PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 371-375 (1975)

The Effect of Parahydroxylation of Diphenylhydantoin on Metaphase Accumulation (38810)

A. A. MACKINNEY, R. VYAS, AND S. S. LEE
(Introduced by C. M. Kunin)

Departments of Neurology and Medicine, VA Hospital and the University of Wisconsin Medical School, Madison, Wisconsin

Diphenylhydantoin is a valuable antiepileptic drug which is associated with a variety of unusual idiosyncratic reactions involving lymphocytes. We have shown that the drug inhibits DNA synthesis of proliferating human lymphocytes (1). Paradoxically, high concentrations of DPH increased mitoses of cultured human lymphocytes. We found that the drug inhibited polymerization of isolated, purified microtubules, verifying a colchicine-like action (2). Of related hydantoins, only its metabolite, 5-[4-hydroxyphenyl]-5-phenyl hydantoin (HPPH) inhibited microtubular polymerization and inhibited completion of mitosis in tissue culture. HPPH appeared more potent than the parent compound.

Since detoxification may enhance certain biologic properties of drugs, we studied the comparative potency of DPH and HPPH in tissue culture of human lymphocytes to determine the spectrum of activity of HPPH. We found that hydroxylation of DPH selectively enhanced metaphase accumulation without significantly affecting the inhibition of DNA or protein synthesis.

Materials and Methods. 5,5-Diphenylhydantoin was obtained from Parke-Davis; 5-[4-hydroxyphenyl]-5-phenylhydantoin (HPPH) was obtained from Aldrich Chemical Company. Colchicine was obtained from Sigma Chemical Company and Fisher Scientific Company. Vincristine was obtained from Lilly and Company, phytohemagglutinin-P (PHAP) and minimal essential medium (MEM) from Difco. Hydantoin compounds were dissolved in water at pH 11 or in 40% propylene glycol, 10% ethanol, pH 11. Previous dose-response studies established that colchicine 1×10^{-5} *M* [4 μg/ml] gave highest mitotic indices and this concentration was used uniformly.

Mitotic accumulation. Standard phytohemagglutinin-P cultures were prepared as follows: multiple 5-ml cultures containing 20% heparinized plasma, 80% MEM, 1000 leukocytes/μl and PHAP 0.1 ml (1:50 dilution) were incubated for 3 days at 37°C in sealed blood culture bottles. Drugs or diluent were added at 3 days when lymphocytes were proliferating maximally. The cells were collected by centrifugation at 400*g* after 2, 4, 6, or 24 hr of further incubation. The cell button was resuspended in 0.85% Na citrate for 20 min, spun, and fixed in acetic methanol 1:3 for 1 hr, blown on glass slides, air dried, and stained with Giemsa. At least 1000 cells were counted at each time interval. Data were analyzed using confidence limits for the mean of the Poisson distribution.

In four experiments, various concentrations of DPH were compared with fixed concentrations of HPPH or colchicine. In three experiments, varying concentrations of HPPH were compared with a fixed concentration of colchicine. In four additional experiments, DPH, colchicine, or vincristine was washed from cultures with three exchanges of 15 ml of warm MEM after 6-hr incubation and metaphase preparations made immediately and at 24 hr to determine the reversibility of drug effect.

DNA and protein synthesis. Drugs or diluent were added to 3-day-old cultures during final 2 hr of incubation. To estimate DNA synthesis, thymidine (methyl ^{3}H, 0.12 mg/mCi, NEN) 1 μCi/ml was then added. Previous studies showed that 65% of the incorporated radioactivity was in DNA of both DPH and control cultures by PCA extraction (1). To estimate protein synthesis, 3 day-old cultures were incubated with l-leucine (4,5^{3}H, 4.3 mg/mCi NEN) 1 μCi/ml for 2 hr. Uridine incorporation was not

studied because DPH inhibits incorporation of this isotope minimally. Cultured cells were washed with cold 0.15 *M* sodium chloride, extracted with cold 10% trichloracetic acid (TCA) for 1 hr, washed three times with 5% TCA on glass filters, followed by a wash with methanol. Methanol was evaporated at 80°C for 20 min. The filter residue was dissolved in Soluene (Packard) 0.5 ml and mixed with 10 ml of Omnifluor (Packard) for liquid scintillation counting. Counting efficiency for tritium was approximately 25%. Disintegrations per minute were determined using external standardization. All determinations were performed in duplicate and averaged. Data were analyzed using *t* test for paired samples.

