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REDUCTION OF INTRACELLULAR pH BY TENIDAP

INVOLVEMENT OF CELLULAR ANION TRANSPORTERS IN THE pH CHANGE

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Abstract—Tenidap [5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1*H*-indole-1-carboxamide], a novel antirheumatic agent, produces a rapid and sustained intracellular acidification when applied to cells in culture. To investigate the mechanism by which this change in ionic homeostasis is achieved, the acidification activities of structural analogs of tenidap were determined, and the movements of [^{14}C]tenidap into and out of cells were explored. The acidification activity of tenidap was enhanced by lowering extracellular pH, suggesting that the free acid species was required for this process. Consistent with this requirement, a non-acidic analog of tenidap did not produce a change in intracellular pH (pHi). In contrast, multihalogenated derivatives of tenidap produced greater changes in pHi than did tenidap, and one analog produced a transient acidification from which the cell recovered; this recovery, however, was blocked by an inhibitor of the Na^+/H^+ antiporter. Fibroblasts incubated with [^{14}C]tenidap achieved within 5 min a level of cell-associated drug that remained constant during longer incubations. Simultaneous addition of the electrogenic ionophore valinomycin or the P-glycoprotein inhibitor 4-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-6,7-dimethoxy-2-quinazolinamine (CP-100,356) caused a time- and concentration-dependent increase in the level of cell-associated [^{14}C]tenidap; other agents tested did not promote this enhanced cellular accumulation. [^{14}C]Tenidap accumulated by fibroblasts in the presence of CP-100,356 subsequently was released when these cells were placed in a tenidap- and CP-100,356-free medium. Importantly, several agents that are known to inhibit anion transport processes, including α-cyano-β-(1-phenylindol-3-yl) acrylate, 5-nitro-2(3-phenylpropylamino)-benzoic acid, and meclofenamic acid, inhibited efflux of [^{14}C]tenidap. In contrast, ethacrynic acid and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid did not impair the efflux process. Likewise, tenidap analogs that produced a sustained intracellular acidification blocked the efflux of [^{14}C]tenidap, but non-acidifying species did not. These data suggest that movements of tenidap into and/or out of cells is a facilitated process subject to pharmacological intervention. Together, the structural selectivity of the acidification response and the evidence of facilitated transport suggest that the pHi modulating activity of tenidap is dependent on its unique physicochemical properties. Due to the dependence of these physicochemical properties on environmental and cellular conditions, *in vivo* expression of the acidification activity is likely to occur only within restricted environments that favor this tenidap-induced process.

Key words: tenidap; intracellular pH; anion transport; drug efflux

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§ Abbreviations: NSAIDs, non-steroidal antiinflammatory drugs; pHi, intracellular pH; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; PMNs, human neutrophils; HBSS, Hanks' Balanced Salt Solution; αMEM, α-Minimal Essential Medium; FBS, fetal bovine serum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; NPPB, 5-nitro-2(3-phenylpropylamino)-benzoic acid; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; UK5099, α-cyano-β-(1-phenylindol-3-yl) acrylate; tenidap, 5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1*H*-indole-1-car-boxamide; CP-72,133, 6-chloro-5-fluoro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1*H*-indole-1-carboxamide; CP-100,829, 6-chloro-5-fluoro-2,3-dihydro-3-[hydroxy-(4-chloro-2-thienyl)methylene]-2-oxo-1*H*-indole-1-carboxamide; CP-236,492, 6-chloro-2,3-dihydro-3-methyl-3-(2-thienylcarbonyl)-2-oxo-1*H*-indole-1-carboxamide; CP-64,912, *N*-(4-chlorophenyl)-2,3-dihydro-3-[hydroxy-(4-fluorophenylmethyl)methylene]-2-oxo-1*H*-indole-1-carboxamide; and CP-100,356, 4-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-6,7-dimethoxy-2-quinazolinamine.

Low molecular weight organic drugs often need to penetrate the plasma membrane of a cell to gain access to their molecular targets. NSAIDs§, for example, must enter a cell to interact with cyclooxygenases, their primary therapeutic targets [1]. Likewise, many anti-cancer drugs enter a cell prior to elicitation of their cytotoxic activity [2]. Movement across the plasma membrane may occur as a result of a passive diffusion process dependent on the lipophilicity, charge, and concentration of the drug. Increasingly, however, examples of facilitated drug transport processes are being reported. Kidney cells, for example, contain a well known *p*-aminohippuric acid transporter that facilitates transport of many different organic anions [3], and a probenecid-inhibitable organic anion transporter has been described in murine peritoneal macrophages [4]. Acquisition of drug resistance during chemotherapy can be mediated, in part, by induction of P-glycoproteins, which facilitate clearance of neutral and/or basic drugs from the cytoplasm [5]. Likewise, methotrexate transport into and out of cells appears to be a carrier-mediated process [6, 7]. Thus, influx and/or efflux of select organic molecules can oc-

cur via transporters that exist within cell membranes, but in many cases the identity of the physiologically relevant substrate for these transporters is unknown.

Tenidap is an antirheumatic agent that has demonstrated a novel therapeutic profile in clinical trials in patients with rheumatoid arthritis (Fig. 1) [8–10]. The efficacy of tenidap is characterized by a reduction in standard clinical endpoints (e.g. a decline in the number of swollen joints) as well as a reduction in several biochemical endpoints indicative of cytokine modulation (e.g. lowering plasma levels of the acute phase protein C-reactive protein) [11, 12]. Although tenidap is a potent inhibitor of cyclooxygenase [13], changes in many of the altered clinical parameters are inconsistent with tenidap's therapeutic activity being derived solely from cyclooxygenase inhibition [12]. Indeed, *in vitro* studies indicate that tenidap possesses activities not shared with reference NSAIDs [14–17]. A most unusual and novel feature that seems to be responsible for many of the *in vitro* activities of tenidap is its ability to reversibly lower pHi. This pHi-lowering activity is rapid in onset, is sustained as long as tenidap remains present within the medium, and is associated with effects on anion transport processes [18, 19]. These tenidap-induced changes in ionic homeostasis, in turn, can alter inflammatory cytokine production and function *in vitro* [19, 20] and may account for the cytokine modulatory activity observed *in vivo* [11, 12]. In contrast to tenidap, the antirheumatic agents chloroquine and hydroxchloroquine are reported to raise pH within cytosolic compartments [21]. Modulation of pHi, therefore, may represent a new and important therapeutic target.

