Cancer Chemother Pharmacol (2000) 45: 142–148

ORIGINAL ARTICLE

N.L. Lehman · P.V. Danenberg

Modulation of RTX cytotoxicity by thymidine and dipyridamole in vitro: implications for chemotherapy

Received: 22 March 1999 / Accepted: 13 August 1999

Abstract *Purpose*: The cytotoxicity of the thymidylate synthase (TS) inhibitor raltitrexed (RTX) is reversed by supraphysiologic thymidine concentrations. Dipyridamole (DP) blocks thymidine salvage (uptake) and potentiates several chemotherapeutic agents in vitro. The purpose of this study was to determine whether DP is capable of potentiating RTX cytotoxicity in the presence of physiologic thymidine concentrations. *Methods*: We examined the effect of DP on RTX cytotoxicity in the presence of various physiologic thymidine concentrations in eight colorectal adenocarcinoma cell lines by colony formation assay, and in p53-defective HL60 S and p53-competent HL60 SN3 promyelocytic leukemia cells by a tetrazolium reduction-based assay. *Results*: In WiDr colon adenocarcinoma cells the cytotoxicity of 1.0 μ*M* RTX (4 h) varied from 0% to >99% within the reported range of human serum thymidine concentrations from 500 to <50 n*M*, respectively. DP potentiated RTX cytotoxicity 2- to >93-fold within this range. Free DP concentrations of 10 to 20 n*M*, achievable with oral dosing, potentiated RTX at thymidine concentrations up to 100 n*M*. Potentiation at higher physiologic thymidine concentrations of ≥200 n*M* required DP concentrations of at least 50 n*M*, or about twice the steady-state DP concentration obtainable from oral and intravenous dosing, but well within that obtainable by intraperitoneal infusion. Maximal potentiation required 72 to 120 h of DP exposure. DP also potentiated RTX in the absence of exogenous thymidine in six of eight colon cell lines and HL60 S and SN3 cells suggesting a second, nonthymidine salvage-dependent mechanism of potentiation. *Conclusions*: Clinically achievable free DP concentrations potentiated RTX cytotoxicity within the range of physiologic serum thymidine concentrations. RTX/DP or DP analogue-based combination therapy should, therefore, be considered for clinical trial. Serum or tumor thymidine concentration determinations may aid in identification of patients likely to respond to TS and nucleoside salvage inhibitors versus alternate, non-TS-directed therapies.

Key words Colorectal cancer · Cytotoxicity · Dipyridamole · Nucleoside salvage · Raltitrexed

N.L. Lehman (✉)[1] · P.V. Danenberg
Norris Comprehensive Cancer Center,
University of Southern California Los Angeles,
CA 90033, USA

N.L. Lehman
Department of Surgery, Huntington Memorial Hospital,
Pasadena, CA 91105, USA

Present address:
[1] Department of Pathology,
Creighton University/St. Joseph Hospital,
601 N. 30th Street, Omaha, NE 68131, USA
e-mail: nlehman@pathology.creighton.edu
Tel.: +1-402-4494630; Fax: +1-402-4495252

Introduction

The antimetabolite drug raltitrexed (RTX, Tomudex, ZD1694) is a quinazoline folic acid analogue and a potent competitive inhibitor of thymidylate synthase (TS), the enzyme responsible for the de novo synthesis of the nucleotide thymidylate [1]. RTX has shown clinical activity, particularly against colorectal cancer, where it is as efficacious as standard fluorouracil plus leucovorin therapy [2].

Like that of other TS inhibitors, such as the prototype quinazoline antifolate CB3717, RTX cytotoxicity can be prevented by supraphysiologic concentrations (10 to 100 μ*M*) of the nucleoside thymidine. This is because extracellular thymidine is taken up by cells and converted to thymidylate by the salvage enzyme thymidine kinase [1], thereby providing a means of bypassing TS.

While a whole array of model systems have been derived for in vitro testing of antitumor drugs, arriving at a realistic estimate of the antitumor activity that could be expected from TS-directed drugs in vivo presents a special challenge because of the effect of thymidine on

their cytotoxicity. For example, the high thymidine levels in mice compared to humans (at least tenfold higher) have been shown to be sufficient to circumvent TS inhibition by CB3717 [3]. On the other hand, in vitro systems using dialyzed serum (in order to prevent salvage of thymidine present in undialyzed serum) or model tumors designed to be deficient in thymidine kinase [4] may present a falsely optimistic picture of TS-directed antimetabolite activity in humans. This is because thymidine salvage is completely absent in such systems. Although the physiologic serum concentrations of purine and pyrimidine nucleosides are known, they are rarely used in the in vitro analysis of antimetabolites.

Because of the possibility of tumor protection from drugs by salvage enzymes, Weber has suggested that antimetabolites targeting de novo pathways should be combined with salvage inhibitors [5]. Dipyridamole (DP, Persantine) is a nucleoside transport inhibitor in clinical use for its antiplatelet and vasodilator activities. DP potentiates the action of several cytotoxic drugs in vitro including the antimetabolites methotrexate [6], fluorouracil [7], and CB3717 [8]. Diverse agents potentiated by DP include: Adriamycin, daunomycin, vincristine [9]; vinblastine [10]; doxorubicin [11]; etoposide [12]; cisplatin [13]; and others. DP's ability to potentiate these drugs has been attributed to both inhibition of nucleoside uptake and blockage of the multidrug-resistance P-glycoprotein membrane pump [9].

