Galactosyl Derivatives of L-Arginine and D-Arginine: Synthesis, Stability, Cell Permeation, and Nitric Oxide Production in Pituitary GH_3 Cells

Daniela Melisi,[‡] Agnese Secondo,[†] Paola Montoro,[§] Sonia Piacente,[§] Maria Grazia Rimoli,[‡] Massimiliano Minale,[†] Paolo de Caprariis,[§] and Lucio Annunziato*[,†]

Division of Pharmacology, Department of Neuroscience, School of Medicine, Federico II University of Naples, Via Sergio Pansini 5, 80131 Naples, Italy, Department of Pharmaceutical and Toxicological Chemistry, Faculty of Pharmacy, Federico II University of Naples, Via Domenico Montesano 49, 80131 Naples, Italy, and Department of Pharmaceutical Science, Faculty of Pharmacy, University of Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy

Received January 3, 2006

Nitric oxide (NO) is critical for the normal physiological regulation of the nervous system and other tissues. L-Arginine, but not D-arginine, is the natural substrate for nitric oxide synthase (NOS), for it is enzymatically converted to NO and L-citrulline. However, recent evidence suggests that D-arginine can also produce NO and NO-derivatives via a different pathway. The aim of the present paper was to raise NO levels in the cells by increasing the cell permeation of its precursors. To this aim, two galactosyl prodrugs, L-arginine-D-galactos-6′-yl ester (L-ArgGal) and D-arginine-D-galactos-6′-yl ester (D-ArgGal) were synthesized. Remarkably, using the HPLC-ESI/MS technique, we found that L-ArgGal and D-ArgGal prodrugs both increased the concentration levels of L- and D-arginine and their derivatives in pituitary GH_3 cells. Furthermore, we found that D-ArgGal (1) penetrated cell membranes more rapidly than its precursor D-arginine, (2) released arginine more slowly and in greater amounts than L-ArgGal, and (3) produced much higher levels of DAF-2 monitored NO and nitrite than did L-ArgGal under the same experimental conditions. In conclusion, these results indicate that an increase in the cell permeation of L- and D-arginine by L-ArgGal and D-ArgGal can lead to an increase in NO levels.

Introduction

In the early 1990s, when scientists discovered that nitric oxide (NO) was implicated in several physiological and pathophysiological functions,[1] it was generally accepted that L-arginine, but not D-arginine, was the natural substrate for NOS, a theory based on the fact that this amino acid enzymatically converts to NO and L-citrulline.[2] However, after these pivotal assumptions, further studies suggested that D-arginine could also bring about an increase in the production of NO and its derivatives.[3,4] Indeed, this D-amino acid (1) reduces angiotensin II-induced increase of systolic blood pressure,[5] (2) protects cells against radical-induced myocardial injury by exerting a superoxide anion ($O_2^{\bullet}$) scavenging activity,[6] (3) exerts an antiapoptotic action,[7] and (4) displays central stimulant properties.[8]

Several theories have been provided to explain why D-arginine is able to increase the production of NO and its derivatives. First, this D-amino acid, as well as L-arginine, can generate NO nonenzymatically thanks to the reaction occurring between hydrogen peroxide, present in the extracellular and intracellular environment, and the guanidine group of D-arginine.[4] Second, D-arginine, like L-arginine, might protect NO from $O_2^{\bullet}$-induced inactivation by exerting a scavenging action on $O_2^{\bullet}$, thus increasing the levels of the gaseous mediator.[9] Third, it is possible that this D-amino acid, which is present in plants and various classes of marine and terrestrial organisms, for example, in the nervous tissue of crustaceans,[10] may be converted to the respective L-isomer by a D-amino acid racemase,[11] as it already occurs for D-serine.[12,13]

There is evidence, however, that although L mojety is the prevailing stereoisoform of arginine, D-arginine, despite having a lower affinity compared to the L-isoform, is equally able to cross the plasma membrane by using the amino acid transporter systems denoted as $b^{0,+}$, $B^{0,+}$, and Y^+L.[14,15] In light of these biochemical and pharmacological properties, the aim of our study was (1) to design a synthetic strategy that would facilitate the penetration of D-arginine and L-arginine across the cell membrane and (2) to provide these two basic amino acids with a carrier group that once penetrated the plasma membrane could be enzymatically cleaved by releasing the functional active molecule, thus leading to an increase in NO production.

To this aim, by conjugating D-arginine and L-arginine to galactose, we obtained two galactosyl prodrugs: L-arginine-D-galactos-6′-yl ester (L-ArgGal) and D-arginine-D-galactos-6′-yl ester (D-ArgGal). Indeed, by virtue of the glucose carrier (GLUTs),[16,17] these prodrugs were able to cross the cell membrane more efficiently. These galactosyl prodrugs yield the best results probably because of their high affinity for GLUT1 transporter[18,19] and because of their capability of being cleaved by esterases.[20,21]

To assess the validity of this pharmacological approach, we used the HPLC-ESI/MS technique to measure the content of arginine and arginine-galactosyl derivatives in GH_3 cells upon extracellular incubation with D-arginine, L-arginine, L-ArgGal, and D-ArgGal; then, we assayed NO-forming properties of D-arginine, L-arginine, L-ArgGal, and D-ArgGal in pituitary GH_3 and bovine aortic endothelial cells.

Results

General Procedure for the Synthesis of L-ArgGal and D-ArgGal. Esterification of the two enantiomeric forms of Boc-N^g-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-arginine (Boc-L-Arg(Pbf)-OH and Boc-D-Arg(Pbf)-OH) with 1,2,3,4-di-*O*-isopropylidene-D-α-galactopyranose (DIPG) in the presence of 1,1′-carbonyldiimidazole (CDI), dissolved in dry dichloromethane/*N*,*N*-dimethylformamide (CH_2Cl_2/DMF, 20 mL, 7:3), yielded 61.8% of **1a** and 56.6% of **2a** (Scheme 1). Protecting

* To whom correspondence and reprint request should be addressed. Tel.: +39−081−7463318; fax: +39−081−7463323; e-mail: lannunzi@unina.it.

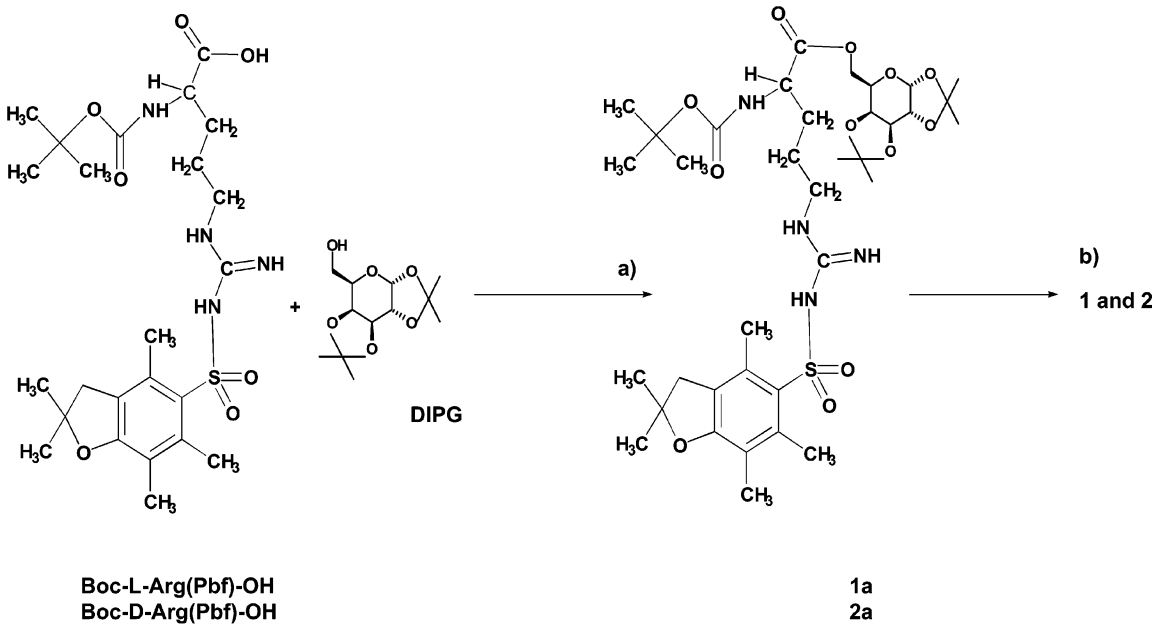
[†] School of Medicine, Federico II University of Naples.