The mechanism by which tenidap lowers pHi is unknown. Intracellular pH is maintained through the concerted action of Na^+/H^+ antiporters, Cl^-/HCO_3^- exchangers, and other channels and/or transporters that exist within the plasma membrane of eucaryotic cells [22]. We demonstrated previously that the intracellular acidification induced *in vitro* by tenidap does not result from inhibition of the Na^+/H^+ antiporter [18]. On the other hand, tenidap inhibited Cl^-/HCO_3^- exchange in human neutrophils, but inhibition of this exchanger by other agents did not necessarily lower pHi. DIDS, for example, inhibits Cl^-/HCO_3^- exchange without acutely lowering pHi [19]. The effect of tenidap on pHi, therefore, cannot result solely from its inhibition of this anion transporter. Most NSAIDs do not produce a sustained pHi-lowering response, indicating that the activity of tenidap is not dependent on cyclooxygenase inhibition [18]. Ionophores, such as nigericin, can lower pHi [23, 24], but tenidap does not dissipate the low pH within lysosomes, indicating that it does not function as a protonophore in intact cells [18]. Likewise, weak organic acids (such as propionic acid) can promote intracellular acidification, but the acidification induced by these agents is not maintained and occurs at concentrations much higher than those observed with tenidap [18, 22, 25]. To cause a sustained lower pHi, therefore, tenidap must either affect an unidentified component of the pHi regulatory apparatus or directly alter pHi as a result of novel physicochemical features. Data presented in this report identify important structural elements of tenidap that affect acidification activity and demonstrate that tenidap's movements across the cell membrane occur via a facilitated process. These results provide a basis for a hypothesis that links the acidification activity of tenidap to its unique physicochemical properties.

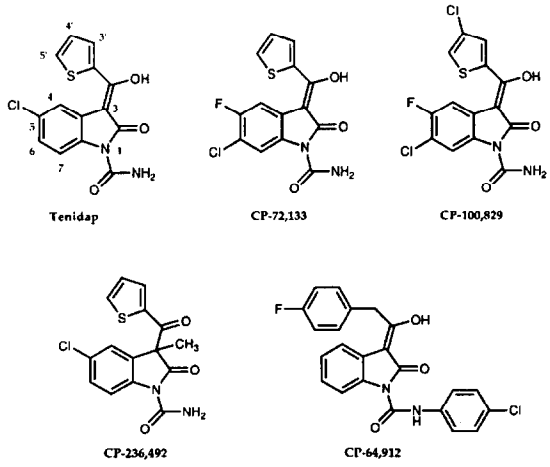

Fig. 1. Chemical structures of tenidap and its analogs.

MATERIALS AND METHODS

Cells and reagents

PMNs were obtained from the blood of normal volunteers by Ficoll-Hypaque centrifugation [26]. PMNs recovered from the gradient were washed twice with HBSS, then were subjected to one round of hypotonic lysis to remove contaminating red blood cells, and subsequently were washed with HBSS. Human fibroblasts (GM03652B) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ) and were maintained in αMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 10% FBS; fibroblasts employed in these studies were utilized prior to their 20th passage. Mouse L-cells were grown as monolayers and were maintained in αMEM, medium and RBL2H3 cells were maintained in RPMI 1640 medium; both media were supplemented with penicillin/streptomycin and 10% FBS.

Agents used in these studies that were synthesized at Pfizer Central Research included tenidap, UK5099, CP-72,133, CP-100,829, CP-236,492, CP-64,912, and CP-100,356. Agents obtained from commercial sources included valinomycin, nigericin, monensin, A23187, CCCP, DIDS, ethacrynic acid, DNP, probenecid, cycloheximide, verapamil, and vincristine from Sigma (St. Louis, MO), and meclofenamate and NPPB from Biomol (Plymouth Meeting, PA).

Intracellular pH measurements

PMNs (5×10^7 cells) were collected by centrifugation and suspended in 2 mL of modified Dulbecco's medium (20 mM HEPES, pH 7.2, 137 mM NaCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.9 mM $CaCl_2$, 5 mM glucose, 5 mM $NaHCO_3$, 1% FBS). To this suspension, 6 μL of a 1 mg/mL solution (in DMSO) of BCECF acetoxymethyl ester (Molecular Probes, Portland, OR) was added, and the cell suspension was incubated at 37° for 10 min. This suspension was diluted with 48 mL of cold modified Dulbecco's medium, and the cells were collected by centrifugation, then suspended in 1 mL of

modified Dulbecco's medium, and stored on ice. RBL2H3 and L-cells were loaded with BCECF in a similar protocol. Human fibroblasts were seeded into 3.5-cm dishes at a density of 2.5×10^5 cells/mL in 2 mL of medium; each dish contained two glass coverslips designed to fit into a spectrofluorometer cuvette. After 5 days of growth, an individual coverslip containing a confluent monolayer of fibroblasts was rinsed twice with modified Dulbecco's medium, then placed in 2 mL of modified Dulbecco's medium that contained 6 μL of 1 mg/mL BCECF acetoxymethyl ester, and incubated for 10 min at 37°. Next, the coverslip was rinsed three times with modified Dulbecco's medium.

0.1-mL sample of a BCECF-loaded PMN, RBL2H3, or L-cell suspension (5×10^6 cells) was added to 1.4 mL of modified Dulbecco's medium in a cuvette within a model 8000 SLM/Aminco spectrofluorometer. Alternatively, a coverslip containing BCECF-loaded fibroblasts was positioned in the cuvette with 3 mL of modified Dulbecco's medium. Fluorescence was recorded at a single excitation (500 nm) and emission (525 nm) wavelength. At appropriate times, a test agent was introduced into the cuvette by injection of a concentrated stock solution; the spectrofluorometer cuvette contained a magnetic stir bar to facilitate mixing. Measurements that employed PMNs were performed at 37°; fibroblast and RBL2H3 cell studies were performed at 20° to minimize dye leakage.

