Biochemical Pharmacology, Vol. 25, pp. 2755–2757. Pergamon Press, 1976. Printed in Great Britain.

Inhibition by trimethoprim of some effects of epidermal growth factor on human fibroblasts in tissue culture

(*Received* 17 *May* 1976; *accepted* 3 *August* 1976)

Trimethoprim (2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl) pyrimidine, TP) is an antibacterial chemotherapeutic agent with an affinity approximately 50,000 times greater for bacterial dihydrofolate reductase than for the corresponding mammalian enzyme [1]. TP inhibits DNA synthesis in human lymphocytes stimulated to transform into blasts and divide by the plant mitogen concanavalin A [2] and there is some evidence for its efficacy as an immunosuppressive agent [3]. We were therefore interested to determine whether this inhibition is specific to lymphocytes or related to a more general phenomenon. Epidermal growth factor (EGF) is a small polypeptide, of mol wt 6500, extracted from mouse submaxillary gland which stimulates both DNA and RNA synthesis in contact-inhibited human fibroblast cultures *in vitro* [4]. Although evidence is lacking for a specific hormonal function of EGF, a substance with similar immunological properties has been detected in human urine [5] and it may thus be representative of factors present in serum and tissues which promote and modulate normal cell growth.

Normal human diploid fibroblasts, obtained from newborn foreskin or punch-biopsy specimens from adult forearm skin, were cultured in monolayer at 37° in Eagle's minimal essential medium containing Earle's balanced salts with 2 mM glutamine supplemented with 10% (v/v) foetal calf serum. Cultures were incubated at 37° in 5% carbon dioxide in air. Cells were subcultured with 0.25% (w/v) trypsin which was diluted at least 1:40 after the dispersion of the parent monolayer and eliminated in subsequent feedings. No culture had undergone more than twenty passages before use. For experiments cells were subcultured into 16-mm disposable multi-well trays (Linbro Chemical Co. FB 16-25-TC), each well receiving approximately 5×10^4 trypsinised cells in 1 ml medium. After 48–72 hr incubation the medium was replaced by one entirely free of trypsin and after a further 48–72 hr this was replaced by one containing only 5% (v/v) foetal calf serum. Experiments were carried out not earlier than 5 days subsequent to this final feeding after visual inspection of each well to ensure confluency and uniformity of growth. The appropriate EGF and drug concentration were added and the trays were then returned to the incubator. For each well 20 μl of a 1:200 dilution (v/v) in 5% albumin in saline (w/v) EGF was used. The EGF was an electrophoretically homogenous specimen prepared from fresh frozen male mouse submaxillary glands [6] containing 0.47 mg protein/ml and was a kind gift of Dr. M. D. Hollenberg. In all experiments controls were run with the concentration of ethanol used as a vehicle for TP. Since the incubation medium contained 10% foetal calf serum (v/v) TP would presumably be bound to the extent of 42–44% [7]. After 22 hr incubation in the presence of EGF and drug, 1 μCi of [^{3}H]methyl thymidine (6.7 Ci/mM, New England Nuclear Corporation) was added. Following a further 2 hr incubation, the monolayers were washed three times with successive 2-ml aliquots of ice-cold Hank's buffered saline, twice with 2-ml portions of ice-cold 5% trichloracetic acid and once with 2 ml methanol. The remaining precipitated cellular material, which adhered firmly to the well, was solubilised at 70° for 15 min in 0.5 ml of 1 M Hyamine (in methanol) and transferred to scintillation vials with 3 ml ethanol. Radioactivity was measured by liquid scintillation counting, the efficiency for tritium being approximately 20%.

TP at concentrations ranging from 1×10^{-7} to 1×10^{-3} M produced a concentration-dependent inhibition of the increase in [^{3}H]thymidine incorporation produced by EGF as shown in Fig. 1.

The concentration of EGF employed in these experiments produced an approximately 5-fold increase in [^{3}H]-thymidine incorporation compared to control cultures not exposed to EGF. Cell viability was assessed by removal of the supernatant medium and replacement by 0.5 ml of 0.04% trypsin (w/v) in 0.2% (w/v) versene. The cells were freed by agitation and 0.1 ml of 0.4% trypan blue in physiological saline (w/v) added, the viable cells which excluded dye were counted in a haemocytometer. Viability assessed by this technique proved to be in excess of 99 per cent in both control and TP-treated cultures. Cell numbers were estimated by vital staining of the trypsinised cells with crystal violet and counting in the haemocytometer. Each well contained approximately 10,500 cells and no significant difference was found between control cultures and those exposed to 5×10^{-4} TP.

