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EFFECTS OF SELECTED ARYLHYDROXAMIC ACIDS ON LYMPHOCYTE MITOGENESIS*

GLEN R. GALE and JAMES E. CARNES

Veterans Administration Hospital and Department of Pharmacology, Medical University of South Carolina, Charleston, S.C. 29403, U.S.A.

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Abstract—Twenty arylhydroxamic acids were investigated as potential antimitogenic agents using phytohemagglutinin-stimulated human lymphocytes in culture. 4-Hydroxybenzoylhydroxamic acid (4-HBHA) was active (82 per cent inhibition at 10^{-4} M) against the blastogenic transformation as assessed by thymidine-methyl-^{3}H incorporation over a 2-hr interval initiated 46 hr after addition of phytohemagglutinin. Five other 4-substituted derivatives of benzoylhydroxamic acid were much less active. 2,3-Dihydroxybenzoylhydroxamic acid (2,3-DHBHA) was also antimitogenic (87 per cent inhibition at 10^{-4} M), while the 2,4-, 2,5-, and 2,6-dihydroxy analogs were relatively inactive. 4-Hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were inactive at 10^{-4} M. Differences observed between the two active compounds as regards dose–response relationships, temporal variations in onset of action, and degrees of reversibility of inhibition indicated different action mechanisms for 4-HBHA and 2,3-DHBHA in spite of their apparent structural similarities.

EARLIER reports from this laboratory described a number of arylhydroxamic acids which inhibit selectively, to varying degrees, the biosynthesis of DNA in Ehrlich ascites carcinoma cells *in vitro*.[1,2] One of these compounds, 4-hydroxybenzoylhydroxamic acid (4-HBHA), when administered at 400 mg/kg/day for 9 days, increased up to 57 per cent the survival times of BDF_1 mice bearing the L1210 lymphoid leukemia (Mr. N. H. Greenberg, Cancer Chemotherapy National Service Center, National Cancer Institute, personal communication). Another of these compounds, 2,3-dihydroxybenzoylhydroxamic acid (2,3-DHBHA), at 340 mg/kg/day for 10 days, effected a highly significant reduction of the thymus/body weight ratio in immature rats, with no notable actions on other organs examined.[3] Histologically, the thymuses of animals so treated were pale-staining, there was a loss of distinction between cortex and medulla, and germinal follicles were indistinguishable. Thymic lymphocytes were sparse, and there was considerable lympholysis and lymphocytic necrosis, similar to the well known cortisone effect.

There is adequate evidence that various alkyl- and arylhydroxamic acids do not merely mimic the actions of hydroxylamine on biological systems, since different R substitutions of RCONHOH confer quite dissimilar pharmacological properties.[4–7] An amino-substituted member of this class of compounds, hydroxyurea, is known to suppress blastogenic transformation in cultured lymphocytes[8,9] and to prolong skin graft survival on rabbits.[10] It was thus considered of interest to examine the actions of a number of other hydroxamic acids on this transformation, since the lymphocyte culture system is considered to be a sensitive measure *in vitro* of the effects of potential

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immunosuppressive agents on the lymphoid organ.[11] This initial study was limited to certain of those aryl derivatives described in earlier reports[1,2] along with two congeners prepared more recently.

MATERIALS AND METHODS

The source or method of synthesis of each of the arylhydroxamic acids has been described in previous reports[1,2] with the exception of 2,4-dihydroxybenzoylhydroxamic acid and 2-hydroxy-4-aminobenzoylhydroxamic acid. The former was synthesized* by the method of Scott and Kearse,[12] and the latter by the method of Priewe and Rutkowski.[13] Hydroxyurea was obtained from Squibb and phytohemagglutinin-P (PHA) from Difco.

