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INHIBITION OF PRECURSOR INCORPORATION INTO NUCLEIC ACIDS OF MAMMALIAN TISSUES BY ANTIMALARIAL AMINOQUINOLINES

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1 Chloroquine, primaquine and ethidium inhibited thymidine incorporation into deoxyribonucleic acid of rat tissues when administered concurrently with the labelled precursor.

2 Chloroquine and primaquine inhibited the incorporation of uridine and adenine, but not orotate, into various ribonucleic acid fractions of liver of rats and mice. These drugs had no effect on leucine incorporation into hepatic protein in rats or mice.

3 Although chloroquine and primaquine are active against different stages in the life cycle of the malarial parasites, the two aminoquinolines exert similar effects in rodent tissues.

Introduction

The antimalarial 4-aminoquinolines (e.g., chloroquine) and 8-aminoquinolines (e.g., primaquine) interact with deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and nucleoproteins of mammalian cells (Holbrook, Whichard, Morris & White, 1971; Washington, White & Holbrook, 1973). The interaction with DNA may result in the inhibition of replication or transcription, and the interaction with RNA may result in the inhibition of translation. Chloroquine and primaquine inhibit the incorporation of radioactive precursors into DNA and/or RNA in non-mammalian eukaryotic cells (Schellenberg & Coatney, 1961; Polet & Barr, 1968; Van Dyke, Szustkiewicz, Lantz & Saxe, 1969; Conklin & Chou, 1972a) and in cultured mammalian cells (Gabourel, 1963; Epstein, Fukuyama & Epstein, 1971). *In vitro*, chloroquine and primaquine inhibit DNA and RNA polymerases from bacterial sources (Cohen & Yielding, 1965; O'Brien, Olenick & Hahn, 1966; Whichard, Washington & Holbrook, 1972; Marquez, Cranston, Ruddon & Burckhalter, 1974) and from *Tetrahymena pyriformis* (Conklin, Heu and Chou, 1973). Moreover, various steps in polypeptide synthesis in eukaryotic cells are inhibited by chloroquine or primaquine (Ilan & Ilan, 1969; Landez, Roskoski & Coppoc, 1969; Roskoski & Jaskunas, 1972; Lefler, Lilja & Holbrook, 1973). Modification of other processes by aminoquinolines may indirectly decrease the incorporation of precursors into DNA, RNA or protein (Beaudoin & Aikawa, 1968; Skelton, Pardini, Heidker & Folkers, 1968; Carter & Van Dyke, 1972; Conklin & Chou, 1972a & b).

The effects of chloroquine and primaquine on precursor incorporation into DNA, RNA and protein have been studied in mammalian tissues to examine their possible mechanisms of toxicity.

Methods

Animals

Male albino rats (Sprague-Dawley strain) were purchased from Zivic-Miller Laboratories and male albino mice (CF-1 strain) from Carworth Farms. Adrenalectomies were performed at least 48 h before drug treatment. All injections of radioactive precursors and of drugs were given intraperitoneally. In studies of the incorporation of radioactive precursors, all drugs were administered at doses appreciably below lethal amounts; no deaths occurred.

Incorporation of thymidine into DNA and into perchloric acid-soluble fraction

The tissue was homogenized in cold 1 M $HClO_4$ and centrifuged. An aliquot of the supernatant was used for radioactivity measurement in the acid-soluble fraction. The remainder of the supernatant was neutralized with KOH and, after precipitation of $KClO_4$,

was used for thin layer chromatography. RNA in the original tissue precipitate was hydrolyzed in 0.5 M NaOH (2.5 h, 70°C). DNA and protein were reprecipitated with cold $HClO_4$. DNA was hydrolyzed in 0.5 M $HClO_4$ (20 min, 90°C) and determined by ultraviolet absorbance at 268 nm.