As RTX is a relatively pure TS inhibitor whose activity is reversible by thymidine and DP efficiently blocks cellular thymidine uptake, its cytotoxicity should be enhanced by DP. However, a rational proposal to use this combination clinically should be based on data indicating the anticipated effect of clinically achievable levels of DP on the activity of RTX at thymidine levels approximating those found in humans. Here we examined the effects of various combinations of DP and physiologic thymidine concentrations on RTX cytotoxicity in different cell lines.

Materials and methods

Reagents

RTX (Tomudex) was provided by Zeneca (Wilmington, Del.). Fetal calf serum (FCS) and dialyzed fetal calf serum (dFCS) were from Gibco-BRL (Gaithersburg, Md.). RPMI-1640 culture medium was from Irvine (Santa Anna, Calif.). *l*-Folinic acid was generously provided by Dr. C. Paul Spears. Trypsin (0.05%)/EDTA-4Na (0.02%) without Ca^{++} and Mg^{++} was from JRH (Lenexa, Kan.). Methanol was from Fisher (Fair Lawn, N.J.) and ethanol was from Gold Shield (Hayward, Calif.). All other chemicals were purchased from Sigma (St. Louis, Mo.).

Colony formation assays

Cell culture was carried out in standard water-jacketed incubators at 37 °C in an atmosphere containing 5% CO_2. Colon adenocarcinoma cells were plated at 200 to 4000 cells per 60-mm culture dish (Corning, Grand Island, N.Y.) in 5.0 ml of medium with 10% v/v dFCS or FCS, and 24 h later treated with RTX for 4 h in the presence or absence of DP. A 4-h RTX exposure was used to approximate cellular exposure time from the 15-min intravenous infusion used clinically where a triphasic decline of plasma RTX concentration shows a mean β-phase half-life of 1.3 to 2.6 h [14]. Following each drug treatment cells were washed with Dulbecco's phosphate-buffered saline and the medium replaced with fresh medium. The medium was otherwise changed every 3 to 4 days with or without drug treatment. Control dishes were manipulated exactly as treated dishes. Added thymidine and/or folates were present during the entire experiment where indicated. Dishes were stained with 0.5% methylene blue w/v in methanol 10 to 14 days after all RTX or DP treatments were completed. Visible colonies were counted with a Lab-Line (Melrose Park, Ill.) model 1590 colony counter under 2× magnification. Percent survival was calculated as the ratio of the mean number of colonies formed in triplicate treatment dishes to the mean number of colonies in three or more control dishes.

MTS assays

Logarithmically growing HL60 S and SN3 suspension culture cells were seeded into 24-well culture plates (Becton Dickinson, Franklin Lakes, N.J.) at 100,000 cells/ml and exposed to RTX with or without DP for 24, 48, or 72 h. Cell viability was then measured in triplicate by spectrophotometric determination of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reduction using a CellTiter 96 AQ_{ueous} assay kit (Promega, Madison, Wis.).

Results

Raltitrexed cytotoxicity in colon tumor cell lines

RTX was potently cytotoxic against colon adenocarcinoma cell lines in medium containing dFCS. RTX at a concentration of 1 μ*M* resulted in approximately 1.0% survival or less in SW480, HT29, WiDr and RKO cells (Fig. 1). LoVo and Colo 205 cells were more sensitive with 1.0 μ*M* RTX resulting in less than 0.1% survival, while H630 and SW48 cells were relatively resistant to 1.0 μ*M* RTX showing about 55% and 6.0% survival, respectively (Fig. 1).

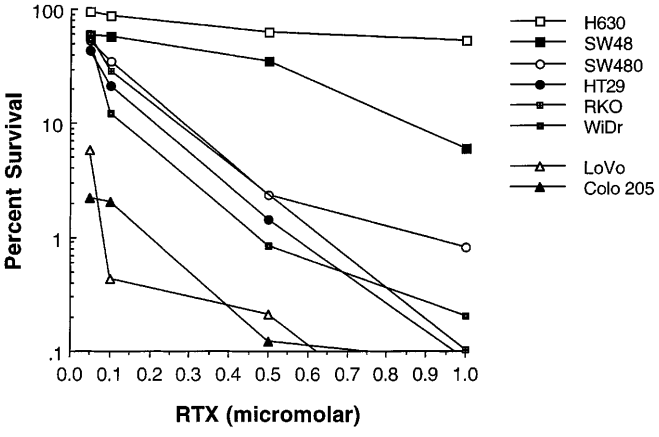

Fig. 1 Cytotoxicity of increasing concentrations of RTX in colon adenocarcinoma cell lines in medium supplemented with dFCS. Cytotoxicity is expressed as the average percent survival of control colonies formed after a 4-h RTX treatment. The mean of triplicate values from two experiments are shown

Thymidine modulation of raltitrexed cytotoxicity in WiDr cells

In medium containing dFCS and added thymidine at 50 n*M* or less, 1.0 μ*M* RTX produced substantial cytotoxicity (0.2% to 4.2% survival, Table 1). This cytotoxicity was partially negated by 100 n*M* thymidine (43.9% survival), nearly completely negated by 200 n*M* thymidine (80.6% survival) and obliterated in the presence of FCS (Table 1).

