New insights in pyrimidine antagonist chemotherapy

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 09-11-2018
Gemcitabine and epirubicin plasma concentrations and excretion in saliva in non-small cell lung cancer patients.

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Submitted
Abstract

Aim The excretion in saliva of gemcitabine and its metabolite 2',2'-difluorodeoxyuridine (dFdU) as well as epirubicin and its metabolite epirubicinol was studied in non-small cell lung cancer patients, treated with gemcitabine plus epirubicin.

Methods Patients (n=12) were treated with gemcitabine 1125 mg/m², followed by epirubicin 100 mg/m². Blood, saliva and oral mucosa cells were collected during 22 h for analysis of gemcitabine, epirubicin and their metabolites. Gemcitabine, dFdU, epirubicin and epirubicinol were quantified by High Performance Liquid Chromatography (HPLC).

Results Gemcitabine was rapidly cleared from plasma and undetectable after 3 h in all patients. Gemcitabine was detectable in saliva only during the first hour after infusion. The $C_{max}$ in saliva was 0.66 ± 0.61 mg/L and the saliva/plasma ratio (S/P ratio) was 0.038 ± 0.037. Epirubicin displayed a triexponential plasma concentration-time profile. The concentration in saliva at t=6 h after administration was 55 ± 27 µg/L and decreased to 28 ± 14 µg/L at t=22 h. The corresponding S/P ratios were 1.28 ± 0.73 and 1.72 ± 1.00. The amount of epirubicin in mucosal cells ranged from 135-598 ng/10⁶ cells at t=3 h and decreased to 33-196 ng/10⁶ cells at t=22 h.

Conclusion Gemcitabine and epirubicin as well as their main metabolites dFdU and epirubicinol are excreted in detectable amounts in saliva, although their absolute amounts remain relatively low.

Introduction

Gemcitabine and epirubicin, respectively a pyrimidine antagonist and an anthracycline analogue, are both extensively used in the treatment of solid tumors. Both agents are rapidly distributed to tissues and quickly cleared from plasma immediately after intravenous administration. The clearance of gemcitabine is primarily determined by its degradation into 2',2'-difluorodeoxyuridine (dFdU) by the enzyme cytidine deaminase [1]. Epirubicin is cleared by liver metabolism and excretion into bile [2]. The main metabolite is epirubicinol, which also has cytotoxic activity. The plasma pharmacokinetics of both drugs have been described previously [3-5]. Hardly any information however is available with respect to the excretion of gemcitabine and epirubicin into saliva. Previously, the excretion in saliva of the pyrimidine antagonist cytarabine and that of the anthracycline analogue doxorubicin have been reported [6-8]. Saliva is secreted by the parotid, sublingual and submaxillary glands, with the parotid and submaxillary glands accounting for 90% of the approximately 1200 mL of saliva that is secreted each day [9]. The blood supply to the glands is provided by the external carotid artery with the direction of the arterial flow being countercurrent to the direction of salivary flow within the ductal system. The excretion of cytotoxic drugs in saliva may be related to the development of side effects.
such as oral mucositis and diarrhea. The extent of salivary secretion is mainly determined by the lipophilicity and pKa of the drug, the level of protein binding of the drug in plasma and the pH of saliva. Drugs are believed to enter saliva predominantly via passive diffusion, a process that is limited to the un-ionized drug [9]. The aim of the current study was to determine the excretion of gemcitabine and epirubicin and respectively their main metabolites dFdU and epirubicinol in saliva. In addition, for these drugs the importance of salivary excretion as a route of elimination was evaluated.

Patients and methods

Patient selection
This study was performed as a site study of a phase III trial of gemcitabine plus epirubicin versus gemcitabine plus cisplatin in advanced NSCLC patients [10]. Patients had to meet the inclusion and exclusion criteria of the main study. In short, patients were included if they had stage IIIB/IV NSCLC. No prior chemotherapy was allowed. An adequate bone marrow reserve, normal renal and liver function were required. Patients were excluded if they had active infections, second primary malignancies, uncorrected hypercalcaemia or a left ventricular ejection fraction (LVEF) less than 45%. A detailed description of the inclusion and exclusion criteria was published earlier [10]. The local medical ethics committee of the hospital approved the protocol. All patients gave informed consent before study entry. The gemcitabine and dFdU plasma pharmacokinetic data presented in this study were also used to explore the interaction of gemcitabine and epirubicin in another trial. The results of this interaction study are shown in chapter 9. The toxicity was scored before chemotherapy and on days 8, 15 and 21 according to the Common Toxicity Criteria, version 2.0.

