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Comparative cytotoxicity of folate-based inhibitors of thymidylate synthase and 5-fluorouracil ± leucovorin in MGH-U1 cells

Charles Erlichman[1, 2], **Biserka Mitrovski**[1]

[1] Division of Experimental Therapeutics, The Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario, M4X 1K9, Canada
[2] Department of Medicine, Pharmacology and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

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Abstract. Thymidylate synthase (TS) is a critical enzyme in the synthesis of DNA and an important target for cancer chemotherapy. 5-Fluorouracil (5FU) combined with leucovorin (LV) has been used to inhibit TS, and inhibition is dependent on the formation of a ternary complex between a folate cofactor, TS, and 5-fluorodeoxyuridine monophosphate (FdUMP), a metabolite of FU. The folate-based TS inhibitors CB3717, its analogs, and BW1843U89 have been synthesized as specific inhibitors of TS that do not require activation or the presence of a cofactor. We have compared the cytotoxicity of 5FU ± LV with that of these folate-based TS inhibitors in human bladder cancer MGH-U1 cells using a colony-forming assay. After a 6-h exposure, FU+LV, CB3717, dCB3717, or C2 methyl dideazafolate analogs demonstrated similar cytotoxic potency that was 0.96 to 2.9 times that of 5FU alone. A 24-h exposure did not increase the potency of 5FU+LV relative to 5FU alone, but there was a marked increase in the cytotoxicity of the dideazafolates as compared with 5FU+LV. Similarly, BW1843U89 was more cytotoxic than 5FU+LV. This was reflected in a 3.2- to 1333-fold decrease in the 50% inhibitory concentration (IC_{50}). Simultaneous exposure to LV and thymidine (TdR) protected MGH-U1 cells from the cytotoxicity of CB3717, its analogs, and BW1843U89. We conclude that (a) the folate-based TS inhibitors are more potent than 5FU+LV after a 24-h exposure, (b) protection by LV and TdR indicates that TS inhibition is the primary site of action, and (c) BW1843U89 is more potent than D1694 in MGH-U1 cells.

Introduction

Thymidylate synthase (TS) plays a central role in maintaining pools of deoxythymidine monophosphate (dTMP) necessary for DNA synthesis and cell growth. Inhibition of TS by 5-fluorodeoxyuridine monophosphate (FdUMP) binding in the presence of excess methylene tetrahydrofolate (CH_2FH_4) is a major mechanism of 5-fluorouracil (5FU)-mediated cytotoxicity [7, 27]. This has led to the use of 5FU combined with leucovorin (LV) in patients with metastatic colorectal cancer [5, 21, 23] and to ongoing evaluation of this treatment in the adjuvant setting. 5FU is metabolized to fraudulent nucleotides that mediate its cytotoxic effects [22]. Fluorouridine triphosphate (FUTP) incorporation into RNA results in translational errors. Fluorodeoxyuridine triphosphate (FdUTP) incorporation into DNA may cause DNA strand breaks. FdUMP inhibition of TS with subsequent cessation of DNA synthesis is dependent on adequate quantities of folate cofactor, CH_2FH_4. Administration of LV increases intracellular pools of CH_2FH_4 [6, 16] and prolongs inhibition of TS via the formation of a stable ternary complex. The extensive metabolism of two prodrugs and competition with natural intracellular substrates have been identified as mechanisms of resistance to 5FU+LV in patients [25].

Analogs of CH_2FH_4, folate-based TS inhibitors, that have demonstrated experimental activity are undergoing clinical trials. CB3717 and other quinazoline antifolates were synthesized to bind TS at the CH_2FH_4 binding site and to compete with CH_2FH_4 [11–15, 17, 19, 20]. Folate-based TS inhibitors may be more effective inhibitors of TS than 5FU+LV because they do not require anabolism or a cofactor, they do not appear to be catabolized, there is limited accumulation of the competing natural substrate, CH_2FH_4, and they are not incorporated into DNA or RNA. They are polyglutamated [9, 24], leading to intracellular retention and increased inhibition of TS. Some quinazoline antifolates appear to be transported by the high-capacity membrane folate-binding protein [10, 26]. BW1843U89 is a benzoquinazoline TS inhibitor that is noncompetitive with CH_2FH_4 for binding to TS [3]. LV protection of growth inhibition by D1694 or BW1843U89 is concentration-dependent, with greater protection occurring against D1694 than against BW1843U89 at the same LV concentrations.

