The Protective Effect of Pyrimidine Nucleosides on Human HaCaT Keratinocytes Treated with 5-FU

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Abstract. Background: Therapy with 5-fluorouracil (5-FU) and capecitabine is often complicated by skin toxicity (hand-foot syndrome, HFS). Topical application of uridine ointment is beneficial for alleviating HFS and other pyrimidine nucleosides have been described as 5-FU toxicity modulators. We tested pyrimidine nucleosides and their combinations to find the best combination for topical therapy of HFS. Materials and Methods: Cellular viability was measured by the real-time cell analyser and methyl thiazol tetrazolium (MTT) assay in order to evaluate the effect of pyrimidine nucleosides on HaCaT keratinocytes treated with 5-FU. The results were confirmed by evaluation of the cellular colonization by microphotography. Results: Cytidine and uridine protected keratinocytes to the same extent. Thymidine enhanced the protective effect when added to cytidine or uridine. Deoxycytidine did not have any protective effect. Conclusion: Our findings support the rationale for using uridine or cytidine in combination with thymidine in ointment for HFS treatment.

In European and US guidelines, 5-fluorouracil (5-FU) in bolus or continuous administration, as well as the oral 5-FU prodrug capecitabine, are cornerstones of chemotherapeutic regimens in the treatment of colorectal and other types of cancers (1). Continuous infusion of 5-FU or oral administration of capecitabine leads to better tolerability with a different adverse effect profile than bolus administration of 5-FU. In this setting, less haematological and gastrointestinal toxicity occurs and hand-foot syndrome (palmar-plantar erythrodysesthesia, HFS) becomes one of the most remarkable problems (2). Although HFS is mostly regarded as a non-severe adverse effect, it worsens the patient’s quality of life and may lead to a dose reduction or even interruption of the otherwise effective therapy (3, 4). A case report describing death following sepsis due to HFS development was published recently (5).

HFS can be characterized as non-specific keratinocyte reaction to cytotoxic drug presence in the skin (6). 5-FU toxicity is mediated by incorporating the fluorinated nucleosides into nucleic acids and by the thymidylate synthase (TYMS) inhibition. The correlation of the latter mechanism with the clinical response of colorectal cancer to 5-FU treatment has been described by Noordhuis et al. (7). A high TYMS expression also correlates with tumor resistance to 5-FU-based therapy (8). Uridine was shown to reduce growth inhibition in some human and murine cell lines treated with 5-FU by preventing 5-FU incorporation into RNA (9, 10) and 10% uridine ointment is already used in empirical clinical practice in Germany, Poland and the Czech Republic for reducing HFS (11).

Thymidine has been widely studied as a 5-FU therapy-modulating agent. It has been shown to have its own cytotoxic activity and to increase 5-FU incorporation into RNA by several mechanisms which leads to increased 5-FU toxicity (12). On the other hand, it can also abrogate TYMS inhibition and reverse the toxicity of the selective TYMS inhibitor tomudex (13). Thymidine is used as a cell division blocking agent for synchronisation of the cell cycle (14). This occurs because thymidine blocks deoxycytidine synthesis via allosteric inhibition of ribonucleotide reductase, which leads to the depletion of the deoxycytidine pool and disruption of DNA synthesis. This can be prevented in human leukemia T-lymphocytes by deoxycytidine supplementation (15).

Very little is known about the influence of cytidine on 5-FU toxicity. In one study, increased intracellular levels of...
atmosphere at 37˚C and 3.5% CO2. The cells were maintained in a humidified box supplemented with sodium pyruvate, 1 g/l NaHCO3, 10% bovine serum, 2% foetal MEM) supplemented with non-essential amino acids, 0.12 g/l streptomycin). The cells were maintained in a humidified atmosphere at 37˚C and 3.5% CO2.

In our study, we tested uridine, cytidine, thymidine, deoxycytidine and their combinations for their ability to protect human skin cells against 5-FU-induced damage. For this purpose we used the human keratinocyte cell line HaCaT (17). We measured the cell viability by Real-Time Cell Analyser (Roche, Prague, Czech Republic) which allows a continuous evaluation of cell viability (18). The results were confirmed by the methyl thiazol tetrazolium (MTT) assay (19) and by evaluating the cell density after May-Grünwald and Giemsa-Romanowski staining. We used microphotography images for the evaluation of changes in cell morphology.

