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RESEARCH PAPER

The use of the Chandler loop to examine the interaction potential of NXY-059 on the thrombolytic properties of rtPA on human thrombi *in vitro*

NJ Mutch[1,5], NR Moore[1], C Mattsson[2], H Jonasson[3], AR Green[4,6] and NA Booth[1]

[1]*Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK;* [2]*AstraZeneca R&D Mölndal, Mölndal, Sweden;* [3]*AstraZeneca R&D Södertälje, Södertälje, Sweden and* [4]*AstraZeneca R&D Charnwood, Loughborough, UK*

Background and purpose: Recombinant tissue-type plasminogen activator (rtPA) is the only globally approved treatment for acute ischaemic stroke. Other potential treatments might be administered with rtPA, making it important to discover whether compounds interfere with rtPA-induced lysis. We evaluated methods for examining the effect of the neuroprotectant NXY-059 on the lytic property of rtPA.
Experimental approach: Plasma clot formation and lysis in the presence of rtPA and NXY-059 was measured as the change in plasma turbidity. The effect of NXY-059 on rtPA-induced lysis was similarly assessed on preformed clots. Lysis of the thrombus formed in a Chandler loop measured release of fluorescent-tagged fibrinogen that had been incorporated during thrombus formation. Thrombi were exposed to both rtPA and NXY-059 throughout lysis in the presence of 80% autologous plasma and the release of label during lysis was measured.
Key results: Data interpretation is limited in the clot lysis experiments because either the rtPA was present during clot formation or the drug was added to a clot formed in static conditions. In contrast, thrombi were formed in dynamic flow conditions in the Chandler loop and the time course of lysis in plasma was examined. rtPA increased thrombolysis and the antifibrinolytic trans-4-(aminomethyl) cyclohexane carboxylic acid (AMCA) inhibited lysis. Lysis induced by rtPA was unaltered by NXY-059.
Conclusions and implications: The Chandler loop method provides a reliable technique for examining the effect of compounds on rtPA-induced lysis *in vitro* and demonstrated that NXY-059 does not alter rtPA-induced lysis at clinically relevant concentrations of either drug.
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Keywords: recombinant tissue-type plasminogen activator; NXY-059; rtPA; thrombolysis; Chandler loop

Abbreviations: AMCA, *trans*-4-(aminomethyl) cyclohexane carboxylic acid; ANOVA, analysis of variance; FITC, fluorescein isothiocyanate; NXY-059, disodium 2,4-disulphophenyl-*N*-tert-butylnitrone; rtPA, recombinant tissue-type plasminogen activator

Introduction

Administration of recombinant tissue-type plasminogen activator (rtPA) is an approved treatment for acute ischaemic stroke in many countries. Other compounds have been investigated both experimentally and clinically for treating acute ischaemic stroke, most of these being potential neuroprotective agents that are compounds that interfere with the biochemical mechanisms involved in ischaemia-induced neuronal cell death in the brain (Green and Shuaib, 2006). To date, no neuroprotective agent has been found to demonstrate convincing efficacy in a large clinical trial (O'Collins *et al*., 2006), but novel compounds are still being developed (Green and Shuaib, 2006). It has been suggested that the combination of a neuroprotective agent to protect neurons, together with rtPA to restore blood flow, might enhance the penetration of the neuroprotective agent into the ischaemic region and prove efficacious (Wagner and Jauch, 2004; Ly *et al*., 2006). Furthermore, given the clinical

Correspondence: Dr AR Green, Institute of Neuroscience, School of Biomedical Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK.
E-mail: richard.green@nottingham.ac.uk
[5]Current address: Faculty of Biological Sciences, University of Leeds, Leeds, UK.
[6]Current address: Institute of Neuroscience, School of Biomedical Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK.
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efficacy of rtPA in acute ischaemic stroke (NINDS t-PA Stroke Study Group, 1995), it is reasonable to suppose that in future most potential neuroprotectants will be administered to stroke patients either concurrently or sequentially with rtPA in most clinical trials.

Disodium 2,4-disulphophenyl-*N*-tert-butylnitrone (NXY-059) is a novel nitrone compound with free radical trapping properties (Maples *et al*., 2001; Green *et al*., 2003; Williams *et al*., 2007) that has been demonstrated to be an effective neuroprotectant in both rodent and primate models of acute ischaemic stroke (Kuroda *et al*., 1999; Marshall *et al*., 2001, 2003; Sydserff *et al*., 2002). In the course of the clinical development of NXY-059 (see Green and Ashwood, 2005), it became clear that an investigation was required into whether this compound would interfere with the lytic properties of rtPA. A lack of interaction would both increase the confidence of clinical investigators in the safety of combining administration of the thrombolytic rtPA with NXY-059 and meet the requirements of the FDA that an *in vitro* study be undertaken with human thrombi before the drug combination be given to human subjects. Primarily, it was necessary to ascertain whether NXY-059 would be likely to either increase the thrombolytic properties of rtPA, which might increase the rate of cerebral haemorrhage transformation, a problem that occurs in the treatment of stroke patients (Wardlaw *et al*., 1997) or, alternatively, decrease the thrombolytic properties of the drug thereby decreasing its effectiveness in restoring cerebral blood flow.