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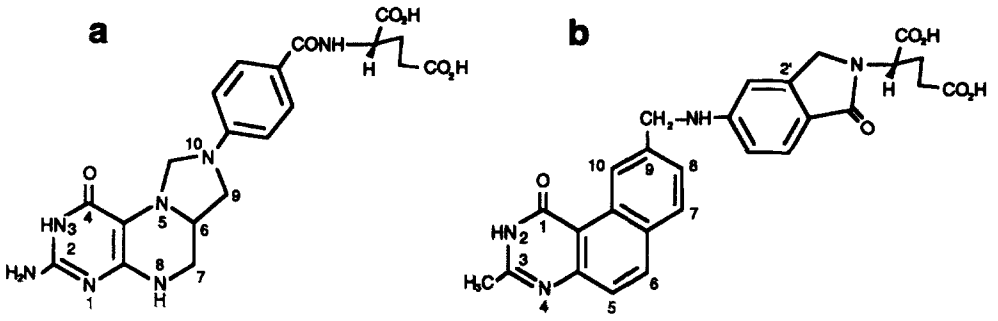
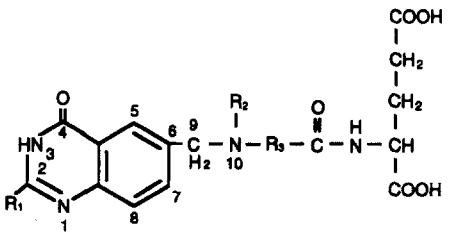
Correspondence to: C. Erlichman, Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada





Compound	R_1	R_2	R_3
CB3717	NH_2	$CH_2C\equiv CH$	
dCB3717	H	$CH_2C\equiv CH$	
1	CH_3	$CH_2C\equiv CH$	
2	CH_3	$CH_2C\equiv CH$	
3	CH_3	$CH_2C\equiv CH$	
4	CH_3	C_2H_5	
5	CH_3	CH_3	
D1694	CH_3	CH_3	

Fig. 1. Structure of (**a**) 5,10-methylene tetrahydrofolate, (**b**) BW1843U89, and (**c**) CB3717 and its analogs

The purpose of these studies was to compare the cytotoxicity of FU±LV with that of CB3717, its analogs, and BW1843U89 (Fig. 1); to determine the relationship between cytotoxic potency and modifications to the chemical structure of CB3717; and to determine whether TdR or LV could rescue cells from the cytotoxicity of these compounds.

Materials and methods

Chemicals. LV, FU, and TdR were purchased from Sigma Chemical Co, (St. Louis, Mo.). CB3717 and dCB3717 were provided by Dr. V. Narayanan, Drug Synthesis and Chemistry Branch, NCI (Bethesda, Md.). The C2-methyl quinazoline analogs of CB3717 were a gift from ICI Pharmaceuticals (Alderly Park, Macclesfield, Cheshire, UK). BW1843U89 was a gift from Burroughs Wellcome (Research Triangle Park, N.C., USA). The drugs were dissolved in 0.15 *M* $NaHCO_3$ (CB3717, dCB3717), 0.4 *M* $NaHCO_3$ (CB3717 analogs), or a 2:1 molar ratio of NaOH:BW1843U89 adjusted to pH 7. Drugs were protected from light and dilutions were made in phosphate-buffered saline (PBS). Media, antibiotics, trypsin, and plasticware were obtained from Gibco (Grand Island, N.Y.).

Cell line. A human bladder-cancer cell line, MGH-U1, originally obtained from Dr. G. R. Prout (Boston, Mass.) was maintained as a monolayer in α-MEM supplemented with 0.01% streptomycin, 0.01% penicillin, and 10% fetal calf serum (FCS; Whittaker, Walkerswille and Bockneck, Toronto) at 37° C in a humidified atmosphere containing 5% CO_2 and was subcultured three times a week. Under these conditions, the doubling time of cells growing exponentially was 20 h.

Cytotoxicity assay. Clonogenic survival of drug treated cells was determined as previously described [2]. One million cells were seeded in an 83-cm^2 flask in 10% DFCS nucleoside-free α-MEM. After 2 days, the exponentially growing cells were exposed to various drug concentrations for the desired periods. For rescue studies, cells were exposed to the drug and TdR or LV simultaneously. Cell survival was expressed as a fraction relative to the control value. Concentrations yielding 50% and 90% inhibition of colony growth (IC_{50} and IC_{90} values) were determined from graphs of survival versus the logarithms of drug concentration.

