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The Protective Effect of Pyrimidine Nucleosides on Human Keratinocytes HaCaT 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. Matherials 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 cancer (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 nonspecific 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 tumour 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 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

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cytidine triphosphate (CTP) were associated with 5-FU resistance in V79 Chinese hamster cells (16).

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 human keratinocyte cell line HaCaT (17). We measured the cell viability by Real-Time Cell Analyser (Roche s. r. o., 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 $NaHCO_3$, 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% CO_2.

The tested substances. Uridine, cytidine, thymidine and deoxycytidine were obtained as >99% powder in cell-culture suitable quality from Sigma-Aldrich (SIGMA-ALDRICH s. r. o., Prague, Czech Republic) and further diluted as described below. 5-FU was obtained from local hospital pharmacy as a 50 mg/ml solution in water for injection (EBEWE Pharma Ges. m. b. H. Nfg. KG, Unterach, Austria).

Real-time cell analyser (xCELLigence (R), Roche s. r. o., 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 have found that exponential growth of the cell index curve occurs when the cells are slightly subconfluent. The cell index value was recorded every 15 minutes. 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 s. r. o., Prague, Czech Republic), 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 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 prolong the cell survival of cells treated with 5-FU significantly. Uridine and cytidine at the same concentration prolonged cell survival for approximately two days (52 and 64 hours, 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

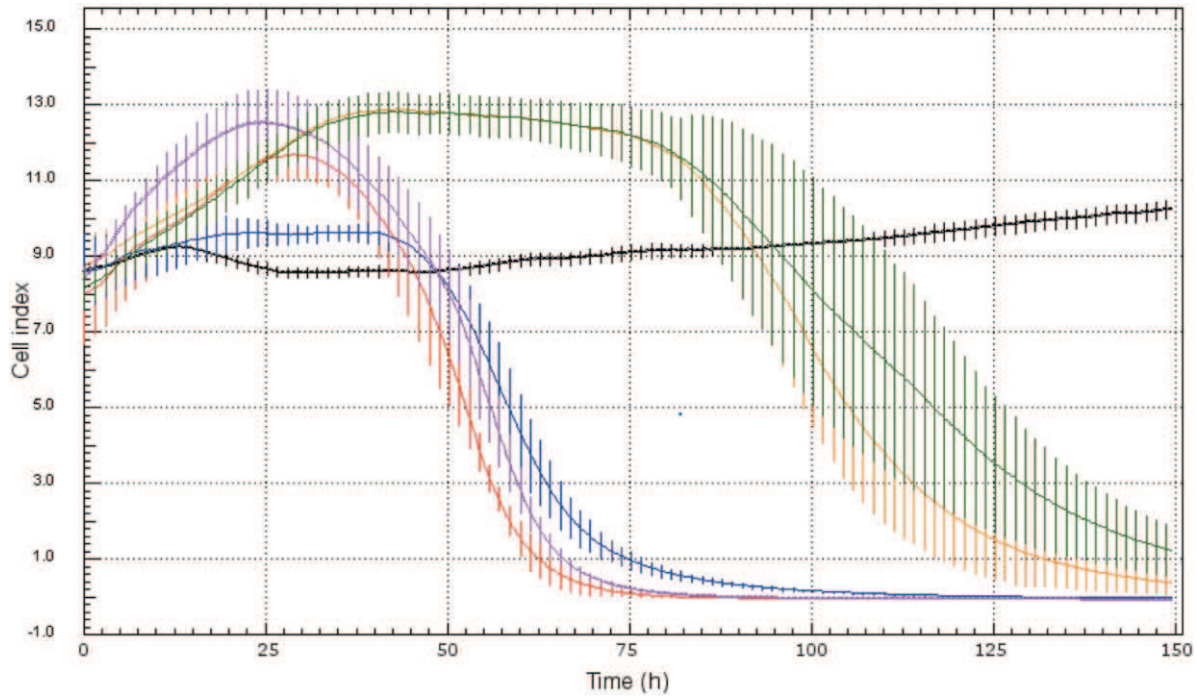

Figure 1. *Real-time cell analyser measurement. Comparison of the protective effect of nucleosides in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of HaCaT cell viability (cell index values) after 150 hours of exposure to 5-FU (7.5 μg/ml) only (red curve) or with protective 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.*

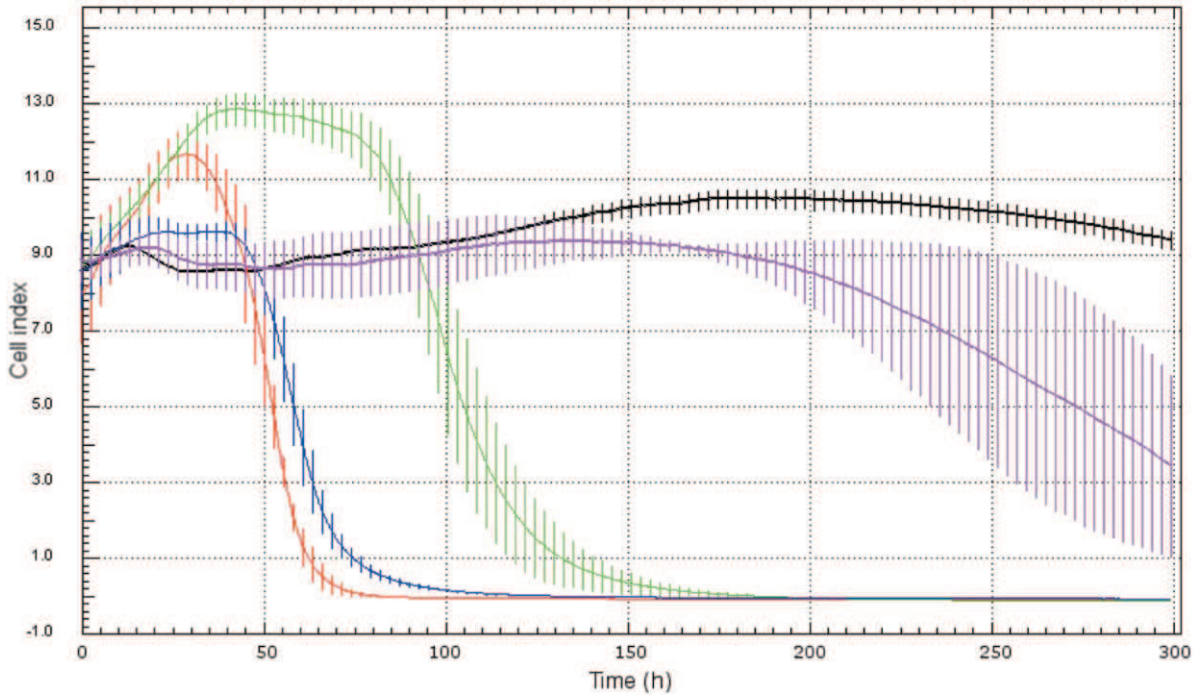

Figure 2. *Real-time cell analyser measurement. Comparison of the protective effect of the uridine, thymidine and uridine-thymidine combination in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of the HaCaT cell viability (cell index values) after 300 hours of exposure 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.*

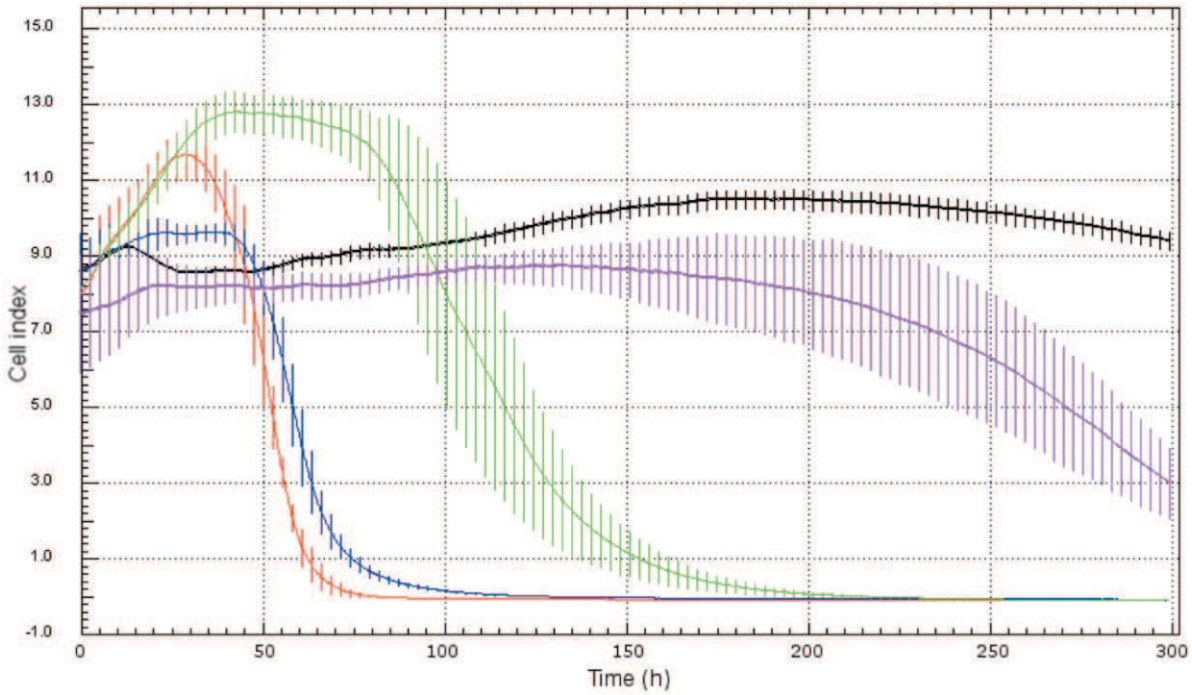

Figure 3. *Real-time cell analyser measurement. Comparison of the protective effect of the cytidine, thymidine and cytidine-thymidine combination in the presence of 7.5 μg 5-fluorouracil (5-FU)/ml. The curves represent the course of HaCaT cell viability (cell index values) after 300 hours of exposure 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.*