We now describe the *in vitro* investigations undertaken, using human blood and plasma, to examine whether NXY-059 altered the fibrinolytic properties of rtPA. Preliminary studies examined plasma clots formed in a static system. The main investigation used the Chandler loop (Chandler, 1958) where, unlike clots formed under static conditions, the resulting thrombi have striking morphological and biochemical similarity to those formed *in vivo* (Stringer *et al*., 1994; Robbie *et al*., 1997; Mutch *et al*., 2003, 2007). This confers a degree of validity to the method and enhances its physiological relevance to humans. While biochemical mechanisms of the fibrinolytic system have been investigated using the Chandler loop, it does not appear to have been previously used to examine drug interaction effects. We therefore evaluate it here in some detail to assist others who might consider using it in future studies.

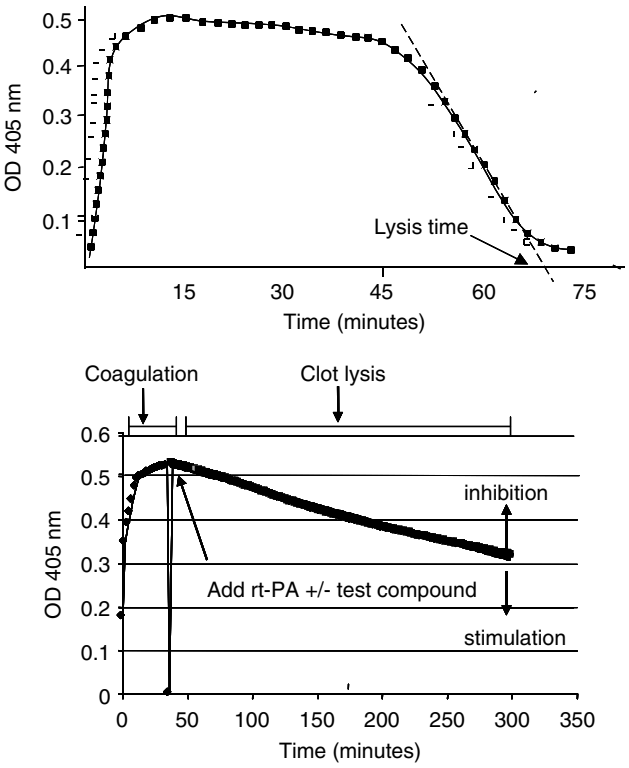

Figure 1 Top: A typical plasma clot lysis curve induced by recombinant tissue-type plasminogen activator (rtPA) (0.6 nmol l^{-1}) added to the system before clot formation. Bottom: a typical coagulation and lysis curve when rtPA was added to the system after the clot has been formed. OD, optical density.

Methods

Effect of rtPA and NXY-059 on plasma clot formation and lysis
Plasma clot lysis was studied in a system using thrombin/Ca^{2+} to induce clotting and rtPA to induce clot lysis. These events were monitored in a microtitre plate reader (Spectra MAX Plus, Molecular Devices, CA, USA) by measuring the change in turbidity at 405 nm at 37 °C. Human plasma, previously collected, pooled and stored at −70 °C, was thawed for 10 min at 37 °C and kept on ice until the start of the assay. NXY-059 (final concentration 0–1600 μmol l^{-1}) was first added to the microtitre plate wells, immediately followed by the addition of (final assay concentration) thrombin (1.8 nmol l^{-1}), rtPA (0.6 or 0.3 nmol l^{-1}) and $CaCl_2$ (15 mmol l^{-1}). The clotting reaction was started by the addition of 50 μl human pooled plasma and the reaction volume was adjusted to 150 μl by the addition of 50 mmol l^{-1} Tris buffer and 106 mmol l^{-1} NaCl, pH 7.4. Changes in turbidity were monitored and time to clot formation was defined as the time taken to reach the midpoint of maximum turbidity. Time to lysis was obtained from the inflection point between the tangent of the turbid-to-clear transition curve and the baseline turbidity (see Figure 1a). Statistical evaluation was by the Astute Statistical programme (DDU Software, University of Leeds, UK). The function 'Linear regression' was used to evaluate whether the slope of a fitted dose–response curve was significantly different from zero.

