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INHIBITION OF NUCLEIC ACID SYNTHESIS BY THE DI-*n*-BUTYL ESTER OF METHOTREXATE

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Abstract—The effects of dibutyl methotrexate (DBMTX) on the incorporation into DNA of labelled deoxynucleosides have been studied in a variety of cultured cell lines. In serum-free conditions DBMTX consistently inhibited thymidine and deoxyuridine incorporation. Prior incubation of DBMTX in 10% mouse serum abolished the inhibition of thymidine incorporation but did not influence the inhibition of deoxyuridine incorporation. Identical results were observed in cell lines resistant to methotrexate because of increased levels of dihydrofolate reductase or altered drug transport characteristics. Citrovorum factor prevented only partially the effects of DBMTX in serum-free cultures.

The clinical effectiveness of prolonged methotrexate (MTX) therapy is frequently limited by the development of drug resistance. Impaired cellular transport of MTX and changes in activity or conformation of dihydrofolate reductase (DHFR) have been identified in cultured cells resistant to MTX, but the basis for clinical resistance is poorly understood [1–3]. Experimental evidence indicates that uptake of MTX by cells occurs via a carrier-mediated active transport process. A number of MTX analogues with increased lipid solubility have been synthesized with the hope that they would pass more readily through the cell membrane thereby circumventing a major mechanism of drug resistance [4–6]. In this paper we report the effects of the di-*n*-butyl ester of MTX (DBMTX) [6] on the incorporation of nucleosides into DNA by a number of cultured mammalian cell lines.

MATERIALS AND METHODS

MTX and Citrovorum Factor, the latter as the racemic compound leucovorin (*d,l*-N^5-tetrahydrofolate), were obtained from Lederle Laboratories, Pearl River, N.Y., and were diluted to the appropriate concentration in Hanks' balanced salt solution (HBSS) (Grand Island Biological Company, Grand Island, N.Y.). DBMTX and diethyl MTX were synthesized in our laboratories by methods already described [6]. Deoxyuridine, deoxyadenosine and thymidine were obtained from Sigma Chemical Corporation (St. Louis, Mo.) and [^{3}H]thymidine ([^{3}H]TdR) (54 Ci/m-mole); [^{3}H]deoxyuridine ([^{3}H]UdR) (17 Ci/m-mole) were obtained from Schwartz-Mann Company (Orangeburg, N.Y.). Reagent chemicals were obtained from Aldrich Chemical Company (Milwaukee, Wi.), and analytical grades were used when available. Radioisotopic counting was performed in a Beckman LS 335 scintillation counter using Aquasol scintillation fluid (New England Nuclear, Boston, Mass.).

The maintenance of CCRF-CEM cells and MTX-sensitive L1210 cells (L1210/S) has previously been described [7]. An MTX-resistant L1210 subline with increased levels of DHFR (L1210/DHFR) and an MTX transport-resistant L1210 (L1210/TR) subline were obtained from Mr. I. Wodinsky (A. D. Little, Inc. Cambridge, Mass.).

Prior to study, cells in log phase growth were counted, collected by centrifugation at 300 *g* for 20 min, and the cell pellet resuspended in Eagle's minimal essential medium (MEM) at pH 7.4 to give a final concentration of 10^6 cells/ml. In experiments using L1210 cells, MEM was supplemented with 0.05 m-moles 2-mercaptoethanol. Aliquots of the cell suspension (10^6 cells) were added to triplicate tubes containing 10 μl of drug solution, and the tubes incubated at 37° with gentle shaking. Thirty min before the reaction was terminated, 10 μl of either [^{3}H]TdR (100 μCi/ml, 6×10^{-6} M) or [^{3}H]UdR (100 μCi/ml, 2×10^{-6} M) were added and the incubation continued. The reaction was terminated by placing the tubes in ice and adding 0.5 ml of ice-cold 10^{-3} M TdR or UdR as appropriate. The tube contents were filtered on Nitrocellulose filters (PHW PO 2400, Millipore Corporation, Bedford, Mass.), rinsed three times with ice cold HBSS, three times with ice cold 10% perchloric acid, and finally once again with HBSS. Filters were dried and left in Aquasol at 4° overnight. For each series of experiments, controls were run in which no drug but equal volumes of saline were added to the tubes. All results of labelled nucleoside incorporation into acid precipitable material were expressed as percentage of control. Internal experimental error was less than 10 per cent. Cell clumping and pH changes throughout the course of an experiment were minimal, and cell viability, assessed by Trypan blue dye exclusion, was in the order of 90 per cent.