[‡] Faculty of Pharmacy, Federico II University of Naples.

[§] University of Salerno.

10.1021/jm060005s CCC: $33.50
Published on Web 07/19/2006

Scheme 1[a]



[a] (a) CDI, dry CH_2Cl_2/DMF; (b) TFA, dry CH_2Cl_2

groups (Boc, Pbf, and ketals) were completely removed with trifluoroacetic acid (TFA), dissolved in dry dichlorometane, so as to obtain 98% of L-arginine-D-galactos-6′-yl ester **1** and 99% of D-arginine-D-galactos-6′-yl ester **2** (Figure 1).

HPLC-ESI/MS Analyses of L**-ArgGal and** D**-ArgGal.** HPLC-ESI/MS analyses of L-arginine, D-arginine, L-ArgGal, D-ArgGal, and L-arginine ethoxilate, used as the internal standard, are shown in Figure 2, whereas the chromatographic conditions are described in the Experimental Section. A better interpretation of the TIC (total ion current) chromatogram results was achieved when the reconstructed ion chromatograms were developed for the three *m*/*z* values of interest (*m*/*z* 175 protonated molecular ion $[M+H]^+$ for arginine, *m*/*z* 203 protonated molecular ion $[M+H]^+$ for arginine ethoxide, and *m*/*z* 337 protonated molecular ion $[M+H]^+$ for arginine galactose) (Figure 2a and 2b).

Stability Test of L**-ArgGal and** D**-ArgGal.** Figure 3 shows the calibration curves of both L-arginine and D-arginine, as well as the stability curves of both L-ArgGal and D-ArgGal. Under dark conditions and at room temperature, L-ArgGal, dissolved in PBS buffer solution at pH 7.4, appeared stable for 7 h but decreased from 1 μM to 0.8 μM thereafter (Figure 3a). By contrast, under the same experimental conditions, and for the same amount of time, D-ArgGal appeared to be less stable than L-ArgGal, decreasing from 1 μM to 0.68 μM thereafter (Figure 3b).

Detection of Intracellular Drug Concentrations for L**-Arginine,** D**-Arginine,** L**-ArgGal, and** D**-ArgGal and Quantification of Arginine Released from** L**-ArgGal and** D**-ArgGal in Homogenates from Pituitary GH_3 Cells.** When GH_3 cells were incubated for 30 min with increasing concentrations of L-arginine, L-ArgGal, D-arginine, and D-ArgGal solutions (3 mM, 10 mM, and 30 mM), different levels of cell permeation were yielded (Figure 4a). In particular, within the same range of concentrations, L-ArgGal and D-ArgGal crossed the plasma membrane more easily than the two free amino acids. In addition, they crossed the plasma membrane in a dose-dependent way. Indeed, evidence that the increased amount of arginine released from L-ArgGal and D-ArgGal was concentration-dependent is that its progressive increase was closely associated with the increase in the concentration levels (3, 10, and 30 mM) of the two prodrugs (Figure 4b). In addition, L-ArgGal and D-ArgGal remained stable in the cells, since the concentrations of the two prodrugs were higher than the ones released by arginine (Figure 4a and 4b). Figure 5a shows the time-course (from 15 to 240 min) of the two prodrugs and their respective free amino acids. D-Arginine, L-arginine, and D-ArgGal penetrated GH_3 cells in a time-dependent way, whereas L-ArgGal reached an intracellular peak value 15 min after it was added to the incubation medium. Interestingly, L- and D-arginine entry into the cells did require at least 30 min (Figure 5a). In addition, D-ArgGal was able to release D-arginine in a time-dependent way and in a larger amount than did L-ArgGal under the same experimental conditions (Figure 5b).

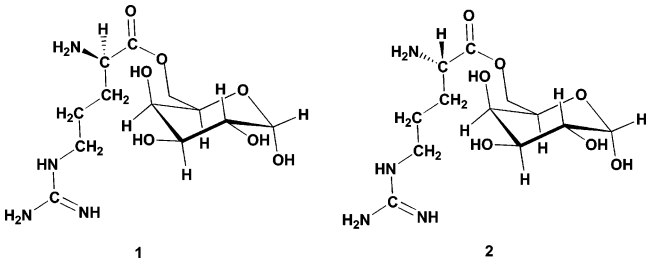


Figure 1. Representation of L-ArgGal (1) and D-ArgGal (2) chemical structures.

DAF-2 Monitored NO and Nitrite Production after L**-Arginine,** D**-Arginine,** L**-ArgGal, and** D**-ArgGal Incubation in Pituitary GH_3 Cells.** When L-arginine, L-ArgGal, D-arginine, and D-ArgGal were incubated for 30 min together with pituitary GH_3 cells, a dose-dependent increase in intracellular DAF-2 monitored NO and nitrite levels in the extracellular medium was detected (Figure 6a and 6b). However, after 30 min of incubation, D-arginine produced a greater increase in both DAF-2 monitored NO and nitrite levels, as compared with the L-enantiomer. Furthermore, L-arginine and D-arginine increased DAF-2 monitored NO and nitrite levels depending on the incubation period (Figure 7a and 7b). By contrast, L-ArgGal displayed the maximal production of both DAF-2 monitored NO and nitrites already

