td>...</td>
<td>...</td>
<td>No inhibition at 0.2 μmol/L and higher†</td>
</tr>
<tr>
<td>C1924–81</td>
<td>1.2</td>
<td>47 000</td>
<td>58 000</td>
<td>Partial inhibition at 20 μmol/L</td>
<td>No inhibition at 0.7 μmol/L.</td>
</tr>
<tr>
<td>C2112–42</td>
<td>1.4</td>
<td>2800</td>
<td>...</td>
<td>...</td>
<td>No inhibition at 1.3 μmol/L.</td>
</tr>
<tr>
<td>C2104–75</td>
<td>1.4</td>
<td>5000</td>
<td>3100</td>
<td>Inhibitory at 10 μmol/L</td>
<td>No inhibition at 7 μmol/L.</td>
</tr>
</tbody>
</table>

All six compounds studied have similar Ki values to FXa. Prothrombinase inhibition in plasma, expressed as an extension in time to maximum thrombin generation, was 5- to 10-fold better with benzamidines than with the other classes of compounds. Thrombin generation in PRP was inhibited by the benzamidines at nanomolar concentrations, but micromolar amounts were required by the aminoisoquinoline or the neutral compound tested. Whole blood assays showed inhibition of thrombin generation markers that required concentrations of inhibitor 10 to 20 times that of the aminoisoquinoline or neutral over the benzamidines. Thrombin inhibition in an in vivo model of thrombosis was only demonstrated by the benzamidines.

*Significant inhibition of thrombosis compared with vehicle controls (P<0.05, Student’s t test).
†C2072–24 was tested at two doses, 0.8 and 2.4 mg/kg. Technical difficulties with LC/MS/MS protocol prevented us from accurately determining rabbit plasma concentrations at the higher dose.

**Thrombin Generation in Reconstituted Prothrombinase Complex**

Data for inhibition of thrombin generated by a purified, reconstituted human prothrombinase complex is shown in Table 2. The rank order of inhibition by the studied compounds is similar to that seen in the 3 human plasma/blood assays.

**Evaluation in Animal Model of Thrombosis**

To test the 6 inhibitors in an in vivo setting, we modified a model of venous thrombosis previously used for the positive evaluation of specific inhibitors of fXa, fIXa, and thrombin. In the original model, enoxaparin at a plasma concentration of 4 U/mL completely inhibited thrombus growth. The same dose (200 U/kg plus 2 U/kg per min) attains a lower level of inhibition (50% to 55%) in the

**Figure 3.** Tissue factor-mediated thrombin generation in human PRP. A representative control (n=5) profile for thrombin activity in PRP followed by profiles for each chemical class of inhibitor at 10, 100, or 10 000 nmol/L is shown. The bolded line depicts thrombin activity over time as measured by fluorescent peptide substrate cleavage. The change in relative fluorescence units over time (the derivative) is also shown. The area under the curve was calculated from the derivative of thrombin generation.
TABLE 2. Inhibition of Prothrombinase Activity in a Reconstituted System

<table>
<thead>
<tr>
<th>Concentration (nmol/L)</th>
<th>C1942–36</th>
<th>C2092–38</th>
<th>C1942–75</th>
<th>C1921–81</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>53.9±2.5</td>
<td>35.2±9.4</td>
<td>16.6±11.0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>91.4±0.8</td>
<td>85.4±2.7</td>
<td>41.0±11.6</td>
<td>19.3±9.6</td>
</tr>
<tr>
<td>1000</td>
<td>98.6±0.4</td>
<td>97.5±2.0</td>
<td>87.6±0.7</td>
<td>65.0±4.0</td>
</tr>
</tbody>
</table>

Cleavage of prothrombin 2 by the reconstituted human FXa/va complex on phospholipid membranes is used as a measure of prothrombinase activity. Results are shown as average ± SEM (n = 4).

TABLE 3. Evaluation in a Rabbit Model of Venous Thrombosis

<table>
<thead>
<tr>
<th>Total Dose of C2104–75 (mg/kg)</th>
<th>No. of Animals</th>
<th>Inhibition of Thrombosis, %</th>
<th>Plasma Concentration, μmol/L</th>
<th>PT, fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.914</td>
<td>7</td>
<td>9†</td>
<td>0.98</td>
<td>1.07</td>
</tr>
<tr>
<td>2.338</td>
<td>3</td>
<td>19†</td>
<td>3.2</td>
<td>1.20*</td>
</tr>
<tr>
<td>5.076</td>
<td>3</td>
<td>0†</td>
<td>7</td>
<td>1.25*</td>
</tr>
</tbody>
</table>

Efficacy results for an escalation in administration of C2104–75 are shown. Increased dosing of compound gave a plasma level that reached 7 μmol/L. Despite the change in ex vivo clotting parameters, there was no inhibition of thrombus weight.

†Statistically increased compared with vehicle control group (P<0.05).

Generation of thrombin is an integral part of platelet activation and fibrinogen cleavage at the site of vascular injury. Thrombin is the product of a cascade of proteolytic activity that is highly regulated by enzymatic complex formation. FXa, the enzymatic component of the prothrombinase complex, is capable of generating thrombin as an independent enzyme and also as a part of the FVa-bound complex. The complex-bound enzyme is 300 000-fold more efficient in prothrombin cleavage, so a potential drug candidate has to inhibit both solution-phase FXa and the prothrombinase complex. Ideally it would be advantageous to screen several natural products and synthetic inhibitors against the real target, ie, prothrombinase assembled on activated platelets. The technical complexity of setting up robust screening assays that incorporate human cells has prevented this from being the protocol of choice. Even when the assays are set up using phospholipid membranes, a system that involves a membrane-bound enzymatic complex cleaving a membrane-bound substrate is poorly amenable to generating kinetic parameters in a screening assay. We have measured the inhibitory activity of 4 of the inhibitors studied here in a reconstituted purified prothrombinase assay (Table 2). The results support our assertion that we are measuring prothrombinase activity in our plasma/whole blood assays because the compounds inhibit in a similar rank order.

