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Simvastatin Improves Clinical Scores In A Rabbit Multiple Infarct Ischemic Stroke Model: Synergism With A ROCK Inhibitor, But Not The Thrombolytic Tissue Plasminogen Activator

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Abstract

Statins have pleiotropic neuroprotective effects in the central nervous system. In this study, we assessed the pharmacological effects of simvastatin on measures of behavior in New Zealand white rabbits embolized using a suspension of small-sized blood clots. For these studies, simvastatin was administered up to 3 hours following embolization, and behavior was measured 48 hours following embolization in order to calculate the dose of emboli (P50 in mg) that produces neurological deficits in 50% of the rabbits. A treatment is considered neuroprotective if it significantly increases the P₅₀ compared to control.

Simvastatin treatment (20mg/kg, bolus subcutaneous injection) significantly improved clinical function and increased the P₅₀ by 143% when administered 1 hour following embolization, but was ineffective at 3 hours. In combination studies with the thrombolytic, tissue plasminogen activator (tPA) using a standard intravenous dose of 3.3 mg/kg (20% bolus, 80% infused), we found that simvastatin could be safely administered with tPA to improve clinical scores, however the maximum behavioral improvement with the combination treatment was similar to either monotherapy alone, both of which significantly improved behavior ($p < 0.05$).

It has been proposed that Simvastatin neuroprotection may be related to a variety of signaling pathways including Rho-kinase (ROCK). To determine if a ROCK mechanism is involved in simvastatin-induced neuroprotection following embolic strokes, we used pharmacological intervention with the ROCK inhibitor, FASUDIL. When FASUDIL was administered 30 minutes prior to simvastatin (given at 1 hour), there was an additional significant ($p = 0.0217$) synergistic increase in behavioral function. However, FASUDIL as a monotherapy did not affect behavioral function in embolized rabbits. The study suggests that there may be an interaction between simvastatin treatment and the ROCK signaling pathway that should be further explored. Our results suggest that simvastatin treatment may have clinical benefit when used alone or in the presence of tPA, but the therapeutic window using a single dose regimen is narrow.

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Keywords

neuroprotection; embolism; acute ischemic stroke; clinical; behavior; toxicity; translational science

1. Introduction

Statins, 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors were initially designed as cholesterol-lowering drugs for the primary and secondary prevention of both coronary artery disease(CAD) and acute ischemic stroke(AIS) (Delanty and Vaughan, 1997; Hebert et al., 1997; Kashyap, 1997). According to the recent JUPITER report, more than 33.5 million Americans 50 year of age or older are currently taking a statin(Spatz et al., 2009). There has been some interest in using statins to treat ischemic and hemorrhagic stroke(Kramer et al., 2008; Meier et al., 2009; Montaner, 2005; Naval et al., 2008; Uyttenboogaart et al., 2008) because of the pleiotropic effects of statins, which include protective effects on the vascular compartment and neuroprotective effects on both neurons and glial cells(Bays, 2006; Endres, 2005; Halcox and Deanfield, 2004; Lapchak and Han, 2009; Liao, 2002; Moonis and Fisher, 2004; Rutishauser, 2006; Schmeer et al., 2006; Trinkl et al., 2006; Vivancos-Mora and Gil-Nunez, 2005; Waldman and Kritharides, 2003; Werner et al., 2002).

In animal models of stroke, in particular rodent stroke models, there has been some interesting, albeit confusing, observations made regarding the pharmacology and neuroprotective effects of high dose statins. The original study by Endres and colleagues(Endres et al., 1998) demonstrated that chronic 14 day pre-treatment with simvastatin at doses in the range of 0.2-20 mg/kg reduced infarct volume and decreased neurological score (i.e. improved neurological function) in mice following insertion of a silicone-coated monofilament into the internal carotid artery. Subsequently, Laufs et al. (Laufs et al., 2002) showed that rosuvastatin could also reduce infarct volume 50–56% in mice also using a monofilament occlusion model, and that the neuroprotective effect was independent of an effect on serum cholesterol and triglyceride levels. It has also been documented that atorvastatin may reduce cell death due to suppressed apoptosis and anti-inflammatory actions(Jung et al., 2004). Cimino and colleagues(Cimino et al., 2005) also found that simvastatin was protective and could reduce infarct volume when the drug was administered 3 hours after ischemia induced by electrocoagulation of the MCA. A post-ischemia effect of simvastatin (20mg/kg) on infarct volume was also demonstrated by Sironi et al.(Sironi et al., 2003). Prinz et al. showed that rosuvastatin could reduce infarct and improve volume if given by either an intravenous or intraperitoneal injection within 4 hours of filament occlusion(Prinz et al., 2008). However, Zhang et al.(Zhang et al., 2007) found that acute atorvastatin treatment had no effect on infarct volume when given 4 hours after a stroke, but the same group found that atorvastatin given daily for 2 weeks, starting 1 day after an MCAO, showed some functional recovery(Chen et al., 2005). In contrast, Shimamura et al. found no effect of fluvastatin on infarct volume when the initiation of treatment was delayed 7 days post MCAO(Shimamura et al., 2007) and treatment was continued for 2–3 months. Chen et al.(Chen et al., 2006) also showed that a 1 week treatment regimen of atorvastatin after a brain insult could improve functional outcome in “old” rats. Thus, the existing data from a series of diverse studies using various statins, treatment regimens and preclinical rodent models, suggest that there is a biological signal following statin treatment and that statins should be further pursued as a possible treatment strategy for AIS.

Taken together, the preclinical rodent stroke studies described above indicate that statins can either be used as an acute neuroprotective treatment or as a strategy to promote long-term recovery. In fact, based upon rodent data, the NeuSTART clinical trial studied the safety of lovastatin in stroke patients when administered within 24 hours of a stroke (Elkind et al., 2008; Elkind et al., 2009). However, even though a clinical trial has been designed and initiated, at this stage in the translational development of statins to treat AIS, neuroprotective effects of statins have yet to be demonstrated in a species other than rodent.

