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# DUP 785 (NSC 368390): SCHEDULE-DEPENDENCY OF GROWTH-INHIBITORY AND ANTIPYRIMIDINE EFFECTS

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**Abstract**—DUP 785 (NSC 368390; Brequinar sodium) is a new inhibitor of pyrimidine *de novo* biosynthesis with antitumor activity against several experimental tumors. DUP 785 inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, blocking the conversion of dihydroorotate to orotate. We examined the influence of exposure time to DUP 785 on its growth-inhibitory effects in L1210 murine leukemia and WiDR human adenocarcinoma cells and the effects of pyrimidine (deoxy) nucleosides on reversal of growth-inhibition. The results were correlated with changes in intracellular pyrimidine nucleotide pools and cell cycle distribution. In L1210 cells, a continuous exposure to 25 μM DUP 785 up to 96 hr caused complete growth inhibition. A 2 hr exposure of cells to the drug did not affect growth. In WiDR cells, exposure to the drug for 1–24 hr, followed by cultivation in drug-free medium resulted in recovery of growth. However, cells exposed to the drug for 48 hr or longer were not able to resume growth when recultured in drug-free medium. Reversal studies were performed to know whether selective depletion of one of the pyrimidine (deoxy) nucleotides might be related to the growth-inhibitory effects of DUP 785. Neither thymidine, deoxycytidine alone, deoxycytidine plus tetrahydrouridine; nor cytidine plus tetrahydrouridine added after 24 hr were able to reverse cell growth inhibition induced by 25 μM DUP 785. However, uridine and cytidine alone reversed growth inhibition. UTP and CTP pools in L1210 cells decreased to about 30–40% of control levels after 4 hr of drug exposure, while dTTP and dCTP pools decreased to about 30% of control levels. There were no significant changes in purine nucleotide pools. In WiDR cells, UTP and CTP pools decreased rapidly after drug exposure and were substantially depleted after 24 hr. Reculture of cells in drug-free medium resulted in a significant recovery of UTP and CTP levels only for cells exposed to DUP 785 for 1–24 hr. For cells exposed to the drug for 48 and 72 hr recovery of nucleotide pools was minimal. In L1210 cells, a 12-hr exposure to the drug caused an accumulation of cells in the early *S*-phase. In WiDR cells, there was a clear accumulation of cells in the *S*-phase of the cell cycle after 24 hr drug exposure. After culture in drug-free medium cells continued to traverse through the cell cycle. Our results demonstrate that prolonged exposure of cells to DUP 785 was necessary for a long-lasting depletion of pyrimidine nucleotides and a substantial suppression of RNA and DNA synthesis. Selective repletion of one of the (deoxy) ribonucleotides did not restore cell growth. Only repletion of all nucleotides by uridine and/or cytidine restored cell growth. The failure of short-term drug exposure to cause effects may be explained by the rapid recovery of pyrimidine nucleotide pools in drug-free medium, presumably allowing cells to continue RNA and DNA synthesis and to reverse the *S*-phase block in the cell cycle.

During the past years, various inhibitors of pyrimidine *de novo* biosynthesis have been developed. Acivicin, PALA‖ and pyrazofurin are examples [1–3]. These compounds are capable of blocking specific steps in the formation of UMP, leading to a depletion of pyrimidine nucleotide pools and impaired RNA and DNA synthesis (Fig. 1).

Pyrimidine *de novo* biosynthesis involves six sequential steps. The first three enzymes exist in one enzyme complex, leading to a chanelling of substrates towards the synthesis of DHO [4]. The first step consists of the synthesis of carbamyl phosphate from glutamine, ATP and $CO_2$. This reaction is catalyzed by carbamyl phosphate synthetase II and can be inhibited by acivicin [1]. The enzyme is also subject to feedback inhibition by UTP and is stimulated by PRPP [4]. The conversion of carbamyl phosphate to *N*-carbamyl aspartate is catalyzed by carbamyl aspartate transcarbamylase and can be inhibited by PALA [2]. Dihydroorotase is the enzyme responsible for the conversion of *N*-carbamyl aspartate to DHO. Recently, it has been reported that this enzyme can be inhibited by boronic acid analogs of amino acids [5].

DHO-DH is the fourth enzyme of the pyrimidine *de novo* pathway, catalyzing the oxidation of DHO to orotate [6]. This enzyme is different from the other pyrimidine enzymes by its localization in the mitochondria at the outer site of the inner membrane [4, 7].

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‖ Abbreviations used: DHO-DH, dihydroorotate dehydrogenase; OPRT, orotate phosphoribosyltransferase; ODC, orotidine 5′-monophosphate decarboxylase; DHO, dihydroorotate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PALA, *N*-phosphonacetyl-L-aspartate; HPLC, high pressure liquid chromatography; THU, tetrahydrouridine.

