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INHIBITION OF THE LEUKOTRIENE SYNTHETASE OF RAT BASOPHIL LEUKEMIA CELLS BY DIETHYLCARBAMAZINE, AND SYNERGISM BETWEEN DIETHYLCARBAMAZINE AND PIRIPROST, A 5-LIPOXYGENASE INHIBITOR

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Abstract—Diethylcarbamazine inhibited the formation of sulfidopeptide leukotrienes in rat basophil leukemia (RBL) cells (50% inhibitory concentration, EC_{50}, 3 mM). Similar concentrations also inhibited the formation of leukotriene C_4 (LTC_4) by LTC synthetase, a detergent-solubilized cell free particulate enzyme from RBL cells which is capable of coupling LTA_4 to glutathione. By contrast, the conversion of LTA_4 to LTC_4 using enzymes from rat liver was at least ten times less sensitive to this inhibitor. The EC_{50} for inhibition of the leukotriene C synthetase of RBL cells was directly proportional to the LTA_4 concentration in the incubations, ranging from 1.5 mM at 10 μM LTA_4 to over 40 mM at 500 μM LTA_4. Kinetic analysis revealed that the inhibition of the leukotriene C synthetase reaction by diethylcarbamazine was competitive with respect to LTA_4. In contrast to diethylcarbamazine, piriprost (U-60,257; 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1), which inhibits the formation of sulfidopeptide leuktrienes in RBL cells at the 5-lipoxygenase step (EC_{50} 5 μM), did not inhibit the leukotriene synthetase of these cells. On the other hand, low concentrations of piriprost, which had no demonstrable inhibitory activity on leukotriene formation by themselves, markedly synergized the inhibitory activity of diethylcarbamazine. These results are consistent with the interpretation that both piriprost and diethylcarbamazine inhibit leukotriene formation but that they act on sequential steps in the biosynthetic pathway in such a manner as to synergistically interfere with the availability or utilization of LTA_4 in the leukotriene C synthetase reaction.

The observation that diethylcarbamazine (DEC) can inhibit the formation of slow-reacting substance of anaphylaxis (SRS-A; [1]) predates the determination of the structure of the leukotrienes (LT) by at least 10 years and was initially based on somewhat anecdotal clinical observations, suggesting that this antifilarial agent had beneficial effects with respect to the asthma of a patient who also had filariasis [2]. After the structures of the leukotrienes became known, there developed a concerted effort to find selective inhibitors of their formation and it was not surprising that DEC once again received attention [3]. One of the publications which resulted from this renewed interest [4] suggested that extremely low concentrations of DEC inhibited the formation of both LTB_4 and the sulfidopeptide leukotrienes in mastocytoma cells while actually stimulating the formation of 5-hydroxyeicosatetraenoic acid (5-HETE), suggesting that the site of action of DEC in inhibiting leukotriene formation may be the leukotriene A_4 synthetase reaction.

We were interested in finding out whether DEC might also inhibit the conversion of LTA_4 to LTC_4 by the solubilized leukotriene C synthetase which we had been studying [5]. This paper describes the results of our experiments and demonstrates that DEC is a competitive inhibitor with respect to LTA_4 in the leukotriene C synthetase reaction.

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MATERIALS AND METHODS

Chemicals. 2,4-Dinitrochlorobenzene (DNCB), 3,4-dichloronitrobenzene (DCNB) and sodium sulfobromophthaleine were purchased from the Aldrich Chemical Co., Milwaukee, WI; diethylcarbamazine citrate and glutathione (GSH), from the Sigma Chemical Co., St. Louis, MO; Triton X-100 and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (ENPP) from Eastman Kodak, Rochester, NY; epoxy-activated Sepharose 6B, chromatofocusing reagents and adsorbents from Pharmacia Fine Chemicals, Piscataway, NJ; and the calcium ionophore, A23187, from Calbiochem-Behring, San Diego, CA. The lithium salt of leukotriene A_4 (LTA_4) and piriprost (U-60,257; 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1) were prepared by Drs. Douglas R. Morton, Jr. and Herman W. Smith, respectively, of these laboratories by published procedures [6, 7]. Glutathionyl sepharose was also prepared by published procedures [8].

Tests with intact rat basophil leukemia (RBL) cells and with solubilized LTC synthetase. RBL cells were cultured in Minimal Essential Medium (Eagle) which was supplemented with glutamine, streptomycin, penicillin and gentamycin as described [9]. The procedures for the measurement of inhibition of leukotriene synthesis have also been described previously [9]. Briefly, washed cells [1.87×10^7 cells/ml in modified Tyrode's buffer, which was 10 mM with respect to 4-(2-hydroxyethyl)-1-piperazine-ethane-

sulfonic acid (HEPES), pH 7.0] were distributed after a brief preincubation at 30° (0.2 ml/tube) into 12 × 75 mm plastic culture tubes which contained 0.2 ml of the same buffer containing double strength inhibitor solutions. After 2.5 min of further incubation, 50 μl of a neutralized 0.1 M cysteine solution was added to each tube, and this was followed in another 2.5 min by 50 μl of a 120 μg/ml solution of the calcium ionophore. Incubation was continued for 20 min at which time the reaction was stopped by chilling the tubes in an ice bath and adding 2 ml per tube of absolute methanol to dissociate any bound leukotrienes and to precipitate the proteins. After standing (30 min, 0°) supernatant fractions were collected by centrifugation, were dried under vacuum at 45° in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY), and were stored at −80° until assay.

