

## The antiangiogenic agent Neovastat (Æ-941) stimulates tissue plasminogen activator activity

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**Key words:** Neovastat, Æ-941, angiogenesis, t-PA, endothelial cells

### Summary

The plasminogen activator/plasmin system represents a key component of the proteolytic machinery underlying angiogenesis. In this work, we investigated the effect of Neovastat (Æ-941), a naturally occurring multifunctional antiangiogenic agent that is currently in Phase III clinical trials, on tissue and urokinase plasminogen activator activities. We found that *in vitro*, Neovastat at 100 µg/ml markedly stimulates t-PA-mediated plasmin generation, while it slightly inhibits the generation of plasmin mediated by uPA. The stimulatory effect of Neovastat on t-PA activity was markedly increased by a heat treatment, resulting in a 15-fold increase in the rate of activation of plasminogen. Neovastat did not directly stimulate the activity of t-PA or plasmin towards exogenous substrates, suggesting that its effect requires the presence of plasminogen. Accordingly, kinetic analysis showed that Neovastat increases both the  $k_{cat}$  of t-PA as well as its affinity for plasminogen by 10-fold. The stimulation of t-PA activity by Neovastat was also correlated with a direct interaction of Neovastat with plasminogen as monitored by the surface plasmon resonance technology. Overall, these results identify Neovastat as a potent stimulator of t-PA-dependent activation of plasminogen, further emphasizing its pleiotropic mechanism of action on several molecular events involved in angiogenesis.

**Abbreviations:** MMP, matrix metalloproteinase; t-PA, tissue plasminogen activator; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor

### Introduction

It is well established that tumor-induced neovascularization, angiogenesis, is an absolute requirement for the growth of tumors and of their metastases [1]. During angiogenesis, endothelial cells undergo stimulation by tumor-derived cytokines, such as vascular endothelial growth factor (VEGF), leading to increased proliferation, extracellular matrix breakdown and formation of new blood vessels near the tumor cells [2]. Numerous studies have provided evidence that proteolytic degradation of the extracellular

matrix (ECM) by both matrix metalloproteinases (MMPs) and plasminogen activators (PA)/plasmin systems represent a key event in angiogenesis [3].

The PA/plasmin system comprises two major types of PA, tissue PA (t-PA) and urokinase PA (uPA) that both specifically convert circulating plasminogen to the active proteinase plasmin by cleavage of the Arg<sup>561</sup>–Val<sup>562</sup> peptide bond [4]. Plasmin is a trypsin-like protease with broad specificity that is capable of degrading most components of the ECM either directly or through activation of MMPs or elastases [5]. While uPA and t-PA share a common

substrate, there is evidence that the physiological roles of the two proteins are distinct. The binding of uPA to its cell-surface receptor (uPAR) is involved in pericellular proteolysis and is associated with cell locomotion [5,6]. Interference with the binding of uPA to its receptor results in an inhibition of tumor angiogenesis [7] thus suggesting that uPA/uPAR interaction plays an important role in neovascularization. In contrast, the role of t-PA in angiogenesis remains unclear. t-PA is mainly synthesized in endothelial cells where it is stored in Weibel–Palade bodies and released following stimulation of the cells by several stimuli [8]. The released t-PA is a key enzyme in fibrinolysis due to its ability to significantly increase the cleavage of fibrin-bound plasminogen into plasmin, leading to fibrin degradation [4]. How this fibrinolytic activity contributes to angiogenesis remains unknown.

In addition to their role in the promotion of angiogenesis, there is considerable evidence that some components of the PA/plasmin system may inhibit angiogenesis. Recently, plasmin has been suggested to be involved in the generation of anti-angiogenic proteins, such as angiostatin [9], and therefore to play an additional role in the angiogenesis cascade. Although the role of PA in this process remains unclear, recent evidence suggests that the ability of several tumor cell lines to generate angiostatin is correlated with their content of either uPA or t-PA [10]. Moreover, high t-PA levels correlate with good prognosis in various tumors [11,12] whereas lower t-PA levels have been associated with malignant tumors [13]. While such a positive role of t-PA in tumor progression remains unexplained, it has been proposed that the overstimulation of plasmin generation by t-PA may induce the degradation of the pro-angiogenic fibrin matrix, resulting in the inhibition of angiogenesis [14].