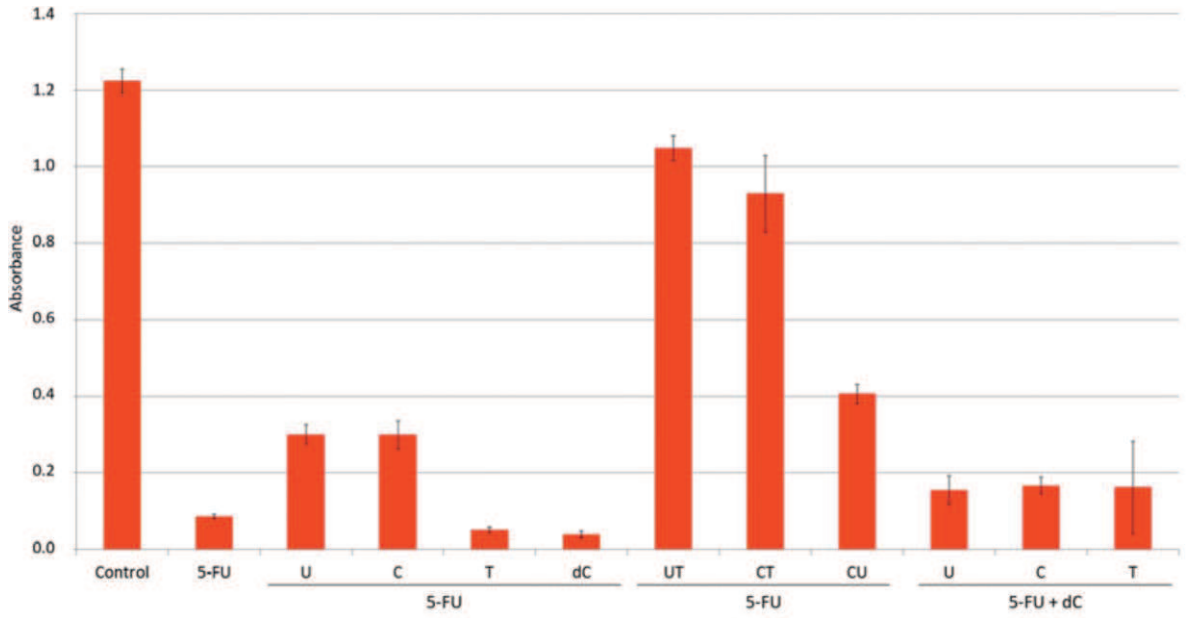

Figure 4. *Methyl thiazol tetrazolium assay (MTT). HaCaT cell line. Comparison of the cellular metabolic activity represented by absorbance of formazane produced from MTT after four days of treatment with 7.5 μg 5-fluorouracil (5-FU)/ml alone or 7.5 μg 5-FU/ml together with single pyrimidine nucleosides (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.*

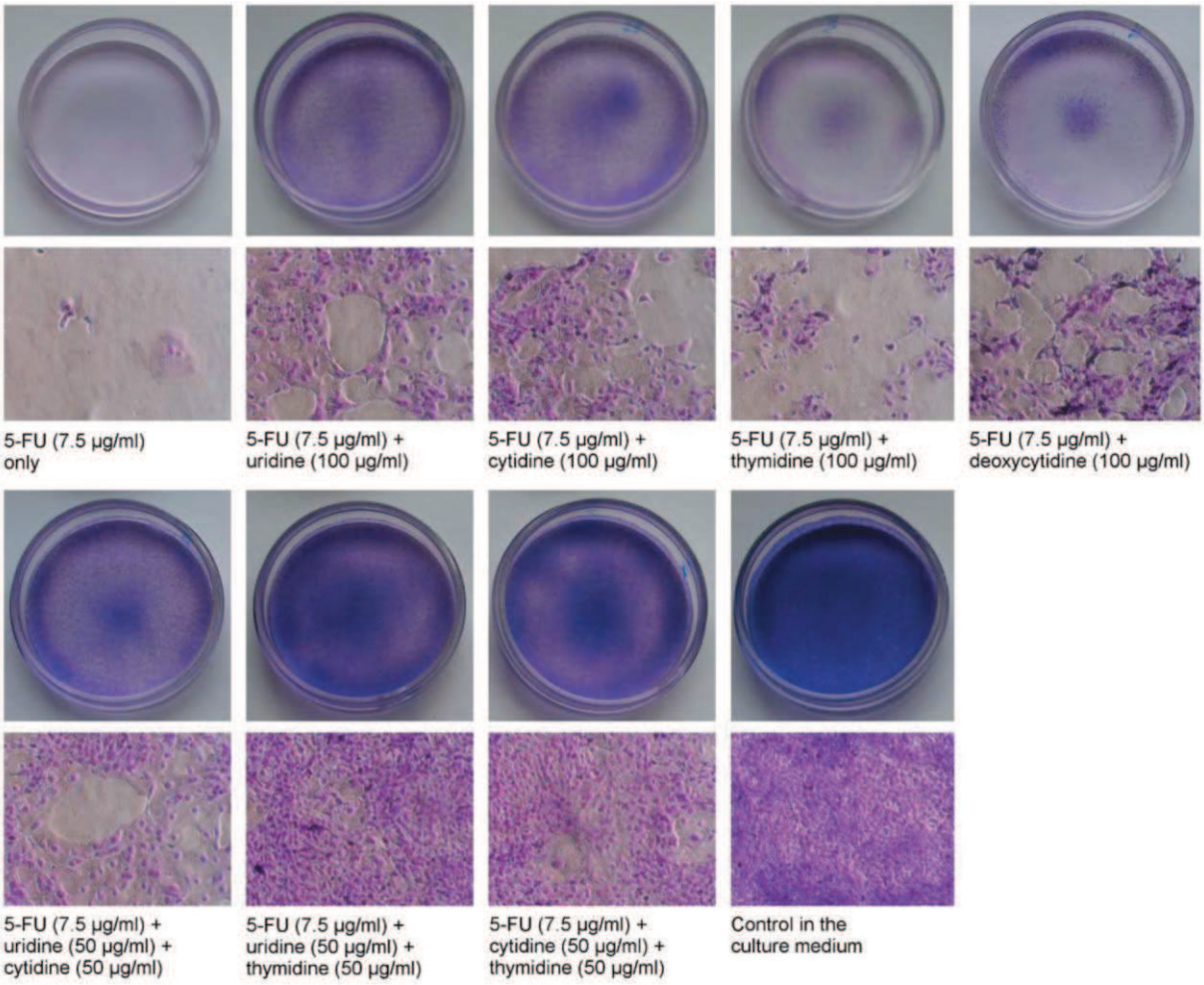

Figure 5. *Images of HaCaT cells cultured in Petri dishes and microphotographs of HaCaT cells cultivated with 5-fluorouracil (5-FU) and various combinations of protective nucleosides. The cells are stained by May-Grünwald and Giemsa-Romanowski staining, ×100 magnification. The darker the bottom of the dish, the greater the cell density the and therefore the higher the viability of the cells. The microphotograph below each dish illustrates the morphology of the cells. In the control dish without 5-FU, no signs of toxicity can be seen and a very similar appearance is clear for the uridine-thymidine and cytidine-thymidine dishes. All other combinations and single nucleosides resulted in less viability. The dish for cells treated with 5-FU alone is almost completely free of cells.*

5-FU 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 the 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 as 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 proceed to the similar level to that for cells treated with 5-FU only but continues 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 the cells

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).*

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

[a]Cell index is an indicator of cell viability repeatedly recorded by the real-time cell analyser.

treated with 5-FU alone. The cells protected by thymidine-cytidine or thymidine-uridine combination survived more than 200 hours longer than the cells without any protective agent (220 and 218 hours 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 the 5-FU and various combinations of nucleosides. The viability can be estimated according to the fraction of the cells that remained attached to the culture dish which are stained to dark blue and according to the cell morphology. In comparison with control cells, 5-FU-treated cells exhibited a reduction in the density of colonies and signs of apoptosis in the microphotographs. While only insignificant signs of apoptosis and low reduction in density are apparent for dishes with uridine-thymidine and cytidine-thymidine combinations, more severe damage can be seen in the presence of the uridine-cytidine combination and uridine or cytidine alone. Thymidine and deoxycytidine had only a slight protective effect and no cells were present in dish with cells treated with 5-FU alone (Figure 5).

