Formulation and Delivery Mode Affect Disposition and Activity of Tyrphostin-Loaded Nanoparticles in the Rat Carotid Model


Abstract—Poor drug residence in the arterial wall hinders clinical implementation of local drug delivery strategies for the treatment of restenosis. A rat carotid model of vascular injury and intraluminal delivery of tyrphostin-containing polyactic acid (PLA) nanoparticles (NPs) were used to determine the relationship between residence properties and biological activity of different formulations and administration modes. The effects of delivery modes (denudation and delivery time) and formulation variables (adsorbed vs encapsulated drug, and NP size) on arterial drug/NP retention were examined. Antirestenotic effects of large (160 nm) and small (90 nm) tyrphostin-containing NPs, surface-absorbed tyrphostin, and systemic treatment were compared. Fluorescent NPs were used to study the spatial distribution of the carrier in the arterial wall. The decrease in arterial tyrphostin level over time fitted a biexponential model. Delivery time and pressure, endothelium integrity, particle size, and drug-polymer association affected local pharmacokinetics and the antirestenotic results after 14 days. The PLA-based tyrphostin NP formulation ensured a prolonged drug residence at the angioplasty site after single intraluminal application. Several readily adjustable formulation and procedural factors considerably modified arterial ingress of the drug-loaded NPs and governed their subsequent redistribution, tissue binding, elimination, and ensuing antirestenotic effect. (Arterioscler Thromb Vasc Biol. 2001;21:1434-1439.)

Key Words: restenosis ■ nanoparticles ■ local delivery ■ tyrphostins ■ controlled release

The main focus in treatment strategies targeting postinterventional vasculoproliferative disorders has recently shifted from systemic to locally delivered therapeutics. The latter approach has an important advantage of decreasing the total drug burden while greatly augmenting the drug concentration in the target site of the artery. Moreover, various polymer-based drug delivery systems allow for a prolonged drug presence in the delivery site, thus making possible the use of unstable drugs that are rapidly degraded when administered in the unprotected free form. Several groups have investigated the local pharmacokinetics of free8,9 and polymer-embedded10–14 drugs delivered to the balloon-injured arterial segments of animals. Other researchers have sought to determine the effects of particle size15–17 and surface characteristics17–20 on the interactions with the vessel wall. However, a systematic quantitative study of the pharmacokinetics after local drug delivery has not yet been described. Additionally, several important variables related to the delivery technique itself, such as delivery duration, pressure, and the degree of endothelial layer damage, were not previously addressed in the context of local arterial pharmacokinetics.

In the present study, the impact of the formulation and delivery procedure on arterial disposition and vasculoprotec-
tive activity of AG-1295, a tyrophostin compound with proven antirestenotic activity,4,21 was characterized after intramural delivery of AG-1295–containing nanoparticles (NPs) to the rat carotid artery.

Methods

Preparation of NPs

The detailed procedure of NP preparation is described elsewhere.4 The mean tyrophostin concentration in the final suspension of NPs was 530 µg/mL, with 1.5% (wt/wt) drug in the polymer. The NP size and size distribution were found to be 160±25 and 90±20 nm for larger and smaller particles, respectively.

Animal Procedures

Animals

Male rats of the Sabra strain weighing 350 to 420 g were obtained from Harlan Laboratories (Jerusalem, Israel). Animals were handled in accordance with the standards of the Hebrew University of Jerusalem, in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC, 1996). All in vivo experiments were made under general anesthesia (National Research Council, Washington, DC, Laboratory Animals 1996). All in vivo experiments were made under general anesthesia (National Research Council, Washington, DC, Laboratory Animals 1996).

Animal Procedures

Group A: Reperfusion Time

A 50-µL volume of NP suspension (160-nm average size) with an AG-1295 content of 350±50 µg/mL was delivered to the balloon-injured segment of the carotid artery. After a 15-minute delivery period (2 atm), the remainder of the suspension was evacuated (typically ~25 µL), and circulation across the injured segment was restored. The animals were euthanized by ether suffocation at 5 minutes (n=5), 90 minutes (n=9), 6 hours (n=4), 1 day (n=5), 7 days (n=6), and 14 days (n=8) after blood flow restoration.