Materials and Methods

Cultivation of HaCaT keratinocytes. HaCaT cells were kindly provided by professor Dr. J. Bereiter-Hahn, Kinematic Cell Research Group, Institute for Cell Biology and Neurosciences, Goethe University Frankfurt am Main, Germany. HaCaT cells are a spontaneously immortalized human epithelial cell line that maintains full epidermal differentiation capacity (17). The cells were cultured in HEPES-buffered Minimum Essential Medium (H-MEM) supplemented with non-essential amino acids, 0.12 g/l sodium pyruvate, 1 g/l NaHCO3, 10% bovine serum, 2% foetal bovine serum and antibiotics (200 U/ml penicillin and 100 μg/ml streptomycin). The cells were maintained in a humidified atmosphere at 37˚C and 3.5% CO2.

The tested substances. Uridine, cytidine, thymidine and deoxycytidine were obtained as ≥99% powder in cell-culture suitable quality from Sigma-Aldrich (SIGMA-ALDRICH, Prague, Czech Republic) and further diluted as described below. 5-FU was obtained from local hospital pharmacy as a 50 mg/ml solution in 10% sodium dodecyl sulphate (SDS) solution in distilled water. After overnight incubation, formazan production was stopped by addition 100 μl of 10% sodium dodecyl sulphate (SDS) solution in distilled water. After overnight incubation, the plates were analysed by an enzyme linked immunosorbent assay (ELISA) reader (570 nm test wavelength and 630 nm background wavelength). The mean values of absorbance from the wells with the same concentration of the tested agents (three for each setting) were considered as indicators of the cellular metabolic activity.

Real-time cell analyser (xCELLigence (R), Roche, Prague, Czech Republic). Fourteen thousands cells in 100 μl medium were plated into each of the wells of a 96-well plate. Each well has gold electrodes on the bottom surface. The measurable impedance between these two electrodes increases when the cells are growing and dividing. The cell surface changes and the adhesive and morphological changes also influence the measurable impedance. As a result, a cell index is derived from the aforementioned cellular properties. The cell index can generally be considered as an indicator of cellular viability, with some limitations (18). The results presented in this study are not obtained from measurements of the outer wells, which frequently exhibit considerably different results because of cells being affected by evaporation.

The tested substances (5-FU and pyrimidine nucleosides) diluted in 100 μl of the medium were added when the cell index plot curves were growing exponentially. The final concentration of 5-FU was 7.5 μg/ml. This concentration was selected after several tests of different 5-FU concentrations. For nucleosides, 100 μg/ml was selected as the concentration of uridine or cytidine clearly protecting the cells against a cytotoxic damage induced by 7.5 μg/ml 5-FU. When using the combination of two nucleosides, 50 μg/ml of each nucleoside were used in order to eliminate the effect of a higher nucleoside concentration in the medium.

We found that exponential growth of the cell index curve occurs when the cells are slightly subconfluent. The cell index value was recorded every 15 min. Time 0 represents the time of adding the tested compounds. Methyl thiazol tetrazolium (MTT) assay. Methyl thiazol tetrazolium was obtained as 98% powder from Sigma-Aldrich (SIGMA-ALDRICH), diluted in phosphate buffered saline (PBS) to obtain 5 mg/ml concentration and filtered through 0.22 μm sterile filter. We performed the classical endpoint MTT test described by Mosman (19). The cells were plated into 96-well plate (14,000 cells per well in 100 μl of the medium without phenol red). The outer wells were not seeded with cells but filled with sterile water for injection. When cellular layers were almost confluent, 100 μl of the tested agents dissolved in the medium were added to the wells. The obtained concentrations of tested agents were the same as used for real-time cell analyser test. For each of 12 different agents settings (5-FU only or in combination with pyrimidine nucleosides or their combinations), three wells were used (total number of 36 wells). The metabolic activity was measured after four days of cultivation. 10 μl of MTT (5mg/ml) was added to the wells. After six hours of incubation, formazan production was stopped by addition 100 μl of 10% sodium dodecyl sulphate (SDS) solution in distilled water. After overnight incubation, the plates were analysed by an enzyme linked immunosorbent assay (ELISA) reader (570 nm test wavelength and 630 nm background wavelength). The mean values of absorbance from the wells with the same concentration of the tested agents (three for each setting) were considered as indicators of the cellular metabolic activity.

Microphotographs and photographs of cells cultured in Petri dishes. We plated cells into 35 mm Petri dishes (100,000 cells per Petri dish in 4 ml of medium with phenol red). When the cell cultures nearly reached confluency, the medium was replaced by 4 ml of the test drug solution dissolved in medium. The concentrations of tested agents were the same as those used for real-time cell analyser test. After four days, the medium was washed out and the cells were stained by May-Grünwald and Giemsa-Romanowski solutions. Photographs of the whole dish show the density of the cell colonization, and the microscopic morphology shows the degree of cell damage (20).