Table II. *Methyl thiazol tetrazolium (MTT) 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.*

	Absorbance[a]	% of control
Control in the culture medium	1.22±0.03	100.0±2.5
5-FU (7.5 μg/ml) + U(50 μg/ml) + T(50 μg/ml)	1.05±0.03	85.6±2.6
5-FU (7.5 μg/ml) + C(50 μg/ml) + T(50 μg/ml)	0.93±0.10	76.0±8.2
5-FU (7.5 μg/ml) + C(50 μg/ml) + U(50 μg/ml)	0.41±0.03	33.2±2.0
5-FU (7.5 μg/ml) + U(50 μg/ml) + dC(50 μg/ml)	0.15±0.04	12.6±3.0
5-FU (7.5 μg/ml) + C(50 μg/ml) + dC(50 μg/ml)	0.17±0.02	13.7±1.8
5-FU (7.5 μg/ml) + T(50 μg/ml) + dC(50 μg/ml)	0.16±0,12	13.3±9.8
5-FU (7.5 μg/ml) + U(100 μg/ml)	0.30±0.03	24.5±2.0
5-FU (7.5 μg/ml) + C(100 μg/ml)	0.30±0.04	24.5±3.0
5-FU (7.5 μg/ml) + T(100 μg/ml)	0.05±0.01	4.2±0.5
5-FU (7.5 μg/ml) + dC(100 μg/ml)	0.04±0.01	3.3±0.7
5FU only (7.5 μg/ml)	0.09±0.01	7.1±0.5

[a]The absorbance is directly proportional to the metabolic activity of the cells.

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). Here 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) has the same potential 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 evidence. The addition of 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 (Figure 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 converses 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-fluorouridine 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 tumour-bearing mice (9). They showed that a co-administration of uridine to these mice enabled increase of the maximal tolerated 5-FU bolus dose, therefore augmenting TYMS inhibition in the tumour. 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 tumour 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 a block in cell division (14). This can be bypassed by deoxycytidine supplementation (15). Nevertheless we did not prove that thymidine-deoxycytidine combination leads to 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 preclinical data on the protective activity of uridine against 5-FU-induced keratinocyte damage. Furthermore, by three different methods, we have shown 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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Research Article

Local Treatment of Hand-Foot Syndrome with Uridine/Thymidine: *In Vitro* Appraisal on a Human Keratinocyte Cell Line HaCaT

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5-fluorouracil (5-FU) is one of the most commonly used antineoplastic drugs in the anticancer therapy. The hand-foot (HF) syndrome (palmar-plantar erythrodysesthesia) is an adverse effect frequently related to long-term i.v. administration of 5-FU or its orally applicable prodrug capecitabine. Its severity can even lead to interruption of the otherwise effective anticancer therapy. Tentative practice in some clinics has shown that topical application of 10% uridine ointment is beneficial for calming down the HF syndrome. This study is focused on verifying the alleged protective activity of uridine in the *in vitro* model of cultured human keratinocyte cell line HaCaT. We also tested the protective effects of thymidine alone or uridine-thymidine combination. The cellular viability time progression was measured in order to evaluate the effect of protective agents by three different types of cytopathogenicity tests—NTCA test (non-destructive test of cellular activity), modified MTT test and RTCA (real-time cell analyser, Roche). All three methods proved the ability of uridine and uridine-thymidine combination to protect keratinocytes against 5-FU damage *in vitro*. While thymidine alone did not show any remarkable effect, the thymidine-uridine combination demonstrated enhanced protective activity compared to uridine alone. Our findings provided the supporting rationale for using uridine or uridine-thymidine ointments in the HF syndrome local therapy.

1. Introduction

5-fluorouracil (5-FU) is one of the most frequently used chemotherapeutic agents in the treatment of various tumour diseases such as breast, oesophagus, and colorectal cancer [1, 2]. There is hardly any therapeutic schedule for colorectal cancer not containing 5-FU or its orally administered prodrug capecitabine.

5-FU continuous infusion shows better tolerability and efficacy than its bolus administration [3]. One of the most remarkable adverse effects accompanying long-term 5-FU administration is the hand-foot syndrome (palmar-plantar erythrodysesthesia), which represents locally misplaced activity of 5-FU systemic therapy [3, 4]. It is also one of the most frequent adverse effects of capecitabine [5], which often replaces i.v. 5-FU in therapeutic schedules. It can be characterized as a nonspecific toxic reaction of keratinocytes to the presence of a cytotoxic agent, not necessarily fluoropyrimidine only. The rate of clinical manifestation can be divided into four grades from slight dysesthesia to

desquamation, blistering, and ulceration [6], with possible resulting interruption of an otherwise effective therapy of the main disease [6, 7].

Empirical clinical practice in Germany, Poland, and Czech Republic showed that topical application of 10% uridine ointment can help prevent the HF syndrome by local cancellation of the 5-FU effect [8–11]. The protective effect of uridine was also reported in the study of mice treated with 5-FU in which uridine infusions significantly reduced the systemic toxicity of 5-FU [12]. This was not confirmed by another study in which uridine after i.v. administration to the patients was rapidly catabolized and did not prevent the side effects of 5-FU therapy [13]. On the other hand, orally administered uridine triacetate, which increased the intracellular concentration of uridine, was successfully used as an antidote in cases of 5-FU overdose [14].

Another agent modulating 5-FU toxicity is thymidine. It abrogates the inhibition of thymidylate synthase (TS) by 5-FU [15, 16]. In some studies, the combination of 5-FU and thymidine led to the decreased 5-FU toxicity *in vitro*, but in other studies the outcome was opposite [17, 18].

This study is focused on finding out whether there is also convincing evidence for the alleged protective activity of uridine and thymidine in the *in vitro* model of cultured human skin cells. We also additionally examined the combination of these two agents. For that purpose we used human keratinocyte cell line HaCaT [18] and three different methods for evaluating cell viability *in vitro*. NTCA test is based on evaluating the cell morphology and cell density of surviving cells [19], the modified MTT test shows metabolic activity in time [20], and a rather novel technology of Real-Time Cell Analyzer (RTCA, Roche) continuously evaluates cell viability [21] and was already successfully used for the study of HaCaT cells [22].

2. Materials and Methods

2.1. Cultivation of HaCaT Keratinocytes. HaCaT cells are defined as a spontaneously immortalized human epithelial cell line that maintains full epidermal differentiation capacity [18]. The cells were cultured in H-MEM medium supplemented with nonessential amino acids, 0.12 g/L sodium pyruvate, 1 g/L NaHCO3, 10% bovine serum, 2% fetal bovine serum, and antibiotics (200 U/mL penicillin and 100 μg/mL streptomycin). The cells were maintained in humidified atmosphere at 37°C and 3.5% CO_2.

2.2. NTCA Test. As the first method for measuring cell viability we used the non-destructive test of cellular activity NTCA [19]. The cells were plated into the 24-well plate (40,000 cells per well in one mL of the medium with phenol red). When cellular layers nearly reached confluence, one ml of the tested agents dissolved in the medium was added. After 5 days, the medium was washed out and the cells were stained by May-Grünwald and Giemsa-Romanowski solutions. The photographs of the whole plates show the presence of the cells in the wells and microscopic morphology shows the degree of damage. The viability was estimated according to the fraction of the cells that remained attached to the culture dish and according to the cell morphology.

2.3. RTCA (xCELLigence). For the second cell survival measurements, we used a Real-Time Cell Analyser produced by Roche Applied Sciences (xCELLigence). Seven thousand cells in 100 μL medium were plated into each of the wells of a 96-well plate. Each well is provided with golden electrodes on the bottom surface. The measurable impedance between these two electrodes grows when the cells are growing and dividing. Cell surface changes, adhesion, and morphology also play a role in this measurement. As a result, we obtain the "cell index" derived from the above-mentioned cellular properties. The cell index can be generally considered as the cellular viability indicator with some limitations [21].

When the cell index plot curves were growing exponentially, the tested substances diluted in 100 μL of the medium were added. We have found that exponential growth of the cell index curve occurs when the cells are slightly subconfluent. The cell index value was recorded every 15 minutes. In the presented plot, time 0 represents the time of adding the tested compounds.

2.4. MTT Assay Modified for Measuring Time Progression of Cellular Metabolic Activity. The third method used was the classical endpoint MTT test [20] modified for measuring the time progression of cellular metabolic activity. The cells were plated into 96-well plates (12,000 cells per well in 100 μL of medium without phenol red). The outer wells were not seeded with cells but filled with sterile water for injection. Two plates were used for each experiment. When cellular layers reached confluence, 100 μL of the tested agents dissolved in the medium were added to the wells. One plate was used for two different settings (different tested substances or their combinations). We recorded the absorbance values every day for six days. Each day 10 μL of MTT (5 mg/mL, dissolved in PBS) was added to one column in the plate. After six hours of incubation, the formazan production was stopped by 100 μL of 10% SDS solution in distilled water. After overnight incubation, the plates were analysed by 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 concentration and each day) were considered as indicators of the cellular metabolic activity.

3. Results

The NTCA test was performed to find out whether uridine can protect keratinocytes against 5-FU damage and, if so, which concentrations of 5-FU and uridine are meaningful for further testing.

The results of NTCA test proved the ability of uridine to protect the cells from damage caused by 5-FU. Therefore, we tested several concentrations of 5-FU and uridine (Figure 1). The lowest concentration (7.5 μg 5-FU/mL) is still higher than steady plasma concentration of any dosage schedule of this drug or its prodrugs used in clinical praxis [23–25].