Neovastat is a naturally occurring inhibitor of angiogenesis derived from marine cartilage (dogfish) [15]. Based on results from Phase II clinical trials in non-small cell lung cancer and in renal cell carcinoma [16,17], this compound is currently undergoing Phase III clinical trials for the treatment of refractory renal cell carcinoma and non-resectable non-small cell lung cancer and is also tested in a Phase II pivotal clinical trial for the treatment of recurrent multiple myeloma [17]. There is now considerable evidence that the clinical benefits observed upon Neovastat treatment rely on the presence of multiple angiogenesis inhibitors, including inhibitors of MMP activities

[18] and of VEGF-mediated signaling events [19], as well as to the presence of an endothelial-specific proapoptotic activity [20]. These antiangiogenic activities are likely to be responsible for the antitumor and antimetastatic properties of Neovastat observed in *in vivo* models [21].

In this work, we investigated the effects of Neovastat on plasminogen activator activities. We found that *in vitro*, Neovastat markedly stimulates t-PA activity and that this stimulation is likely due to a direct interaction of the drug with plasminogen, resulting in an increased catalytic efficiency of t-PA-mediated plasmin generation.

## Material and methods

### Materials

Neovastat<sup>TM</sup> (Æ-941) was obtained from Æterna Laboratories (Québec City, QC, Canada) [22]. Human recombinant single-chain t-PA and plasminogen activator inhibitor (PAI)-1 were from Calbiochem. Plasminogen, uPA, the plasmin substrate Chromozym<sup>®</sup>PL (tosyl-glycyl-prolyl-lysine-4-nitrilide acetate) and the t-PA substrate Chromozym<sup>®</sup>t-PA (*N*-methylsulfonyl-D-phenylalanyl-glycyl-arginine-4-nitrilide acetate) were from Roche Biochemicals (Laval, QC, Canada). Plasmin, thrombin,  $\epsilon$ -amino-*n*-caproic acid and other biochemical reagents were from Sigma (St Louis, MO). Sensor chips (CM5) were from BIAcore (Piscataway, NJ).

### Plasminogen activation assay

The kinetics of t-PA- and uPA-mediated plasminogen activation was determined by measuring amidolytic activity of the plasmin generated by activation of plasminogen. The reaction was performed in a final volume of 200  $\mu$ l in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 50 mM CaCl<sub>2</sub>. Plasmin generation was monitored at 405 nm for 1 h using a Molecular Devices microplate reader (SpectraMax Plus). Under these conditions, if a constant rate of plasminogen activation is assumed, the concentration of plasmin increases linearly with time and that of *p*-nitroaniline follows a parabolic curve [23]. Initial rates of plasmin generation were calculated using linear regression analysis of plots of  $A_{405\text{ nm}}$  versus time<sup>2</sup>, as described [23].

For the measurement of plasmin activity, similar conditions were used except that t-PA and plasminogen were omitted from the buffer and the reaction was started by the addition of plasmin. t-PA amidolytic activity was measured under these conditions using Chromozym<sup>®</sup>t-PA as the t-PA substrate.

#### *Kinetic analysis*

The steady-state rate of plasminogen activation that is, the t-PA turnover number ( $k$ ), was calculated at different plasminogen concentrations using values of initial rates of plasmin generation ( $A_{405\text{ nm}}/\text{min}^2$ ) and the equation:

$$k = A_{405\text{ nm}}/0.5\Delta\varepsilon_{405} k_1[\text{t-PA}]t^2 \quad (1)$$

where  $\Delta\varepsilon_{405} = 10,500$ ,  $[\text{t-PA}] = 1\text{ nm}$  and  $k_1$ , the plasmin turnover number  $= 8.5\text{ s}^{-1}$  [24]. Nonlinear regression analysis of the data was performed using the Kaleidagraph 3.5 software (Synergy software, Reading, PA).

#### *Preactivation of Neovastat*

Preactivation of Neovastat was performed by standard procedures [25]. Briefly, for acid or alkali treatments, Neovastat was incubated for 30 min at 37 °C in either 0.1 N HCl or 0.1 N NaOH in sealed glass tubes then drug-mixtures were neutralized. Heat activation was carried out by incubating Neovastat for 30 min at either 60 or 95 °C and the samples were allowed to cool down at room temperature prior to the assay. Neovastat was also incubated for 30 min in either 6 M guanidine-HCl or 100 mM dithiothreitol (DTT), followed by dialysis against several changes of buffer (20 mM Tris-HCl, pH 7.4). Neovastat was also incubated in the presence of 0.1% sodium dodecyl sulfate (SDS) or treated with 2.5  $\mu\text{g}/\text{ml}$  plasmin or thrombin for 30 min at room temperature and enzyme activities were terminated with 1 mM phenylmethylsulfonyl fluoride (PMSF). Neovastat incubated without enzymes but with 1 mM PMSF was used as the control. The mixtures were dialyzed overnight against 20 mM Tris-HCl pH 7.4 and tested for their capacity to stimulate plasmin generation.