Dipyridamole potentiation of raltitrexed cytotoxicity

A 24-h exposure to DP concentrations of up to 5.0 μ*M* produced no appreciable toxicity in WiDr cells in medium containing FCS (93.3 ± 3.1% survival, Fig. 2). When 2.5 μ*M* DP was present during RTX treatment of WiDr cells in medium containing FCS and for a total of 24 h, RTX cytotoxicity was restored to that seen in medium containing dFCS without added thymidine (Table 1, Fig. 2). DP also enhanced RTX cytotoxic potency in the presence of physiologic thymidine concentrations of 50 to 200 n*M* (Fig. 3). Both RTX potency and potentiation by low-dose DP increased with decreasing thymidine concentrations. DP at a concentration of 2.5 μ*M* potentiated RTX (at 50 n*M*, 100 n*M* or 1.0 μ*M*) by at least twofold even in medium containing dFCS without added thymidine in six of the eight cell lines tested (Table 2). Among the eight cell lines the average RTX potentiation from 2.5 μ*M* DP was about 3.6-fold with a range of zero (0.9-fold) to 16.3-fold (Table 2). $p53^-$ HL60 S promyelocytic leukemia cells, which do not express functional p53, were resistant to RTX cytotoxicity compared to $p53^+$ HL60 SN3 cells, which express transfected functional p53 [15] (Fig. 4). DP at a concentration of 2.5 μ*M* potentiated RTX cytotoxicity in HL60 S and HL60 SN3 cells both in the presence of 200 n*M* thymidine and in its absence (Fig. 4).

Time course of the dipyridamole effect

Treatment of WiDr cells with 50 n*M* DP continuously for 72 h after initiation of RTX exposure resulted in 98% of maximal cell kill. The maximal effect (0% survival) occurred with between 5 and 10 days of DP exposure (Fig. 5).

Table 1 Cytotoxicity of RTX with various thymidine concentrations. WiDr cells were treated with 1.0 μ*M* RTX for 4 h and cultured in medium containing 10.0% dFCS alone or plus various concentrations of thymidine, or 10.0% FCS alone. The mean ± SEM of triplicate values from separate experiments (*n*) are shown

Medium supplement	Survival (%)
dFCS alone	0.2 ± 0.08 (*n* = 3)
dFCS + 50 n*M* thymidine	4.2 ± 1.0 (*n* = 3)
dFCS + 100 n*M* thymidine	43.9 ± 11.5 (*n* = 3)
dFCS + 200 n*M* thymidine	80.6 ± 6.4 (*n* = 5)
dFCS + 500 n*M* thymidine	105.5 (*n* = 1)
FCS alone	97.2 ± 1.6 (*n* = 2)

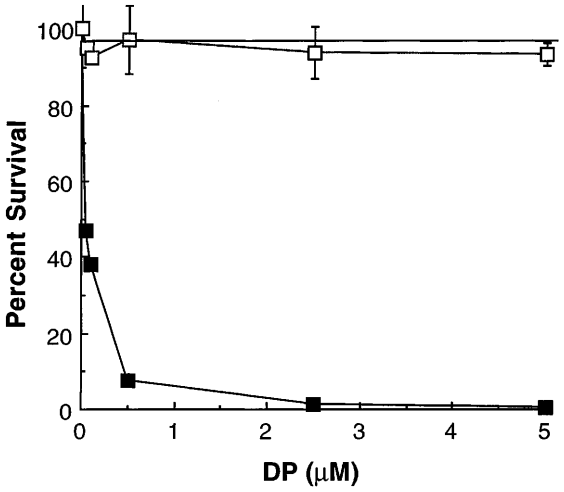

Fig. 2 DP potentiation of RTX cytotoxicity in medium containing FCS. WiDr cells were treated with DP (24 h) in medium containing non-dialyzed FCS alone (□) or with a 1 μ*M* RTX (4 h) cotreatment (■). The *solid line* indicates 1 μ*M* RTX alone. The mean ± SEM of triplicate values from two experiments are shown

Effect of concomitant versus sequential dipyridamole cotreatment

In WiDr cells the cytotoxicity of 50 n*M* RTX with concomitant DP cotreatment (10 n*M* to 2.5 μ*M*) essentially did not differ from sequential DP treatment occurring just after RTX exposure (Table 3).

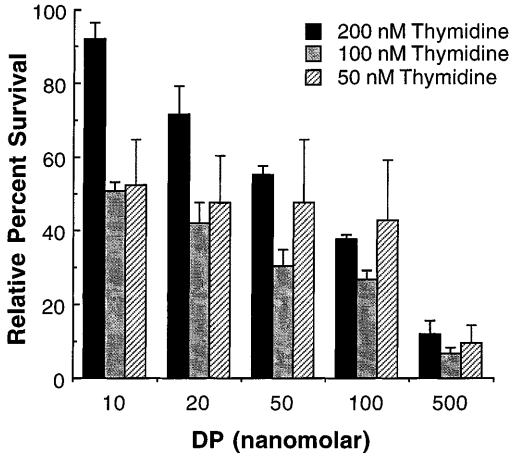

Fig. 3 RTX potentiation in the presence of various concentrations of thymidine and DP. WiDr cells were treated with 1.0 μ*M* RTX (4 h) in the presence of various concentrations of thymidine with and without various concentrations of DP (24 h). The values shown are the mean ± SEM percent survival from two experiments relative to RTX without DP. RTX alone produced 92.2 ± 4.2% survival in the presence of 200 n*M* thymidine, 49.2 ± 8.0% survival in the presence of 100 n*M* thymidine and 4.5 ± 1.7% survival in the presence of 50 n*M* thymidine

