RENAL-SELECTIVE DELIVERY AND ANGIOTENSIN-CONVERTING ENZYME INHIBITION BY SUBCUTANEOUSLY ADMINISTERED CAPTOPRIL-LYSOZYME

Jai Prakash, Annemiek M. van Loenen-Weemaes, Marijke Haas, Johannes H. Proost, Dirk K. F. Meijer, Frits Moolenaar, Klaas Poelstra, and Robbert J. Kok

Department of Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration, University of Groningen, Groningen, the Netherlands (J.P., A.M.V.L.W., J.H.P., D.K.F.M., F.M., K.P., R.J.K.); and BioMaDe Technology Foundation, Groningen, the Netherlands (M.H.)

Received October 29, 2004; accepted January 25, 2005

ABSTRACT:

In previous studies, we have demonstrated that the low molecular weight protein lysozyme can be used as a renal-selective drug carrier for delivery of the angiotensin-converting enzyme (ACE) inhibitor captopril. Typically, such macromolecular drug-targeting preparations are administered intravenously. In the present study, we investigated the fate of captopril-lysozyme following subcutaneous administration, a convenient route for long-term treatment. The absorption from the subcutaneous injection site and renal uptake of lysozyme were determined by gamma scintigraphy in rats. Bioavailability, renal accumulation, and stability of the captopril-lysozyme conjugate were evaluated by high performance liquid chromatography analysis and by ACE activity measurements.

Lysozyme was absorbed gradually and completely from the subcutaneous injection site within 24 h and accumulated specifically in kidneys. After subcutaneous injection of the captopril-lysozyme conjugate, higher renal captopril levels and lower captopril-lysozyme levels in urine indicated the improved renal accumulation in comparison with intravenous administration of the conjugate, as well as its stability at the injection site. After both treatments, captopril-lysozyme conjugate effectuated renal ACE inhibition, whereas plasma ACE was not inhibited. In conclusion, our results demonstrate that we can use the subcutaneous route to administer drug delivery preparations like the captopril-lysozyme conjugate.

Drug-targeting technology aims at delivering higher amounts of drug specifically to the desired site of action and at minimizing potential adverse effects in nontarget organs. In recent years, we have evaluated this concept using low molecular weight proteins (LMWPs) as drug carriers for selective delivery, to the kidneys, of various drugs, such as the angiotensin-converting enzyme (ACE) inhibitor captopril or the nonsteroidal anti-inflammatory agent naproxen (Haas et al., 2002). Although these drugs are being used routinely for different indications without serious side effects in the majority of patients, renal delivery may improve their effectiveness by increasing drug concentrations locally within the kidney without effectuating systemic effects such as blood pressure reduction. Eventually, this approach may help in understanding the contribution of organ-specific effects to the overall therapeutic profile of the drug. In the kidneys, LMWPs are freely filtered through the glomerulus and reabsorbed by receptor-mediated endocytosis into the proximal tubular cells, and subsequently catabolized intralysosomally into small peptides and single amino acids (Christensen and Nielsen 1991; Christensen and Birn 2001). Our studies demonstrated that drug-lysozyme conjugates selectively accumulated in the kidneys after intravenous administration (Haas et al., 1997; Kok et al., 1999). However, it is not feasible to administer such conjugates intravenously for long-term therapy in animal or human subjects. Although several routes of administration, such as oral, nasal, and pulmonary route, have been used for oligopeptides and proteins, the inconsistent and low bioavailability, local irritation, and immunogenicity imply major limitations (Wolff, 1998; Cleland et al., 2001). We now propose subcutaneous administration as an alternative dosage regimen for macromolecular drug-targeting conjugates. In fact, this route of administration has been used for the systemic delivery of LMWPs and other macromolecules such as insulin and heparin and has proven to be valid for extensive periods of time without serious side effects. Yet, the local stability at the subcutaneous site of injection and the rate of absorption of proteins from the subcutaneous tissue can be quite unpredictable and variable (Porter and Charman, 2000; Cleland et al., 2001; Porter et al., 2001).