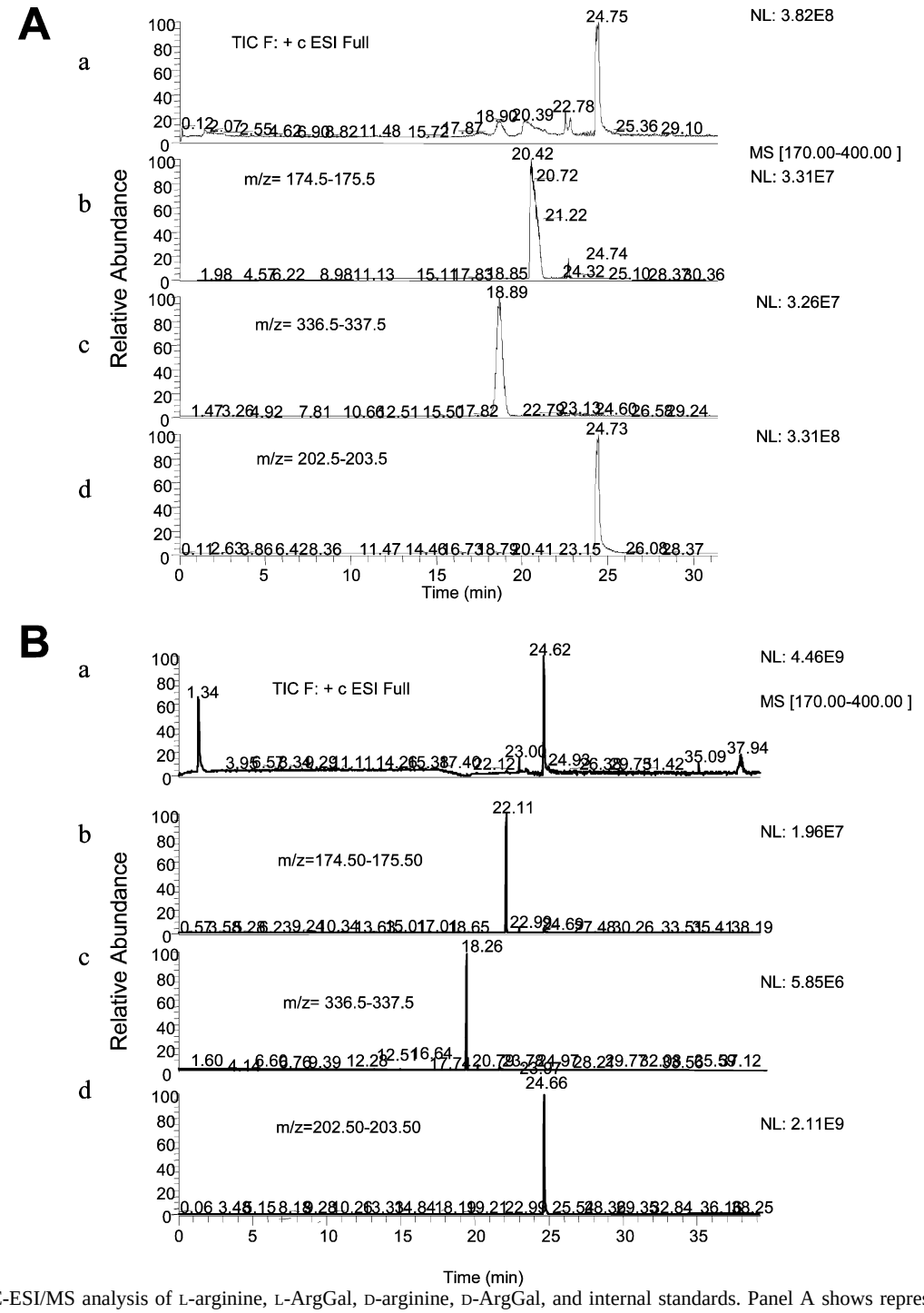


Figure 2. HPLC-ESI/MS analysis of L-arginine, L-ArgGal, D-arginine, D-ArgGal, and internal standards. Panel A shows representative traces of HPLC-ESI/MS analysis of L-arginine, L-ArgGal, and internal standard: 2Aa, total ion current chromatogram; 2Ab, reconstructed ion chromatogram of L-arginine; 2Ac, reconstructed ion chromatogram of L-ArgGal; 2Ad, reconstructed ion chromatogram of internal standard. Panel B depicts representative traces of HPLC-ESI/MS analysis of D-arginine, D-ArgGal, and internal standard: 2Ba, total ion current chromatogram; 2Bb, reconstructed ion chromatogram of D-arginine; 2Bc, reconstructed ion chromatogram of D-ArgGal; 2Bd, reconstructed ion chromatogram of internal standard. The chromatographic conditions for HPLC-ESI/MS analysis is described in the Experimental Section.

at 30 min. No further increases in NO were detected thereafter. Intriguingly, D-ArgGal significantly delayed the increase in DAF-2 monitored NO and nitrite production, as compared with all the other experimental groups (Figure 7b). Accordingly, the area under the curve (AUC), obtained by plotting nitrite values with the periods of incubation, was (in nmol/mL*min) as follows: 480 ± 24,2 for L-arginine, 378 ± 6.5 for L-ArgGal, 851 ± 36,1 for D-arginine, and 1338 ± 18 for D-ArgGal.

DAF-2 Monitored NO Production after the Incubation of L-Arginine, D-Arginine, L-ArgGal, and D-ArgGal in Bovine Aortic Endothelial (BAEC) Cells. As already demonstrated for GH_3 cells, when L-arginine, L-ArgGal, D-arginine, and D-ArgGal were incubated at 10 mM for 30 min, a massive increase in intracellular DAF-2 monitored NO levels in BAEC cells was detected (Figure 8). Consistently, in these cells, D-ArgGal elicited the greatest NO production, as compared with L-arginine, L-ArgGal, and D-arginine (Figure 8).

Discussion

The results of the present paper provide substantial support to our hypothesis that galactosyl prodrugs can increase cell permeation thanks to their high affinity for GLUT1, the major

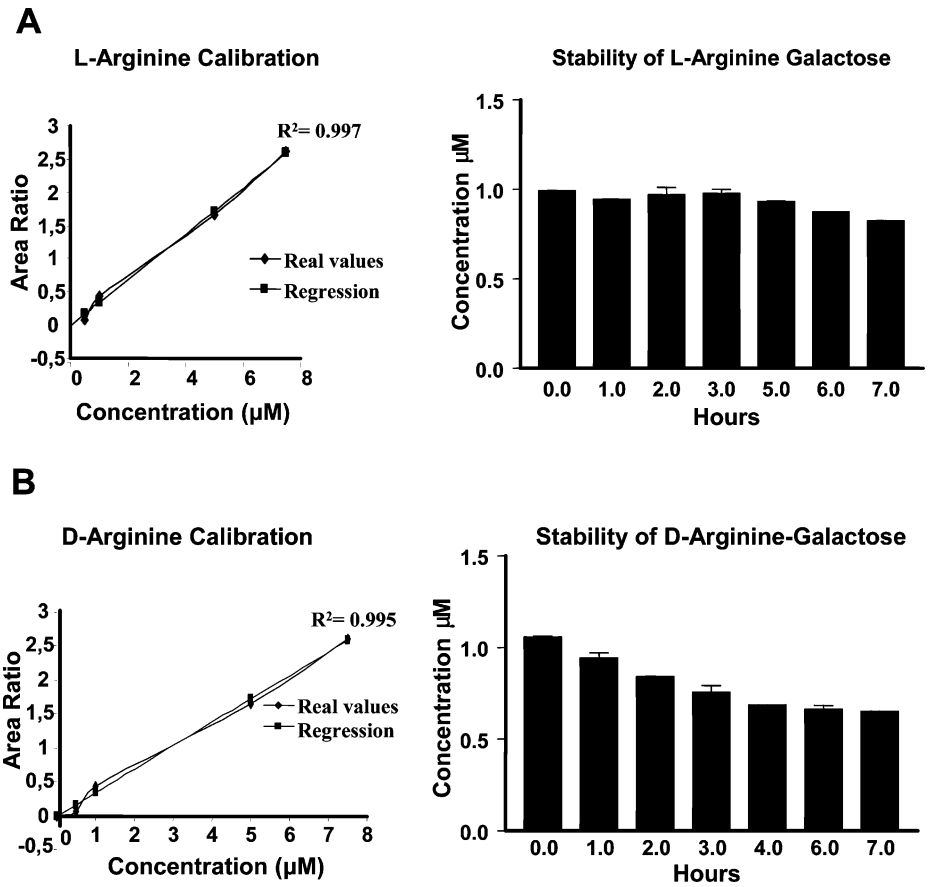


Figure 3. Representation of L-arginine and D-arginine calibration plots and L-arginine galactose and D-arginine galactose stability curves. Panel A shows a representative L-arginine calibration plot (left) and a representative L-arginine-galactose stability curve studied at different times (0−7 h) (right). Panel B shows a representative D-arginine calibration plot (left) and a representative D-arginine-galactose stability curve studied at different times (0−7 h) (right). The experimental conditions used to perform these curves is described in the Experimental Section.

isoform of the exose transporter expressed in mammalians, especially in the blood-brain barrier (BBB).[22] In particular, the present paper demonstrates that D-ArgGal (1) penetrates cell membranes more rapidly than its precursor D-arginine, (2) releases greater quantities of arginine than L-ArgGal, and (3) produces much higher levels of both DAF-2 monitored NO and nitrite than does L-ArgGal under the same experimental conditions.

