Abstract

Purpose: In search for a new drug for intravesical use in superficial urothelial cell carcinoma of the bladder, a pig model is used for pharmacokinetics and toxicity measurements after intravesically administered pemetrexed.

Experimental Design: In the dose escalation phase, two groups of two pigs received 5 and 10 mg/kg pemetrexed intravesically; four groups of three pigs received 15, 20, 25, and 30 mg/kg, respectively. The well-being of the animals was monitored. Blood was studied for pharmacokinetic analysis and signs of myelosuppression. Posttreatment urine samples were collected to measure the concentration of pemetrexed after instillation. Twenty-four hours posttreatment, the animals were cystectomized and sacrificed. Histopathologic examination of the bladder wall was done. In the second study phase, five pigs were instilled weekly with the maximum tested dose for 6 weeks. The same methods were applied.

Results: All doses (5-30 mg/kg) in the first study phase were well tolerated, enabling the use of 30 mg/kg in the second study phase. In both study phases, the pigs' well-being was not influenced. Full blood counts showed no sign of myelosuppression. Systemic absorption was not observed. Urine pemetrexed concentrations remained almost unchanged. Histopathologic examination of the bladder wall did not reveal significant abnormalities. Bladder mucosa remained intact at any time, without hemorrhage.

Conclusions: Intravesically administered pemetrexed in pigs is well tolerated, not absorbed systemically, and causes no bladder wall toxicity.

Superficial urothelial cell carcinoma of the bladder has a high incidence and even higher prevalence due to a high recurrence rate after primary transurethral resection. Adjunctive intravesical instillations are used to lower the recurrence rate and the chance of progression to muscle-invasive disease. For intravesical instillations, the options are in principle 2-fold: chemotherapy or immunotherapy. Intravesical chemotherapy has modest effect on recurrence rate and, moreover, has repeatedly shown to have no influence on tumor progression. Intravesical immunotherapy, mostly bacillus Calmette-Guerin, clearly reduces recurrence rate significantly more compared with intravesical chemotherapy, although at a cost of inducing more systemic side effects (1). In addition, bacillus Calmette-Guerin is able to delay bladder tumor progression (2). Ultimately, in the treatment of patients with superficial bladder cancer, a new drug should be able to combine the benefits of existing intravesical therapies, with higher efficacy and less toxicity.

Pemetrexed (Alimta, LY231514, MTA; Eli Lilly and Company, Indianapolis, IN) is a multitargeted antifolate with structural similarity to methotrexate, the most commonly used antifolate today. It inhibits at least three enzymes involved in folate metabolism and purine and pyrimidine synthesis. Used systemically, pemetrexed has shown broad antitumor activity in human phase II (3–5) and III (6, 7) trials in a variety of solid tumors. Moreover, in advanced bladder cancer patients, the safety and efficacy of pemetrexed was explored in a phase II study (8). The antitumor activity observed in the trial was encouraging and identified pemetrexed as a potent drug against urothelial cancer.

We carried out a pig study consisting of a dose escalation and 6-week instillation phase. We studied animal well-being, potential myelosuppression, drug absorption, and bladder wall histology.

Materials and Methods

Approval was granted from the Committee for Animal Experiments (Radboud University Nijmegen Medical Centre, the Netherlands) before the study was undertaken. The animals used for this study were 3-month-old sows (crossbred York × York/Dutch pig). Rationale for the female pig was its close resemblance to humans with regard to the urogenital tract and the ease of transurethral catheterization (9). The sows were housed in special swine stainless steel battery cages and fed...
with universal swine food (Hendrix UTD B.V., Boxmeer, the Netherlands). The first part of the study contained the dose escalation phase. Procedures were done under general anesthesia. Premedication contained a mixture of 10 mg/kg ketamine, 0.5 to 1.0 mg/kg midazolam, and 1 mL atropine i.m. in one shot. Sedation maintenance was done by the same mixture in half the dosage, without atropine. Sixteen pigs were split in six smaller groups and received in increasing dose a single intravesical instillation of pemetrexed (Eli Lilly and Company) dissolved in 50 mL 0.9% NaCl. First, a French Foley 10ch catheter with luer lock system was inserted, and the bladder was emptied. Posttreatment and posttreatment urine was tested by pH indicator sticks. Respectively, and 5 and 10 mg pemetrexed per kg body mass was instilled in two groups of pigs and 15, 20, 25, and 30 mg/kg in four groups of three pigs. The solution remained in the bladder for 1 hour, after which the bladder was emptied, and the urine was collected; 1.8 mL was directly frozen in cryovials at −80°C for analysis of pemetrexed concentration (Taylor Technology, Inc., Princeton, NJ). This was done by a liquid chromatography tandem mass spectrometry method. The analyte was extracted from pig urine by precipitation of proteins with 7% perchloric acid. The supernatant was then chromatographed under reverse-phase conditions on a YMC Basic (100 Å 30 mm, 2.0 × 50 mm/50°C) column that used a gradient system with water and acetonitrile containing 0.2% formic acid. [2H4]pemetrexed was used as the internal standard. The compounds were detected and quantified by tandem mass spectrometry with electrospray ionization. The liquid chromatography tandem mass spectrometry method for determination of pemetrexed in both urine and plasma were validated for the concentration range of 1,000 to 200,000 ng/mL using 0.5 mL of pig urine/plasma, respectively. A 12.5-fold dilution was validated to show the ability of the assay to analyze samples at higher concentrations.