#### *BIAcore analysis*

Real time protein–protein interactions were examined on a BIAcore instrument (BIAcore X). t-PA or plasminogen were immobilized on different flow cells of

a CM5 sensor chip using an amine-coupling kit (Amersham Biosciences). Briefly, the chip surface was first activated by injection of 35  $\mu\text{l}$  of 1:1 mixture of 0.4 M *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide hydrochloride and 0.1 M *N*-hydroxysuccinimide. t-PA and plasminogen (50  $\mu\text{g}/\text{ml}$ ) in 20 mM acetate buffer pH 4.0 and 5.5, respectively, were immobilized on one flow cell. An additional flow cell was prepared as a blank background by activating it under the same buffer conditions. Remaining activated groups on each flow cell were blocked by injection of 35  $\mu\text{l}$  of 1 M ethanolamine-HCl, pH 8.5. Using these coupling conditions, about 6000–8000 RU of either t-PA or plasminogen were immobilized on the sensor chip surface. Finally, the system was primed with the running buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM  $\text{CaCl}_2$ ). Fifty microliters of Neovastat proteins diluted in running buffer were injected at a flow rate of 10  $\mu\text{l}/\text{min}$  and the interaction with the immobilized proteins was monitored over a 10–15 min period.

## **Results**

#### *Stimulation of t-PA-dependent conversion of plasminogen to plasmin by Neovastat*

Neovastat is a natural, multifunctional antiangiogenic agent produced from shark cartilage extracts. In the course of studies aimed at the identification of activities that may contribute to its antiangiogenic properties, we tested the effect of the drug on both the tissue- and urokinase-PA/plasmin systems. As shown in Figure 1, addition of Neovastat at 100  $\mu\text{g}/\text{ml}$  in the incubation medium increases the generation of plasmin mediated by t-PA by 4.3-fold (Figure 1). This effect was only observed when both t-PA and plasminogen were present in the reaction mixture and therefore was not related to the presence of endogenous t-PA or plasminogen within the drug. This stimulatory effect was specific for t-PA-dependent plasmin generation since Neovastat had no stimulatory effect on uPA-dependent plasmin generation and was even inhibitory at higher concentrations (data not shown).

We then examined the possibility that Neovastat might directly affect either the t-PA or the plasmin activities by monitoring the effects of the drug on the activities of these enzymes toward synthetic substrates. As shown in Table 1, Neovastat had negligible effects on both t-PA and plasmin amidolytic activities,

as recently reported [18]. In the presence of t-PA and plasminogen, the increase in plasmin generation induced by Neovastat was abolished by low concentrations (50 nM) of the lysine analog  $\epsilon$ -amino-*n*-caproic

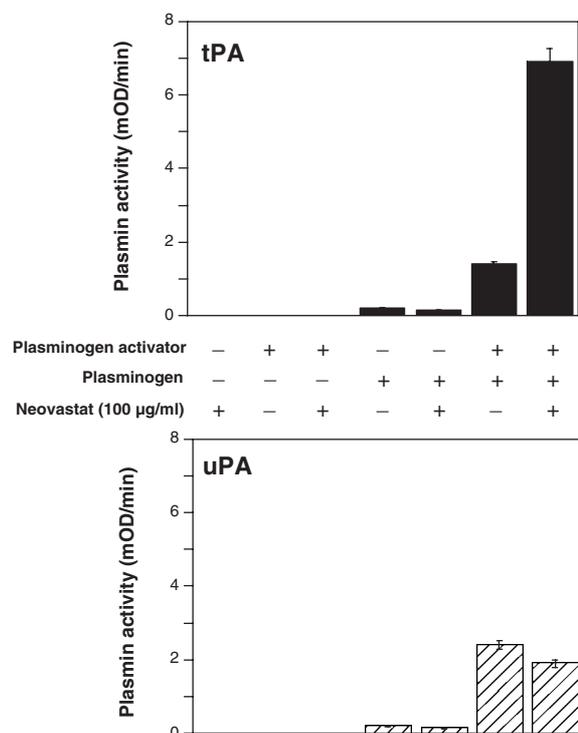


Figure 1. Neovastat stimulates the t-PA-dependent activation of plasminogen. Plasminogen activation by t-PA (upper panel) or uPA (lower panel) was monitored by measuring amidolytic activity of the plasmin generated by activation of plasminogen in the absence or in the presence of 100 µg/ml of Neovastat. Results represent the means  $\pm$  SD of four experiments.