Lymphocytes for culture were obtained from venous blood of various presumably normal donors and were separated as described by Ohno and Hersh,[11] using dextran (mol. wt. = 204,000) for erythrocyte sedimentation. Cultures were prepared in Eagle's minimum essential medium with Hanks' balanced salt solution and the total volume of each was 2·0 ml. Each ml contained 0·2 ml of autochthonous serum, 100 units of penicillin G, 100 μg streptomycin, 14·0 mg of supplemental $NaHCO_3$, 0·005 ml PHA, 0·05 ml dimethylsulfoxide with or without the appropriate hydroxamic acid, and 0·5 to 2·0 $\times$ 10^6 lymphocytes. This concentration of dimethylsulfoxide was itself without effect on the rate of thymidine incorporation, and a sufficient number of preliminary experiments showed that optimal deoxyriboside incorporation occurred in cell populations within the 4-fold range used. PHA was added at the beginning of the culture period, and incubation was at 37° in 5% CO_2–95% air. The hydroxamic acids were added either immediately after the addition of PHA or at selected intervals over the subsequent incubation period, depending upon the design of each of the several experiments. A terminal pulse-labeling period was then initiated by the addition (in 0·2 ml of 0·9% NaCl) of thymidine-methyl-^{3}H (15 c/m-mole), uridine-5-^{3}H (20 c/m-mole), or L-leucine-^{14}C (250 mc/m-mole) (New England Nuclear Corp.) to yield final activities of 1·0, 1·0, and 0·1 μc/ml respectively. After 2 hr of further incubation, the cultures were chilled, the cells were washed with cold 0·9% NaCl, resuspended in 2·0 ml of 0·9% NaCl, and the acid-insoluble material was precipitated with 2·0 ml of cold 10% trichloroacetic acid (TCA). After two washings with cold 5% TCA, the precipitated material was solubilized in 2·0 ml of 1·0 M hydroxide of Hyamine in methanol, transferred quantitatively to 15 ml of a toluene-based phosphor,† and radioactivity was measured with a liquid scintillation spectrometer (Nuclear Chicago Corp., Mark I). Statistical evaluation of triplicate control cultures in each experiment showed that the standard deviation, in terms of per cent inhibition, was less than 5 per cent in all cases. However, among various experiments, the values of the control tubes ranged from about 3500 to just over 8000 counts/min with cells from separate donors.

RESULTS

Initial experiments were designed to assess the relative activities of the 20 chosen compounds at a final concentration of 10^{-4} M when added after PHA. Incubation

* We gratefully acknowledge the assistance of Dr. J. B. Hynes, School of Pharmacy, Medical University of S.C., in preparing these two compounds.

† PPO–POPOP: 2,5-diphenyloxazole and 1,4-bis-2-(4-methyl 5-phenyloxazolyl)benzene.

followed for 46 hr and, in turn, was succeeded by a pulse-labeling period of 2 hr with thymidine-methyl-^{3}H. Table 1 lists the compounds used and the percentage inhibition obtained with each. Comparative data on hydroxyurea and hydroxylamine are also included. The two most notable features of this survey are: (1) of the six derivatives of benzoylhydroxamic acid substituted singly in the 4-position, only 4-HBHA conferred appreciable inhibition; and (2) of the four derivatives of the same compound with dihydroxy substitutions, only 2,3-DHBHA was notably active in this regard. The concentration of 10^{-4} M of each compound used in the initial survey corresponds to just over 15 μg/ml of 4-HBHA, and to about 17 μg/ml of 2,3-DHBHA. Even though this may be considered a reasonably high degree of potency, activity does not quite approach that of chloroquine, which completely inhibits PHA-induced transformation at 1–10 μg/ml, or about 0·03 to 0·3 $\times$ 10^{-4} M.[14] Activity of each of the two active derivatives did, however, exceed that of either hydroxyurea or hydroxylamine. Further, the degree of inhibition obtained with hydroxyurea in the present study agreed rather well with that obtained by Topping *et al.*,[8] who reported that this compound yielded 50 per cent inhibition of thymidine incorporation when used at 1·1 to 1·2 $\times$ 10^{-4} M in cultures of lymphocytes stimulated with pokeweed mitogen.

TABLE 1. INHIBITION OF LYMPHOCYTE MITOGENESIS BY SELECTED ARYLHYDROXAMIC ACIDS, HYDROXYUREA AND HYDROXYLAMINE*

Hydroxamic acid	Inhibition (%)	Hydroxamic acid	Inhibition (%)
Benzoyl-	4	2,3-Dihydroxybenzoyl-	87
2-Hydroxybenzoyl-	11	2,4-Dihydroxybenzoyl-	31
2-Fluorobenzoyl-	10	2,5-Dihydroxybenzoyl-	21
Acetylsalicyl-	0	2,6-Dihydroxybenzoyl-	22
3-Aminopyrazino-2-	10	3,5-Diaminobenzoyl-	17
4-Hydroxybenzoyl-	82	2-Hydroxy-3-methylbenzoyl-	13
4-Fluorobenzoyl-	15	2-Hydroxy-4-aminobenzoyl-	0
4-Chlorobenzoyl-	19	2-Hydroxy-3,5-diisopropylbenzoyl-	14
4-Bromobenzoyl-	26	3,4,5-Trimethoxybenzoyl	23
4-Nitrobenzoyl-	21	Hydroxyurea	43
4-Aminobenzoyl-	0	Hydroxylamine	40

* Each compound was present at a final concentration of 10^{-4} M and was added immediately after phytohemagglutinin. A 2-hr pulse-labeling period with thymidine-methyl-^{3}H, 1·0 μc/ml, was initiated at 46 hr. Each value represents the average of at least two experiments, each of which was done in triplicate.