In the present study, we determined the absorption of the renal-selective carrier lysozyme from the subcutaneous injection site and studied the renal handling in the physiological and pathological (proteinuria) state using gamma camera scintigraphy in healthy and doxorubicin (Adriamycin)-induced proteinuric rats. In these studies, we used a radiolabeling technique [123I-tyramine cellobiose (TC) labeling], which ensures entrapment of the radiolabel in the cells in which the product is internalized and degraded, thus allowing clear visualization of the distribution of the protein to the organs. In addition, we studied the pharmacokinetic fate of captopril-lysozyme conjugate following subcutaneous and intravenous injections. The captopril...
concentrations in kidneys and urine were estimated to assess the extent of renal uptake and loss of the conjugate in the urine, and plasma and renal ACE activities were determined to assess the formation of pharmacologically active drug from the captopril-lysozyme conjugate. Together, these analyses allowed us to evaluate the feasibility of the subcutaneous administration route as well as the fate of the targeted drug once accumulated in the kidneys.

Materials and Methods

Radiolabeling of Lysozyme. As described previously, lysozyme was labeled with radioactive iodine via TC, a label that is retained intracellularly after lysosomal degradation of the protein (Haas et al., 1993). Briefly, the synthesis of TC was performed by reductive amination of cellulose with tyramine (Pittman et al., 1983). Cellulose (10 mmol), 10 mmol tyramine hydrochloride, and 10 mmol propionic acid were dissolved in 40 ml of methanol. Sodium cyanoborohydride (12 mmol) was dissolved in 15 ml of methanol and added drop-wise. The mixture was refluxed overnight. Acetone (300 ml) was added and the precipitate was filtered. The precipitate was dissolved in water overnight and applied to a cation exchange column (Dowex W50-X4; size 0.25 × 20 cm). The column was eluted with 0.5 M ammonia and the absorbance measured at 279 nm. The first peak was collected and lyophilized twice to remove traces of ammonia. The yield was about 40%. For gamma camera studies, 123I labeling of TC and, subsequently, coupling to lysozyme (Sigma-Aldrich, St. Louis, MO) was carried out. In short, in an iodogen-coated tube, 10 ml of TC (0.01 M) in phosphate buffer (0.02 M, pH 7.2) and 80 MBq Na123I were incubated for 30 min at room temperature. The reaction was stopped by addition of 10 ml of sodium sulfite (0.05 M) and 5 ml of potassium-iodide (0.1 M). The solution was transferred to a clean tube to couple the iodinated TC to lysozyme. Cyanuric chloride (30 nmol) in 20 ml of acetone and 5 ml of NaOH (0.01 M) were added and the mixture was vortex-mixed for 30 s. Next, 10 ml of lysozyme solution (100 mg/ml in 10 mM sodium carbonate buffer, pH 9.0) was added and the solution was mixed gently. The 123I-TC-lysozyme complex was purified by Sephadex G25 gel filtration. After gel filtration, the radiolabeled protein contained less than 5% free 123I as determined by protein precipitation with purified lysozyme. After gel filtration, the radiolabeled conjugate was lyophilized and stored at −80°C. ACE activity was determined in kidney homogenates and plasma samples by a method of Hip-His-Leu conversion to His-Leu as described elsewhere (Oliveira et al., 2000). Total captopril (captopril–SH analysis after reduction of disulfide adducts) concentrations were estimated in all the samples by HPLC as described previously (Kok et al., 1997). In brief, samples were treated with the reducing agent 0.1% tributylphosphine for 20 min to reduce the dithio (–S–S–) bond between the thiol group of captopril and other thiol-containing molecules such as the linker used in the captopril-lysozyme conjugate. The formed captopril was separated by HPLC and detected by on-line postcolumn derivatization with o-phthalaldehyde, as measured by fluorescence of the captopril-o-phthalaldialdehyde adduct. In urine samples, the relative amounts of captopril-lysozyme and non-conjugate-bound captopril were also measured by precipitating intact captopril-lysozyme with methanol (3:1 v/v), followed by total captopril analysis in the supernatant and precipitate as described above. In another study, rats (n = 3) were injected with free captopril (1 mg/kg dissolved in 1 ml of 5% glucose) via the subcutaneous route. Rats were placed in metabolic cages and sacrificed at 2 h after administration of the drug. Urine samples were collected and analyzed for total captopril levels as mentioned above.