The LTC synthetase was solubilized from the high speed particulate fraction of RBL cell homogenates using 0.3% Triton X-100 and assayed as previously described [5, 9]. Briefly, reaction mixtures (0.45 ml) consisting of solubilized enzyme, 3 mM GSH and inhibitors in suspending buffer (137 mM NaCl, 2.6 mM KCl, 0.36 mM NaH_2PO_4, 10 mM HEPES, 1 mM EDTA, pH 7.0) were equilibrated at 37° for 2 min, and the reaction was initiated by adding 50 μl of 0.1 mM LTA_4, lithium salt in suspending buffer which also contained 10 mg/ml of bovine serum albumin (BSA). Incubations were continued for 10 min since we have reported previously that the reaction was linear for this period [5]. In certain experiments where the effect of the LTA_4 concentration on leukotriene generation was to be measured, the incubations were scaled down to a volume of 0.1 ml in order to conserve substrate. In these experiments, the final BSA concentration was increased to 10 mg/ml because preliminary results had shown that this was necessary to achieve a good dependence on the LTA_4 dose. Incubations were stopped as described above. After drying, the samples were rehydrated to their original volume and applied to 300 μl microcolumns of Dowex 50 (H^+). The columns were extensively washed with water and with 80% aqueous methanol, and the sulfidopeptide leukotrienes were eluted in 5% aqueous pyridine [5]. The samples were again dried to remove the pyridine and were bioassayed after rehydration for LTC_4 on the guinea pig ileum using synthetic leukotriene reference standards. The reference standard was LTC_4 when LTC synthetase assays were performed and a mixture of equal parts of LTC_4 and LTD_4 when samples from incubations of intact cells were assayed. Recovery of synthetic LTC_4 through this procedure was 20.6 ± 1.4% (S.E.M.) for samples containing 1–20 ng LTC_4.

In certain experiments, the production of LTC_4 and LTD_4 was monitored by two newly-developed radioimmunoassays (G. J. White, personal communication). Both assays are based on the use of rabbit antisera which are selective for the respective leukotriene or group of leukotrienes. Cross-reactivity to other sulfidopeptide leukotrienes was as follows: anti-LTC_4, less than 10% for LTD_4 and less than 1% for LTE_4; anti-LTD_4, about 14% for LTC_4 and 79% for LTE_4. The assay procedure has been described previously [10] and is based on the sequestration of bound leukotrienes by dextran-coated charcoal at the end of the incubation.

Isolation and characterization of rat liver glutathione S-transferases. The procedures employed for the isolation of the glutathione *S*-transferase (GSH ST) fractions from rat liver cytosol have been described elsewhere [11]. Briefly, GSH ST activity in the 100,000 *g* supernatant fraction from rat liver homogenates was isolated by adsorption to and elution from glutathionyl sepharose and was then fractionated by serial chromatofocusing at increasing pH. The fractions which were eluted were characterized for their activity using both DNCB and DCNB [12], and the fraction which was primarily used in the present studies (elution peak centering on pH 9.0) defined a peak of activity in which biologic activity with the two substrates and adsorption in the ultraviolet at 280 nm appeared to coincide. The particulate GSH ST from rat liver was a suspension of a washed microsomal fraction which was isolated between 20,000 and 100,000 *g*, and which was stored at −80° beween uses. Protein concentrations were determined by a method employing a Folin–Ciocalteau reagent [13].

Kinetic studies and statistical treatment of the results. It was necessary to normalize the results from LTC synthetase assays before they could be used for kinetic analysis. To accomplish this, the results for all the inhibitor concentrations in a given experiment, for which activity fell between 15 and 85% of control (the linear portion of the log dose vs response plot), were used to compute the regression line for this relationship by the method of least squares. Normalized values for the extent of inhibition at each of the inhibitor concentrations that had been used were then interpolated from this regression curve and were used for the kinetic estimates. This method permitted us to reduce the scatter in the experimental points by relying on all the values on the curve rather than having to rely on the individual values with their larger associated errors. The same procedure was also used to obtain estimates of the 50% inhibitor concentration (EC_{50}).

The evaluation of the kinetics of the inhibition of the GSH STs was carried out under conditions where one of the substrates (GSH) was present at saturating concentration and the concentration of the other substrate was varied around the apparent K_m. The results were analyzed by the method of Lineweaver and Burk [14].

