Folinic Acid Enhanced Cytotoxicity of Fluoropyrimidines in Human Breast Cancer Cells*

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Summary

This report describes the effects of the natural diastereoisomer of 5-formyltetrahydrofolate (6S)- and of the mixture of both the natural and unnatural diastereoisomers (6R,S)-5-HCO-H₄PteGlu (folinate) on the cytotoxicity of 5-fluoro-2'-deoxyuridine (FdUrd) and 5-fluorouracil (FUra) in MCF-7 and MDA-MB 231, human breast cancer cell lines. Continuous exposure of exponentially growing cells to various concentrations of FUra for 72 h caused a 50% inhibition of growth with IC₅₀ at concentrations of 3.40 ± 0.16 and 29 ± 1.40 μ M in MCF-7 and MDA-MB 231, respectively. Preincubation of the cells for 24 h with (6S)-5-CHO-H₄PteGlu (5 μ M) prior to addition of FUra decreased the IC₅₀ values by 2.4-2.6-fold in both cell lines. A quantitatively similar effect was achieved by preincubation with 10 μ M of (6R,S)-5-HCO-H₄PteGlu. The cytotoxicity of FdUrd was markedly more pronounced than that of FUra exhibiting IC₅₀ values at a range of 0.045-0.050 μ M for both cell lines. Preincubation with (6S)-5-HCO-H₄PteGlu increased the susceptibility to the drug by about 3.5-fold. The optimal dose of (6S)-5-CHO-H₄PteGlu for achieving the lowest IC₅₀ value for FdUrd in MCF-7 cells was 5 μ M, whereas that in MDA-MB 231 was 20 μ M. Similar increase of the cytotoxicity of FdUrd was obtained with about a 2-fold higher concentration of (6R,S)-5-HCO-H₄PteGlu.

Cells pretreated for 24 h with FUra or FdUrd respectively, exhibited enhanced incorporation of [³H] thymidine into the TCA-precipitable fraction. Presence of (6S)-5-HCO-H₄PteGlu (10 μ M) doubled the amount of incorporated thymidine, which reflects depletion of intracellular thymidylate pools due to enhanced formation and stabilization of the complex formed between thymidylate synthase, fluorodeoxyuridylylate and 5,10-methylenetetrahydrofolate.

Key words: Folinic acid, Cytotoxicity, Fluoropyrimidines, Human breast cancer cell lines

Introduction

Fluoropyrimidines, mainly 5-fluorouracil (FUra) and 5-fluoro-2'-deoxyuridine (FdUrd) are important chemotherapeutic drugs used extensively in the treatment of carcinomas of the gastrointestinal tract, breast, ovary and other tumors. Studies *in vitro* have shown a marked potentiation of the cytotoxicity of FdUrd in mouse leukemia L1210 cells by 5-formyltetrahydrofolate (5-HCO-H₄PteGlu) (1), known as folinate or leucovorin, a mixture of the natural (6S) and unnatural (6R)-diastereoisomers. The folinate-

enhanced cytotoxicity of FUra or FdUrd was observed later in mouse S-180 sarcoma and human Hep-2 carcinoma (2), in Friend erythroleukemia (3), murine and human leukemia cells (4, 5), human colorectal (6), squamous cell carcinoma (7) and recently in several human lung cancer cell lines (8). Thus, folinate acts as a modulator of the cytotoxicity of fluoropyrimidines and has been exploited as such to increase the clinical effectiveness of these drugs (9).

The cytotoxicity of fluoropyrimidines is based upon the following main mechanisms of action: inhibition of thymidylate synthase (TS) by 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), analogized from FdUrd or FUra (10), incorporation of

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The purpose of the present study was to examine the effects of folinate, (racemic 6R,S)-, and of the natural (6S)-isomer on the cytotoxicity of FUra and FdUrd in MCF-7 and MDA-MB 231, human breast cancer cell lines exposed to a continuous drug treatment. To further elucidate the effect of these fluorinated pyrimidines with and without 5-HCO-H₄PteGlu on the cellular pool of thymidylate, we followed the incorporation of [³H] thymidine into the cold TCA-precipitable fraction of cells pretreated with various combinations of the mentioned drugs.

Materials and Methods

Chemicals

Folinic acid (calcium salt), FUra, FdUrd and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co., St Louis, MO. (6S)-HCO-H₄PteGlu was a product of Sapec S.A. Barbengo, Switzerland. [³H] Thymidine, specific activity 22 Ci/m mol was purchased from Amersham International, Buckinghamshire, England.