[^{14}C]Tenidap partitioning

Monolayers of mouse L-cells or human fibroblasts in 3.5-cm tissue culture dishes were incubated in 1 mL of αMEM, 1% FBS that contained 34 μM unlabeled tenidap and 16 μM [^{14}C]tenidap (52.4 mCi/mmol); where indicated, a test agent also was added to the medium. Cells were incubated at 37° for the indicated time periods after which the dishes were placed on ice, media were removed, and the cells were washed three times with 2 mL of cold αMEM, 20 mM HEPES, pH 7.2, 1% FBS. Washed cell monolayers then were extracted with 1 mL of 25 mM HEPES, pH 7, 150 mM NaCl, 1% Triton X-100, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and aliquots of these cell lysates were transferred to scintillation vials for determination of their radioactivity content. [^{14}C]Tenidap was labeled in the benzene ring and was prepared at Pfizer.

For efflux studies, the medium from cells loaded with [^{14}C]tenidap was discarded, and 1 mL of αMEM, 1% FBS was added immediately to the monolayers and they were incubated at 37° for 5–30 min. Where indicated, the chase medium also contained a test agent. At the end of this incubation, cells were harvested, and the cell-associated radioactivity was determined as described above. Test agents were prepared as concentrated stock solutions in DMSO and diluted into medium such that the final DMSO concentration did not exceed 0.2%; valinomycin was prepared as a 10 mM stock in ethanol.

All experiments reported were performed multiple times with comparable results; within an individual experiment, the number of replicate samples is indicated in the figure legends. Fluorescence tracings shown represent individual runs; every condition was repeated at least twice.

RESULTS

Effect of extracellular pH on the acidification response of tenidap

When tenidap was introduced into the medium surrounding BCECF-loaded human neutrophils, a rapid and sustained decrease in the fluorescence intensity was observed; this decrease is indicative of a lower cytoplasmic pH (Fig. 2A). Weak acids, such as propionic acid, are known to lower pHi by diffusion of the conjugate acid species across the plasma membrane and subsequent dissociation within the cytosol [22]. Unlike the tenidap-induced pH change, however, the acidification induced by propionic acid is not sustained [18, 22]. Tenidap also is a weak organic acid as a result of the dissociation of the enol proton (Fig. 1); its pK_a is estimated to be 3*. Assuming that the acidification induced by tenidap requires entry of its conjugate acid, then lowering extracellular pH should enhance the acidification response by causing a higher percentage of tenidap within the medium to exist as the protonated membrane-permeant species. Indeed, decreasing the pH of the medium surrounding BCECF-loaded RBL2H3 cells resulted in an enhanced initial reduction in the tenidap-induced fluorescence quenching (Table 1). Thus, at an extracellular pH of 8, 50 μM tenidap caused an 11% decrease in BCECF fluorescence intensity. At an extracellular pH of 5.1, on the other hand, the same concentration of tenidap caused a 43% decline in fluorescence intensity. At pH 7.2, the change in pHi promoted by 50 μM tenidap was estimated to correspond to an absolute change of 0.1 units [18].

Influence of structural features on the acidification response of tenidap

The enhanced acidification observed at lower extracellular pH suggested that the conjugate acid form of tenidap entered the cell and mediated the pHi change. Additional evidence that the acidic proton of tenidap was involved in this response was obtained by analyzing the pHi-altering activity of a non-acidic analog, CP-236,492 (Fig. 1). Placement of a methyl group at the 3 position of the oxindole ring prevents enolization of the ketone and renders the molecule non-acidic; in all other respects, CP-236,492 is identical to tenidap. This compound produced no significant change in pHi when applied to BCECF-loaded human PMNs (Fig. 2C). Therefore, the initial cytoplasmic acidification observed in the presence of tenidap appears to result from entry of the conjugate acid and its subsequent dissociation within the cytosol to liberate a proton and the tenidap anion.

Halogenation of tenidap improved its acidification activity. Thus, CP-72,133 and CP-100,829, which possess 2 and 3 halogen atoms, respectively (Fig. 1), produced greater acidifications than did tenidap (Fig. 2, D and E). With both analogs, their potency was not significantly different from that of tenidap, but the extent of acidification was greater at concentrations above 50 μM as the degree of halogenation increased (Fig. 3). The extent of

* The pK_a of tenidap was determined by a reversed-phase HPLC method, and log P values were determined based on partitioning between octanol and aqueous 50 mM sodium monobasic phosphate buffer at pH 4.9; Dr. E. Fiese (Pfizer Central Research), unpublished data. Cited with permission.