Effect of rtPA and NXY-059 on preformed clots
In this modification of the method, plasma (50 μl) was added to the wells and diluted with 80 μl Tris buffer, 50 mmol l^{-1}, containing 106 mmol l^{-1} NaCl, pH 7.4. The clotting reaction was started by the addition of thrombin (5 μl) and $CaCl_2$ (5 μl) to a final assay concentration of 1.8 nmol l^{-1} and 15 mmol l^{-1}, respectively. After allowing the clots to form for 30 min, NXY-059 in saline (5 μl) was added on top of the clots in the microtitre plate wells, immediately followed by the addition of 5 μl rtPA (final concentration 2.0, 4.0 and 6.0 nmol l^{-1}). NXY-059 was tested at 0 mmol l^{-1} (baseline control) and at 250 and 625 μmol l^{-1}. In a separate

experiment, NXY-059 was replaced by the antifibrinolytic *trans*-4-(aminomethyl) cyclohexane carboxylic acid (tranexamic acid or AMCA, 31.8 μmol l^{-1}) (see Krishnamurti *et al*., 1994; Weide *et al*., 1996) with the addition of rtPA (2.5 nmol l^{-1}). Turbidity was continuously monitored for 300 min, but since lysis is not complete at that time due to a small contact surface between rtPA and its substrate (plasminogen inside the clot), the slope of the lysis curve was used to determine the rate of clot lysis instead of 'time to lysis'.

Figure 1b shows a typical clot lysis curve from the programe SOFTmax PRO 3.1.1. The study parameter was the lysis rate (arbitary unit min^{-1}), which is defined as the slope of the linear lysis curve. Statistical evaluation where rtPA concentration was the dependent variable was performed with one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. All other results were evaluated by the use of one-way ANOVA, with NXY-059 concentration as the dependent variable and using Dunnett's *post hoc* test.

Chandler loop

Thrombolysis was monitored by incorporating fibrinogen, labelled with a fluorescent tag, into the forming thrombus and measuring the amount of label released as fibrin degradation products during lysis in autologous plasma. The thrombi were exposed to exogenous rtPA and test substances throughout lysis.

Potential interaction effects of NXY-059 and rtPA were investigated in a repeated measures two-way factorial design with four NXY-059 concentrations (0, 600, 2000 and 6000 μmol l^{-1}) and four rtPA concentrations (0, 2.86, 14.3 and 71.5 nmol l^{-1}). Five healthy human volunteers (2 male, 3 female; age range: 23–50 years) donated blood from which model thrombi were made and subsequently exposed to the 16 combinations of NXY-059 and rtPA concentrations. On the day of an experimental session, a blood sample (approximately 50 ml) was collected into 0.13 mol l^{-1} trisodium citrate (ratio = 9 ml blood:1 ml citrate). Model thrombi (24 from each donor) were prepared as described below. Plasma was prepared from the remainder of the citrated blood sample by centrifugation at 1850 *g* for 30 min at 4 °C. The plasma prepared by this method was stored at 4 °C until needed (approximately 90 min later).

Fluorescein isothiocyanate (FITC)-labelled fibrinogen (Kabi L fibrinogen labelled in-house, final concentration: 75 μg l^{-1}, FITC:fibrinogen ratio; 4.4:1) was added to 0.9 ml of citrated whole blood. The sample was recalcified by addition of $CaCl_2$ (10.9 mmol l^{-1}), giving a final volume of 1.15 ml and calculated free calcium concentration of 3 mmol l^{-1}. Each sample was placed in vinyl tubing (inner diameter = 3 mm, external diameter = 4.2 mm, length = 33 cm; Portex, Hythe, Kent) and the open ends were joined using a short sleeve of larger tubing (internal diameter = 4 mm, external diameter = 6.8 mm) to form a Chandler loop with a diameter of 10.5 cm. Loops were attached to a horizontal axle which passed through the centre of the loops, and the axle rotated at 30 r.p.m., giving a shear rate of 428 s^{-1} (Poole, 1959; Gardner, 1974), for 90 min at ambient temperature. A single thrombus, approximately 1 cm in length, was formed in the flowing blood in the loop. This was removed, rinsed in 0.9% (w/v) NaCl, blotted with filter paper and transferred to a tube containing autologous plasma (0.40 ml) to which rtPA solution (0.05 ml) and NXY-059 solution (0.05 ml), or rtPA solution (0.05 ml) and AMCA solution (0.05 ml), had been added at the concentration detailed in the Results section. Tubes were incubated at 37 °C and samples (5.0 μl) of the plasma mixture were removed at 15 and 30 min, and at 30 min intervals thereafter up to 300 min. Each sample was added to a tube containing 0.245 ml phosphate-buffered saline (PBS), mixed and the released fluorescence was measured in a fluorescence plate reader at excitation:485 nm and emission: 530 nm (Mutch *et al*., 2003). Blanks containing PBS gave a measure of background fluorescence.

In the presence of rtPA, lysis proceeded at a generally constant rate for approximately 90 min. Between 90 and 300 min, the rate of lysis decreased relative to the initial rate so that the fluorescence data as a function of time were curvilinear over the 300 min incubation period. Time-dependent changes in fluorescence values were therefore analysed in two ways: first, the rate of lysis was computed by linear regression of the fluorescence data as a function of time for the first 90 min; second, the curvilinear characteristics of the data for the entire 300 min lysis period were represented by the first- and second-order rate parameters determined by quadratic regression.