Pharmacokinetics and Statistical Calculations. The statistical analyses were performed using Student’s t test with p < 0.05 as the minimal level of significance unless indicated otherwise. SigmaStat Version 1 software (SPSS Inc., Chicago, IL) was used to analyze the statistical parameters. Results are presented as mean ± S.E.M. Pharmacokinetic analysis of the plasma captopril concentrations was performed using the Multitfit program (Department of Pharmacokinetics and Drug Delivery, University of Groningen, The Netherlands). The pharmacokinetic parameters for subcutaneous administration were calculated by performing simultaneous analysis of the data derived from the plasma captopril concentrations-time curve of intravenous and subcutaneous administrations. The two-compartment model and first-order kinetics were used to analyze these data. A log-normal distribution of the plasma concentration measurement errors was assumed, and log-transformed concentration data were used in the fitting procedure. The correctness of the latter assumption was tested by visual inspection of the graphs of the residuals plotted against time and against concentration. Goodness-of-fit was evaluated from visual inspection of the measured and calculated data points and of the residuals.

Downloaded from dmj.aspetjournals.org on June 26, 2017
plotted against time and against concentration. The choice between the one- and two-compartment model was based on the lowest value of Akaike’s information criterion (AIC).

Results

Gamma Camera Imaging. The external counting of $^{123}$I was performed using a gamma camera to study the absorption profile of lysozyme from the subcutaneous injection site, and the time course and total extent of renal uptake of lysozyme after a single subcutaneous or intravenous injection of radiolabeled lysozyme. This technique clearly demonstrated that lysozyme was gradually and almost completely absorbed from the site of injection in 24 h. After both subcutaneous and intravenous administration, lysozyme accumulated in the kidneys, as illustrated in Fig. 1. These pictures (Fig. 1, A to E) demonstrate that the subcutaneously administered lysozyme accumulated continuously in the kidneys, resulting in a gradual shifting of the radioactivity from the site of injection into two hot-spots at the renal locations. In the case of intravenous administration, $^{123}$I-TC-lysozyme was rapidly and primarily deposited into the kidneys within 15 min as shown in Fig. 1F. From the gamma camera data, we quantified the cumulative renal uptake of $^{123}$I-TC-lysozyme at the time points 0, 3, 6, 12, and 24 h (Fig. 2, solid lines). Approximately 90% of the dose of $^{123}$I-TC-lysozyme accumulated in the kidneys within 15 min after intravenous administration. A similar extent of renal accumulation was found after subcutaneous administration, but now the maximum renal content was reached only after 12 h.

In addition, we investigated the influence of total lysozyme dose on the renal uptake of radiolabeled lysozyme. Renal uptake of $^{123}$I-TC-lysozyme was decreased gradually and significantly with increasing dose of unlabeled lysozyme after intravenous administration. However, this decrease was statistically significant at the highest dose in the case of subcutaneous administration (Table 1). Of note, the values of renal uptake of $^{123}$I-TC-lysozyme were found to be significantly ($p < 0.01$) higher at the dose of 500 mg/kg after subcutaneous administration compared with that of intravenous administration.

Similar experiments were conducted in Adriamycin-induced nephrotic rats to observe the effect of proteinuria on the renal uptake of radiolabeled lysozyme. We found that the renal accumulation of $^{123}$I-TC-lysozyme was reduced at the pathological state by 30% ($p < 0.05$) both after intravenous and subcutaneous administration in comparison with healthy rats (Fig. 2, dashed lines). No hot-spots except the kidneys were detected by the gamma camera, whereas the radioactivity excreted in the urine during the 24-h period was increased (data not shown). Similar to healthy rats, the dose-dependent decrease in renal uptake was also found in the proteinuric rats; the renal uptake of $^{123}$I-TC-lysozyme was clearly lower at higher doses of unlabeled lysozyme (Table 1). Yet the subcutaneous administration displayed a better renal uptake profile, since uptake of lysozyme in the kidneys was higher with all three tested doses, compared with intravenous injection.