Blood samples were taken for pharmacokinetic analysis and full blood count. In the right jugular groove, 2 cm above the manubrium, the cephalic vein or internal, external, or communal jugular vein was punctured, depending on puncture angle and depth of needle penetration. The samples for pharmacokinetic analysis were taken before instillation of pemetrexed, 30 and 60 minutes after instillation and 30, 60, and 120 minutes after emptying the bladder. Blood was collected in 3 mL lithium heparin tubes with gel divider and transferred on ice to the laboratory for plasma processing within 30 minutes. At 4°C, the blood was centrifuged for 12 minutes at 3,000 rpm. Plasma was then transferred with a pipette into a 1.8-mL cryovial, stored at −80°C, and shipped on dry ice for analysis (Taylor Technology). This was done in a similar way as described above.

Samples for full blood count (i.e., signs of myelosuppression) were collected in a 5-mL potassium EDTA tube 60 minutes and 24 hours (just before cystectomy) after instillation. The samples were analyzed within 2 to 4 hours in the laboratory, using Advia 120 Hematology System (Bayer, Munich, Germany) and ADVIA multi species software version 3.1.8. Posttreatment, an experienced staff (veterinarian, animal keepers, and K.H.) examined the pigs’ well-being by a meticulously selected protocollary list of possible signs and symptoms of toxicity (Table 1). Twenty-four hours after instillation, the pigs were once more anaesthetized as described above. Mouth and vulva were inspected for signs of mucosal toxicity. Subsequently, the pigs received a mixture of O2, N2O, and isoflurane by inhalation and were cystectomized. After cystectomy, sacrifice was executed using an overdose of pentobarbital. Bladder biopsies of 1 cm² were taken from dome, trigone, right lateral wall, and left lateral wall and transferred into 10% formalin in PBS for 24 hours. Then the biopsies were embedded in paraffin, sectioned, and stained with H&E. The fresh slides were consistently evaluated by two observers (C.A.H.K. and K.H.) for signs of inflammation and allergic reaction in submucosa and mucosa. Quantification on a 0 to 3 scale corresponded with, respectively, no reaction to extreme reaction. Two unaffected bladders were gathered from a trauma course that used comparable pigs and used as histopathologic controls.

The second part of the study contained the 6-week instillation and observation phase. Five pigs received weekly instillations of pemetrexed, with the maximum tested treatment dose of the first phase of the study. Plasma for pharmacokinetic analysis was taken weekly 60 minutes after pemetrexed instillation (i.e., just before emptying the bladder). The puncture for full blood count at 24 hours was the only act for which the animals did not receive anesthesia. Again, the well-being of the animals was observed accurately. Posttreatment urine was collected and tested for pH. Twenty-four hours after the sixth instillation the animals were cystectomized and sacrificed.

### Results

Sixteen pigs were studied the first part of the study with a mean weight of 38.4 kg (range, 36.0–41.7 kg). After an average of five housing days, the pig’s weight lessened to 35.7 kg (range, 32.3–39.6 kg) just before instillation of pemetrexed, with hardly any weight difference 24 hours later at cystectomy. This weight loss is what can be expected by pig distress due to moving. The five pigs in the second phase all lost on average 2 kg up till the third week but then gained 3.8 kg in the last weeks. The mean instillation time for both phases of the study was 62 minutes (range, 59–72 minutes). Throughout both study phases, the animals showed no deterioration of their well-being. Inspection of the vulva revealed a hyperemic urethral orifice in one pig treated with 30 mg/kg. In the second study phase, this was seen in two pigs at the fifth instillation and in the other three pigs at the sixth instillation. No additional signs of mucosal toxicity were observed. Pig 13 (25 mg/kg) accidentally had a traumatic catheterization and did not receive pemetrexed. Its bladder was used for reference histopathology.

Posttreatment urinary pH (mean, 6.3; range, 5.3–8.0) was not influenced by the instillation of pemetrexed and comparable with pretreatment urinary pH (mean, 6.5; range, 5.0–8.5). Posttreatment urinalysis showed high amounts of pemetrexed (Table 2). This is more obvious when the urine concentration of pemetrexed is multiplied by end-treatment urine volume (Fig. 1). No difference was seen between a single dose of 30 mg/kg and six weekly instillations with 30 mg/kg.

