Lack of contribution of dihydrofluorouracil and α-fluoro-β-alanine to the cytotoxicity of 5′-deoxy-5-fluorouridine on human keratinocytes

Jean-Louis Fischel[a], Patricia Formento[a], Joseph Ciccolini[b], Marie-Christine Etienne-Grimaldi[a] and G. Milano[a]

Capecitabine (Xeloda) is a very active oral fluoropyrimidine (colon and breast cancers) whose clinical use is complicated by the presence of hand–foot syndrome (HFS). This cutaneous toxicity is less frequently encountered with other oral fluoropyrimidines containing a dihydropyrimidine dehydrogenase (DPD) inhibitor. The HFS is thus attributed to the presence of the main 5-fluorouracil (5-FU) metabolites, dihydrofluorouracil (5-FUH_2) and α-fluoro-β-alanine (FBAL), but without strong pharmacological arguments. The aim of the present study was to closely examine this latter hypothesis. Capecitabine generates 5′-deoxyflourouridine (5′-DFUR) which is transformed into 5-FU at the cellular target site through the intermediary of thymidine phosphorylase (TP). The cytotoxic effects (MTT test, 4-day exposure) of 5′-DFUR, 5-FU, 5-FUH_2 and FBAL were tested against the spontaneously immortalized human keratinocyte cell line (HaCaT) and the human cancer colon cell line WiDr as a control. Mean IC_{50}s on HaCaT and WiDr were, respectively, 1.3 and 10 μM for 5′-DFUR, 0.2 and 3.3 μM for 5-FU, 13.4 and 560 μM for 5-FUH_2, and greater than 650 and 6500 μM for FBAL. The respective 5′-DFUR IC_{50}s values were not different when cells were exposed to 5′-DFUR alone or in combination with 5-FU, 5-FUH_2 and FBAL in both cell lines, the relative proportion of each drug reflecting known pharmacokinetic data for capecitabine (5′-DFUR 12.4%, 5-FUH_2 6.4%, 5-FU 1.2% and FBAL 80%). This latter finding demonstrates the relative lack of significant cytotoxic activity of 5-FUH_2 and FBAL on human keratinocytes. TP activity was particularly high in HaCaT cells and DPD activity was very low in both cell lines. These data strongly suggest that the presence of 5-FU metabolites does not play a major role in the HFS generated by capecitabine and that it can probably be attributed to particularly high TP activity in keratinocytes. This observation may have important clinical consequences such as a possible local pharmacological inhibition of TP for controlling HFS. *Anti-Cancer Drugs* 15:969–974 © 2004 Lippincott Williams & Wilkins.

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[a]Centre Antoine-Lacassagne, Nice, France and [b]Pharmacy School, Marseille, France.

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Correspondence to G. Milano, Oncopharmacology Unit, Centre Antoine Lacassagne, 33 Avenue de Valombrose, 06189 Nice Cedex 2, France.
Tel: +33 (4) 92 03 15 53; fax: +33 (4) 93 81 71 31;
e-mail: gerard.milano@nice.fnclcc.fr

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Introduction

The use of oral fluoropyrimidines is expanding [1]. This is due to patient convenience combined with satisfactory treatment efficacy [2]. There are two main categories of oral fluoropyrimidines which can be distinguished by the presence or not of a dihydropyrimidine dehydrogenase (DPD) inhibitor [3]. DPD is the rate-limiting enzyme for the extensive rate of 5-fluorouracil (5-FU) catabolism [4]. DPD inhibition allows direct absorption of 5-FU [5] and increases 5-FU half-life [6], thus allowing less frequent intakes of oral fluoropyrimidines. The pharmacodynamics of both oral fluoropyrimidines and continuous infusion of 5-FU is characterized by the presence of a more or less frequent and severe hand–foot syndrome (HFS) [7]. The occurrence of HFS is never life threatening, but can develop into a debilitating condition that may interfere with quality of life [8]. Of note, HFS is almost absent with oral fluoropyrimidines including a DPD inhibitor, but is, in contrast, more frequent with capecitabine, a major oral fluoropyrimidine which does not contain a DPD inhibitor [9]. The cause of this difference in HFS frequency between oral fluoropyrimidines including or not DPD inhibitor has not been clearly established. Among the hypotheses currently formulated there is the assumption that, during oral fluoropyrimidine treatment, the lack of DPD inhibition triggers continuous exposure, in biological fluids and particularly the sweat, to relatively elevated concentrations of the main 5-FU metabolites, dihydrofluorouracil (5-FUH_2) and α-fluoro-β-alanine (FBAL) whose cytotoxicity has been previously reported on experimental bases [10,11]. The high proportion of 5-FUH_2 and FBAL in comparison to 5-FU and 5′-deoxyflourouridine (5′-DFUR) encountered in plasma of patients treated by capecitabine [12] supports the

