The mechanism of action of 8-HQ has not been explored. However, possible targets for its activity as a chelating agent might be mitochondrial cytochromes as well as ribonucleotide reductase. Our results with the *in vivo* anti-leukaemic activity found for 8-HQ derivatives [7], suggest that hyroxyquinoline derivatives should be further evaluated for their antitumour activities.

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Role of Hypoxanthine and Thymidine in Determining Methotrexate plus Dipyridamole Cytotoxicity

Thomas C.K. Chan and Stephen B. Howell

The nucleoside transport inhibitor dipyridamole can potentiate the cytotoxicity of methotrexate by a mechanism that was thought to be related to the inhibition of thymidine salvage. In human ovarian carcinoma cells thymidine only partly reversed the *in vitro* cytotoxicity of methotrexate plus dipyridamole at sub-millimolar concentrations, above which the cytotoxicity of thymidine itself became evident. Hypoxathine with thymidine, or hypoxanthine alone at a higher concentration, completely reversed methotrexate and methotrexate plus dipyridamole cytotoxicity. The effects of dipyridamole on cellular cyclic adenosine monophosphate (cAMP) levels and on ^{3}H-methotrexate efflux in 2008 cells were examined. At 10 μmol/l, dipyridamole did not alter cAMP content or methotrexate influx in ovarian carcinoma cells, but reduced the rate of efflux of ^{3}H-methotrexate by 25%. In Chinese hamster ovary cells and their folylpolyglutamyl synthase-deficient variant AUX B1, the reduced methotrexate efflux by dipyridamole was not due to increased polyglutamation, since increased retention was observed in both cell lines. The data support the hypothesis that dipyridamole potentiated the activity of methotrexate by inhibiting the salvage of hypoxanthine, and to a lesser extent, that of thymidine. The ability of dipyridamole to increase the cellular retention of methotrexate was probably a non-specific action of dipyridamole on the cell membrane, and may have a role in the observed synergy.
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INTRODUCTION

THE ABILITY to salvage preformed metabolites by tumour cells may be an important determinant of tumour sensitivity to antimetabolite chemotherapy [1–4]. We and others have demonstrated that dipyridamole, a potent membrane nucleoside transport inhibitor, can potentiate the anti-tumour activity of both purine and pyrimidine antimetabolites [5–9]. Although the effects of anti-metabolite potentiation can be demonstrated easily in cytotoxicity assays and in animal models, the mechanisms by which dipyridamole acts are not clearly known. In addition to its ability to inhibit nucleoside transport across the plasma membrane, dipyridamole inhibits phosphodiesterase activity

[10] which can elevate cellular cyclic adenosine monophosphate (cAMP) in cultured tumour cells [11]. Methotrexate cytotoxicity is generally thought of as the result of inhibition of dihydrofolate reductase, leading to purine and possibly pyrimidine depletion [12]. Although dipyridamole augmented the cytotoxicity of methotrexate by preventing thymidine salvage in Chinese hamster ovary (CHO) cells [7] and in human colon cancer cells [13], there are also reports of dipyridamole increasing both methotrexate retention [14] and its polyglutamate synthesis [15]. The different mechanisms reported by these studies can be partly explained by the concentration of dipyridamole used, with the higher concentrations altering drug retention and metabolism and the lower concentrations inhibiting nucleoside salvage activity. The relative contribution of each of these mechanisms on cytotoxicity, however, has not been defined in a single tumour model.

We have investigated the mechanism of cytotoxicity of methotrexate and the contribution of the different mechanisms of synergy between dipyridamole and methotrexate in a human ovarian carcinoma cell line.

MATERIALS AND METHODS

Drugs and chemicals

Dipyridamole was supplied by Boehringer Ingelheim. Methotrexate, fetal bovine serum (FBS), phosphate-buffered saline (PBS) and all nucleosides and nucleotides were purchased from Sigma. (8-^{3}H)-hypoxanthine and (methyl-^{3}H)-thymidine were purchased from ICN Radiochemicals and (L-glutamyl-3,4-^{3}H)-methotrexate was purchased from New England Nuclear. All other chemicals and the scintillation cocktail ('BioHP') were purchased from Fisher Scientific.