Thus, per STAIR criteria, which recommend that new treatments for stroke be studied in multiple species (STAIR, 1999), we studied the effects of the lipophilic statin, simvastatin in the rabbit small clot embolic stroke model (RSCM), which is a useful translational model and possibly a predictor of treatments that may show efficacy in human clinical trials (Lapchak, 2010a; Zivin et al., 1985). The RSCM was the preclinical animal model used in the development and FDA-approval of tissue plasminogen activator (tPA), the development profile which has recently been reviewed by Lapchak (Lapchak, 2010a). The RSCM uses embolization to cause multifocal cerebral ischemia, which results in graded behavioral deficits that can be quantified (Lapchak et al., 2008; Lapchak and Zivin, 2009; Lapchak, 2010b). The primary endpoint in the RSCM is behavioral functional, which is based upon motor function components of the National Institutes of Health Stroke Scale (NIHSS) for stroke in humans (Broderick et al., 2000; Clark et al., 2000; Lyden et al., 1994; Lyden et al., 1999). While the RSCM is a superior translational model to study behavioral effects of pharmacological agents (Lapchak, 2010a), it is not a model where infarct volume can be measured using conventional techniques due to the presence of widespread ischemic cores in multiple brain areas, thus, the present study utilized functional analysis as the primary endpoint.

Using the RSCM, the present study tested the hypothesis that simvastatin applied as a single dose following embolization would attenuate embolism-induced behavioral deficits. Moreover, since optimal doses of tPA do not eliminate all brain damage, even though tPA does increase cerebral reperfusion (reviewed in (Lapchak, 2002)), we investigated the interaction between tPA and simvastatin to determine whether there are any positive or synergistic interactions when the drugs are combined. Last, we studied a potential proposed mechanism of action of simvastatin in the RSCM, by testing simvastatin in the presence of the Rho-kinase (ROCK) inhibitor FASUDIL, since it has previously been suggested that a ROCK pathway may mediate the effects of simvastatin (Ma et al., 2009).

2. Results

2.1 Simvastatin Therapeutic Window Analysis

For therapeutic window studies, simvastatin was acutely administered to rabbits as a single injection up to 3 hours following embolization using a previously published dose of simvastatin (Lapchak and Han, 2009; Laufs et al., 2002; Sugawara et al., 2008a). For this series of experiments, we used a cumulative control group because we have previously documented that the baseline P_{50} value for embolized rabbits is quite consistent over time (Lapchak et al., 2008; Lapchak and Zivin, 2009; Lapchak, 2010b) and the use of the cumulative control allows us to reduce the usage of large numbers of rabbits. Simvastatin was beneficial and improved behavior and P_{50} values when given 5 minutes and 1 hour following embolization, P_{50} values of 2.05 ± 0.31 ($n=20$, $p<0.05$) and $P_{50}=2.39 \pm 0.23$ mg ($n=21$, $p<0.05$), respectively, but not 3 hours following embolization [$P_{50}=1.49 \pm 0.45$ mg ($n=24$, $p>0.05$)], compared to the vehicle control group, which had a P_{50} value of 0.95 ± 0.18 mg ($n=32$). Figure 1 shows a graphical representation of quantal analysis curves for the 1 hour treatment study. Raw behavioral data is superimposed on the theoretical statistically fit quantal analysis curves. The triangles are raw data for the simvastatin group, whereas the

circles are raw data points for the vehicle control group. Figure 2 provides the therapeutic window profile for simvastatin in rabbits following embolic strokes when given up to 3 following embolization as an acute treatment.

2.2 Simvastatin/tPA Combination Studies

In this series of studies, we examined the effect of administration of simvastatin in combination with tPA to determine whether the combination would have an additive or synergistic effect on behavioral outcome. In previous studies, we have found that combination therapy could be administered safely to provide substantial benefit (Lapchak et al., 2002; Lapchak et al., 2004; Lapchak, 2006; Lapchak, 2007). Using an add-on design, quantal analysis curves for tPA and the Simvastatin/tPA combination were constructed. tPA significantly improved behavior and increased the group P_{50} value of 2.96 ± 0.41 mg (n=19, $p<0.05$) compared to the vehicle control group (Figure 3). In combination studies, when both simvastatin and tPA were administered 1 hour following embolization, the group P_{50} value measured 24hour following embolization was 2.66 ± 0.31 mg (n=22), a P_{50} that was significantly different from control ($p<0.05$). There was no synergistic or additive effect of the drug combination when used at their maximum therapeutic window.

2.3 Simvastatin/FASUDIL Combination Studies

In this series of studies, we determined if simvastatin-induced neuroprotection and behavioral improvements were sensitive to intravenously administered FASUDIL, a Rho-kinase (ROCK) inhibitor, since previous studies have suggested that simvastatin pretreatment may suppress Rho signaling (Relja et al., 2009; Wang et al., 2006). However, the acute effects of a ROCK inhibitor in combination with simvastatin have not previously been studied in a preclinical stroke model. FASUDIL administered 30 minutes following embolization had no effect on behavior measured 48 hours later (Figure 4); the P_{50} value was 1.18 ± 0.15 mg (n=17), whereas the control group P_{50} value was 1.09 ± 0.23 mg (n=20). In combination studies, when both simvastatin and FASUDIL were administered following embolization, the group P_{50} value measured 24hour following embolization was 3.42 ± 0.21 mg (n=18), a P_{50} that was significantly different from control ($p<0.05$) and significantly different from simvastatin alone ($p=0.0217$). The behavioral effect of FASUDIL given in combination with simvastatin was synergistic (an additional 42% increase) compared to simvastatin alone, which produced a 121% increase in the P_{50} value.

3. Discussion

In the present study, we studied the effects of single bolus dose administration of simvastatin on behavioral function in rabbits following cerebral ischemia induced by injecting a flurry of small-sized blood clots into the cerebral vasculature. The study shows that simvastatin administration is neuroprotective in embolized rabbits when administered following the insult. Moreover, when simvastatin was applied following treatment with a ROCK inhibitor, there was a synergistic effects of the 2 drugs suggesting that a ROCK signaling pathway may be involved in the behavioral improvement following embolic strokes.

Our data is an important confirmation of neuroprotection and extension of previous preclinical studies using various rodent MCA models (Cimino et al., 2005; Endres et al., 1998; Laufs et al., 2002; Prinz et al., 2008) and is the first translational study using non-rodent research animals. Even though we have found that simvastatin improved clinical function, it must be pointed out that there are many differences between the rodent and rabbit studies. First, in the majority of rodent studies, other forms of statins, such as atorvastatin, fluvastatin and rosuvastatin were used. While they are all primarily 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, there may be pharmacological

differences between different types of statin compounds, which may be related to structure, lipophilicity and dosing regimens. For instance, Karki(Karki et al., 2009) et al showed that there were quantitative difference between the effects of atorvastatin and simvastatin on histological measures following a stroke when both drugs were administered chronically at the same dose (2mg/kg). Moreover, Sironi and colleagues found that simvastatin was not protective in stroke prone rats, while rosuvastatin was neuroprotective, when anti-inflammatory effects of the statins were studied(Sironi et al., 2005). Maeda (Maeda et al., 2010) showed that some pharmacological effects of statins directly correlate with the ability of the statin to cross membranes(i.e. comparing lipophilic atorvastatin with hydrophilic pravastatin). Last, even in clinical studies, differences have been noted. In the IDEAL study, Tikkanen(Tikkanen et al., 2009) clearly showed that the effects of atorvastatin (80 mg) were similar to simvastatin (20–40 mg) using cardiovascular endpoints, but the doses required to achieve the similar results were very different. Thus, based upon the cited examples, it is possible that the extent or efficacy of the secondary pharmacological actions of statins would also be related to structure, BBB penetration and dosing regimens.