Results

Single nucleosides protective effect measured by real-time cell analyser. Thymidine or deoxycytidine at 100 μg/ml did not significantly prolong the cell survival of cells treated with 5-FU. Uridine and cytidine at the same concentration prolonged cell survival for approximately two days (52 and 64 h, respectively). 5-FU without any protective nucleoside first stimulated the cells to reach higher cell index values than was observed for the control cells and then the curve decreased rapidly. A very similar curve progression was shown when deoxycytidine was added to the 5-FU-treated cells. After reaching the same cell index values as the cells treated with 5-FU only, the curves of cells treated with 5-FU
Figure 1. Real-time cell analyser measurement. Comparison of the protective effect of nucleosides in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of HaCaT cell viability (cell index values) after 150 h of exposure to 5-FU (7.5 μg/ml) only (red curve) or with protective agents: 5-FU + uridine (100 μg/ml, orange), 5-FU + cytidine (100 μg/ml, green), 5-FU + thymidine (100 μg/ml, blue) and 5-FU + deoxycytidine (100 μg/ml, violet). The black curve represents control cells in culture medium.

Figure 2. Real-time cell analyser measurement. Comparison of the protective effect of the uridine, thymidine and uridine-thymidine combination in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of the HaCaT cell viability (cell index values) after 300 h of exposure to the 5-FU (7.5 μg/ml) only (red curve) or with protective agents: 5-FU + uridine (100 μg/ml, green), 5-FU + thymidine (100 μg/ml, blue), 5-FU + thymidine and uridine (50 μg/ml for each, violet). The black curve represents control cells in the culture medium.
Figure 3. Real-time cell analyser measurement. Comparison of the protective effect of the cytidine, thymidine and cytidine-thymidine combination in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of HaCaT cell viability (cell index values) after 300 h of exposure to the 5-FU (7.5 μg/ml) only (red curve) or with protective agents: 5-FU + cytidine (100 μg/ml, green), 5-FU + thymidine (100 μg/ml, blue) and 5-FU + thymidine and cytidine (50 μg/ml for each, violet). The black curve represents control cells in the culture medium.

Figure 4. Methyl thiazol tetrazolium assay (MTT). HaCaT cell line. Comparison of the cellular metabolic activity represented by absorbance of formazane produced from MTT after four days of treatment with 7.5 μg 5-fluorouracil (5-FU)/ml alone or 7.5 μg 5-FU/ml together with single pyrimidine nucleosides (100 μg/ml of uridine (U), cytidine (C), thymidine (T) or deoxycytidine (dC)) or their combinations (50 μg/ml for each one of the combination). The absorbance is directly proportional to the metabolic activity of the cells. Control: Untreated cells.
and uridine or cytidine proceeded for approximately two days at the same level and then they decreased rapidly. Interestingly, the curve for 5-FU and thymidine-treated cells did not reach such high cell index values as for other compounds and progressed with similar values to those of the control cell curve. This was followed by a rapid decrease in the same time as that for cells treated with 5-FU only (Figure 1, Table I).

**Nucleoside combinations protective effect measured by real-time cell analyser test.** The curves of the 5-FU and thymidine-treated cells reach high cell index values as the control cells and they decreased rapidly in the same time as the cells treated with 5-FU alone (Figures 1 and 2). When uridine (Figure 1) or cytidine (Figure 2) was added to 5-FU-treated cells, the curves proceeded to a similar level to that for cells treated with 5-FU-only but continued in proceeding in this level for two more days after 5-FU-only treated cells died. The cell viability curves for cells treated with 5-FU and thymidine-uridine combination (Figure 2) or 5-FU and thymidine-cytidine combination (Figure 3) are similar to the control curve, without reaching such high values as cells treated with 5-FU-alone. The cells protected by thymidine-cytidine or thymidine-uridine combination survived more than 200 h longer than the cells without any protective agent (220 and 218 h respectively; Table I). Other nucleoside combinations prolonged cell viability less than the thymidine-cytidine or thymidine-uridine combination (Table I).
**Methyl thiazol tetrazolium (MTT) test.** The results of the MTT test presented in Figure 4 and Table II show the metabolic activity of the cells after four days of cultivation in the medium with different combinations of 5-FU and pyrimidine nucleosides. In agreement with the results from the RTCA analysis, the best protection was given by the uridine-thymidine and cytidine-thymidine combinations. In comparison with control cells, the metabolic activity measured by absorbance was 85.6±2.6% and 76.0±8.2% for uridine-thymidine and cytidine-thymidine combinations respectively. Uridine and cytidine alone showed much less protective activity in comparison with control (24.5±2.0% and 24.5±3.0%, respectively) and the lowest protective activity was measured for thymidine and deoxycytidine alone (4.2±0.5% and 3.3±0.7%, respectively) (Figure 4, Table II).

