Biochemical Pharmacology, Vol. 31, No. 2, pp. 263-266, 1982.
Printed in Great Britain.

0006-2952/82/020263-04$03.00/0

PRELIMINARY COMMUNICATIONS

EVIDENCE THAT AT "HIGH" EXTRACELLULAR METHOTREXATE CONCENTRATIONS THE TRANSPORT BARRIER IS UNLIKELY TO BE AN IMPORTANT MECHANISM OF DRUG RESISTANCE

Bridget T. Hill[1], S. Dedhar and J.H. Goldie

Cellular Chemotherapy Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX[1] [BTH] and Division of Advanced Therapeutics, Cancer Control Agency of British Columbia, 2656 Heather Street, Vancouver, B.C. V5Z VJ3, Canada.

(Received 26 October 1981; accepted 4 November 1981)

Goldie et al. [1] recently showed that methotrexate (MTX)-resistant sub-lines of L5178Y cells contain two forms of folate reductase. The principle form is similar to that present in MTX-sensitive cells, binding firmly to the affinity chromotography column and being very sensitive to MTX inhibition. The second form, present in much lower amount, is characterised by poor binding with MTX-sepharose and by marked insensitivity to MTX inhibition with a Ki increased 100,000 fold. The significance of this variant form of folate reductase in relation to other proposed mechanisms of resistance, including increased levels of the enzyme and impaired drug transport [2-4], needs to be established. Whilst MTX-resistant L5178Y cells have been shown to have a transport defect for MTX [5,6], these studies utilised relatively low extracellular drug concns approx (10^{-6}M) which corresponded to the pharmacologically achieved concns used in drug protocols during the 1950's and 1960's. However, it was noted that as extracellular MTX levels increased differences in influx and net transport between drug-resistant and -sensitive cells decreased [6], suggesting the value of high extracellular MTX levels in generating free intracellular drug as an approach to overcoming the permeability defect. Indeed recent clinical studies have employed very marked dose escalations of MTX [7,8]. Since significant inhibition of the low affinity insensitive enzyme in drug-resistant cells occurs only at MTX concns greatly in excess of those required to inhibit the "sensitive" enzyme, impaired MTX transport would further compound attempts to kill these resistant cells. We have therefore examined the uptake of isotopically labelled MTX in drug-resistant L5178Y cells using a "high" extracellular concn of 10^{-4}M, approximately twice that of the ID_{50} (defined as the concn of MTX required to inhibit cell proliferation by 50%) for this cell line. Our results indicate that at these "high" drug concns the transport barrier is overcome in the resistant cells. This suggests that at high MTX levels other properties, such as the amount and type of folate reductase present, may be more important in determining drug sensitivity than the extent of drug uptake.

Materials and Methods

Cell culture - details of the origin and maintenance of these L5178Y cells have been provided recently [1], which differ in a few respects from those used earlier [6]. The parent drug sensitive line was designated S and the resistant line derived from it R3. For logarithmically-growing cells the ID_{50} values for MTX differed by a factor of approximately 1000 being 3×10^{-8}M and 4×10^{-5}M for S and R3 cells respectively. The R3 cells had an elevated level of folate reductase, being 7-fold higher than the S line where the activity, measured as described earlier [1], was 1.0 Units/mg protein.

[1]These studies were initiated during a sabbatical period at the Cancer Control Agency of British Columbia.

Transport studies - The method previously described [9] was used, except that (i) ^{3}H-MTX, sp. act. 250 mCi/mmole (Amersham-Searle Corporation, Illinois, U.S.A.) was added to cells suspended in Fisher's medium without serum and (ii) radioactivity was determined using a Searle Mk.III Liquid Scintillation Spectrometer with a 40% counting efficiency for ^{3}H. MTX was purchased from Lederle Products, Montreal, Canada, DL-N-5-formyl tetrahydrofolic acid (folinic acid) was supplied by ICN Pharmaceuticals, Plainview, N.Y. and DL-N-5-methyl tetrahydrofolate acid (5-CH_3FH_4) and pCMS were obtained from Sigma Chemicals, St. Louis, MO.