Microscopic examination of cells treated with 4-HBHA or 2,3-DHBHA revealed a rather severe cytotoxic action, indicating that the reduced rate of thymidine incorporation in the presence of each compound was not merely a result of differential degradation of the deoxyriboside or of variations of pool sizes (Fig. 1).

The rate of thymidine incorporation into lymphocytes, when assessed at graded concentrations of each of the two most active inhibitors, yielded a satisfactorily linear relationship when the probits of the per cent of control values were plotted against the logarithms of inhibitor concentrations (Fig. 2), indicating a standard distribution of cells with varying sensitivities within the population. The concentrations required for

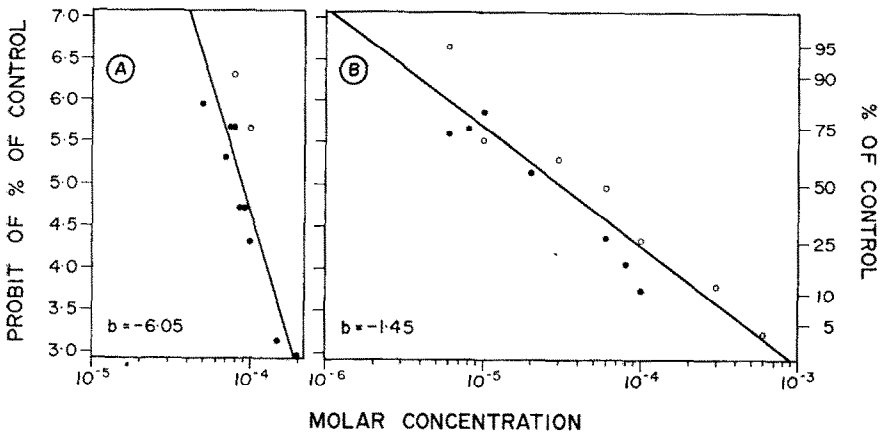

FIG. 2. Effects of graded concentrations of (A) 4-hydroxybenzoylhydroxamic acid and (B) 2,3-dihydroxybenzoylhydroxamic acid on DNA synthesis in phytohemagglutinin-stimulated human lymphocytes. Phytohemagglutinin and each inhibitor were added at zero time; a terminal pulse-labeling period of 2 hr with thymidine-methyl-^{3}H, 1·0 μc/ml, was initiated after 46 hr of incubation. Different symbols indicate separate experiments.

50 per cent inhibition were 9×10^{-5} M 4-HBHA and 3×10^{-5} M 2,3-DHBHA. An unexpected feature which emerged from regression analysis of these experiments was the rather sizable difference between the slopes of the two lines. The slope of the response obtained with 4-HBHA was so abrupt that unusually small concentration increments were necessary to yield useful probit values between 3·0 and 7·0. The numerical slope of the response obtained with 2,3-DHBHA was less than one-fourth of that found with 4-HBHA. This was particularly surprising in view of the apparent structural similarities between the compounds, since finding dissimilar values of *b* computed from probit–log dose plots is generally construed as indicative of dissimilar modes of pharmacological action.[15] In spite of this apparent difference between the two inhibitors, the position of the ring substitution was not the sole determinant of biological activity, since 4-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were devoid of such activity at 10^{-4} M.

Another indication of a mechanistic difference in the actions of the two compounds was found when assessing the time course of onset of action against thymidine incorporation. Replicate identical cultures were prepared as before with PHA added at zero time. A concentration of each inhibitor was chosen which, by reference to Fig. 2, would yield about 95 per cent inhibition when added to the cells 46 hr before initiation of the 2-hr pulse-labeling period. These concentrations corresponded to 2×10^{-4} M 4-HBHA and 6×10^{-4} M 2,3-DHBHA. Each compound was then added at various times during the 46-hr pre-pulse interval and the per cent inhibition was measured as before. Figure 3 shows that the onset of action of 2,3-DHBHA was quite rapid, and virtually maximum inhibition was observed even when the inhibitor was added immediately prior to the 2-hr pulse period. With 4-HBHA, however, a preincubation period of over 30 hr was required to attain the same degree of impairment of thymidine incorporation.