Cell culture and cytotoxicity assays

Human ovarian carcinoma cells (2008) were maintained in exponential growth in 'RPMI 1640' supplemented with 10% FBS and 1% L-glutamine in a humidified incubator at 37°C in a 5% CO_2 atmosphere. CHO cells and a folylpolyglutamyl synthase-deficient mutant (AUX B1) were cultured in 'MEM-alpha' with deoxyribonucleosides and 10% FBS under the same incubation conditions.

Synergy between methotrexate and dipyridamole in 2008 cells was tested with clonogenic cells in which exponentially dividing cells were harvested by trypsinization, washed, resuspended in fresh medium and plated in triplicate onto 60 mm plastic culture dishes (Falcon) at 300 cells per plate. Varying concentrations of methotrexate and dipyridamole were added to the plates with the control cultures receiving the same volume of normal saline. The plates were then incubated under 7% CO_2 at 37°C for 10–14 days. Cells were washed once with PBS, fixed in methanol and stained with Giemsa. Each cluster of greater than 50 cells was counted as one colony, and the control plates usually had 100–120 colonies with less than 5% variation in triplicates [6]. In the CHO and AUX-B1 cells, methotrexate and dipyridamole synergy was tested with a 72 h growth rate assay [6]. The results were expressed as a percentage of the growth rate in untreated cells.

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Methotrexate uptake and retention in cultured cells

Exponentially growing cells were harvested, washed and resuspended in their respective growth media containing 100 μmol/l methotrexate (1856×10^3 Bq/ml) in the presence and absence of dipyridamole (1 and 10 μmol/l). Equal portions of cells were removed at timed intervals for up to 1 h, washed twice in chilled PBS and the intracellular radioactivity measured by digesting the cells in 0.1 mol/l NaOH followed by liquid scintillation counting. For radiolabelled methotrexate retention studies, cells were similarly incubated. At the end of the 1 h incubation period, cells were washed twice in chilled PBS and replated into growth medium containing no methotrexate but the same concentrations of dipyridamole during the incubation periods. Equal portions of cells were removed from the different treatment groups, washed twice in chilled PBS, digested and the intracellular radioactivity measured.

Changes in cellular cAMP pool

Exponentially growing cells were harvested and resuspended at 10^7/ml in growth media containing varying concentrations of dipyridamole, 500 μmol/l dibutyryl-cAMP (positive control) or equal volumes of PBS. 0.5 ml was removed at selected times and rapidly dispersed into tubes containing chilled 0.8 mol/l perchloric acid and left on ice for 10 min after vortexing. The precipitates were removed by centrifugation and the supernatants were neutralized with 2.2 mol/l potassium bicarbonate. A second centrifugation for 5 min at 2000 *g* removed all particulate matter and measured volumes of the clear supernatants were injected into a high-performance liquid chromatography (HPLC) system. Cell viability at the end of the experiment was checked by trypan blue staining and haemocytometer counting.

The HPLC system used was a Waters 6000A pump, a Waters 490 variable wavelength multichannel ultraviolet detector set at 254 nm, 280 nm, and 254/280 ratioplot, a Waters Z-module fitted with a C-18 μ 'Bondapak' cartridge with a guard column of the same packing material. The isocratic mobile phase was 0.2 mol/l $NH_4H_2PO_4$ pH 3.0 and 5% methanol eluting at 2.0 ml/min. The typical retention time for cAMP was 14 min, and peak identity was confirmed by co-elution with a purified standard [16]. Cellular cAMP contents were calculated from integrated peak areas and extrapolation from standard curves, and were normalized to mol/10^6 cells.

Nucleoside and nucleobase transport studies

Nucleoside and nucleobase transport (zero-trans = transport of nucleosides into cells within the first 60s of exposure and before significant intracellular metabolism has occurred) into 2008 cells was measured by a modified oil-stop method. 100 μl of uptake medium containing radiolabelled nucleosides or nucleobases was layered onto 100 μl of an oil mixture of nine parts of silcone oil (Aldrich 17563-3) to one part of paraffin oil (Fisher 0121-1) in a 1.5 ml Eppendorf microcentrifuge tube. Transport measurements were started by the addition of 100 μl cell suspension and the reactions were stopped at timed intervals between 0 and 60 s by pelleting the cells through the oil cushion at 12 000 *g* for 30 s. The oil was then aspirated and the cell pellet digested with 0.1 mol/l NaOH and the radioactivity measured [17]. The radioactivity in the cell pellet not associated with dipyridamole inhibitable transport was estimated by pre-incubating cells in medium containing 10 μmol/l dipyridamole and starting the reaction with the transport inhibitor present.