Numerous studies have found that pre-stroke administration of statins produced a neuroprotective effect either on infarct volume, behavior or both endpoints(Endres et al., 1998; Nicholas et al., 2008; Shabanzadeh et al., 2005; Trinkl et al., 2006). However, in clinically applicable studies where treatment was delayed, Cimino found that statin was effective within 3 hours following a stroke(Cimino et al., 2005). Sironi et al(Sironi et al., 2003), also found a therapeutic effect when simvastatin was administered for 3 consecutive days after permanent MCAO. In contract to studies cited above, the Zhang studies(Zhang et al., 2007; Zhang et al., 2009) found there was no neuroprotective effect of the statin at 4 hours(Liu et al., 2006; Zhang et al., 2005; Zhang et al., 2009). Our current study shows that there is a beneficial effect of simvastatin if applied as a single dose bolus injection within 1 hour of an embolic stroke, but not thereafter. The observation that the therapeutic window is shorter in the rabbit embolic stroke model compared to the rodent models, using the single high dose treatment regimen is not surprising. In previous studies, which have been reviewed in the scientific literature (Bath et al., 2009; Lapchak and Araujo, 2007; Lapchak, 2010a), it appears that efficacy and therapeutic windows derived from rodent models may overestimate the translational efficacy in stroke patients. Nevertheless, the data, taken together, supports the observation that statins are neuroprotective and should be further pursued in randomized double-blind clinical trials.

It is important to distinguish between the pharmacological effects of chronic statin treatment in humans as a means of attempting to prevent CAD and AIS, which typically use statin doses in the range of 5–80mg per person as opposed to the higher doses of statins that are required to exert a neuroprotective effects in animal model of AIS. Low dose statin therapy is effective at inhibiting HMG-CoA reductase by hindering the access of the substrate HMG-CoA to the enzyme. The enzyme inhibition constant (K_i) is approximately 1nM for statins(Istvan, 2003) including Type 1 statins such as simvastatin which exhibit binding to the reductase via a decalin ring structure, whereas the majority of preclinical studies show that statin doses in the range of 2–20 mg/kg are required for neuroprotective effects. This suggests that the “mechanisms” involved in statin-induced neuroprotection, and behavioral improvement as is the case in this study, are less sensitive to statin than the primary original pharmacological action of statins.

Since tPA is the first line of treatment for stroke patients(Bluhmki et al., 2009; Lansberg et al., 2009; Lapchak, 2010a), based upon the safety and efficacy profile of simvastatin in combination with tPA in this study and our previous study (Lapchak and Han, 2009), the clinical trial could include a simvastatin-tPA arm. Moreover, recent rodent studies with atorvastatin in combination with tPA (i.e.: atorvastatin at 4 h, followed by tPA at 6h) have

purported(Zhang et al., 2009) that the statin can dramatically extend the therapeutic window for tPA possibly by enhanced dissolution of the embolus at the origin of the MCA, and improving microvascular patency while promoting cell survival. The same group showed that combining atorvastatin and tPA (Zhang et al., 2005) at 4 hours following MCA occlusion could have the same effect. All studies taken together suggest that statins can be used in combination with tPA, either to promote neuroprotection (this study), increase the therapeutic window for tPA(Zhang et al., 2005; Zhang et al., 2009), or reduce tPA-induced intracerebral hemorrhage(Lapchak and Han, 2009). However, the dosing regimen and administration time required for a beneficial effect in AIS patients remains to be established.

The clinical benefit of simvastatin therapy given post-embolization may be attributed to the statins effect on one or more signaling pathways in brain, effects that are unrelated to its primary effect of reducing serum lipids and inhibiting cholesterol production(Chmielewski et al., 2002; Corsini et al., 1999; Kolovou et al., 2008). The benefit may be associated with one of the following molecular pathways: Rho kinase (ROCK) (Ma et al., 2009; Wang et al., 2006), PI3K/Akt (Sugawara et al., 2008b), or JAK-STAT signaling pathway(Wu et al., 2006). Based upon the report by Ma and colleagues(Ma et al., 2009), which suggested that a ROCK pathway may mediate the effects of simvastatin, we tested the hypothesis that combination therapy with simvastatin and a ROCK inhibitor (FASUDIL) would further enhance the efficacy of simvastatin using behavior as a measure. In the study, we found that combination therapy was synergistic, although FASUDIL was ineffective as a monotherapy at the dose used in the study. The lack of efficacy of monotherapy may be related to the compartment-specific (vascular) effects of the hydrophilic ROCK inhibitor used in the study(Davies et al., 2000) or that the ROCK pathway did not lead to a critical pathophysiological cascade in this model, even though ROCK has been suggested as a viable target for neurodegenerative diseases(Mueller et al., 2005). This is in contrast to the study of Toshima and colleagues who found that fasudil improved neurological deficits following sodium laurate-induced microemboli in pentobarbital anesthetized rodents(Toshima et al., 2000). Nevertheless, the effects of the combination therapy suggest that ROCK may be a possible simvastatin mechanism of neuroprotection in the embolic stroke model. However, it is possible that in the presence of simvastatin, the ROCK inhibitor effect was unmasked, which resulted in pharmacological synergism. Since both simvastatin(van der Most et al., 2009) and FASUDIL(Olson, 2008) are compounds with multiple diverse mechanisms of action, it is possible that multiple cellular mechanisms may be involved in brain neuroprotection. There is substantial literature suggesting that many other mechanisms may contribute to the neuroprotective effects of statins(van der Most et al., 2009). Not only are the above mentioned signaling pathways involved, but there is a role for nitric oxide(Laufs et al., 2002), tumor necrosis factor(Dolga et al., 2008), and nuclear factor-kappa B(Dolga et al., 2008) in statin-induced neuroprotection (van der Most et al., 2009). Clearly, now that we have established simvastatin and ROCK inhibitor effects, additional studies are required to elucidate other pathways that may be involved in the pharmacological effects of simvastatin in the embolic stroke model.