Incorporation of precursors into RNA

The liver homogenate was mixed with an equal volume of cold 2 M $HClO_4$. The supernatant was used to determine the radioactivity of the *acid*-soluble fraction. The *total* cellular RNA was obtained from the pellet. Nuclei were isolated from an aliquot of the original liver homogenate by centrifugation through a layer of 48% sucrose. The nuclear RNA was extracted from the pellet. In some studies in mice, a liver sample was homogenized and centrifuged (10 min at 28,000 *g*-max). Sodium deoxycholate (final concentration of 0.7%) was added to the supernatant suspension. The suspension was placed over a discontinuous gradient with layers containing 1.6 M and 2.0 M sucrose and centrifuged for 3 h at 129,000 *g*-av. The pellet contained the ribosomal RNA.

After washing with cold 0.5 M $HClO_4$, the pellets containing the RNA fractions were incubated with 0.3 M NaOH (37°C, 1 hour). Unhydrolyzed material was reprecipitated with cold $HClO_4$. After centrifugation, aliquots of the supernatant were used for radioactivity measurements and for the determination of RNA by the ultraviolet absorbance at 260 nm or by the orcinol reaction.

Incorporation of leucine into protein

Nucleic acids were hydrolyzed in 0.5 M $HClO_4$ (90°C, 20 minutes). The protein was collected by centrifugation and washed in succession with 0.5 M $HClO_4$, 95% ethanol, a warm ethanol-diethyl ether (3:1) mixture, and finally, warm ether. Pellets were dissolved in 1 M NaOH. Aliquots were used for radioactivity measurement and for protein analysis (Lowry, Rosebrough, Farr & Randall, 1951). In studies on the effects of chloroquine and primaquine on leucine incorporation into the total cellular protein in rat and mouse liver, the aminoquinolines and radioactive leucine were injected at 3 h and 1 h, respectively, before removal of the tissue.

Chromatography of cold $HClO_4$-soluble fraction

The cold $HClO_4$-soluble fraction from thymidine-injected rats was subjected to thin layer chromatography on precoated polyethyleneimine-cellulose sheets (Macherey-Nagel MN-Polygram Cel 300-PEI obtained, from Brinkmann Instruments). Development of the chromatograms with 0.02 M ammonium acetate: 95% ethanol mixture (1:1, v/v) caused the migration of thymidine plus thymine near the solvent front whereas the thymidine phosphates remained at or near the origin.

Materials

[8-^{14}C]-adenine (sp. act. 50 mCi/mmol) and [methyl-^{3}H]-thymidine (3.0 Ci/mmol) were obtained from Schwarz/Mann; [2-^{14}C]-uridine (55 mCi/mmol) and [5-^{3}H]-uridine (5.0 Ci/mmol) from Amersham/Searle; [6-^{14}C]-orotic acid (53 mCi/mmol) from New England Nuclear; chloroquine diphosphate, primaquine diphosphate, and ethidium bromide were obtained from Sigma Chemical Co.

Table 1 Effect of chloroquine and primaquine on thymidine incorporation and uptake[a]

Treatment (μmol/kg)	Number of rats	Liver		Spleen		Kidney	
		DNA[b]	Acid-soluble[b]	DNA[b]	Acid-soluble[b]	DNA[b]	Acid-soluble[b]
Control	20[c]	100 ± 18	100 ± 5	100 ± 11	100 ± 8	100 ± 15	100 ± 6
Chloroquine, 50	8–9	48 ± 12*	84 ± 8	54 ± 14*	76 ± 8*	40 ± 10**	79 ± 12
Chloroquine, 100	12[d]	47 ± 6**	119 ± 8†	79 ± 11	121 ± 14	71 ± 11	117 ± 8†
Primaquine, 100	8–9	62 ± 13†	85 ± 6†	53 ± 9**	77 ± 4*	54 ± 10*	74 ± 9*
Primaquine, 150	12–13	71 ± 16	90 ± 8	68 ± 14†	86 ± 9	49 ± 9**	90 ± 10