acid, a molecule that competes for the lysine-binding sites of plasminogen and t-PA (Table 1) [26]. Moreover, the addition of equimolar concentration of PAI-1 also completely abolished plasmin generation, further emphasizing the effect of Neovastat on t-PA activity rather than on plasmin. Overall, these results suggest that Neovastat directly stimulates the t-PA-dependent activation of plasminogen.

To confirm this hypothesis, we measured the effect of Neovastat on the initial rate of plasmin generation. Since the acceleration of plasmin formation was constant over time under these assay conditions, plotting the experimental data of absorbance as a function of time square allows the determination of the initial rate of plasmin formation [23]. As shown in Figure 2A, addition of increasing concentrations of Neovastat promoted a marked increase in the initial rate of plasmin generation that was linear over time. Using the values of the slopes obtained at each Neovastat concentration and Equation 1, we estimate that Neovastat stimulates the t-PA turnover number ( $k$ ) in a dose-dependent manner, half-maximal stimulation occurring at 20 µg of Neovastat (Figure 2B).

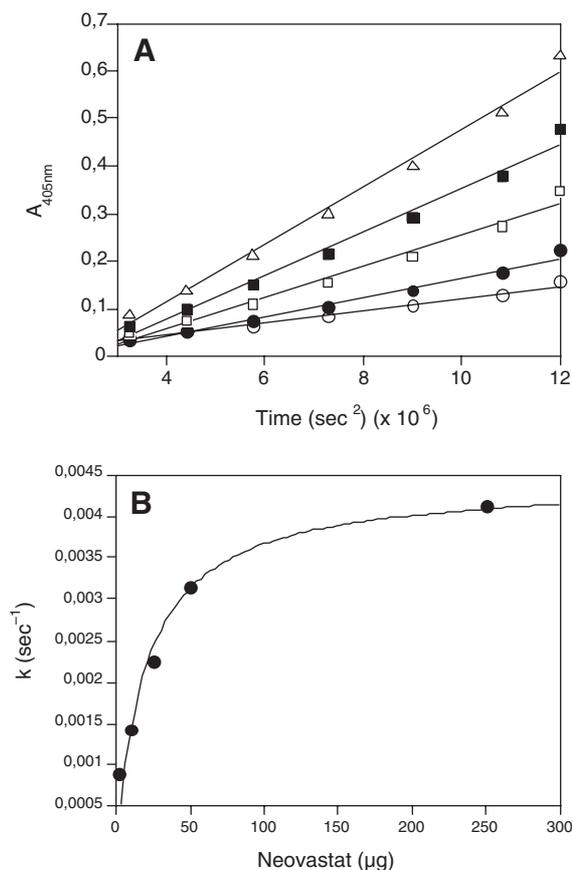
#### Preactivation of Neovastat increases its t-PA-promoting effect

We next investigated whether a partial denaturation of the protein components within Neovastat could increase its capacity to stimulate t-PA activity, as it has been reported for several proteins [25,27,28]. Neovastat was pretreated by a variety of treatment including heat, acid or alkali conditions, addition of detergent or sulfhydryl reagents and enzymatic digestion for 30 min, before testing its t-PA-promoting

Table 1. Neovastat stimulates the t-PA-dependent activation of plasminogen without affecting either t-PA or plasmin activity

	Formation of <i>p</i> -nitroaniline (mOD/min)		
	Control	Neovastat	Stimulation ( <i>x</i> -fold)
t-PA	2.1 $\pm$ 0.2	1.9 $\pm$ 0.2	0.9
Plasmin	1.8 $\pm$ 0.2	1.7 $\pm$ 0.08	0.9
t-PA + plasminogen	1.5 $\pm$ 0.1	6.4 $\pm$ 0.3	4.3
+ $\epsilon$ -amino- <i>n</i> -caproic acid (50 nM)	1.1	1.2	1.1
+ PAI-1 (5 nM)	0.22	0.24	1.1

The t-PA-dependent generation of plasmin was measured as described in Figure 1, in the absence or in the presence of the tested inhibitors. t-PA and plasmin amidolytic activities were assayed using Chromozym-t-PA and Chromozym-PL, respectively, as substrates, in the absence or in the presence of 100 µg/ml of Neovastat. Results are the means  $\pm$  SD of three experiments performed in triplicate.