Tests for synergism were carried out with intact RBL cells which were incubated, simultaneously, with various concentrations of both DEC and piriprost. The presence of synergism in any given combination of the two inhibitors was established qualitatively as follows: Inhibition was expressed as the percent of control leukotriene synthesis activity which was found in the presence of any given inhibitor or combination of inhibitors. The predicted activity of a given combination is the geometric sum of the activities of the inhibitors used singly. Therefore, if the observed

$$[(\text{percent of control})_{\text{combined inhibitors}}]$$

is smaller than the product

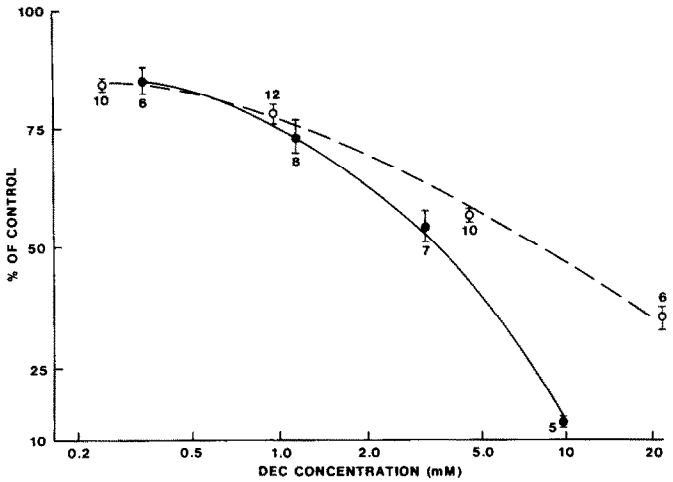

Fig. 1. Dose dependence of the inhibition of leukotriene formation by DEC. Key: closed circles, solid line, intact cells (uninhibited production was approximately 50 pmoles LTC_4 equivalents/10^6 cells in each of eight separate experiments); vertical bars, standard error of the mean. Open circles, broken line, cell-free production (uninhibited production averaged 23 pmoles LTC_4/min in the incubations in twelve different experiments). Results are corrected for leukotriene production in the absence of ionophore (intact cells) or in the absence of added enzyme (cell free). These corrections represented less than 10% of the leukotriene production in the most highly inhibited incubations.

$[(\text{percent of control})_{\text{inhibitor A}}]$ $[(\text{percent of control})_{\text{inhibitor B}}]$, synergism is demonstrated.

RESULTS

Inhibition of leukotriene formation in intact cells by DEC and piriprost. The dose-dependence of the inhibition of leukotriene formation by DEC in RBL cells that were challenged with the calcium ionophore A23187 is shown in Fig. 1. The EC_{50} value was 3 mM. Under the assay conditions employed, DEC concentrations below 0.3 mM in the assay bath did not affect the bioassay for leukotrienes. This concentration corresponds to a DEC concentration of 60 mM in the RBL cell incubations.

We have reported previously [15] that piriprost is a potent inhibitor of leukotriene formation in ionophore-challenged rat mononuclear cells (EC_{50} 4.6 μM). The EC_{50} for the RBL cells is comparable and ranged between 5 and 3.6 μM in multiple experiments (unpublished results).

Effects of DEC and piriprost on LTC synthetase. The assay for the LTC synthetase has been described previously [5], and it was demonstrated that the Triton X-100-solubilized enzyme is devoid of gamma glutamyl transpeptidase activity so that the only sulfidopeptide leukotriene which can be found is LTC_4. It is for this reason that the incubations were bioassayed using an LTC_4 standard rather than the mixed LTC_4 and LTD_4 standard which was used when mixtures of the two leukotrienes were expected to be present. DEC inhibited the LTC synthetase at concentrations which were similar to those which inhibited leukotriene formation in intact cells (Fig. 1). The EC_{50} was 7.5 mM. We have reported previously [15] that piriprost is a potent inhibitor of the cytosolic GSH STs of rat liver. However, when the effect of piriprost on the solubilized LTC synthetase was studied, no inhibition was found at concentrations ranging up to 0.35 mM in five independent experiments.