[a] The aminoquinoline and the [^{3}H-methyl]-thymidine (18 μCi/100 g body weight) were injected concurrently 1.0 h before removal of the tissues. [b] Units: data are expressed as percentage of the mean control (mean ± s.e.). DNA (ct min^{-1} μmol^{-1} DNA-nucleotide) of controls were 2160, 3520 and 906 in liver, spleen and kidney, respectively. Acid-soluble fraction (total ct/min in acid-soluble fraction derived from tissue containing 1 μmol DNA-nucleotide) of controls were 20.5, 3.17 and 15.6 in liver, spleen and kidney, respectively. [c] 25–26 rats for DNA samples. [d] 17–19 rats for DNA samples.
Statistical analysis (Student's *t*-test): **, $P < 0.01$; *, $P < 0.05$; †, $0.05 < P < 0.10$.

Results

Thymidine incorporation

Chloroquine and primaquine decreased thymidine incorporation into DNA of liver, spleen and kidney (Table 1). In some instances, the aminoquinolines decreased thymidine uptake into the acid-soluble fraction but such decreases were appreciably less than the corresponding decreases in thymidine incorporation into the DNA (Table 1). Thus, it appears that a decreased thymidine uptake into tissues may have contributed to the decreased thymidine incorporation into DNA in a few instances. After the cold-$HClO_4$-soluble fraction of liver or spleen had been subjected to thin layer chromatography, 60 to 75% of the radioactivity was within the area of the thymidine plus thymine in both control and primaquine-treated rats and essentially all of the remaining radioactivity on the chromatograms was within the area of the thymidine phosphates. Thus, primaquine did not alter either the percentage distribution of radioactivity in the pools of the thymidine plus thymine and the thymidine phosphates, or the conversion of thymidine to its phosphorylated products.

The concurrent administration of ethidium (50, 100, 150 or 200 μmol/kg) with radioactive thymidine decreased the thymidine incorporation into DNA of liver by 31 to 36% and into DNA of spleen by 39 to 47% (data not tabulated), the effect showing no graduation with dosage. Ethidium had no effect on thymidine uptake into the acid-soluble fraction.

Incorporation of uridine, orotate and adenine

In experiments in which various doses and durations of treatment were used, chloroquine and primaquine markedly inhibited uridine incorporation into total cellular RNA, total RNA of isolated ribosomes, and nuclear RNA of mouse liver (Table 2). When chloroquine was administered daily for three days, uridine incorporation into total cellular RNA and RNA of

Table 2 Effects of chloroquine and primaquine on uridine incorporation into RNA of mouse liver

Expt	Amino-quinoline	Duration of treatment	Dose (μmol/kg)	Uridine incorporation period (h)	Mean % inhibition of incorporation of uridine (cf. Control)		
					Total cellular RNA	RNA of ribosomes	Nuclear RNA
A	Chloroquine	3 days	38	2	44†	61*	
			56		20	54†	
			75		51*	74**	
			94		54**	64*	
B	Primaquine	3 days	46	2	1	—	
			69		29†	27	
			92		46*	52*	
			116		39**	55*	
C	Primaquine	18 h	46	2	26	39†	
			69		22†	17	
			92		30*	34†	
			116		53**	55**	
D	Primaquine	3 h	46	2	47*	51**	
			69		39*	51*	
			92		35**	40*	
			116		54**	63**	
E	Primaquine	3 h	92	1	53*	60*	47*
			116		68*	51	76**
		3 h	92	0.5	34†	72*	40*
			116		61*	75*	60**
		3 h	92	0.25	38†	52	46

There were 6–7 and 22 control mice in experiments A and B–D, respectively (each using [^{3}H]-uridine); and 8, 3 and 5 control mice in the incorporation studies for 1, 0.5, and 0.25 h, respectively, in experiment E (using [^{14}C]-uridine). There were 3–4 mice in each drug-treated group. In expts A and B, the drugs were administered for 3 days (i.e., at 70, 46 and 22 h before [^{3}H]-uridine administration) with each daily dose at the indicated dose level. The statistical analysis and symbols are as given in Table 1.