For the linear regression (0–90 min), a least-squares linear regression line was fitted (SAS computer program) to the background-corrected fluorescence data as a function of time for all individual thrombi. The linear equation has the form

$$\text{fluorescence units} = (m \times \text{time}) + \text{intercept}$$

The linear slope parameter m is a direct metric of lysis rate during the 0–90 min interval and this linear rate parameter was a dependent variable that was analysed with inferential statistics.

For the quadratic regression (0–300 min), a least-squares quadratic regression curve was fitted (SAS computer program) to data as a function of time for all individual thrombi. The quadratic equation has the form

$$\text{fluorescence units} = (a \times \text{time}^2) + (b \times \text{time}) + \text{intercept}$$

The second- and first-order rate parameters a and b, respectively, were computed for all individual donors in all experimental conditions. The b parameter is a metric of the overall linear rate vector of the function, whereas the a parameter is a metric of the curvature of the function. The first- and second-order rate parameters were dependent variables that were analysed separately with inferential statistics.

In the primary analysis of NXY-059 × rtPA interaction for the 0–90 min subset of fluorescence data, the linear rate parameter m was analysed by repeated measurement ANOVA. The ANOVA, with random effect for donor, tested for an overall main effect of rtPA concentration, a main effect of NXY-059 concentration and an rtPA × NXY-059 interaction. The analysis plan for the quadratic regression rate metrics a and b was to trigger contrasts comparing the different concentrations of NXY-059 relative to control only if a main

effect of NXY-059 or an NXY-059 × rtPA interaction was statistically significant.

Analysis of the data for the 0–300 min interval proceeded in an analogous fashion, but the quadratic regression parameters *a* and *b* were analysed by separate repeated-measures ANOVAs. Each ANOVA tested for a main effect of rtPA concentration, a main effect of NXY-059 concentration and rtPA × NXY-059 interaction. The analysis plan for the quadratic regression rate metrics ***a*** and ***b*** was to trigger contrasts comparing the different concentrations of NXY-059 relative to control only if a main effect of NXY-059 or an NXY-059 × rtPA interaction was statistically significant. Nevertheless, despite the lack of significance of the overall ANOVAs that indicated that there was no effect of NXY-059 on rtPA-induced lysis (see later), additional secondary statistical analyses were conducted to characterize potential relationships between rtPA and NXY-059. The three NXY-059 conditions versus control for each of four rtPA conditions were analysed using separate repeated measurement ANOVA models with fixed effects for NXY-059 condition and random effect for donor. Just as would have been done if the overall ANOVA contained significant effects, the α criterion was adjusted for multiple comparisons. For each parameter (*m*, *a* and *b*) from the regression functions, there were 12 comparisons (three NXY-059 conditions versus control for each of four rtPA conditions). To maintain an overall false positive rejection rate of 0.05, each of the 12 comparisons for a given parameter involved a Bonferroni correction to adjust α to $1-(0.95)^{(1/12)} = 0.004265$. The secondary analyses were conducted in analogous ways for the rate parameter *m* derived from linear regression of the 0–90 min data-subset and for the first- and second- order rate parameters (*b* and *a*, respectively) derived from quadratic regression of the 300 min data set.

Materials

AMCA and FITC were obtained from Sigma and NXY-059 was supplied by AstraZeneca R&D Södertälje.

Results

Formation and lysis of plasma clots

Disodium 2,4-disulphophenyl-*N*-tert-butylnitrone alone did not influence the time to clot formation, which normally occurs at 4–5 min (accumulated reference data). NXY-059, at concentrations up to 1600 μmol l^{-1}, did not affect *in vitro* clot lysis time induced by the thrombolytic agent rtPA at 0.3 or 0.6 nmol l^{-1} (Figure 2). The higher rtPA concentration decreased lysis time compared with that at the lower concentration (Figure 2).

Lysis of preformed clots

As expected, analysis of the rate of lysis at rtPA concentrations of 0–6 nmol l^{-1} revealed a concentration-dependent effect on lysis rate ($F(3,19) = 25.96$; $P < 0.0001$) in the control group (Table 1). The lysis rate of -0.70 ± 0.07 ($n = 6$) induced by rtPA (2.5 nmol l^{-1}) was significantly ($P = 0.002$) reduced by AMCA (31.8 μmol l^{-1}) to -0.57 ± 0.01 ($n = 6$).

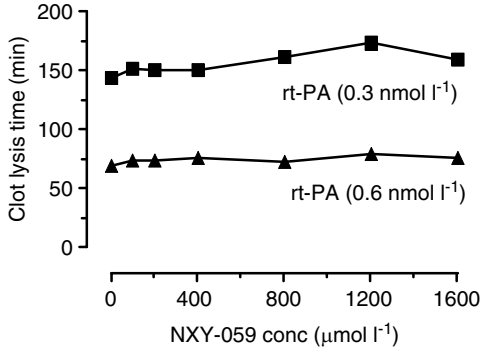

Figure 2 Plasma clot lysis time in human plasma induced by rtPA (0.3 and 0.6 nmol l^{-1}) added to the system before clot formation and in the presence of NXY-059 (0–1600 μmol l^{-1}). Each point is the mean of two observations.