Our experience with the LTC synthetase is that, in general, this enzyme is less sensitive to inhibition than are the cytosolic GSH STs of rat liver or RBL cells [9, 16]. We therefore wondered whether DEC might also be an inhibitor of the latter enzymes. Three affinity-purified fractions of rat liver GSH ST, eluting in chromatofocusing separations at pH 6.75, 8.10 and 8.65, were unaffected by DEC concentrations up to 13 mM. One fraction, which eluted at pH 9.0, was inhibited in a concentration-dependent manner, EC_{50} 14 mM. This fraction had the following specific activities (pmoles/mg protein/min): DNCB, 12.6; DCNB, 1.41; ENPP, 0.468; ethacrynic acid, 0.0331; bromsulfophthalein, 0.235; *trans*-4-phenyl-3-buten-2-one, 0.0344; and cumene hydroperoxide, 0.809. It also catalyzed the conversion of LTA_4 to LTC_4 (0.375 pmole/mg protein/min). This activity profile did not correspond to any of the profiles for the published GSH STs [17]. The microsomal GSH ST of rat liver also was virtually unaffected by DEC. An extrapolation of the minimal inhibition which was observed (25% inhibition at 13 mM) suggests an EC_{50} in excess of 50 mM. When the ability of DEC to inhibit the generation of LTC_4 by the microsomal rat liver GSH ST (specific activity, 1.6 nmoles/mg/min) and by the cytosolic fraction eluting at pH 9.0 was examined, there was again little inhibition at DEC concentrations up to 26 mM. Extrapolation of the minimal inhibition which was seen with the latter enzyme is that the EC_{50} must be above 50 mM.

Kinetics of the inhibition of leukotriene formation. The effect of increasing concentrations of LTA_4 on the EC_{50} for DEC in inhibiting the LTC synthetase of RBL cells is summarized in Fig. 2. It was necessary to decrease the incubation volumes in order to conserve the LTA_4 and to increase the BSA con-

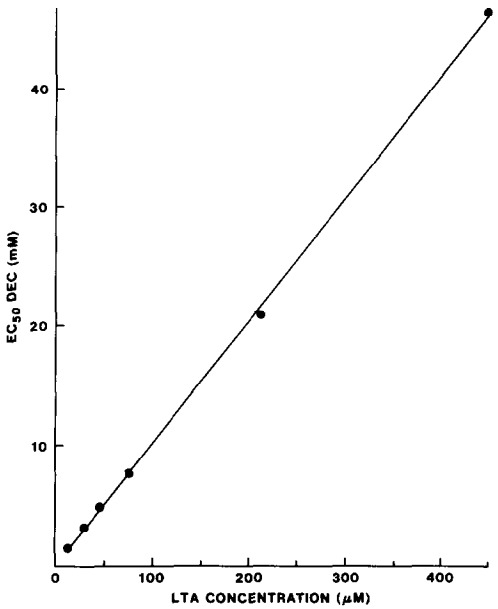

Fig. 2. Effect of LTA_4 concentration on the EC_{50} for DEC in the cell free generation of LTC_4 by solubilized enzyme from RBL cells. Results are averaged from two experiments in which the EC_{50} was determined by the method of least squares using at least three concentrations of DEC within the log-linear dose range. Uninhibited production of LTC_4 ranged from 35 pmoles/min at 30 μM LTA_4 to 167 pmoles/min at 167 μM LTA_4.

centration to 10 mg/ml in order to stabilize the higher concentrations of LTA_4 [18]. It will be noted that there was a direct correlation between the EC_{50} and the LTA_4 concentration.

Before a more careful kinetic analysis could be performed, it was necessary to obtain an estimate of the apparent K_m for LTA_4 in the LTC synthetase reaction. The effect of the LTA_4 concentration on the activity of the solubilized LTC synthetase of RBL cells in the presence of a saturating (3 mM) concentration of GSH is summarized in Fig. 3. The apparent K_m was approximately 0.1 mM.

Experiments were therefore designed to measure the kinetics of the inhibition of the LTC synthetase reaction by DEC at LTA_4 concentrations which were

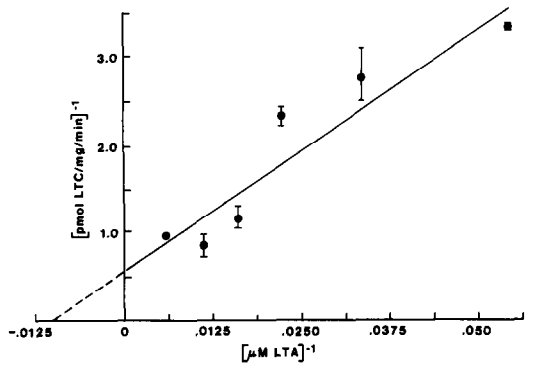

Fig. 3. Lineweaver–Burk plot for the effect of LTA_4 concentration on the production of LTC_4 by solubilized LTC synthetase of RBL cells. Results are derived from four independent experiments.

near the apparent K_m rather than at the 10 μM concentration which we employ for routine assays of this enzyme. A Lineweaver–Burk plot of the results from two such experiments is shown in Fig. 4. The lines defined by the double reciprocals of the data points (regression coefficient 0.98 or greater) all intersect on the ordinate, suggesting that the inhibition was competitive. A secondary plot of the slope of these lines against the concentration of DEC defines a straight line (regression coefficient of 0.979) and an estimated K_i of 3.75 mM. It was not possible to measure the kinetics of the inhibition by DEC with respect to changes in GSH concentration because when GSH concentrations were reduced at or near the optimal LTA_4 concentration leukotriene production declined too abruptly.