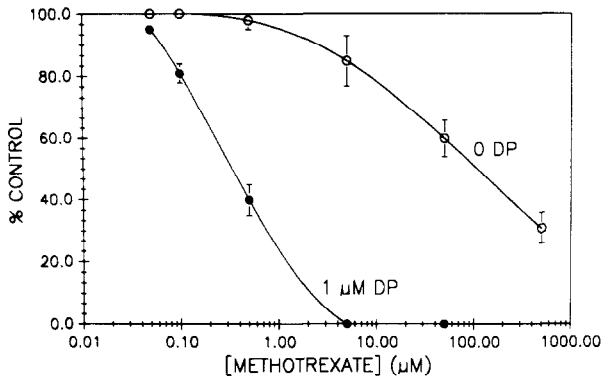

Fig. 1. Clonal survival of 2008 cells exposed to varying concentrations of methotrexate in the absence and presence of 1 μmol/l dipyridamole (Dp). Mean (S.D.) of five experiments.

RESULTS

Methotrexate and dipyridamole synergy

Methotrexate alone was moderately cytotoxic to 2008 cells in clonogenic assays with a mean IC_{50} of 102 (S.D. 48) μmol/l. Dipyridamole at 1 μmol/l augmented the cytotoxicity of methotrexate by reducing the IC_{50} to 0.2 (± 0.15) μmol/l and achieved total cell kill (0 colony) at 5 μmol/l (Fig. 1). Dipyridamole alone was minimally cytotoxic, the reduction of colony count was 4% and 9% for 1 and 10 μmol/l, respectively.

These IC_{50} values were used in the testing of the ability of endogenous nucleosides and nucleobases to antagonize the toxicity of methotrexate alone and in combination with dipyridamole. Table 1 summarizes our results from a complicated series of experiments. In cells exposed to methotrexate alone, the inclusion of thymidine at 100 μmol/l partly antagonized methotrexate cytotoxicity. Higher concentrations of thymidine (500 μmol/l) reduced colony counts, probably because its ability to protect cells from methotrexate was offset by the cytotoxicity of this nucleoside at such a high concentration (25% reduction of colony count). The combination of thymidine and hypoxanthine (100 μmol/l) completely antagonized the cytotoxicity of methotrexate in these cells. Interestingly, hypoxanthine alone demonstrated a concentration-dependent protection of cells from methotrexate, with complete antagonism observed at 500 μmol/l. Exposure to hypoxanthine alone up to a concentration of 1 mmol/l was not cytotoxic.

Table 1. Ability of thymidine and hypoxanthine to protect 2008 cells from methotrexate plus dipyridamole cytotoxicity

Treatments	Drug concentrations (μmol/l) MTX*	DP	HYP	THY	% Control (n = 4)
Control	0	0	0	0	100
MTX alone	200	0	0	0	40 (7)†
MTX/THY	200	0	0	100	83 (9)
MTX/THY	200	0	0	500	65 (12)
MTX/HYP	200	0	100	0	87 (8)
MTX/HYP	200	0	500	0	101 (5)
MTX/HYP/THY	200	0	100	100	99 (4)
DP	0	1	0	0	96 (2)
MTX/DP	0.2	1	0	0	49 (6)
MTX/DP/THY	0.2	1	0	100	52 (7)
MTX/DP/THY	0.2	1	0	500	30 (5)
MTX/DP/HYP	0.2	1	100	0	71 (9)
MTX/DP/HYP	0.2	1	500	0	87 (10)
MTX/DP/HYP/THY	0.2	1	100	100	92 (6)
MTX/DP/HYP/THY	0.2	1	500	100	102 (4)
THY	0	0	0	500	75 (5)
HYP	0	0	500	0	98 (3)

*MTX = methotrexate, DP = dipyridamole, HYP = hypoxanthine and THY = thymidine.
†Mean (S.D.).