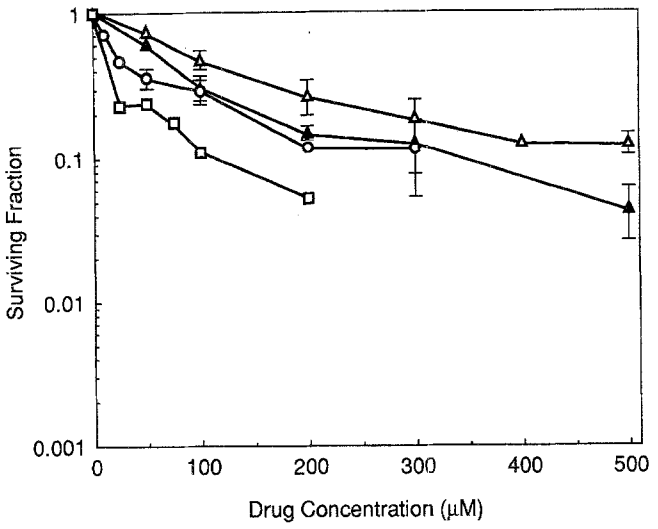


Fig. 2. Clonogenic survival of MGH-U1 cells treated for 6 h with 5FU (△), 5FU with 100 μ*M* LV (▲), CB3717 (○), and compound 3 (□). The points represent the mean of triplicate samples from at least 2 experiments

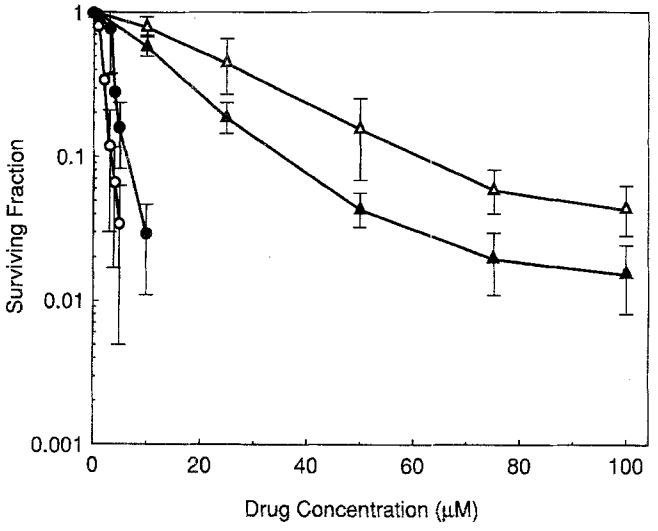


Fig. 3. Survival of MGH-U1 cells after 24 h exposure to 5FU (△), 5FU+ 100 μ*M* LV (▲), CB3717 (○), and dCB3717 (●). The points represent the mean of triplicate samples from at least 3 experiments

Results

The chemical structures of CB3717, its analogs and BW1843U89 are shown in Fig. 1. Modifications were made in the C2 position, the N10 position and the benzamide moiety of the *p*-aminobenzoylglutamate of CB3717 in compounds 1–5 and D1694. BW1843U89 differs in that a benzoquinazoline moiety replaces the pteridine ring in the folate structure and an isoindolinyl group replaces the benzamide moiety of the *p*-aminobenzoylglutamate side chain. Each compound has a terminal glutamate. Survival curves for a 6-h exposure to FU alone, FU+LV, CB3717, and compound 3 are shown in Fig. 2. LV had no effect on

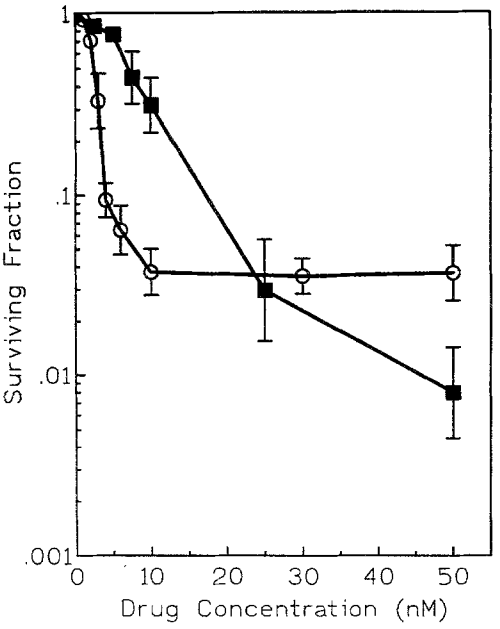


Fig. 4. Clonogenic survival of MGH-U1 cells treated with D1694 (■) and BW1843U89 (○) for 24 h. The points represent the mean of triplicate samples from at least 3 experiments

Table 1. IC_{50}[a] (μ*M*) for MGH-U1 cells exposed to FU±LV, CB3717, and folate-based TS inhibitors for 6 or 24 h

Compound[b]	6 h	24 h
5FU	96	22
5FU+LV	64	12
CB3717	40	1.6
dCB3717	22	3.7
1	–	0.06
2	100	0.2
3	25	0.01
4	35	0.01
5	30	0.02
D1694	–	0.06
BW1843U89	–	0.03

–, Not determined

[a] Average of 3 determinations; SE <10% on each value

[b] For compound structures refer to Fig. 1

MGH-U1 survival on its own. A 1.5- to 2-fold increase in the cytotoxicity of FU was observed when 100 μ*M* LV was added. The cytotoxicity of CB3717 was similar to that of FU+LV, whereas compound 3 appeared more potent than FU+LV during a 6-h exposure.