Table 2 Potentiation of RTX cytotoxicity by DP in colon adenocarcinoma cell lines. Cells were exposed to RTX (4 h) in medium containing 10% dFCS with or without 2.5 μ*M* DP (24 h). Average values from 2 or more experiments are shown. The percent survival of DP alone ranged from 72.1 to 112.6% for all cell lines, mean = 92.5 ± 3.8 (n = 10)

Cell line	Survival (%)								
	50 n*M* RTX			100 n*M* RTX			1.0 μ*M* RTX		
	Without DP	With DP	Fold potentiation	Without DP	With DP	Fold potentiation	Without DP	With DP	Fold potentiation
WiDr	27.4	6.1	4.5	12.8	3.1	4.2	–	–	–
HT29	43.7	12.5	3.5	21.3	4.9	4.3	–	–	–
RKO	9.7	3.5	2.8	25.7	7.6	3.4	–	–	–
Colo 205	2.2	2.6	0.9	2.0	1.4	1.4	–	–	–
SW480	53.5	45.3	1.2	35.4	32.0	1.1	–	–	–
SW48	59.1	30.0	2.0	57.9	31.3	1.9	–	–	–
LoVo	5.9	0.4	16.3	0.4	0.1	3.1	–	–	–
H630	–	–	–	–	–	–	53.8	15.14	3.5

Effects of exogenous folates on dipyridamole potentiation of raltitrexed

In WiDr cells folic acid led to partial reversal of RTX cytotoxicity (3.2-fold reduction), and to a lesser degree, DP potentiation of RTX cytotoxicity (2.3-fold reduction), whereas folinic acid produced full reversal of both RTX and DP-potentiated RTX cytotoxicity (Table 4).

Discussion

In this study, we sought to determine: (a) the extent to which thymidine concentrations approximating those found in human serum would obviate the activity of the TS-directed antifolate RTX; and (b) the extent to which clinically achievable concentrations of DP could reverse the negative effects of thymidine on RTX activity. Reported mean human serum thymidine concentrations range from 130 to 360 n*M* in normal subjects and 160 to 200 n*M* in cancer patients, and individual values range from less than 40 to 870 n*M* [16, 17]. As shown in Table 1, a thymidine concentration of 100 n*M*, which is in the low to mid portion of the range found in humans, was sufficient to increase cell survival in the presence of