RESULTS

Marked inhibition of [^{3}H]UdR incorporation into DNA in the CCRF-CEM cell line occurred with both MTX and DBMTX at two concentrations (10^{-5} M and 10^{-6} M) (Fig. 1). In this cell line, inhibition of

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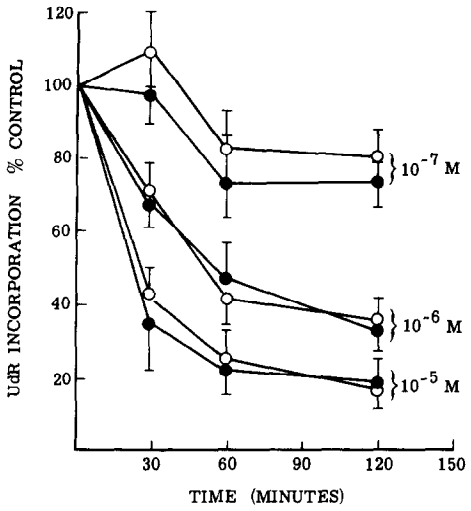

Fig. 1. Inhibition of [^{3}H]UdR incorporation into DNA by MTX (○——○) and DBMTX (●——●) at three drug concentrations (10^{-5}, 10^{-6}, 10^{-7} M) in the CCRF-CEM human lymphoblastic leukaemia cell line. Data from three identical experiments. Bars indicate S.E.M.

[^{3}H]UdR incorporation by either drug at a concentration of 10^{-7} M was minimal. At all concentrations, the effects of MTX and DBMTX were similar and a clear dose–response curve was obtained. Essentially identical data were found in L1210/S (data not shown).

Table 1 shows the kinetics of inhibition of [^{3}H]UdR incorporation in MTX sensitive and resistant L1210 sublines. DBMTX inhibited [^{3}H]UdR incorporation in all three sublines at stated drug concentrations. In the L1210/DHFR cells, inhibition to 39 per cent of control value occurred after 30 min of exposure to DBMTX at 10^{-5} M concentration, and in L1210/TR, the inhibition was to 27 per cent of control value. In resistant sublines, MTX caused no significant inhibition of [^{3}H]UdR incorporation.

Effects of citrovorum factor. Both MTX and DBMTX at a concentration of 10^{-5} M inhibited [^{3}H]UdR incorporation in the L1210/S cell line to less than 40 per cent of control value by 30 min and to less than 10 per cent at 60 and 120 min (Fig. 2). The addition of 10^{-3} M CF at time zero totally prevented the inhibition of [^{3}H]UdR incorporation caused by MTX. However, prevention of DBMTX induced inhibition was only partial and at 120 min, [^{3}H]UdR incorporation was 30 per cent of control. Similar findings were observed with the CCRF-CEM subline (data not shown).