Results. Table I compares the inhibition of DNA and protein synthesis by DPH and HPPH in 2-hr incubations. The effect of these compounds in 2-hr incubation is less than that cited for 72 hr (1). No significant differences were found between the activity of the parent and its metabolite, although HPPH appeared to be more inhibitory to protein synthesis than did DPH. The mean control leucine incorporation was 20,229 dpm ± 7, 511 [1 SEM]; the mean control thymidine incorporation was 268,137 dpm ± 19,181.

Table II shows that DPH enhanced metaphase accumulation significantly at 3.6×10^{-4} *M* but was weaker than colchicine 1×10^{-5} *M* and HPPH 3.6×10^{-4} *M*. In nine time-matched studies the mean ratio of mitotic indices of HPPH/DPH 3.6×10^{-4} *M* was 3.0. HPPH (Table III) had colchicine-like activity at 1.8×10^{-4} *M*. At 3.6×10^{-4} *M* mitotic frequency was not significantly different from colchicine. In 28 time-matched studies the mean ratio of mitotic indices of HPPH 3.6×10^{-4} *M*/colchicine 1×10^{-5} *M* was 0.97.

Table IV shows that HPPH effect on mitosis was reversed when cells were washed free of drug. In contrast, colchicine effects were not reversed by washing but rather appeared enhanced. Vincristine washout significantly reduced the mitotic index but did not return the mitotic index to control values.

Discussion. Our studies show that the first step in detoxification of diphenylhydantoin (DPH) increased its power to accumulate metaphases 3-fold. This phenolic compound [5-[4-hydroxyphenyl]-5-phenylhydantoin (HPPH)], 3.6×10^{-4} *M*, was as efficient in accumulating metaphases as colchicine 1×10^{-5} *M*. On the other hand, hydroxylation had no effect on degree of inhibition of DNA synthesis and a minor effect on inhibition of protein synthesis.

DPH is hydroxylated and excreted as the glucuronide by the liver. Unconjugated HPPH is usually found in small amounts in the urine and, therefore, is believed to be

TABLE I. EFFECT OF HYDROXYLATION OF DIPHENYLHYDANTOIN ON INHIBITION OF DNA AND PROTEIN SYNTHESIS. PERCENT OF CONTROL.

	DNA synthesis				Protein synthesis			
	HPPH[a]		DPH[b]		HPPH		DPH	
Mol.	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM
3.6×10^{-6}	100[c]	4.0	96[d]	0.7	118[e]	20	105[f]	1.4
3.6×10^{-5}	98	3.9	104	4.8	86	3.9	105	5.3
7.2×10^{-5}	92	7.2	89	7.3	84	3.2	100	6.0
1.8×10^{-4}	86	2.3	94	5.1	87	6.9	99	8.9
3.6×10^{-4}	79	2.4	80	3.9	74[g]	4.4	93[g]	9.2

[a] 5-[4-hydroxyphenyl]-5-phenylhydantoin.
[b] 5,5-diphenylhydantoin.
[c] n = 3.
[d] n = 5.
[e] n = 3.
[f] n = 4.
[g] *P* = >0.05.

TABLE II. TIME-COURSE OF METAPHASE ACCUMULATION BY VARYING CONCENTRATIONS OF DIPHENYLHYDANTOIN. MITOSES PER THOUSAND.

	Hours			
	2	4	6	24
Control	6	5	6	7
	(3–9)[a]	(5–7)	(5–8)	(5–9)
DPH 3.6×10^{-6} *M*	5	6	9	6
	(2–8)	(5–10)	(7–10)	(4–8)
3.6×10^{-5} *M*	3	6	7	6
	(2–6)	(4–10)	(5–9)	(3–8)
7.2×10^{-5} *M*	8	9	9	7
	(7–10)	(2–10)	(7–10)	(5–9)
1.8×10^{-4} *M*	8	8	11	7
	(6–13)	(6–10)	(10–13)	(6–9)
3.6×10^{-4} *M*	13[b]	11[b]	16[b]	15[b]
	(10–17)	(10–13)	(12–21)	(10–22)
7.2×10^{-4} *M*	14	18	24[c]	27[c]
	(11–18)	(18–19)	24, 25	(18–41)
HPPH 3.6×10^{-4} *M*	15	20[d]	36[d]	46[c,d]
	(13–17)	16, 23	27, 44	(24–58)
Colch. 1×10^{-5} *M*	11	26	42	67
	(8–13)	16–23	35–53	(30–103)

[a] Range.
[b] Differs from control $P < 0.05$.
[c] Differs from colchicine $P < 0.05$.
[d] Different from DPH 3.6×10^{-4} *M*.
n = 4.