Pharmacokinetics of Captopril-Lysozyme. After determining the renal uptake of the carrier lysozyme, we compared the pharmacokinetics of the drug-lysozyme conjugate captopril-lysozyme after intravenous and subcutaneous administration in healthy rats. For this purpose, we determined captopril concentrations after reduction of disulfide bonds, thus analyzing the total concentration of captopril that can be regenerated from the captopril-lysozyme conjugate together with captopril–SH and captopril disulfide metabolites already formed in vivo. Since captopril disulfides are interchanged readily in vivo to free captopril–SH, all of these species of captopril can become pharmacologically active and are considered as the total sum of potentially active drug. We will therefore refer to these concentrations as total captopril concentrations. Figure 3 shows the plasma levels of total

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Dose of Unlabeled Lysozyme (mg/kg)</th>
<th>Healthy Rats</th>
<th>Adriamycin-Nephrotic Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous</td>
<td>Intravenous</td>
</tr>
<tr>
<td>0</td>
<td>89 ± 4.4</td>
<td>88 ± 1.9</td>
</tr>
<tr>
<td>100</td>
<td>75 ± 4.7</td>
<td>63 ± 3.6***</td>
</tr>
<tr>
<td>500</td>
<td>63 ± 3.5**</td>
<td>35 ± 5.7***</td>
</tr>
</tbody>
</table>

---

FIG. 1. Gamma camera images of the body distribution of lysozyme (indicated by $^{123}$I) after injection of 2 MBq of $^{123}$I-tyramine cellobiose-lysozyme in the healthy rat. A to E, at 0, 3, 6, 12, and 24 h after subcutaneous injection; F, at 15 min after intravenous injection.
Fig. 3. Total captopril concentrations (captopril-SH concentrations after reduction of disulfides) in plasma at different time points after single subcutaneous (closed symbols) and intravenous (open symbols) injection of captopril-lysozyme (equivalent to 1 mg/kg captopril) in healthy rats. The continuous and dashed lines show the pharmacokinetic data fit for intravenous and subcutaneous administration, respectively. Data represent the mean ± S.E.M. for n = 4 in both administrations.

captopril at various time points after a single subcutaneous or intravenous injection of the conjugate. The pharmacokinetic parameters derived from these plasma disappearance curves are shown in Table 2. To calculate pharmacokinetic parameters, one-compartment and two-compartment models were tested for the best fit according to AIC. The two-compartment model (AIC = −22.7) fitted our data significantly better than the first-compartment model (AIC = 8.0). The renal levels of captopril after subcutaneous injection gradually increased until 6 h, indicative of sustained absorption from the injection site, whereas after intravenous injection, they already reached a maximum after 1 h and subsequently decreased gradually (Fig. 4). Captopril concentrations in the kidneys following subcutaneous administration were significantly higher at 6, 12, and 24 h in comparison to the intravenous administration. In addition, to compare the efficiency of captopril-lysozyme accumulation in the kidneys, we calculated the renal AUC from 0 to 24 h (AUC0–24) after subcutaneous administration (242.8 ± 8.93 μg · h/g) was significantly higher than after intravenous administration (166.11 ± 7.29 μg · h/g). In accordance with the gamma camera studies with lysozyme, this experiment demonstrated that subcutaneous administration of the captopril-lysozyme conjugate resulted in a slow but sustained release from the site of injection and a concomitant accumulation in the kidneys in time. In urine samples, total and non-protein-bound captopril were measured and captopril-lysozyme excretion levels were calculated from those data. The protein-bound captopril (captopril-lysozyme conjugate) in urine at different time points shows that the conjugate was excreted in significantly higher amounts after intravenous injection than after subcutaneous injection (Fig. 5A). In addition, the constant levels of the conjugate in urine after both administration routes demonstrate that there was no further direct excretion of the conjugate in the urine after 4 h. Ure excretion of low-molecular weight captopril (free captopril plus captopril disulfide) after intravenous or subcutaneous injection was not significantly different except at 6 h after dosing (Fig. 5B). Moreover, Fig. 5B shows that subcutaneously administered free captopril was rapidly eliminated from the body. Total captopril levels after administering free captopril were significantly higher than after captopril-lysozyme intravenously or subcutaneously at all time points.