Synergism between DEC and piriprost. It seemed to us that the weak inhibitory activity of DEC might be enhanced if this inhibitor were combined with another agent which would reduce the amount of LTA_4 available to the LTC synthetase of the challenged cells. An inhibitor of the 5-lipoxygenase would be expected to have this effect. We chose piriprost as a model inhibitor since it is known to affect the formation of 5-HETE, LTB_4 and the sulfidopeptide leukotrienes [15] but not other branches of the arachidonate cascade. Furthermore, studies in human neutrophils have suggested that piriprost is an inhibitor of the 5-lipoxygenase [19]. We therefore set up experiments to test the effects of combinations of DEC and piriprost on the formation of sulfidopeptide leukotrienes in ionophore-challenged RBL cells. Table 1 presents the averaged results of two such experiments in which the same inhibitor concentrations were tested. The agreement between the results of the two experiments was within 10%. It will be noted that concentrations of piriprost which had no significant effect on the generation of leukotrienes by themselves markedly potentiated the inhibitory activity of DEC in this system. Indeed, the observed activities of the combinations of the two drugs were greater than the predicted activity of the sum of the two drugs in all of the combinations which are shown in the table.

We were interested in determining whether the observed synergism was expressed primarily by one of these drugs acting on the second or whether the effects were actually bi-directional. To gain some insight into this, we recalculated the results in Table 1, expressing the activities in the tubes which contained mixtures of the two drugs relative to the activity when either drug in the mixture was present by itself. Table 2 summarizes the activities relative to those in the absence of piriprost but in the presence of increasing concentrations of DEC, and Table 3 summarizes the activities relative to those in the absence of DEC but in the presence of increasing concentrations of piriprost. These relative activities permit the calculation of the apparent EC_{50} values for each inhibitor in the presence of increasing concentrations of the other, and these EC_{50} values are also shown in the tables. It will be noted that, while increasing concentrations of DEC had little effect on the EC_{50} for piriprost, increasing concentrations of piriprost had a profound effect on the EC_{50} for DEC. This is shown in Fig. 5 where the ratios of the EC_{50}

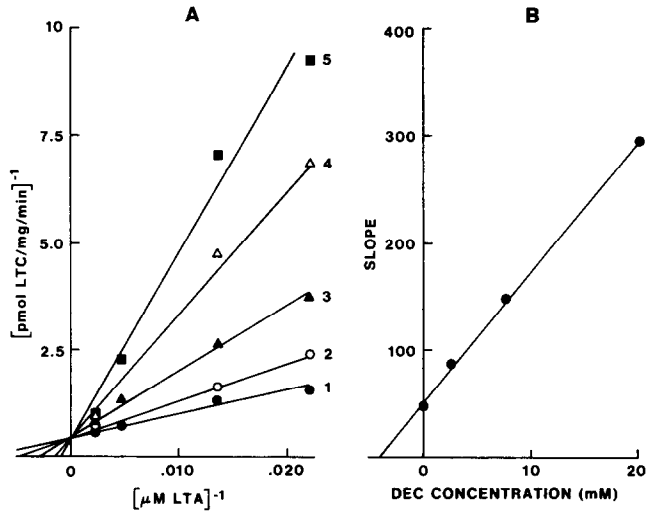

Fig. 4. (A) Lineweaver–Burk plot for the effect of DEC on the synthesis of LTC_4 at various LTA_4 concentrations (glutathione concentration at 3 mM) by solubilized LTC synthetase from RBL cells. Results are from two independent experiments. Solid circles, line 1, no DEC; open circles, line 2, 2.6 mM DEC; closed triangles, line 3, 7.9 mM DEC; open triangles, line 4, 20 mM DEC; and closed squares, line 5, 26 mM DEC. (B) Plot of the slope of the lines in panel A against the concentration of DEC. The slopes were computed after forcing the lines through a common intercept on the ordinate.

values in the absence of the second inhibitor to those in its presence are plotted against the concentration of the second inhibitor. Both interactions resulted in changes in EC_{50} which best fit a log–log plot, but the range of changes in the EC_{50} for piriprost did not exceed 10-fold, while the potentiation of the action of DEC was greater than 100-fold at the highest piriprost concentration.

A corollary to our interpretation, that the synergism between piriprost and DEC is due to the inhibitory activities of these compounds on different steps in the sequence leading to the biosynthesis of leukotrienes, is that there should not be any synergism manifested by combinations of DEC and piriprost in the LTC synthetase reaction. Table 4 shows the results of an experiment in which this possibility was tested. It is clear that, if anything, there was a slight antagonism rather than a synergism between these inhibitors.