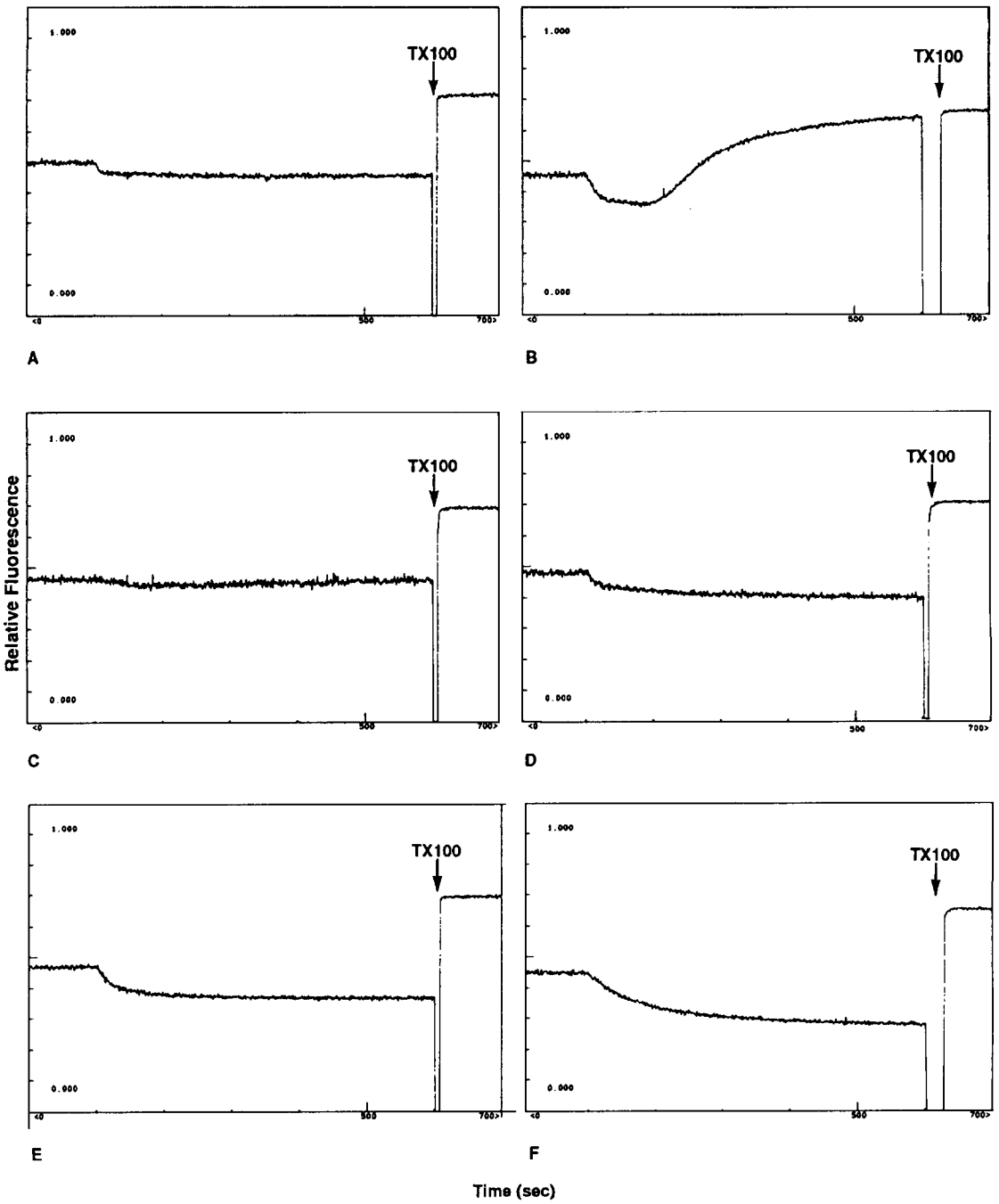

Fig. 2. Comparison of tenidap analogs as intracellular acidifiers. BCECF-loaded PMNs were treated with 50 μM concentrations of tenidap (A), CP-64,912 (B), CP-236,492 (C), CP-72,133 (D), CP-100,829 (E), and CP-64,912 plus 20 μM 5-*N*,*N*-diethylamiloride to inhibit the Na^+/H^+ antiporter (F); the test agents were introduced at 120 sec. At 600 sec, the cuvette chamber was opened and 0.02 mL of 20% Triton X-100 was added to lyse the cells and equilibrate the BCECF probe to the pH of the medium. In the absence of 5-*N*,*N*-diethylamiloride, the CP-64,912-treated cells were visibly swollen in appearance, but in the presence of this agent a normal morphology was maintained after CP-64,912 exposure. The absolute decline in fluorescence intensity (as a percentage of the initial resting intensity) was 9.4, 17, 22, and 1.4% for tenidap, CP-72,133, CP-100,829, and CP-236,492, respectively.

acidification appeared to approach a plateau value at the higher concentrations of all three agents, but this value differed (Fig. 3). The pHi change induced by both CP-72,133 and CP-100,829 was rapid in onset and sustained (Fig. 2).

Importantly, not all tenidap analogs produced a sustained acidification. CP-64,912, where the thiophene ring of tenidap is replaced with a fluorophenylmethyl group and the free carboxamide nitrogen group (at position No. 1) of tenidap is substituted with a 4-chlorophenyl group (Fig. 1), produced a rapid acidification when applied to BCECF-loaded human PMNs, but the acidi-

Table 1. Effect of extracellular pH on the pHi-lowering activity of tenidap

Extracellular pH	% Decrease in BCECF fluorescence
8	10.6
7.6	10.9
7.2	15.3
6.8	16.6
6.4	21.2
6.0	29
5.6	34
5.1	43

BCECF-loaded RBL2H3 cells were suspended within a cuvette of a spectrofluorometer in a Dulbecco's buffer containing 1% FBS at the indicated pH; the medium was buffered with 20 mM HEPES (pH values of 8 to 6.8) or 20 mM MES (pH values 6.4 to 5.1). Tenidap (50 μM) subsequently was introduced, and the resulting initial change in fluorescence intensity was determined and is indicated as a percentage of the fluorescence intensity immediately prior to the addition of tenidap. A decrease in fluorescence intensity is indicative of an intracellular acidification. Only the immediate change in fluorescence intensity observed after tenidap addition was monitored in this experiment because lowering extracellular pH also reduced pHi independently of tenidap but on a slower time scale.

fied state was not maintained (Fig. 2B). Within several minutes of its addition, the fluorescence intensity of the CP-64,912-treated cells increased and achieved a level greater than that observed in the untreated cells. The CP-64,912-induced pHi change, however, was sustained if an inhibitor of the Na^+/H^+ antiporter, 5-*N*,*N*-diethylamiloride [27], was included within the cell suspension (Fig. 2F). PMNs treated with CP-64,912, therefore, demonstrated only a transient pHi reduction in the absence of a Na^+/H^+ antiport inhibitor.