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hypothesis that 5-FU metabolites may play a role in HFS. The aim of the present study was to try to shed some light on this current question of the interaction between HFS and the presence of 5-FU metabolites during capecitabine treatment [13]. We adopted an experimental approach using a relevant human keratinocyte cell line HaCaT so as to examine the cytotoxic activity of 5-FUH_2 and FBAL. The HaCaT cell line is the best characterized and the most used of the few spontaneously human immortalized keratinocyte cell lines [14]. WiDr was taken as a 5-FU-sensitive control tumor cell line. WiDr was shown to be, in our hands, the most sensitive to 5-FU among a panel of different colon cancer cell lines [15]. Particular care was taken when testing drugs in proportions respecting clinical pharmacokinetic knowledge of capecitabine [12] (Fig. 1).

Material and methods

Chemicals

5-FUH_2 and FBAL were kindly provided by Roche (Neuilly-sur-Seine, France) and working solutions (10^{-1} M) were prepared in PBS and aliquots frozen at −30°C. 5-FU was obtained from Pharmacy Hospital, 5′-DFUR, MTT and DMSO were purchased from Sigma (St Quentin Fallavier, France). Tritiated 5′-DFUR (3.2 Ci/mmol) came from Moravek Biochemicals (Brea CA). Dulbecco's modified Eagles medium (DMEM) and glutamine were purchased from Biowhittaker (Verviers, Belgium). Fetal bovine serum (FBS) was obtained from Dutscher (Brumath, France), and penicillin and streptomycin were from Meyrieux (Lyon, France).