The in vitro protein binding values in Figure 1 were used to calculate free inhibitor concentrations, and the results were compared among the 3 chemical classes of inhibitors. The
differences in efficacy among the inhibitors tested cannot be explained solely on protein binding, because several measured inhibitor concentrations vastly exceed their putative therapeutic levels.

There are several compromises made in setting up the ex vivo prothrombinase assays investigated in this report. The TF used in both TF-mediated assays (plasma and PRP) is a recombinant purified protein produced in \textit{Escherichia coli}. The recombinant TF is truncated and is artificially lipidated by being combined with synthetic phospholipids. It is the synthetic lipid that most likely accounts for the concentration requirement for added TF being dependent on the presence or absence of platelets. In the absence of platelets (Figure 2), 640 pmol/L TF is used to initiate thrombin generation in reptilase-treated plasma; in the PRP assay (Figure 3), 0.8 pmol/L TF is sufficient for detecting thrombin activity. In addition, in the PRP assay, effects of TF and Pefabloc FG are related to the aggregometer tube being siliconized (Sigma-coted). When comparing the onset of detectable thrombin levels in nonsiliconized versus siliconized tubes in the absence of TF, a greatly increased lag time is noted in a sigmacoted tube. Tubes that have not been siliconized are most likely triggering thrombin generation by TF as well as by the contact pathway initiated on the surface of the tube. In siliconized tubes, the effect of varying concentrations of tissue factor led to dose-dependent changes in thrombin activity, whereas the relationship was variable in noncoated tubes. In addition, for assays carried out in coated tubes, omission of TRAP significantly dampens the rate of thrombin generation. The combination of TRAP and magnetic stirring assures that reproducible platelet aggregation takes place in platelet preparations from different donors. In the thrombin generation assays described here, the clotting of fibrinogen can interfere with detection of signal. In the plasma assay (Figure 2), reptilase treatment is used to remove most of the fibrinogen and thereby prevent clot formation that can interfere with detection of paranitroanilide cleavage. Because clot-bound thrombin is important in propagating the clotting cascade and subsequent platelet activation, this feedback mechanism is completely ignored in the plasma assay. In the PRP assay, the agent Pefabloc FG and stirring are required to prevent clotting, because this leads to irregular fluorescent tracings. When following thrombin generation markers in whole blood, stirring at a consistent rate is required to enable reproducible sampling over the time course of experimentations. Both the PRP and whole-blood assay (Figures 3 and 4, Table 1) are sensitive to rates of stirring, and reproducibility of stirring had to be ensured between different experiments.

In the series of prothrombinase inhibitory assays summarized in Table 1, it is clear that higher concentrations of inhibitor are required for inhibition of thrombin generation than is needed for inhibition of \( \text{fXa} \) cleavage of tripeptide substrates. This is true for all 3 classes of inhibitors examined in this study. In the plasma assay, the issue of competing with 1.4 \( \mu \text{mol/L} \) prothrombin (thezymogen concentration in plasma) may be a contributing factor to the reduced potency across different chemical classes. We have observed that for P1 benzamidines, which are relatively potent in the presence of prothrombin (0.4 \( \mu \text{mol/L} \) in the plasma assay and 0.1 \( \mu \text{mol/L} \) in the PRP assay), the concentration of inhibitor required for half-maximal inhibition is at least 100-fold greater than the requirements for inhibition of tripeptide substrate cleavage.

Krishnaswamy and Betz\cite{1} have reported that the recognition of prothrombin results from an initial interaction at an exosite that is distinct from the protease active site. P-amino benzamide, an active site inhibitor, behaves as a competitive inhibitor of cleavage of peptide paranitroanilide substrate by \( \text{fXa} \). When the same inhibitor is evaluated for its capacity to inhibit prothrombin 2 cleavage by prothrombinase, the kinetics of inhibition follow those of a classical noncompetitive inhibitor. Betz and Krishnaswamy have also shown that inhibition of macromolecular substrate cleavage can be achieved by interfering with exosite binding without blocking the enzyme active site.\cite{2} Bothrojaracin, a snake venom protein that binds to the proexosite I on prothrombin, can inhibit prothrombin activation by prothrombinase complexes assembled on platelet membranes.\cite{3} The inhibitory effect of Bothrojaracin can only be detected when the assays are carried out using the prothrombinase complex and not \( \text{fXa} \) alone. The results provide additional proof that exosites are involved in productive recognition of prothrombin as substrate. It is possible that the alterations in \( \text{fXa} \) conformation and activity, which stem from its cofactor binding and incorporation into prothrombinase, have an effect on the inhibition profile of the active site-directed inhibitors being evaluated for drug development. When newly synthesized compounds are screened against purified \( \text{fXa} \), the emerging SAR of a particular series is often compared with that of other chemically divergent series. It is important to remember that SAR identified by inhibitory activity against solution phase \( \text{fXa} \) may be significantly different from the SAR for prothrombinase complex inhibition. This in turn would yield varied correlation with whole blood assays and animal models. Thus, for drug development projects, it may be prudent to consider \( \text{fXa} \) inhibitory potency as a screening criteria and to use inhibition of prothrombinase as a more predictive tool for identification of candidates for additional evaluation.

References


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