Biochemical Pharmacology, Vol. 21, pp. 891–894. Pergamon Press, 1972. Printed in Great Britain

SHORT COMMUNICATIONS

Depressed incorporation of purine derivatives into malarial parasite ribonucleic acid by known ribonucleic acid polymerase-inhibiting drugs*†

(*Received* 18 *June* 1971; *accepted* 22 *October* 1971)

STUDIES of mammalian and bacterial RNA polymerase have yielded valuable chemotherapeutic information. However, extracted and purified enzyme often manifests artifactual and methodological problems (including disarrangement of control proteins, templates and polymerases), which obscure the interpretation of many studies on the synthesis of RNA.[1] To avoid the problems inherent in enzyme purification procedures, we developed a system[2] utilizing malarial parasites (*Plasmodium berghei*) released from their host cells (reticulocytes) to study plasmodial nucleic acid metabolism. Previous studies[3] of traditional antimalarials suggested that drugs which intercalate[4–6] into DNA were potent inhibitors *in vitro* of plasmodial nucleic acid anabolism. This report describes studies on the ion dependency of the incorporation of tritiated purines into parasite RNA, as well as experiments designed to measure the potency of various known inhibitors of bacterial and mammalian RNA polymerase in the erythrocyte-free parasite system.[2]

Materials and Methods

P. berghei-infected rat red blood cells (maintenance of parasites in rats reported previously)[2,3,7] were lysed in a solution (0·1% w/v) of saponin in Krebs' phosphate buffer (calcium ion omitted) according to procedures detailed earlier[2,3] so that 1 cc of packed parasitized red blood cells was incubated with 40 cc of saponin solution. Incubation mixtures consisted of the following: 1 ml of free parasite solution; 2·5 μc of AMP-8-^{3}H (17·2 c/m-mole) in Krebs' phosphate buffer (KB); 1 ml of drug dissolved in KB; or 1 ml of KB. Optimal incubation conditions (pH 7·4, temperature 37°) and linear conditions for the incorporation of radioactivity into RNA (determined in previous work[2]) were used in the present experiments. Thus, incubations were terminated with trichloroacetic acid (10% w/v, TCA) after 15 min (midway on the linear portion of the time course of incorporation of radioactivity into RNA). Details of the washing procedure for TCA-insoluble material, specific degradation of RNA by KOH (1 N) hydrolysis and degradation of DNA by acid-heat hydrolysis (as modified from standard biochemical methods),[8] as well as counting of radioactivity by liquid scintillation spectrophotometry, were the same as in previous work.[2,3,7] It should be noted that the choice of precursor (AMP-8-^{3}H) was made following experiments[2] on the whole series of adenine derivatives and that, in the serum containing incubation medium, the nucleotide appears to serve as a protected, storage form of the nucleoside (adenosine-8-^{3}H) which penetrates the parasite membrane. Chromatographic studies on intraerythrocytic malaria[9] and erythrocyte-free parasites[2] indicate that very little metabolic interconversion of adenosine occurs and that 95 per cent of total incorporation of radioactive purine is into parasite RNA with only 5 per cent of total incorporation into parasite DNA.

Results and discussion

A valuable concept in chemotherapy is the inhibition of pathogen nucleic acid anabolism exerted by certain drugs which insert between the base pairs of the DNA helix and thus seem to interfere with DNA-dependent RNA polymerization through the mechanism of intercalation.[4–6] It is important to note that in the present system nearly all of the incorporation of label from exogenous AMP-8-^{3}H is associated with parasite RNA (95 per cent of total) while only a small portion of the total incorporation is associated with DNA (5 per cent).[2,10] Further, as indicated by autoradiographic[9,10] and biochemical[7] studies, the malarial parasite is the only source in this system to which one may

* This study represents a portion of the work carried out by C. H. Lantz in partial fulfillment of the requirements for the degree of Doctor of Philosophy at West Virginia University.

† This work was supported by a West Virginia University School of Medicine Institutional General Research Support Grant and by a West Virginia University Senate Grant. This investigation received financial support from the World Health Organization.

attribute incorporation of radioactivity from exogenous AMP-8-^{3}H (since white blood cells and reticulocytes are not significantly active in this regard). It is possible that some compounds (e.g. EDTA) may interfere with nucleotidase activity, preventing the dephosphorylation of AMP-8-^{3}H to adenosine-8-^{3}H and thereby causing a decrease in the uptake of labeled precursor into the free parasites. However, we feel that this alternative is unlikely and furthermore, none of the drugs studied inhibit phosphorylation or interfere with other processes which might reflect a decreased incorporation of label into parasite RNA.[11]