Results and Discussion

Fig. 1 shows the uptake of MTX into S and R3 cells at an extracellular concn of 10^{-6}M (Fig.1A) or 10^{-4}M (Fig.1B). At the "low" drug concn the pattern of uptake by S cells is similar to that reported previously [5,6], being rapid during the first 5 min of incubation and then achieving a steady-state level after 20 min. In contrast, MTX uptake by the R3 cells was greatly impaired. Under these conditions, the mean influx for MTX in the S cells was 9.1μM with a Vmax of 0.4 n mole/min 10^{9} cells. We also confirmed (data not included) earlier studies [6] showing that MTX influx into S cells was temperature sensitive and both the initial rate of influx and the steady-state concentration were sensitive to inhibition by pCMS, 5-CH_3FH_4 and folinic acid. These studies could not be carried out using R3 cells because of their limited rate and extent of MTX uptake.

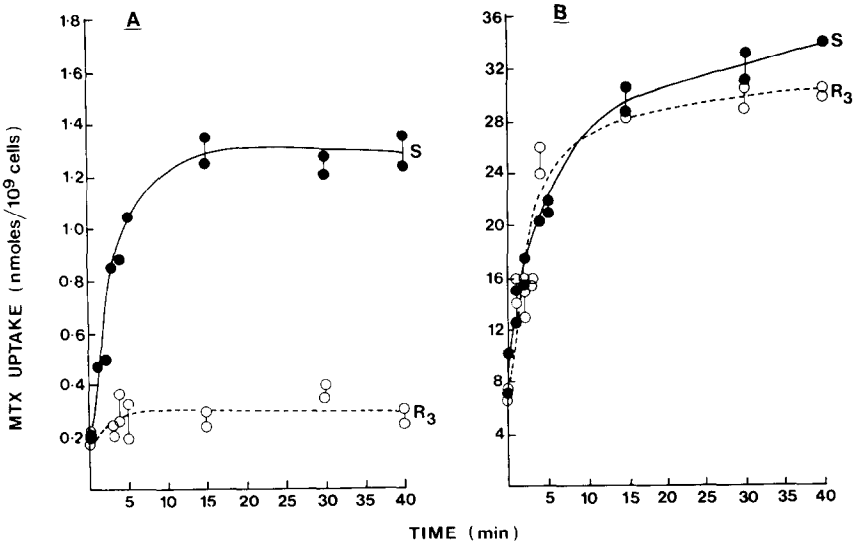

Fig. 1. Time course of uptake of ^{3}H-MTX in MTX sensitive cells (S) and MTX-resistant cells (R3) after exposure to extracellular concns of: A to 10^{-6}M drug and B. to 10^{-4}M drug. Each point represents the range of 5 determinations.

At the "high" drug concn Fig.1B shows that significant amounts of MTX entered both cell lines. At all points measured the amount of MTX associated with both S and R3 cells was nearly identical. At this 10^{-4}M MTX concn not only are higher intracellular levels achieved, but there is also a considerable quantity of drug associated with the cells at

zero time, which is consistent with earlier reports [2,6]. This is considered to represent the component bound to the cell surface or an exceedingly rapid uptake component not measurable under our experimental conditions. Whilst this manuscript was in preparation a publication raised the possibility that the initial, rapid uptake of ^{3}H-MTX might not be MTX but rather a ^{3}H-p-aminobenzoylglutamate, even when the ^{3}H-MTX was greater than 97% homogeneous [10]. In our studies at "high" extracellular concns of MTX the contribution of this initial component was similar in both S and R3 cells. So, even if one were to attempt to correct for the presence of such an impurity, the significant observation that when cells were exposed to 10^{-4}M MTX after 30 min incubation an equivalent amount of radioactivity was associated with both cell types is still valid.

When S or R3 cells were exposed to 10^{-4}M MTX for 2 or 30 min drug uptake was not significantly influenced by 70μM pCMS, 10^{-4}M 5-CH_3FH_4 or 10^{-4}M folinic acid (Table 1). This is in contrast to the marked effects seen in the S cells at the "low" MTX concns where influx was inhibited by 89%, 75% and 60% respectively by these three compounds.

Table 1. Effects of pCMS, 5-CH_3FH_4 or folinic acid addition on ^{3}H-MTX uptake.