Group B: Delivery Modes

Modifications of the basic technique (protocol for Group A) were as follows: Group B1, noninjected arteries (ie, without balloon injury [n=6]); Group B2, delivery times of 3 minutes (n=7) and 1 hour (n=5) for NP delivery duration; and Group B3, low-pressure delivery, ie, reduced pressure (0.5 atm) during drug delivery (n=5).

Group C: Encapsulated vs Adsorbed Drug

Rats were treated with empty NPs with AG-1295 adsorbed on their surface rather than encapsulated into the polymeric matrix (230 µg AG-1295 per mL of empty NP suspension). Rats treated with tyrophostin-adsorbed NPs were humanely killed at 5 minutes (n=4), 90 minutes (n=3), and 1 day (n=3) after blood flow restoration.

Group D: NP Size

Rats were treated with large (160±25 nm) and small (90±20 nm) AG-1295–containing NPs (530 µg of the drug per mL of the particle suspension). After completion of the standard injury/delivery protocol, the treated arterial segments were retrieved after 5 minutes (n=4), 90 minutes (n=4), 1 day (n=5), and 14 days (n=8).

Group E: Fluorescent NPs

To visualize NP localization in the arterial wall, NPs containing Nile Red (500 µg/mL) were formulated (the physicochemical properties of Nile Red are similar to those of AG-1295). After the standard balloon-injury procedure, rats were euthanized at 5 minutes (n=3), 1.5 hours (n=3), 1 day (n=3), 7 days (n=3), and 14 days (n=3) after delivery of Nile Red–containing NPs.

Group F: Encapsulated vs Free Fluorescent Marker

The arterial residence time of fluorescent NPs was compared with that of the free dye. Owing to the negligible solubility of AG-1295 and Nile Red in water, these experiments were performed with rhodamine B (150 µg/mL of suspension) and an equimolar solution of rhodamine B in phosphate-buffered saline. The rats were treated with both rhodamine B formulations according to the standard delivery protocol. The animals were humanely killed 90 minutes (n=6), 8 hours (n=6), and 24 hours (n=6) after delivery, and the tissues were processed as in protocol D.

Group G: Therapeutic Efficacy

The common carotid arteries of rats treated with different AG-1295 formulations were examined 2 weeks after balloon injury to assess the antirestenotic potential of the AG-1295–containing NPs. The animal groups were designated as follows: (1) local delivery of AG-1295–containing large NPs (530 µg/mL, n=10); (2) local delivery of AG-1295–containing small NPs (530 µg/mL, n=10); (3) local delivery of the empty, large NPs with adsorbed AG-1295 (230 µg/mL, n=10); (4) systemic administration of AG-1295 dissolved in polyethylene glycol-300 delivered by osmotic pumps (Alzet model 2 ML4, Alza Corp) at a dose of 250 µg/d (n=6); (5) local delivery of the empty, large NPs (n=10); and (6) no treatment (n=10). Morphometric procedures were performed as described previously.2-5

High-Performance Liquid Chromatography Assay

The arterial segments were lyophilized, weighed, and ground under LN2. After addition of the internal standard (AG-1296 21), the tissue powder was suspended in water and acetonitrile (2:3, vol/vol). The drug was extracted in chloroform and determined by an HPLC (Packard) with the use of a phenyl column (Spherisorb, Phenomenex). The eluent consisted of a 35:65 (vol/vol) mixture of acetonitrile/phosphate buffer (pH 3.5), and the detector was set to 350 nm. Linearity of the method was demonstrated for a concentration range of 0.5 to 1000 ppm. The amount of drug found in each artery was normalized to the dry weight of the explanted arterial segment.