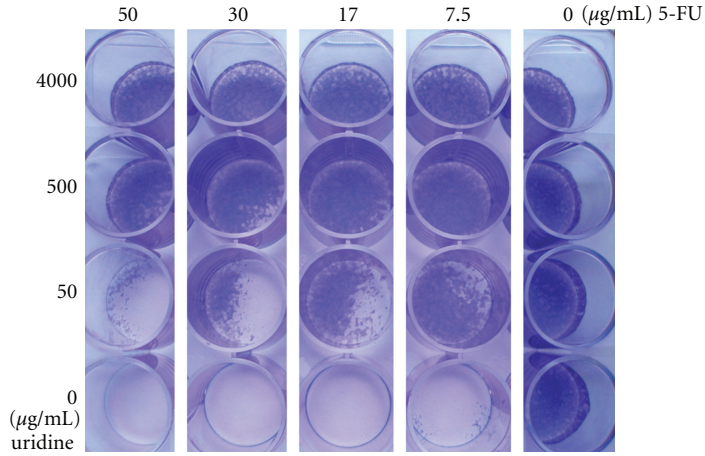

Figure 1: NTCA test. HaCaT cells after 5-day exposition to 5-FU and uridine. 40,000 cells were seeded to each well of the plate. After reaching confluency, 5-FU and uridine were added in different concentrations. After 5 days, the medium was washed out and the cells were stained by May-Grünwald and Giemsa-Romanowski solutions.

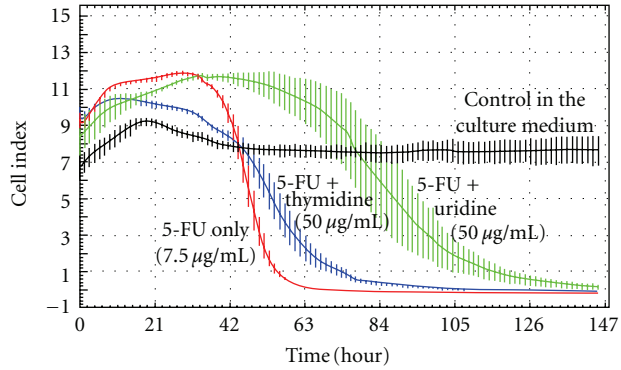

Figure 2: RTCA measurement. Comparison of the protective effect of uridine and thymidine in the presence of 7.5 μg 5-FU/mL. The curves represent the course of HaCaT cell viability after 147 hours of exposure to 5-FU (7.5 μg/mL) only (red curve) or with protective agents: green (5-FU + uridine 50 μg/mL), blue (5-FU thymidine 50 μg/mL). The black curve represents control cells in the culture medium.

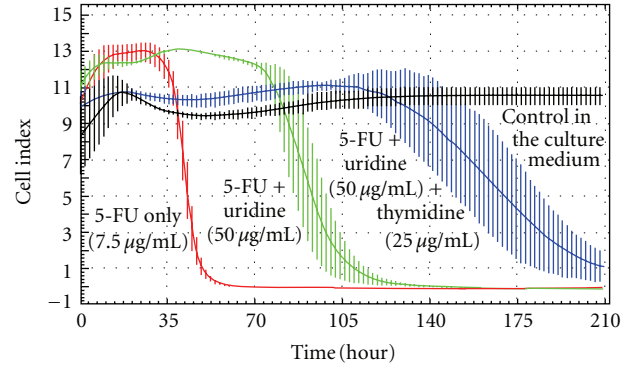

Figure 3: RTCA measurement. Comparison of the protective effect of uridine only and uridine together with thymidine in the presence of 7.5 μg 5-FU/mL. The curves represent the course of HaCaT cell viability after 210 hours of exposure to 5-FU (7.5 μg/mL) only (red curve) or with protective agents: green (5-FU + uridine 50 μg/mL), blue (5-FU + uridine 50 μg/mL and thymidine 25 μg/mL). The black curve represents control cells in the culture medium.

Wide range of concentrations was chosen for uridine. The cultivation was stopped after 5 days.

Figure 1 shows that all the cells died when no uridine was added in all concentrations of 5-FU applied (7,5–50 μg 5-FU/mL). The differences between uridine concentrations are clearly expressed in the cell survival. In the concentration 50 μg/mL of uridine, the cells showed less viability in wells with higher concentrations of 5-FU. In higher concentrations of uridine, the majority of cells survived no matter how high the 5-FU concentration was.

The results from NTCA test clearly visually demonstrates the uridine protection efficacy for the cells treated with 5-FU but did not provide the information about time progression of this protective effect.

For further confirmation of uridine protective efficacy and monitoring its time progression we used RTCA measurements (Figures 2 and 3) and modified MTT test (Figure 4). According to results from NTCA we used 50 μg/mL of uridine and 7.5 μg/mL of 5-FU. Furthermore, we tested also thymidine to compare its protective effect with uridine.

Figure 2 shows the comparison of protective effect of uridine and thymidine in the concentration 50 μg/mL. Thymidine showed much lower protective ability.

Another RTCA measurement shows that combination of uridine and thymidine together in the ratio 2 : 1 (50 μg of uridine/mL and 25 μg of thymidine/mL) protects the cells better than uridine only. This ratio was chosen according to our findings from former testing of several ratios of these two agents in three different 5-FU concentrations (results not presented).

The results of RTCA test presented in Figure 3 show clearly that the cells with uridine protection survived 60 hours longer than the cells without any protective agent. The cells with uridine-thymidine combination survived 120 hours longer than the cells without any protective agent.

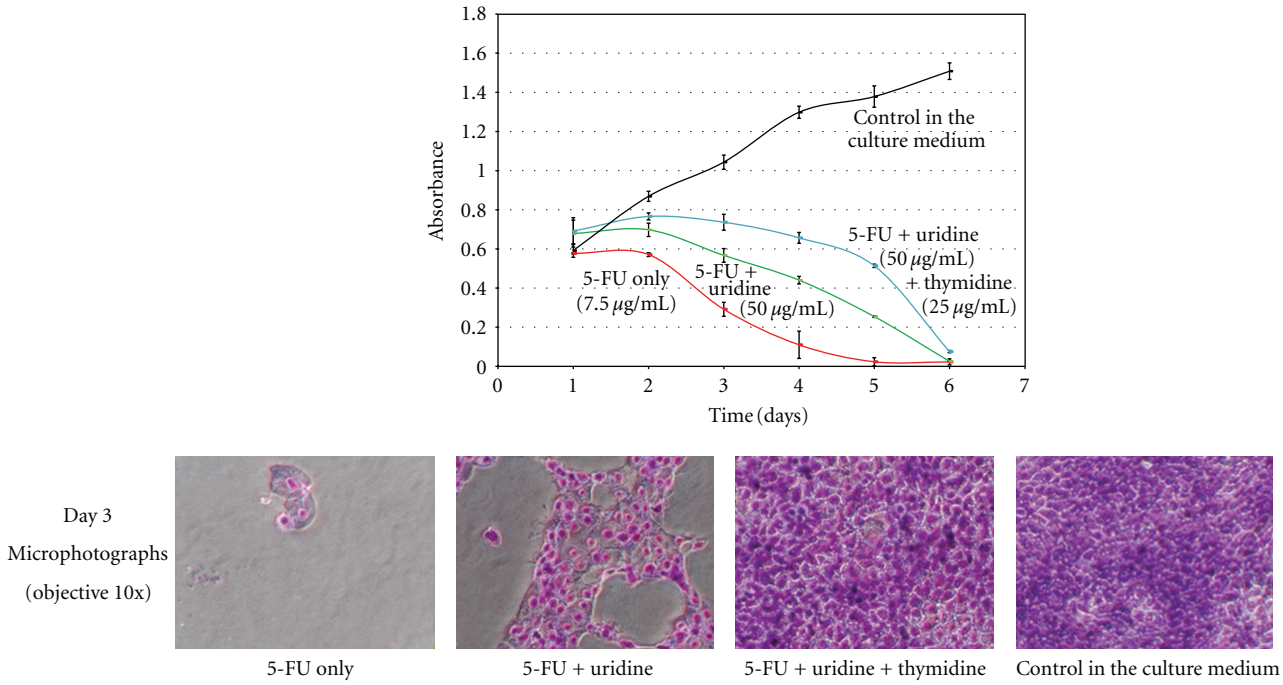

Figure 4: MTT test. Comparison of the protective effect of uridine only and uridine together with thymidine in the presence of 7.5 μg 5-FU/mL. The curves represent the course of HaCaT cell viability after 6 days of exposure to 5-FU (7.5 μg/mL) only (red curve) or with protective agents: green (5-FU + uridine 50 μg/mL), blue (5-FU + uridine 50 μg/mL and thymidine 25 μg/mL). The black curve represents control cells in the culture medium. Microphotographs of HaCaT cells stained with May-Grünwald/Giemsa-Romanowski (day 3, objective 10x) demonstrate the resultant state of the outgrowth of the cell population.

The results of MTT test presented in Figure 4 confirm that uridine is able to protect the cells against 5-FU damage and that the combination of uridine and thymidine protects the cells better than uridine only until day 5.

4. Discussion

The hand-foot syndrome often represents a serious complication of fluoropyrimidine-based chemotherapy, accompanying especially its long-term administration and anticancer treatment. There are no generally accepted recommendations for effective treatment of the HF syndrome. One of the approaches, already clinically tested on a small scale, is topical application of 10% uridine ointment [8, 9, 11].