In particular, the two prodrugs displayed different pharmacological properties. In fact, D-ArgGal showed a slower kinetic of cell membrane penetration compared with that of L-ArgGal. This property can be attributed to the different affinity of the two prodrugs for the exose transporter, a characteristic possibly linked to their different spatial conformation. Another difference between the two prodrugs is the reduced stability of the D-Arg-Gal compared with that of L-Arg-Gal. The lower stability of D-ArgGal produces a time-dependent increase in the NO levels, thus providing a useful property for a prodrug approach.

As regards the experimental techniques, two innovative aspects should be underscored: the synthesis of L-arginine-D-galactos-6′-yl ester and D-arginine-D-galactos-6′-yl ester and the use of mass spectrometry for the quantification of amino acids in cell homogenates.

Indeed, to determine the amount of L-arginine and D-arginine and their derivatives in GH_3 cells, after their transport across the cellular membrane, mass spectrometry detection was used. One of the critical factors concerning the chromatographic separation of amino acids is that their low molecular weight and ionic nature do not yield good separation results with the reverse-phase liquid chromatography (RP-HPLC). Accordingly, recent works entailing the elution of amino acids have adopted ion-exchange[23,24] or ion-pair chromatography.[25−27]

Specifically, to separate proteins and peptides, perfluorinated carboxylic acids with longer *n*-alkyl chains have already been employed as an alternative to trifluoroacetic acid (TFA). Recently, these perflurinated surfactants were proposed by Petritis et al. for the analysis of nonderivative polar amino acids.[28] The advantage resulted in a reasonable retention time of most polar amino acids, including arginine, in RP-HPLC.

Pentadecafluorooctanoic acid (PDFOA) gave the best results with this amino acid. The method developed for arginine, containing the internal standard arginine ethoxilate, was also used for galactosilated arginine. Notably, when it was applied to an HPLC-ESI/MS system (high-performance liquid chromatography− electrospray−mass spectrometry), it yielded good results in terms of sensitivity, selectivity, and reproducibility.[29]

In conclusion, the present study clearly shows that once galactosyl prodrugs penetrate the cell membrane they are enzymatically cleaved by releasing the functional active molecule, thus leading to an increase in NO production. More important, this paper shows that D-ArgGal can penetrate the cell membranes of GH_3 cells more rapidly than its precursor D-arginine and can increase NO levels more slowly than L-ArgGal.

Furthermore, our findings suggest that arginine supplementation through D-ArgGal might be a novel strategy for the enhancement of NO availability in cells and, therefore, might constitute a pharmacological model for those neuropathological conditions in which a higher and prolonged level of the gaseous mediator could have beneficial therapeutic effects.

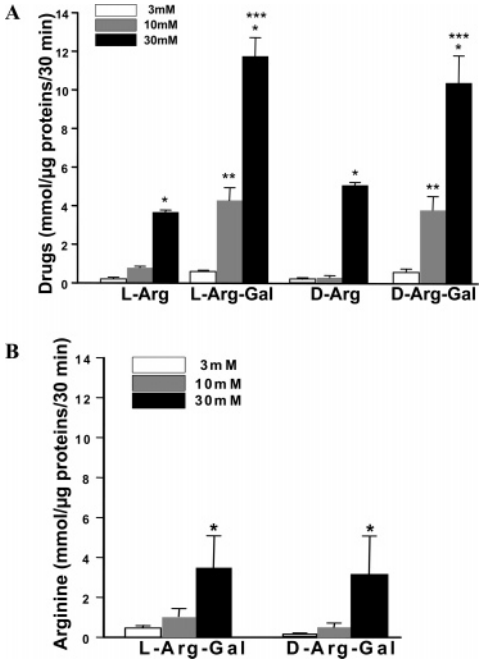


Figure 4. Detection of intracellular drug concentrations after incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal in pituitary GH_3 cells. The bar graph depicts the amount of drugs measured in cell homogenates after 30 min incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at 3, 10, and 30 mM. Samples were analyzed in triplicate in at least three different experimental sessions. The results were reported as mmol/μg of total proteins. * $p < 0.05$ versus 3 mM and 10 mM respective group values; ** $p < 0.05$ versus 3 mM and 10 mM of L-Arg and D-Arg group values; *** $p < 0.05$ versus 10 mM and 30 mM L-Arg and D-Arg group values.

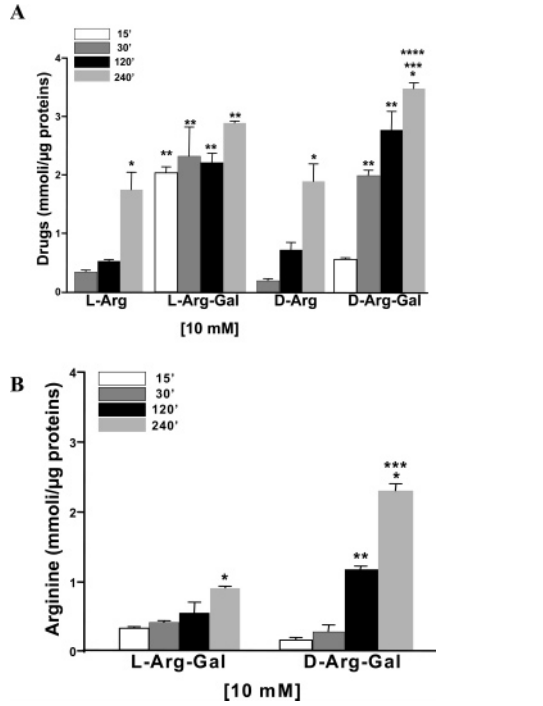


Figure 5. Detection of intracellular drug concentrations after the incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal in pituitary GH_3 cells. The bar graph shows the quantification of drug amount measured in GH_3 cell homogenates after 15, 30, 120, and 240 min incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at 10 mM. The chromatographic conditions for this analysis are described in the Experimental Section. Each experimental group was studied in at least three different experimental sessions. Panel A: * $p < 0.05$ versus 30 and 120 min respective groups; ** $p < 0.05$ versus 30 and 120 min of L-Arg and D-Arg treatments; *** $p < 0.05$ versus 120 and 240 min of L-Arg, L-ArgGal, and D-Arg treatments; **** $p < 0.05$ versus 15, 30, and 120 min groups of D-ArgGal. Panel B: * $p < 0.05$ versus 15, 30, and 120 min respective groups; ** $p < 0.05$ versus 15, 30, and 120 min of L-ArgGal treatments; *** $p < 0.05$ versus 240 min of L-ArgGal treatment.

Experimental Section

Drugs and Chemicals. L-Arginine, D-arginine, sodium phosphate monobasic, sodium phosphate dibasic, paraformaldheyde, and sodium chloride were obtained from Sigma Chemical Co. (Sigma Italia, Milano, Italy). L-Arginine ethoxilate, used as the internal standard, was purchased from Chem Progress (Sesto Ulteriano, MI, Italia). Set-Pack C-18 cartridges were obtained by Waters Corporation (Milford, MA). DAF-2DA was obtained from Calbiochem (La Jolla, CA). L-arginine and D-arginine were dissolved in normal Krebs solution (10 mM), and stock solutions were kept at −20 °C. Appropriate dilutions were prepared daily.