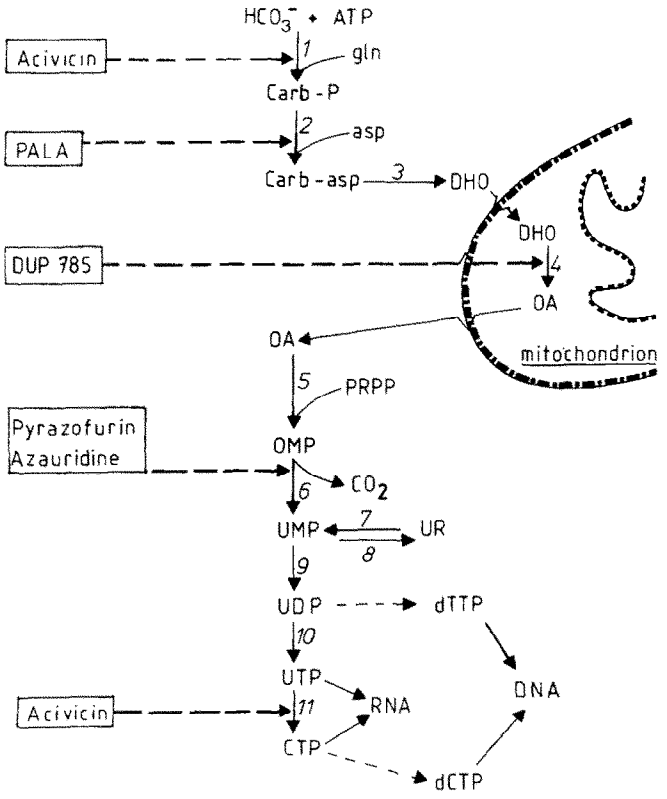


Fig. 1. Schematic outline of pyrimidine *de novo* and salvage pathways. The enzymes catalyzing these reactions are: 1, carbamyl-phosphate synthase II (EC 6.3.5.5); 2, aspartate transcarbamylase (EC 2.1.3.2); 3, dihydroorotase (EC 3.5.2.3); 4, DHO-DH (EC 1.3.3.1); 5, OPRT (EC 2.4.2.10); 6, ODC (EC 4.1.1.23); 7, uridine-cytidine kinase (EC 2.7.1.48); 8, 5′-nucleotidase; 9, nucleoside monophosphate kinase (EC 2.7.4.14); 10, nucleoside diphosphate kinase (EC 2.7.4.6.); 11, CTP synthetase (EC 6.3.4.2). Carb-P, carbamyl phosphate; Carb-asp, carbamyl-aspartate; gln, glutamine; asp, aspartate; OA, orotic acid. The site of action of various inhibitors of pyrimidine *de novo* biosynthesis is also represented.

There are three general classes of inhibitors of DHO-DH. The first class consists of orotate and analogs of DHO and orotate [4]. The second class of inhibitors are the naphthoquinone analogs, such as dichloroallyl lawsone and lapachol [8, 9]. The third class includes compounds that are inhibitors of the electron transfer chain such as cyanide and 2-4-dinitrophenol and interfere non-specifically with the activity of DHO-DH [10].

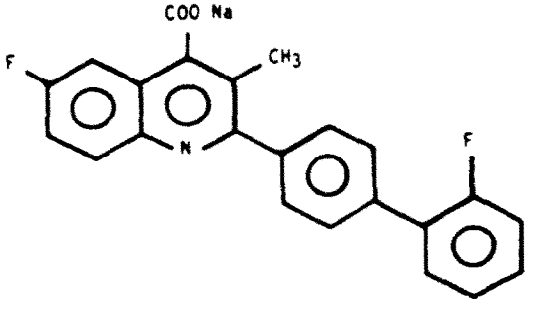


Fig. 2. The chemical structure of DUP 785 (NSC 368390; Brequinar sodium); [6-fluoro-2-(2′-fluoro-1-1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid, sodium salt].

The next two enzymes also form an enzyme complex called UMP synthase [4] and catalyze the conversion of orotate to OMP, and subsequently to UMP. UMP synthase consists of OPRT and ODC, respectively. The complex can be inhibited by 5′-phosphate derivatives of pyrazofurin [11] or azauridine [12].

DUP 785 (NSC 368390) is a 4-substituted quinoline carboxylic acid (Fig. 2), which was shown to be a potent inhibitor of DHO-DH [13, 14], with apparent $K_i$ values for various systems ranging between 23 and 100 nM. The mode of enzyme inhibition in rat mitochondria was described as of linear mixed type [14]. DUP-785 lacks structural similarity with the substrate, the product or the cofactor of the biochemical reaction catalyzed by DHO-DH.

DUP 785 has a broad spectrum of activity against several murine tumors and human tumor xenografts [15]. *In vitro* studies with L1210 murine leukemia demonstrated that exposure of cells to DUP 785 causes a strong inhibition of DHO-DH activity. However, enzyme activity recovered when cells were cultured in drug-free medium after a short incubation with DUP [14]. At prolonged exposure of cells to the drug for 24–48 hr, inhibition of DHO-DH is retained and associated with pronounced growth-inhibitor effect [14].

Uridine and cytidine can prevent the growth-inhibition caused by DUP 785 [13, 14]. In order to know whether a selective depletion of one of the pyrimidine (deoxy) ribonucleotides is responsible for the growth-inhibitory effects of DUP 785, we performed reversal studies with the various pyrimidine (deoxy) ribonucleosides.