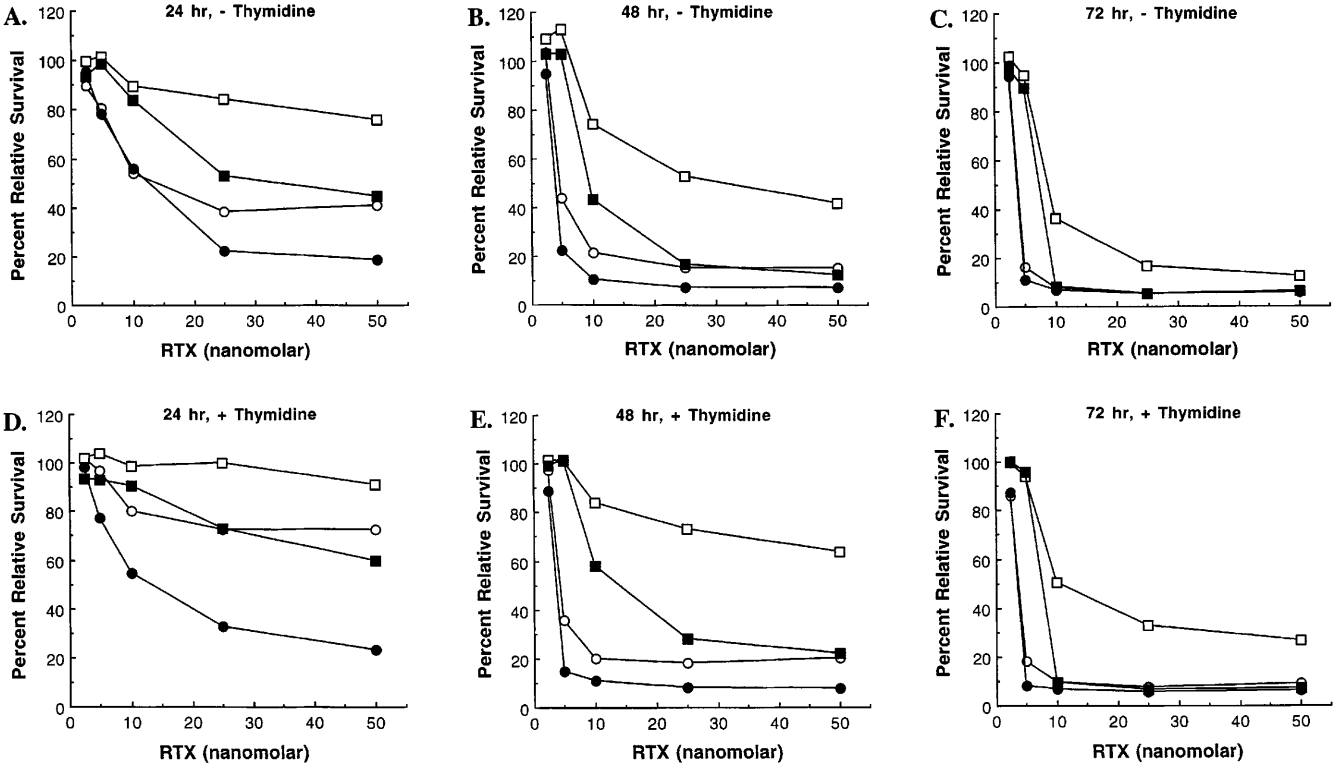

Fig. 4A–F RTX cytotoxicity in p53$^-$ and p53$^+$ HL60 cells in the absence (**A–C**) and presence (**D–F**) of 200 n*M* thymidine, with and without 2.5 μ*M* DP (□ HL60 S cells without DP, ■ HL60 S cells with DP, ○ HL60 SN3 cells without DP, ● HL60 SN3 cells with DP). The mean of triplicate values are shown

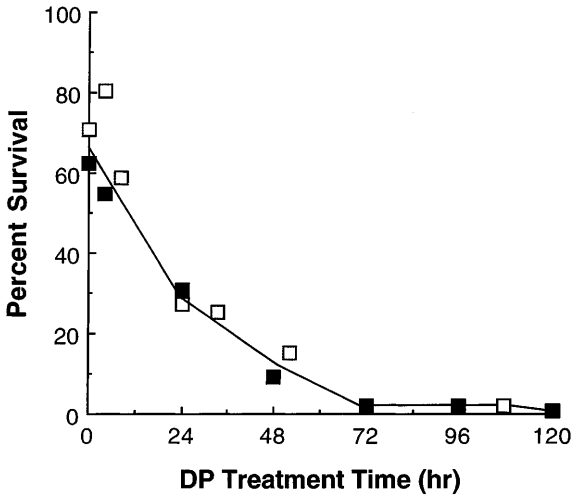

Fig. 5 Time-course of the effect of DP on RTX cytotoxicity. WiDr cells were treated with 1.0 μ*M* RTX and 50 n*M* DP for 4 h in medium supplemented with dFCS and 200 n*M* thymidine. Identical medium containing 50 n*M* DP without RTX was replaced and incubation continued for various lengths of time. The means of triplicate values from two experiments are shown (□, ■)

Table 3 Potentiation of RTX cytotoxicity by concomitant vs. sequential DP treatment. WiDr cells were treated with 50 n*M* RTX for 4 h in medium containing 10% dFCS with or without 200 n*M* thymidine. DP was added during RTX treatment (*concomitant*) or just following RTX treatment (*sequential*) and continued for 72 h

Treatment	Fold potentiation	
	Concomitant	Sequential
RTX + 10 n*M* DP	1.6	2.0
RTX + 20 n*M* DP	2.1	2.0
RTX + 2.5 μ*M* DP	2.8	3.2
RTX + 2.5 μ*M* DP + 200 n*M* thymidine	3.2	3.8

1.0 μ*M* RTX from near zero without added thymidine to almost 50%. As thymidine concentrations increased toward the high end of the human serum range, cells were completely protected against RTX (Table 1). These results indicate that thymidine levels found in humans can influence the activity of RTX, and, considering the previously observed variability of human serum thymidine concentrations, suggest that in order to predict the efficacy of TS-directed agents such as RTX, serum or tumor thymidine levels need to be taken into account along with tumor TS levels. This may be more important with pure TS inhibitors such as RTX, in contrast to fluoropyrimidines, for example, since fluorouracil's toxicity has also been partially attributed to direct effects on RNA [18].

Previous studies have shown that TS expression levels are important in determining response to fluorouracil in gastrointestinal cancers [19]. Tumors with high TS expression have been found to be resistant to fluorouracil-based therapy, but many tumors with low TS levels, which would normally be a favorable condition for response, are also nonresponsive. The findings of this study suggest the possibility that some fraction of tumors with low TS levels do not respond to fluorouracil because those patients have sufficiently high thymidine levels to decrease the effectiveness of TS inhibition. Moreover, it has been shown that RTX treatment itself can lead to increased thymidine kinase activity and nucleoside transporter expression in vitro [20], thereby increasing the activity of the salvage pathway.