Boc-L-Arg(Pbf)-OH and Boc-D-Arg(Pbf)-OH were obtained from Advanced Biotech Italia Srl (Seveso, MI, Italy). HPLC grade acetonitrile was obtained by J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

Chemistry. The course of reactions and purity of products were controlled by TLC (silica gel 60F254s; Merck), and spots were detected by UV radiation and exposition to a ninidrine ethanolic solution. Flash chromatography was performed on Merck silica gel (0.040−0.063 mm). Melting points (mp) were determined with a Kofler hot stage microscope and were uncorrected. Elemental analyses, indicated by the symbols of the elements, were performed on a Perkin-Elmer model 240 elemental analyzer and were within ±0.4% of theoretical values. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on a Buker AMX-500 spectrometer equipped with a Bruker X-32 computer. Chemical shift values are reported in δ units (ppm) relative to TMS, used as the internal standard.

Diacetone 6′-*O*-[Boc-N^g-(2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine]-α,β-D-galactopyranoside (1a). First, 1:1′-carbonyldiimidazole (185 mg; 1.14 mmol) was added to a stirred solution of Boc-L-Arg(Pbf)-OH (400 mg; 0.76 mmol) that was dissolved in dry CH_2Cl_2−DMF (20 mL, 7:3). Then, after 12 h, 1,2,3,4-di-*O*-isopropylidene-α-D-galactopyranose (198 mg; 0.76 mmol), dissolved in dry CH_2Cl_2 (10 mL), was added dropwise to this mixture for more than 30 min and was stirred continuously for 24 h. Evaporation of the solvent gave a residue which was taken up in ethyl acetate and washed with water. The organic layer was dried on Na_2SO_4, which was filtered and then evaporated in a vacuum. The obtained residue, which underwent flash chromatography by elution with $CHCl_3$, yielded the title compound as a white solid (360 mg; 61.8%); mp: 290−291 °C. ^{1}H NMR ($CDCl_3$): δ 1.28, 1.30 (2s, 6H, 2″-CH_3); 1.34, 1.35, 1.40, 1.41 (4s, 12H, ketals); 1.53 (s, 9H, Boc); 1.70 (m, 2H, 4-H); 1.81 (m, 2H, 3-H); 2.06 (s, 3H, 6″-CH_3); 2.50 (s, 3H, 7″-CH_3); 2.54 (s, 3H, 4″-CH_3); 2.91 (s, 2H, 3″-H); 3.20 (m, 2H, 5-H); 4.00 (m, 1H, 4′-H); 4.18 (m, 2H, 5′-H); 4.21 (m, 1H, 6′-H); 4.30 (m, 1H, 2-H); 4.42 (m, 1H, 2′-H); 4.60 (m, 1H, 3′-H); 5.50 (m, 1H, 1′-H). ^{13}C NMR ($CDCl_3$): δ 13.5 ($2CH_3$-2″); 18 and 20 ($4CH_3$-ketals); 25 (CH_3-6″); 25.4 (CH_3-7″); 25.5 (CH_3-4″); 26.6 (C-4); 28.9 ($3CH_3$-Boc); 29.2 (C-3); 33.9 (C-3″); 41.5 (C-5); 44 (C-2); 65 (C-6′); 66.7 (C-4′); 71 (C-5′); 71.5 (C-2′); 72 (C-3′); 81 (C-Boc); 86.5 (C-2″); 97 (C-1′); 109 and 111-(C-ketals); 117.4 (C-7″); 125 (C-3″a); 132.8 (C-4″); 133.4 (C-6″); 138.9 (C-5″); 156 (C-7″a); 159.1 (C-6); 170 (CO-Boc); 172.5 (CO). Anal. ($C_{36}H_{56}N_4O_{12}S$) C, H, N, S.

Diacetone 6′-*O*-[Boc-N^g-(2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl)-D-arginine]-α,β-D-galactopyranoside (2a). The ester **2a** (330 mg; 56.6%), prepared as a white solid, was obtained from Boc-D-Arg(Pbf)-OH (400 mg; 0.76 mmol) and 1,2,3,4-di-*O*-isopropylidene-α-D-galactopyranose (198 mg; 0.76 mmol) in the presence of 1:1′-carbonyldiimidazole (185 mg; 1.14 mmol), following the same procedure used for making compound

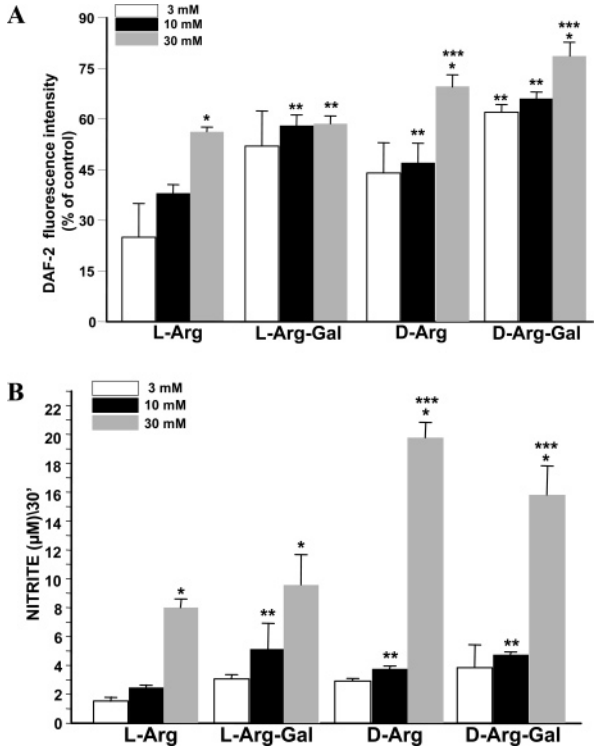


Figure 6. Dose-dependence of DAF-2 monitored NO and nitrite production after the incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal in pituitary GH_3 cells. Panel A: The bar graph depicts DAF-2 monitored NO production measured as fluorescence intensity in GH_3 cells after 30 min incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at the concentration of 3, 10, and 30 mM. Each bar represents the mean ± S.E. of three values obtained in three different experimental sections. All data are reported as % of control fluorescence * $p < 0.05$ versus 3 mM and 10 mM respective groups; ** $p < 0.05$ versus 3 mM and 10 mM values of L-Arg group; *** $p < 0.05$ versus 10 mM and 30 mM values of L-Arg and L-ArgGal treatments. Panel B: The bar graph depicts the quantification of nitrite production obtained from GH_3 cells after 30 min incubation with the L-arginine, L-ArgGal, D-arginine, and D-ArgGal at the concentration of 3, 10, and 30 mM. Nitrite production was measured in the incubation medium by Griess reaction, as reported in the Experimental Section. Each bar represents the mean ± SE of six values obtained in three different experimental sections: * $p < 0.05$ versus 3 mM and 10 mM respective groups; ** $p < 0.05$ versus 3 mM and 10 mM values of L-Arg group; *** $p < 0.05$ versus 10 mM and 30 mM values of L-Arg and L-Arg-Gal treatments.