Recent work[12] indicated that some species of malarial parasites may have a higher base pair ratio of A:T than G:C in their DNA. Actinomycin-D which is an intercalating antibiotic[13] with an apparent high affinity for guanosine binding[5] or G:C base pair binding is the most potent inhibitor (Fig. 1)

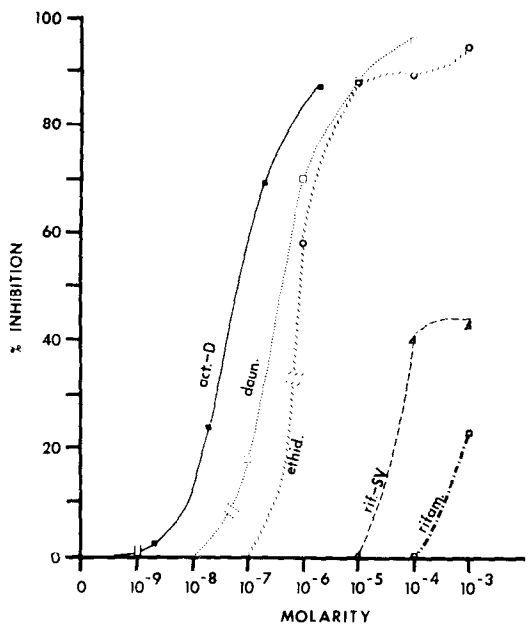

FIG. 1. Inhibition of incorporation of radioactivity from AMP-8-^{3}H into RNA of erythrocyte-free *P. berghei* by actinomycin-D (act.-D), daunomycin (daun.), ethidium bromide (ethid.), rifamycin SV (rif.-SV) or rifampicin (rifam.). Incubation (15 min) of 1 ml of erythrocyte-free parasites at 37° and pH 7·4 with 2·5 μc of AMP-8-^{3}H and 1 ml of Krebs' phosphate buffer yielded 248,900 dis./min incorporated into RNA.

of incorporation of label into plasmodial RNA among numerous traditional antimalarials[14] and experimental compounds tested in this system. Note that extremely small concentrations for this system (2×10^{-9} to 2×10^{-8} M) begin to inhibit incorporation into RNA (by 25 per cent) and that marked inhibition (70 per cent) occurs in the presence of 2×10^{-7} M actinomycin-D. The postulated mechanism of action of actinomycin-D involved a binding to the DNA template with steric inhibition[15] of RNA polymerase.[16] Recent X-ray crystallographic studies[5] confirm such a binding, and we believe the potency of this drug against plasmodial nucleic acid anabolism to be a result of intercalation.[4] The logical extension of such a conclusion is that, in the absence of drug, the added tritiated nucleotide is utilized for DNA-dependent RNA polymerization.

Rifampicin, a drug which appears to bind to bacterial RNA polymerase and thus inhibits it directly,[17] is remarkably less potent against precursor incorporation into plasmodial RNA when compared to the three intercalating drugs (Fig. 1). Extremely high concentrations of rifampicin (10^{-3} M) are necessary to produce inhibition of precursor incorporation by 25 per cent. Similarly, rifamycin SV is relatively ineffective since inhibition begins at a dose between 10^{-5} to 10^{-4} M but only reaches its maximum (42 per cent) in the presence of 10^{-3} M drug. It is noteworthy that both of these direct inhibitors do exert depression of plasmodial RNA anabolism to some extent. However, their actions at relatively high doses are in marked contrast to the potent actions of actinomycin-D, daunomycin

or ethidium (all intercalators), which begin to inhibit at much lower concentrations. Although our measurements were against an "apparent" RNA polymerase activity, comparison of rifamycin SV potency in a "specific" *E. coli* polymerase system[17] shows 50 per cent inhibition of the bacterial enzyme at a concentration of 2×10^{-8} M. This drug is obviously much less potent against incorporation of label into RNA of erythrocyte-free parasites (and presumably against the plasmodial enzyme responsible for this activity). Thus, the ability of the drug to interact with the plasmodial enzyme may differ in some respect from the bacterial case.

The chelating agent, ethylenediamine tetra-acetic acid (EDTA), was studied to determine if removal of endogenous, divalent ions would inhibit precursor incorporation into plasmodial RNA.