Effects on thymidine incorporation. In the L1210/S cell line, MTX had no inhibitory effect on [^{3}H]TdR incorporation into DNA (Fig. 3). However, profound inhibition of [^{3}H]TdR incorporation was seen with DBMTX; 10^{-5} DBMTX reduced [^{3}H]TdR incorporation to 10 per cent of control value by 30 min, and this inhibition persisted throughout the 2-hr test period. With 10^{-6} M DBMTX, [^{3}H]TdR incorporation was less than 60 per cent of control value. This was sustained throughout the test period and was not influenced by 1×10^{-4} M CF, 1×10^{-4} M folic acid or 2×10^{-5} M adenosine. Similar inhibition of [^{3}H]TdR incorporation was observed in CCRF-CEM cells with DBMTX and with the diethyl ester of MTX. Hydrolysis products of DBMTX (MTX and butanol) did not inhibit [^{3}H]TdR incorporation and their effects were equivalent to MTX alone. Moreover, pre-incubation of DBMTX for 1 hr at 37° in 10% pooled AKR mouse serum (which has high ester-

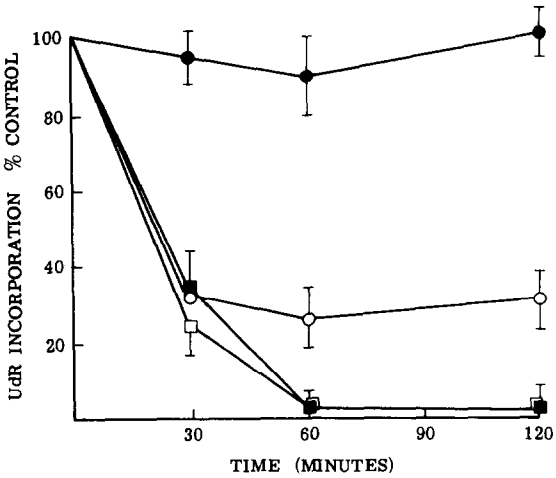

Fig. 2. Effect of 10^{-5} M MTX (□——□) and 10^{-5} M DBMTX (■——■) on [^{3}H]UdR incorporation in the L1210/S cell line. CF (10^{-3} M) was added to two groups of tubes (MTX + CF: ●——●; DBMTX + CF ○——○). Data from four identical experiments. Bars indicate S.E.M.

Table 1. Incorporation into DNA of [^{3}H]UdR in sensitive and resistant L1210 cells in the presence of MTX and DBMTX

Cell line	Concentration (M)	[^{3}H]UdR incorporation (% of control value) Duration of exposure to drug (min)					
		DBMTX			MTX		
		30	60	90	30	60	90
L1210/S	10^{-5}	45	19	22	66	35	25
	10^{-6}	80	33	42	78	38	39
L1210/DHFR	10^{-5}	39	32	20	110	115	78
	10^{-6}	87	76	58	129	89	92
L1210/TR	10^{-5}	27	33	29	82	91	96
	10^{-6}	38	35	42	90	104	92

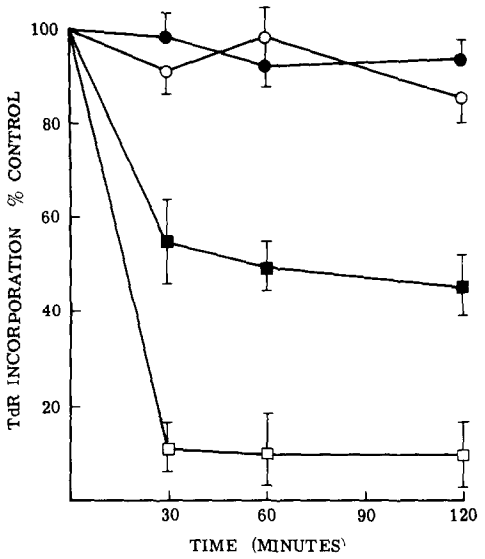

Fig. 3. Effect of MTX and DBMTX on [^{3}H]TdR incorporation in the L1210/S subline. Drug concentrations were MTX 10^{-4} M (●——●); MTX 10^{-5} M (○——○); DBMTX 10^{-5} M (□——□); DBMTX 10^{-6} M (■——■). Data from four identical experiments. Bars indicate S.E.M.

