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A Comparison of Potency of Hydantoins in Metaphase Arrest and Inhibition of Microtubular Polymerization

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SUMMARY

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The effects of 11 hydantoins on the mitotic apparatus were compared in two assays: metaphase arrest and tubulin polymerization. Drugs were fitted into three of four classes: (1) metaphase arrest and inhibition of tubulin polymerization [DPH, HPPH, MPPH]; 2) inhibition of tubulin polymerization only (1,3-DiAc-DPH and 3-AcO-DPH); (3) no effect on either system (mesantoin, DHDH, 3-OH-DPH, coord, hydantoin, nirvanol); and (4) metaphase arrest only (no drugs found). Structure–function relationships favored an intact hydantoin ring and two benzene rings for activity in both assays. Although complete correlation was not found between the two assays, it appears that inhibition of tubulin polymerization is a necessary feature of metaphase arrest by hydantoins.

INTRODUCTION

We recently reported that 5,5-diphenylhydantoin (DPH) and its metabolite, 5-(4-hydroxyphenyl)-5-hydantoin (HPPH), inhibited metaphase completion of cultured human lymphocytes. The effect was dose and time dependent and reversible (1). HPPH was three times more potent than DPH while 5-phenyl-5-ethylhydantoin (nirvanol), 3-methyl-5-phenyl-5-ethylhydantoin (mesantoin), 3-hydroxy-5,5-diphenylhydantoin (3-OH-DPH) and hydantoin had no effect on metaphase arrest (2).

We further evaluated these compounds by assessing their ability to inhibit the polymerization of purified microtubular protein (tubulin). Tubulin is the principal structural protein of the mitotic spindle apparatus. These studies showed that active hydantoins inhibit tubulin polymerization (viscosity or electron microscopy) but do not degrade or depolymerize microtubules (sedimentation). DPH and HPPH were equally potent in the assay of microtubular polymerization by viscosimetry. 3-OH-DPH, mesantoin, and nirvanol had minor effects (3). In order to further clarify the relationship between metaphase arrest and inhibition of tubulin polymerization, we enlarged the study to include eleven hydantoins and colchicine using these two assay systems. One hydantoin with a methoxy derivative was synthesized to mimic the colchicine-like drugs, all of which have at least one methoxy group (4).

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MATERIALS AND METHODS

3-Allyl-5-isobutyl-2-thiohydantoin (coord), 5,5-diphenyl-4-oxotetrahydroglyoxalene (DHDH, SKF 2599), 5-phenyl-5-ethylhydantoin (nirvanol), and 3-methyl-5-phenyl-5-ethylhydantoin (mesantoin) were gifts from Dr. Harold Booker. 3-Hydroxy-5,5-diphenylhydantoin (3-OH-DPH) was a gift from Dr. Ludwig Call (5). 5,5-Diphenylhydantoin (DPH) was obtained from Parke–Davis Company. 5-(4-Hydroxyphenyl)-5-phenylhydantoin (HPPH) was obtained from the Aldrich Chemical Company; hydantoin was from Eastman Organic Chemicals; colchicine was from Sigma Chemical Company and Fisher Scientific Company. 1-Acetyl-3-acetoxy-5,5-diphenylhydantoin (3-AcO-DPH) was synthesized by the Aldrich Chemical Company using the method of Call (5). 1,3-Diacetyl-5,5-diphenylhydantoin (1,3-DiAc-DPH) was synthesized as previously described (6).

3-Methyl-5-(4-methoxyphenyl)-5-phenylhydantoin (MPPH) was synthesized as follows: 540 mg HPPH, 2 parts diethyl ether and 1 part methanol, were combined with enough diazomethane to maintain a yellow color. The mixture was incubated for 7 days at −20°, then decolorized with 1 N HCl, evaporated under vacuum, and crystallized from diethyl ether. The melting point was 126–128°. The molecular ion peak was 297 on mass spectrography and NMR confirmed the presence of a methoxy group and an *N*-methyl group. Table 1 lists the 11 hydantoins with their substituents.