A valid measure of the extent of reversibility of the action of each inhibitor was

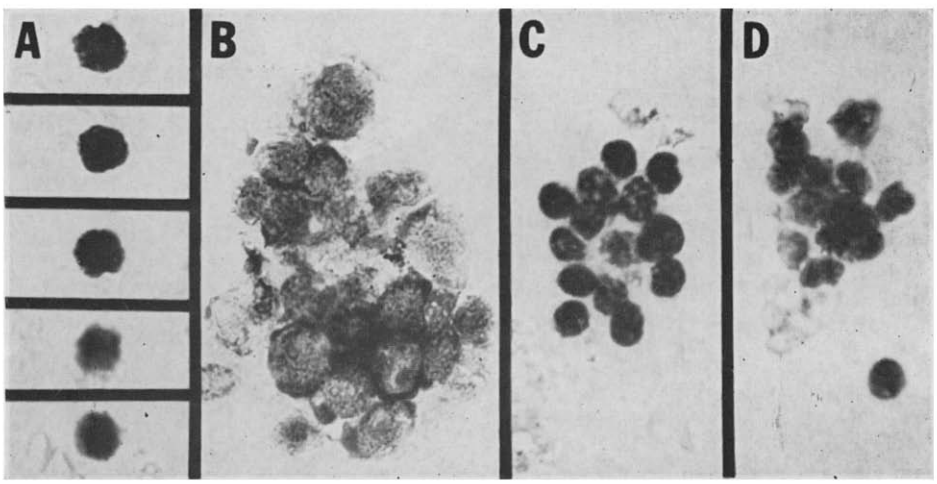

FIG. 1. Effects of certain arylhydroxamic acids on morphology of human lymphocytes in culture. (A) (Composite), no stimulation; (B) phytohemagglutinin (PHA) only; (C) PHA and 4-hydroxybenzoylhydroxamic acid (2×10^{-4} M); (D) PHA and 2,3-dihydroxybenzoylhydroxamic acid (6×10^{-4} M). PHA and each inhibitor were added at zero time, and cells were stained with Wright's stain after 48 hr of incubation. Magnification, × 720.

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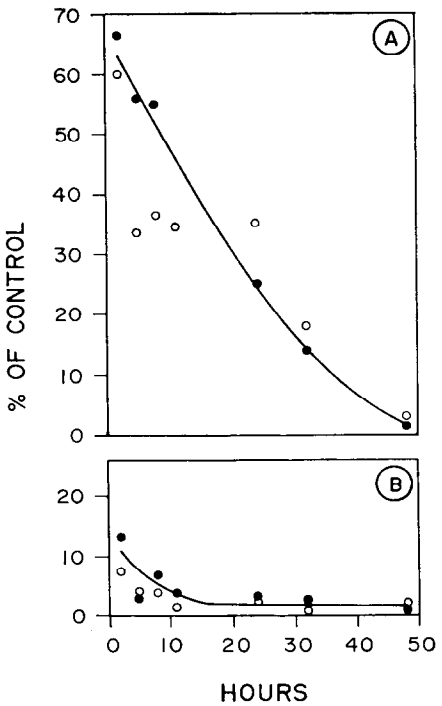

FIG. 3. Effects of duration of exposure of phytohemagglutinin-stimulated human lymphocytes to (A) 4-hydroxybenzoylhydroxamic acid, 2×10^{-4} M, and (B) 2,3-dihydroxybenzoylhydroxamic acid, 6×10^{-4} M, on the subsequent rate of DNA synthesis as measured by a 2-hr pulse-labeling period with thymidine-methyl-^{3}H, 1·0 μc/ml. Different symbols indicate separate experiments.