Treatment and sample collection
Gemcitabine (Gemzar®, Lilly, Nieuwegein, The Netherlands) in a dose of 1125 mg/m² in 250 mL 0.9% NaCl solution was administered as a 30 min infusion on day 1 and day 8. Epirubicin (Farmorubicine®, Pharmacia, Woerden, The Netherlands) in a dose of 100 mg/m² in 50 mL 0.9% NaCl solution was administered as an intravenous bolus injection over 5 min, immediately after the end of the gemcitabine infusion, on day 1 of each 21-day cycle. For pharmacokinetic sampling, a cannula was placed intravenously in the arm of the patient contralateral to the side of drug administration. Blood samples of 9 mL were collected in heparinized tubes containing 0.25 mg tetrahydrouridine (THU) in 50 µL water, just before chemotherapy and at t = 25, 40, 50, 60, 75, 90, 105, 120, 150, 180, 270, 360, 540 and 1320 min after start of the gemcitabine infusion. Unstimulated whole saliva was collected over 5 min periods in plastic cups, containing 0.25 mg THU in 50 µL water, just before chemotherapy and at t = 25, 40, 50, 60, 90, 120, 180, 270, 360, 540 and 1320 min after start of the gemcitabine
infusion. The saliva pH was measured with a calibrated pH meter on the day of sample collection. Harvesting of oral mucosa cells was performed before chemotherapy, and 180 min and 1320 min after start of the gemcitabine infusion by thorough mouth rinsing during a 30 sec period with 5 mL 0.9 % NaCl solution. The cell suspension was collected in plastic cups, containing 0.25 mg THU in 50 µL water, and subsequently placed on ice. Within 1 h after collection, the cell suspension was centrifuged during 10 min at 190 g at 4 ºC. The supernatant was discarded and cells were washed in 10 mL ice cold Phosphate Buffered Saline, pH=7.40 (PBS) and centrifuged during 10 min at 190 g at 4 ºC. The pellet was resuspended in 1 mL ice cold PBS and a 50 µL aliquot of this cell suspension was mixed with 50 µL tryptan blue solution (0.4% in a 8.77 g/L NaCl solution) for microscopic cell counting. The viability was estimated by measuring the percentage of cells able to exclude tryptan blue. The remaining suspension was transferred in a microcentrifuge cup and shortly (15 s) centrifuged at 10,000 g. The supernatant was discarded and the pellet was kept frozen at – 80 ºC until further analysis.

**HPLC analysis**

Epirubicin hydrochloride and epirubicinol hydrochloride were obtained from Pharmacia & Upjohn (Milano, Italy). Doxorubicin hydrochloride was purchased from Rhône-Poulenc Pharma (Hoovelaken, the Netherlands). Gemcitabine hydrochloride and dFdU, were obtained from Lilly Co., (Indianapolis, IN). Tetrahydouridine was purchased from Calbiochem (La Jolla, CA). All other chemicals were of standard analytical grade.

The extraction and analysis of epirubicin and epirubicinol from plasma and saliva was carried out according to the method previously described by Dodde et al. [11].

The analysis of gemcitabine and dFdU in plasma was carried out as described by Freeman et al. [12]. For the analysis in saliva, a slight modification on this method was performed as follows: Individual 200 µL aliquots of plasma or 1 mL aliquots of saliva standards, controls and subject samples were pipetted into 10 mL glass tubes. For plasma sample concentrations above 10 mg/L, sample volumes from 20 - 100 µL were used and diluted to 200 µL with THU treated plasma. A 50 µL aliquot of working internal standard solution was added to each tube. The samples were briefly vortex-mixed. Then 1 mL isopropanol was added and the samples were vortex-mixed again. After 5 min a 2.5 mL aliquot of ethylacetate was added and vortex-mixed. The samples were centrifuged at 2,500 g for 10 min. The supernatant was transferred to a fresh tube and evaporated at 40 ºC under a stream of nitrogen. The samples were reconstituted with 250 µL of mobile phase.