Table 1 Slope of plasma clot lysis curve in the presence of rtPA and NXY-059

rtPA (nmol^{-1})	*Control*	*NXY-059 (250 μmol l^{-1})*	*NXY-059 (625 μmol l^{-1})*
0	—	0.052 ± 0.001	0.046 ± 0.001
2	−0.67 ± 0.08	−0.73 ± 0.12	−0.69 ± 0.22
4	−0.85 ± 0.11	−0.86 ± 0.09	−0.92 ± 0.30
6	−1.02 ± 0.14	−1.02 ± 0.28	−1.02 ± 0.16

Abbreviations: NXY-059, disodium 2,4-disulphophenyl-*N*-tert-butylnitrone; rtPA, recombinant tissue-type plasminogen activator.
Results are reported as mean ± s.d. ($n = 6$). No significant effect of NXY-059 on the slope of the rtPA-induced lysis curves was observed.

NXY-059 at a concentration of 250 or 625 μmol l^{-1} had essentially no lytic effect in itself and there was no significant effect of NXY-059 on the slope of the lysis curves produced by any of the rtPA concentrations examined using ANOVA analysis and Dunnett and Tukey *post hoc* tests (Table 1).

Lysis of model thrombi formed in the Chandler loop

In the absence of rtPA, there was a low rate of spontaneous lysis (Figure 3). During the initial 90 min of incubation, rtPA increased the rate of lysis, although the rate for the highest concentration of rtPA (71.5 nmol l^{-1}) was similar to that for 14.3 nmol l^{-1}.

The rate of rtPA-induced lysis declined after approximately 90 min. The decline in lysis rate was concentration dependent with a greater decrease at 71.5 nmol l^{-1} rtPA than at 14.3 or 2.86 nmol l^{-1}. Indeed, the total amount of lysis at 14.3 nmol l^{-1} exceeded that at 71.5 nmol l^{-1} after approximately 120 min of incubation, and after 300 min the total amount of lysis with 71.5 nmol l^{-1} was approximately the same as that with 2.83 nmol l^{-1} (Figure 3). Thrombolysis induced by 14.3 nmol l^{-1} rtPA was inhibited by AMCA (318 μmol l^{-1}) (Figure 3).

In the absence of rtPA, the slow rate of spontaneous lysis was unaffected by the presence of NXY-059 at any of the

concentrations examined (Figure 4). Further, lysis induced by each of the concentrations of rtPA that were examined was unaffected by any concentration of NXY-059 during the first 90 min. This was supported by statistical evaluation since it was found that the linear rate parameter m for the initial 90 min of incubation increased with rtPA concentration, and this lytic effect of rtPA was highly significant ($F(3,60) = 134.58$; $P < 0.0001$). However, the main effect of NXY-059 was not significant ($F(3,60) = 0.09$; $P = 0.9642$), and the NXY-059 × rtPA interaction was also not significant ($F(9,60) = 0.83$; $P = 0.5911$).

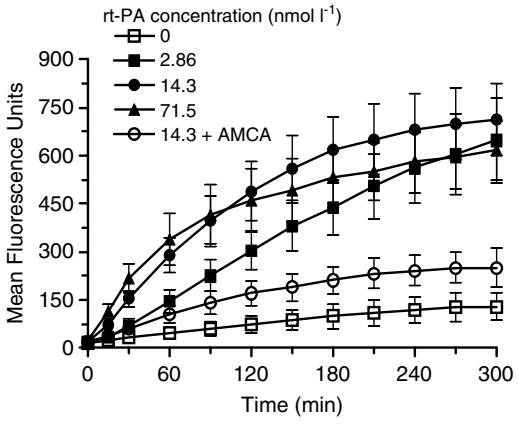

Figure 3 The concentration-response for the effect of recombinant tissue-type plasminogen activator on lysis (measured in fluorescence units) over 5 h. Data are presented as mean (± s.d.) of the response of thrombi prepared from five subjects and measured on two separate occasions. The effect of antifibrinolytic trans-4-(aminomethyl) cyclohexane carboxylic acid (AMCA) (318 μmol l^{-1}) on the response to rtPA (14.3 nmol l^{-1}) is also shown. The effect of AMCA (318 μmol l^{-1}) on the thrombolytic effect of rtPA (14.3 nmol l^{-1}) is significant over the period 0–90 min ($F(1,4) = 119.74$, $P = 0.0004$) and the period 0–300 min ($F(1,4) = 82.14$, $P = 0.0008$).