Photographs of whole Petri dish cultures and cell microphotographs. Figure 5 shows photographs of whole Petri dish cultures and microphotographs of the cells cultivated in the presence of 5-FU and various combinations of nucleosides. In agreement with the results from the RTCA analysis, the best protection was given by the uridine-thymidine and cytidine-thymidine combinations. In comparison with control cells, the metabolic activity measured by absorbance was 85.6±2.6% and 76.0±8.2% for uridine-thymidine and cytidine-thymidine combinations respectively. Uridine and cytidine alone showed much less protective activity in comparison with control (24.5±2.0% and 24.5±3.0%, respectively) and the lowest protective activity was measured for thymidine and deoxycytidine alone (4.2±0.5% and 3.3±0.7%, respectively) (Figure 4, Table II).

**Discussion**

In our previous study as well as here, we confirmed that uridine can protect against 5-FU toxicity in HaCaT cells and that the uridine-thymidine combination prevents 5-FU damage even better than uridine alone (21). Herein we showed that cytidine has the same protective effect against 5-FU as uridine and the cytidine-thymidine combination (Figures 3-5, Tables I and II) as the uridine-thymidine combination (Figures 2, 4 and 5; Tables I and II). This was confirmed by three different methods, which provide a strong level of confidence. The addition of

### Table I. Real-time cell analyser test results. HaCaT cell line. Comparison of the protective effect of uridine (U), cytidine (C), thymidine (T), deoxycytidine (dC) and their combinations in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. Comparison of the maximal cell index values, times when maximal cell index values were reached and times to cell death (cell index of 5).

<table>
<thead>
<tr>
<th></th>
<th>Maximal cell index value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time when maximal cell index was reached (h)</th>
<th>Time to death (cell index of 5) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells in culture medium</td>
<td>10.4±0.3</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>5-FU only (7.5 μg/ml)</td>
<td>11.6±0.6</td>
<td>30</td>
<td>53</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (100 μg/ml)</td>
<td>12.8±0.4</td>
<td>43</td>
<td>105</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (100 μg/ml)</td>
<td>12.7±0.5</td>
<td>43</td>
<td>117</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + T (100 μg/ml)</td>
<td>9.6±0.2</td>
<td>37</td>
<td>55</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + dC (100 μg/ml)</td>
<td>12.4±0.8</td>
<td>25</td>
<td>56</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (50 μg/ml) + T (50 μg/ml)</td>
<td>9.3±0.4</td>
<td>139</td>
<td>273</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (50 μg/ml) + C (50 μg/ml)</td>
<td>11.6±0.3</td>
<td>42</td>
<td>97</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (50 μg/ml) + C (50 μg/ml)</td>
<td>12.8±0.8</td>
<td>32</td>
<td>81</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + T (50 μg/ml)</td>
<td>8.7±0.4</td>
<td>132</td>
<td>271</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + dC (50 μg/ml)</td>
<td>10.8±1.7</td>
<td>29</td>
<td>84</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + dC (50 μg/ml) + T (50 μg/ml)</td>
<td>6.9±0.8</td>
<td>22</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell index is an indicator of cell viability repeatedly recorded by the real-time cell analyser.

### Table II. Methyl thiazol tetrazolium (MTT) test results for the HaCaT cell line. Comparison of the protective effect of uridine (U), cytidine (C), thymidine (T), deoxycytidine (dC) and their combinations in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml.