Mitosis. Phytohemagglutin-stimulated human lymphocytes were cultured for 3 days as previously described

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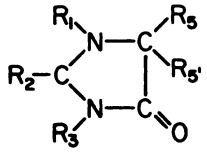
TABLE 1

Substituents of hydantoin molecule



Group	R_1	R_2	R_3	R_5	R_5'
(1) DPH	—H	=O	—H	Phenyl	Phenyl
HPPH	—H	=O	—H	4-OH-phenyl	Phenyl
MPPH	—H	=O	CH_3—	4-CH_3O-phenyl	Phenyl
(2) 3-AcO-DPH	$CH_3C(=O)$—	=O	$CH_3C(=O)$—O—	Phenyl	Phenyl
1,3-DiAc-DPH	$CH_3C(=O)$—	=O	$CH_3C(=O)$—	Phenyl	Phenyl
(3) HYD	—H	=O	H	H	H
3-OH-DPH	—H	=O	—OH	Phenyl	Phenyl
MES	—H	=O	CH_3—	Ethyl	Phenyl
NIRV	—H	=O	H	Ethyl	Phenyl
DHDH	—H	—H_2	H	Phenyl	Phenyl
Coord	—H	=S	Allyl	Isobutyl	H

(3). After 3 days, when proliferation was optimal, the cultures were exposed to drug or diluent in 0.1 ml volume/5 ml culture for 2–24 hr. Dose–response studies were performed on 8 of 11 compounds in the range 3.6×10^{-6} to 3.6×10^{-4} M. These limits were based on pharmacologic blood levels (3.6×10^{-5} M), solubility, and culture toxicity. Cells were processed for metaphase study, and mitoses per 1000 cells enumerated. Metaphase accumulation ratio was calculated as the ratio of mitoses per 1000 in test cultures divided by diluent control. Data were analyzed using estimates based on the Poisson distribution. Values of 12/1000 were significantly greater than controls of 7/1000.

Tubulin polymerization. Tubulin was purified from pig brain by repeated polymerization and depolymerization as previously described (2). It was incubated at 37° in the presence of drug or diluent in Ostwald viscosimeters and the specific viscosity (η_{sp}) was assayed at 1-min intervals for 15 min. The plateau of polymerization at 12–15 min was used as the endpoint. Percentage inhibition of polymerization was based on the formula

$$\frac{\text{test plateau} - T_0 \text{ control} \times 100}{\text{control plateau} - T_0 \text{ control}},$$

in which T_0 control was the average of initial (30-sec) η_{sp} values. All hydantoins were assayed at 3.6×10^{-5} and 3.6×10^{-4} M and full dose–response curves were obtained on four of the active compounds. Values greater than 30% inhibition were positive. Due to the variability in control plateau readings from experiment to experiment, test plateau measurements were adjusted in an analysis of covariance for the value of the concurrently measured control plateau. Using a total significance probability of 0.05, simultaneous comparisons using the Bonferroni method were made of the 12 adjusted treatment plateau means versus adjusted control means (7).

RESULTS

Table 2 summarizes the changes in mitotic indices and tubulin polymerization by hydantoins at 3.6×10^{-4} M. Dose–response curves showed that all active compounds

TABLE 2

Inhibition of microtubule polymerization and metaphase arrest by hydantoins[a]

Group	Microtubular polymerization			Mitotic index		
	$\bar{x}$	SEM	n	$\bar{x}$	Ratio to control	N
(1) DPH	0.214[b]	0.063	8	16[c, d]	2.3[e]	4
HPPH	0.229	0.032	6	43[d]	5.4	4
MPPH	0.239	0.025	5	50[d]	5.6	4
(2) 3-AcO-DPH	0.104	0.007	4	10	1.3	10
1,3-DiAc-DPH	0.306	—	2	11	1.6	5
(3) HYD	0.481	0.055	3	8	1.1	4
3-OH-DPH	0.390	—	2	6	0.9	4
MES	0.352	—	2	7	1.0	4
NIRV	0.393	0.083	4	7	1.0	4
DHDH	0.287	—	2	6	1.3	3
Coord	0.346	—	2	11	1.3	5
Colchicine	0.058	0.020	8	40	5.7	9
Control	0.322	0.066	23	—	—	—