For the highest concentration of rtPA, there appeared to be a slight increase in lysis at the highest concentrations of NXY-059 examined at the end of the 300 min incubation (Figure 4). Analysis over the full 300 min showed that the main effect of rtPA was highly significant in the overall ANOVA for both the first-order lysis rate parameter b ($F(3,60) = 117.80$; $P < 0.0001$) and the second-order lysis rate parameter a ($F(3,60) = 113.44$; $P < 0.0001$). However, the main effect of NXY-059 was not significant for either the first-order parameter ($F(3,60) = 0.49$; $P = 0.6914$) or the second-order parameter ($F(3,60) = 0.95$; $P = 0.4222$). Likewise, the NXY-059 × rtPA interaction was not significant for either the first-order ($F(9,60) = 0.98$; $P = 0.4663$) or the second-order ($F(9,60) = 0.93$; $P = 0.5030$) lysis rate metrics.

Although these statistical data indicated no interaction between the two compounds, visual evaluation of the curves shown in Figure 4 suggested a possible increase in lysis with increasing concentration of NXY-059 for thrombi in the highest rtPA concentration (71.5 nmol l^{-1}), and this

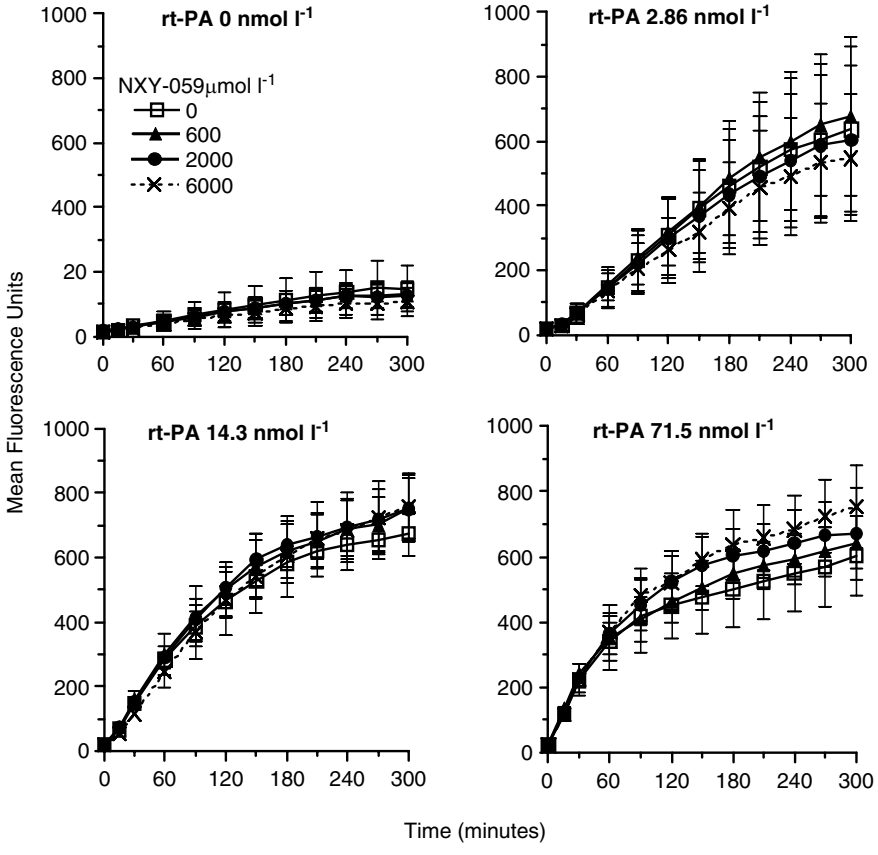

Figure 4 Effect of NXY-059 on rtPA-induced lysis. Lysis was measured in fluorescence units over a 5 h period using thrombi from five donors and data are presented as mean ± s.d. NXY-059, disodium 2,4-disulphophenyl-N-tert-butylnitrone.