In this paper, we extended the studies on the *in vitro* antipyrimidine effects of DUP 785 in L1210 leukemia and WiDR human adenocarcinoma cells. We examined which nucleotide pools are important for the growth-inhibitory effects of DUP 785, as well as the influence of exposure time to the drug on cell growth in relation to the retention of the depletion of pyrimidine nucleotide pools. These results were correlated with DNA flow cytometry studies performed in cells cultured in the presence of DUP 785.

## MATERIALS AND METHODS

*(a) Materials.* DUP 785 was obtained from DuPont Biomedical Products, E.I. du Pont de Nemours Inc. (Wilmington, DE) U.S.A. RPMI-1640, Dulbecco's minimum essential media, and heat-inactivated fetal bovine serum were obtained from Flow Laboratories, Irvine, Scotland. Nucleotide standards were obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were from standard quality commercially available.

*(b) Cell culture.* L1210 murine leukemia cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 60 μM 2-mercaptoethanol as described previously [14]. Cells were exposed to 25 μM DUP 785 for increasing time intervals and cell number was estimated at 0, 24, 48 and 96 hr using a Sysmex electronic cell counter CC-110 (TOA Medical Electronics Co., Ltd., Kobe, Japan).

WiDR human adenocarcinoma cells were cultured in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum as described previously [16]. Cells were allowed to attach to the plate for 24 hr and cultured in the presence of 25 μM of DUP 785 during various time intervals; and then recultured in drug-free medium. After trypsinization, cell number was estimated as described above; and cell viability was studied by the Trypan blue exclusion test [17].

For reversal studies, cells were cultured in the presence of 25 μM of DUP 785 during 24 hr and then nucleosides were added to the cultures without removal of DUP 785: uridine (1 mM), cytidine (1 mM), thymidine (0.1 and 1 mM), deoxycytidine (1 mM); cytidine (1 mM) plus THU (0.1 mM and 1 mM); and deoxycytidine (1 mM) plus THU (0.1 mM and 1.0 mM). THU was added to inhibit deamination of (deoxy) cytidine [18]. The effect of nucleosides on the growth of cells cultured in drug-free medium was also examined. Cell number was estimated at 0, 24, 48 and 72 hr as described above.

*(c) Nucleotide measurements.* Nucleotide measurements were performed with L1210 and WiDR cells by HPLC methods as described previously [16, 19]. Briefly, harvested cells were extracted with cold 10% trichloroacetic acid and the supernatant was neutralized with a trioctylamine–Freon (1:4) mixture. Ribonucleotides and deoxyribonucleotides were separated on a strong anion exchange Partisil SAX column (Whatman Inc., Clifton, NJ) using a Waters (Etten-Leur, Netherlands) HPLC equipped with a dual-wavelength UV detector. For deoxyribonucleotide separation, extracts were first subjected to periodation to eliminate interfering ribonucleotides [20, 21].

*(d) Cell-cycle distribution.* Cell-cycle distribution studies were performed with L1210 and WiDR cells by DNA flow cytometry as previously described [22]. Briefly, ethanol-fixed cells were stained with ethidium bromide and Hoechst 33258 stain and analyzed with a Phywe II pulse cytophotometer. In L1210 cells, DNA flow cytometry was performed in non-treated cells and in cells exposed to 25 μM DUP 785 for 12 hr, whereas in WiDR cells experiments were performed in non-treated cells, in cells exposed to the drug for 24 and 48 hr and in cells recultured in drug-free medium for 24 and 48 hr.

## RESULTS

### *(a) Effect of drug exposure on cell growth*

The effect of DUP 785 on the growth of L1210 cells was estimated at 24, 48 and 96 hr after incubation of cells at a 25 μM concentration (Fig. 3). In these cells, continuous exposure to DUP 785 up to 96 hr caused almost complete growth-inhibition. A 2-hr exposure of cells to the drug did not affect growth.

The effect of DUP 785 on growth-inhibition of WiDR cells was studied after exposure of the cells for various time periods to 25 μM of the drug (Fig. 4). In these cells, drug exposure for 1–24 hr with reculture of cells in drug-free medium was followed by recovery of growth. However, for cells exposed to DUP 785 for 48 hr or longer, growth was not resumed after reculture of cells in drug-free medium. Cell viability decreased from 96% at the start of experiments to 46% at 72 hr. This result suggests that the cytostatic effects of DUP 785 are followed by cytocidal effects after prolonged drug exposure.

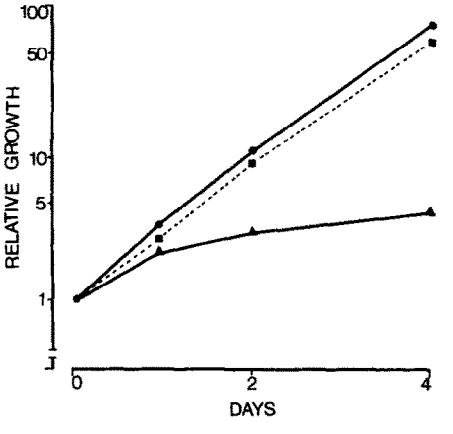


Fig. 3. Growth-inhibition of L1210 cells in the presence of DUP 785. ●—●, control; ■—■, 2 hr exposure; and ▲—▲, continuous exposure to 25 μM of DUP 785. One representative experiment out of 4 (duplicates) is shown.