Effect of other agents on [^{14}C]tenidap accumulation

To explore whether the cellular partitioning of tenidap was sensitive to pharmacological intervention, cells were exposed to [^{14}C]tenidap in the presence of various effector agents; fibroblasts were employed for these studies because their adherence to culture plates facilitated washing procedures necessary to remove non-cell-associated ligand, and they previously were shown to display an acidification response when treated with tenidap [18]. In the absence of an additional effector, the level of cell-associated [^{14}C]tenidap reached an apparent steady state within 5 min after which no further increase occurred (Fig. 4). Addition of the electrogenic ionophore valinomycin caused a concentration- and time-dependent increase in the level of mouse L-cell-associated tenidap (Fig. 4); valinomycin is expected to hyperpolarize the membrane potential as a result of its movement of K^+ out of the cell down its concentration gradient [23]. Valinomycin also dramatically stimulated [^{14}C]tenidap accumulation by human fibroblasts (Table 2). Nigericin, an electroneutral K^+/H^+ exchanging ionophore, increased mouse L-cell-associated tenidap 3-fold and monensin, an electroneutral Na^+/H^+ exchanger, produced a 1.6-fold increase (Table 2). Nigericin and monensin also enhanced accumulation slightly by human fibroblasts (Table 2). The Ca^{2+} ionophore A23187 and the protonophore CCCP did not affect the level of [^{14}C]tenidap (Table 2); the latter agent is expected to uncouple mitochondria, an activity shared with valinomycin [23]. Likewise, the mitochondrial uncoupler DNP did not affect tenidap accumulation, nor did the protein synthesis inhibitor cycloheximide (Table 2). Ionophores are known to affect P-glycoprotein activity [28]; therefore, three additional inhibitors of P-glycoprotein function were analyzed for their effects on tenidap accumulation. Two classical inhibitors of P-glycoprotein activity, verapamil and vincristine [5], did not enhance tenidap's accumulation. In contrast, in the presence of the diaminoquinazoline P-glycoprotein inhibitor CP-100,356 [29], the level of cell-associated tenidap approached that observed with valinomycin (Table 2). The differential effect of these three agents suggests that CP-100,356 acted to alter tenidap's cellular partitioning independently of

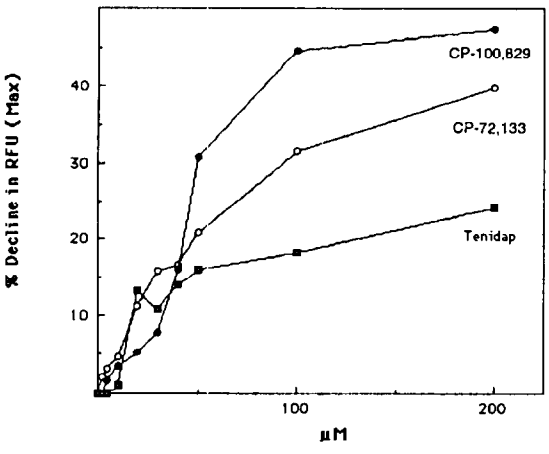

Fig. 3. Comparison of the concentration–response profiles of tenidap, CP-72,133, and CP-100,829 as intracellular acidifiers. BCECF-loaded mouse L-cells were treated with the indicated concentration of test agent, and the maximum decline in relative fluorescence units (RFU) that occurred during the initial 8 min of treatment is indicated as a percentage of the initial resting intensity.

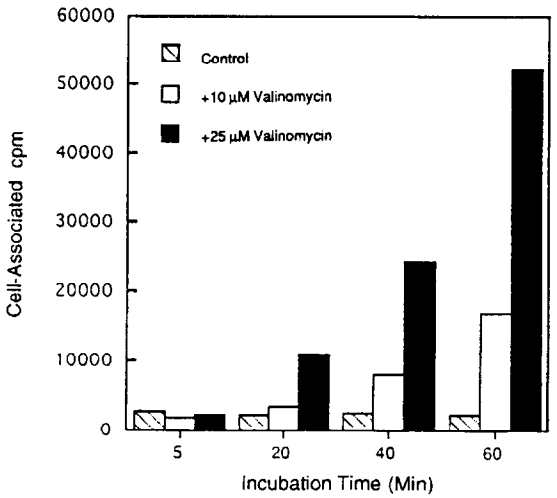

Fig. 4. Effect of valinomycin on cell-associated tenidap. Mouse L-cells were incubated for the indicated time with ^{14}C-labeled tenidap (50 μM) in the presence or absence of 10 or 25 μM valinomycin. Cell-associated radioactivity (average of duplicate wells) is indicated as a function of time of treatment.

Table 2. Identification of agents that affect the cellular accumulation of tenidap

Test agent	Concentration (μM)	Cell-associated [^{14}C]tenidap (% of control) L-cells	Human fibroblasts
Valinomycin	25	748	1760
Nigericin	25	316	146
Monensin	20	NT*	187
	25	164	NT
A23187	25	107	NT
CCCP	25	97	NT
DNP	25	99	NT
	500	NT	103
Cycloheximide	25	100	NT
Probenecid	250	81	120
	2000	NT	100
Verapamil	100	NT	75
	1000	66	NT
Vincristine	250	NT	124
CP-100,356	25	560	1560

Mouse L-cells or normal human fibroblasts were incubated with 50 μM [^{14}C]tenidap in 1 mL of αMEM, 20 mM HEPES, pH 7.2, 1% FBS and the indicated effector molecule. After a 60-min incubation at 37°, the dishes were placed on ice and the monolayers were rinsed immediately with cold medium to remove non-cell-associated tenidap. Washed monolayers subsequently were solubilized by detergent extraction, and aliquots of the cell extracts were analyzed by liquid scintillation counting. Each condition was performed in duplicate, and the average value is indicated relative to a matched control (tenidap alone). Data were compiled from a number of separate experiments; control values (tenidap alone) for mouse L-cells ranged from 1860 to 2740 cpm and for human fibroblasts from 830 to 3000 cpm. Between experiments, no adjustment for cell number differences or for specific activity differences between lots of [^{14}C]tenidap were applied.

* NT = not tested.

P-glycoprotein activity. Unlike valinomycin, CP-100,356 did not promote $^{86}Rb^+$ efflux from mouse L-cells preloaded with this K^+ analog, suggesting that it is not a potassium ionophore (data not shown). CP-100,356, however, caused a cytoplasmic acidification (data not shown); the mechanism by which this non-acidic compound caused a change in pHi is not known but P-glycoprotein function has been associated with changes in pHi [30, 31].

Identification of agents that inhibit the efflux of tenidap

Agents that promote an increase in the cellular accumulation of tenidap may do so by stimulating influx of the drug and/or by inhibiting its efflux. Loss of cell-associated [^{14}C]tenidap, therefore, was measured as a means to investigate whether the efflux mechanism was sensitive to pharmacological intervention. Cells loaded with tenidap in the presence of 10 μM valinomycin accumulated 4-fold greater amounts of radioactivity than did cells maintained in the absence of this ionophore (Fig. 5). The majority of [^{14}C]tenidap accumulated by L-cells in the absence of valinomycin was released into the medium within 5 min of suspension in tenidap-free medium (Fig. 5). [^{14}C]Tenidap accumulated in the presence of valinomycin, however, was released only slowly (Fig. 5). Removal of valinomycin from the chase medium caused a slightly greater rate of tenidap efflux, but the majority of the radiolabel still remained cell-associated after 30 min of chase (Fig. 5). Non-reversibility of the valinomycin effect is consistent with previous studies indicating that this hydrophobic ionophore is not readily displaced from biological membranes [32] and that the release of tenidap from cells is impaired by valinomycin.