	MTX-sensitive cells	MTX-resistant cells
Rate of influx after 2 min incubation with 10^{-4}M ^{3}H-MTX (n moles/min/10^{9} cells)	3.9 ± 0.4	3.6 ± 0.4
Inhibition by pCMS (70μM)	0%	6%
" " 5-CH_3FH_4 (10^{-4}M)	11%	0%
" " folinic acid (10^{-4}M)	0%	7%
Steady-state level after 30 min incubation with 10^{-4}M ^{3}H-MTX (n moles/min/10^{9} cells)	32.3 ± 1.2	29.5 ± 1.3
Inhibition by pCMS (70μM)	0%	15%
" " 5-CH_3FH_4 (10^{-4}M)	10%	5%
" " folinic acid (10^{-4}M)	10%	15%

Each point represent the mean of at least 3 estimations.

Therefore, whilst we have confirmed that at relatively low extracellular MTX concns drug uptake in S cells appears to occur via the carrier mediated active transport process demonstrated in other cell types [2,6,11], at high MTX concns (10^{-4}M), corresponding to levels achieved with high-dose MTX clinical protocols, a different situation is apparent. Under these conditions both drug-sensitive and -resistant lines took up equivalent amounts of MTX. There was also little competition for uptake between MTX and 5-CH_3FH_4 or folinic acid, strongly suggesting that most of the MTX being taken up was not via the physiological carrier that accounts for most transport at low extracellular concns [2]. The different nature of the process of uptake at these high drug concns is also indicated by the markedly reduced sensitivity to pCMS observed.

It is apparent, therefore, that at high MTX concns the transport barrier to drug uptake is unlikely to be an important mechanism of resistance. However, the resistant cells are still markedly less susceptible to MTX cytotoxicity despite the fact that potentially cytocidal intracellular concns of drug can be achieved. As shown earlier [9] a 48 hr exposure to 10^{-4}M MTX produced a 2.6 log cell kill in S cells, but only a 1.5 log cell kill in resistant cells. Therefore, the two further factors known to contribute to drug resistance in these cells, namely (i) the 7-fold increase in levels of intracellular folate reductase and (ii) the presence of an additional variant enzyme characterised by markedly reduced

sensitivity to MTX inhibition, assume increased importance at higher MTX concns. Thus attempts to design new drugs capable of overcoming clinical resistance to MTX might include the preparation of inhibitors effective not only against the "conventional" folate reductase but also against this variant enzyme, if its presence is confirmed in other tumour types.

In summary, the use of high extracellular concns of MTX can overcome the drug transport defect of MTX-resistant cells. However these resistant cells remain less responsive than the parent line to the cytotoxic effects of MTX even after exposure to these very high drug levels. Therefore factors other than drug uptake, for example the presence of the altered form of folate reductase, appear important in assessing response to MTX under these conditions.

Acknowledgements

J.H. Goldie acknowledges the support of th Cancer Control Agency of British Columbia and the National Cancer Institute of Canada.

References

1. J.H. Goldie, G. Krystal, D. Hartley, G. Gudauskas and S. Dedhar, Eur. J. Cancer, 16, 1539 (1980)
2. I.D. Goldman, Cancer Chemother. Rep. 6, (3) 63 (1975)
3. R.C. Jackson, L.I. Hart, and K.R. Harrap, Cancer Res. 36, 1991 (1976)
4. R.J. Kaufman, P.C. Brown and R.D. Schimke, Proc. Nat. Acad. Sci. U.S.A. 76, 5667 (1979)
5. K.R. Harrap, B.T. Hill, M.E. Furness and L.I. Hart, Ann. N.Y. Acad. Sci. 186, 312,(1971)
6. B.T. Hill, B.D. Bailey, J.C. White, and I.D. Goldman, Cancer Res. 39, 2440 (1979)
7. J.H. Goldie, L.A. Price, and K.R. Harrap, Eur. J. Cancer, 8, 409 (1972)
8. W.A. Bleyer, Cancer Treat. Rev. 4, 87 (1977)
9. J.H. Goldie, S. I. Harrison, L.A. Price, and B.T. Hill, Eur. J. Cancer, 11, 627 (1975)
10. B.A. Kamen, A.R. Cashmore, R.N. Dreyer, B.A. Morrison, P. Hsieh, and J.R. Bertino, J. Biol. Chem. 255, 3254 (1978)
11. D. Niethammer and R.C. Jackson, The Chemistry of Biology of Pteridines, L. Kisliuk and G.M. Brown (eds.), Elsevier, p.197 (1978)