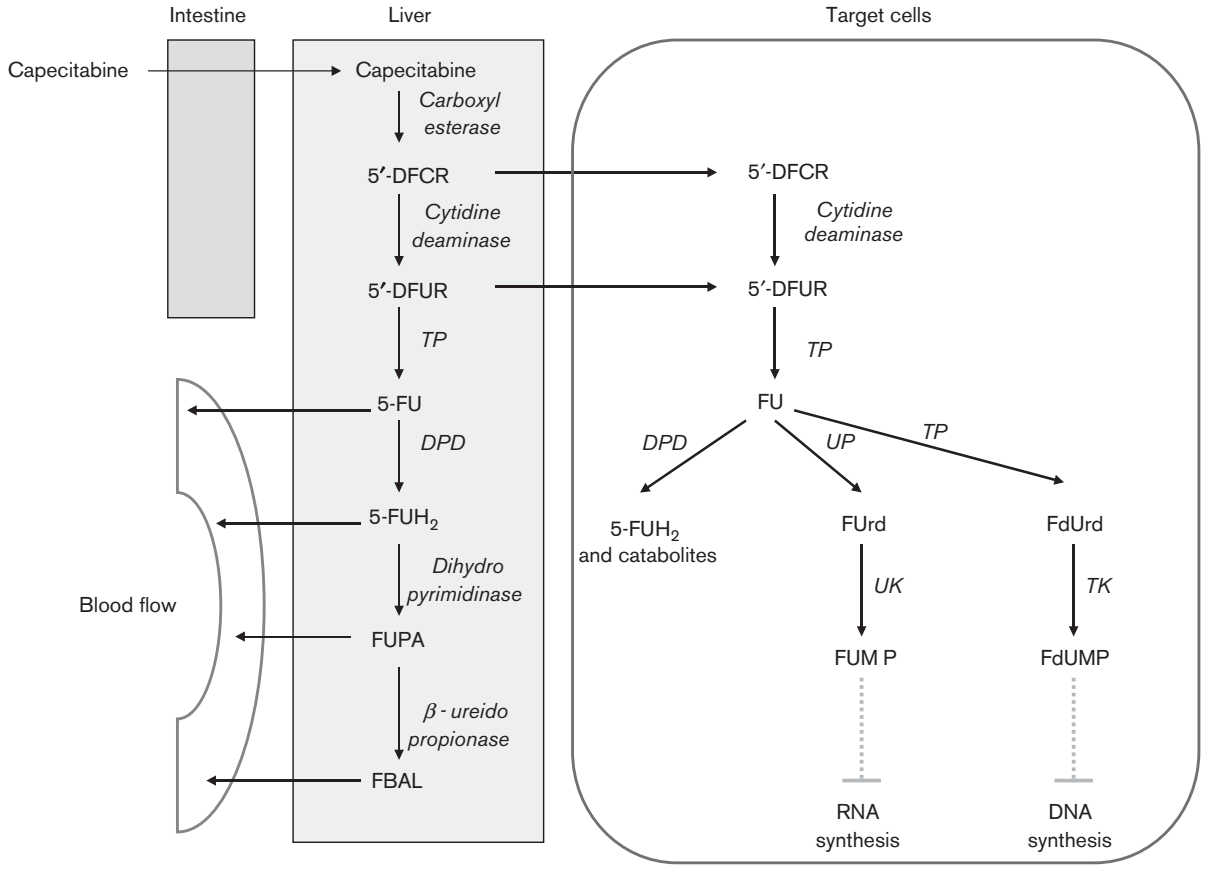
Cell lines

The human colon cancer cell line WiDr was provided by the EORTC PAMM group and the human spontaneously immortalized normal epithelial cell line HaCaT was kindly provided by Dr Aberdam, (INSERM U385).

Evaluation of 5′-DFUR, 5-FU, 5-FUH_2 and FBAL cytotoxicity

Cells were seeded in 96-well microtitration plates (100 µl/well) to obtain exponential growth for the duration of experiments (initial cell density 2500

Fig. 1



Global distribution of capecitabine and metabolites. 5′-DFCR=5′-deoxyfluorocytidine, 5′-DFUR=5′-deoxyflourouridine, 5-FU=5-fluorouracil, 5-FUH_2=dihydrofluorouracil, FUPA=α-fluoro-β-ureidopropionate, FBAL=α-fluoro-β-alanine, FdUrd=fluorodeoxyuridine, FdUMP=fluorodeoxyuridine monophosphate, FUrd=flurouridine, FUMP=fluorouridine monophosphate, TK=thymidine kinase, TP=thymidine phosphorylase, UP=uridine phosphorylase, UK=uridine kinase.

cells/well for both cell lines). At 48 h after seeding, cells were exposed to 5′-DFUR, 5-FU, 5-FUH_2 and FBAL given alone or in association during 96 h.

The same 11 concentrations were tested for each drug given alone or in combination corresponding to published pharmacokinetic data [13], i.e. 5′-DFUR 12.4%, 5-FUH_2 6.2%, 5-FU 1.2% and FBAL 80%.

WiDr: 5-FU: 0.01–100 μM; 5′-DFUR: 0.1–1000 μM; 5-FUH_2: 0.05–500 μM; FBAL: 0.6–600 μM.

HaCaT: 5-FU: 0.001–10 μM; 5′-DFUR: 0.01–100 μM; 5-FUH_2: 0.005–50 μM; FBAL: 0.06–600 μM.

Growth inhibition was assessed at the end of drug exposure (144 h after cell seeding) in fresh medium by the MTT test [16] as follows: cells were washed in fresh medium and incubated with MTT; after 2 h of exposure MTT was released and coloration was revealed by the addition of DMSO (100 µl). Absorbance at 540 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Cell sensitivity to the tested drugs or combinations was expressed by IC_{50}, which was determined using Prism software (GraphPad, San Diego, CA). In the particular case of the 5-FU-related species (FRS) mixture, IC_{50} represents the concentration of the 5′-DFUR component only in the mixture which diminishes the growth by 50%; the concentrations of other FRS could easily be determined from their relative, above-described, proportions. Experiments were performed in triplicate.

Enzyme activity measurements

TP activity was assayed as described previously, with minor modifications [17]. Aliquots of 50 µl of cytosol were added to the reaction mixture (10 mM thymidine, 10 mM KH_2PO_4, pH 8.4) and incubated at 37°C for 4 h. The reaction was stopped by adding 800 µl of ice-cold NaOH (0.2 N). The amount of thymine formed was then determined at 300 nm using a calibration curve. TP activity was finally expressed as pmol thymine formed/µg protein/h. The limit of detection for TP activity was 50 pmol thymine formed/µg protein/h.