Clinically achievable free DP plasma levels are limited by extensive serum protein binding, largely to α_1-acid glycoprotein, the levels of which vary in cancer patients and are a potentially important determinant of obtainable free DP concentrations [21]. Hendrix et al. [22] reported free DP peak plasma concentrations of 18.4 to 30.8 n*M* (mean 24.1 n*M*) from 450 mg DP divided into six oral doses daily, concentrations similar to steady-state free DP plasma levels (mean 27.8 n*M*) obtainable by continuous intravenous infusion [23]. To address the question as to whether clinically achievable free DP levels could be expected to alter the effect of thymidine on RTX activity in vivo, we measured the effect of DP on restoration of RTX sensitivity of cells at various DP doses. In culture medium supplemented with 10% FCS, 75–100% of DP is non-protein bound, i.e. free [24]. DP potentiated RTX in the presence of physiologic thymidine concentrations from less than 50 n*M* to 200 n*M* or more[1] (Figs. 2–5). This potentiation was quite sensitive to DP, being observable even at 10 n*M* (Fig. 3). Thymidine concentrations in serum may underestimate those found in areas of high cell turnover, such as tumor margins. Conversely, a large tumor burden of actively dividing cells with a large salvage capacity could deplete local thymidine in a manner similar to a large number of proliferating cells in culture [3]. Nevertheless, our data show that 20 to 30 n*M* DP could be expected to potentiate the activity of RTX at thymidine concentrations up to 100–200 n*M*, which is within the range of thymidine levels found in humans.

Higher free DP levels capable of potentiating RTX at higher thymidine concentrations may be obtained via localized infusions. Intraperitoneal infusion of DP and methotrexate has been used to treat advanced ovarian and gastrointestinal cancers predominantly confined to the peritoneal cavity with which a mean steady-state free DP concentration of 11.7 μ*M* has been achieved [25]. A combination of RTX and DP as a localized infusion may prove effective for compartmentalized disease such as intraperitoneal carcinomatosis, primary bladder tumors, or intrathecal or paraventricular neurological tumors. Kelland et al. [26] found no cross-resistance between RTX and platinum-based drugs in RTX- and cisplatin-resistant cell lines and suggested these drugs be considered for combination chemotherapy. Since DP enhances cisplatin activity, a regimen including DP with RTX and a platinum-based drug should be highly potent.

[1] We estimate that RPMI-1640 medium supplemented with 10% FCS contains 300 to 500 n*M* thymidine

Table 4 Effects of folic acid and folinic acid on RTX cytotoxicity and potentiation by DP. WiDr cells were treated with RTX (4 h) alone or with DP (24 h) with or without added folic or folinic acid. In the folic and folinic acid controls the survival was 111.8 ± 6.9% (n = 3) and 118.6 ± 0.9% (n = 2), respectively. The mean ± SEM of the values from two experiments are shown

Treatment	Survival (%)
50 nM RTX	14.0 ± 0.8 (n = 6)
50 nM RTX + 2.5 μM DP	4.3 ± 0.4 (n = 5)
50 nM RTX + 25 μM folic acid	44.3 ± 2.6 (n = 4)
50 nM RTX + 25 μM folic acid + 2.5 μM DP	9.7 ± 0.8 (n = 3)
50 nM RTX + 10 μM folinic acid	103.5 ± 8.0 (n = 5)
50 nM RTX + 10 μM folinic acid + 2.5 μM DP	98.9 ± 7.3 (n = 4)

The potentiation of RTX by DP was highly time-dependent (Fig. 5). The time-course for the sensitization effect of DP is similar to that of "thymineless death" produced by RTX against WiDr cells reported previously in medium containing dFCS [27] and represents the time-course of irreversible cytotoxicity resulting from interruption of thymidylate synthesis and thymidine salvage. The lack of substantial difference between concomitant or sequential RTX-DP cotreatment (Table 3) suggests that DP does not significantly interfere with RTX metabolism or uptake. Clinical DP loading could therefore occur prior to or during RTX exposure. Drug administration sequence is important with other compounds potentiated by DP, e.g. cisplatin, cytosine arabinoside, or tiazofurin [13, 28, 29], and would have to be considered in multiple drug regimens including those agents.

Interestingly, we found that DP potentiated RTX even in the absence of exogenous thymidine in six of the eight colon adenocarcinoma cell lines and in HL60 promyelocytic leukemia cells (Tables 2, 3; Fig. 4A–C). This suggests a second non-thymidine salvage-dependent mechanism of action active in most, but not all, of the colon adenocarcinoma cell lines. DP restored the RTX sensitivity of $p53^-$ HL60 S cells to that of $p53^+$ HL60 SN3 cells in the presence or absence of added thymidine (Fig. 4). In fact, the ratios of cytotoxicities of RTX + DP over RTX alone were greater for $p53^-$ cells, i.e. potentiation was greater, indicating a possible selectivity of DP-RTX potentiation for $p53^-$ cells.

The data in Table 3 show that both folic acid and folinic acid protected cells against RTX, but the protection by folic acid was reversed by DP whereas that of folinic acid was not. Although a direct effect of DP on folic acid transport remains to be shown, these observations tend to indicate that the mechanism of the non-salvage potentiation effect of DP may involve decreased intracellular folate pools due to inhibition of folic acid uptake via the membrane folate binding protein. Lower levels of folic acid metabolites in cells would be expected to enhance the efficiency of RTX polyglutamation and binding to TS.

Serum levels of thymidine normally found in humans can substantially decrease the antitumor activity of RTX, thereby attesting to the importance of inhibiting thymidine salvage for optimal therapeutic use of this drug. The nucleoside transport inhibitor DP at its clinically achievable plasma levels can be expected to resensitize tumors to RTX in a subset of patients with thymidine levels toward the low to mid range found in humans. In the presence of higher thymidine levels a DP analogue such as BIBW 22, which is reportedly sevenfold more potent than DP at blocking nucleoside transport [30], may be more effective. Salvage might be further inhibited by the addition of the thymidine kinase inhibitor azidothymidine (AZT) to an RTX/DP regimen. In vitro AZT potentiates fluorouracil plus DP and enhances RTX cytotoxicity [31, 32]. The addition of RTX/DP to AZT might be effective against AIDS-related neoplasms or enhance inhibition of viral replication [22]. However, as with all cytotoxic therapies in AIDS, bone marrow suppression could be problematic.