ribosomes was inhibited by approximately 40 to 60% (Table 2, Expt A). Primaquine, at its higher doses, inhibited uridine incorporation into total cellular or ribosomal RNA by one-third to two-thirds when the primaquine was administered either daily for 3 days (Table 2, Expt B), once at 16 h (Expt C), or only 1 h before the [^{3}H]-uridine injection (Expt D). At lower doses, primaquine significantly inhibited uridine incorporation only when given 1 h prior to the uridine, a finding which may be related to the moderately rapid metabolism of primaquine in mammalian tissues. At shorter intervals of uridine incorporation (Table 2, Expt E), the inhibition by primaquine of incorporation into nuclear RNA was approximately equal to the inhibition observed in total cellular RNA and total RNA of ribosomes. Furthermore, it is apparent that primaquine greatly inhibited the synthesis of messenger RNA or its release from the nucleus since essentially all of the radioactive RNA in the ribosomes after labelling periods of 0.25 to 0.5 h represents messenger RNA.

In subsequent studies, chloroquine inhibited uridine incorporation into nuclear RNA and total cellular RNA in liver of adrenal-intact and adrenalectomized rats (Table 3) in keeping with the results of Magus, Harrison & King (1971). A similar trend was observed in mice (Table 3). Moreover, the administration of chloroquine did not appear to alter the uptake of [^{3}H]-uridine into the acid-soluble fraction of liver. The median uptake into the acid-soluble fraction in chloroquine-treated rats was equal to, or slightly greater than that in control animals whether the animals had been adrenalectomized or not (data not shown). In contrast, chloroquine did not inhibit the incorporation of radioactive orotate into nuclear RNA or total cellular RNA of rat or mouse liver. Likewise, primaquine did not inhibit orotate incorporation into RNA in adrenal-intact or adrenalectomized rats (Table 3). Orotate and uridine are pyrimidine precursors. Although the purine adenine, like orotate, is converted to nucleoside phosphates predominantly by reaction with phosphoribosylpyrophosphate, adenine incorporation into total cellular RNA of mouse liver was inhibited by both chloroquine and primaquine (Table 3).

Leucine incorporation

In rats, chloroquine (100 μmol/kg) or primaquine (100 μmol/kg) did not alter significantly the incorporation of leucine into hepatic protein (results not tabulated). Similarly in mice, chloroquine (100 or 150 μmol/kg)

Table 3 Effects of chloroquine and primaquine on precursor incorporation into RNA of liver

Precursor	*Animals*	*Treatment (μmol/kg)*	*Number of animals*	*Total cellular RNA*[a]	*Nuclear RNA*[a]
Uridine	Rats	Control	8	49.1 ± 4.0	146 ± 16
		Chloroquine, 100	7–8	31.0 ± 5.4*	68 ± 12**
		Control (Adx)[b]	8	57.9 ± 7.1	131 ± 18
		Chloroquine, 100 (Adx)	8–9	39.6 ± 10.0	90 ± 23
Uridine	Mice	Control	24	173 ± 25	720 ± 103
		Chloroquine, 100	24	121 ± 15†	561 ± 65
Orotate	Rats	Control	16	489 ± 67	2250 ± 350
		Chloroquine, 100	9	615 ± 47	2930 ± 310
		Primaquine, 100	6	492 ± 92	2480 ± 450
		Control (Adx)[b]	11	686 ± 29	2810 ± 270
		Primaquine, 100 (Adx)	6	645 ± 54	2810 ± 250
Orotate	Mice	Control	9	89.2 ± 9.5	519 ± 58
		Chloroquine, 100	10	97.8 ± 10.6	633 ± 68
Adenine	Mice	Control	43	75.6 ± 4.1	
		Chloroquine, 100	19	58.9 ± 6.7	
		Chloroquine, 125	16	62.0 ± 3.6*	
		Primaquine, 100	16	58.9 ± 4.7*	
		Primaquine, 150	6	31.3 ± 10.3**	

Statistical symbols: as in Table 1. In all experiments, the chloroquine or primaquine was injected 3 h before removal of the tissue. In rats, the duration of incorporation of uridine and orotate were 2 h and 1 h, respectively. In mice, the durations of incorporation of uridine, orotate and adenine were 1 h, 2 h and 2 h, respectively.