Addition of TP 1 hr before [^{3}H]thymidine after incubation of the monolayers for 21 hr, resulted in significant inhibition only at the highest drug concentrations tested, viz. 1×10^{-4} and 5×10^{-4} M, when the mean incorpor-

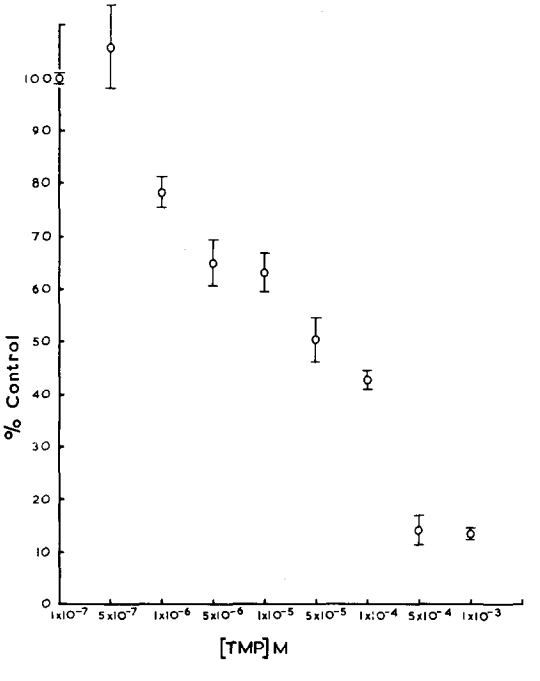

Fig. 1. Incorporation from a 2-hr pulse of [^{3}H]thymidine into fibroblast cultures after 24 hr incubation in the presence of EGF and different TP concentrations. Incorporation expressed as percentage of the control incorporation in the absence of the drug. Points represent the means for 9–15 replicate cultures; bars represent S.E.M.

Table 1. Removal of TP from medium after 22 hr incubation and replacement by either plain medium or TP-containing medium followed by 2-hr incubation with 1 μCi [^{3}H]thymidine

	cpm	S.E.M.	N	% Control
Control	3016	605	6	100
1×10^{-4} M TP	1165	281	6	39
1×10^{-4} M TP washed off	2337	206	6	77
5×10^{-5} M TP	1848	400	6	61
5×10^{-5} M TP washed off	3687	617	6	122

ations were 62 and 38 per cent of control (c.f. 43 and 14 per cent when drug was present throughout incubation). This observation is also indirect evidence that TP does not inhibit thymidine uptake by competition for cellular transport for if this were the case then the inhibition in these two circumstances might be expected to be similar. The effect of TP is at least partially reversible. Cells were incubated in the presence of TP and then the drug-containing medium removed and replaced by fresh medium which in some cultures contained the same concentration of TP as had been present for the preceding 22 hr, so that apart from the brief wash period TP had been present throughout. The results of subsequent labelling with a 2-hr pulse of [^{3}H]thymidine are shown in Table 1.

An early event in the stimulation of fibroblasts by EGF is the transport of amino acids into the cell which occurs some hours before thymidine incorporation becomes maximal. This can be followed by determining the uptake of 2-aminoisobutyric acid, which is not metabolised by the cell. In these experiments the fibroblast monolayers were washed with 1 ml of 0.1 M pH 7.4 Tris–HCl buffer in Earle's balanced salt solution containing 0.1% albumin (w/v) and then incubated for 2 hr at 37°. This medium was aspirated and replaced by 0.5 ml of the same mixture containing a suitable EGF concentration. TP was added at this juncture and the cells again incubated for 2 hr at 37°. One μCi [^{3}H]aminoisobutyric acid (3.5 Ci/mM: New England Nuclear Corporation) was added for a 12-min pulse in room air at 37°. Controls with and without EGF were inserted at the beginning and end of each series of experiments to allow for any possible alteration in pH resulting from the transfer from the incubator to room air which might alter the uptake of aminoisobutyric acid. The uptake was terminated by washing the monolayers three times with 1 ml ice-cold Earle's salt solution. The cells were dissolved by heating for 10 min at 70° with 0.4 ml 0.2 M sodium hydroxide and the solution transferred to scintillation vials with 0.4 ml of 0.2 M hydrochloric acid. The radioactivity was counted following addition of scintillation fluid and the results are shown in Table 2.