[a] Lymphocyte cultures and tubulin preparations were incubated with hydantoins at 3.6×10^{-4} M or colchicine (1×10^{-5} M) except that 3-AcO-DPH (3.6×10^{-5} M) was used for cultures.
[b] η_{sp}, plateau at 15 min.
[c] Mitoses/1000 after 6 hr incubation with drug.
[d] Different from control, $P < 0.05$.
[e] Ratio of mitotic index to diluent control.

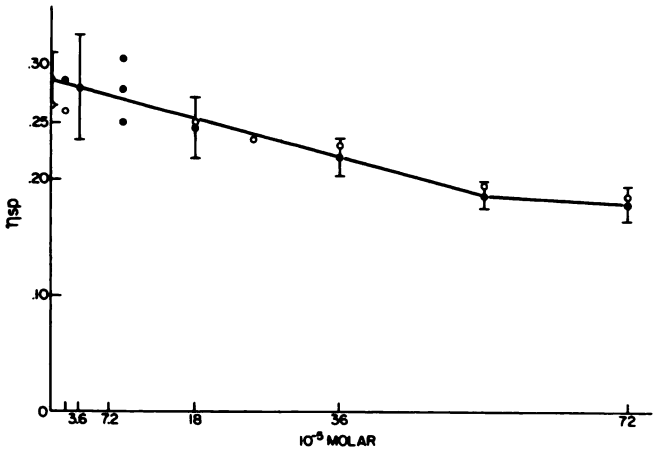


FIG. 1. *Dose-response plot of inhibition of tubulin polymerization by DPH (●) and its derivative MPPH (○)*
MPPH points fall within 1 SEM of DPH values.

had increased potency with increasing concentrations of drug. DPH, HPPH, and probably MPPH inhibited microtubular polymerization 50% at 3.6×10^{-4} M. MPPH did not differ from DPH in inhibiting tubulin polymerization over the range of concentrations (Fig. 1). Other hydantoins were less active, except AcO-DPH which inhibited polymerization 90%. Colchicine inhibition of polymerization was essentially complete at 4 μg/ml. Figure 2 illustrates the dose-response curve of MPPH with colchicine as a single concentration showing complete inhibition.

MPPH was more potent than HPPH in metaphase accumulation at 50 μg/ml ($P < 0.025$) and both MPPH and HPPH were more potent than DPH at 100 μg/ml ($P < 0.05$) (Fig. 3).

DPH, HPPH, MPPH, and colchicine were active in both assays. Aco-DPH and 1,3-DiAc-DPH inhibited polymerization of tubulin, but had no effect on mitosis. Hydantoin, mesantoin, nirvanol, 3-OH-DPH, coord, and DHDH were inert in both systems. [See Appendix for statistical treatment of polymerization data.] Figure 4 shows that there was a positive correlation between metaphase arrest and antitubulin effect in 9 of 12 compounds (data derived from Table 2).

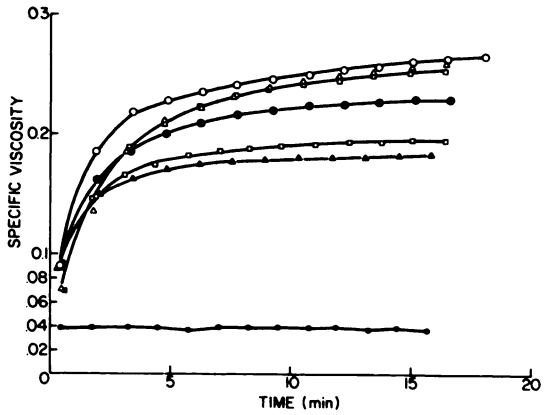


FIG. 2. *Effect of MPPH on tubulin viscosity (η_{sp})*
Symbols from top to bottom: (○) control; (△) 10 μg/ml; (□) 50 μg/ml; (●) 100 μg/ml; (□) 150 μg/ml; and (▲) 200 μg/ml. Colchicine (4 μg/ml) is shown as a single line at the bottom for comparison (•).