<table>
<thead>
<tr>
<th></th>
<th>Absorbance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control in the culture medium</td>
<td>1.22±0.03</td>
<td>100±2.5</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (50 μg/ml) + T (50 μg/ml)</td>
<td>1.05±0.03</td>
<td>85.6±2.6</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + T (50 μg/ml)</td>
<td>0.93±0.10</td>
<td>76.0±8.2</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + U (50 μg/ml)</td>
<td>0.41±0.03</td>
<td>33.2±2.0</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (50 μg/ml) + C (50 μg/ml) + dC (50 μg/ml)</td>
<td>0.15±0.04</td>
<td>12.6±3.0</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + dC (50 μg/ml)</td>
<td>0.15±0.04</td>
<td>12.6±3.0</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + C (50 μg/ml) + dC (50 μg/ml) + T (50 μg/ml)</td>
<td>0.17±0.02</td>
<td>13.7±1.8</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (100 μg/ml)</td>
<td>0.30±0.03</td>
<td>24.5±2.0</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + U (100 μg/ml) + C (100 μg/ml)</td>
<td>0.30±0.04</td>
<td>24.5±3.0</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + T (100 μg/ml) + dC (100 μg/ml)</td>
<td>0.05±0.01</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>5-FU (7.5 μg/ml) + dC (100 μg/ml)</td>
<td>0.04±0.01</td>
<td>3.3±0.7</td>
</tr>
<tr>
<td>5-FU only (7.5 μg/ml)</td>
<td>0.09±0.01</td>
<td>7.1±0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The absorbance is directly proportional to the metabolic activity of the cells.
thymidine either to uridine or to cytidine led to better results than the combination of uridine and cytidine together, even though thymidine-alone did not have any protective activity at all (Figures 4 and 5; Tables I and II).

The protective effect of cytidine may be caused by its conversion into uridine by CTP synthetase 2 (CTPS2), an enzyme which converts UTP to CTP and vice versa. CTPS2 inhibition has been described to have a similar protective effect against 5-FU as uridine addition to human tumour cell lines and xenografts. This is probably due to an accumulation of UTP after CTPS2 inhibition and a subsequent competition of UTP with 5-flourouridine for RNA incorporation (22).

TYMS inhibition and subsequent toxicity can be abrogated by thymidine (13). The absence of any thymidine protective activity on the HaCaT cells when not combined with other nucleosides (Figures 1-5, Tables I and II) may indicate that RNA incorporation of 5-FU is the main toxicity pathway of 5-FU in this cell line, rather than TYMS inhibition. RNA incorporation of 5-FU can be abrogated by uridine, which is supported by our results and by a previous study by Codacci-Pisanelli et al. in C26-10 tumor-bearing mice (9). They showed that a co-administration of uridine to these mice enabled an increase of the maximal tolerated 5-FU bolus dose, therefore augmenting TYMS inhibition in the tumor. This also shows that the mechanism of toxicity may differ between tumourous and healthy cells. Moreover, another study with ‘delayed uridine rescue’ in mice describes a treatment toxicity reduction without reducing antitumor efficacy (23). The different toxicity mechanisms of 5-FU in tumor and healthy tissues may be used for the tissue-selective treatment with 5-FU. In the case of HFS, the tissue-specific treatment is also achieved by topical application of uridine by means of ointment which is already used in clinical praxis (11).

The lack of protective potential for thymidine when not in combination with uridine or cytidine can also be explained by its own toxicity which leads to blocking cell division (14). This can be by-passed by deoxycytidine supplementation (15). Nevertheless we did not prove that thymidine-deoxycytidine combination leads to a better protection than thymidine itself (Figure 4, Table I and II). This indicates that inhibition of cell division by thymidine probably plays a less important role in the toxicity mediation in HaCaT cells when thymidine was added to 5-FU, at least at 7.5 μg/ml 5-FU concentration, and when treated cells are nearly confluent. The RTCA viability curves of cells treated with 5-FU and thymidine or 5-FU and thymidine in combinations with other pyrimidine nucleosides did not reach as high values of the cell index as the curves for the viability of cells without thymidine (whether 5-FU alone or in combination with other pyrimidine nucleosides). This may be due to an inhibition of cell division by thymidine, but against this conclusion is the fact that the control curve proceeds to the similar cell index values as the curves of cells protected by thymidine (Figure 1-3, Table I).

Dihydropyridine dehydrogenase (DPD) is the rate-limiting enzyme of 5-FU degradation pathways. The role of DPD in HFS development was suggested in literature (24). This may indicate the potential role of 5-FU degradation products in HFS pathogenesis. Nevertheless, the study on HaCaT cells did not prove that the 5-FU catabolites produced by DPD cause particular toxicity to human keratinocytes (25).

Conclusion

HFS often causes serious complication of fluoropyrimidine-based chemotherapy, especially in case of long-term administration. In the summary of the product characteristics of capecitabine (Xeloda), withdrawal or dose reduction is recommended for HFS treatment (4). Uridine ointment is a promising way of ameliorating 5-FU-induced skin toxicity without influencing the anticancer efficacy of the treatment. The presented study supports this therapeutic approach by pre-clinical data on the protective activity of uridine against 5-FU-induced keratinocyte damage. Furthermore, by three different methods, we showed that uridine can be replaced by cytidine without loss of efficacy and that an addition of thymidine to either of these two ribonucleosides would probably improve treatment efficacy.

Acknowledgements

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