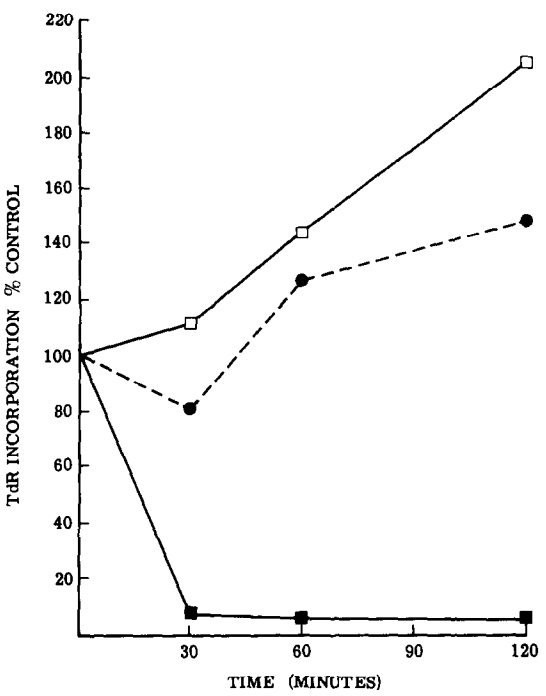

Fig. 4. Effect of serum incubation on [^{3}H]TdR incorporation in the L1210/DHFR subline exposed to DBMTX and MTX. MTX 10^{-5} M (□——□), DBMTX 10^{-5} M (■——■); DBMTX 10^{-5} M preincubated for one hour in 10% pooled AKR serum (●——●).

ase levels) prevented the inhibition of [^{3}H]TdR incorporation (Fig. 4). DBMTX did not affect either [^{3}H]deoxyadenosine incorporation into DNA or [^{3}H]leucine uptake into acid precipitable protein (data not presented).

DISCUSSION

Striking differences between the effects of MTX and DBMTX were observed in these studies. Unlike MTX, DBMTX consistently inhibited [^{3}H]TdR incorporation into DNA in all cell lines. Inhibition of [^{3}H]UdR incorporation in MTX-resistant cells was also observed and this effect was only partially prevented by CF. Preincubation of DBMTX with 10 per cent pooled mouse serum for 1 hr abolished the inhibition of [^{3}H]TdR incorporation caused by DBMTX but the hydrolysis products of DBMTX (MTX and butanol) caused no inhibition of [^{3}H]TdR incorporation.

These results suggest that DBMTX in serum-free conditions penetrates cells and affects [^{3}H]nucleoside metabolism before the drug is completely hydrolysed. The absence of a suppression of [^{3}H]deoxyadenosine incorporation by DBMTX indicates that there is no general disturbance of nucleoside transport in drug-treated cells, and the failure of CF to reverse DBMTX inhibition of [^{3}H]UdR (and [^{3}H]TdR) incorporation suggests that the mechanism of action of DBMTX is not directly related to depletion of reduced folates.

Previous studies by Johns *et al.* [8] suggest that the di-*n*-alkyl esters of MTX exhibit anti-tumour activity in the mouse due to rapid hydrolysis to the parent drug. The plasma esterase activity varies very greatly between species, rodents having very high levels and man lower levels. The experiments reported here indicate that DBMTX may affect [^{3}H]nucleoside metabolism by different mechanisms than MTX. Moreover, our results show that DBMTX inhibits nucleoside metabolism in cells resistant to MTX because of increased DHFR levels and altered drug transport characteristics. Thus, esters of MTX which are active in MTX-resistant cells may have an important clinical role since intrinsic or acquired resistance to MTX frequently limits the usefulness of this drug.

A recent study has reported that a lipid-soluble antifolate drug was active in patients with lung cancer [9]. Moreover, it has recently been reported that MTX esters applied locally are more active than MTX in the inhibition of psoriatic epidermal cell DNA synthesis [10]. The lower plasma esterase level in man suggests rapid hydrolysis of MTX ester is unlikely. Thus the results reported here may have clinical implications with particular reference to MTX-resistant tumours. Further studies are required to determine the mechanism or action of DBMTX, but our results suggest that esters of MTX have a mechanism of action quite different from other antifolate drugs.

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