The extraction of epirubicin and epirubicinol from cells was performed as follows: Frozen cell pellets were thawed and resuspended in 150 µL ultrapure water using a vortex mixer. Protein denaturation was carried out by addition of 50 µL trichloroacetic acid solution 50%. The mixture was allowed to stand on ice during 20 min. Subsequently, the suspension was centrifuged during 2 min at 10,000 g. The supernatant was transferred into a clean cup and 1 mL of trioctylamine : 1,1,2-trichlorotrifluoroethane (1:5) was added.
Table 1  Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(n=12)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td></td>
<td>10/2</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td>65 ± 7</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td>77 ± 13</td>
<td></td>
</tr>
<tr>
<td>Length (cm)</td>
<td></td>
<td>176 ± 4</td>
<td></td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td></td>
<td>1.93 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Serum-Creatinine (µmol/L)</td>
<td></td>
<td>86 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

Mean values are presented ± the standard deviation

Table 2  Toxicity CTC score during the first treatment cycle

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Grade 0 n</th>
<th>Grade 1-2 n</th>
<th>Grade 3-4 n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Neutro-/Granulocytes</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Platelets</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Nausea</td>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Vomiting</td>
<td>11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mucositis</td>
<td>5</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3  Epirubicin pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Epirubicin</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg)</td>
<td>190 ± 15</td>
<td>1880 ± 578</td>
<td>1483 ± 565</td>
</tr>
<tr>
<td>$AUC_{0→22,\text{h}}$ (µg.h/L)</td>
<td></td>
<td>414 ± 164</td>
<td>16.0 ± 4.1</td>
</tr>
<tr>
<td>$V_{\text{distribution}}$ (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI (L/h)</td>
<td>90 ± 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{saliva},6,\text{h}}$ (µg/L)</td>
<td>55 ± 27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/P ratio $6,\text{h}$</td>
<td>1.28 ± 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{saliva},22,\text{h}}$ (µg/L)</td>
<td>28 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/P ratio $22,\text{h}$</td>
<td>1.72 ± 1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values ± the standard deviation are presented.
mixture was vortexed during 1 min and subsequently centrifuged for 2 min at 10,000 g. The supernatant was divided over 2 autosampler tubes for HPLC analysis of epirubicin and epirubicinol. The lower limit of quantitation was 5 µg/L for epirubicin and 2 µg/L for epirubicinol.

Pharmacokinetic analysis
Pharmacokinetic data were analyzed in Mw\Pharm (version 3.5; MediWare, Groningen, The Netherlands) using KinFit. For both gemcitabine and epirubicin and their metabolites, the Area Under the Curve (AUC; trapezoid rule) and elimination half-lifes were calculated by non-compartment analysis. Additionally, the epirubicin and epirubicinol data were also analyzed in a three compartment pharmacokinetic model. The saliva/plasma ratios were calculated by dividing saliva and plasma concentrations at each time point.

Statistical analysis
Subject data were analyzed for descriptive statistics in the SYSTAT 7.0 statistical program (SPSS inc. 1997). Blood, saliva and mucosal cellular levels and saliva/plasma ratios on different time points were compared with a paired Student’s t-test. Pairs consisted of data obtained from the same individual.

Results

Patients
Between November 2000 and September 2002 we included 12 patients. Patient characteristics are represented in table 1. All patients had normal liver and renal function parameters. Treatment related toxicity, measured during the first cycle, is represented in table 2. Nearly all patients experienced hematological toxicity. The main non-hematological toxicity was grade 1/2 mucositis in 7 out of 12 patients.

Pharmacokinetics
Mean pharmacokinetic curves of gemcitabine and dFdU in saliva are represented in figure 1. Gemcitabine and dFdU plasma data, also reported in detail in chapter 9, were used for saliva/plasma ratio (S/P ratio) calculations and for this reason included in figure 1. Gemcitabine was rapidly cleared from plasma and undetectable after 3 h in all patients. Both gemcitabine and dFdU data were fitted in a parent drug – metabolite model, comprising one compartment for gemcitabine and two compartments for dFdU pharmacokinetics. Gemcitabine was detectable in saliva only during the first hour after infusion. The $C_{max}$ in saliva was $0.66 \pm 0.61$ mg/L and the S/P ratio was only $0.038 \pm 0.037$. This was in line with the theoretical S/P ratio, calculated for gemcitabine.

Mean pharmacokinetic curves of epirubicin and epirubicinol in plasma and saliva are
Figure 1  Mean concentration-time profiles of gemcitabine and its metabolite dFdU in plasma and saliva after a 30 min infusion of 1125 mg/m² gemcitabine. Error bars represent the s.d. values.