Many of the preclinical studies support the study of statins in AIS patients, and this is being done in the NeuSTART trials(Elkind et al., 2008; Elkind et al., 2009), but trials should be designed to take into consideration the results of the SPARCL trial. The SPARCL trial tested the effects of atorvastatin (80mg per day) in patients with a history of recent stroke or transient ischemic attack. The trial showed that atorvastatin reduced the overall incidence of strokes and cardiovascular events, but caused an increase in hemorrhage (Amarenco et al., 2006). Goldstein et al (Goldstein et al., 2008) showed that there was a correlation between hemorrhagic stroke reports in patients with a hemorrhagic stroke as an entry event into the trial and it also was higher in patients with Stage 2 hypertension(JNC-7). Select exclusion criteria to include the above are advised in future statin stroke trials.

In conclusion, we have demonstrated that simvastatin administered following small clot embolic strokes can significantly improve clinical behavioral. Our pharmacological data suggests that a ROCK pathway may be involved in the beneficial effects of simvastatin following a stroke, because we were able to demonstrate synergism when embolized rabbits were treated with both simvastatin and FASUDIL. Last, we have shown that simvastatin can be safely administered with the current first line of stroke therapy, tPA, although there was no additional beneficial effect of the combination therapy at the time point studied.

It is important to emphasize that there was no detrimental effect of combining simvastatin with tPA and our previous study showed that simvastatin may also protect the vasculature and block tPA-induced intracerebral hemorrhage (Lapchak and Han, 2009). Overall, our study suggests that lipophilic statins may be effective neuroprotective treatments for embolic stroke to improve clinical function, however, the optimal treatment regimen for use within the longest therapeutic window remains to be established.

4. Experimental Procedures

4.1 Animals and animal welfare

Male New Zealand white rabbits weighing 2 to 2.5 Kg were purchased from Rabbit Source Farms (Ramona, CA). Rabbits were supplied food (alfalfa cubes) and water ad libitum while under quarantine in an enriched environment for at least 5 days prior to experimental use. Surgery was done in a sterile controlled environment with a room temperature between 22.8–23.2°C. Institutional Animal Care and Use Committee (IACUC) approved the surgical and treatment procedures used in this study. Extreme care prior to and post-surgery and embolization was used to minimize adverse effects in study rabbits. However, per the IACUC-approved protocol, rabbits were euthanized if they displayed extreme discomfort, or if they were unable to reach food or water post-embolization.

4.2 Surgery and embolization

Surgical procedures were done as described previously (Lapchak and Han, 2009; Lapchak and Zivin, 2009; Lapchak and De Taboada, 2010). Briefly, rabbits were anesthetized with isoflurane in oxygen via a face-mask, 5% in 3 l/min at induction, and 2-3% in 3 l/min as a maintenance dose and placed on a QuantumHeat™ heat-pack. The right internal carotid artery was exposed, and the external carotid artery and the common carotid artery were ligated. A Becton, Dickinson and Company (B-D) plastic catheter oriented toward the brain was inserted into the common carotid artery positioned toward the brain and secured with ligatures. The incision was closed around the catheter so that the distal end was accessible outside. The catheter was filled with 0.2 ml of heparinized saline (33 units/ml) and plugged with injection caps. The animals were allowed to recover from anesthesia for at least 2–3 hours before embolization and were normothermic during the process.

Blood is drawn from one or more donor rabbits and allowed to clot for 3h at 37°C. The large blood clots are then suspended in phosphate-buffered saline pH 7.4 (PBS) with 0.1% bovine serum albumin (BSA) and Polytron-generated fragments are sequentially passed through sizing screens. Small-sized blood clots (100–250µm) were suspended in PBS containing 1% bovine serum albumin and were then spiked with ⁵⁷Co-labeled microspheres (Perkin-Elmer Inc.) to allow for tracking into the brain. An aliquot of the solution was removed for the determination of specific activity. For embolization, rabbits are placed in a restrainer and 1 ml of a clot particle suspension is injected through carotid catheter, which is then flushed with 5 ml of sterile saline. Rabbits are fully awake during the embolization procedure and they are self-maintaining (i.e.: they do not require artificial respiration or other external support). This allows for immediate observation of the effects of embolization on behavior

at the time of embolus injection and thereafter. After the embolization process was completed, the catheter was heat ligated close to the neck leaving a small portion protruding from the skin.

4.3 Clinical Rating Score Behavioral and Quantal Dose-Response Analysis

The use of clinical rating scores is an appropriate primary endpoint to use when a treatment is being developed to support a clinical trial. Clinical scores in combination with quantal analysis is a sophisticated statistical analysis method to determine how a large population of stroke “patients” in this case, rabbits, will respond to a treatment (Lapchak and Han, 2009; Lapchak and Zivin, 2009; Lapchak and De Taboada, 2010). To evaluate the quantitative relationship between clot dose lodged in brain and behavioral deficits or clinical scores, logistic sigmoidal (S-shaped) quantal analysis curves were fit to dose-response data as originally described by Waud (Waud, 1972) and thereafter. To construct a quantal analysis curve, a wide range of clot doses were injected in this study in order to produce a spectrum of behaviorally normal to abnormal animals, which included death on the continuum of embolization-induced effects. In the absence of a neuroprotective treatment regimen, small numbers of microclots cause no grossly apparent neurologic dysfunction. However, when large numbers of microclots are injected, they invariably caused encephalopathy. In this study, less than 5% of animals died due to embolization, but in the event that embolization did result in a rapid death, there was a positive correlation with a high clot dose measured in brain and the animal was represented as an abnormal (or dead) on the quantal analysis curve if the rabbit received treatment. Abnormal rabbits with encephalopathy include those with one or more of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of limb/ facial sensation and occasionally, hind-limb paraplegia. Using a simple dichotomous rating system, with a reproducible composite result and low inter-rater variability (<5%), each animal was rated as either behaviorally normal or abnormal by an observer naïve to the study design and/or treatment groups.

Using quantal analysis, we are to detect behavioral changes following pharmacological intervention. A separate quantal curve was generated for each treatment condition and a statistically significant increase in the P_{50} value (or the amount of blood clots in brain that produce neurologic dysfunction in 50% of a group of animals) compared to a control curve is indicative of neuroprotection or a behavioral improvement in the study population.