Figure 1. Line graph depicting focal arterial levels (logarithmic scale) of AG-1295 delivered from PLA NPs (160 nm, 530 µg/mL) as a function of time after delivery. NPs were delivered intraluminally for 15 minutes at 2 atm to balloon-injured rat carotid arteries. Arterial segments were retrieved 5 minutes, 90 minutes, 6 hours, 24 hours, 7 days, and 14 days after completion of the delivery protocol. AG-1295 arterial content was determined by HPLC. Insert: Confocal images of rat carotid arteries after local delivery of Nile Red–loaded fluorescent NPs (500 µg/mL, 160 nm). The arteries were harvested 5 minutes (a), 90 minutes (b), 1 day (c), 7 days (d), and 14 days (e) after 15 minutes of intraluminal delivery and 6 hours after delivery (f). Discrete, granular, fluorescent foci of particle aggregates are clearly distinguished in f. L, M, N, and A indicate the lumen, media, neointima, and adventitia, respectively.
Another set of experiments examined the residence profile of smaller (90 nm) vs larger (160 nm) NPs. Analysis of the 5-minute time point disclosed 3.4 times higher drug concentrations in the arterial segments treated with the smaller particles (Table 2). However, assessment of drug levels 90 minutes after delivery demonstrated markedly higher drug concentrations in the arterial segments treated with the larger NPs. In contrast, 1 day after delivery, drug concentration was considerably higher in the vascular tissue of animals treated with the smaller NPs (Table 2).

Five minutes after a single 15-minute intramural delivery, Nile Red was abundant in the subendothelial space and between the elastin membranes of the media, whereas the adventitia was devoid of this fluorescent dye (Figure 1a). One and one-half hours after completion of fluorescent marker administration, Nile Red–containing NPs were evenly distributed between 2 discrete pools: in the innermost media and in the adventitia (Figure 1b). The same 2 pools of labeled NPs were seen in the rat carotid arteries 24 hours after delivery; however, the intensity of fluorescence was markedly reduced after 1.5 hours (Figure 1c). One week after intramural delivery, location of the particles was restricted to several foci on the border of the media and adventitia (Figure 1d). The same localization of Nile Red NPs was observed 2 weeks after delivery, albeit the intensity of fluorescence was further reduced (Figure 1e). It is noteworthy that at all studied time points, the fluorescence in the arteries had a distinct punctuate pattern corresponding to the size characteristics of NP aggregates rather than to individual particles (Figure 1f).

A nonsignificant difference between elimination of rhodamine B in NPs vs in solution was found at the earlier time point (1.5 hours, Figures 2A and 2D). Nevertheless, the difference in fluorescence intensity between the 2 delivery modes was marked after 8 hours and in particular, after 24 hours (Figures 2C and 2F). Treatment with drug-loaded NPs of both sizes decreased neointimal formation in comparison with the control groups, but statistical significance was obtained only for the smaller NPs (Figure 3).

### Discussion

Site-specific vascular delivery of pharmaceuticals is plagued by the inherent problem of rapid drug elimination from the treated site. Several groups have demonstrated the residence time$^{10,11}$ and antirestenotic effects$^{10,12–14}$ of particulate therapeutics. However, the local arterial pharmacokinetic ...

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### Table 1. AG-1295 Concentration in Rat Carotid Arteries After Intraluminal Administration of Drug-Containing NPs as a Function of Delivery Time, Pressure, and Prior Endothelial Denudation

<table>
<thead>
<tr>
<th>Injury/Delivery Type</th>
<th>Concentration, ng/mg Dry Tissue Weight</th>
<th>Particle Size and Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short delivery (3 min)</td>
<td>85.38±29.37*</td>
<td>Encapsulated Drug: 160 nm 90 nm Adsorbed Drug: 160 nm</td>
</tr>
<tr>
<td>Standard delivery (15 min)</td>
<td>224.70±36.56</td>
<td></td>
</tr>
<tr>
<td>Prolonged delivery (1 h)</td>
<td>603.29±146.08†</td>
<td></td>
</tr>
<tr>
<td>No denudation</td>
<td>97.96±24.76*</td>
<td></td>
</tr>
<tr>
<td>Low pressure (0.5 atm)</td>
<td>60.81±20.77*</td>
<td></td>
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Arterial Pharmacokinetics of AG-1295 NPs

Delivery efficiency, defined as the ratio of drug levels in the arterial wall at the earliest examined time point (5 minutes) to the total amount of delivered drug, was 2.3% for the 160-nm NPs. This value is 230 times higher than the delivery efficiency calculated for colchicine solution \(11\) and \(15\) times higher than the fractional intramural delivery of radioactive latex microparticles \(26\) applied through a porous balloon catheter in the atherosclerotic rabbit femoral model. Although the higher initial uptake in our study could be due to differences in arterial substrate and delivery device, it is most likely that this high uptake was derived from the smaller size of the NPs.