The 5-FU concentrations in the skin of the palms and soles of patients with 5-FU-induced HF syndrome are difficult to predict. The fact that this syndrome occurs almost exclusively with 5-FU continual administration suggests that some concentration mechanism may be involved. On the other hand, Fischel and Formento showed that 5-FU and its prodrugs and metabolites are more toxic to the HaCaT keratinocytes than to colorectal cancer cells [26].

In RTCA and MTT experiments we used the 5-FU concentration of 7.5 μg/mL. This concentration is higher than the steady-state plasma concentration of any dosage schedule of 5-FU or its prodrugs used in clinical practice, which ranges from 9 ng/mL to 950 ng/mL [23–25]. We proved the ability of uridine to protect *in vitro* cultured keratinocytes against cytotoxic damage caused by 5-FU exposure. The results were proved by three different *in vitro* methods (NTCA, RTCA, and MTT). The phase contrast microscope also showed differences in the cell damage.

This brings up the question of potential systemic effect of uridine absorbed from the ointment. The partition coefficient $\text{Log P}_{\text{octanol/water}}$ for uridine is -1.98 whilst partition coefficients between 1 and 3 refer to molecules that are suitable for systemic absorption through the skin [27]. It is therefore unlikely that uridine could be systemically absorbed from the ointment in a significant amount. Furthermore, the i.v. administered uridine has a distribution volume approximately similar to whole body water and is catabolized rapidly with 2-hour half-life [13]. Therefore, we do not expect that absorption of a small amount of uridine from the locally administered 10% ointment should affect the antineoplastic therapy.

We also tested the combination of uridine and thymidine in the ratio 2 : 1. This combination results in markedly better protection than uridine alone. The rationale for this may be that uridine competes with 5-flurouridine for RNA incorporation and thymidine does the same in the case of DNA. Moreover, the inhibition of TS is abrogated by thymidine substitution, and therefore the effect of this nucleoside deficiency leading to an inappropriate DNA synthesis is prevented [15].

5. Conclusion

We succeeded in prolonging the time of onset of cell death by 5-6 days using uridine or uridine-thymidine treatment in the presence of 7.5 μg/mL of 5-FU. This is in accordance with the effect observed in empirical clinical practice using the application of uridine ointment [8, 9, 11]. The presented results provide preclinical confirmation of the uridine ointment usage meaningfulness and rationale for further large-scale clinical testing. These results also show that the HaCaT keratinocyte model is suitable for measuring the effect of protective agents on skin integrity at least in the case of 5-FU-induced HF syndrome.

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PREKLINICKÉ TESTY PODPORUJÍCÍ LÉČBU PALMÁRNÍ-PLANTÁRNÍ ERYTHRODYSESTHESIE 10% URIDINOVOU MASTÍ

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Oddělení klinické farmacie, Nemocnice Na Bulovce

Palmární-plantární erythrodysesthesie neboli hand-foot syndrom (HF sy), o kterém již byla na stránkách Onkologické Farmacie zmínka[1], je poměrně častý nežádoucí účinek doprovázející léčbu capecitabinem, případně kontinuálně podaným 5-fluorouracilem (5-FU)[2), 3)]. Jedná se o zarudnutí až ulceraci dlaní a plosek nohou, které je v literatuře především spojováno s lokálním toxickým působením 5-FU (příp. jiných cytostatik). Lokalizace na výše zmíněné oblasti je zdůvodňována poškozením kapilár a uvolněním cytostatika do kůže v místech mechanicky namáhaných, případně exkrecí cytostatika potem v místě vyššího výskytu potních žláz a následnou absorpcí[2)].

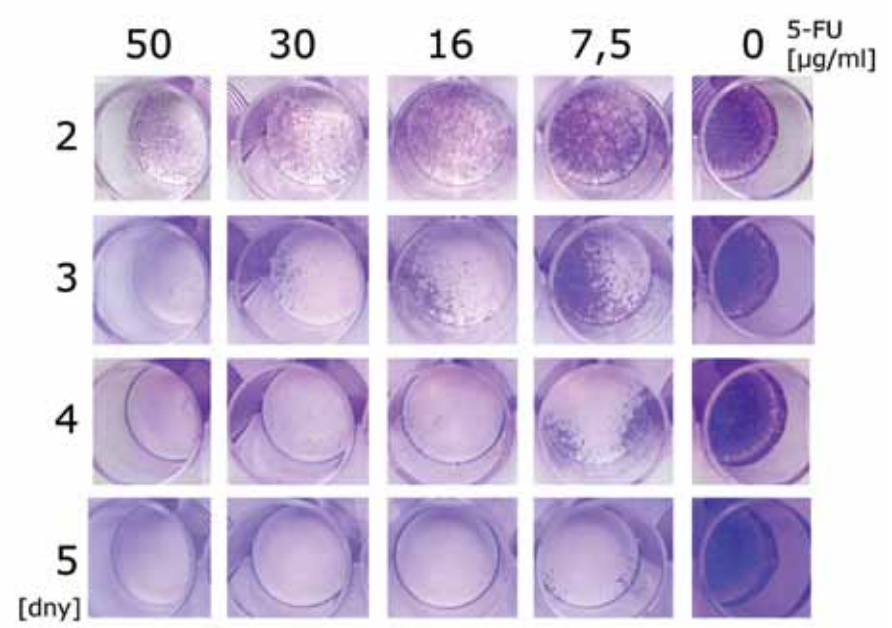
Již delší dobu byla na různých pracovištích ve střední a západní Evropě, za účelem zmírnění tohoto nepříjemného efektu, který může ve svých nejhorších projevech znemožnit další léčbu fluoropyrimidiny, používána 10% uridinová mast (viz tab. 1). V současnosti jsou dostupné určité skromně publikované informace o kladných klinických zkušenostech s touto léčbou[4), 5)], nicméně dosud chybí jednoznačné potvrzení účinku uridinu *in vitro*, které by pomohlo rozlišit terapeutický efekt tohoto nukleotidu od možného vlivu masťového základu, případně placebo efektu. Za účelem získání těchto informací byl podán grant *NS 9786-4/2008* na Ministerstvu zdravotnictví ČR. Část tohoto výzkumu je prezentována v následujícím textu.

TABULKA 1: SLOŽENÍ 10% URIDINOVÉ MASTI

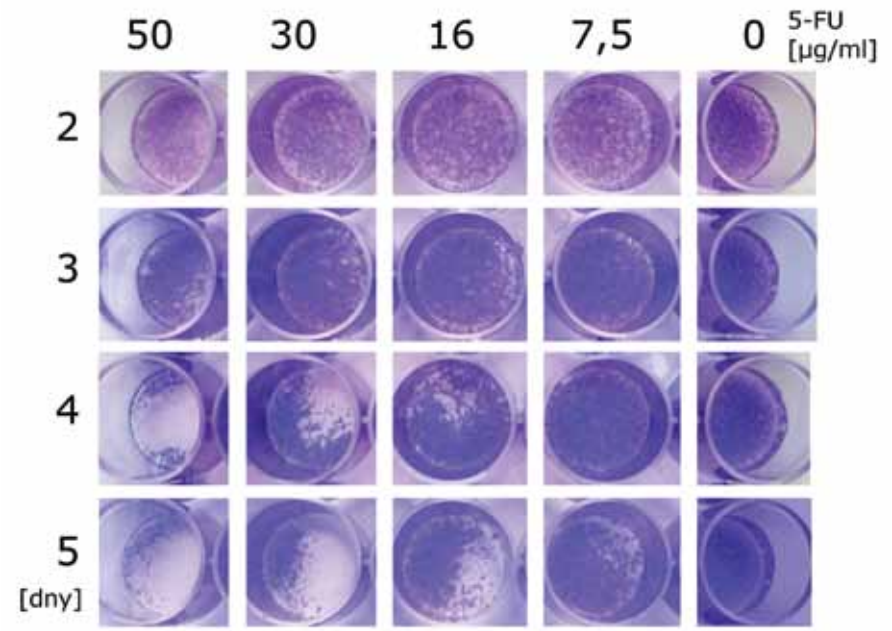
10% uridinová mast	
Uridini	10
Paraffini liq.	q.s.
Cremoris neoaquasorbi	ad 100

Především šlo o to zjistit, zda je uridin schopen ochránit keratinocyty před toxickým účinkem 5-FU. K tomuto studiu byla použita buněčná linie HaCaT, která je odvozena od lidských keratinocytů spontánní imortalizací a zachovává velkou míru původní diferenciace[6], neboli jedná se o poměrně přesný model lidských kožních buněk, které je možno neomezeně kultivovat na Petriho miskách či platech.

Obr. 1 a 2 znázorňují výsledky z tzv. NTCA testů (nedestruktivní testy buněčné aktivity)[7]. Buňky byly ponechány, aby porostly téměř souvislou vrstvou dna jednotlivých jamek, následně byly přidány testované látky a po určité době kultivace byly buňky zafixovány a obarveny dle Giemsy-Romanowského. V průběhu kultivace mrtvé buňky opouštějí povrch jamky, a proto nejsou na výsledku zachyceny (je vidět nesouvisle porostlá či prázdná jamka).