Cell culture

The cell lines MCF-7 and MDA-MB 231 were cultivated in RPMI 1640 medium obtained from Biological Industries, Bet Haemek, supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). For growth inhibition studies, exponentially growing cells were dispersed into 24-well plates and incubated in a humidified incubator at 37°C, under an atmosphere of 5% CO₂ for 24 or 48 h for MDA-MB 231 and MCF-7 cells respectively. The exponentially growing cells were then incubated with or without (6S)-5-HCO-H₄PteGlu for 24 h. Subsequently, drugs were added and the cultures were re-incubated for an additional 72 h. The growth was terminated by two washes with phosphate buffered saline (PBS) and to obtain a single-cell suspension, the monolayers were incubated at 37°C for 10 min with 0.1% EDTA in PBS without calcium and magnesium. The cells were quantitated by counting in a hemocytometer.

MTT colorimetric assay

Cell survival was assessed by an assay (15), based on the reduction of the MTT to a water-insoluble formazan in mitochondria of living cells. 10 µl of MTT at a concentration of 5 mg/ml in PBS were added into each well containing 100 µl medium and the cell monolayer and the plates were incubated for 4 h in a humidified incubator at 37°C in a 5% CO₂ atmosphere. Subsequently the medium was removed, the formazan crystals produced by the viable

Figure 1. Effect of FUra and (6S)-5-HCO-H₄PteGlu on growth of MCF-7 and MDA-MB 231 cells. Experimental conditions were as described in Materials and Methods. MCF-7 cells without (●) and with (○) 5-HCO-H₄PteGlu; MDA-MB 231 cells without (▲) and with (△) 5-HCO-H₄PteGlu. Points represent the mean value of three experiments in triplicate and the S.E. are within the symbols.

Figure 2. Effect of FdUrd and (6S)-5-HCO-H₄PteGlu on growth of MCF-7 and MDA-MB 231 cells. Experimental conditions and symbols were as in Fig. 1. Points represent the mean value of three experiments in triplicate with S.E. within the symbols.

Table 1. Effect of increasing concentrations of (6S)- and (6R,S)-5-HCO-H₄PteGlu on the cytotoxicity of FdUrd in MCF-7 and MDA-MB 231 cell lines.

	FdUrd IC ₅₀ (nM)	
	MCF-7	MDA-MB 231
(6S)-5-HCO-H ₄ PteGlu (μM)		
—	45.1 ± 5.2	49.3 ± 7.1
0.1	43.3 ± 6.0	47.8 ± 6.3
0.5	23.4 ± 3.3	42.1 ± 6.0
2.0	15.6 ± 2.3	21.3 ± 3.2
5.0	12.3 ± 1.6	13.6 ± 2.0
10.0	12.5 ± 1.4	11.5 ± 1.3
20.0	12.4 ± 1.3	9.2 ± 1.1
(6R,S)-5-HCO-H ₄ PteGlu (μM)		
5.0	15.1 ± 2.0	22.4 ± 3.3
10.0	12.5 ± 1.8	13.9 ± 1.9
20.0	12.4 ± 1.5	10.9 ± 1.3
50.0	12.5 ± 1.4	8.9 ± 1.1

Cells (3×10^5) were plated into 96-well plates and incubated for 24 h in RPM 11640 medium supplemented with 10% dialyzed fetal bovine serum and the indicated concentrations of (6S)- or (6R,S)-5-HCO-H₄PteGlu. Subsequently various concentrations of FdUrd were added and the cultures were reincubated for 48 h with MDA-MB 231 and for 72 h with MCF-7 cells. Cell growth was measured by a MTT colorimetric assay. Values are means ± S.E. of three experiments in triplicate.

cells were solubilized in 150 μl of dimethyl sulfoxide and the optical density was read at 550 nm in a scanning multiwell spectrophotometer (SLT-Labinstruments, Austria).

Table 2. Effect of increasing concentrations of (6S)- and (6R,S)-5-HCO-H₄PteGlu on the cytotoxicity of Fura in MCF-7 and MDA-MB 231 cell lines.

	Fura IC ₅₀ (μM)	
	MCF-7	MDA-MB 231
(6S)-5-HCO-H ₄ PteGlu (μM)		
—	3.46 ± 0.28	31.21 ± 2.83
0.1	2.97 ± 0.24	28.53 ± 2.55
1.0	2.01 ± 0.19	16.57 ± 1.81
5.0	1.51 ± 0.13	11.21 ± 1.30
10.0	1.40 ± 0.12	10.03 ± 1.23
20.0	1.52 ± 0.14	9.10 ± 1.02
(6R,S)-5-HCO-H ₄ PteGlu (μM)		
1.0	2.53 ± 0.19	24.03 ± 2.11
5.0	1.91 ± 0.15	18.11 ± 1.75
10.0	1.54 ± 0.12	11.95 ± 1.20

Experimental details are as in Table 1. Values are means ± S.E. of three experiments in triplicate.