Analysis of the drug elimination curve (Figure 1) shows that arterial tyrphostin concentration decreased steeply up to 24 hours after delivery and was slowly reduced afterward. Given the short half-life of AG-1295 in the circulation (20 minutes, authors’ unpublished results, 2000), this 2-phase kinetics apparently reflects 2 distinct processes. Because our in vitro data \(4\) demonstrated the sustained-release pattern of AG-1295 from polylactic acid (PLA) NPs for 30 days, it seems unlikely that drug release per se could account for the 60-fold decrease in drug levels during the first 24 hours after delivery. Therefore, it seems that the marked reduction of arterial drug levels was due to NP loss from the arterial wall, presumably by either direct washout into the lumen or through the adventitial vasa vasorum. Indeed, a progressive decrease in the number of polymeric particles residing in the arterial wall after intraluminal delivery was previously demonstrated in the rat carotid model. \(12\) This explanation was also confirmed by the experiment that compared arterial drug levels in rats treated with drug-encapsulated NPs with those treated with drug-adsorbed NPs (Table 2). After 90 minutes, arterial drug levels exhibited a similar degree of reduction (59% to 60%) in animals treated with both types of NPs. However, 1 day after delivery, drug levels in the adsorbed-drug group were 4.4-fold lower (Table 2). Because a faster release rate was obtained for NPs with surface-adsorbed drug in comparison with encapsulated drug (authors’ unpublished data, 2000), this kinetic profile is expected if the leading mechanism of drug elimination at early time points after delivery is NP washout. Commencing after 24 hours, when the process of particle removal is essentially completed, the actual release of the drug from the NP dominates the elimination kinetics of the drug.

The relationship between prior balloon injury and arterial delivery yield is not obvious, because injury both destroys the potential binding sites in the endothelium and causes medial tears permitting deeper drug/vehicle penetration into the arterial wall. \(27\) Apparently, the final outcome depends on the specific animal model, the type of angioplasty and delivery catheters, and the nature of the delivered vehicle. In our study, the preceding injury augmented drug uptake, probably reflecting better ingress of NPs through the damaged elastic membranes of the media (Table 1).

Our study unequivocally demonstrated an increase of AG-1295 arterial content when delivery time was prolonged from 3 minutes to 1 hour (Table 1). This finding might be attributed to the anatomic characteristics of the rat artery and the overall prolonged local exposure (15 minutes vs 1 minute \(20\)) of the injured arteries to drug-containing NPs in our study. These data are of obvious clinical importance, because if arterial drug concentrations are delivery time dependent,
then the design of catheters used in a clinical setting should allow optimal distal perfusion during prolonged delivery sessions.

Drug levels obtained in vascular tissue with delivery pressures <2 atm were 3.5-fold higher than those observed after incubation under lower pressure (0.5 atm) (Table 1). Apparently, high pressure gradients across the vessel wall enhance the convective forces that drive deeper penetration of the drug molecules or of drug-containing particulate carriers, resulting in a slower and lessened washout phase of drug elimination.

A striking difference in residence properties of large (160 nm) and small (90 nm) NPs was found in our study (Table 2). The smaller particles are characterized by better ingress into arterial tissue, reflected by a 3.4 times higher initial drug concentration. Rapid elimination in the first 90 minutes after intraluminal administration is more pronounced for the smaller particles than for the larger ones. The early faster elimination of the smaller particles is probably due to easier migration to the adventitia, facilitating their elimination through the vasa vasmoris, as demonstrated with fluorescent NPs and the work of other investigators.10,12,14 The reduced late washout of the smaller NPs is probably due to their better penetration into deep arterial structures and creation of a drug depot that is relatively inaccessible to leaching by blood flow, the larger cumulative surface area of the smaller NPs, the direct cellular uptake of smaller particles, or some combination thereof. Given that the low tissue drug levels at advanced chronicity thereof. Given that the low tissue drug levels at advanced stages were 3.5-fold higher than those observed in elastic-type carotid arteries, attained in the muscular-type femoral arteries were uniformly higher than those observed in elastic-type carotid arteries, although the difference did not exceed 60%.