Figure 2  Mean concentration-time profiles of epirubicin and its metabolite epirubicinol in plasma and saliva after a bolus dose of 100 mg/m² epirubicin. The error bars represent the s.d. values.
represented in figure 2. Data of epirubicin and epirubicinol were fitted separately in a three-compartment pharmacokinetic model. Table 3 represents the pharmacokinetic parameters of both compounds in plasma and saliva, including S/P ratios at t=6 h and t=22 h after epirubicin administration. Figure 3 displays the S/P ratios at t=6 h (upper panel) and t=22 h (lower panel) as function of saliva pH. There was no consistent effect of pH on the S/P ratio. Nearly all S/P ratios of epirubicin exceeded 1, which was above the theoretical maximum value calculated for epirubicin.

Mucosal cells for quantification of intracellular epirubicin were available from 7 patients. The amount of epirubicin ranged from 135-598 ng/10⁶ cells at t=3 h and decreased to 33-196 ng/10⁶ cells at t=22 h (p<0.05, paired t-test; see figure 4).
Excretion of gemcitabine in saliva occurred in negligible amounts. Low S/P ratios were measured and gemcitabine was only detectable in small amounts during the first hour after infusion. The low S/P ratios were in line with our expectations, since gemcitabine is a hydrophilic molecule and ionized at physiological pH. Based on the pK\(_a\) and fraction unbound in plasma, for each drug the S/P ratio can be calculated. For weak acidic drugs the general formula is [13]:

\[
\frac{S}{P} = \frac{1 + 10^{(pH_s - pK_a)}}{1 + 10^{(pH_p - pK_a)}} \times \frac{f_p}{f_s}
\]

Where \(f_p\) and \(f_s\) represent the fractions of unbound drug in plasma and saliva and pH\(_s\) and pH\(_p\) represent the salivary and plasma pH values.

Gemcitabine has a pK\(_a\) of 3.6 and is hardly bound to proteins [14]. This results in theoretical S/P ratios from 0.0006 to 0.13 depending on saliva pH over a range from 4 to 7.4. Low S/P ratios have also been reported for the pyrimidine antagonist cytarabine [6].

Following the administration of epirubicin, a triexponential disappearance of epirubicin from plasma was observed with a rapid distribution phase and a prolonged elimination phase. This pharmacokinetic profile is typical for anthracyclines [5]. The prolonged elimination is consistent with extensive drug retention within peripheral tissues and

Figure 4 Amounts of epirubicin in oral mucosa cells (n=7) measured at t = 3 h and at t = 22 h after a bolus dose of 100 mg/m\(^2\) epirubicin. Values from the same individual are paired with a closed line.
gradual release thereafter. Anthracyclines are accumulated within cells against a gradient, because of the intracellular presence of high affinity receptors such as DNA. Compared to gemcitabine, much larger S/P ratios were observed for epirubicin, which were in line with previous findings for doxorubicin [8]. The pKₐ of epirubicin is 7.7 and the fraction unbound in plasma 0.8, which results in theoretical S/P ratios ranging from 0.13–1.00 depending on saliva pH values from 4.0–7.4 [15]. The S/P ratios of epirubicin increased significantly with time during the 22 h period after dosing, mostly due to decrease of plasma levels. The absolute amount of epirubicin excreted in saliva remained however low, as saliva concentrations never exceeded 220 µg/L. As a result, in patients with a normal saliva production of 1.2 – 1.5 L per day, the total amount epirubicin excreted over 24 h is only 0.3 mg, which corresponds to about 0.1–0.15 % of the administered dose. The mean gemcitabine C_max in saliva was about 0.7 mg/L and rapidly dropped to undetectable levels within 2 h. The total amount excreted in saliva during this period was less then 0.1 mg, which is 0.05 % of the gemcitabine dose.

These calculations indicate that the gastrointestinal exposure to epirubicin and gemcitabine as a result of excretion in saliva is limited. Although more than 50% of all patients in our study experienced grade 1/2 mucositis, we believe that it is indeed not very likely that the mucositis is caused by a direct effect of the small amounts of gemcitabine excreted in saliva during only few hours. On the other hand, we are less sure about the role of epirubicin. After an initial distribution phase, the epirubicin concentrations were generally higher in saliva than in plasma and epirubicin remained detectable in saliva until at least 22 h after intravenous administration. The prolonged exposure of oral mucosal cells to both epirubicin in saliva and epirubicin from blood in the extravascular fluid may have added to the development of mucositis.

We conclude that gemcitabine and epirubicin as well as their main metabolites dFdU and epirubicinol are excreted in detectable amounts in saliva, although their absolute amounts remain relatively low.
References


14. Gemzar package insert (Lilly Canada), 12/12/96.