Clinical trials of combination therapy consisting of RTX, DP (or a DP analogue) and a third agent potentiated by DP, such as cisplatin or vinblastine, appear warranted, particularly against refractory malignancies such as colorectal, gastric, pancreatic, and non-small-cell lung cancers where in vivo DP potentiation of fluorouracil-based chemotherapy has been clinically suggested [33–37]. Studies comparing patient serum thymidine levels to clinical response to RTX, RTX plus a DP-like agent, or even fluorouracil, may also be warranted, since patients identified as having high thymidine levels may respond more favorably to alternate, non-TS-directed therapies.

Acknowledgements This work was supported in part by grant R01 CA60859 from the National Cancer Institute. We thank Dr. L. David Ormerod and Dr. John W. Bishop for critical review of this manuscript.

References

1. Jackman AL, Farrugia DC, Gibson W, Kimbell R, Harrap KR, Stephens TC, Azab M, Boyle FT (1995) ZD1694 (Tomudex): a new thymidylate synthase inhibitor with activity in colorectal cancer. Eur J Cancer 31A: 1277–1282
2. Cunningham D, Zalcberg JR, Rath U, Olver I, van Cutsem E, Svensson C, Seitz JF, Harper P, Kerr D, Perez-Manga G, Tomudex Colorectal Cancer Study Group (1996) Final results of a randomised trial comparing 'Tomudex' (raltitrexed) with 5-fluorouracil plus leucovorin in advanced colorectal cancer. Ann Oncol 7: 961–965
3. Jackman AL, Taylor GA, Calvert AH, Harrap KR (1984) Modulation of anti-metabolite effects. Effects of thymidine on the efficacy of the quinazoline-based thymidylate synthetase inhibitor, CB3717. Biochem Pharmacol 33: 3269–3275
4. Radparvar S, Houghton PJ, Germain G, Pennington J, Rahman A, Houghton JA (1990) Cellular pharmacology of