complicated by the fact that such an extensive period of preincubation with 4-HBHA was required to achieve a high degree of inhibition at a reasonably low concentration. For experiments designed to detect reversibility, concentrations of each compound were chosen as described above (2×10^{-4} M 4-HBHA; 6×10^{-4} M 2,3-DHBHA) and the period of preincubation with each compound was that which was necessary to yield about 90 per cent inhibition of thymidine incorporation, by reference to Fig. 3. Replicate cultures were prepared and PHA was added at zero time. At 16 hr, 4-HBHA was added to the appropriate number of cultures; at 46 hr, 2,3-DHBHA was added to an equivalent number. At 46 hr, one-third of the cultures, control and experimental, were pulse labeled for 2 hr to determine the per cent inhibition extant at 48 hr. One-third of the remaining cultures containing each inhibitor were centrifuged at 48 hr along with an equal number of control cultures; the lymphocytes were washed three times with fresh medium, and all were restored to the original volume with medium devoid of either inhibitor. The remaining one-third of the cultures, control and experimental, were merely incubated without washing for the total duration of the experiment. At 70 hr of elapsed time, the terminal labeling period was initiated, after which the reaction was terminated as before. Table 2 shows that the inhibitory action of 2,3-DHBHA was almost totally abolished by washing the cells, but the action of 4-HBHA was only partially attenuated by this procedure. It is likely that the

total period of exposure of the cells to this latter compound prior to washing may have played a role in its persistence of action; however, as indicated by Fig. 3, this prolonged exposure was required in order to achieve its maximum inhibitory action at a reasonably low concentration.

A substantial margin of selectivity of 2,3-DHBHA against DNA synthesis has been noted previously; using Ehrlich ascites carcinoma cells *in vitro*, the rate of incorporation of thymidine was virtually totally suppressed, while the incorporation of uridine

TABLE 2. DIFFERENT DEGREES OF PERSISTENCE OF INHIBITORY ACTIONS OF 4-HYDROXYBENZOYLHYDROXAMIC ACID (4-HBHA) AND 2,3-DIHYDROXYBENZOYLHYDROXAMIC ACID (2,3-DHBHA) ON DNA SYNTHESIS IN PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES AFTER WASHING AND RESUSPENDING THE CELLS IN FRESH MEDIUM DEVOID OF EITHER INHIBITOR*

Incubation period (hr)	Washed at 48 hr	Inhibition† (%)	
		4-HBHA‡	2,3-DHBHA§
48	–	79	87
72	–	67	99
72	+	42	11

* Phytohemagglutinin was added at 0 time.

† Values are averages of two experiments, each of which was done in duplicate. Standard deviations of the counts per minute were less than 5 per cent in terms of per cent inhibition.

‡ 2×10^{-4} M, added at 16 hr.

§ 6×10^{-4} M, added at 46 hr.

TABLE 3. EFFECTS OF 4-HYDROXYBENZOYLHYDROXAMIC ACID (4-HBHA) AND 2,3-DIHYDROXYBENZOYLHYDROXAMIC ACID (2,3-DHBHA) ON THE INCORPORATION OF URIDINE AND L-LEUCINE INTO THE ACID-INSOLUBLE FRACTION OF PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES

Expt.	Precursor	Inhibitor*	Counts/min	Inhibition (%)
A	Uridine	None	8630	
	Uridine	4-HBHA	1450	83
	Uridine	2,3-DHBA	630	93
	L-leucine	None	2260	
	L-leucine	4-HBHA	425	81
	L-leucine	2,3-DHBHA	360	84
B	Uridine	None	8620	
	Uridine	4-HBHA	740	91
	Uridine	2,3-DHBHA	4490	48
	L-leucine	None	1960	
	L-leucine	4-HBHA	150	92
	L-leucine	2,3-DHBHA	940	52

* Final concentrations: 4-HBHA, 2×10^{-4} M; 2,3-DHBHA, 6×10^{-4} M. Each inhibitor was added immediately after PHA; a 46-hr incubation preceded the 2-hr terminal pulse period.

or L-leucine was not altered appreciably.[1] In that study, however, the cells were in contact with the inhibitor for only up to 2 hr and, since the medium was not supplemented with protein, the cells were not replicating. In that same study, 4-HBHA was found to be a rather weak and nonselective inhibitor. To assess the degree of selectivity in the lymphocyte culture system, the rates of incorporation of uridine-5-^{3}H and L-leucine-^{14}C were measured. At the same concentration of each inhibitor as that used in the time course and reversal experiments above, 4-HBHA manifest no selectivity whatsoever; the rates of RNA and protein synthesis were as severely retarded as was that of DNA synthesis. An occasional and nominal degree of selectivity was seen with 2,3-DHBHA, and representative values which were obtained are listed in Table 3. Since the lymphocyte culture system differs from the Ehrlich ascites carcinoma system *in vitro* (alluded to above) in both duration of incubation and composition of the medium, it seems probable that secondary events in the lymphocyte cultures may have supervened to annul any early selectivity of 2,3-DHBHA against DNA synthesis. The possibility that a greater margin of selectivity of 2,3-DHBHA could be demonstrated by shortening the period of incubation with the inhibitor prior to pulse labeling was not explored.