In no case was the uptake of amino-isobutyric acid significantly affected by TP.

Inhibition of dihydrofolate reductase would reduce the availability of thymidine for DNA synthesis and also block mitochondrial protein synthesis which requires formyl-methionyl-t-RNA. Both processes are prerequisites for normal mitotic division in fibroblasts [8]. An indirect estimate of dihydrofolate reductase activity *in vivo* was made by

Table 2. Effect of TP on amino-isobutyric acid uptake by human fibroblasts after 2 hr stimulation by EGF

TP	% Control uptake	SEM	N
5×10^{-4} M	109.5	5.1	10
1×10^{-4} M	109.0	8.2	13
5×10^{-5} M	114.9	4.5	11
1×10^{-5} M	108.9	10.6	10

Results expressed as percentage of control uptake determined during that experimental run.

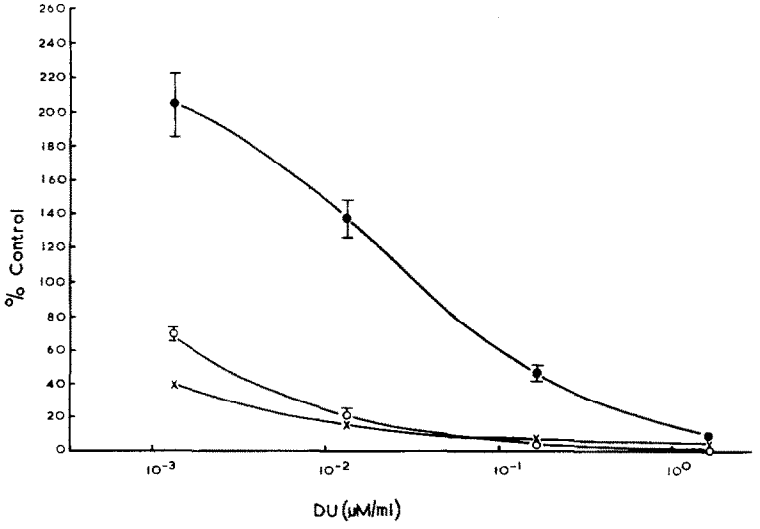

Fig. 2. Incorporation of [^{3}H]thymidine into EGF stimulated fibroblasts in the presence of different deoxyuridine concentrations. Cultures were incubated for 1 hr with deoxyuridine in the presence of 1×10^{-6} M methotrexate (●——●), 1×10^{-4} M TP (×——×) or diluent (○——○). The ordinate is [^{3}H]thymidine uptake expressed as a percentage of uptake in the absence of deoxyuridine; the abscissa is deoxyuridine concentration. Points are means of 4–6 replicate cultures; bars represent S.E.M.

the method of Metz *et al.* [9] in which varying concentrations of 2-deoxyuridine were added to monolayer cultures stimulated 22 hr previously with EGF in the presence of methotrexate or TP. After 1 hr incubation at 37°, 1 μCi [^{3}H]thymidine was added and after a further 2 hr incubation the cells were processed and harvested as described previously. The results are shown in Fig. 2.

Methotrexate is a pseudo-irreversible inhibitor of dihydrofolate reductase with high affinity for the enzyme so that the synthesis of thymidylate from deoxyuridine is suppressed thereby reducing the size of the intracellular thymidine pool. Consequently the incorporation of the exogenous [^{3}H]thymidine is increased. TMP on the other hand, does not significantly change the incorporation of [^{3}H]thymidine in this system. In two studies on normal human bone marrow a small inhibitory effect on dihydrofolate reductase has been noted at 1×10^{-4} M TP [10, 11] although another study only detected inhibition at 1×10^{-1} M [12].

This preliminary data would indicate that TP does not act to inhibit thymidine incorporation into EGF-stimulated human fibroblasts by virtue of an inhibition of dihydrofolate reductase. TP is a lipophilic compound and could well act directly at the cell membrane: if this is the case however it apparently does not affect the active uptake of amino acids which occurs during cell stimulation by EGF.

Acknowledgements—We are indebted to Dr. Morley Hollenberg for the gift of EGF and for much helpful advice and discussion. H. J. R. was a Wellcome travelling research fellow of the M.R.C.