Incorporation of [³H] thymidine

The drug-exposed cells were washed twice with 1 ml PBS; [³H] thymidine was added in 1 ml of serum-free medium and after incubation for 1 h at 37°C in a humidified incubator, the cells were washed three times with 2 ml of ice-cold PBS and the radioactivity was determined in cold 5% trichloroacetic acid-precipitate solubilized in 1N NaOH. Samples, neutralized with HCl were counted in a Triton-toluene scintillation fluid.

Results

Inhibition of growth of MCF-7 and MDA-MB 231 cells by Fura and FdUrd in the presence and absence of (6S)-5-HCO-H₄PteGlu.

Exposure of the cells to increasing concentrations of Fura caused inhibition of growth with IC₅₀ values of 3.4 ± 0.16 and 29 ± 1.8 μM for MCF-7 and MDA-MB 231, respectively (Fig. 1). Preincubation for 24 h with 5 μM (6S)-5-HCO-H₄PteGlu prior to addition of the drug caused a 2.4-2.6-fold decrease of the IC₅₀ values in both cell lines. 5-HCO-H₄PteGlu by itself did not affect growth.

The susceptibility of both cell lines to FdUrd is markedly higher than to Fura, exhibiting almost the same IC₅₀ values, namely 45 ± 2.8 and 49 ± 3.9 nM for MCF-7 and MDA-MB 231 respectively. The effect of the reduced folate was more pronounced than with Fura and caused an about 3.5-fold increase in cytotoxicity (Fig. 2).

Table 3. Incorporation of [^3H] thymidine into DNA of MCF-7 and MDA-MB 231 cells pretreated with FUra and FdUrd in the presence and absence of (6S)-5-HCO-H₄PteGlu.

	[^3H] Thymidine Incorporated (%)	
	MCF-7	MDA-MB 231
Control	100.0 $\pm$ 5.2	100.0 $\pm$ 5.8
(6S)-5-HCO-H ₄ PteGlu	100.0 $\pm$ 5.4	100.0 $\pm$ 3.5
FUra (5 μM)	147.2 $\pm$ 9.2	128.9 $\pm$ 8.3
FUra (5 μM)+(6S)-5-HCO-H ₄ PteGlu	242.6 $\pm$ 12.4	226.6 $\pm$ 15.3
FUra (10 μM)	196.9 $\pm$ 13.6	151.3 $\pm$ 10.2
FUra (10 μM)+(6S)-5-HCO-H ₄ PteGlu	245.4 $\pm$ 15.1	231.2 $\pm$ 14.8
FdUrd (0.02 μM)	115.1 $\pm$ 8.1	118.1 $\pm$ 9.7
FdUrd (0.02 μM)+(6S)-5-HCO-H ₄ PteGlu	250.5 $\pm$ 20.2	195.6 $\pm$ 19.3
FdUrd (0.05 μM)	186.9 $\pm$ 14.0	205.4 $\pm$ 12.6
FdUrd (0.05 μM)+(6S)-5-HCO-H ₄ PteGlu	266.9 $\pm$ 24.1	265.1 $\pm$ 13.9

Drugs were added into exponentially growing cells at almost confluency in 24-well plates and the cultures were incubated for 24 h. The concentration of (6S)-5-HCO-H₄PteGlu added was 10 μM . The drug treatment was terminated by two washes with PBS. [^3H] thymidine (60 pmol/0.4 μCi) was added in 1 ml of serum free medium and the cells were incubated for 1 h at 37°C. Further details are as described in Materials and Methods. Values are mean $\pm$ S.E. of three experiments in triplicate.

Effect of increasing concentrations of (6S) and (6R,S)-5-HCO-H₄PteGlu on the cytotoxicity of FdUrd and FUra in MCF-7 and MDA-MB 231 cell lines.

As depicted in Table 1, exposure of the cells for 24 h to increasing concentrations of the natural isomer of 5-HCO-H₄PteGlu or to folinate caused increasing cytotoxicity of FdUrd. The maximal enhancement of MCF-7 and MDA-MB cell kill was at 5 and 20 μM respectively. Since the cytotoxicity achieved at 5 μM of the (6S)-isomer corresponds to that at 10 μM of (6R,S)-5-HCO-H₄PteGlu, it could be assumed that the unnatural isomer does not contribute to enhancement of cytotoxicity. MDA cells incubated with 100 μM folinate did not exhibit further enhancement of the cytotoxicity of FdUrd (not shown).