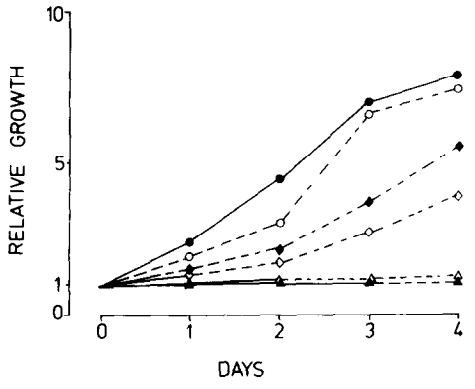


Fig. 4. Growth-inhibition of WiDR cells in the presence of DUP 785 (25 μM) during increasing intervals of time and further culture in drug-free medium (indicated by broken lines); ●—●, controls; ○—○, 1 hr; ◆—◆, 12 hr; ◇—◇, 24 hr; △—△, 48 hr; and ▲—▲, 72 hr exposure. One experiment out of 3 (triplicates) is shown.

*(b) Reversal studies*

Reversal of the growth-inhibition of DUP 785 was studied by the addition of uridine, cytidine, thymidine or deoxycytidine; or the addition of cytidine and deoxycytidine plus THU to the cultures after 24 hr, without removal of DUP 785 (Fig. 5).

Neither uridine, cytidine or deoxycytidine (1 mM) alone affected cell growth significantly. However, thymidine (1 mM) or THU (1 mM) alone caused partial growth-inhibition and therefore a lower concentration (0.1 mM) was chosen for subsequent reversal studies. Analysis of culture media with reversed phase HPLC [25] at the end of the incubation showed that 0.1 mM THU inhibited deamination.

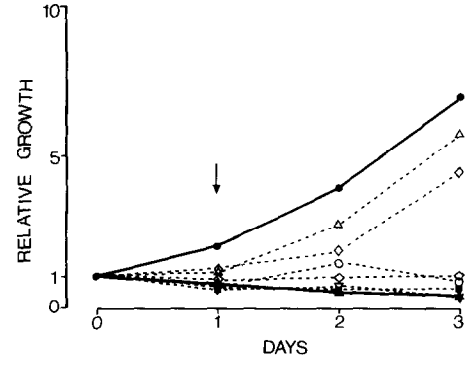


Fig. 5. Growth-inhibition of WiDR cells in the presence of DUP 785 either alone or after the addition of nucleosides at 24 hr. ●—●, control; ▲—▲, 25 μM DUP 785; △—△, DUP 785 and 1 mM uridine (24 hr); ◇—◇, DUP 785 and 1 mM cytidine (24 hr); ■—■, DUP 785 and 1 mM cytidine plus 0.1 mM THU (24 hr); ○—○ DUP 785 and 0.1 mM thymidine (24 hr); ▽—▽, DUP 785 and 1 mM deoxycytidine (24 hr); or ◇—◇, DUP 785 and 1 mM deoxycytidine plus 0.1 mM THU (24 hr). One representative experiment out of 2 (triplicates) is shown. The arrow indicates the time of addition of nucleotides.

The addition of uridine or cytidine at 24 hr after DUP 785 restored cell growth. However, cytidine plus THU, thymidine, deoxycytidine alone, deoxycytidine plus THU and cytidine plus THU did not restore cell growth in the presence of the drug.

*(c) Nucleotide measurements*

UTP, CTP, dCTP and dTTP concentrations were measured in L1210 cells before and after 2- and 4-hr exposure to 25 μM of DUP 785 (Fig. 6). UTP and CTP concentrations decreased to about 30–40% of control levels after 2 hr. This was accompanied by a proportional depletion of dTTP and dCTP down to about 30% of control levels after the same exposure time. Addition of 1 mM of uridine to the cultures prevented the decrease of both UTP and CTP as well as dTTP and dCTP (data not shown).

There were no significant changes in the concentration of purine nucleotides during the same interval, except for a minor increase in the concentration of ATP during the first 2 hr that followed drug exposure.

WiDR cells were exposed to 25 μM of DUP 785 for time-periods varying from 1 to 72 hr, followed by reculture in drug-free medium. After 1 hr, UTP and CTP decreased to about 30% and 70% of control levels, respectively (Fig. 7). After 24 hr, pyrimidine nucleotides were depleted almost completely. The reculture of cells in drug-free medium resulted in a partial recovery of UTP and CTP concentrations only for cells exposed to DUP 785 for 1–24 hr, which were also capable of resuming growth (Fig. 4). For those cells exposed to the drug for 48–72 hr, only a minimal or no recovery of UTP and CTP levels was observed, which explains why growth remained completely inhibited.

With continuous drug exposure, the depletion of CTP was less than that of UTP, which is reflected by the lower UTP/CTP ratio (Table 1). The value after 3 hr, however, is higher probably because of a delayed effect on the cytosine nucleotides. At each time point after a 12-hr exposure or longer, UTP/CTP ratios were lower than the control, but it increased when cells were cultured in drug-free medium.