Table 2. Cellular methotrexate levels in 2008 cells

Time (min)	Total cellular radioactivity* Control	1 μmol/l DP	10 μmol/l DP
Influx phase			
0	232 (27)	248 (25)	217 (20)
10	2788 (65)	2645 (38)	2692 (51)
30	4913 (49)	5032 (59)	4967 (75)
60	5120 (76)	4941 (68)	5081 (83)
Efflux phase			
0	5178 (94)	5077 (81)	5003 (70)
10	4322 (77)	4675 (90)†	4605 (98)†
30	4011 (62)	4284 (56)†	4328 (77)†
60	3896 (43)	4072 (82)†	4101 (91)†

*Mean (S.D.) of four experiments, normalized to counts per min per 10^6 cells.
†Significantly different from control values at same time point, $P < 0.05$, paired t test.

In cells exposed to the combination of methotrexate and dipyridamole, thymidine alone was ineffective in antagonizing cytotoxicity. Hypoxanthine alone demonstrated concentration-dependent antagonism of methotrexate plus dipyridamole cytotoxicity but the protection was incomplete, even at 1 mmol/l. The combination of 500 μmol/l hypoxanthine and 100 μmol/l thymidine completely antagonized the cytotoxicity of methotrexate plus dipyridamole. Comparable results were obtained from the CHO and AUX B1 cells with respect to the methotrexate/dipyridamole synergy in 72 h growth assays, except that the reduction of IC_{50} for methotrexate was 200 fold in the presence of 1 μmol/l dipyridamole (data not shown).

Methotrexate uptake and retention

Dipyridamole had no effect on the influx of radiolabelled-methotrexate into 2008 cells, within the concentration range tested (Table 2). The influx was linear for the first 20–30 min before reaching a plateau. The efflux of methotrexate, however, was moderately inhibited by dipyridamole in the 2008 cells. The cellular level of radiolabel after incubation with radioactive methotrexate for 1 h was consistently higher by 10% in cells re-incubated in media containing 1 or 10 μmol/l dipyridamole (Table 2). Although the moderate increase in cellular methotrexate level was unlikely to be the sole basis for the dramatic synergy between these two drugs, it is nonetheless informative about the mechanism responsible for increased methotrexate retention.

To test whether dipyridamole affects the polyglutamation of methotrexate intracellularly, hence increasing its retention, we studied methotrexate fluxes in CHO and AUX B1 cells [18]. Dipyridamole did not alter the influx of methotrexate into either (data not shown). After equilibration in medium containing

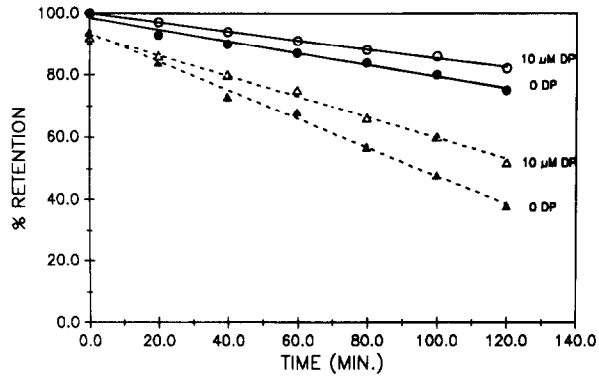

Fig. 2. Radiolabelled methotrexate retention in CHO (○, ●) and AUX B1 (△, ▲) cells in presence and absence of 10 μmol/l dipyridamole. Values were normalized as % of radioactivity in CHO cells at 0 min in absence of dipyridamole and represent mean of four experiments. Standard deviations were less than 5% of mean in all cases.