4.4 Drug Treatment

For test substance administration, rabbits were placed in a Plexiglas restrainer (Plaslabs Inc.) for the duration of the treatment. For all experiments in this study, rabbits were randomly allocated into treatment groups before the embolization procedure, with concealment of the randomization guaranteed by using an independent party. The randomization sequence was not revealed until all postmortem analyses were complete.

(A) Therapeutic window analysis—For therapeutic window analysis, following embolization, rabbits were given a bolus SC injection of vehicle (100% HPLC grade DMSO, Fisher Sci.) or unmodified simvastatin [20mg/kg simvastatin (Lapchak and Han, 2009; Laufs et al., 2002; Sironi et al., 2003; Sugawara et al., 2008a)] at the nape of the neck. Pharmaceutical grade (United States Pharmacopeia) simvastatin was a gift to MKH from Chong Kun Dang Pharmaceutical Co., Ltd (Seoul, South Korea).

(B) Thrombolytic Combination studies—For thrombolytic studies, tPA (3.3mg/kg) was given IV 1 hour post-embolization using the marginal ear vein for administration, with 20% as a bolus injection over one minute, followed by the remaining 80% infused over 30 min (Lapchak and Han, 2009; Lapchak, 2010b). Clinical grade tPA lyophilized in 50 mg

configurations, containing 50 mg tPA (29 million IU), 1.7mg L-arginine, 0.5g phosphoric acid and less than 4 mg Polysorbate 80, that was then reconstituted with sterile water at a dose of 1 mg/ml was purchased from Genentech, Inc. (South San Francisco, CA) for the studies.

(C) ROCK Inhibitor Interaction Studies—To test whether Rho-kinase (ROCK) was involved in the neuroprotective effect of simvastatin, we used the ROCK inhibitor FASUDIL monohydrochloride salt (purchased from LCL, USA) dissolved in saline at a dose of 10 mg/kg (Kondoh et al., 1991) for IV injection. FASUDIL was given 30 minutes prior to simvastatin treatment in the mechanism of action combination study to ensure distribution. In a separate group of rabbits, FASUDIL was also administered in the absence of simvastatin to determine the effects of the drug alone.

4.5 Power and Statistical Analysis

Power analysis of quantal dose-response curves indicates that, assuming $\alpha=.05$ and $\beta=.90$, a coefficient of variation of 15% and a difference between means of 20%, a sample size of 14 animals are required per group. Our previous experience is that we actually need 18-20 animals per group due to premature loss caused by technical difficulties or death following embolization. Animals that do not survive until treatment is given were excluded from the final analysis. The behavioral data are presented as P_{50} (Mean $\pm$ SEM) in mg clot for the number of rabbits in each group (n), which were calculated using an iterative curve fitting algorithm described previously (Waud, 1972). They were analyzed for significance using the t-test with SigmaStat 3.5.

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5. Literature Cited

- Amarenco P, Bogousslavsky J, Callahan A 3rd, Goldstein LB, Hennerici M, Rudolph AE, Sillese H, Simunovic L, Szarek M, Welch KM, Zivin JA. High-dose atorvastatin after stroke or transient ischemic attack. *N Engl J Med*. 2006; 355:549–59. [PubMed: 16899775]
- Bath PM, Gray LJ, Bath AJ, Buchan A, Miyata T, Green AR. Effects of NXY-059 in experimental stroke: an individual animal meta-analysis. *Br J Pharmacol*. 2009; 157:115–71.
- Bays H. Statin safety: an overview and assessment of the data--2005. *Am J Cardiol*. 2006; 97:6C–26C.
- Bluhmki E, Chamorro A, Davalos A, Machnig T, Sauce C, Wahlgren N, Wardlaw J, Hacke W. Stroke treatment with alteplase given 3.0–4.5 h after onset of acute ischaemic stroke (ECASS III): additional outcomes and subgroup analysis of a randomised controlled trial. *Lancet Neurol*. 2009; 8:1095–102. [PubMed: 19850525]
- Broderick JP, Lu M, Kothari R, Levine SR, Lyden PD, Haley EC, Brott TG, Grotta J, Tilley BC, Marler JR, Frankel M. Finding the most powerful measures of the effectiveness of tissue plasminogen activator in the NINDS tPA stroke trial. *Stroke*. 2000; 31:2335–41. [PubMed: 11022060]
- Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, Katakowski M, Lu M, Chopp M. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab*. 2005; 25:281–90. [PubMed: 15678129]
- Chen J, Zacharek A, Li A, Zhang C, Ding J, Roberts C, Lu M, Kapke A, Chopp M. Vascular endothelial growth factor mediates atorvastatin-induced mammalian achaete-scute homologue-1 gene expression and neuronal differentiation after stroke in retired breeder rats. *Neuroscience*. 2006; 141:737–44. [PubMed: 16730914]