Both purine ribonucleotides showed an initial increase in their intracellular concentrations which was followed by a late depletion (Fig. 8). ATP increased about 20% after the first hours of drug exposure but decreased to about 80% of control levels after prolonged culture. GTP also increased about 20% after drug exposure but decreased to about 50% of control levels after culture in drug-free medium.

*(d) Cell-cycle studies*

If DUP 785 acts mainly as an antimetabolite, it should have a characteristic cell-cycle specificity. We performed DNA flow cytometry studies in control cells and those treated with DUP 785 (Fig. 9). In L1210 cells, 12-hr exposure of cells to 25 μM of DUP 785 caused an accumulation of cells in early *S*-phase. In WiDR cells, a 24-hr exposure of cells to the drug resulted in a clear accumulation in the *S*-phase, but reculture of cells in drug-free medium allowed cells to pass through the cycle.

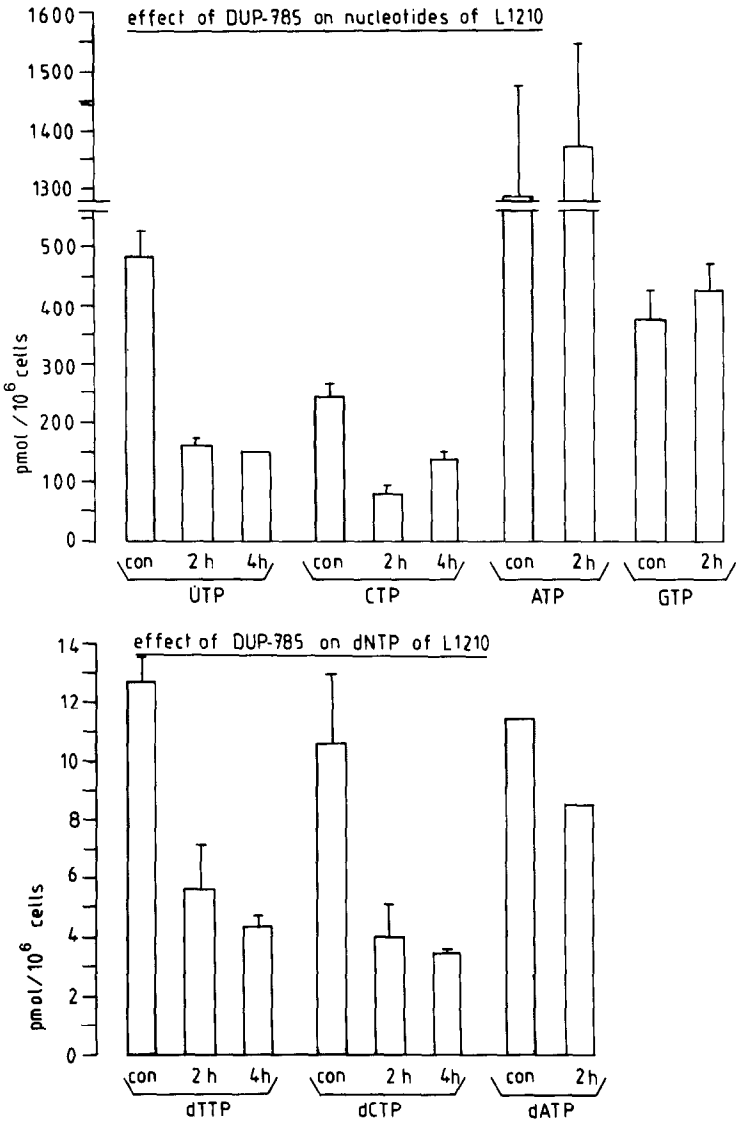


Fig. 6. (A) Intracellular concentrations of ribonucleotides in L1210 cells exposed to 25 μM DUP 785 for 2 hr and 4 hr. Bars represent means SE of 3 separate experiments. (B) Intracellular concentrations of deoxyribonucleotides in L1210 cells exposed to 25 μM DUP 785 for 2 and 4 hr. Bars represent means SE of 3 separate experiments.

Table 1. UTP/CTP ratios in WiDR cells cultured in the presence of DUP 785

| Time (hr) | DUP 785* exposure-time | | | | |
|---|---|---|---|---|---|
| | Continuous | 1 hr | 24 hr | 48 hr | 72 hr |
| 0 | 3.92 ± 0.20 | | | | |
| 1 | 2.50 ± 0.13 | | | | |
| 3 | 4.49 ± 0.93 | 1.69 ± 0.33 | | | |
| 12 | 2.02 ± 0.24 | 3.62 ± 0.36 | | | |
| 24 | 1.05 ± 0.09 | 3.86 ± 0.68 | | | |
| 48 | 1.72; NC | 3.28 ± 0.21 | 3.20 ± 2.06 | | |
| 72 | 0.80; NC | | | | |
| 96 | | | | 3.83; 3.21 | |
| 120 | | | | | 1.46; NC |

Values are means ± SE of at least 3 separate experiments.
NC = one of the values was not calculated because it was below detection limit by HPLC.
* 25 μM.