A 24-h exposure to these drugs was associated with an increase in cytotoxicity. The survival curves for FU, FU+LV, CB3717, and dCB3717 are shown in Fig. 3 and those of D1694 and BW1843U89 are plotted in Fig. 4. The IC_{50} values for 6- and 24-h exposures to each drug are summarized in Table 1. The potency of these analogs was 3- to ~1333-fold greater than that of FU±LV for a 24-h exposure. Furthermore, the potency of the quinazoline analogs increased from 6- to ~4000-fold when the exposure time was increased from 6 to 24 h. BW1843U89 was 2 times more potent than D1694 in this cell line with a 24-h exposure.

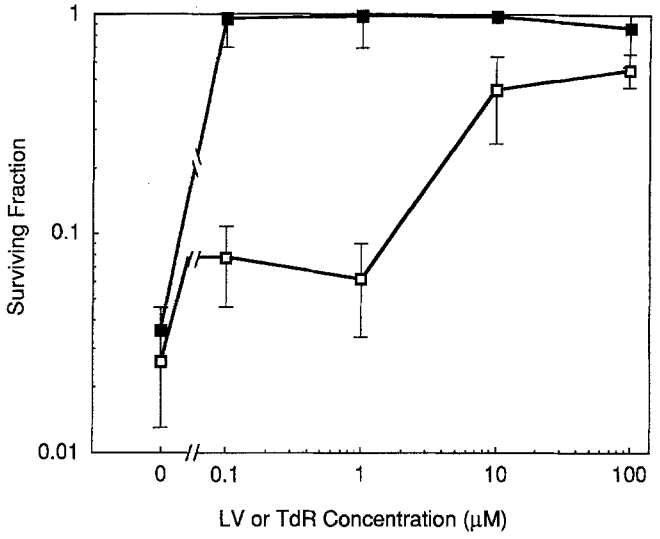


Fig. 5. Cytotoxicity of D1694 (10 n*M*) after a 24-h coincubation with increasing concentrations of LV (■) or TdR (□). The points represent the mean of triplicate samples from 3 separate experiments

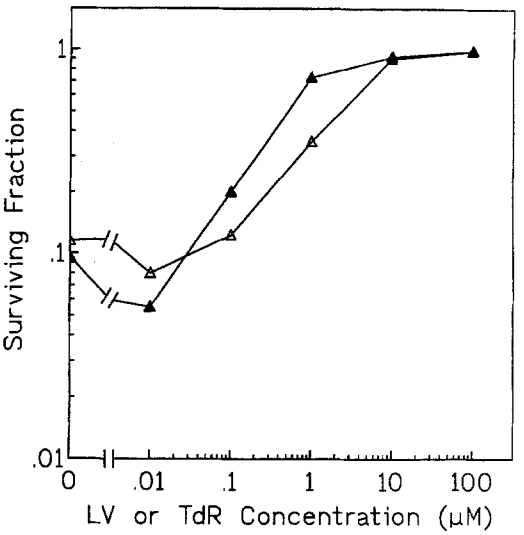


Fig. 6. Cytotoxicity of BW1843U89 (5 n*M*) after a 24-h coincubation with increasing concentrations of LV (▲) or TdR (△). The points represent the mean of triplicate samples from 2 separate experiments

To determine whether LV or TdR could protect MGH-U1 cells from drug cytotoxicity, cells were exposed to 10 n*M* D1694 (Fig. 5) and 5 n*M* BW1843U89 (Fig. 6) for 24 h, which resulted in a surviving fraction of ~0.1. Cells were completely protected from D1694 cytotoxicity by simultaneous exposure to 0.1 μ*M* LV, whereas 10 μ*M* LV was required to protect MGH-U1 cells completely from BW1843U89. Prevention of cytotoxicity by TdR occurred at higher concentrations of 10 and 100 μ*M*. Similar results were achieved with CB3717 and dCB3717 (data not shown).