DPD activity was determined as previously published [18]. The cytosol was incubated with [6-^{14}C]5-FU (26.5 µM final; Amersham, Little Chalfont, UK), NADPH (120 µM final), magnesium chloride (6.5 mM final) and nicotinamide (1.0 µM final) in sodium phosphate buffer (pH 8.0) at 37°C for 30 min (total volume was 200 µl) of ice-cold ethanol. The incubation mixture was kept on ice for 30 min, and then was filtered on a Whatman polypropylene microfilter (0.45 µm) and centrifuged (4000 r.p.m., 15 min). Samples were stored at −20°C until the HPLC analysis. The catabolite 5-FUH_2 was separated from the substrate 5-FU by the HPLC technique with a Merck Hitachi LichroGraph HPLC system according to Sommadossi *et al.* [19]. The radioactivity of the catabolite was measured by a Beckman liquid scintillation spectrometer. DPD activities were expressed as the amount of 5-FUH_2 formed/min/mg cytosolic protein (pmol/min/mg protein).

Analysis of intracellular metabolism of 5′-DFUR

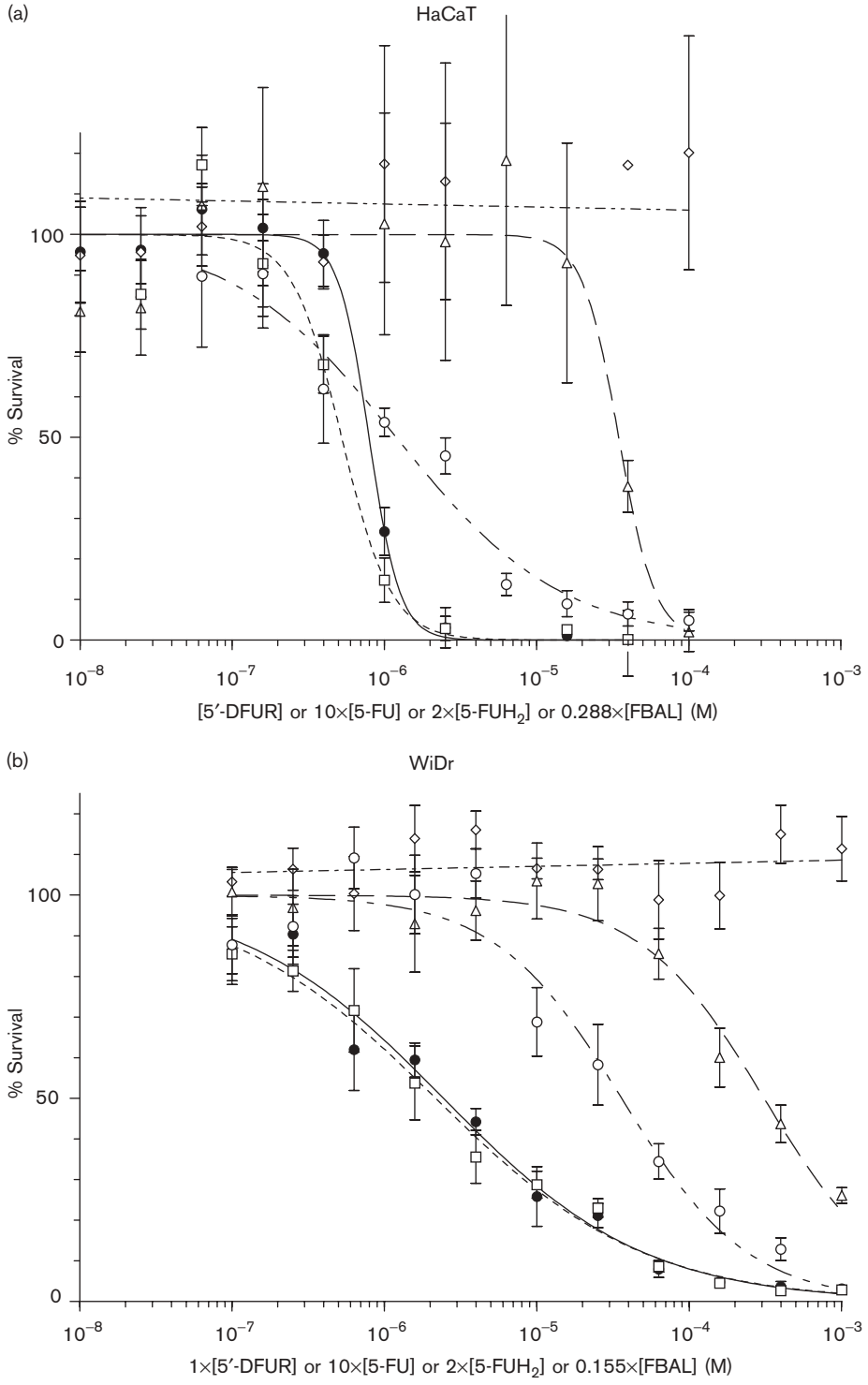
Cells were seeded in T75 plates and left attached overnight. Cells in exponential phase were exposed to 50 µCi of [^{3}H]5′-DFUR (final concentration: 2 µM) for 4 h. Cells were then washed, trypsinized and resuspended in PBS buffer prior to centrifugation at 800 r.p.m. for 10 min. Several washing/centrifugation cycles were performed. Cell pellet was isolated and lysed by vigorous shaking in 60% methanol for 30 min. Cells were kept at −80°C overnight and cytosol was next isolated by centrifugation at 15 000 *g* for 35 min. Cytosol were evaporated at 37°C under nitrogen and reconstituted in 100 µl of mobile phase. Separation and detection of tritiated 5′-DFUR, 5-FU and 5-FU metabolites was performed by HPLC (HP1090, France) equipped with a radioactive flow detector (Flo-One; Packard, Issy les Moulineaux, France). The mobile-phase consisted of 50 mM KH_2PO_4 (pH 6.8) containing 5 mM tetrabutyl ammonium nitrate and 5–30% gradient methanol (0–25 min). Chromatography was performed using a Lichrospher 100 RP18 5 µm column (Interchim, Montluçon, France).