Table 2 NXY-059 and rtPA interaction: quadratic regression[a] rate parameters

rtPA ($nmol\,l^{-1}$)	*NXY-059* ($\mu mol\,l^{-1}$)	*Second-order parameter a*		*First-order parameter b*	
		Mean ± s.d.	*ANOVA*[b]	*Mean ± s.d.*	*ANOVA*[b]
0.0	0	−0.0008 ± 0.0009	NA	0.703 ± 0.505	NA
	600	−0.0007 ± 0.0006	$F(1,12) = 0.05$ $P = 0.8322$	0.609 ± 0.244	$F(1,12) = 0.44$ $P = 0.5177$
	2000	−0.0007 ± 0.0007	$F(1,12) = 0.00$ $P = 0.9681$	0.619 ± 0.301	$F(1,12) = 0.35$ $P = 0.5635$
	6000	−0.0007 ± 0.0006	$F(1,12) = 0.09$ $P = 0.7678$	0.524 ± 0.312	$F(1,12) = 1.62$ $P = 0.2272$
2.86	0	−0.0029 ± 0.0013	NA	3.07 ± 1.17	NA
	600	−0.0026 ± 0.002	$F(1,12) = 0.37$ $P = 0.5550$	3.12 ± 1.19	$F(1,12) = 0.09$ $P = 0.7663$
	2000	−0.0026 ± 0.0014	$F(1,12) = 0.29$ $P = 0.6002$	2.86 ± 1.14	$F(1,12) = 1.48$ $P = 0.2468$
	6000	−0.0022 ± 0.0012	$F(1,12) = 1.56$ $P = 0.2350$	2.55 ± 1.01	$F(1,12) = 9.46$ $P = 0.0096$
14.3	0	−0.0086 ± 0.001	NA	4.71 ± 0.47	NA
	600	−0.0086 ± 0.0027	$F(1,12) = 0.00$ $P = 0.9721$	4.89 ± 1.04	$F(1,12) = 0.21$ $P = 0.6545$
	2000	−0.0091 ± 0.0012	$F(1,12) = 0.23$ $P = 0.6431$	5.12 ± 0.64	$F(1,12) = 1.11$ $P = 0.3123$
	6000	−0.0072 ± 0.0035	$F(1,12) = 1.46$ $P = 0.2495$	4.65 ± 1.31	$F(1,12) = 0.02$ $P = 0.8810$
71.5	0	−0.0077 ± 0.0026	NA	3.89 ± 1.08	NA
	600	−0.0077 ± 0.0019	$F(1,12) = 0.00$ $P = 0.9985$	4.04 ± 0.67	$F(1,12) = 0.41$ $P = 0.5348$
	2000	−0.010 ± 0.0018	$F(1,12) = 26.81$ $P = 0.0002$	4.91 ± 0.70	$F(1,12) = 18.61$ $P = 0.0010$
	6000	−0.009 ± 0.0021	$F(1,12) = 12.56$ $P = 0.0040$	4.94 ± 0.89	$F(1,12) = 19.85$ $P = 0.0008$

Abbreviations: NA, not applicable; NXY-059, disodium 2,4-disulphophenyl-N-tert-butylnitrone; rtPA, recombinant tissue-type plasminogen activator; s.d., standard deviation.
[a]Fluorescence units $= (a \times \text{time}^2) + (b \times \text{time}) + \text{intercept}$.
[b]ANOVA Contrasts compared each NXY-059 level to control (0 $\mu mol\,l^{-1}$ NXY-059) within each rtPA concentration.

prompted additional statistical evaluation. For both the first- and second-order rate parameters, 10 of the 12 contrasts that compared each NXY-059 condition to control (NXY-059: 0 $\mu mol\,l^{-1}$) exceeded the adjusted α criterion of 0.0043. Those comparisons therefore provide no evidence for a treatment-related effect of NXY-059 (Table 2). Two experimental conditions (NXY-059 at a concentration of 2000 and 6000 $\mu mol\,l^{-1}$ when combined with the highest concentration of rtPA) were statistically significant within this additional testing (Table 2) and are discussed below when assessing potential relevance to clinical use.

Discussion

The initial studies on plasma clots formed in a static system suggested that NXY-059 had no effect on the thrombolytic properties of rtPA. Our judgement was that these studies, and any like them, have distinct limitations. In the first approach, both rtPA and NXY-059 were present during the clotting phase. This is not a clinically relevant situation in stroke where the infarct has always occurred before treatment is initiated. While the method using preformed plasma clots was more relevant, the clot had still been produced in a static situation. Despite these limitations, there was a concentration-dependent lysis produced by rtPA and the effect of rtPA was inhibited by AMCA, a known inhibitor of rtPA-induced lysis (Krishnamurti *et al*., 1994). It should also be noted that the rtPA concentration required to produce lysis of a preformed clot was much higher than that needed when it was present during clotting, presumably because the drug can only act on the outer surface of the clot.

In contrast, the flow conditions of the Chandler loop mimic the shear rates of those found in larger arteries (400–600 s^{-1}), thus producing a thrombus with a biochemical and structural morphology that is markedly similar to that produced *in vivo* (Stringer *et al*., 1994; Robbie *et al*., 1997; Mutch *et al*., 2003). The subsequent lysis of these thrombi by rtPA can be monitored in detail over time and has recently been used to successfully evaluate the efficacy of endogenous inhibitors on fibrinolysis (Mutch *et al*., 2007) and in the study of fibrinolytic surfaces for use in biomaterials (McClung *et al*., 2007). The assay reliably detected the inhibitory effect of AMCA on fibrinolysis, thereby increasing confidence in the validity of the experimental technique. Crucially, the assay also showed the expected effect of rtPA on the rate of thrombolysis. During prolonged exposure to rtPA alone, the total amount of lysis with 71.5 $nmol\,l^{-1}$ was approximately the same as that with 2.83 $nmol\,l^{-1}$ and less than that with 14.3 $nmol\,l^{-1}$. This reduction in activity of rtPA at high concentrations has been observed by others and is attributed to a diminution of free plasminogen and leaching of clot-bound plasminogen into the plasminogen-depleted phase of the lysis system (Sobel *et al*., 1990; Torr *et al*., 1992).