Table 2 depicts a similar experiment performed with FUra. With this drug, as well, the most effective concentration of the (6S)-isomer appears to be at 5 μM for MCF-7 cells which corresponds to the effect obtained with 10 μM of (6R,S)-5-HCO-H₄PteGlu. The maximal enhancement of the cytotoxicity of FUra for MDA-MB 231 cells was at 20 μM of the (6S)-5-HCO-H₄PteGlu.

Incorporation of [^3H] thymidine into DNA of MCF-7 and MDA-MB 231 cells treated with fluoropyrimidines in the presence and absence of (6S)-5-HCO-H₄PteGlu.

To determine the relation between enhancement of the cytotoxicity of fluoropyrimidines by folinate and depletion of the intracellular thymidylate pool, we examined the incorporation of [^3H] thymidine into

cells pretreated with various combinations of the above drugs. As shown in Table 3, exposure to FUra at a concentration of 5 μM increased the incorporated radioactivity by 47.2% in MCF-7 and by 28.9% in MDA-MB 231 cells, which correlates with the lower susceptibility of the latter to FUra. Cells treated with 10 μM FUra exhibited an increased incorporation of thymidine and at both drug concentrations, the incorporation was enhanced by (6S)-5-HCO-H₄PteGlu.

Treatment with FdUrd at a 250 times lower concentration than that of FUra markedly enhanced the incorporation of thymidine, which was also augmented by the presence (6S)-5-HCO-H₄PteGlu.

Discussion

We have shown that (6S)-5-HCO-H₄PteGlu enhanced the cytotoxicity of FdUrd and FUra in MCF-7 and MDA-MB 231, human breast cancer cell lines (Figs. 1, 2), which is in agreement with the data obtained in studies with other tumor cell lines (1-8). Folate, a mixture of the natural and unnatural isomers, exhibited a 50% activity of that of the natural isomer (Tables 1, 2). Thus, the (6R) isomer does not act as a modulator of the cytotoxicity of fluoropyrimidines, and does not interfere with the enhancing activity of the natural isomer, as also recently shown in an ileocecal carcinoma cell line (16). Although the (6R)-isomer is not biologically inert and inhibits competitively the uptake of the (6S)-compound in human lymphoblastic leukemia CCRF-CEM cells, the apparent K_i value is relatively high and the cellular accumulation markedly lower than

that of the natural isomer (17).

The susceptibility of both cell lines to FdUrd is markedly higher than to FUra and so is the enhancement of the cytotoxicity by the reduced folate (Figs. 1, 2). Similar phenomenon was found in mouse and human leukemia cells (4, 5), and in several human lung cancer cell lines (8). This difference in cytotoxicity, as well as in the enhancing effect of folinate, may be explained, at least partially by a more efficient anabolism of FdUrd into FdUMP, a potent inhibitor of TS. FdUMP binds to the enzyme and 5,10-CH₂-H₄PteGlu forming a ternary complex, which is also stabilized by this reduced folate (13, 14). Formation of the stable ternary complex results in inhibition of thymidylate synthesis and thus presumably, depletion of its intracellular pool. The data depicted in Table 3, support this hypothesis by showing that exposure of MCF-7 and MDA-MB 231 cells to the fluorinated pyrimidines results in increased incorporation of [³H]-thymidine, which is also markedly augmented by the presence of (6S)-5-HCO-H₄PteGlu.

The results of this study indicate that MCF-7 and MDA-MB 231 cells are able to metabolize the 5-HCO-H₄PteGlu into 5,10-CH₂-H₄PteGlu and the fluorinated pyrimidines into FdUMP, which are crucial components of the ternary complex with TS. The recently published paper by Boarman *et al.* strengthens this assumption by demonstrating that increasing concentrations of 5-HCO-H₄PteGlu in the growth medium of MCF-7 cells, increased the intracellular 5,10-CH₂-H₄PteGlu pool (18).

Another important factor in the modulation of the cytotoxicity of fluoropyrimidines by folinate appears to be the availability of the polyglutamates of the 5,10-CH₂-H₄PteGlu. As recently shown, the growth inhibitory effect of fluoropyrimidines on CCRF-CEM cells increased in the presence of folinate, but no such effect could be found in a subline of these cells with an impaired ability to form poly-

glutamates (19).

The results of the presented study suggest that a prolonged exposure to folinate, prior to that of fluoropyrimidines will supply a pool of 5,10-CH₂-H₄PteGlu and its polyglutamates required for a rapid formation of the ternary complex with TS, leading to inhibition of DNA synthesis and thus achieving optimal therapeutic effects.

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