We measured the renal and plasma ACE activities at chosen time points to determine whether the subcutaneously administered captopril-lysozyme would effectuate a renal-selective profile similar to that of the intravenously administered conjugate. As shown in Fig. 6A, the plasma ACE activity was not significantly reduced at any time point after subcutaneous or intravenous administration. In contrast, the renal ACE activity was significantly inhibited until 12 h and subsequently increased to normal after intravenous administration. Similarly, following subcutaneous administration, renal ACE activity was also inhibited significantly until 12 h after injection (Fig. 6B), although the decline was more gradual as compared with intravenous injection. The difference between subcutaneous and intravenous injection of the renal-specific drug was only significant at t = 1 h.

Discussion

Many drug-targeting preparations, for instance, the drug-protein conjugate presented in this study, are macromolecular constructs that have to be administered parenterally. Commonly, these types of therapeutics are administered intravenously. When aiming for a chronic treatment protocol, however, even in laboratory animal studies, repeated intravenous injections are not convenient and may lead

**Table 2**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kp (per h)</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>F</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>Vm (ml/kg)</td>
<td>477 ± 89</td>
</tr>
<tr>
<td>Cl (ml/kg/h)</td>
<td>150 ± 14</td>
</tr>
<tr>
<td>t1/2 α (h)</td>
<td>0.46 ± 0.12</td>
</tr>
<tr>
<td>t1/2 β (h)</td>
<td>3.32 ± 0.23</td>
</tr>
</tbody>
</table>

Kp, absorption rate constant; F, bioavailability; Vm, volume of distribution at steady-state level; Cl, plasma clearance; t1/2 α, distribution half-life; t1/2 β, elimination half-life.
to adverse effects. Subcutaneous administration is in principle an attractive alternative route of administration. Data from the present study suggest that drug-targeting preparations can be administered subcutaneously, resulting in sustained and complete absorption in the target organ. In addition, the sustained release of lysozyme after subcutaneous administration restricts the dose-dependent and protein-uria-dependent loss of the carrier protein in the urine. The subcutaneous administration of captopril-lysozyme conjugate also leads to a sustained release of the intact conjugate from the site of injection, which results in a more prolonged residence time in kidneys compared with intravenous administration.

LMWPs are reabsorbed in the kidneys via the megalin/gp 330 receptor that is expressed specifically on the brush-border of proximal

Fig. 5. Urine excretion of intact captopril-lysozyme conjugate (A) and unbound captopril (captopril–SH and captopril –S–S-captopril) (B) at different time points after single subcutaneous (fine crossed bars) and intravenous (striped bars) injection of captopril-lysozyme (equivalent to 1 mg/kg captopril) in healthy rats. Data represent the mean ± S.E.M. for n = 4 in both administrations of the conjugate. Differences between subcutaneous and intravenous administration of captopril-lysozyme are presented as *, p < 0.05; and **, p < 0.01. The filled bar at 2 h (B) shows the urinary excretion of captopril after administering 1 mg/kg free captopril subcutaneously in rats (n = 3). At all time points, subcutaneously administered free captopril excretion is significantly (#, p < 0.05) higher than the captopril excretion (lysozyme bound and unbound) after subcutaneous or intravenous administration of the conjugate.

Fig. 6. Plasma (A) and renal (B) ACE activity after administering a single subcutaneous (closed symbols) and intravenous (open symbols) injection of captopril-lysozyme (equivalent to 1 mg/kg captopril) in healthy rats. One unit (U) of ACE activity represents nmol His-Leu/min. Data represent the mean ± S.E.M. for n = 4 in both administrations. The control values of renal ACE activity (55.82 ± 4.75 U/g; n = 7) and plasma ACE activity (50.3 ± 2.0 U/ml; n = 7) are shown as a dashed line. Plasma ACE activities were not inhibited significantly in both treatment groups. Renal ACE activities were inhibited significantly after captopril-lysozyme subcutaneously (4–12 h) and intravenously (1–12 h). At 1 h, renal ACE activity differed significantly between subcutaneous and intravenous administration of captopril-lysozyme (*, p < 0.05).
tubular cellular (Hysing et al., 1990). In addition, renal disease will damage the glomerulus, leading to filtration of plasma proteins, which subsequently are also reabsorbed by these receptors (Leheste et al., 1999). High concentrations of lysozyme or plasma proteins in the urine may saturate the megalin receptors and reduce the efficiency of the reabsorption process (Haverdings et al., 2001). Indeed, we observed this phenomenon when we administered different doses of lysozyme in healthy and proteinuric rats. Strikingly, this less efficient renal uptake was much less apparent after subcutaneous administration. From this result, it can be inferred that the slow release from the subcutaneous injection site reduces the levels of lysozyme in the kidneys, and, consequently, the degree of saturation due to high dose or plasma proteins is lower. We therefore conclude that the lysozyme carrier can deliver drugs to the kidney even at pathological conditions, and that the renal delivery is more efficient when using the subcutaneous route of administration.