1a; mp: 290−291 °C. ^{1}H NMR ($CDCl_3$): δ 1.28, 1.30 (2s, 6H, 2″-CH_3); 1.34, 1.35, 1.40, 1.41 (4s, 12H, ketals); 1.53 (s, 9H, Boc); 1.70 (m, 2H, 4-H); 1.81 (m, 2H, 3-H); 2.06 (s, 3H, 6″-CH_3); 2.50 (s, 3H, 7″-CH_3); 2.54 (s, 3H, 4″-CH_3); 2.91 (s, 2H, 3″-H); 3.20 (m, 2H, 5-H); 4.00 (m, 1H, 4′-H); 4.18 (m, 2H, 5′-H); 4.21 (m, 1H, 6′-H); 4.30 (m, 1H, 2-H); 4.42 (m, 1H, 2′-H); 4.60 (m, 1H, 3′-H); 5.50 (m, 1H, 1′-H). ^{13}C NMR ($CDCl_3$): δ 13.5 ($2CH_3$-2″); 18 and 20 ($4CH_3$-ketals); 25 (CH_3-6″); 25.4 (CH_3-7″); 25.5 (CH_3-4″); 26.6 (C-4); 28.9 ($3CH_3$-Boc); 29.2 (C-3); 33.9 (C-3″); 41.5 (C-5); 44 (C-2); 65 (C-6′); 66.7 (C-4′); 71 (C-5′); 71.5 (C-2′); 72 (C-3′); 81 (C-Boc); 86.5 (C-2″); 97 (C-1′); 109 and 111(C-ketals); 117.4 (C-7″); 125 (C-3″a); 132.8 (C-4″); 133.4 (C-6″); 138.9 (C-5″); 156 (C-7″a); 159.1 (C-6); 170 (CO-Boc); 172.5 (CO). Anal. ($C_{36}H_{56}N_4O_{12}S$) C, H, N, S.

L-Arginine-D-galactos-6′-yl Ester (1). A solution consisting of **1a** (360 mg; 0.47 mmol) and trifluoroacetic acid (2 mL) was continuously stirred in dry CH_2Cl_2 (5 mL) for 36 h at room temperature. Evaporation of the solvent, which produced a residue purified by precipitations with diethyl ether, gave the title compound **1** (156 mg; 98%); mp: 302−303 °C. ^{1}H NMR (D_2O): δ 1.55 (m, 2H, 4-H); 1.84 (m, 2H, 3-H); 3.10 (m, 2H, 5-H); 3.65 (m, 1H, 4′-H); 3.80 (m, 1H, 5′-H); 4.05 (m, 2H, 6′-H); 4.20 (m, 1H, 2′-H); 4.30 (m, 1H, 2-H); 4.40 (m, 1H, 3′-H); 5.09 (m, 1H, 1′-H). ^{13}C NMR (D_2O): δ 25.2 (C-4); 28.8 (C-3); 41.7 (C-5); 55.6 (C-2);

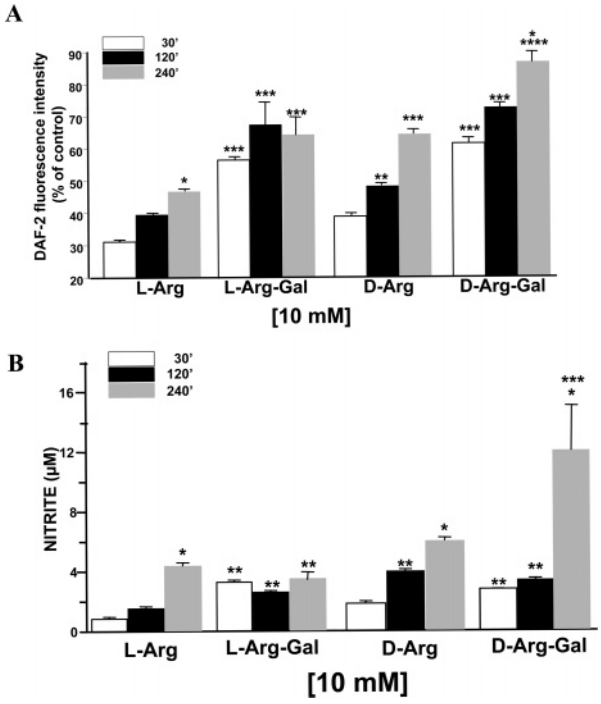


Figure 7. Time-course of DAF-2 monitored NO and nitrite production after incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal in pituitary GH_3 cells. Panel A: The bar graph depicts DAF-2 monitored NO production measured as fluorescence intensity in GH_3 cells after 30, 120, and 240 min incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at 10 mM. Each bar represents the mean ± S.E. of three values obtained in three different experimental sections. All data are reported as % of control fluorescence. * $p < 0.05$ versus 30 min and 120 min of respective groups; ** $p < 0.05$ versus 30 and 120 min groups of L-Arg treatments and $p < 0.05$ versus 30 min of D-Arg treatment; *** $p < 0.05$ versus 30, 120, and 240 min of L-Arg treatments and $p < 0.05$ versus 30 and 120 min of D-Arg treatments; **** $p < 0.05$ versus 30, 120, and 240 min of L-Arg, D-Arg, and L-ArgGal treatments. Panel B: The bar graph shows the quantification of the nitrite production obtained from GH_3 cells after 30, 120, and 240 min incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at 10 mM. Each bar represents the mean ± S.E. of six values obtained in three different experimental sections: * $p < 0.05$ versus 30 min and 120 min of respective groups; ** $p < 0.05$ versus 30 and 120 min groups of L-Arg treatments and $p < 0.05$ versus 30 min of D-Arg treatment; *** $p < 0.05$ versus 30, 120, and 240 min of L-Arg, D-Arg, and L-ArgGal treatments.

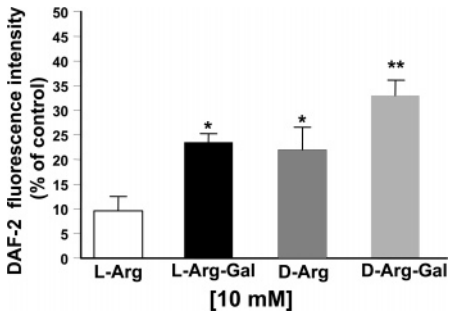


Figure 8. DAF-monitored NO levels after the incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal in BAEC cells. The bar graph shows DAF-2 monitored NO production measured as fluorescence intensity in BAEC after 30 min of incubation with L-arginine, L-ArgGal, D-arginine, and D-ArgGal at 10 mM. Each bar represents the mean ± S.E. of three values obtained in three different experimental sections. All data are reported as % of control fluorescence. * $p < 0.05$ versus L-arginine; ** $p < 0.05$ versus all treatments.

65.5 (C-6′); 68 (C-4′); 70 (C-5′); 73.1 (C-2′); 74.9 (C-3′); 94 and 99 (C-1′); 157 (C-6); 170 (CO). Anal. ($C_{12}H_{24}N_4O_7$) C, H, N.

D-Arginine-D-galactos-6′-yl Ester (2). A solution consisting of **2a** (330 mg; 0.43 mmol) and trifluoroacetic acid (2 mL) was continuously stirred in dry CH_2Cl_2 (5 mL) for 36 h at room temperature. Evaporation of the solvent, which produced a residue purified by precipitations with diethyl ether, gave the title compound **2** (144 mg; 99%); mp: 302−303 °C. ^{1}H NMR (D_2O): δ 1.55 (m, 2H, 4-H); 1.84 (m, 2H, 3-H); 3.10 (m, 2H, 5-H); 3.65 (m, 1H, 4′-H); 3.80 (m, 1H, 5′-H); 4.05 (m, 2H, 6′-H); 4.20 (m, 1H, 2′-H); 4.30 (m, 1H, 2-H); 4.40 (m, 1H, 3′-H); 5.09 (m, 1H, 1′-H). **^{13}C NMR** (D_2O): δ 25.2 (C-4); 28.8 (C-3); 41.7 (C-5); 55.6 (C-2); 65.5 (C-6′); 68 (C-4′); 70 (C-5′); 73.1 (C-2′); 74.9 (C-3′); 94 and 99 (C-1′); 157 (C-6); 170 (CO). Anal. ($C_{12}H_{24}N_4O_7$) C, H, N.