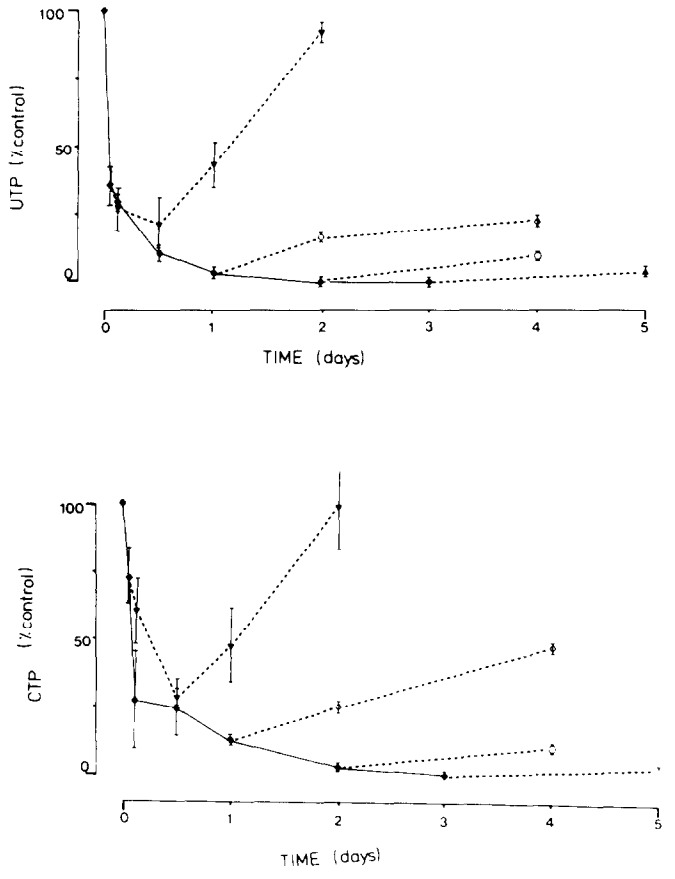


Fig. 7. The effect of 25 μM DUP-785 on the intracellular concentration of pyrimidine ribonucleotides in WiDR cells. Cells were continuously exposed to DUP 785 (◆—◆, solid lines) for various periods of time (∇—∇, 1 hr; ◇—◇, 24 hr; ○—○, 48 hr; and △—△, 72 hr exposure) followed by culture in drug-free medium (broken lines). Results are means SE of at least 3 separate experiments. Absolute concentrations of UTP and CTP at $t = 0$ were 2510 42 and 645 28 pmol/10 cells, respectively (means SD).

## DISCUSSION

The results of this study underscore the importance of continuous exposure of cells to DUP 785 to achieve maximal growth-inhibitor effects. These effects appear to be dependent on a prolonged depletion of the intracellular pyrimidine nucleotide pools, leading to an accumulation of cells in *S*-phase and thus inhibition of DNA synthesis. Reculture of cells in drug-free medium allowed cells to leave the *S*-phase and resume cell-cycle progression as a result of the recovery of pyrimidine nucleotide biosynthesis.

Our experiments using L1210 leukemia and WiDR adenocarcinoma cells demonstrated that growth-inhibition was more pronounced as exposure time was increased, without recovery of growth after prolonged drug exposure (48 hr or longer). These findings are in agreement with previous studies using Clone A human colon tumor cells which showed that exposure of cells to 25–72 μM of DUP 785 for 48–72 hr caused maximal cytotoxic effects in the clonogenic assay [13]. In that study, it was also observed that continuous exposure of Clone A human colon tumor and L1210 leukemia cells to 25 μM of DUP 785 caused a decrease of UTP and CTP to 50% of control levels by 3 hr, and to undetectable levels after 15 hr [13].

In another study, it was shown that the concentrations of dichloroallyl lawsone and lapachol, other inhibitors of DHO-DH, required to cause 50% growth inhibition of L1210 cells over a period of 48 hr were 19 μM and 4 μM, respectively, which are higher than the values for DUP 785 [14]. These compounds had little or no effect on ATP and GTP levels but significantly decreased intracellular pyrimidine nucleotide levels. After 4 hr exposure to dichloroallyl lawsone or lapachol the UTP levels were about 60 and 40% of control levels, respectively [8]. Our results with DUP 785 in L1210 cells were comparable to those described above, and showed a decrease in both pyrimidine ribo- and deoxyribonucleotide levels to about 30–40% of controls after 4 hr exposure to DUP 785. These results correlate with previous findings on the retention of DHO-DH inhibition by prolonged exposure to DUP 785 [14] and also demonstrate that with this drug a depletion of UTP and CTP was accompanied by a