Validation of the bioassay procedure. One of the shortcomings of the quantitation of results by bioassay employing the guinea pig ileum is the ever-present danger that the inhibitors which are being studied might interfere with the bioassay. This could take at least two forms. The inhibitors might affect the response of the bioassay or they might influence

Table 1. Synergism between DEC and piriprost

Piriprost concn (μM)	Test of synergism: Diethylcarbamazine concentration (mM) 0.0	0.026	0.079	0.254	0.791	2.64
0.0	**100**	**100**	**100**	**99.3**	**76.6**	**56.8**
0.2	**101**	0.87	0.76	0.69	0.70	0.69
0.7	**99.5**	0.85	0.57	0.76	0.41	0.38
2.3	**62.8**	0.83	0.64	0.49	0.35	0.27
7.0	**29.3**	0.67	0.38	0.17	0.14	0.11

RBL cells were incubated with combinations of DEC and piriprost of various concentrations and challenged with the calcium ionophore in the presence of cysteine. See Materials and Methods for details. The results represent the averages of duplicate incubations in two independent experiments and are expressed relative to the uninhibited controls which are taken as 100%. The "predicted" activity in the presence of the combinations is obtained by deriving the least square line for the log of the concentration against activity for the inhibitors taken singly, and then forming the product, $[(\%Control)_A \cdot (\%Control)_B]$, where A and B are piriprost and DEC respectively. The table gives the ratio of the observed activity to the predicted activity. Values that are smaller than 1.0 indicate the presence of synergism. Uninhibited leukotriene production was 0.56 nmole LTD_4 equivalents/10^6 cells.

Table 2. Effect of increasing concentrations of DEC on the inhibition by piriprost of leukotriene formation in ionophore-challenged RBL cells

Piriprost concn (μM)	Leukotriene production (%)					
	Diethylcarbamazine concentration (mM)					
	0.00	0.026	0.079	0.264	0.791	2.64
0.0	100	100	100	100	100	100
0.2	101	88.1	77.7	70.3	71.0	70.0
0.7	99.5	84.3	56.7	75.1	46.7	38.1
2.3	62.8	51.8	40.1	30.6	21.8	16.8
7.0	29.3	19.6	11.2	5.9	4.0	3.6
EC_{50} (μM)	3.53	2.12	1.00	0.98	0.58	0.47
Regression coeff.	0.999	0.953	0.991	0.921	0.999	0.987

The data are taken from Table 1. For each concentration of DEC, the activity in the absence of piriprost was taken as $100\%_{relative}$ and the activity in the presence of combinations of piriprost and DEC was then expressed relative to this value. These activities were then used to compute the EC_{50} for piriprost at the respective concentrations of DEC by the method of least squares. The regression coefficients for these computed lines are also shown.

the conversion of LTC to LTD and of LTD to LTE so that the differential sensitivity of the bioassay to these substances [20] might result in apparent changes in the total amount of sulfidopeptide leukotrienes which was produced when, in fact, there was merely a shift in the relative proportions of these substances. We eliminated the first of these pitfalls by demonstrating that there was no interference in the bioassay when DEC, piriprost, or combinations of these were present in the samples to be assayed. We sought to eliminate the second possibility by examining the effect of the inhibitors, singly or in combination, on the ratios of the sulfidopeptide leukotrienes which were produced by means of selective radioimmunoassays. We employed DEC concentrations of 0.77, 2.7 and 7.7 mM and piriprost concentrations of 2.3 and 7.0 μM. In addition, we studied the combination of 1 μM piriprost with the DEC concentrations above. Through all these conditions, the molar ratio of LTC to (LTD plus LTE) was 2.08 ± 0.23 (S.D.). It is theoretically possible to estimate the LTE concentration by solving simultaneous equations using the cross reactivity of the antibody to LTD and the relative activities of the three leukotrienes in the bioassay. However, when we used the conversion constants which we had determined on numerous occasions ([21]; 31.2 units/pmole for LTC, 62 units/pmole for LTD and 21.1 units/pmole for LTE), only uninterpretable results were obtained. We suspect that this may be due to interference between the leukotrienes in the bioassay when they are present as a mixture.

Table 3. Effect of increasing concentrations of piriprost on the inhibition by DEC of leukotriene formation in ionophore-challenged RBL cells

DEC concn (mM)	Leukotriene production (%)				
	Piriprost concentration (μM)				
	0.0	0.2	0.7	2.3	7.0
0.000	100	100	100	100	100
0.026	100	86.7	84.7	82.5	66.9
0.079	100	76.5	57.0	63.8	38.2
0.264	99.3	62.0	67.6	43.6	15.3
0.791	76.6	52.6	30.8	26.1	(10.2)*
2.640	56.8	41.0	22.8	(15.9)*	(6.8)*
EC_{50} (mM)	6.58	1.02	0.31	0.18	0.052
Regression coeff.	0.985	0.999	0.975	0.999	0.996

For each concentration of piriprost, the activity in the absence of DEC was taken as $100\%_{relative}$ and the activity in the presence of combinations of piriprost and DEC was then expressed relative to this value. These activities were then used to compute the EC_{50} for DEC at the respective concentrations of piriprost by the method of least squares. The regression coefficients for these computed lines are also shown.