- Chmielewski M, Zdrojewski Z, Rutkowski B. Benefits and menaces related to the use of statins in patients after renal transplantation. *Ann Transplant*. 2002; 7:6–10. [PubMed: 12416466]
- Cimino M, Balduini W, Carloni S, Gelosa P, Guerrini U, Tremoli E, Sironi L. Neuroprotective effect of simvastatin in stroke: a comparison between adult and neonatal rat models of cerebral ischemia. *Neurotoxicology*. 2005; 26:929–33. [PubMed: 15923039]
- Clark WM, Albers GW, Madden KP, Hamilton S. The rtPA (alteplase) 0- to 6-hour acute stroke trial, part A (A0276g) : results of a double-blind, placebo-controlled, multicenter study. Thrombolytic therapy in acute ischemic stroke study investigators. *Stroke*. 2000; 31:811–6. [PubMed: 10753980]
- Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol Ther*. 1999; 84:413–28. [PubMed: 10665838]
- Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000; 351:95–105. [PubMed: 10998351]
- Delanty N, Vaughan CJ. Vascular effects of statins in stroke. *Stroke*. 1997; 28:2315–20. [PubMed: 9368582]
- Dolga AM, Nijholt IM, Ostroveanu A, Ten Bosch Q, Luiten PG, Eisel UL. Lovastatin induces neuroprotection through tumor necrosis factor receptor 2 signaling pathways. *J Alzheimers Dis*. 2008; 13:111–22. [PubMed: 18376053]
- Elkind MS, Sacco RL, MacArthur RB, Fink DJ, Peerschke E, Andrews H, Neils G, Stillman J, Corporan T, Leifer D, Cheung K. The Neuroprotection with Statin Therapy for Acute Recovery Trial (NeuSTART): an adaptive design phase I dose-escalation study of high-dose lovastatin in acute ischemic stroke. *Int J Stroke*. 2008; 3:210–8. [PubMed: 18705902]
- Elkind MS, Sacco RL, Macarthur RB, Peerschke E, Neils G, Andrews H, Stillman J, Corporan T, Leifer D, Liu R, Cheung K. High-dose lovastatin for acute ischemic stroke: results of the phase I dose escalation neuroprotection with statin therapy for acute recovery trial (NeuSTART). *Cerebrovasc Dis*. 2009; 28:266–75. [PubMed: 19609078]
- Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, Liao JK. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*. 1998; 95:8880–5. [PubMed: 9671773]
- Endres M. Statins and stroke. *J Cereb Blood Flow Metab*. 2005; 25:1093–110. [PubMed: 15815580]
- Goldstein LB, Amarenco P, Szarek M, Callahan A 3rd, Hennerici M, Sillesen H, Zivin JA, Welch KM. Hemorrhagic stroke in the Stroke Prevention by Aggressive Reduction in Cholesterol Levels study. *Neurology*. 2008; 70:2364–70. [PubMed: 18077795]
- Halcox JP, Deanfield JE. Beyond the laboratory: clinical implications for statin pleiotropy. *Circulation*. 2004; 109:II42–8. [PubMed: 15173062]
- Hebert PR, Gaziano JM, Chan KS, Hennekens CH. Cholesterol lowering with statin drugs, risk of stroke, and total mortality. An overview of randomized trials. *Jama*. 1997; 278:313–21. [PubMed: 9228438]
- Istvan E. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. *Atheroscler Suppl*. 2003; 4:3–8. [PubMed: 12714031]
- Jung KH, Chu K, Jeong SW, Han SY, Lee ST, Kim JY, Kim M, Roh JK. HMG-CoA reductase inhibitor, atorvastatin, promotes sensorimotor recovery, suppressing acute inflammatory reaction after experimental intracerebral hemorrhage. *Stroke*. 2004; 35:1744–9. [PubMed: 15166393]
- Karki K, Knight RA, Han Y, Yang D, Zhang J, Ledbetter KA, Chopp M, Seyfried DM. Simvastatin and Atorvastatin Improve Neurological Outcome After Experimental Intracerebral Hemorrhage. *Stroke*. 2009
- Kashyap ML. Cholesterol and atherosclerosis: a contemporary perspective. *Ann Acad Med Singapore*. 1997; 26:517–23. [PubMed: 9395824]
- Kolovou GD, Katerina A, Ioannis V, Cokkinos DV. Simvastatin: two decades in a circle. *Cardiovasc Ther*. 2008; 26:166–78. [PubMed: 18485137]
- Kondoh Y, Mizusawa S, Murakami M, Nagata K, Nakamichi H, Watanabe K. Effects of HA1077, an intracellular calcium antagonist, on neurotransmitter metabolism in rat brain in vivo. *Metab Brain Dis*. 1991; 6:111–24. [PubMed: 1779923]

- Kramer AH, Gurka MJ, Nathan B, Dumont AS, Kassell NF, Bleck TP. Statin use was not associated with less vasospasm or improved outcome after subarachnoid hemorrhage. *Neurosurgery*. 2008; 62:422–7. discussion 427–30. [PubMed: 18382320]
- Lansberg MG, Bluhmki E, Thijs VN. Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a metaanalysis. *Stroke*. 2009; 40:2438–41. [PubMed: 19478213]
- Lapchak PA. Development of thrombolytic therapy for stroke: a perspective. *Expert Opin Investig Drugs*. 2002; 11:1623–1632.
- Lapchak PA, Araujo DM, Song D, Wei J, Zivin JA. Neuroprotective effects of the spin trap agent disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (generic NXY-059) in a rabbit small clot embolic stroke model: combination studies with the thrombolytic tissue plasminogen activator. *Stroke*. 2002; 33:1411–5. [PubMed: 11988623]
- Lapchak PA, Song D, Wei J, Zivin JA. Coadministration of NXY-059 and tenecteplase six hours following embolic strokes in rabbits improves clinical rating scores. *Exp Neurol*. 2004; 188:279–85. [PubMed: 15246827]
- Lapchak PA. 3alpha-OL-5-beta-pregnan-20-one hemisuccinate, a steroidal low-affinity NMDA receptor antagonist improves clinical rating scores in a rabbit multiple infarct ischemia model: synergism with tissue plasminogen activator. *Exp Neurol*. 2006; 197:531–7. [PubMed: 16300757]
- Lapchak PA. The phenylpropanoid micronutrient chlorogenic acid improves clinical rating scores in rabbits following multiple infarct ischemic strokes: synergism with tissue plasminogen activator. *Exp Neurol*. 2007; 205:407–13. [PubMed: 17439814]
- Lapchak PA, Araujo DM. Advances in ischemic stroke treatment: neuroprotective and combination therapies. *Expert Opin Emerg Drugs*. 2007; 12:97–112. [PubMed: 17355216]
- Lapchak PA, Kirkeby AG, Zivin JA, Sager TN. Therapeutic window for nonerythropoietic carbamylated-erythropoietin to improve motor function following multiple infarct ischemic strokes in New Zealand white rabbits. *Brain Res*. 2008
- Lapchak PA, Han MK. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin reduces thrombolytic-induced intracerebral hemorrhage in embolized rabbits. *Brain Res*. 2009; 1303:144–50. [PubMed: 19781532]
- Lapchak PA, Zivin JA. The lipophilic multifunctional antioxidant edaravone (radicut) improves behavior following embolic strokes in rabbits: a combination therapy study with tissue plasminogen activator. *Exp Neurol*. 2009; 215:95–100. [PubMed: 18851964]
- Lapchak PA. Translational stroke research using a rabbit embolic stroke model: a correlative analysis hypothesis for novel therapy development. *Translational Stroke Research*. 2010a10.1007/s12975-010-0018-4
- Lapchak PA. Efficacy and safety profile of the carotenoid trans sodium crocetin administered to rabbits following multiple infarct ischemic strokes: a combination therapy study with tissue plasminogen activator. *Brain Res*. 2010b; 1309:136–45. [PubMed: 19891959]
- Lapchak PA, De Taboada L. Transcranial near infrared laser treatment (NILT) increases cortical adenosine-5'-triphosphate (ATP) content following embolic strokes in rabbits. *Brain Res*. 2010; 1306:100–5. [PubMed: 19837048]
- Laufs U, Gertz K, Dirnagl U, Bohm M, Nickenig G, Endres M. Rosuvastatin, a new HMG-CoA reductase inhibitor, upregulates endothelial nitric oxide synthase and protects from ischemic stroke in mice. *Brain Res*. 2002; 942:23–30. [PubMed: 12031849]
- Liao JK. Statins and ischemic stroke. *Atheroscler Suppl*. 2002; 3:21–5. [PubMed: 12044582]
- Liu XS, Zhang ZG, Zhang L, Morris DC, Kapke A, Lu M, Chopp M. Atorvastatin downregulates tissue plasminogen activator-aggravated genes mediating coagulation and vascular permeability in single cerebral endothelial cells captured by laser microdissection. *J Cereb Blood Flow Metab*. 2006; 26:787–96. [PubMed: 16177809]
- Lyden P, Brott T, Tilley B, Welch KM, Mascha EJ, Levine S, Haley EC, Grotta J, Marler J. Improved reliability of the NIH Stroke Scale using video training. NINDS TPA Stroke Study Group. *Stroke*. 1994; 25:2220–6. [PubMed: 7974549]
- Lyden P, Lu M, Jackson C, Marler J, Kothari R, Brott T, Zivin J. Underlying structure of the National Institutes of Health Stroke Scale: results of a factor analysis. NINDS tPA Stroke Trial Investigators. *Stroke*. 1999; 30:2347–54. [PubMed: 10548669]