In contrast, [^{14}C]tenidap was released from cells that were loaded in the presence of CP-100,356. L-cells accumulated approximately 8-fold more [^{14}C]tenidap in the presence of 25 μM CP-100,356 than in its absence (Fig. 5). Efflux of radiolabeled tenidap from non-CP-100,356-treated cells again was a rapid process (Fig. 5). Importantly, efflux of [^{14}C]tenidap from cells that were loaded in the presence of CP-100,356 also was a rapid process, and the majority of the accumulated tenidap was externalized within 5 min of chase (Fig. 5). Cells that were loaded with tenidap in the presence of CP-100,356 and then chased in the continued presence of this agent, on the other hand, retained a significant amount of the radiolabel after 5 min of chase and >30 min was required for these cells to release the bulk of their [^{14}C]tenidap (Fig. 5). These data suggest, therefore, that the enhanced tenidap accumulation observed in the presence of CP-100,356 is due, in part, to inhibition of the efflux of tenidap from the cell.

Based on this reversible accumulation, an efflux assay was developed to assess whether agents known to affect various transport processes influenced the exit of tenidap from cells. Fibroblasts were loaded with [^{14}C]tenidap in the presence of CP-100,356 after which they were chased in tenidap- and CP-100,356-free medium supplemented with a potential effector molecule. Since tenidap was shown previously to inhibit anion transport processes [18, 19], several known anion transport inhibitors were assessed as inhibitors of tenidap's efflux. The stilbene derivative DIDS did not affect the efflux (Fig. 6). On the other hand, UK5099 impaired [^{14}C]tenidap efflux from normal human fibroblasts (Fig. 6). Cells chased in the presence of UK5099 retained >50% of the initial [^{14}C]-labeled tenidap after a 12-min chase compared with <15% retained by cells in the absence of this agent (Fig. 6). Likewise, meclofenamate and NPPB, but not ethacrynic acid, caused retention of [^{14}C]tenidap (Table 3). All of these agents are reported to inhibit various anion transport processes [33–37]. The P-glycoprotein inhibitor verapamil did not inhibit the efflux of tenidap from fibroblasts, nor did the monovalent cation ionophores monensin and nigericin or the protonophore CCCP (Table 3).

The ability of tenidap analogs to inhibit [^{14}C]tenidap efflux also was analyzed. Concentrations of 50 and 100 μM CP-100,829 were equally effective at inhibiting [^{14}C]tenidap efflux (Fig. 7). In the presence of 50 μM CP-100,829, release of a small amount of cell-associated [^{14}C]tenidap was observed within the first 6 min of chase after which the level of cell-associated radioactivity remained relatively constant (Fig. 7). CP-72,133 and unlabeled tenidap also blocked [^{14}C]tenidap efflux; 100 μM tenidap was more effective than 50 μM (Fig. 7). In contrast, CP-236,492 and CP-64,912 did not produce significant inhibition of [^{14}C]tenidap efflux (Fig. 7). The ability of these oxindoles to inhibit cellular efflux of