**Figure 2.** Dose-dependent increases in the initial rate of plasmin generation by Neovastat. (A) t-PA-dependent plasminogen activation was measured in the presence of 2.5 ( $\circ$ ), 10 ( $\bullet$ ), 25 ( $\square$ ), 50 ( $\blacksquare$ ) and 250 ( $\triangle$ )  $\mu\text{g}$  of Neovastat as described in the Methods section and the experimental absorbance values were plotted as a function of time square [23]. (B) Slopes from Panel A, which represent the initial rates of plasmin generation obtained following addition of the indicated amount of Neovastat, were used to calculate the t-PA turnover number using Equation 1. A representative experiment is shown.

activity. As shown in Table 2, acidic treatment (HCl 0.1 N) of Neovastat or its short exposure to 60 °C, markedly increased the initial rate of plasmin generation, and resulted in a 15-fold increase of t-PA-dependent plasminogen activation. However, in contrast with data reported on several proteins [26], the increase in t-PA activity associated with thermal treatment was maximal after treatment at 60 °C, and was strongly decreased at higher temperatures (Table 2). An increase in t-PA activity was also observed following incubation of Neovastat in various chaotropic conditions such as 0.1% SDS, or in the presence of 6 M guanidine-HCl. However, incubation in alkali (0.1 N NaOH) or with a reducing agent (100 mM DTT) did

**Table 2.** Preactivation of Neovastat increases its capacity to stimulate t-PA-dependent plasmin generation

Treatment	t-PA-dependent plasmin generation (x-fold)
None	4.3 $\pm$ 0.3
60 °C	16.2 $\pm$ 0.2
95 °C	2.4 $\pm$ 0.1
0.1 N HCl	15.6 $\pm$ 0.3
0.1 N NaOH	4.2 $\pm$ 0.3
0.1% SDS	8.6 $\pm$ 0.1
6 M guanidine	14.1 $\pm$ 0.2
100 mM DTT	4.1 $\pm$ 0.4
Plasmin (2.5 $\mu\text{g/ml}$ )	3.8 $\pm$ 0.5
Thrombin (2.5 $\mu\text{g/ml}$ )	2.7 $\pm$ 0.4

The t-PA-dependent generation of plasmin was performed as described in the Materials and methods section, except that Neovastat was either untreated or treated for 30 min with the indicated conditions prior to the measure of t-PA-dependent plasmin generation. The results are means  $\pm$  SD of two distinct experiments performed in triplicate.

not modulate the t-PA-dependent plasmin generation. Proteolytic digestion of Neovastat with plasmin (or thrombin) did not alter the activity of Neovastat, suggesting that the stimulatory effect does not require hydrolysis by the plasmin produced by t-PA activity.

#### *Neovastat stimulates t-PA-dependent plasminogen activation through an increase in the catalytic efficiency of the enzyme*

We next examined the effects of both native and heat-activated Neovastat on the kinetic parameters for the activation of plasminogen by t-PA. Initial rates of plasmin generation were calculated at several plasminogen concentrations using slopes of  $A_{405}$  versus  $t^2$  plots and the steady-state rate of plasminogen activation, that is, the t-PA turn-over number ( $k$ ), was calculated using Equation 1. The resulting experimental data were fitted using nonlinear regression analysis. As shown in Figure 3, the kinetics of plasminogen activation were markedly increased in the presence of Neovastat and particularly of heat-activated Neovastat. In both cases, the best fits of the experimental data were obtained using the Michaelis–Menten equation ( $\text{rms} = 0.98$ ), whereas fitting of the data according to Hill equation ( $k = k_{\text{cat}}/1 + (K_m/s)^n$ ) was poor ( $\text{rms} = 0.63$ ). The kinetic constants obtained with the