Results

Figure 2(a and b) depicts the dose–effect curves for the different compounds tested, and their combination on HaCaT and WiDr, respectively. The respective IC_{50} values are given in Table 1. It appears that FBAL has a very limited, if any, cytotoxic effect and 5-FUH_2 a much smaller one than that of 5-FU or 5′-DFUR. 5-FUH_2 was found to be, on average, 10-fold less active than 5-FU on HaCaT cells and more than 50-fold less active than 5-FU on WiDr cells. The immortalized keratinocyte cell line HaCaT was more sensitive than WiDr to drug cytotoxic effects whatever the drug species considered. Of note, the addition of the other species to 5′-DFUR, the relative proportion of each drug reflecting known pharmacokinetic data, did not change 5′-DFUR cytotoxic effects as compared to the single cytotoxic activity generated by 5′-DFUR alone.

WiDr cells and HaCaT cells did not exhibit a detectable DPD activity (below 0.02 nmol/min/mg protein): this suggests that 5-FUH_2 cytotoxic effect is direct and cannot be mainly attributable to a reverse transformation of 5-FUH_2 into 5-FU through the intermediary of DPD.

Fig. 2



Cytotoxic effects of 5′-DFUR (□), 5-FU (○), 5-FUH$_2$ (△), FBAL (◇) and the drug mixture (●) on HaCaT (a) and WiDr (b). The *x*-axis allows a direct comparison of the dose effect between each drug taken alone and the drug mixture.

This was confirmed by the absence of measurable 5-FU concentration in the culture medium during experiments testing the effect of 5-FUH$_2$.

There was a marked difference in TP activity between the two cell lines. HaCaT exhibited a TP activity (156 ± 15 pmol/h/mg protein) which was 5 times greater

Table 1 Sensitivity of tested cell lines to 5-FU-related species (mean ± SD)

	HaCat IC_{50} (μM)	WiDr IC_{50} (μM)	IC_{50} WiDr:IC_{50} HaCat
5′-DFUR	1.3 ± 1	10 ± 8	10.5 ± 11
5-FU	0.2 ± 0.1	3.3 ± 0.4	36.3 ± 30
5-FUH_2	13.4 ± 5	560 ± 423	56 ± 66
FBAL	>650	>6500	
5′-DFUR, 5-FUH_2, 5-FU and FBAL mixture[a]	1.9 ± 1.6	4.2 ± 2.5	2.5 ± 0.7

[a]For the respective proportions of the drugs, see Material and methods.
IC_{50} values are relative to 5′-DFUR.

than that of WiDr (30 ± 8 pmol/h/mg protein). This was confirmed by the results on the intracellular metabolism of 5′-DFUR: the 5-FU/5′-FUDR ratio of HaCaT was 3-fold higher than the WiDr one. The higher sensitivity to 5′-DFUR and 5-FU for HaCaT as compared to WiDr could be attributable to the presence of high TP activity in HaCaT cells.