TABLE III. TIME-COURSE OF METAPHASE ACCUMULATION BY VARYING CONCENTRATIONS OF HPPH. MITOSES PER THOUSAND.

	Hours			
	2	4	6	24
Control	6	5	6	7
	1–9[a]	3–7	5–8	6–9
HPPH 3.6×10^{-6} *M*	2	6	4	11
	1–4	4–8	2–5	10–11
3.6×10^{-5} *M*	8	7	8	11
	6–10	4–9	7–9	7–16
7.2×10^{-5} *M*	10	10	8	10
	6–15	8–13	6–9	5–14
1.8×10^{-4} *M*	16[b]	17[b]	22[b]	20[b]
	11–19	16–19	18–28	17–24
3.6×10^{-4} *M*	13	29	34	69
	10–21	24–35	30–38	60–75
7.2×10^{-4} *M*	13	31	36	77
	—	—	—	—
Colch. 1×10^{-5} *M*	9	31	48	80
	7–10	22–24	36–42	72–87

n = 3.
[a] Range.
[b] Different from controls $P < 0.05$.

TABLE IV. REVERSIBILITY OF HPPH, COLCHICINE, AND VINCRISTINE EFFECTS ON METAPHASE ACCUMULATION.

	Drug 0–6 hr	Drug 0–24 hr	Drug removed 6–24 hr	Drug replaced 6–24 hr
Diluent	5	8	12	12
	(1–13)[a]	(4–10)	(9–16)	(9, 16)
HPPH 3.6×10^{-4} *M*	34	50[b]	14[b]	36
	(17–47)	(29–76)	(10–16)	(34, 38)
Colchicine 1×10^{-5} *M*	46	94[c]	182[c]	102
	(29–63)	(63–122)	(146–228)	(80, 183)
Vincristine 2.4×10^{-7} *M*	51	86[d]	56[d]	237
	(28–81)	(32–143)	(28–95)	—

[a] Range.
[b] $P < 0.05$.
[c] $P < 0.05$.
[d] $P < 0.05$.
n = 4.

present in low concentration in blood and other fluid compartments (3). However, a significant amount of HPPH was found in the urine of one of the 10 subjects studied by Chang and Glazko (3). The observation suggests that glucuronide formation may vary and free HPPH may circulate in occasional patients.

We have presented evidence that HPPH is biologically active in tissue culture of human lymphocytes. HPPH was previously reported to be an inactive compound *in vivo* but estimates of function were limited to neurotoxicity studies in mice and dogs (4). Probably the central nervous system effects of anticonvulsants are independent of colchicine-like properties. In our previous studies, nirvanol and mesantoin, two effective anticonvulsants, did not promote metaphase accumulation. Furthermore, we cannot be certain that the glucuronide is inert. Although we have not been able to test the glucuronide, conjugation of HPPH with acetate in our laboratory yielded a compound which was equivalent to DPH in metaphase accumulation (unpublished data).

In contrast to colchicine, HPPH effect on mitosis was reversible. Reversibility was shown by decrease in mitotic index to control values 18 hr after the drug had been removed. Although colchicine action is said to be reversible (5), washout enhanced mitotic accumulation. Vincristine effect, on the other hand, was partially reversed by removing the drug. These findings do not correlate with the association constants of these drugs for microtubular protein: vincristine has a higher association constant than colchicine (6) and should be less easily removed from its binding sites.

The biological relevance of DPH colchicine-like action is suggested by endocrine effects. DPH inhibits secretion of insulin (7) and other hormones (8, 9). Colchicine also inhibits secretory functions (10, 11). Since both drugs inhibit microtubular polymerization, it is attractive to attribute inhibition of secretion to this property. Membrane effects of these drugs, however, have not been excluded (12). We note that neither DPH or HPPH resemble colchicine-like compounds structurally (13). We suspect that more active hydantoins will be discovered or synthesized.

Summary. DPH has a colchicine-like action on metaphase arrest of cultured human lymphocytes. The first step in detoxification of DPH increased its power to accumulate metaphases 3-fold. This hydroxy derivative [5-[4-hydroxyphenyl]-5-phenylhydantoin, HPPH] 3.6×10^{-4} *M* was equivalent to colchicine 1×10^{-5} *M* in its power to inhibit metaphase completion. The effect of HPPH on mitosis was reversible; colchicine effect was not reversed and vincristine effect was partially reversed by washing drug from the medium. Hydroxylation of DPH did not change its inhibition of DNA synthesis and

enhanced inhibition of protein synthesis to a minor degree. Detoxification increases the colchicine-like action of DPH.

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Received: Jan. 13, 1975. P.S.E.B.M., 1975, Vol. 149.