5-fluorouracil in a human colon adenocarcinoma cell line selected for thymidine kinase deficiency. Biochem Pharmacol 39: 1759–1765
5. Weber G (1983) Biochemical strategy of cancer cells and the design of chemotherapy. In: Clowes GHA (ed) Memorial lecture. Cancer Res 43: 3466–3492
6. Chan TCK, Howell SB (1990) Role of hypoxanthine and thymidine in determining methotrexate plus dipyridamole cytotoxicity. Eur J Cancer 26: 907–911
7. Grem JL, Fischer PH (1985) Augmentation of 5-fluorouracil cytotoxicity in human colon cancer cells by dipyridamole. Cancer Res 45: 2967–2972
8. Curtin NJ, Harris AL, Aherne GW (1991) Mechanism of cell death following thymidylate synthase inhibition: 2′-deoxyuridine-5′-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and dipyridamole. Cancer Res 51: 2346–2352
9. Asoh K-I, Saburi Y, Sato S-I, Nogae I, Kohno K, Kuwano M (1989) Potentiation of some anticancer agents by dipyridamole against drug-sensitive and drug-resistant cancer cell lines. Jpn J Cancer Res 40: 475–481
10. Shalinsky DR, Andreeff M, Howell SB (1990) Modulation of drug sensitivity by dipyridamole in multidrug resistant tumor cells in vitro. Cancer Res 50: 7537–7543
11. Yung BY, Chang FJ, Luo KJ (1991) Dipyridamole enhancement of doxorubicin-induced translocation of nucleophosmin and inhibition of cell growth in HL-60 cells. Int J Cancer 49: 592–597
12. Turner RN, Curtin NJ (1996) Dipyridamole increases VP16 growth inhibition, accumulation and retention in parental and multidrug-resistant CHO cells. Br J Cancer 73: 856–860
13. Barberi-Heyob M, Griffon G, Merlin JL, Weber B (1993) Sequence-dependent growth-inhibitory effects of the in vitro combination of fluorouracil, cisplatin, and dipyridamole. Cancer Chemother Pharmacol 33: 163–170
14. Clarke SJ, Ward J, de Boer M, Planting A, Verweij J, Sutcliffe F, Azab M, Judson IR (1994) Phase I study of the new thymidylate synthase inhibitor Tomudex (ZD1694) in patients with advanced malignancy. Ann Oncol 5 [Suppl 5]: A240
15. Banerjee D, Lenz H-J, Schnieders B, Manno DJ, Ju JF, Spears CP, Hochhauser D, Danenberg K, Danenberg P, Bertino JR (1995) Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. Cell Growth Differ 6: 1405–1413
16. Holden L, Hoffbrand AV, Tattersall MHN (1980) Thymidine concentrations in human sera: variations in patients with leukemia and megaloblastic anemia. Eur J Cancer 16: 115–121
17. Howell SB, Mansfield SJ, Taetle R (1981) Thymidine and hypoxanthine requirements of normal and malignant human cells for protection against methotrexate cytotoxicity. Cancer Res 41: 945–950
18. Ghoshal K, Jacob ST (1997) An alternate molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. Biochem Pharmacol 53: 1569–1575
19. Johnston PG, Lenz H-J, Danenberg KD, Leichman CG, Allegra CJ, Danenberg PV, Leichman L (1995) Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 55: 1407–1412
20. Pressacco J, Mitrovski B, Erlichman C, Hedley DW (1995) Effects of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. Cancer Res 55: 1505–1508
21. Curtin NJ, Harris AL (1988) Potentiation of quinazoline antifolate (CB3717) toxicity by dipyridamole in human lung carcinoma, A549, cells. Biochem Pharmacol 37: 2113–2120
22. Hendrix CW, Flexner C, Szebeni J, Kuwahara S, Pennypacker S, Weinstein JN, Lietman PS (1994) Effect of dipyridamole on zidovudine pharmacokinetics and short-term tolerance in asymptomatic human immunodeficiency virus-infected subjects. Antimicrob Agents Chemother 38: 1036–1040
23. Wilson JK, Fischer PH, Tutsch K, Alberti D, Simon K, Hamilton RD, Bruggink J, Koeller JM, Tormey DC, Earhart RH, Ranhosky A, Trump DL (1988) Phase I clinical trial of a combination of dipyridamole and acivicin based upon inhibition of nucleoside salvage. Cancer Res 48: 5585–5590
24. Szebeni J, Weinstein JN (1991) Dipyridamole binding to proteins in human plasma and tissue culture media. J Lab Clin Med 117: 485–492
25. Goel R, Cleary SM, Horton C, Balis FM, Zimm S, Kirmani S, Howell SB (1989) Selective intraperitoneal biochemical modulation of methotrexate by dipyridamole. J Clin Oncol 7: 262–269
26. Kelland LR, Kimbell R, Hardcastle A, Aherne GW, Jackman AL (1995) Relationships between resistance to cisplatin and antifolates in sensitive and resistant tumour cell lines. Eur J Cancer 31A: 981–986
27. Smith SG, Lehman NL, Moran RG (1993) Cytotoxicity of antifolate inhibitors of thymidylate and purine synthesis to WiDr colonic carcinoma cells. Cancer Res 53: 5697–5706
28. Yang JL, White JC, Capizzi RL (1990) Enhanced retention of cytosine arabinoside and its metabolites and synergistic cytotoxicity by sequential treatment with dipyridamole in L5178Y leukemia. Cancer Chemother Pharmacol 26: 135–138
29. Jayaram HN, Murayama K, Pillwein K, Zhen W, Weber G (1992) Schedule-dependent synergistic action of tiazofurin and dipyridamole on hepatoma 392 A cells. Cancer Chemother Pharmacol 31: 93–96
30. Chen H-X, Bamberger U, Heckel A, Guo X, Cheng Y-C (1993) BIBW 22, a dipyridamole analogue, acts as a bifunctional modulator on tumor cells by influencing both P-glycoprotein and nucleotide transport. Cancer Res 53: 1974–1977
31. Zhen Y-S, Taniki T, Weber G (1992) Azidothymidine and dipyridamole as biochemical modifiers: synergism with methotrexate and 5-fluorouracil in human colon and pancreatic carcinoma cells. Oncol Res 4: 73–78
32. Pressacco J, Erlichman C (1993) Combination studies with 3′-azido-deoxythymidine (AZT) plus ICI D1694. Cytotoxic and biochemical effects. Biochem Pharmacol 46: 1989–1997
33. Isacoff WH, Eilber F, Jacobs AD, Taylor O (1994) A phase II trial of continuous infusion (CI) 5-fluorouracil (5-FU), calcium leucovorin (LV), mitomycin-C (Mito-C), and dipyridamole (D) in patients with advanced colon cancer. Proc Am Soc Clin Oncol 13: A651
34. Isacoff WH, Reber H, Tompkins R, Zinner MJ, Brody M, Quilici P, Taylor O (1995) Continuous infusion (CI) 5-fluorouracil (5-FU), calcium leucovorin (LV), mitomycin-C (MC), and dipyridamole (D); treatment for patients with locally advanced pancreatic cancer. Proc Am Soc Clin Oncol 14: A471
35. Mitsugi K, Yasutake T, Etoh T, Ishimaru T, Ueda A, Nakano S, Niho Y (1993) Successful treatment of recurrent multiple lung metastasis from colon cancer with combination chemotherapy using methotrexate, 5-fluorouracil, and high-dose leucovorin: a case report. Jpn J Cancer Chemother 20: 2387–2390
36. Nomura M, Nakano S, Kudou J, Ishibashi O, Niho Y (1990) Successful treatment of advanced gastric cancer with multiple liver metastasis by combination chemotherapy using mitomycin C, 5-fluorouracil, and high-dose leucovorin: a case report. Jpn J Cancer Chemother 17: 2097–2100
37. Sugimori H, Nakano S, Chifu Y, Mitsugi K, Kudo J, Ishida T, Niho Y (1993) 5-Fluorouracil + low-dose leucovorin and cisplatin sequential chemotherapy with dipyridamole for advanced nonresectable squamous cell carcinoma of the lung: a case report. Jpn J Cancer Chemother 20: 287–290