* Not used in computations.

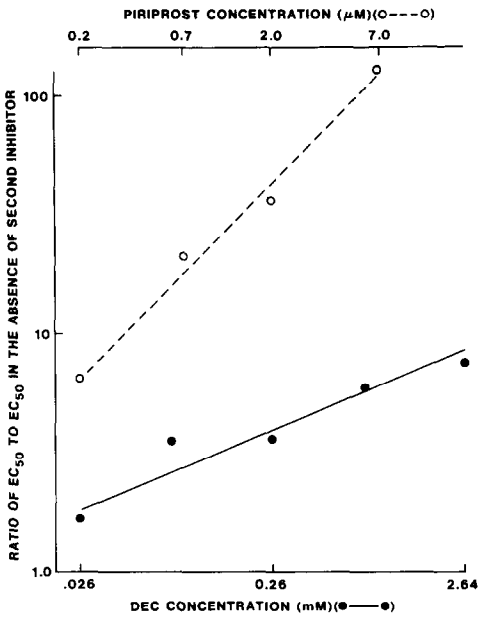

Fig. 5. Effect of increasing concentrations of DEC on the EC_{50} for piriprost (closed circles, solid line) and of increasing concentrations of piriprost on the EC_{50} for DEC (open circles, broken line). The data are derived from Tables 2 and 3 and are expressed as the ratio of the EC_{50} in the absence of the second inhibitor to the EC_{50} at the concentration shown. Note that both scales are logarithmic.

DISCUSSION

This paper addresses two related issues. First, it demonstrates that DEC, which has been known to inhibit the synthesis of SRS-A for many years [1], is an inhibitor of the terminal step in the synthesis of the sulfidopeptide leukotrienes. Second, it shows that this activity of DEC can be markedly potentiated by the simultaneous presence in the incubations of low doses of piriprost, a compound which has been reported to be a selective inhibitor of the 5-lipoxygenase.

The paper also addresses two ancillary questions which, while they may be of some interest in their own right, are necessary for the validation of the main points of this paper. These are first, the validation of the assay procedures which were employed and, second, the examination of the selectivity of action of DEC for the LTC synthetase compared to other GSH STs.

One of the perennial issues which must be addressed in any study of inhibitors is that of possible trivial explanations for the observed inhibition. In our case, where the quantitation of the leukotrienes was based on bioassay results, this is particularly important since compounds which interfere with the assay might be confused with compounds which interfere in the biosynthesis of the mediators. Thus, testing for such interference in the assay has become a routine procedure. The possibility of a more subtle interference, for example, by shifting the ratios of the three sulfidopeptide leukotrienes, is much more difficult to exclude until truly selective assays of sufficient sensitivity for the individual components of the mixture become available. We are only able to do this in part at the moment since we do not yet have selective assays for LTD and LTE and can only measure the concentration of a mixture of the two. Our results, thus far, make it unlikely, however, that shifts in the proportions of the three sulfidopeptide leukotrienes could explain the observed results.

The results presented in this paper clearly indicate that DEC is an inhibitor of the LTC synthetase of RBL cells. It differs from other inhibitors of this enzyme which we have examined, such as sulfasalazine [9], in that it appears to be more selective toward the LTC synthetase of RBL cells compared to the GSH STs in rat liver, whereas the selectivity of all the other inhibitors we have examined thus far was in the opposite direction [16]. The ability to identify selective inhibitors of LTC synthetase is of potential practical importance since one would not wish to encumber potential therapeutically-useful inhibitors of leukotriene production with the hepatotoxicity which might accompany the inhibition of the hepatic GSH STs.

The question therefore arises if the observed inhibition of LTC synthetase by DEC is sufficient to account for the inhibition of leukotriene formation in the intact RBL cells. We cannot rule out an effect of DEC on the 5-lipoxygenase or, perhaps, on the LTA synthetase as suggested by Mathews and Murphy [4], but the similarity in EC_{50} values for inhibition of leukotriene production by intact cells and for the

Table 4. Test of synergism between DEC and piriprost in inhibiting the LTC synthetase reaction using solubilized enzyme

	Leukotriene production (%)				
DEC concn (mM)	Piriprost concentration (μM) 0.0	0.26	0.77	2.6	7.7
0.0	100	100	83.5	66.9	52.9
23.5	100	99.4	89.5	87.4	60.9
118.0	100	96.7	113.3	88.5	79.3

See Materials and Methods for details. Results are the average percent of control activity which was found in the presence of the inhibitor combinations shown, where control activity was 0.18 nmole/mg protein/min based on dose–response interpolations using four different enzyme concentrations.

isolated LTC synthetase suggests that the inhibition of this enzyme may represent a major portion of the inhibition of leukotriene formation in RBL cells. However, there is the possibility that the mastocytoma cells which were used by Mathews and Murphy, and which were apparently uniquely sensitive to this inhibitor, had an abnormal LTA synthetase which was more sensitive to DEC than the corresponding enzyme in RBL cells.