Signs of myelosuppression were not observed (Figs. 2 and 3). Full blood count remained within the reference range. Only pig 1 in the second study phase had a reversible major platelet drop towards the second instillation (Fig. 3), without additional changes in its peripheral blood count. In all plasma samples, the concentration of pemetrexed was below the detection limit of 1,000 ng/mL.
The resected bladders showed no macroscopic abnormalities. For quantification of microscopic abnormalities, a mean score was calculated per group (the 0, 1, 2, and 3 scores counted and divided by the number of biopsies; Table 3). The mucosa remained intact without signs of erosion or hemorrhage, with the exception of three pre-erosive lesions attributed to mechanical damage by the catheter. Mucosal atypia or detrusor muscle changes were not observed. Neutrophil and lymphocyte infiltrations were mild and not related to dosage. Submucosal edema, hyperemia, and hemorrhage were mild.

Discussion

To lower superficial bladder cancer recurrence and progression rates and reduce the side effects of intravesical therapy as seen with the currently used intravesical agents, the search for new intravesical drugs remains legitimate.

Pemetrexed is a pyrrolo-pyrimidine analogue of folic acid that inhibits multiple folate-dependent enzyme targets involved in both purine and pyrimidine synthesis. Polyglutamated to the active pentaglutamid, which is the predominant intracellular form, it is a potent direct inhibitor of thymidylate synthase (10). The polyglutamation process of pemetrexed is more efficient than, for example, methotrexate, resulting in increased cellular retention of pentaglutamate and a 60-fold more potent inhibition of thymidylate synthase than its parent compound (11). Inhibition of the enzymes dihydrofolate reductase and glycaminid ribonucleotide formyltransferase seems to be of lesser importance.

Pemetrexed in its systemic application has shown a broad spectrum of clinical activity in multiple tumor types in phase II and III studies, including mesothelioma (7, 12), colorectal (13–16), breast (17), non–small cell lung (5, 6), pancreatic, head and neck (18), and cervical cancers (19). Several dosing schedules have been tested systemically in phase I studies (3). In an every-21-day schedule, 600 mg/m^2 seemed to be the maximum tolerated dose. Treating 20 patients with 600 mg/m^2, a mean maximum plasma concentration of 137 μg/mL was attained with a harmonic mean half-life of 3.1 hours (range, 2.2-7.2 hours). The mean clearance value was similar to that of creatinine clearance in the age range of the patients, and the

![Fig. 1. Total amounts of pemetrexed. Intravesically administered pemetrexed (dose multiplied by pig weight) compared with end-treatment amount of pemetrexed (measured end-treatment concentration multiplied by urine volume).](fig1)

![Fig. 2. WBC count in five pigs during six weekly instillations.](fig2)

Table 2. Per treatment group, the administered and measured end-treatment amount of pemetrexed

<table>
<thead>
<tr>
<th>Treatment dose (mg/kg)</th>
<th>Administered</th>
<th></th>
<th>End treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (mg/mL)</td>
<td>Total amount (mg)</td>
<td>Concentration (mg/mL)</td>
<td>Urine volume</td>
</tr>
<tr>
<td></td>
<td>3.2 (2.8-3.6)</td>
<td>59 (56-61)</td>
<td>188 (154-221)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.8 (5.5-6.0)</td>
<td>66 (64-68)</td>
<td>380 (373-386)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0 (4.7-8.8)</td>
<td>53 (46-61)</td>
<td>367 (248-449)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.2 (9.6-10.9)</td>
<td>59 (49-65)</td>
<td>607 (492-617)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.9 (7.9-9.9)</td>
<td>82 (78-85)</td>
<td>727 (613-841)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4 (7.1-16.6)</td>
<td>89 (70-126)</td>
<td>866 (507-1163)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>12.8 (6.2-27.1)</td>
<td>100 (49-167)</td>
<td>1051 (830-1327)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.8 (7.2-25.9)</td>
<td>81 (58-143)</td>
<td>1105 (640-1994)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15.8 (14.6-18.0)</td>
<td>72 (42-116)</td>
<td>1128 (635-1693)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.3 (10.6-24.4)</td>
<td>65 (51-86)</td>
<td>1184 (699-1806)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>21.4 (19.3-20.7)</td>
<td>1009 (966-1035)</td>
<td>1056-1089</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.1 (22.6-23.6)</td>
<td>1155 (1128-1182)</td>
<td>1104-1194</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>23.1 (22.1-23.9)</td>
<td>1155 (1104-1194)</td>
<td>1104-1194</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Mean concentration and total amount (lowest and highest values). The values were not rounded for the calculations that are made.
volume of distribution reflects limited distribution outside the bloodstream (20). The disposition of pemetrexed did not change after multiple doses, and no accumulation seemed to occur with multiple courses. Various phase II studies copied this every-21-day schedule with 600 mg/m² but often required dose reductions to 500 mg/m² due to toxicity (3), particularly when used in combination with other cytotoxic agents (13). Among the mostly seen side effects were grade 4 neutropenia and grade 4 thrombocytopenia, respectively, in 12% to 39% and 8% to 55% of the cases. These percentages were higher when also grade 3 toxicities were included. Other side effects included rash, mucositis, nausea, vomiting, fatigue, anorexia, elevation of liver transaminases, and febrile neutropenia. Patients at risk for these toxicities were found to have elevated pretreatment levels of either total plasma homocysteine concentrations, methylmalonic acid, or both (21, 22). To minimize the risk of severe toxicity, it was decided in 1999 to add folic acid and vitamin B₁₂ supplementation to all patients receiving the drug. This intervention, death rates and incidence of (non)hematologic toxicities decreased across all courses. Among the mostly seen side effects were grade 4 neutropenia and grade 4 thrombocytopenia, respectively, in 12% to 39% of the cases, with also significantly reduced mean baseline total plasma homocysteine concentration levels. There was no evidence for loss of efficacy of pemetrexed.