DISCUSSION

The number of compounds which have been assessed for activity against the blastogenic transformation of lymphocytes is rather vast. Ling[14] reviewed much of the literature prior to 1967 and discussed briefly certain chracteristics of inhibition obtained with the more active agents such as chloroquine, corticosteroids, nitrogen mustard, salicylate, mercaptoethanol, actinomycin, puromycin, cycloheximide, and 5-fluoro-2′-deoxyuridine. Brief allusion was also made to those agents which are active, if at all, only at pharmacologically unrealistic concentrations; among this group are thalidomide, tetracycline, chloramphenicol, indomethacin, biogastrone, phenytoin, mefenamic acid, and flufenamic acid. Agents with appreciable activity which were either not discussed by Ling or appeared in the later literature include L-asparaginase,[16] amantadine hydrochloride,[17] isopentenyladenosine,[18] chlorpromazine,[19] ouabain,[20] and hydroxyurea.[8,9] Caffeine at 10^{-4} M produces erratic effects, generally enhancing the rate of mitosis of lymphocytes from female subjects while suppressing or having no effect on cells from males.[21] Neither lysergic acid diethylamide nor marihuana produces remarkable effects on cultured lymphocytes from subjects who received the drugs.[22]

A consideration of the structures of the two active arylhydroxamic acids revealed by the present study evokes a comparison with hydroxyurea, which shares the hydroxamic acid group, and with salicylate, which is structurally similar to both of the aryl inhibitors.

Studies of the mode of action of hydroxyurea indicate that its principal action on susceptible cells is inhibition of DNA synthesis by suppression of the activity of ribonucleoside diphosphate reductase;[23–25] this inhibition is ameliorated in various cell systems by the addition of the four deoxyribosides to the medium.[26,27] The inhibitory action of 2,3-DHBHA is only partially annulled under experimental conditions and deoxyriboside concentrations which completely reverse the action of hydroxyurea, suggestive of different action mechanisms.[27] 4-HBHA was not included

in that study since its action *in vitro* against the test system was quite weak and was not selective for DNA synthesis.[1]

A comparison of the actions of 4-HBHA and 2,3-DHBHA with the action of salicylate permits no valid conclusion regarding similarity of actions. Gantner and Zuckner[28] found that lymphocytes obtained from subjects who had received therapeutic doses of aspirin or sodium salicylate failed to undergo transformation when cultured subsequently *in vitro* with PHA, and this effect persisted for 24–36 hr after a single dose. The addition of salicylates to lymphocyte cultures *in vitro* showed "characteristic dosage cutoff points in different individuals", but no quantitative data were presented. Forbes and Smith,[29] using human lymphocytes in a medium devoid of protein and (apparently) PHA, found that salicylate at 500 μg/ml (about 3×10^{-3} M) inhibited the incorporation of leucine-^{14}C an average of only about 24 per cent (range, 0–44 per cent). Concentrations of 4-HBHA and 2,3-DHBHA approximately an order of magnitude lower than this inhibited L-leucine-^{14}C incorporation up to 92 per cent in the present study.

In regard to one of the most potent known inhibitors of PHA-induced mitogenesis, a stark contrast exists between the actions of 4-HBHA and 2,3-DHBHA and that of chloroquine. The latter drug at 10 μg/ml,[30] and even at 1·0 μg/ml,[14] strongly suppresses the PHA-mediated blastogenesis, but only if added to the cultures within the first 4 hr of a 3-day culture period. This severe temporal dependence is presumably a reflection of its mode of action, which is thought to be a stabilization of lysosomal membranes, thereby preventing the release of enzymes required for initiation of mitogenesis.[14]

Thus, there appear to be fundamental differences between the action mechanisms of each of the two active hydroxamic acids reported here, as well as differences between these and other known suppressant drugs in the lymphocyte culture system. Certainly more extensive evaluation of these compounds is warranted to determine their potential as useful immunosuppressive agents.

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