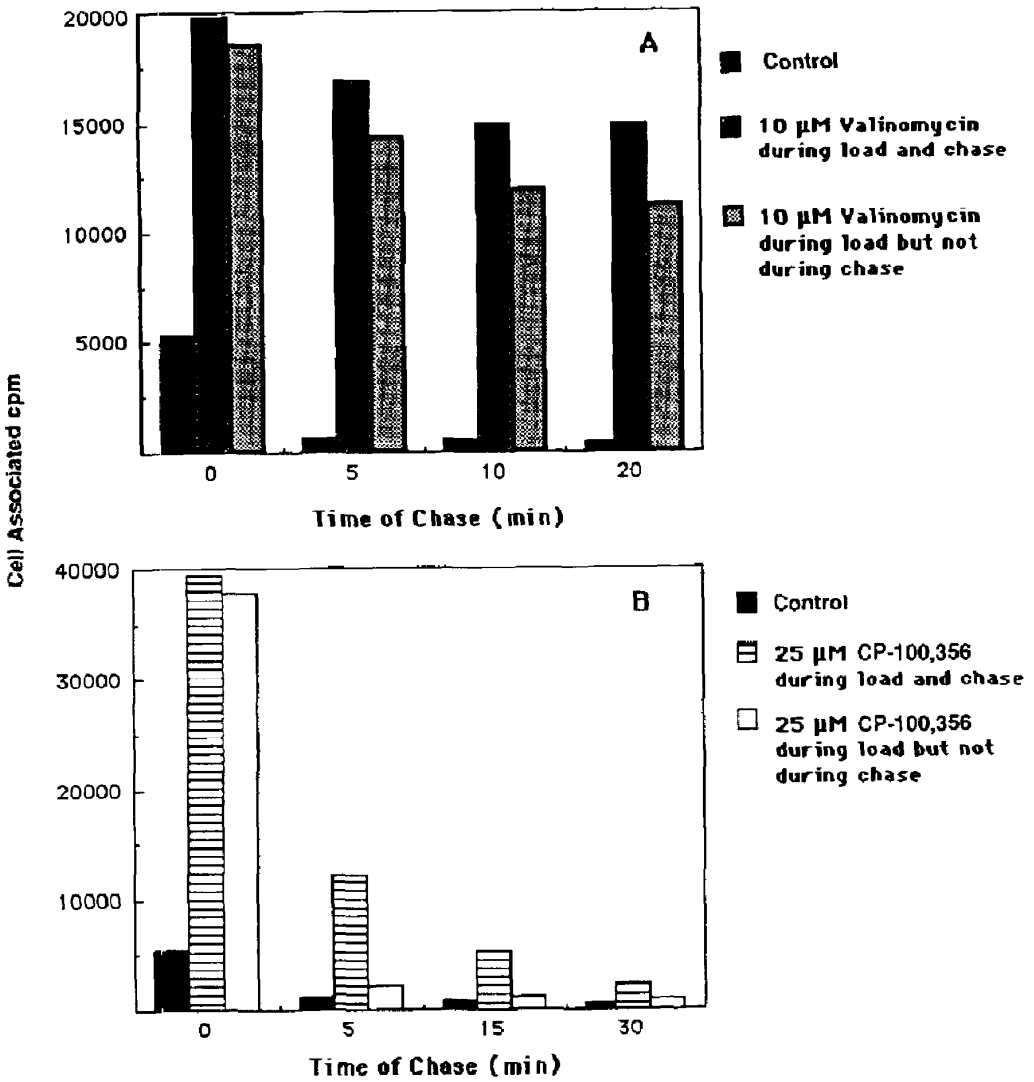

Fig. 5. Inhibition of tenidap's cellular dissociation by valinomycin and CP-100,356. Mouse L-cells were loaded with [^{14}C]tenidap for 60 min in the presence of 10 μM valinomycin (A) or 25 μM CP-100,356 (B) after which they were washed to remove non-cell-associated radioactivity and suspended in fresh tenidap-free medium in the absence or presence of valinomycin or CP-100,356. Following incubation at 37° for the indicated time, the cells were harvested and cell-associated radioactivity was determined; each data point is the average of duplicate determinations.

[^{14}C]tenidap, therefore, correlated with their ability to produce a sustained cytoplasmic acidification in human PMNs (Fig. 2).

Effect of valinomycin on the acidification response of tenidap

Valinomycin on its own did not produce a significant change in pHi (data not shown). Cells that were pretreated with valinomycin, however, demonstrated a greater tenidap-induced acidification response than did non-valinomycin-treated cells (Table 4). Valinomycin did not increase the potency of tenidap as concentrations in excess of 10 μM still were required to promote a significant change in pHi (data not shown). Rather, the potassium ionophore increased the efficacy of the acidification response, as evidenced by the greater degree of BCECF quenching obtained with 50 μM tenidap. Valinomycin is expected to hyperpolarize the membrane potential as a result of a net movement of K^+ out of the cell [23, 32]. To test the hypothesis that the extent of tenidap's acidification was dependent on the membrane potential, cells were exposed to tenidap in a depolarizing (high potassium) medium. Under these conditions tenidap still produced a rapid and sustained cytoplasmic acidification, although the magnitude of the tenidap-induced fluorescence quenching in high K^+ medium was reduced slightly relative to that observed in normal NaCl-containing medium (Table 4). Changes in membrane potential, therefore, may affect the magnitude of the acidification response, but tenidap produced a sustained acidification even under conditions where the membrane potential was minimized. The ability to alter the acidification response indicates that the magnitude of the pHi change is not fixed and is dependent on the physiological state of the cell.

DISCUSSION

The cytoplasmic acidification induced by tenidap is rapid in onset, sustained, and reversible [18], and does

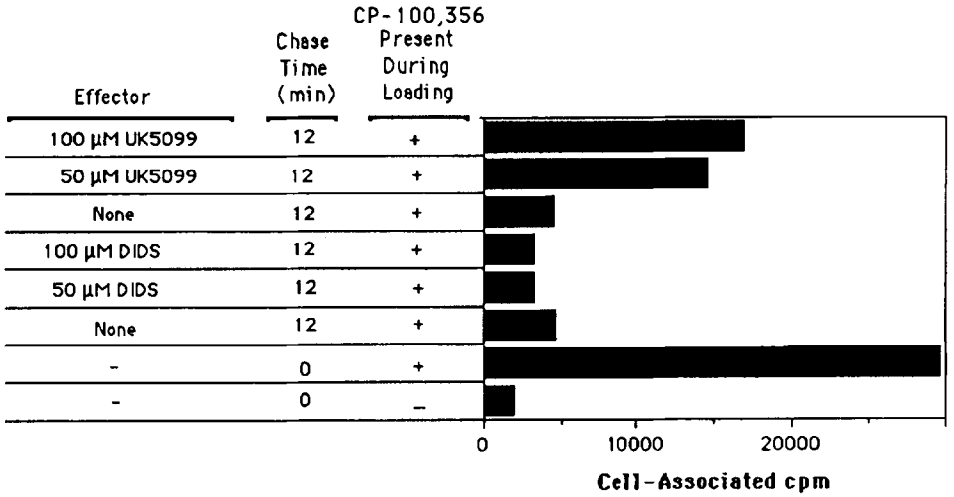

Fig. 6. Inhibition of [^{14}C]tenidap efflux by UK5099 but not by DIDS. Human fibroblasts were incubated with 50 μM [^{14}C]tenidap for 60 min in the presence (+) or absence (–) of 25 μM CP-100,356. After loading, these cells either were harvested immediately (0 min chase) or incubated for 12 min at 37° in the presence of the indicated effector agent prior to harvesting; no CP-100,356 or tenidap was present during the chase. The level of cell-associated radioactivity is indicated (average of duplicate determinations) as a function of the treatment.

not appear to result from an effect on key components of a cell's pHi regulatory apparatus; Na^+/H^+ antiport activity is not blocked by tenidap [18], and inhibition of Cl^-/HCO_3^- exchange by tenidap is not sufficient to produce the pHi change. The tenidap-induced intracellular pH change, however, is influenced by extracellular pH, and analogs possessing larger membrane partition coefficients produce greater acidifications. In addition, export of tenidap from cells appears to be facilitated by membrane-bound transporters. Based on these observations, therefore, it seems likely that the ability of tenidap to promote a sustained cytoplasmic acidification results from its physicochemical properties rather than a direct effect on a component of the pHi regulatory apparatus.