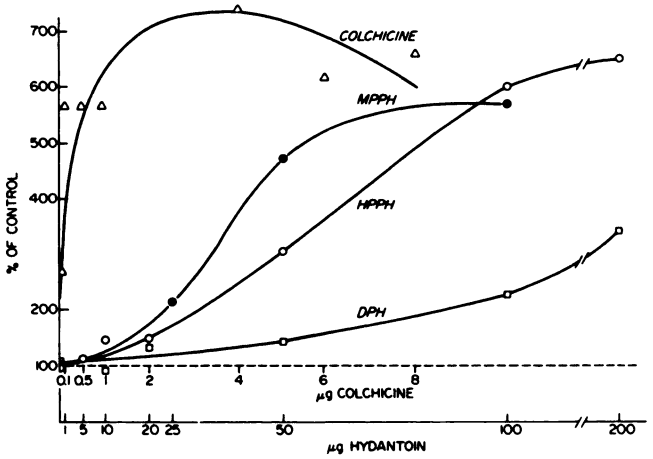


FIG. 3. *Dose-response plot of metaphase arrest by DPH, HPPH, MPPH, and colchicine*
At 50 μg, DPH, HPPH and MPPH are significantly different from each other. At 100 μg, HPPH and MPPH are significantly different from DPH ($P < 0.05$).

DISCUSSION

The study of structure–function relationships led to the following observations: (1) the requirements for metaphase-arresting activity were more stringent than those for antitubulin activity, and (2) no compound had metaphase-arresting properties without antitubulin activity. Three compounds arrested cells in metaphase (Group 1): DPH, along with two derivatives modified at the *para* position by hydroxylation (HPPH) or methoxylation (MPPH). Both modifications increased the potency of DPH in metaphase arrest without altering the antitubulin effect. Therefore, methoxy groups may contribute to metaphase-arresting activity but are not required for compounds in this class, although another recently described metaphase arrestor followed the general rule (8). The disparity between antitubulin and metaphase-arresting properties may be explained by differences in cell membrane transport.

Two compounds active only against tubulin (Group 2) had substituents on N_3, a feature shared by one drug each in Groups 1 (MPPH) and 3 (mesantoin), and con-

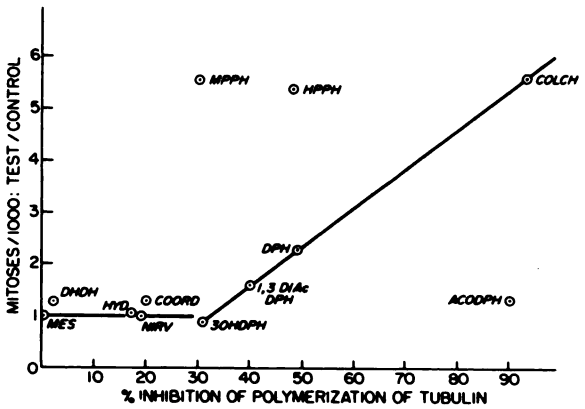


FIG. 4. *Correlation of metaphase arrest and tubulin polymerization inhibition*
The line expresses a relationship between mitosis and tubulin inhibition using the data from Table 2.

TABLE A1

Adjusted treatment means

Treatment	$\hat{\alpha}$[a]	$\hat{S}_{\hat{\alpha}}$[b]
DPH	−0.4867	0.0709
HPPH	−0.5406	0.0729
MPPH	−0.3690	0.0797
3-OH-DPH	−0.2944	0.0720
3-AcO-DPH	−1.3099	0.0800
1,3-DiAc-DPH	−0.4070	0.0794
HYD	−0.1359	0.0720
MES	−0.1963	0.0814
NIRV	−0.1675	0.0702
DHDH	−0.2009	0.0904
Coord	−0.2502	0.0806
Colchicine	−1.9629	0.0708
Control	−0.1618	0.0673

[a] Adjusted mean of ln (plateau).
[b] Estimated standard error of $\hat{\alpha}$.

sequently of little predictive value. They both shared acetyl groups at N_1. Five of the inactive compounds in Group 3 had altered hydantoin rings: (coord, DHDH) or substitutions at C_5 (mesantoin, nirvanol, and hydantoin).