Discussion

Clinical studies have demonstrated that 5FU+LV increases response rates in patients with metastatic colorectal cancer. However, 5FU and LV must be activated to the critical metabolites that bind to TS, and many factors involved in metabolism or binding may lead to resistance. Folate-based TS inhibitors have the potential to be more effective than 5FU+LV. They offer the advantage of a single site of action, a limited need for activation, and no need for a cofactor. Thus, we compared the cytotoxicity of CB3717, a series of C2-desamino C2-methyl analogs, and BW1843U89 with that of 5FU alone or combined with 100 μ*M* LV in MGH-U1 cells treated for 6 or 24 h.

As previously reported [4], 5FU cytotoxicity in MGH-U1 cells can be increased by a factor of 1.5–2 when 100 μ*M* LV is added during a 6-h treatment. The 4-fold increase in cytotoxicity seen with 5FU alone or 5FU+LV after a 24-h exposure is consistent with the phase-specific nature of 5FU cytotoxicity. The relative potency of 5FU+LV/5FU did not differ between a 6-h and a 24-h exposure, suggesting that other factors that might potentiate cytotoxicity further, such as polyglutamation of the cofactor, synthesis of FdUMP, or binding to TS by FdUMP, do not increase between 6 and 24 h. Alternatively, an increase in the synthesis of TS protein during this period could offset any increase in FdUMP formation.

For 6-h exposures we found a marginally greater potency of all TS inhibitors except compound 2 as compared with FU+LV. The relative potency ranged from 0.96 to 2.9-fold (Table 1). After 24-h exposures the folate-based TS inhibitors showed a marked increase in cytotoxicity relative to FU + LV ranging from a factor of ~3 for dCB3717 to that of >1000 for compound 4. Furthermore, we observed a 6- to ~4000-fold increase in cytotoxicity when drug exposure was increased from 6 to 24 h for the folate-based TS inhibitors. These results indicate that the antifolate TS inhibitors are more cytotoxic than FU+LV to MGH-U1 cells during a prolonged exposure and are consistent with increased polyglutamation of these compounds with an associated increase in TS binding and cellular retention of the drugs [9, 24]. The increased potency of BW1843U89 as compared with D1694 is consistent with the observation that it is a better substrate for folylpolyglutamate synthetase (FPGS) [3].

Cytotoxicity of CB3717, its analogs, and BW1843U89 was completely prevented by LV. Duch et al. [3] demonstrated that prevention of growth inhibition by BW1843U89 required 10- to 100-fold higher concentrations of LV than did that by D1694, and we observed a similar difference using clonogenic survival instead of growth inhibition as a measure of cell kill. This supports the contention that the inhibitor BW1843U89 is noncompetitive with CH_2FH_4 for TS. The mechanism of LV protection from cytotoxicity may be by CH_2FH_4 competition with the folate-based TS inhibitors at the folate cofactor-binding site on TS or competition for transport into the cell. A third possible mechanism for LV protection from cytotoxicity is LV competition with the folate-based TS inhibitors for FPGS. If polyglutamated forms of these

analogs bind to TS with higher affinity than that of the monoglutamates, competition at FPGS may be an important mechanism whereby cytotoxicity is prevented. The prevention of cytotoxicity by TdR supports the contention that TS is the major site of drug effect.

A comparison of the potencies for cytotoxicity of CB3717, dCB3717, compounds 1–5, and D1694 gives some insight into the chemical determinants that are important in mediating drug activity. The addition of a C2 methyl group increases cytotoxicity significantly. Modification of the benzyl ring to a thiazole or thiophene contributes a moderate effect to cytotoxicity. Alterations in the N10 position had little effect on cytotoxicity. These results are consistent with the structure activity studies reported previously [11–15, 17, 19] using L1210 cells. The relative potency of these TS inhibitors is also influenced by their substrate specificity for FPGS [18]. Different potencies among the analogs may indicate that some are better substrates for FPGS. CB3717, dCB3717, and compound 1 are good substrates for FPGS and their polyglutamate derivatives are up to 200 times more potent as TS inhibitors [1, 8, 19] than is the monoglutamate. Thiazole substitution of the benzyl moiety increases the substrate affinity for FPGS further [17]. Both D1694 and BW1843U89 appear to be excellent substrates for FPGS [3].

In conclusion, we have demonstrated that the folate-based TS inhibitors appear to have potency comparable with that of 5FU+LV when a short exposure is used but are much more effective with longer exposure times. D1694 and BW1843U89 have comparable potency in this cell line with a 24-h exposure. Protection from the antitumor effects of D1694 and BW1843U89 can be achieved with pharmacologic concentrations of either LV or TdR.

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