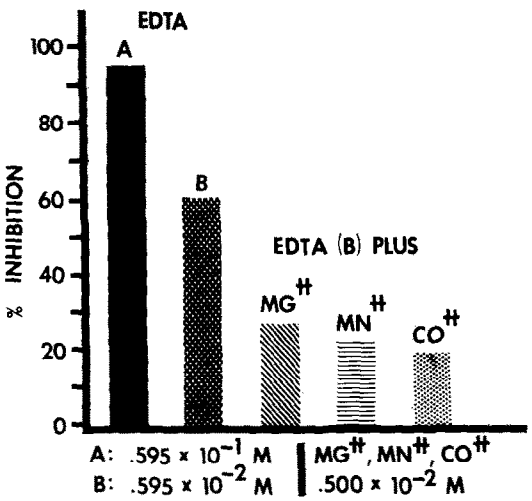

FIG. 2. Inhibition of incorporation of label from exogenous AMP-8-^{3}H into RNA of erythrocyte-free *P. berghei* by the chelating agent ethylenediamine tetra-acetic acid (EDTA). EDTA was present in A: $5{\cdot}95 \times 10^{-2}$ M and B: $5{\cdot}95 \times 10^{-3}$ M concentrations. In the last three bars, EDTA (concentration B) and one exogenous ion (Mg^{2+}, Mn^{2+}, or Co^{2+} at $0{\cdot}5 \times 10^{-2}$ M) were present. Incubation was at 37°, pH 7·4, for 15 min. The control (no EDTA or exogneous divalent ion) incorporated 245,340 dis./min into RNA.

Figure 2 shows that incubation of free parasites with a high concentration of EDTA (bar A) essentially eliminates incorporation into plasmodial RNA (95 per cent inhibition). Incubation with a lesser concentration (bar B) of this chelating agent produces 60 per cent inhibition. Addition of Mg^{2+} to the reaction mixture reduces inhibition by concentration B of EDTA to only 28 per cent. Still less inhibition (21 per cent) of incorporation into RNA by EDTA occurs when Mn^{2+} is added. The least inhibition (19 per cent) by EDTA occurs in the presence of exogenous Co^{2+}. Although it is possible that the inhibition of incorporation of label into RNA by EDTA results from decreased nucleotidase activity (thereby reducing the uptake of nucleoside into parasites), we feel that this alternative is unlikely. The potency of Co^{2+}, Mn^{2+}, and Mg^{2+} in reversing EDTA inhibition of precursor incorporation into plasmodial RNA does not differ greatly. However, it appears that this reversal may be greatest when exogenous Co^{2+} is added. This finding may be somewhat in contrast to the ion sensitivity of mammalian nuclear aggregate[18] and bacterial polymerase[16] in which Mn^{2+} and Mg^{2+} were more stimulatory.

P. berghei is often a reliable model for predicting the effectiveness of drugs against human malaria,[19] which persists as an infectious disease that burdens many developing countries throughout the world.[20] The erythrocyte-free malarial parasite system used in this study makes possible new and potentially useful chemotherapeutic observations about the ion and drug sensitivity of this model's nucleic acid anabolism.

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Biochemical Pharmacology, Vol. 21, pp. 894–896. Pergamon Press, 1972. Printed in Great Britain

Apparent increases in tumour NAD^+ levels induced by treatment with vitamin K_1 or its synthetic substitutes

(*Received* 11 *August* 1971; *accepted* 8 *November* 1971)

IT HAS been shown that treatment with Synkavit (tetrasodium salt of 2 methyl 1,4-naphthohydroquinone diphosphate), a synthetic substitute for the natural vitamin K_1, causes a statistically significant rise in the phosphate levels of tumours.[1] Further work has now shown that both vitamin K_1 and Menadione (2 methyl 1,4-naphthoquinone bisulphite) will also cause temporary increases in the phosphate levels of a variety of transplanted tumours. Similar peaks in phosphate levels can be induced by agents, such as nicotinamide or 3 acetyl pyridine, which cause a new synthesis of NAD^+ (nicotinamide adenine dinucleotide, diphosphopyridine nucleotide or coenzyme I).[2] Since new NAD^+ synthesis and increased phosphate levels have been associated with increased radio-responsiveness[2] we have been prompted to examine the effects of vitamin K_1, Synkavit and Menadione on tumour NAD^+ levels as all three compounds are claimed radiosensitizing agents.

All experiments have been carried out with Balb/c or CBA mice aged 12–14 weeks. Animals of either sex were used according to availability. Solid tumours were transplanted in the flanks of animals and only used when they had grown to a volume of 1 cm^3. The ascites tumour was used 5 days after the intraperitoneal transplant of 1×10^6 cells.

Details of the tumours used are given in Table 1.

TABLE 1. TUMOURS USED

Mouse strain	Tumour	Tumour type
Balb/c	NK/Ly/R	Lymphoma (ascitic) Resistant form of Lymphoma NK/Ly
Balb/c	ADJ/PC5	Plasma cell tumour
Balb/c	PL 64	Carcinoma (skin)
Balb/c	H.P.	Harding Passey melanoma
Balb/c	S.180	Crocker sarcoma
Balb/c	Bp 64/12	Sarcoma
CBA	Bp 65/2	Spindle celled sarcoma