* Address reprint requests to Dr. H. J. Rogers, Department of Pharmacology, Guy's Hospital Medical School, St. Thomas' Street, London, SE1 9RT.

Division of Clinical Pharmacology, Department of Medicine and Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.

HOWARD J. ROGERS*
PAUL S. LIETMAN

REFERENCES

1. J. J. Burchall, *Ann. N.Y. Acad. Sci.* **186,** 143 (1971).
2. H. J. Rogers and P. S. Lietman, *Clin. Pharmac. Ther.* **17,** 242 (1975).
3. M. W. Ghilchik, A. S. Morris and D. S. Reeves, *Nature, Lond.* **227,** 393 (1970).
4. M. D. Hollenberg and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **70,** 2964 (1974).
5. R. H. Starkey, S. Cohen and D. N. Orth, *Clin. Res.* **22,** 350A (1974).
6. C. R. Savage and S. Cohen, *J. biol. Chem.* **247,** 7609 (1972).
7. D. E. Schwartz and W. H. Ziegler, *Postgrad. med. J.* **45,** Suppl. 32, (1969).
8. U. Mittwoch, D. J. Kirk and D. Wilkie, *J. med. Genet.* **11,** 260 (1974).
9. J. Metz, A. Kelly, V. C. Swett, S. Waxman and V. Herbert, *Br. J. Haematol.* **14,** 575 (1968).
10. J. Sive, R. Green and J. Metz, *J. clin. Path.* **25,** 194 (1972).
11. J. Koutts, M. B. Van der Weyden and M. Cooper, *Aust. N.Z. J. Med.* **3,** 245 (1973).
12. S. Waxman, *Clin. Res.* **19,** 434 (1971).

Biochemical Pharmacology, Vol. 25, pp. 2757–2759. Pergamon Press, 1976. Printed in Great Britain.

Subcellular site of acetaldehyde oxidation in monkey liver

(*Received* 17 *March* 1976; *accepted* 11 *June* 1976)

Until recently acetaldehyde was believed to be oxidized in the cytosol of the liver [1,2]. Evidence has now accumulated indicating that its oxidation occurs in mitochondria. Mitochondrial activities have been demonstrated in quantity and with kinetic characteristics adequate to account for acetaldehyde oxidation under most conditions [3–6]. Parrilla *et al.* [7], from changes in the state of reduction of cytosolic and mitochondrial nucleotides when acetaldehyde is utilized by perfused liver and isolated liver cells, concluded that acetaldehyde is oxidized to acetate predominantly in mitochondria. Rognstad and Clark [8], using isolated liver cells, and our laboratory [9], using liver slices and tracing with ^{3}H the fate of the *R* and *S* hydrogens of ethanol, have also arrived at this conclusion. Similarly Grunnet [10], from incorporation by hepatocytes of ^{3}H from [1,1-^{3}H-]ethanol into lactate and β-hydroxybutyrate, have concluded that acetaldehyde oxidation occurs in the mitochondria as well as in cytosolic compartments of the liver cell. In all these studies, the rat was the source for liver. Cytosolic and mitochondrial aldehyde dehydrogenases have been demonstrated to be in horse liver [11] and aldehyde dehydrogenases have been purified from livers of other species, including man and mouse [3], but their physiological role is uncertain.

We have, using slices of monkey liver, now obtained evidence that in this primate acetaldehyde oxidation is also predominantly in the mitochondrial compartment. In our approach [9], the *R* hydrogen of ethanol is accepted to be transferred to NAD with the formation of acetaldehyde, catalyzed by cytosolic alcohol dehydrogenase, and the *S* hydrogen to be transferred to NAD by acetaldehyde dehydrogenase either in the cytosolic and/or mitochondrial compartments. We have compared the incorporation of ^{3}H of [*R*1-^{3}H]ethanol and [*S*1-^{3}H]ethanol into lactate, lactate dehydrogenase being a cytosolic enzyme, and water, since NADH is oxidized via the electron transport system of the mitochondrion.

Livers were removed from three *Macaca fasicularis* (monkeys 1–3 of Table 1) and one *Macaca mulatta* (monkey 4 of Table 1). The first two monkeys were killed under ketamine anesthesia with a lethal dose of nembutal, the third was killed under ketamine anesthesia by exsanguination, and the fourth was under nembutal anesthesia at the time the liver was removed. Each was fasted overnight and