Obr. 1: NTCA test – vývoj toxicity 5-FU v průběhu pěti dnů bez přidání uridinu



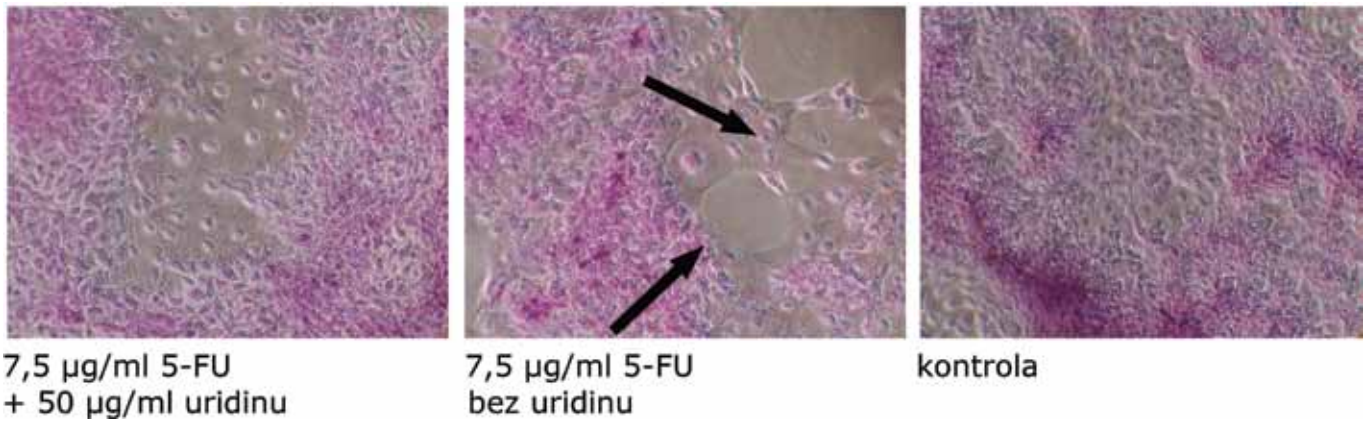
Obr. 2: NTCA test – vývoj toxicity 5-FU v průběhu pěti dnů s přidáním uridinu v konc. 50 µg/ml

Na obr. 1 je možno porovnat vývoj toxicity různých koncentrací 5-FU v průběhu pěti dnů. Na obr. 2 jsou znázorněny buňky ve stejných koncentracích 5-FU, ale do kultivačního média byl přidán uridin v koncentraci 50 µg/ml (0,005 %). Je vidět, že zatímco buňky bez uridinu již čtvrtý den nepřežívaly ani nejnižší koncentraci 5-FU (7,5 µg/ml), buňky, do jejichž média byl přidán uridin, přežily v této koncentraci až do pátého dne a přežívaly lépe i ve vyšších koncentracích 5-FU.

Pro ilustraci je rovněž možno porovnat změny v morfologii buněk na mikrofotografiích (obr. 3). Jedná se o stav zachycený 2. den při nejnižší koncentraci 5-FU, kdy buňky s přidaným uridinem nevykazují ještě morfologické rozdíly oproti buňkám kontroly (souvislost vrstvy není porušena), nicméně u buněk bez uridinu již lze vypozorovat počínající apoptózu (vyznačena šipkami).

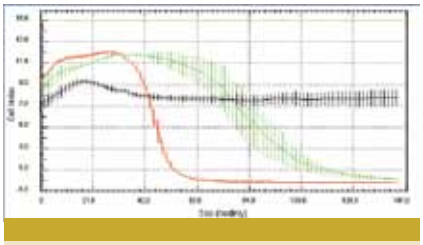
V tuto chvíli je bez dalšího studia těžké odhadnout míru expozice kůže dlaní a plosek nohou 5-fluorouracilem u pacientů s HF sy, ale ustálený stav plazmatické hladiny tohoto cytostatika při dlouhodobém podání se pohybuje v závislosti na léčebném režimu od 9 ng/ml do 950 ng/ml[8)–10)], tedy výrazně méně, než je nejnižší testovaná koncentrace v uváděných pokusech. Nelze však vyloučit, že při patogenezi HF sy se uplatňuje nějaký koncentrační efekt 5-FU.

Pro skutečně průběžné sledování životaschopnosti buněk byla použita RTCA technologie (buněčná analýza v reálném čase – Roche Applied Sciences)[11)]. Jedná se o metodiku založenou na měření odporu mezi zlatými elektrodami na dně jamek 96jamkového plata. Měřená hodnota (tzv. cell index) stoupá v případě růstu a dělení buněk a naopak klesá v případě, že buňky odumírají a odlupují se ode dna. Na grafu č. 1 je vidět průběh cell indexu v případě buněk, v jejichž médiu byl přítomen pouze 5-FU (7,5 µg/ml, červená křivka), a buněk, kde byl přidán 5-FU spolu s uridi-



Obr. 3: 100x zvětšené fázově kontrastní mikrofotografie buněk kultivovaných 2 dny v přítomnosti 7,5 µg/ml 5-FU spolu s uridinem (vlevo), nebo bez uridinu (uprostřed). Vpravo kontrolní jamka bez přidaných testovaných látek. Šipkami je vyznačena počínající apoptóza

nem (50 µg/ml, zelená křivka). Bod 0 na časové ose odpovídá času, kdy byly přidány testované látky. Buňky v přítomnosti uridinu přežily zhruba o 40 hodin déle než buňky se samotným 5-FU. Zároveň je zajímavý počá-

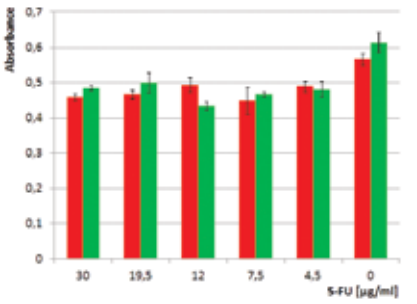


Graf 1: RTCA test. Rozdíl mezi časovým vývojem životaschopnosti (kvantifikované cell indexem) u buněk, v jejichž médiu byl pouze 5-FU (7,5 µg/ml, červená křivka), nebo 5-FU + uridin (50 µg/ml, zelená křivka). Černá křivka zastupuje kontrolu bez testovaných látek

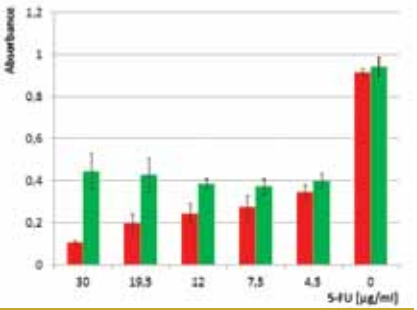
teční vzestup cell indexu u buněk s 5-FU (červená a zelená křivka), který nebyl tak výrazný u kontrolních jamek (černá křivka). Pravděpodobně je zde zaznamenána stresová reakce na přítomnost toxické látky.

Zcela odlišný přístup je měření metabolické aktivity buněk pomocí tzv. MTT testu, který byl proveden za účelem podpořit výsledky odvozené především od adherenčních schopností buněk. Jedná se o velmi klasický způsob zjišťování toxicity látek, který se používá již téměř 30 let a je založen na enzymatické přeměně žluté sloučeniny MTT na tmavé krystalky formazanu buněčnými reduktasami. Reakce je po 6 hodinách inkubace zastavena rozpuštěním buněk i krystalů v 10% laurylsulfátu. Tmavost roztoku v jednotlivých jamkách odpovídá metabolické aktivitě buněk, tedy jejich životaschopnosti, a proměřuje se spektrofotometricky[12]. Pokud např. kultivujeme buňky několik dní v přítomnosti toxické látky, potom přidáme MTT a následně změříme produkci formazanu, zjistíme při porovnání s absorbancí v jamce s buňkami kultivovanými bez přidání toxické látky jedovatost této sloučeniny.

Výsledky opět potvrdily účinnost uridinu v ochraně buněk před poškozením 5-FU (viz grafy 2 a 3), i když rozdíl nebyl tak výrazný jako v případě NTCA testu. 2. den nelze vyčíst z grafu signifikantní rozdíl mezi metabolickou aktivitou buněk, v jejichž médiu byl přítomen uridin, a buněk bez něj (graf 2). Čtvrtý den již je rozdíl patrný (graf 3).
Absorbance jamek s uridinem klesla za 2 dny poměrně nepatrně, zhruba o 0,074 (14,21 %). Oproti tomu pokles absorbance u buněk bez uridinu se pohyboval v závislosti na koncentraci 5-FU mezi hodnotami 0,354–0,144 (77,05–29,40 %) s průměrem 0,238 (50,70 %) a byl výraznější ve vyšších koncentracích 5-FU. Obě kontroly bez 5-FU (s uridinem a bez něj) výrazně zvýšily absorbanci o 0,348 (61,60 %), resp. 0,330 (53,83 %), což při srovnání s buňkami, v jejichž médiu byl 5-FU přítomen, ukazuje, že uridin není schopen buňky kompletně ochránit před stresem vyvolaným tímto cytostatikem (neboť absorbance u buněk s 5-FU by v takovém případě musela také stoupat), nicméně prokazatelně napomáhá

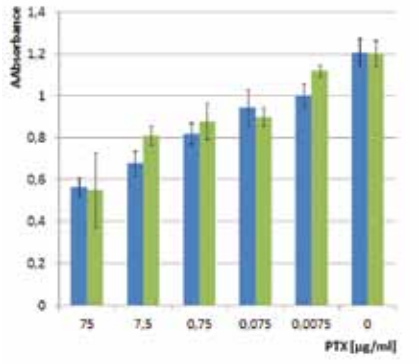


Graf 2: MTT test – porovnání metabolické aktivity buněk vystavených po 2 dny různým koncentracím 5-FU. Červené sloupce – buňky bez uridinu, zelené sloupce – buňky s uridinem (50 µg/ml)



Graf 3: MTT test – porovnání metabolické aktivity buněk vystavených po 4 dny různým koncentracím 5-FU. Červené sloupce – buňky bez uridinu, zelené sloupce – buňky s uridinem (50 µg/ml)

lepšímu přežívání buněk (absorbance u buněk s 5-FU se snižuje jenom pomalu oproti absorbanci buněk bez uridinu). Tento efekt je výraznější u vyšších koncentrací 5-FU.