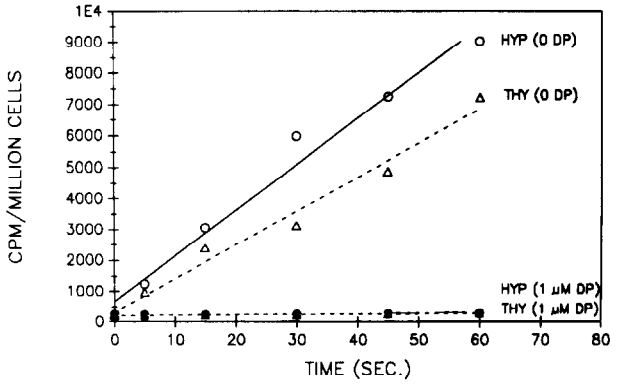

Fig. 4. Zero-trans influx of hypoxanthine (HYP) and thymidine (THY) into 2008 cells in culture media containing 1 μmol/l dipyridamole or PBS. Values are mean of 3 experiments (counts per min per 10^6) and standard errors were less than 8% of mean in all cases.

radiolabelled methotrexate for 1 h, the efflux of radioactivity from the AUX B1 cells was more rapid than that from CHO cells (Fig. 2). The extent of reduction of efflux caused by dipyridamole, however, was the same in both cell lines. These data suggest that polyglutamation in these cells was not affected by dipyridamole and cannot account for increased retention of methotrexate in the presence of dipyridamole.

Cellular cAMP levels

Since dipyridamole inhibits cyclic nucleotide phosphodiesterase, one of its potential actions is to increase cAMP in cells, affecting proliferation and possibly drug retention. We followed the course of changes in cellular cAMP levels in cells exposed to methotrexate with and without dipyridamole, and we included a dibutyryl-cAMP group as a positive control. Although dibutyryl-cAMP consistently resulted in sustained increase in cellular cAMP levels (Fig. 3), this compound was not cytotoxic to the 2008 cells. Methotrexate exposure resulted in a slow decline in cellular cAMP, which was unaffected by the presence of dipyridamole. Dipyridamole alone at 1 and 10 μmol/l did not significantly alter the cellular cAMP pool in the 2008 cells. Our data imply that dipyridamole increases methotrexate retention by mechanisms unrelated to folate polyglutamation or cellular cAMP levels.

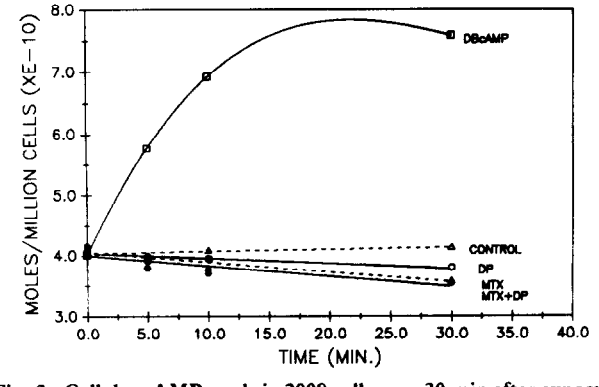

Fig. 3. Cellular cAMP pools in 2008 cells over 30 min after exposure to culture media containing 500 μmol/l dibutyryl-cAMP (DBcAMP), 10 μmol/l dipyridamole, 200 μmol/l methotrexate (MTX) and 10 μmol/l dipyridamole plus 0.2 μmol/l methotrexate. Values are mean of five experiments and standard deviations were less than 5% of mean in all cases.

Nucleoside and nucleobase transport

The protection of methotrexate and methotrexate plus dipyridamole cytotoxicity by hypoxanthine and thymidine requires confirmation that dipyridamole inhibits the transport of these compounds in a concentration-dependent manner in the 2008 cells. Figure 4 summarizes the effect of dipyridamole on the zero-trans influx of thymidine and hypoxanthine. Substantial inhibition of hypoxanthine and thymidine transport was observed at 0.1 μmol/l dipyridamole (data not shown) and complete inhibition was seen at 1.0 μmol/l. The calculated EC_{50} (mean [S.D.]) value for inhibition of thymidine transport was 0.1 (0.06) μmol/l and for hypoxanthine it was 0.4 (0.07) μmol/l (n = 4).