HPLC−ESI/MS. ESI/MS was done by using a Finnigan LCQ Deca ion trap instrument (Thermo Finnigan, San José, CA) equipped with Xcalibur software. To obtain solutions of 1 ng/mL, samples (isolated compounds) were dissolved in methanol and were infused in the ESI source with a syringe pump; the flow rate was 5 μL/min. To study MS parameters, ESI-MS measurements were performed.

After sample preparation, the samples were analyzed by "on-line" LC/ESI/MS using the same instrument described for the ESI/MS, which is equipped with a Spectra System HPLC (Thermo Finnigan, San José, CA). To tune the parameters of the source and analyzer, spectroscopic conditions were optimized for standard L-arginine by ESI-MS analyses.

Separations were done on a 125 × 4 mm (internal diameter of particles 5 μm) (Agilent, Technologies, HP Strasse 8, Waldrom, Germany) column. All the experiments occurred at room temperature at the low rate of 1 mL min^{-1}. For gradient elution, solvent A consisted of 0.1 mM PDFOA (pentadecafluorooctanoic acid), which was diluted in water, whereas solvent B consisted of acetonitrile. Elution started with 10% of solvent B and remained isocratic for 8 min; then, it increased to 20% in 7 min and to 40% in the subsequent 10 min; it passed to 50% in 15 min. During the following 10 min, the last part of the linear gradient ranged from 50% to 60%.

For HPLC/MS analyses, a 50 μL min^{-1} portion of the column eluate was directly injected into the electrospray ion source. Then, the MS spectra were taken and elaborated using the software provided by the manufacturer.

Injections were done with a Termoquest Spectra-System AS3000 autosampler fitted with a 20-μL loop.

Stability Test. For the calibration curve of L-arginine and D-arginine, standard compounds were dissolved in water to obtain four concentration levels for each standard: 0.5, 1, 5, and 7.5 μM. L-Arginine ethoxilate was added to each solution to obtain the final solution (1 μM) of the internal standard (I.S.).

The standard curve for L-arginine standard was prepared at concentration levels ranging from 0.5 to 7.5 μM. These concentrations consisted of four different concentration levels; for each level, triplicate injections were performed. To generate standard curves, the peak area ratios between the area of L-arginine standard or D-arginine standard at each concentration and the area of L-arginine ethoxylate, the internal standard, were calculated and plotted against the corresponding concentrations with weighed linear regression.

To obtain the final concentration of 1 μM, three samples of L-Arg-Gal and D-Arg-Gal were dissolved in PBS (phosphate buffer saline) solution (20 mM) at pH 7.4 and were stored at room temperature in the dark for 7 h. One milliliter of solution was taken at the beginning of the analysis and after each hour. Then, to obtain the final concentrations of Arg-Gal (1 mM) and Arg-Oet (1 mM), the solutions were transferred into an autosampler vial containing 1 nmol of dried internal standard. After agitation on vortex, the samples were directly analyzed with HPLC−ESI−MS. Quantitative data resulted from the external and internal standard. The calibration graphs ($r^2 = 0.997$ and $r^2 = 0.995$) were obtained (1) by plotting the area ratio between the external and internal standard versus the known concentration of each compound and (2) by constructing the regression line with the minimum quadrates method. These graphs were linear within the ranging values of 0.5−7.5 μM.

Cell Culture. GH_3 cells were obtained from Flow Laboratories (Irvine, Scotland) and were grown on plastic dishes containing F10 medium (Gibco, Life Technology) supplemented with 10% FBS, 15% HS (Gibco, Life Technology), 100 I.U. of penicillin/mL, and 100 μg of streptomycin/mL (SIGMA, St. Louis, MO). They were cultured in a humidified 5% CO_2 atmosphere; the culture medium was changed once every 2 days.

Bovine aortic endothelial cells (BAEC) were grown in DMEM supplemented with 10% FBS (Gibco, Life Technology), 100 IU of penicillin/mL, and 100 μg of streptomycin/mL (SIGMA, St. Louis, MO).

Cell Treatments, Nitrite Assay, and Permeability Test Sample Preparation. To study the effects of L-arginine, L-ArgGal, D-arginine, and D-ArgGal (3−30 mM), cells were incubated with the aforementioned treatments in the normal Krebs solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes-NaOH, pH 7.4) in a humidified 5% CO_2 atmosphere at 37 °C. For control experiments, cells were incubated only in normal Krebs solution. Nitrite accumulation was measured in the culture medium by Griess reaction.[30] The area under the curves (AUC), which indicates the time-course of the experiments, was calculated using the trapezoidal rule. For the permeability tests, after drug treatments, cells (4−6 millions) were first washed twice with PBS; then, they were lysed and collected by scraping. Samples were cleared by centrifugation at 15 000 rpm for 20 min at 4 °C, and the supernatants were used for the tests. Protein concentration was determined using the Bradford method.[31]

Nitric Oxide Detection with DAF-2 Assay. After the incubation with the aforementioned drugs in normal Krebs solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes-NaOH, pH 7.4), cells were loaded with 10 μM 4,5-diaminofluorescein-2-diacetate (DAF-2DA) in a humidified 5% CO_2 atmosphere at 37 °C for 30 min. For control experiments, cells were incubated only in normal Krebs solution. Subsequently, fluorescent cells were fixed with 4% (w/v) paraformaldheyde in PBS for 5 min at room temperature. This procedure permits a subsequent densytometric analysis with the fluorescence microscope Nikon Eclipse E400 (Nikon, Torrance, CA) set at an excitation/emission wavelength of 495/515 nm. Fluorescent images are then stored and analyzed with Pro-Plus software (Media Cybernetics, Silver Springs, MD). The data are reported as percentage of the fluorescence for the control group.

Detection of Intracellular Drug Concentrations after Drug Incubation. Standard solutions of l-ArgGal (3, 10, and 30 mM) and L-arginine (3, 10, and 30 mM) were prepared and diluted at 1:100 with water:acetonitrile 1:1. Then, an internal standard was added to obtain 1 μM final concentration.

Standard solutions of D-ArgGal (3, 10, and 30 mM) and D-arginine (3, 10, and 30 mM) were prepared and diluted at 1:100 with water:acetonitrile 1:1. Then, the internal standard was added to these solutions to obtain 1 μM final concentration. The ratio between the arginine, or arginine-galactose, area and the arginine ethoxilate area was obtained as follows: for the former area, a reconstructed ion chromatogram was developed for ion detection at *m*/*z* 175 and at *m*/*z* 337, respectively, whereas, for the latter area, the reconstructed ion chromatogram was developed for ion detection at *m*/*z* 203. This value was considered as 100% of arginine or arginine-galactose. All ion detections corresponded to $[M+H]^+$.

For each sample subsequently analyzed, the ratios between the area of the peak of the compound in the specific reconstructed ion chromatogram and the area of the peak of the internal standard in the specific reconstructed ion chromatogram were measured and divided by the values obtained for the standard, so as to obtain a permeation percentage. The percentage of arginine release was calculated in the same way by referring to the values of arginine standard solution.

To separate drugs from protein residues and to release the drug from the noncovalent bond to the proteins, homogenate samples of GH_3 cells were purified with a SPE C-18 Sep-Pack cartridge. A

preliminary test was done with a cartridge conditioned first with methanol and then with water. To recover the fractions of 1 mL of eluate, the drug solution (10 μM) and the internal standard (1 μM) were deposed and eluted with water−acetonitrile 1:1. More than 98% of the drug, concentrated in the initial five fractions, was recovered. The presence of the internal standard was used to correct errors during the recovery.