Graf 4: MTT test – porovnání metabolické aktivity buněk vystavených po 4 dny různým koncentracím PTX. Modré sloupce – buňky bez uridinu, zelené sloupce – buňky s uridinem (50 µg/ml)

Nárůst absorbance byl srovnatelný jak u kontroly s uridinem, tak u kontroly bez něj. To, spolu s výsledky NTCA testů (obr. 1 a 2), kde je vidět ve všech kontrolních jamkách podobně hustý porost nezávisle na přítomnosti uridinu, dovoluje usuzovat, že jeho přítomnost v médiu buňky nikterak nezatěžuje. V samostatném testu, jehož výsledky zde neuvádíme jsme ověřili že toxická koncentrace tohoto nukleotidu pro buňky linie HaCaT se pohybuje až nad koncentrací 24 mg/ml.

Specificita účinku pro uridin byla ověřena MTT testem s paclitaxelem (PTX – graf č. 4). Rozdíly v toxicitě různých koncentrací PTX jsou na grafu 4 poměrně málo výrazné pravděpodobně z důvodů pozdního přidání cytostatika k buňkám, které již vytvořily souvislou vrstvu (jedná se o mitotický jed a buňky v souvislé vrstvě se dělí méně, než když teprve porůstají dno jamky). Nicméně přesto lze prokázat, že uridin byl v tomto případě neúčinný, neboť po 4 dnech kultivace nebyl naměřen signifikantní, případně pouze nepatrný rozdíl v životaschopnosti mezi buňkami se samotným PTX a jeho kombinací s 50 µg/ml uridinu u žádné z testovaných koncentrací cytostatika. V případě ochrany buněk uridinem před toxickým působením 5-FU se tedy nejedná o nespecifický účinek obohacení buněčného média tímto nukleotidem.

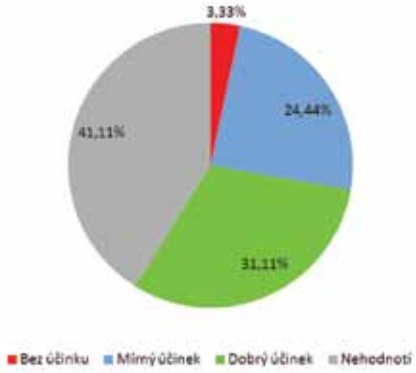
Na základě výše zmíněných in vitro výsledků s testováním uridinu na buněčné linii lidských keratinocytů HaCaT lze tedy 10% uridinovou mast doporučit k léčbě HF sy vyvolaného podáním fluoropyrimidinů. Zjištění o její skutečné účinnosti však může přinést až případná kvalitně navržená klinická studie.

Pro úplnost uvádíme ještě hrubé výsledky monitorování účinku uridinové masti na pracovišti přípravy cytostatik ve VFN zatížené poměrně malou návratností dotazníků. Za období od začátku roku 2007 do začátku roku 2011 na naše dotazy o hodnocení uridinové masti odpověděla pouze asi čtvrtina pacientů (90).

Z tohoto souboru 49 pacientů (55,55 %) hodnotilo přínos léčby jako mírné (22 pacientů – 24,44 %), nebo dobré (27 pacientů – 31,11 %) zlepšení, 3 pacienti (3,33 %) zaškrtli políčko „bez účinku“ a zbytek (38 pacientů – 41,11 %) se nevyjádřil, většinou z důvodu krátké doby trvání léčby. Pro přehled uvádíme výsledky v grafu č. 5.

Na závěr bychom chtěli poděkovat kolektivu pracovníků OPC NL VFN za výraznou pomoc při sběru dat z klinického provozu.

Výzkum byl financován z grantu IGA MZ ČR, NS 9786-4/2008.



Graf 5: Výsledek průzkumu účinku uridinové masti u pacientů formou dotazníků. Odpovědělo celkem 90 pacientů

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PROTECTIVE EFFECT OF URIDINE ON KERATINOCYTES TREATED WITH FLUOROPYRIMIDINES

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Introductin and Aims

Hand-foot (HF) syndrome (palmar-plantar erythrodysesthesia) is an adverse effect that has been described as one of the main complications of capecitabine therapy. Less often it occurs after continuous administration of 5-Fluorouracil[1]. Sometimes it can even lead to dose reduction or discontinuing of an effective therapy. So far this practice is the only proven management of HF syndrome[2].

Uridine has been described as a potential systemic antidote in case of 5-FU overdose in mice[3] but not in human[4]. Nevertheless some studies suggested that local topical application of 10% uridine ointment can help the patients with HF syndrome resulting from administration of 5-fluorouracil (5-FU) or capecitabine[5]. So far only the data from small clinical non-blinded studies are available[6] and no hypothesis of mode of action was verified for this kind of protective therapy. Furthermore no *in vitro* tests confirming the protective effect of uridine were performed on keratinocytes.

To confirm the protective activity of uridine we made set of tests using primary keratinocytes and HaCat cell line (spontaneously immortalized human keratinocytes from adult skin with full epidermal differentiation capacity[7]). Additionally we tested uracil as another potential local antidote for patients with HF syndrome.

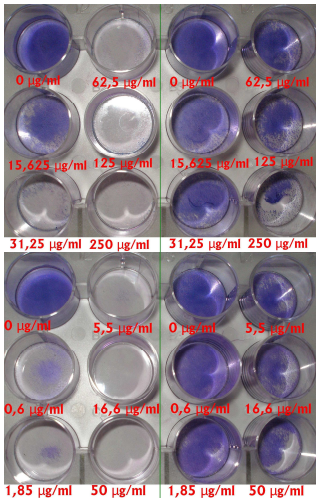
Methods

We used NTCA (Nondestructive Test of Cellular Activity) which was developed as an endpoint test for determination of chemosensitivity. It enables not only testing of viability but also the evaluation of changes in cell morphology[8].

XCELLigence System (Roche Applied Science) measurements were used for continuous monitoring of cell adherence and viability.

Results

We confirmed via NTCA tests that uridine can prevent either HaCat cells or primary keratinocytes against damage resulting from presence of 5-FU in medium:



Primary Keratinocytes

Red numbers – concentration of 5-FU in the medium.

Left side – without uridine.

Right side – with 10 mg/ml of uridine in the medium.

Situation after two days of exposition to 5-FU and uridine.

HaCat cell line

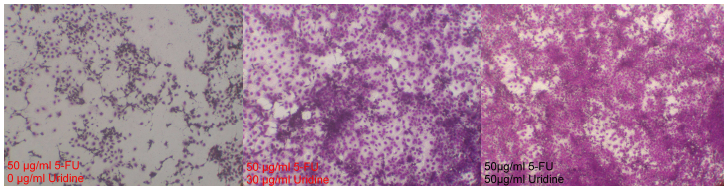
Red numbers – concentration of 5-FU in the medium.

Left side – without uridine.

Right side – with 4 mg/ml of uridine in the medium.

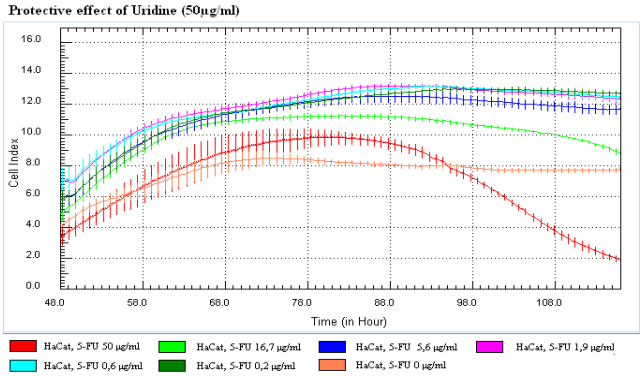
Situation after five days of exposition to 5-FU and uridine.

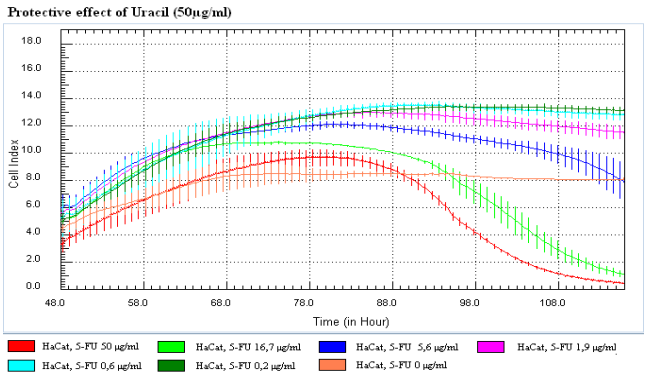
In NTCA tests the lowest sufficiently protective concentration of uridine for HaCat cell line was 50µg/ml:



xCELLigence measurements:

We measured the protective effect of uracil and uridine in concentration 50µg/ml in presence of various concentrations of 5-FU:





Cell index – a value derived from cell number and adherence generally regarded as an indicator of viability.