3-AcO-DPH deserves special notice because of its enhanced potency over 3-OH-DPH and because of its ability to depolymerize microtubules.[1] We interpreted its lack of activity against mitosis to be a result of its short half-life in water ($t_{1/2} = 4$ min). We suspect that no active compound reaches the interior of the cell.

We conclude that the basic DPH structure is necessary for metaphase-arresting activity in this class of compounds and that inhibition of tubulin polymerization is a necessary feature of metaphase arrest. Variability in metaphase-arresting activity may be accounted for by differences in membrane transport of the compounds.

APPENDIX

An analysis of covariance was used to adjust for the variation from experiment to experiment. The natural log of the viscosity measurements was used in the analysis in order to stabilize the variance. The means [of ln (viscosity)] for the 13 groups (12 treatments and 1 control) were adjusted by regression of the plateau measurements on the control plateau observed at the time each experiment was run. The model used was

$$\ln(\text{plateau}) = a \ln(\text{control plateau}) \pm \alpha_i,$$

where the α_i are the effects due to treatment group (chemical or control) adjusted for the average effect of the concurrently observed control plateau.

The regression coefficient a was estimated at $\hat{a} = 0.8579$ (± 0.0571) which is significantly different from 0 ($t_{52} = 15.03$, $P \ll 0.001$) and from 1 ($t_{52} = 2.49$, $P < 0.02$). Estimates of the adjusted treatment means are given in Table A1 with their standard errors. An F test for effect due to treatment was highly significant: $F_{(12, 52)} = 284.95$, $P \ll 0.001$ for the group as a whole.

Paired comparisons were made of the adjusted means

[1] Unpublished results.

TABLE A2

Comparison of adjusted ln (treatment plateau(s)) with adjusted ln (control plateau)

Chemical	$\hat{\psi}$[a]	$\hat{S}_{\hat{\psi}} \cdot t_{52}(0.025/12)$[b]	$\hat{S}_{\hat{\psi}}$[c]
DPH	−0.32490[d]	±0.100071	0.0335808
HPPH	−0.37880[d]	0.120200	0.0403355
MPPH	−0.20720[d]	0.121362	0.0407254
3-OH-DPH	−0.13260	0.190391	0.0638897
3-AcO-DPH	−1.14810[d]	0.149175	0.0500587
1,3-DiAc-DPH	−0.24520[d]	0.181613	0.0609440
HYD	0.02590	0.190391	0.0638897
MES	−0.03450	0.180390	0.0605334
NIRV	−0.00570	0.154038	0.0516905
DHDH	−0.03910	0.179555	0.0602533
Coord	−0.08840	0.180807	0.0606736
Colchicine	−1.80110[d]	0.100072	0.0335811

[a] Adjusted mean ln (treatment plateau) − adjusted mean ln (control plateau).
[b] $t_{52}(0.025/12) = 2.98$.
[c] Standard error of $\hat{\psi}$ (estimated).
[d] Significantly different from zero (total significance probability: $P < 0.05$).

for each treatment group versus the adjusted control mean. Each of these 12 individual comparisons was made using a significance probability of 0.25/12; this guaranteed (by the Bonferroni inequality) that the total significance level for all 12 comparisons was, at most, $\alpha = 0.05$.

According to this criterion, only the adjusted means for DPH, HPPH, MPPH, 3-AcO-DPH, 1,3-DiAc-DPH, and colchicine were significantly different from control. Table A2 presents the 12 differences, their estimated standard errors, and these standard errors multiplied by t_{52} (0.25/12), which equals 2.98.

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