No statistically significant overall effect of NXY-059 was identified on rtPA-induced thrombolysis over either the first 90 min or the entire 300 min incubation period. While two of the 12 secondary statistical analyses were significant, the clinical relevance of these findings was considered to be low. They occurred when NXY-059 at a concentration of 2000 and 6000 $\mu mol\,l^{-1}$ was combined with the highest concentration of rtPA.

The highest concentration of rtPA used in our study (71.5 $nmol\,l^{-1}$) is approximately 50% greater than the peak plasma value reported in humans during the treatment of myocardial infarction (Tanswell *et al*., 1992; Cannon *et al*., 1998). Since both the maximum total dose (100 mg over

90 min) and the maximum bolus dose (15 mg) used for the treatment of myocardial infarction exceed both the maximum total dose (90 mg over 60 min) and maximum bolus dose (9 mg) used for the treatment of acute ischaemic stroke, it is logical to assume that the peak plasma concentration of rtPA in stroke patients will be no greater than that in patients treated for acute myocardial infarction. Moreover, the peak concentration observed in myocardial infarct patients occurs only briefly and not over several hours as in the current *in vitro* study. It is reasonable to suggest therefore that the intermediate concentration of rtPA in the present study ($14.3\,nmol\,l^{-1}$) is the most clinically relevant to the expected plasma concentration during most of the treatment period in patients with acute ischaemic stroke.

Exposure of thrombi to rtPA for 300 min far exceeds the 60 min duration of clinical treatment of acute ischaemic stroke with therapeutic concentrations of rtPA. Elimination of exogenously administered rtPA *in vivo* is rapid, with an initial half-life phase ($t_{1/2\alpha}$) of 4–5 min (Seifried *et al*., 1998; van Griensven *et al*., 1998; Kostis *et al*., 2002). Thirty minutes after cessation of treatment with rtPA, there will be essentially no exogenous rtPA activity in plasma (Grahame-Smith and Aronson, 1992). It is therefore noteworthy that there was no effect of NXY-059 on rtPA-induced lysis in the model thrombi preparation over an incubation period of 90 min even at the highest concentration of both compounds.

Importantly, the lowest concentration of NXY-059 used in this study, $600\,\mu mol\,l^{-1}$, exceeded the target concentration (bound plus unbound drug) in the SAINT 1 efficacy study (Lees *et al*., 2006) in ischaemic stroke by 38% and had no relevant effect on rtPA-induced lysis at any rtPA concentration over the 300 min time period. The higher concentrations of NXY-059 (2000 and $6000\,\mu mol\,l^{-1}$) exceeded the target concentration by respectively 3.3- and 10-fold.

To be clinically relevant, the following conditions would have to be met: (1) exposure to $71.5\,nmol\,l^{-1}$ of rtPA, a value that exceeds the maximum exposure in the clinic by 50% and exceeds the presumed steady-state clinical target by a factor of fivefold, (2) exposure to concentrations of NXY-059 that exceed the transient maximum plasma concentration in the clinic by a factor of more than threefold and (3) an exposure time to rtPA that exceeds not only the duration of clinical treatment with rtPA in acute ischaemic stroke, but also the elimination phase. The concurrence of all three conditions is considered unlikely. Therefore, these series of investigations demonstrated that NXY-059 did not alter the lytic properties of rtPA *in vitro* at the concentrations of either drug to which the patients were subsequently exposed in the clinical trial (Lees *et al*., 2006).

The use of the Chandler loop as described allows investigation to be made of the effect of drugs on the lytic properties of rtPA using physiologically relevant human thrombi. The results of the investigations with NXY-059 show that use of appropriate concentrations of drugs allows interpretation of the *in vitro* data in terms of possible clinical relevance. However, it is acknowledged that there are significant limitations in interpreting such data in terms of a lack of interaction *in vivo*. The fact that the study is performed *in vitro* means that any influence of the endothelium and the rheology of the flowing blood has been eliminated. Additional *in vivo* studies that factor in these parameters are therefore required before any final conclusion about a lack of interaction can be made. The methodological details and review of such approaches can be found elsewhere (Leadley *et al*., 2001; Rebello *et al*., 2001). Nevertheless, the possible value of the current *in vitro* approach as a simple screening method to assess a clinical interaction problem has recently been supported by the large (3200 patients) Phase III trial of NXY-059 in acute ischaemic stroke in which 44% of patients also received rtPA. In that study, no evidence was found to suggest that NXY-059 altered the incidence of symptomatic intracranial haemorrhage that occurred when patients were administered rtPA ($P = 0.56$). This suggests that NXY-059 was not altering the thrombolytic properties of rtPA (Shuaib *et al*., 2007). This clinical *in vivo* study thus validates our approach, being in accord with the *in vitro* observations presented here.

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Conflict of interest

CM, HJ and ARG are or were employees of AstraZeneca. The Chandler loop study was conducted at the University of Aberdeen with financial support from AstraZeneca.

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