Interestingly, the subcutaneous route resulted in higher levels of captopril in the kidneys for a prolonged period of time as compared with the intravenous route. In addition, we found that less conjugate was excreted directly in the urine, confirming our hypothesis that the renal tubular reabsorption process is more efficient after subcutaneous injection of captopril-lysozyme. This higher renal reabsorption of the conjugate after subcutaneous administration is possibly due to the prevention of the saturation of the receptor-mediated uptake process in contrast to intravenous administration. The urine data of the unbound captopril (Fig. 5B) show that both after subcutaneous and after intravenous injection of captopril-lysozyme, the released captopril was excreted continuously until 24 h in the urine. This result indicates that the conjugate accumulates and stays in the kidneys after internalization in the tubular cells until the release of captopril from the conjugate. In contrast, subcutaneous administration of nontargeted free captopril resulted in a rapid excretion of captopril in the urine. Thus, one of the major improvements in captopril disposition after administration of the conjugate is that the drug is available during a prolonged period, which may improve its local effects.

One of the concerns raised when discussing the subcutaneous administration of a drug-LMWP conjugate is the risk that a premature cleavage of the conjugate may occur at the site of injection as well as in the lymphatic fluid that drains the product into the general circulation. As discussed above, this would result in a rapid excretion of free captopril in the urine. Since we only observed minor amounts of such captopril products during the first hours of the experiment, we conclude that the captopril-lysozyme conjugate is accumulated intact within the kidney after subcutaneous administration. This result was corroborated by our results on plasma ACE activity, which showed no significant inhibitions until 24 h after injection of the captopril-lysozyme conjugate. The present data therefore show that the conjugate remained stable at the site of injection as well as in systemic circulation, causing no decline in plasma ACE activity. Within the kidneys, however, the renal ACE activity was reduced gradually and remained significantly inhibited until 12 h after subcutaneous administration, which reflects the release of the active captopril from the conjugate after tubular uptake in the kidneys. Renal ACE activity was inhibited more during the initial hours after intravenous administration of captopril-lysozyme. Obviously, intravenous injections will result in higher initial renal concentrations of the conjugate immediately after administration, whereas the subcutaneous route will result in lower levels at initial time points, but sustained higher levels are reached at later time points. This sustained uptake and release may be quite relevant since free captopril is eliminated rapidly from the kidneys (Kubo and Cody, 1985; Kok et al., 1999).

The present study with captopril-lysozyme resulted in approximately 40 to 50% of renal ACE inhibition after subcutaneous administration during the period of 4 to 12 h. Zoja et al. (2002) have shown the significant improvement of renal function in rats with passive Heymann nephritis after achieving roughly 50% renal ACE inhibition during a long-term treatment with an ACE inhibitor. The observed pharmacological effect in this study after administration of captopril-lysosome is therefore sufficient to reverse or retard kidney damage and proteinuria. Moreover, multiple dosing or higher doses of the conjugate may provide a more pronounced effect. Our data show that subcutaneous administration, which would be more convenient for multiple dosing, can be applied for this type of drug-targeting preparation.

Acknowledgments. We are grateful to R. Boerema for contribution to the gamma camera study. We thank J. Visser for technical assistance in the HPLC method for captopril estimation and J. H. Pol from the Department of Nuclear Medicine for radiolabeling of the proteins.

References


Address correspondence to: Dr. R. J. Kok, Department of Pharmacokinetics and Drug Delivery, University Center for Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands. E-mail: r.j.kok@rug.nl