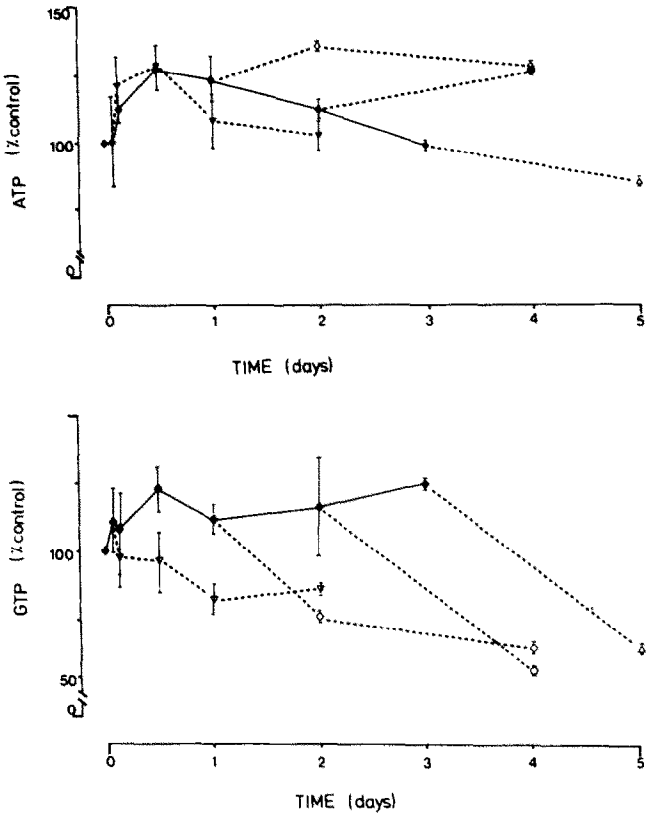


Fig. 8. The effect of 25 μM DUP-785 on the intracellular concentrations of purine ribonucleotides in WiDR cells. Cells were continuously exposed to DUP 785 (◆—◆, solid lines) for various periods of time (∇—∇ 1 hr; ◇—◇ 24 hr; ○—○ 48 hr; and △—△ 72 hr exposure) followed by culture in drug-free medium (broken lines). Results are means SE of at least 3 separate experiments. Absolute nucleotide concentrations of ATP and GTP at $t = 0$ were 4510 58 and 1106 28 pmol/10 cells, respectively (mean SD).

depletion of dTTP and dCTP. This is in agreement with data obtained with other inhibitors of pyrimidine *de novo* synthesis, in which it was shown that depletion of ribonucleotides paralleled those of pyrimidine deoxynucleotides [23, 24].

In order to determine which nucleotide pools are important for DUP 785 cytotoxicity, we performed a series of reversal studies in which the ability of (deoxy) nucleosides to restore cell growth in the presence of a growth-inhibitory concentration of DUP 785 was evaluated. From the deoxynucleosides thymidine and deoxycytidine it is known that they can enhance dTTP and dCTP pools [20] and reverse growth-inhibition induced by depletion of one of these nucleotides [25]. Thus, the toxicity of e.g. 5-fluorouracil which can be mediated by depletion of dTTP [16] can be reversed by thymidine [25]. Toxicity by interference with RNA synthesis can be reversed by uridine [26].

In order to replete selectively one of the (deoxy) ribonucleotide pools, we added nucleosides to DUP 785-treated cells. Furthermore, to selectively replete CTP or dCTP, we also added THU, a potent inhibitor of cytidine deaminase [18] to the cultures in combination with cytidine or deoxycytidine respectively.

Our results suggest that a selective depletion of dTTP or dCTP is not responsible for DUP 785 cytotoxicity, since the addition of cytidine plus THU, thymidine or deoxycytidine (alone or plus THU) did not restore cell growth. Only uridine or cytidine (without THU) was able to restore cell growth. Therefore, the depletion of uridine nucleotides leading to the depletion of CTP, dTTP and dCTP is responsible for the growth-inhibitory effects of DUP 785. The observation that in L1210 cells the addition of uridine to the cultures restored both ribo- and deoxyribonucleotide pools and prevented the toxic effects of DUP 785 supports this conclusion.

In the above aspect DUP 785 appears to be different from, e.g. PALA and pyrazofurin. The toxicity of these two other inhibitors of pyrimidine *de novo* synthesis could be partially reversed by deoxycytidine and thymidine [23] but that of DUP 785 could not. Therefore, UMP depletion plays a central role in DUP 785 cytotoxicity, leading to the inhibition of both RNA and DNA synthesis and growth-inhibition.

The results of this study highlight the importance of continuous exposure of cells to DUP 785 to achieve maximal growth-inhibitory effects. In L1210 cells a 2 hr exposure to the drug did not affect cell

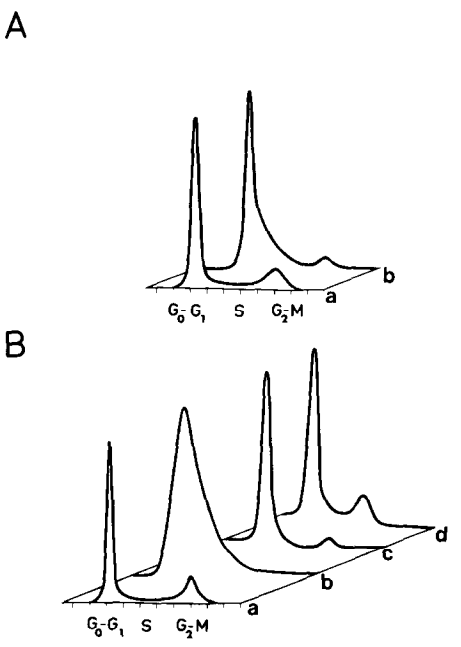


Fig. 9. Cell-cycle distribution by DNA flow cytometry of L1210 and WiDR cells. In A, L1210 control cells (a) and cells cultured in the presence of 25 $\mu$M DUP 785 for 12 hr (b). In B, WiDR control cells (a), cells cultured in the presence of 25 $\mu$M DUP 785 for 24 hr (b) and subsequent reculture in drug-free medium for an additional 24 hr (c) and 48 hr (d).