[a] The specific radioactivities of the RNA are expressed as cpm/A_{260} unit, divided by the μCi of ^{3}H precursor injected/100 g body weight. [b] Adx: adrenalectomized rat.

or primaquine (150 μmol/kg) did not alter leucine incorporation. Thus, treatment with chloroquine or primaquine did not affect protein synthesis in liver *in vivo*.

Discussion

Although chloroquine and primaquine are active against different stages in the life cycle of the malarial parasite, the effects of these drugs on macromolecular synthesis in rodent tissues *in vivo* appear to be similar. The decreased precursor incorporation into nucleic acids is consistent with interaction of the aminoquinolines with the template DNA and the resultant inhibition of mammalian DNA and RNA polymerases (see Introduction). More direct enzymatic studies would be necessary to prove such a mechanism; the inhibition of a bacterial DNA polymerase is due predominantly to action of the aminoquinolines with the polymerase rather than with the template DNA (Whichard *et al.*, 1972).

The lack of effect of chloroquine or primaquine on protein synthesis under the conditions in these studies eliminates inhibition of energy production as a possible cause of this inhibition of precursor incorporation into nucleic acids. The inhibition of thymidine and uridine incorporation by the aminoquinolines was not caused by a major decrease in the entrance of the radioactive precursors into the tissue. Thus, there appear to be major differences in the response to the aminoquinolines by rodent liver and by *Tetrahymena pyriformis* (Conklin *et al.*, 1973).

The half-lives of primaquine in liver and kidney following its intraperitoneal injection into rats are 1.5 to 2 h (J.B. Griffin & D.J. Holbrook, Jr., unpublished data). However, model metabolites of 8-aminoquinolines interact with nucleic acids in a manner similar to the antimalarial aminoquinolines (Morris, Andrew, Whichard & Holbrook, 1970; Holbrook *et al.*, 1971) and may, thereby, inhibit the synthesis of DNA and RNA.

Several states may account for the divergent effects observed in the aminoquinoline effects on uridine and adenine incorporation and on orotate incorporation into RNA. (a) The labelled precursors may enter different intracellular pools (Plagemann, 1971; Kuebbing & Werner, 1975). (b) The major cell types of liver may differ in either the action of the aminoquinolines or the entrance and subsequent utilization of the radioactive precursors. Differences were observed in the incorporation into RNA of orotate and of cytidine by hepatic parenchymal and stromal cells of rats (Bushnell, Whittle & Potter, 1969). In the present study, the two precursors (uridine and adenine) which showed decreased incorporation into RNA are representatives of salvage pathways of nucleotide metabolism but orotate is a representative of the *de novo* synthetic pathway.

The failure to observe a dose-response in the inhibition of thymidine incorporation into DNA by ethidium may be due to marked differential effects of ethidium on different mammalian non-mitochondrial DNA polymerases. One of the mammalian DNA polymerases is not appreciably inhibited by ethidium (Meyer & Simpson, 1969; Berger, Huang & Irvin, 1971) but a second DNA polymerase is sensitive (Sedwick, Wang & Korn, 1972). It is possible that in the present *in vivo* labelling studies, there was almost complete inhibition of the latter DNA polymerase at all doses used but essentially no effect on the former DNA polymerase. The absence of a pronounced dose-response relationship for the effect of chloroquine or primaquine on thymidine incorporation (Table 1) may also be related to a differential effect on the two major mammalian DNA polymerases.

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