- Ma T, Zhao Y, Kwak YD, Yang Z, Thompson R, Luo Z, Xu H, Liao FF. Statin's excitoprotection is mediated by sAPP and the subsequent attenuation of calpain-induced truncation events, likely via rho-ROCK signaling. *J Neurosci*. 2009; 29:11226–36. [PubMed: 19741129]
- Maeda A, Yano T, Itoh Y, Kakumori M, Kubota T, Egashira N, Oishi R. Down-regulation of RhoA is involved in the cytotoxic action of lipophilic statins in HepG2 cells. *Atherosclerosis*. 2010; 208:112–8. [PubMed: 19683239]
- Meier N, Nedeltchev K, Brekenfeld C, Galimanis A, Fischer U, Findling O, Remonda L, Schroth G, Mattle HP, Arnold M. Prior statin use, intracranial hemorrhage, and outcome after intra-arterial thrombolysis for acute ischemic stroke. *Stroke*. 2009; 40:1729–37. [PubMed: 19265056]
- Montaner J. Treatment with statins in the acute phase of ischemic stroke. *Expert Rev Neurother*. 2005; 5:211–21. [PubMed: 15853491]
- Moonis M, Fisher M. HMG CoA reductase inhibitors (statins): use in stroke prevention and outcome after stroke. *Expert Rev Neurother*. 2004; 4:241–7. [PubMed: 15853565]
- Mueller BK, Mack H, Teusch N. Rho kinase, a promising drug target for neurological disorders. *Nat Rev Drug Discov*. 2005; 4:387–98. [PubMed: 15864268]
- Naval NS, Abdelhak TA, Zeballos P, Urrunaga N, Mirski MA, Carhuapoma JR. Prior statin use reduces mortality in intracerebral hemorrhage. *Neurocrit Care*. 2008; 8:6–12. [PubMed: 17701108]
- Nicholas JS, Swearingen CJ, Thomas JC, Rumboldt Z, Tumminello P, Patel SJ. The effect of statin pretreatment on infarct volume in ischemic stroke. *Neuroepidemiology*. 2008; 31:48–56. [PubMed: 18560243]
- Olson MF. Applications for ROCK kinase inhibition. *Curr Opin Cell Biol*. 2008; 20:242–8. [PubMed: 18282695]
- Prinz V, Laufs U, Gertz K, Kronenberg G, Balkaya M, Leithner C, Lindauer U, Endres M. Intravenous rosuvastatin for acute stroke treatment: an animal study. *Stroke*. 2008; 39:433–8. [PubMed: 18162625]
- Relja B, Lehnert M, Seyboth K, Bormann F, Hohn C, Czerny C, Henrich D, Marzi I. Simvastatin Reduces Mortality and Hepatic Injury after Hemorrhage/Resuscitation in Rats. *Shock*. 2009
- Rutishauser J. The role of statins in clinical medicine--LDL--cholesterol lowering and beyond. *Swiss Med Wkly*. 2006; 136:41–9. [PubMed: 16633945]
- Schmeer C, Kretz A, Isenmann S. Statin-mediated protective effects in the central nervous system: general mechanisms and putative role of stress proteins. *Restor Neurol Neurosci*. 2006; 24:79–95. [PubMed: 16720944]
- Shabanzadeh AP, Shuaib A, Wang CX. Simvastatin reduced ischemic brain injury and perfusion deficits in an embolic model of stroke. *Brain Res*. 2005; 1042:1–5. [PubMed: 15823246]
- Shimamura M, Sato N, Sata M, Kurinami H, Takeuchi D, Wakayama K, Hayashi T, Iida H, Morishita R. Delayed postischemic treatment with fluvastatin improved cognitive impairment after stroke in rats. *Stroke*. 2007; 38:3251–8. [PubMed: 17975105]
- Sironi L, Cimino M, Guerrini U, Calvio AM, Lodetti B, Asdente M, Balduini W, Paoletti R, Tremoli E. Treatment with statins after induction of focal ischemia in rats reduces the extent of brain damage. *Arterioscler Thromb Vasc Biol*. 2003; 23:322–7. [PubMed: 12588778]
- Sironi L, Gianazza E, Gelosa P, Guerrini U, Nobili E, Gianella A, Cremonesi B, Paoletti R, Tremoli E. Rosuvastatin, but not simvastatin, provides end-organ protection in stroke-prone rats by antiinflammatory effects. *Arterioscler Thromb Vasc Biol*. 2005; 25:598–603. [PubMed: 15681303]
- Spatz ES, Canavan ME, Desai MM. From Here to JUPITER: Identifying New Patients for Statin Therapy Using Data From the 1999-2004 National Health and Nutrition Examination Survey. *Circ Cardiovasc Qual Outcomes*. 2009; 2:41–48. [PubMed: 20031811]
- STAIR. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke*. 1999; 30:2752–8. [PubMed: 10583007]
- Sugawara T, Ayer R, Jadhav V, Chen W, Tsubokawa T, Zhang JH. Simvastatin attenuation of cerebral vasospasm after subarachnoid hemorrhage in rats via increased phosphorylation of Akt and endothelial nitric oxide synthase. *J Neurosci Res*. 2008a; 86:3635–43. [PubMed: 18683242]