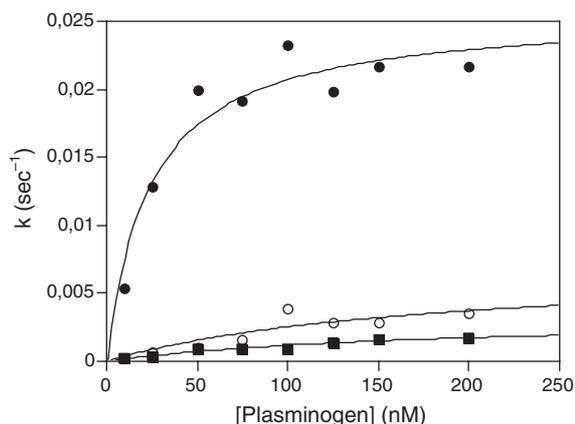


Figure 3. Kinetic analysis of the stimulatory effect of Neovastat on t-PA-dependent plasminogen activation. t-PA-dependent plasminogen activation was assayed using various concentrations of plasminogen in the absence (■) or in the presence of either 100  $\mu\text{g}/\text{ml}$  of native (○) or heat-denatured Neovastat (●). Initial rates of plasmin generation were determined from plots of  $A_{405}$  versus time square, and used to calculate the t-PA turnover number ( $k$ ) as described in the Methods section. Experimental data were fitted using the Michaelis-Menten model. The means of two distinct experiments are shown.

Table 3. Kinetic parameters of t-PA-dependent plasminogen activation in the presence of Neovastat

	Control	Neovastat ( $x$ -fold)	Heated Neovastat ( $x$ -fold)
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	0.003	0.007 (2.5)	0.025 (8.3)
$K_m$ (nM)	320	196 (1.6)	24 (13.3)

Kinetic parameters were measured as described in the legend to Figure 3. Values obtained from a representative experiment are shown.

Michaelis–Menten model showed that Neovastat increased both the  $k_{\text{cat}}$  of t-PA and its affinity for plasminogen (Table 3), these effects being 10-fold stronger when heat-activated Neovastat was used. These results demonstrate that Neovastat stimulates the t-PA-dependent activation of plasminogen by strongly increasing the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of the enzyme.

#### *Stimulation of t-PA-dependent plasminogen activation by Neovastat correlates with its interaction with plasminogen*

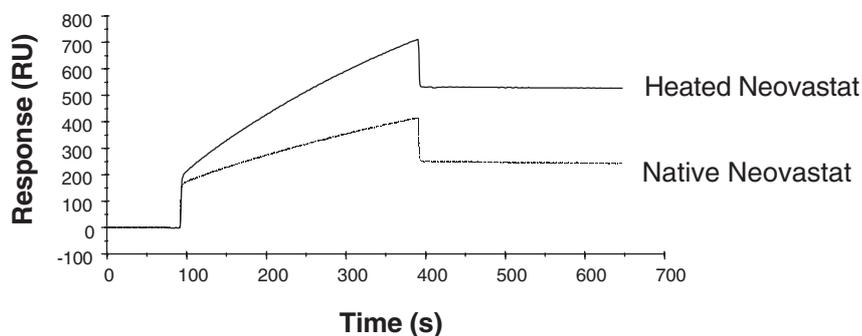
We next investigated whether the increase in t-PA-mediated plasminogen activation involved an interaction of the drug with t-PA or plasminogen,

using biospecific interaction analysis in real-time conditions on a BIAcore apparatus. For BIAcore surface plasmon resonance analyses, t-PA or plasminogen were first immobilized on a sensor chip, different concentrations of Neovastat were individually injected over the chip surface, and sensorgrams were recorded. In these analyses, the sensorgrams, usually show an initial rapid increase in response units upon protein injection followed by the association of injected proteins with molecules immobilized on the chip. After protein injections, a slower dissociation phase takes place characteristic of reversible protein interaction. As shown in Figure 4, injection of Neovastat on the plasminogen-containing chip resulted in a marked interaction of proteins present in the drug with the bound plasminogen (Figure 4A). Heat activation of Neovastat resulted in a two-fold increase in the binding of Neovastat to immobilized plasminogen (Figure 4A), this stimulatory effect being observed at every tested concentrations (Figure 4C). Injection of Neovastat on the t-PA-containing chip also resulted in an interaction of the drug and the immobilized protein, although to a much lesser extent (Figure 4B). In contrast to the results observed with plasminogen, however, the thermal treatment of Neovastat completely abolished its ability to interact with the immobilized t-PA (Figure 4B) even at higher doses of the drug (Figure 4C). Overall, these results indicate that Neovastat interacts preferentially with plasminogen and that the increase in t-PA activity observed after thermal treatment may be related to an increased interaction with plasminogen.