DISCUSSION

Although synergism between dipyridamole and methotrexate in various *in vitro* and *in vivo* models has been reported, the mechanism of synergy has not been defined. This drug combination affects different aspects of cellular metabolism when tested in different experimental systems. Dipyridamole sensitized CHO cells to methotrexate by inhibiting thymidine salvage, but failed to improve methotrexate activity in Ridgeway osteogenic sarcoma and L1210 leukaemia [7]. In sarcoma 180 cells growing in the peritoneal cavity of Swiss mice, dipyridamole enhanced the ability of methotrexate to inhibit ^{3}H-deoxynucleoside incorporation into DNA. Exogenous thymidine completely antagonized that synergy while hypoxanthine had no effect [14]. In a human breast cancer cell line, dipyridamole at 10 μmol/l augmented methotrexate activity by increasing its cellular high-molecular-weight polyglutamate pools [15]. Unfortunately, the role of salvage metabolism was not investigated in that study.

Dipyridamole augmented the cytotoxicity of methotrexate, decreasing the IC_{50} value of the antifolate 1000 times in the 2008 cells. Previous reports have estimated the efficacy of augmentation at low levels [7, 13–15], and one study reported that the mild synergy has minimum effect on survival in a rodent sarcoma model [13]. The human ovarian carcinoma cells used in our study are normally quite resistant to the cytotoxic actions of antimetabolites because of their high salvage capability [5, 6, 17]. Our data suggest that thymidine alone antagonizes the effects of methotrexate in 2008 cells, but was ineffective against methotrexate plus dipyridamole. Hypoxanthine alone antagonized the cytotoxicity of methotrexate, but required the presence of thymidine to reverse the cytotoxicity of methotrexate plus

dipyridamole fully. These data were similar to those reported in mouse L1210 cells treated with 5,10-dideaza-5,6,7,8-tetrahydrofolate, a new antifolate that inhibits folate metabolism at sites other than dihydrofolate reductase: both hypoxanthine and thymidine were needed to antagonize its action [19]. One possible explanation of this similarity is that dipyridamole prolonged the cellular retention of methotrexate (Table 2) making the drug available to cellular targets other than dihydrofolate reductase in the 2008 cells. An alternative explanation for the similarity of actions between methotrexate/dipyridamole and the new antifolate may be that the new drug inhibits enzymes of the salvage metabolic pathway in addition to its antifolate activity.

In our experiments, high concentrations of nucleoside or nucleobase were required to antagonize the methotrexate/dipyridamole synergy. This is consistent with the low binding constant of dipyridamole (nmol/l, hence high affinity) for the nucleoside transporter compared with thymidine and hypoxanthine (μmol/l). Furthermore, our data on the ability of dipyridamole to inhibit hypoxanthine transport agreed with earlier results obtained in S49 T-lymphoblast cells [20], and suggested that the nucleoside transporter plays a role in nucleobase influx.

In view of the finding that dipyridamole increased the retention of radiolabelled methotrexate to the same degree in the CHO cell as in AUX B1 cells, it is unlikely that increased polyglutamation was responsible for the reduced methotrexate efflux observed when dipyridamole was present. It is interesting, however, that the rate of methotrexate efflux from AUX B1 cells was more rapid than that observed in CHO cells. The difference between the two cell lines may therefore reflect the effect of polyglutamation in slowing the loss of methotrexate from cells. Since dipyridamole inhibits cyclic nucleotide phosphodiesterases [10], we monitored changes in cAMP levels in 2008 cells in response to dipyridamole and methotrexate. Our results confirmed that dipyridamole, at the concentrations tested, had no effect on 2008 cellular cAMP pools.

Through a process of elimination, our experiments suggested that dipyridamole potentiated the cytotoxicity of methotrexate in 2008, CHO and AUX B1 cells primarily by inhibiting hypoxanthine salvage. Although thymidine is an important determinant of methotrexate activity, it did not play a major role in modulating the cytotoxicity of methotrexate plus dipyridamole. The dramatic synergy observed here represents an almost 1000 fold increase in cytotoxicity against 2008 cells (the highest we have documented in our laboratory). Our data suggested that this drug combination should be investigated more fully in phase II trials in ovarian cancer patients. Several phase I toxicity trials [21, 22] and an intraperitoneal chemotherapy study have reported that this drug combination was well tolerated by cancer patients [23].

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