Discussion

Previous data from the literature suggest that the main metabolites of 5-FU, 5-FUH_2 and FBAL, can exhibit cytotoxic effects. For instance, an experimental study by Diasio *et al.* [10] on Ehrlich ascites tumor suggested that 5-FUH_2 inhibits cell proliferation and may contribute to the toxicity of 5-FU *in vivo*. The present data corroborate this previous observation with, however, IC_{50} values of 5-FUH_2 several orders of magnitude higher than those of 5-FU or 5′-DFUR. Diasio *et al.* advocated that 5-FUH_2 could be retroconverted into 5-FU through DPD, thus providing plausible explanations for their results [10]. This hypothesis has not, however, been so far confirmed since DPD activity was very low in both tested cell lines. Moreover, the absence of measurable 5-FU in 5-FUH_2-supplemented culture strengthened this observation. On the other hand, FBAL has shown cytotoxic effects on cultured murine cerebellar myelinated fibers [11]. This latter finding was strengthened by the fact that the application of an irreversible DPD inhibitor, 5-ethynyluracil (776C85), prevented 5-FU induced neurotoxicity in dogs [20]. However, other reports suggested an absence of intrinsic cytotoxic activity of FBAL on epithelial cells [10,21]. The present data agree well with this latter observation with an absence of antiproliferative effects of FBAL on WiDr and HaCaT cells.

One of the main objectives of the present study was to examine the impact on human keratinocytes in culture of a mixture of fluoropyrimidines and metabolites representative of that observed in plasma of patients under treatment by capecitabine. This was performed so as to evaluate the contribution of 5-FU metabolites to the cytotoxicity generated by the typical drug combination. HaCaT [14] is the most studied and characterized member of the spontaneously transformed human keratinocyte cell line family (only two others, far less studied members SIK [22] and NIKS [23]). Moreover, HaCaT was presently used as particularly representative of human skin biochemical properties [24] and expressing relevant genes linked to apoptosis [25]. Of note, 5′-DFUR IC_{50} values were not different when cells were exposed to 5′-DFUR alone or in combination with 5-FU, 5-FUH_2 and FBAL, the relative proportion of each compound reflecting human plasma pharmacokinetic data [12]. This observation was done on both HaCaT cells and WiDr cells. This finding suggests the possibility that the marked presence of 5-FUH_2 and FBAL, the main metabolites of 5-FU, are not linked to the development of HFS generated by oral fluoropyrimidines devoid of a DPD inhibitor.

5′-DFUR was the main determinant of cytotoxic activity in the present experimental situation, and it has been shown by others [26–28] and us [17,29] that the intrinsic cellular sensitivity to 5′-DFUR is strongly dependent upon TP activity. Moreover, from human cancer cell xenografts in nude mice it has been shown that intratumoral TP activity correlated well with the degree of capecitabine antitumor efficacy [30]. Other data point out that an enhancement of the efficacy of capecitabine and 5′-DFUR could be achieved through the positive modulation of TP [31]. The presence of a significant TP activity in human skin and human keratinocytes has been previously reported [32], and this must be underlined in the context of capecitabine-related HFS. One can thus make the hypothesis that the HFS encountered with capecitabine may be due, at least in part, to the presence of elevated TP activity in the skin, and particularly in the hand and foot areas where it is known that epidermal renewal is particularly active, thus suggesting in this cutaneous area a high level of cell proliferation and TP activity. This supposition is supported by the demonstration that proliferating keratinocytes readily salvage extracellular thymidine to form nucleotides [33]. This possible link between TP, proliferating keratinocytes and capecitabine HFS does not exclude a plausible explanation for the HFS encountered with other anticancer agents, particularly anthracyclines [34], since it is known that their cytotoxic activity is promoted by the proliferating state of targeted cells. In addition, a possible role of TP in the pathogenesis of HFS under capecitabine is strengthened by the present data where a marked TP activity was found in HaCaT and which, interestingly, was higher than that measured in the WiDr tumoral cells. Finally, this higher TP activity in HaCaT cells was corroborated by the intrinsic cellular capacity to transform 5′-DFUR into 5-FU and by better cytotoxic activity of 5′-DFUR against HaCaT cells as compared to WiDr cells.

We plan further steps which will involve measuring TP activity in palmar–plantar areas as compared with other skin parts which are free of toxicity from capecitabine. The possible availability of specific TP inhibitors [35] could permit application of these compounds on the skin in an effort to prevent HFS induced by capecitabine.

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