- Sugawara T, Jadhav V, Ayer R, Zhang J. Simvastatin attenuates cerebral vasospasm and improves outcomes by upregulation of PI3K/Akt pathway in a rat model of subarachnoid hemorrhage. *Acta Neurochir Suppl.* 2008b; 102:391–4. [PubMed: 19388353]
- Tikkanen MJ, Holme I, Cater NB, Szarek M, Faergeman O, Kastelein JJ, Olsson AG, Larsen ML, Lindahl C, Pedersen TR. Comparison of efficacy and safety of atorvastatin (80 mg) to simvastatin (20 to 40 mg) in patients aged <65 versus ≥65 years with coronary heart disease (from the Incremental DEcrease through Aggressive Lipid Lowering [IDEAL] study). *Am J Cardiol.* 2009; 103:577–82. [PubMed: 19231315]
- Toshima Y, Satoh S, Ikegaki I, Asano T. A new model of cerebral microthrombosis in rats and the neuroprotective effect of a Rho-kinase inhibitor. *Stroke.* 2000; 31:2245–50. [PubMed: 10978059]
- Trinkl A, Vosko MR, Wunderlich N, Dichgans M, Hamann GF. Pravastatin reduces microvascular basal lamina damage following focal cerebral ischemia and reperfusion. *Eur J Neurosci.* 2006; 24:520–6. [PubMed: 16836638]
- Uyttenboogaart M, Koch MW, Koopman K, Vroomen PC, Luijckx GJ, De Keyser J. Lipid profile, statin use, and outcome after intravenous thrombolysis for acute ischaemic stroke. *J Neurol.* 2008; 255:875–80. [PubMed: 18338194]
- van der Most PJ, Dolga AM, Nijholt IM, Luiten PG, Eisel UL. Statins: mechanisms of neuroprotection. *Prog Neurobiol.* 2009; 88:64–75. [PubMed: 19428962]
- Vivancos-Mora J, Gil-Nunez AC. Lipids and stroke: the opportunity of lipid-lowering treatment. *Cerebrovasc Dis.* 2005; 20(Suppl 2):53–67. [PubMed: 16327254]
- Waldman A, Kritharides L. The pleiotropic effects of HMG-CoA reductase inhibitors: their role in osteoporosis and dementia. *Drugs.* 2003; 63:139–52. [PubMed: 12515562]
- Wang S, Lee SR, Guo SZ, Kim WJ, Montaner J, Wang X, Lo EH. Reduction of tissue plasminogen activator-induced matrix metalloproteinase-9 by simvastatin in astrocytes. *Stroke.* 2006; 37:1910–2. [PubMed: 16741180]
- Waud DR. On biological assays involving quantal responses. *Journal of Pharmacological Experimental Theory.* 1972; 183:577–607.
- Werner N, Nickenig G, Laufs U. Pleiotropic effects of HMG-CoA reductase inhibitors. *Basic Res Cardiol.* 2002; 97:105–16. [PubMed: 12002257]
- Wu L, Zhao L, Zheng Q, Shang F, Wang X, Wang L, Lang B. Simvastatin attenuates hypertrophic responses induced by cardiotrophin-1 via JAK-STAT pathway in cultured cardiomyocytes. *Mol Cell Biochem.* 2006; 284:65–71. [PubMed: 16534557]
- Zhang L, Zhang ZG, Ding GL, Jiang Q, Liu X, Meng H, Hozeska A, Zhang C, Li L, Morris D, Zhang RL, Lu M, Chopp M. Multitargeted effects of statin-enhanced thrombolytic therapy for stroke with recombinant human tissue-type plasminogen activator in the rat. *Circulation.* 2005; 112:348–694.
- Zhang L, Zhang ZG, Liu XS, Hozeska-Solgot A, Chopp M. The PI3K/Akt pathway mediates the neuroprotective effect of atorvastatin in extending thrombolytic therapy after embolic stroke in the rat. *Arterioscler Thromb Vasc Biol.* 2007; 27:2470–5. [PubMed: 17717296]
- Zhang L, Chopp M, Jia L, Cui Y, Lu M, Zhang ZG. Atorvastatin extends the therapeutic window for tPA to 6 h after the onset of embolic stroke in rats. *J Cereb Blood Flow Metab.* 2009; 29:1816–24. [PubMed: 19638998]
- Zivin JA, Fisher M, DeGirolami U, Hemenway CC, Stashak JA. Tissue plasminogen activator reduces neurological damage after cerebral embolism. *Science.* 1985; 230:1289–92. [PubMed: 3934754]

Figure 1. Quantal Curves

Effect of simvastatin (20 mg/kg) on clinical function or behavior measured following embolization. For the superimposed graphs, normal animals are plotted on the y-axis at 0 and abnormal animals are plotted at 100. The figure shows that there is positive correlation between the data [circles (vehicle) or triangles (simvastatin)] and the statistically fit sigmoidal quantal curve. Statistical significance (*p<0.05).

Figure 2. Therapeutic Window

P₅₀ (mg clot in brain) as a function of Time post-embolization (minutes). Results are shown as mean $\pm$ SEM (n= 20–32). Significantly different compared to the vehicle-treated control (*p<0.05).

Figure 3. Combination Analysis- Simvastatin & tPA

Abnormal rabbits as a function of clot weight measured in brain. Results are shown as mean $\pm$ SEM for the number of rabbits in each group (n). Both simvastatin (20 mg/kg) and tPA (3.3mg/kg) treatment 1 hour post-embolization significantly ($*p<0.05$) increased P₅₀ values. The combination of simvastatin (1 hour) plus tPA (1 hour) significantly ($*p<0.05$) improved behavior and increased P₅₀ values compared to control. The combination was not significantly different ($p<0.05$) from either simvastatin or tPA monotherapy.

Figure 4. Combination Analysis- Mechanism of Action

Abnormal rabbits as a function of clot weight measured in brain. Results are shown as mean $\pm$ SEM for the number of rabbits in each group (n). Simvastatin (20 mg/kg) treatment 1 hour post-embolization significantly (* $p < 0.05$) increased P_{50} values compared to control. The ROCK inhibitor FASUDIL (HA-1077, 10 mg/kg) was ineffective ($p > 0.05$) as a monotherapy. The combination of simvastatin plus FASUDIL, both administered 1 hour following embolization significantly improved behavior and increased P_{50} values compared to control (* $p < 0.05$) and there was a synergistic effects of the combination therapy (# $p = 0.0217$ compared to simvastatin).