growth and it was accompanied by a rapid recovery of DHO-DH activity [14]. However, marked cytotoxicity occurred with continuous drug exposure. This effect was related to the prolonged inhibition of DHO-DH [14] and a depletion of intracellular pyrimidine nucleotide pools.

From our experiments with WiDR cells, it was evident that increasing exposure time to DUP 785 resulted in a long-lasting depletion of pyrimidine nucleotide which correlated with growth-inhibition. Apparently, the supply of nucleotides was too low to support RNA and DNA synthesis and thus cell growth. For PALA it was shown that depletion of nucleotides was paralleled by inhibition of RNA and DNA synthesis [23]. After short-term exposure to DUP 785, ribonucleotide pools showed clear evidence of recovery, supporting RNA and DNA synthesis and cell growth.

The minor increase in ATP and GTP levels which was observed during drug exposure may be explained by its reduced utilization for RNA and DNA synthesis while UTP and CTP levels were effectively suppressed by DUP 785.

DNA flow cytometry studies in L1210 and WiDR cells showed that cells continously exposed to DUP 785 accumulated in the *S*-phase of the cell cycle, whereas reculture of cells in drug-free medium after a 12–24 hr exposure allowed the reversal of such a block, apparently because cells recovered pyrimidine nucleotide synthesis to such an extent as to be able to resume DNA synthesis. The accumulation of cells in the *S*-phase of the cycle with DUP 785 is more pronounced than with PALA and pyrazofurin [24].

To date, there is no evidence of a mechanism for specific retention of DUP 785 in cells, which could account for a prolongation of drug exposure and perhaps a preferential cytotoxicity to tumor cells. Various other antimetabolites have the capacity for metabolic conversion to active forms which are less membrane-permeable, e.g. polyglutamation of methotrexate [27], and phosphorylation of cytosine arabinoside [28] and 5-fluorouracil [16]. It should be noted that these forms of intracellular drug accumulation have been shown to be important for antitumor activity [27, 29]. Clinical studies of DUP 785 should take into consideration these potential limitations, i.e. the need for prolonged drug exposure and the apparent lack of intracellular retention.

Interestingly, DUP 785, unlike other antimetabolites has its target enzyme located in the mitochondria [4, 7, 10]. This could represent a potential form of cellular retention of the drug by subcellular organelle sequestration. Notably, rhodamine-123, a dye known to be readily taken up by the mitochondria, has been shown to be selectively retained by, and toxic to, cancer cells [30, 31].

Our *in vitro* results cannot be directly extrapolated to the *in vivo* situation. *In vivo*, most cells may predominantly use salvage pathways to maintain their normal nucleotide supply for nucleic acid synthesis [32]. Nucleosides or bases, which are produced by cellular turnover, synthesized in the liver or absorbed from the gastrointestinal tract are important sources of precursors for UTP and CTP synthesis by the pyrimidine salvage pathway [33]. In tumors, the breakdown products of the cellular metabolism in poorly vascularized and necrotic tissues provide an extra source of nucleosides and bases for RNA and DNA synthesis [33]. Therefore, it may be possible that *in vivo* tumor cells are able to circumvent the block of pyrmidine *de novo* biosynthesis by the use of salvage pathways.

Recently, several Phase I trials of DUP 785 have been completed whereby the drug has been given as a short-term intravenous infusion by various schedules to patients with solid tumors. In one study whereby the drug is given every 3 weeks, DUP 785 peak plasma levels of above 1 mM were achieved in patients immediately after the completion of drug infusion, but the levels declined to below 10 $\mu$M after 48–72 hr [34]. Although such concentrations of DUP 785 have been shown to be sufficient to cause *in vitro* growth-inhibitory effects, it may not be enough to produce significant antitumor effects in man. The presence of DUP 785 at effective concentrations over longer periods of time, when tissues might be more dependent on the pyrimidine *de novo* pathway could be more important for antitumor activity. Biochemical modulators such as dipyridamole, which inhibit nucleoside uptake by the cells might be also used to block pyrimidine salvage [35], however, their ability to potentiate the antitumor effects of DUP 785 remains to be seen.

In conclusion, our study demonstrates that UMP depletion is critical for the growth-inhibitory effects of DUP 785 *in vitro*. Prolonged exposure of cells to DUP 785 seems necessary for a long-lasting depletion of intracellular pyrimidine nucleotide pools. This leads to a block in RNA and DNA synthesis and to

the accumulation of cells in the *S*-phase of the cell cycle, causing growth-inhibition. These findings suggest a need for prolonged drug exposure in clinical trials of DUP 785.

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