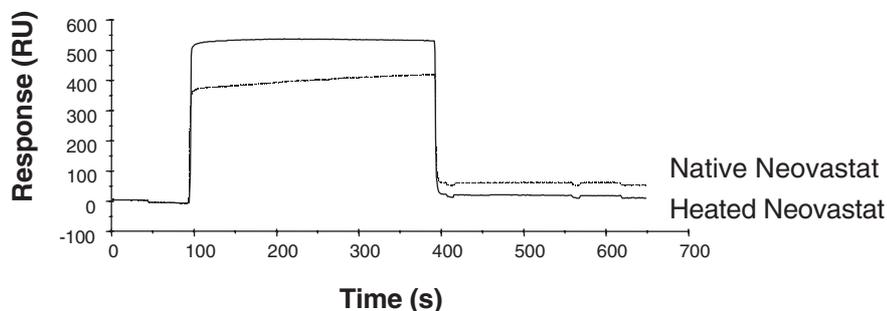
## Discussion

Neovastat is a multifunctional antiangiogenic drug that has reached Phase III clinical trial evaluation. In addition to its anti-MMP [18], anti-VEGF [19] and proapoptotic [20] activities, recent results have suggested that oral administration of the drug to animals bearing C6 glioma, results in an increase in endothelial cell-associated t-PA that correlates with major alterations in tumor characteristics.<sup>1</sup> In this work, we present evidence that Neovastat stimulates t-PA-dependent activation of plasminogen *in vitro* and that this activity may play a key role in the *in vivo* antitumor effects of Neovastat. Biochemical analysis showed that Neovastat was a potent activator of the t-PA-dependent activation of plasminogen,

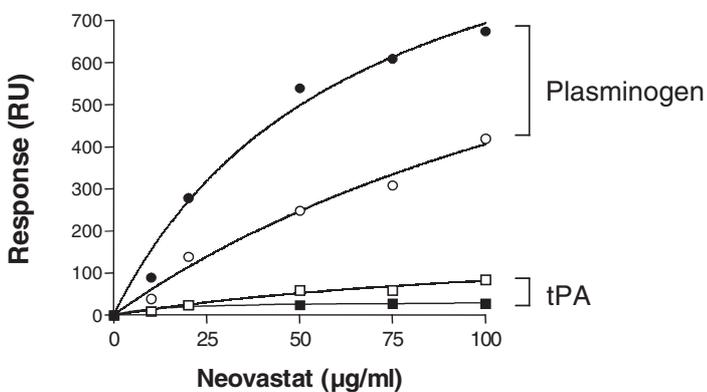
### A. Plasminogen



### B. tPA



### C.



*Figure 4.* Neovastat interacts directly with plasminogen. Plasminogen (A) or t-PA (B) were immobilized on a CM5 sensor chip as described in the Methods section. Native (dashed lines) or heat-activated (full lines) Neovastat ( $50 \mu\text{g/ml}$ ) was injected and the interaction with the immobilized proteins was recorded. (C) Increasing concentrations of either native ( $\circ$ ,  $\square$ ) or heat-denatured ( $\bullet$ ,  $\blacksquare$ ) Neovastat proteins were injected on sensor chips containing plasminogen ( $\circ$ ,  $\bullet$ ) or t-PA ( $\square$ ,  $\blacksquare$ ) and the extent of interaction was monitored as described.

with stimulation of the initial rate of plasmin generation by up to 15-fold. The plots for the activation of plasminogen by Neovastat were linear, suggesting that this stimulatory effect is rapid and does not require an initial activation step by t-PA or plasmin.

This is further supported by the observation that pre-incubation of Neovastat with plasmin had no effect on its stimulatory activity and that a pretreatment with the t-PA inhibitor PAI-1 completely abolish the stimulatory effect of the drug.

Pre-treatment of Neovastat by a variety of chaotic conditions markedly increases its capacity to stimulate t-PA-dependent plasminogen activation. This is an important issue since Neovastat is given to patients orally and that, consequently, drug constituents undergo significant chemical and enzymatic modifications in the gastrointestinal tract. Our results, strongly suggest that the component(s) responsible for the stimulation of t-PA-dependent plasmin generation is (are) likely to be resistant to structural modifications occurring during the gastrointestinal transit, and may even be activated in the stomach. Therefore, these changes may contribute to the clinical antitumor effects of the drug [21].