Adventitial vasa vasmoris are relatively scarce in normal rat arteries. Additionally, the density of vasa vasmoris is increased in atherosclerotic13 and balloon-injured15 compared with nondiseased artery segments. Because the present and other10,12,16 studies have revealed major involvement of the vasa vasmoris in the elimination of particulated matter from the vessel wall, the presence of advanced atherosclerosis might further modify the local pharmacokinetics of NPs. Finally, the principles governing the elimination pattern of particulated therapeutics from arterial tissue disclosed in the present study need to be validated in pig or primate models of arterial remodeling, the stable localization of particulate drug carriers at the medial-adventitial interface could be advantageous.

In the present study, rhodamine B–containing NPs were clearly traced in the rat carotid arteries 24 hours after intraluminal delivery, whereas no fluorescent dye was present in the arterial segments treated with equal amounts of rhodamine B in solution. This result is in accord with those of Dev et al.19 who showed protracted arterial residence of fluorescent microspheres in comparison with the solubilized dye.

Therapeutic Effect of AG-1295 NPs

Neointimal response triggered by balloon injury to rat carotid arteries was significantly reduced by local intramural therapy with 90-nm AG-1295-containing NPs (Figure 3). A reduction in neointimal formation was also shown after administration of the 160-nm NPs with the same tyrphostin content; however, it was statistically nonsignificant. Neither blank NPs with adsorbed tyrphostin nor systemic therapy by osmotic pumps decreased the extent of stenosis in comparison with the control groups of empty NPs and injury alone. Antirestenotic therapy could be effective if a sufficient concentration of the drug can be maintained in the injured arterial site for at least several days31 and possibly for several weeks.32 It was shown that the degree of neointimal inhibition is not dependent on the total amount of the delivered drug but rather on the sustained local concentration of the drug.33 It is noteworthy that the therapeutic effect of different AG-1295 sustained-release formulations is correlated with arterial drug concentration. The impact of procedure-related variables (delivery time and pressure) on the extent of restenosis was not addressed in the present study, but it is reasonable to assume that a prolonged delivery period would further augment the therapeutic effect through the increased local drug concentration.

The extent of neointimal thickening was similar in blank NPs and injury-alone control groups (Figure 3). Thus, additional arterial injury due to pressure-facilitated delivery was not observed in our study. These findings are in agreement with previous work demonstrating no deleterious effect of intraluminally delivered particulate carriers in the NP12 but not in the microparticle size range.

Limitations of the Study

The rat carotid artery is an elastic-type conduit with a higher content of elastin than muscular-type coronary arteries. Therefore, both the distribution and residence of the drug/carrier might be somewhat different in the coronary circulation. The impact of vessel type on arterial drug levels after intraluminal delivery of PLGA NPs was investigated by Song et al.20 in a dog model. In that study, the local drug levels attained in the muscular-type femoral arteries were uniformly higher than those observed in elastic-type carotid arteries, although the difference did not exceed 60%.

Adventitial vasa vasmoris are relatively scarce in normal rat arteries. Additionally, the density of vasa vasmoris is increased in atherosclerotic and balloon-injured compared with nondiseased artery segments. Because the present and other studies have revealed major involvement of the vasa vasmoris in the elimination of particulated matter from the vessel wall, the presence of advanced atherosclerosis might further modify the local pharmacokinetics of NPs. Finally, the principles governing the elimination pattern of particulated therapeutics from arterial tissue disclosed in the present study need to be validated in pig or primate models and encompass a broader range of sizes and drug doses.

Recently, a therapeutic approach exploiting endovascular stents as a platform for local arterial delivery was proposed and implemented in several highly appraised studies.36 However, this treatment modality is limited to situations wherein stent deployment is clinically sound and technically possible. Moreover, severe inflammatory reactions in the arterial wall due to polymer-coated stents were reported.37 Future studies will yield additional information on the advantages and shortcomings of stent-based delivery (tailored to a specific stent) and microparticulated drug delivery (a solution for all angioplasty procedures) for restenosis prevention.
References

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doi: 10.1161/hq0901.095567
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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