Interpretation of the quantitative aspects of the comparison between the activities of DEC on intact cells and on the isolated enzyme is more difficult. On the one hand, the intact cells generate both LTC_4 and LTD_4 and probably LTE_4, and the bioassay which was used to estimate leukotriene production is unequally sensitive to these two agents. In practice, the ratio of LTC_4 to LTD_4 was quite constant under our experimental conditions, both in the current studies and over many years of previous study, so that this is not likely to be a major consideration. Another problem is the effective LTA_4 concentration in our *in vitro* incubations and, therefore, the ability to extrapolate to likely steady-state *in vivo* concentrations of LTA_4. The cell free enzyme incubations were carried out in the presence of both bovine serum albumin (to stabilize the LTA_4) and a non-ionic detergent (to solubilize the enzyme). Both these reagents bind LTA_4 and also bind the sulfidopeptide leukotrienes which are formed. Thus, the available concentration of LTA_4 in the incubations may be much lower than the added concentration while the removal of the LTC_4 which is formed may tend to drive the reaction forward. For this reason, our estimate of the apparent K_m for LTA_4 may be a gross overestimation of the actual value. However, in view of the extreme lability of this intermediate, it was not feasible to carry out the incubations in the absence of stabilizers. Nor would we expect the presence of these stabilizers to affect the qualitative conclusion that DEC is competitive with LTA_4 in its action on the LTC synthetase.

We consider the use of synergism between inhibitors as having a dual function. On the one hand, it points to possible methods for the improved utilization of the inhibitors, but on the other hand it is a valuable tool for dissecting the modes of action of the inhibitors. In the present instance, we have evidence that piriprost does not inhibit the LTC synthetase and that it appears to inhibit the 5-lipoxygenase of a variety of cells. On the other hand, DEC has been reported to be a 5-lipoxygenase inhibitor [3] and, if the observations of Shimizu *et al.* [22] apply to mammalian cells, and the LTA_4 synthetase is in fact one and the same as the 5-lipoxygenase, the observations of Mathews and Murphy [4] would also be explained as an inhibition of this enzyme. Thus, if this were the mode of action of DEC, we would not have expected to see synergism between it and piriprost.

The synergism between DEC and piriprost in inhibiting leukotriene formation thus confirms the fact that these two inhibitors are acting at different sites in the biosynthesis of the leukotrienes. Furthermore, the unusually large synergism suggests that both inhibitors must be affecting the availability of LTA_4 for the synthesis of LTC_4 and LTD_4. Since we have shown that DEC competes with the available LTA_4 at the LTC synthetase, it follows that piriprost must interfere with the formation of LTA_4 which, of course, is consistent with its presumed action as a 5-lipoxygenase inhibitor. The observation that piriprost synergized the action of DEC much more than DEC synergized the action of piriprost (Tables 2 and 3 and Fig. 5) is also consistent with the interpretation that piriprost must be acting earlier in the pathway than is DEC. The results do not, however, exclude the possibility that DEC also inhibits the LTA_4 synthetase as suggested by Mathews and Murphy [4].

It also follows from these observations that the availability of LTA_4 must be limiting the production of the sulfidopeptide leukotrienes in RBL cells. Reference to Fig. 2 indicates that the pool size of LTA_4 in uninhibited cells must be equivalent to the free LTA_4 concentration which is in equilibrium with 10 μM LTA_4 in our incubations, while the presence of 2 μM piriprost apparently reduced this pool size by a factor of 10 or more. It is also clear that a potentiation in the action of an inhibitor such as DEC to the extent which was seen here may afford a means of optimizing the inhibitory activities of inhibitors acting on a common pathway while minimizing unrelated toxic reactions.

The interest in the observations in this paper can come from an interest in the mode of the antifilarial action of DEC, from an interest in its specific site of action in inhibiting leukotriene formation or, finally, from the fact that these results raise the possibility of using combinations of two drugs, both of which affect the 5-lipoxygenase pathway of arachidonate metabolism, to achieve greater selectivity and potency. The biochemical basis for the antifilarial activity of DEC is not completely clear although a number of filarial enzymes have been shown to be inhibited by relatively low concentrations of this drug [23–25]. The results of the current paper further add to the list of publications which document the ability of DEC to inhibit the formation of sulfidopeptide leukotrienes. However, with the exception of the report by Mathews and Murphy [4], the concentrations of DEC which were required to inhibit leukotriene formation were several orders of magnitude greater than the concentrations which reportedly affected the filarial enzymes. Given the potency of DEC in those systems, it is not likely that its relatively low activity in inhibiting LTC_4 production could contribute significantly to its mode of antifilarial action.

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