IC 50 and lethal concentrations of 5-FU and uridine for HaCat cells were determined. In NTCA tests uridine in concentration 12 mg/ml and higher together with 5-FU (50 µg/ml) lost its protective function and elevates its toxicity:

	5-FU (2 days) xCELLigence values	Uridin (7 days) NTCA values	Uridine + 5-FU (3 days) NTCA values
IC 50	5 µg/ml	24,5 mg/ml	12 mg/ml
IC 100	5,56 µg/ml	35 mg/ml	17,15 mg/ml

It was not possible to determine IC 50 and IC 100 for uracil due to its low solubility.

Discussion

Uridine can protect keratinocytes and HaCat cells against damage caused by 5-FU *in vitro*. Uracil can slightly help the cells to survive, but in comparison to uridine this activity was insignificant. We noted higher values of cell index during the xCELLigence measurements when the cells were treated with 5-FU in comparison to control. This occurs even when uridine was present in the medium and it may correspond to the chemical stress of the cells which therefore seems not to be fully prevented by uridine.

Conclusions

We confirmed that application of uridine ointment for preventing hand-food syndrome after a long term administration of fluoropyrimidines is reasonable for its activity *in vitro*. The concentration of uridine presented on the skin should be carefully considerated because of its higher toxicity in the presence of 5-FU.

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THYMIDINE AND URIDINE PROTECTIVE EFFECT ON KERATINOCYTES TREATED WITH FLUOROPYRIMIDINES

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Introduction and Aims:

Hand-foot (HF) syndrome is an adverse effect frequently related with the therapy based on long-term administration of fluoropyrimidines and their prodrugs especially capecitabine[1]. In its extreme manifestation it can lead to disruption of an effective therapy or dose reduction[2,3]. Clinical experience and small clinical trials[4] showed that HF syndrome can be partly or fully cured by topical application of 10% uridine ointment.

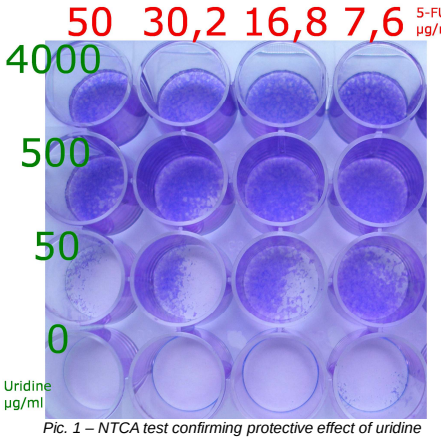
We performed number of *in vitro* tests on HaCat cell line (spontaneously immortalized human keratinocytes[5]) confirming the protective effect of uridine. Additionally we tested uracil and thymidine as another potential local antidotes for patients with HF syndrome.

Methods:

We used NTCA (nondestructive test of cellular activity) tests for cell morphology evaluation[6], MTT tests for evaluation of cellular metabolic activity and xCELLigence System (Roche Applied Science) measurements for continuous monitoring of cell adherence. In all tests we used 5-fluorouracil (5-FU) as a cytotoxic agent.

Results:

We confirmed the protective effect of uridine on cells treated with 5-FU via **NTCA test**:



Pic. 1 – NTCA test confirming protective effect of uridine

HaCat cells after 5 days of exposition to 5-FU and uridine (pic.1).

All the cells in the lowest line, where no uridine was present in the medium died.

Uridine in the concentration 50µg/ml was not able to prevent the cells from cytotoxic damage caused by higher concentrations of 5-FU.

Uridine in the concentration 500µg/ml and 4mg/ml protected the cells against highest tested concentrations of 5-FU (50µg/ml, 30µg/ml - ppc).

Uracil and thymidine showed much lower protective potential as single agents. Nevertheless thymidine together with uridine was able to protect the cells better than solely uridine. This was obvious especially when lower doses of 5-FU were present in the medium.

XCELLigent measurements:

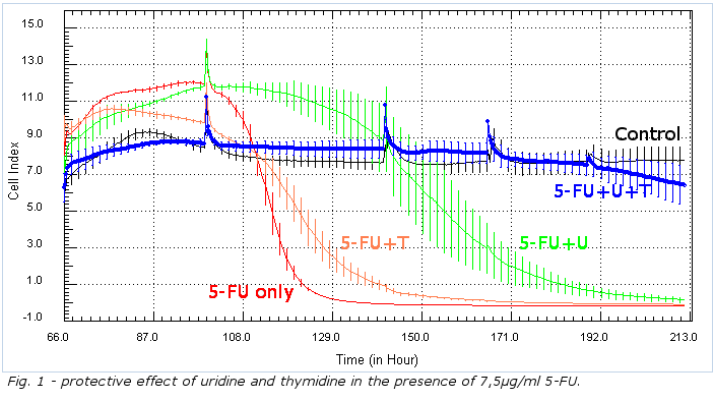


Fig. 1 - protective effect of uridine and thymidine in the presence of 7,5µg/ml 5-FU.

Cell index is value derived from the cell adherence and cell membrane surface changes which can be measured continuously. All cells but the control have 7,5µg/ml 5-FU in the medium (fig.1). It is evident that uridine alone (50µg/ml) can prevent the cells much better, than thymidine alone (50µg/ml). Best protection was reached by combination of these two agents (25µg/ml each).

The peaks refer to the reaction of the cells when the plate was withdrawn from the incubator (this effect occures only in the wells with living cells). It is obvious that the cells without thymidine increases the cell index more than contol for a while, but than the curves rapidly tail off. The curve of the cells with combination of uridine and thymidine proceeds more like the control without so massive reaction to the presence of 5-FU.

The difference between protective effect of solely uridine and uridine-thymidine combination is not so outstanding when higher doses of 5-FU are present in the medium (fig. 2 - 25µg/ml of 5-FU):

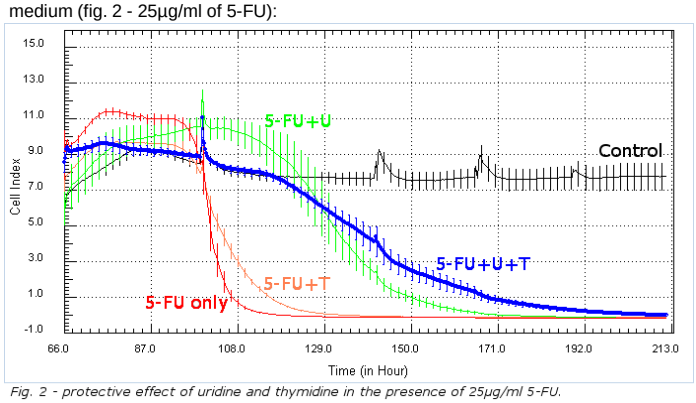


Fig. 2 - protective effect of uridine and thymidine in the presence of 25µg/ml 5-FU.

The protective effect of uridine-thymidine combination was confirmed also by **MTT tests** (fig. 3):

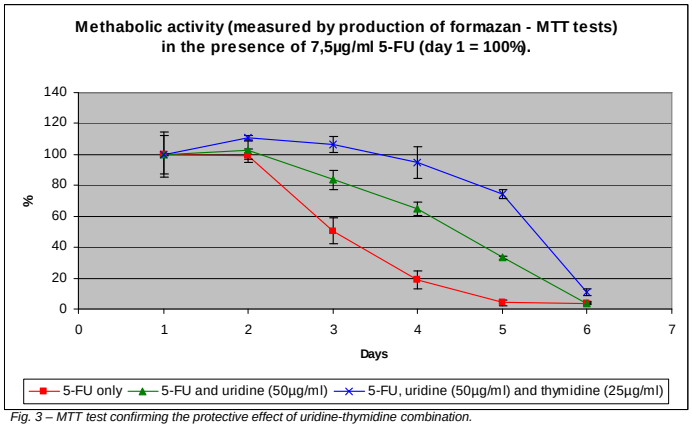


Fig. 3 – MTT test confirming the protective effect of uridine-thymidine combination.

The protective effect of all tested agents developed much better when cells were confluent. Also the difference between uridine it self and combination of uridine and thymidine was obvious only when the cells were confluent (fig. 4).

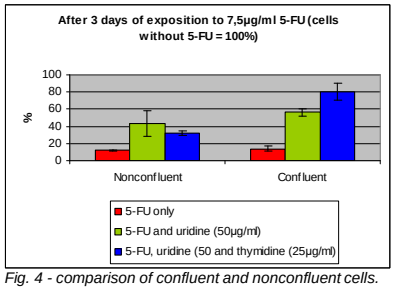


Fig. 4 - comparison of confluent and nonconfluent cells.

Conclusion:

In vitro tests confirmed the ability of uridine to prevent keratinocytes from cytotoxic damage caused by fluoropyrimidine administration and therefore it partly clears up the clinical effect of uridine ointment.

We found out that uridine-thymidine combination works better than uridine it self. This effect occures only in the case of confluent cell cultures. This is probably caused by differences in the methabolism of nonconfluent rapidly dividing cells and confluent layer where the cells are dividing not so rapidly.

It is question weather the cells in the skin of palms and soles are more similar to the confluent or nonconfluent cell culture. The immature keratinocytes are rapidly dividing but the skin itself cannot be considered as nonconfluent layer. Therefore further study of the uridine-thymidine combination in the protective ointment seems to be reasonable.

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