Each homogenate sample, deriving from L-arginine, L-ArgGal, D-arginine, and D-ArgGal permeability tests, produced three fractions of 100 μL. These fractions, together with 10 μL of 1 mM of internal standard solution, were deposited on a cartridge. To obtain a 1:100 dilution, 5 mL of recovered fractions was first dried and reconstructed to 100 μL with water:acetonitrile 1:1 and then was diluted with water:acetonitrile 1:1. The samples, prepared by dissolving L-arginine or d-arginine and the internal standard to obtain a 100% standard solution for arginine, were treated with the same preparatory steps of the sample. Samples, prepared by dissolving arginine-galactose and the internal standard to obtain a 100% standard solution for the drugs, were treated with the same sample preparatory steps of the sample.

These last solutions were transferred into autosampler vials for quantitative analyses.

Statistical Analysis of the Data. The statistical analysis of all data was performed using ANOVA followed by Newman-Keuls test. The threshold for statistically significant differences was set at $p < 0.05$.

References

(1) Moncada, S.; Palmer, R. M. J.; Higges, E. A. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−142.

(2) Beckman, J. S.; Koppenol, W. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol.* **1996**, *271*, C1424−1437.

(3) Chambers, D. C.; Ayres, J. G. Effect of nebulised L-and D-arginine on exhaled nitric oxide in steroid naïve asthma. *Thorax* **2001**, *56*, 602−606.

(4) Nagase, S.; Takemura, K.; Ueda, A.; Hirayama, A.; Aoyagi, K.; Kondoh, M.; Koyama, A. A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine *Biochem. Biophys. Res. Commun.* **1997**, *233*, 150−153.

(5) Wolkart, G.; Stessel, H.; Brunner, F. In vivo administration of D-arginine: effects on blood pressure and vascular function in angiotensin II-induced hypertensive rats. *Atherosclerosis* **2004**, *176*, 219−225.

(6) Lass, A.; Suessenbacher, A.; Wölkart, G.; Mayer, B.; Brunner, F. Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol. Pharmacol.* **2002**, *61*, 1081−1088.

(7) Szende, B.; Tyihak, E.; Trezl, L. Role of arginine and its methylated derivatives in cancer biology and treatment. *Cancer Cell Int.* **2001**, *1*, 3.

(8) Navarro, E.; Alfonso, S. J.; Martìn, F. A.; Castellano, M. A. Toxicological and pharmacological effects of D-arginine. *Basic Clinic. Pharmacol. Toxicol*. **2005**, *97*, 149−154.

(9) Boger, R. H.; Bode-Boger, S. M.; Kienke, S.; Brandes, R.; Dwenger, A.; Frolich, J. C. Supplementation of hypercholesterolaemic rabbits with L-arginine reduces the vascular release of superoxide anions and restores NO production. *Atherosclerosis* **1995**, *117*, 273−284.

(10) Okuma, E.; Abe, H. Simultaneous determination of D- and L-amino acids in the nervous tissue of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high-performance liquid chromatography. *J. Chromatogr., B* **1994**, *660*, 243−250.

(11) D'Aniello, S.; Spinelli, P.; Ferrandino, G.; Peterson, K.; Tsesarskia, M.; Fisher, G.; D'Aniello, A. Cephalopod's vision involves dicarboxylic amino acids: D-Asp, L-Asp and L-Glu. *Biochem. J.* **2005**, *386*, 331−340.

(12) Wolosker, H.; Sheth, K.; Takahashi, M.; Mothet, J. P.; Brady, R. O.; Ferris, C. D.; Snyder, S. H. Purification of serine racemase: Biosynthesis of the neuromodulator D-serine. *Proc. Natl. Acad. Sci. U.S.A.* **1999a**, *96*, 721−725.

(13) Wolosker, H.; Blackshaw, S.; Snyder, S. H. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-*N*-methyl-D-aspartate neurotransmission. *Proc. Natl. Acad. Sci. U.S.A.* **1999b**, *96*, 13409−13414.

(14) Wiesinger, H. Arginine metabolism and the synthesis of nitric oxide in the nervous system. *Prog. Neurobiol.* **2001**, *64*, 365−391.

(15) Devés, R.; Boyd, C. A. Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol. Rev.* **1998**, *78*, 487−545.

(16) Oldendorf, W. H. Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am. J. Physiol.* **1971**, *221*, 1629−1639.

(17) Mueckler, M. Facilitative glucose transporters. *Eur. J. Biochem.* **1994**, *219*, 713−725.

(18) Bonina, F. P.; Arenare, L.; Palagiano, F.; Saja, A.; Nava, F.; Trombetta, D.; de Caprariis, P. Synthesis, stability and pharmacological evaluation of nipecotic acid prodrugs. *J. Pharm. Sci.* **1999**, *88*, 561−567.

(19) Bonina, F. P.; Puglia, C.; Rimoli, M. G.; Melisi, D.; Boatto, G.; Nieddu, M.; Calignano, A.; La Rana, G.; de Caprariis, P. Glycosyl derivatives of dopamine and L-dopa as anti-parkinson prodrugs: Synthesis, pharmacological activity and in vitro stability studies. *J. Drug Targeting* **2003**, *1*, 25−36.

(20) Halmos, T.; Santarromana, M.; Antonakis, K.; Scherman, D. Synthesis of glucose-chlorambucil derivatives and their recognition by the human GLUT-1 glucose transporter. *Eur. J. Pharmacol.* **1996**, *318*, 477−484.

(21) Fernandez, C.; Nieto, O.; Fontenla, J. A.; Rivas, E.; de Ceballos, M. L.; Fernandez-Mayoralas, A. Synthesis and biological studies of glycosyl dopamine derivatives as potential antiparkinsonian agents. *Carbohydr. Res.* **2000**, *327*, 353−365.

(22) Baldwin, S. A. Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta.* **1993**, *1154*, 17−49.

(23) Welch, L. E.; Lacourse, W. R.; Mead, D. A., Jr.; Johnson, D. C. Comparison of pulsed coulometric detection and potential-sweep pulsed coulometric detection for underivatized amino acids in liquid chromatography. *Anal. Chem.* **1989**, *61*, 555−559.

(24) Marioli, J. M.; Sereno, L. E. Electrochemical detection of underivatized amino acids with a Ni Cr Alloy electrode. *J. Liq. Chromatogr. Relat. Technol.* **1996**, *19*, 2505−2515.

(25) Camel, V.; Thiebault, D.; Caude, M.; Dreux, M. Packed column subcritical fluid chromatography of underivatized amino acids. *J. Chromatogr., A* **1992**, *605*, 95−101.

(26) Saurina, J.; Hernandez-Cassou, S. Determination of amino acids by ion-pair liquid chromatography with post-column derivatization using 1,2-naphthoquinone-4-sulfonate. *J. Chromatogr., A* **1994**, *676*, 311−319.

(27) Crommen, J.; Hernè, P. Indirect UV detection of hydrophilic ionized compounds in reversed-phase liquid chromatography by use of a UV-absorbing ion of the same charge. *J. Pharm. Biomed. Anal.* **1984**, *2*, 241−253.

(28) Petritis, K. N.; Chaimbault, P.; Elfakir, C.; Dreux, M. Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent. *J. Chromatogr., A* **1999**, *833*, 147−155.

(29) Chaimboult, P.; Petritis, K.; Elfakir, C.; Dreux, M. Determination of 20 underivatized proteinic amino acids by ion-pairing chromatography and pneumatically assisted electrospray mass spectrometry. *J. Chromatogr., A* **1999**, *855*, 191−202.

(30) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131−138.

(31) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

JM060005S