Kinetic analysis of the t-PA-dependent plasminogen activation showed that Neovastat stimulates t-PA-dependent plasminogen activation by decreasing the  $K_m$  of t-PA for plasminogen and by increasing the  $k_{cat}$  of the enzyme. This stimulatory effect is similar to that induced by several extracellular matrix proteins, including laminin and collagen IV, that also induce activation through a decrease in the  $K_m$  and an increase in the  $k_{cat}$  of t-PA [27]. Since native plasminogen exhibits a tight spiral structure that protects its activation site (Arg<sup>561</sup>-Val<sup>562</sup>) from attack by t-PA, the increase in both the  $k_{cat}$  of t-PA and of its affinity for plasminogen by Neovastat may suggest that Neovastat might induce a conformational change within the plasminogen molecule, and subsequent generation of a conformer that is more susceptible to t-PA-dependent proteolysis. This is strongly supported by the observation that increased t-PA activity is correlated with a direct interaction of Neovastat with plasminogen, as determined by real-time protein interaction analysis using the surface plasmon resonance technology. This interaction is likely to involve lysine residues of plasminogen since the lysine analog  $\epsilon$ -amino-*n*-caproic acid abolished the ability of Neovastat to stimulate t-PA activity. This interaction is also likely to be closely associated with increased t-PA activity since thermal activation of Neovastat markedly increases both its t-PA-promoting activity and its ability to interact with plasminogen. However, since Neovastat does not activate uPA-dependent plasminogen activation, it is also tempting to speculate that the formation of a ternary complex between t-PA, plasminogen and Neovastat proteins may also contribute to the activation process. In this respect, the interaction of t-PA with denatured proteins has been proposed to represent a general mechanism by which plasminogen activation occurs [27,28],

possibly through the interaction of the enzyme with cross- $\beta$  sheets structures present in these proteins [29]. However, although we detected a small but significant interaction of native Neovastat with t-PA, this interaction was abolished after denaturation of the drug suggesting that it does not contribute significantly to the observed increase in t-PA activity.

By contrast to its stimulatory effect on t-PA activity, we observed that Neovastat slightly inhibits uPA-dependent plasmin generation. Although this inhibitory activity was observed at higher concentrations of the drug, it was markedly increased by denaturation of Neovastat (data not shown), suggesting that it may also play a significant role *in vivo*. Given the important role of uPA in tumor cell migration and invasion [5], further studies aimed at the characterization of this uPA inhibitory activity should provide interesting information on its contribution to the antitumor effects of the drug.

By contrast to uPA, the role of t-PA in tumor invasion and angiogenesis remains largely unknown. High t-PA content and activity correlates with good prognosis in melanoma and breast cancer patients and similar correlations were also reported for other types of tumors [11,12 and references therein]. In addition, mice injected with colon cancer cells overexpressing t-PA had a lower number of liver metastases and higher survival rate than those injected with untransfected cells [30], further suggesting that t-PA has a positive role in cancer. Although this functional repercussion of t-PA activity on tumor progression remains unexplained, it has been suggested that overstimulation of t-PA may lead to excessive proteolysis of the provisional fibrin matrix necessary for neovessel formation, and to subsequent endothelial cell apoptosis induced by cell detachment (anoikis) [14]. In this respect, it is noteworthy that plasma from human patients receiving recombinant t-PA in combination with captopril was recently shown to possess potent antiangiogenic activities [31]. Based on these considerations, it is thus tempting to speculate that the stimulation of t-PA-dependent plasmin formation by Neovastat observed in the current work, may impact endothelial cell adhesiveness *in vivo*, and thus, contribute to its antiangiogenic properties. Such a mechanism of action has been proposed for endostatin [14,32] but is distinct from that of angiostatin, which has been reported as an inhibitor of t-PA-dependent plasmin generation [33].

In summary, our results suggest that Neovastat contains potent t-PA-stimulatory factor(s) that through

an ability to interact with plasminogen, lead(s) to an increased catalytic efficiency of t-PA-mediated plasmin generation. Further purification and characterization of the protein(s) responsible for this stimulatory effect are currently underway and should provide interesting new information about the mechanisms involved and their relationship with the antiangiogenic effects of Neovastat.

### Acknowledgments

We would like to thank Drs Patrick Poyet, Violetta Dimitriadou and Muriel Steel for their critical reading of the manuscript. The study was funded by Æterna Laboratories, Québec City, Québec, Canada and by the Canadian Institutes for Health Research.

### Notes

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