In this animal experiment, we hypothesized systemic absorption of pemetrexed through bladder wall passage to be unlikely, due to the antifolate's three charged groups and molecular weight of 597.46 g/mol. Furthermore, we partially aimed at intravesical dosages of pemetrexed that would give toxicity when administered systemically. We locally instilled pemetrexed in a one-time dosage range of 182 to 1,098 mg and serial dosages with the highest amount. In none of the dosages, the pig’s well-being deteriorated. Loss of body weight was already present before instillation with the drug and even improved at the end of the second study phase. Myelosuppression did not occur. Plasma analysis revealed little or no absorption of pemetrexed by the bladder. The remaining high amount of pemetrexed measured in post-instillation urine is in accordance with these findings, although some of the analyzed urine samples contained even higher amounts of pemetrexed than actually given (Table 2; Fig. 1). This can be explained by a minimal spread in validation accuracy and precision of the analysis method. The spread becomes more obvious when multiplying end-treatment urine volumes with urine concentrations of pemetrexed, to enable comparison of total amounts of pemetrexed. Histopathologically, there was a mild degree of inflammation in part of the cases, unrelated to drug dosage. The mucosa remained intact at all times without signs of erosion or substantial hemorrhage.

Due to a lack of formula for a pig’s distribution volume, it is almost impossible to translate a systemic human maximum tolerated dose of mg/m² to a precise intravesical dose of mg/kg in pigs. Studies with other intravesical drugs have indicated that much higher dosages could be given and tolerated, than when given systemically. In those cases, the systemic dose in mg/m² corresponded with the intravesical dose in mg/50 mL (cisplatin, doxorubicin, epirubicin, and gemcitabin). In our study, we have certainly treated with high dosages of pemetrexed but were not able to establish a toxic dose.

Many studies have analyzed the efficacy of pemetrexed when administered i.v. It has shown efficacy against advanced urothelial bladder cancer when administered systemically. To our knowledge, this is the first study in which pemetrexed is used intravesically. Throughout our animal experiment, the drug was safe in all aspects studied, and there was no systemic absorption. These features indicate that pemetrexed could be an attractive chemotherapeutic drug for intravesical instillation in the treatment of urothelial cell carcinoma.

**Acknowledgments**

We thank the personnel support and service of the Animal Laboratory, Radboud University Nijmegen Medical Centre.

**Table 3. Per dose of pemetrexed, the mean histopathologic item score (maximum score per group), based on a 0 to 3 score per biopsy**

<table>
<thead>
<tr>
<th>Treatment dose (mg/kg)</th>
<th>Cases</th>
<th>Total no. biopsies</th>
<th>Submucosa</th>
<th>Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Edema</td>
<td>Hyperaemia</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>12</td>
<td>0.08 (1)</td>
<td>0.08 (1)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>8</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>8</td>
<td>0.63 (1)</td>
<td>0.75 (1)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>12</td>
<td>0.25 (1)</td>
<td>0.42 (1)</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>12</td>
<td>0.08 (1)</td>
<td>0.33 (1)</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>8</td>
<td>0.38 (1)</td>
<td>0.63 (1)</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>12</td>
<td>0.75 (1)</td>
<td>1.08 (2)</td>
</tr>
<tr>
<td>6 × 30</td>
<td>5</td>
<td>20</td>
<td>0.80 (2)</td>
<td>1.15 (2)</td>
</tr>
</tbody>
</table>
Potential and Toxicity of Intravesical Pemetrexed: a Preclinical Study in Pigs
Kees Hendricksen, Paula M.J. Moonen, Antoine G. van der Heijden, et al.


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