Table 3. Search for inhibitors of the efflux of tenidap

Test agent	Concentration (μM)	Cell-associated [^{14}C] tenidap vs control
Experiment 1		
Nigericin	25	0.94
Monensin	20	1.1
Verapamil	100	1.4
Experiment 2		
CCCP	20	1.0
Ethacrynic acid	50	1.1
Meclofenamate	50	2.5
Experiment 3		
NPPB	50	2.8

Human fibroblasts were loaded with [^{14}C]tenidap for 60 min in the presence of 25 μM CP-100,356. These cells then were placed in tenidap- and CP-100,356-free medium that contained the indicated effector molecule. After a 5-min incubation at 37°, cell-associated radioactivity was determined; the level of cell-associated cpm observed in the presence of an effector was normalized to that associated with matched control cells (maintained in the absence of this agent) after the same efflux period. Control cpm values for cell-associated radioactivity in experiments 1, 2, and 3 were 5460, 3860, and 580, respectively. Each condition was performed in duplicate, and the average value was utilized in the calculation.

A close structural analog of tenidap that contains a methyl substituent at the 3-position of the oxindole ring (CP-236,492) does not possess an acidic proton, and this compound did not produce a change in pHi. The inactivity of CP-236,492 indicates that a dissociable proton is a key element to the acidification response of tenidap. Likewise, lowering the pH of the medium favored the tenidap-induced intracellular acidification response. As medium pH is decreased, formation of the free acid form of tenidap, the likely membrane permeant species, will increase. Thus, this dependence on medium pH further suggests that the free acid species of tenidap is important in the acidification response. Exposure of cells to a weak acid such as propionic acid also leads to a cytoplasmic acidification. The neutral free acid species (HA) is thought to enter the cytoplasm where it dissociates to an anion (A^-) and a H^+, resulting in a rapid fall in pHi [22]. The change in pHi subsequently slows as the concentration of HA inside the cell equals its concentration extracellularly. The acidified state, however, generally is not maintained as acid extrusion systems are activated; in many cells activation of the Na^+/H^+ antiporter at the lower cytosolic pHi values removes acid equivalents in exchange for extracellular Na^+ [25, 38]. Export of the anion determines the ultimate level of intracellular pHi change. When A^- is impermeant and not transportable, such as the propionate anion, pHi returns to its initial value. However, when A^- is exportable, the new steady-state pHi value will be less than the initial value. The weak acid CO_2, for example, produces a sustained lower pHi in salamander renal proximal tubule cells because after entry it is converted to $HCO_3^- + H^+$ and the HCO_3^- anion subsequently is exported at a rate that matches the acid extrusion mechanism of the cell [22].

At concentrations of tenidap that lowered neutrophil pHi, this type of simple "weak acid" effect cannot adequately account for the observed sustained acidification. The internal buffering capacity of human neutro-

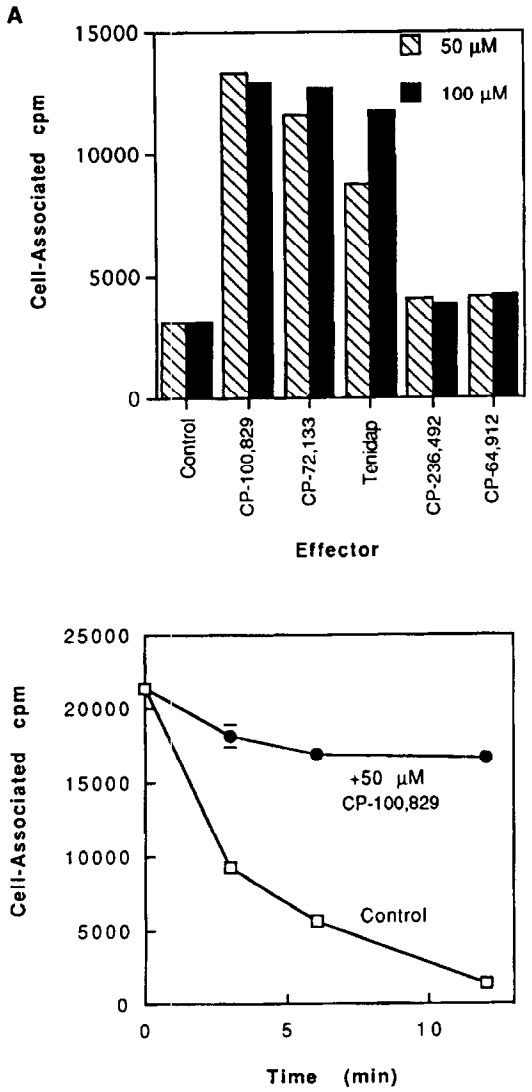

Fig. 7. Inhibition of [^{14}C]tenidap efflux by acidifying oxindoles. Human fibroblasts were loaded with [^{14}C]tenidap in the presence of 25 μM CP-100,356 for 60 min. (A) Cells were washed and suspended in fresh medium containing a 50 or 100 μM concentration of the indicated oxindole after which they were incubated at 37° for 5 min and cell-associated radioactivity was determined. Each data point is the average of duplicate determinations. (B) Time course of [^{14}C]tenidap efflux in the absence (control) or presence of 50 μM CP-100,829; each point is the mean of three separate determinations. In this experiment, after the 60-min loading with [^{14}C]tenidap, 5% of the total input radioactivity was cell-associated.

phils is estimated to be 27–50 mM/pH unit [38, 39]. Thus, a 10 mM concentration of propionic acid is required to produce a pHi change of 0.1 to 0.2 units in these cells [38]. In contrast, 50 μM concentrations of extracellular tenidap and several of its analogs produced a 0.1 unit change in pHi. These agents are stronger acids than propionic acid and possess pK_a values near 3.0 (compared with a pK_a of 4.7 for propionic acid). Based on the volume of a neutrophil (3.6×10^{-10} mL [39]) and the internal buffering capacity noted above, a cytosolic pH change of 0.1 units can be estimated to require 0.9 to 1.65×10^{-12} mmol of H^+ equivalents/cell; this is likely to be an overestimate since cytoplasm does not occupy

Table 4. Effect of valinomycin on the acidification response of tenidap

Condition	N	% Decrease in intensity
50 μM Tenidap	5	16.6 ± 3.5
50 μM Tenidap following valinomycin pretreatment	3	27 ± 1.1
50 μM Tenidap in depolarizing medium	2	13

BCECF-loaded human fibroblasts (on coverslips) were treated with 50 μM tenidap in modified Dulbecco's medium or in a depolarizing medium composed of 20 mM HEPES, pH 7.2, 0.9 mM $CaCl_2$, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 140 mM KCl, 5 mM glucose, 5 mM $NaHCO_3$, and 1% FBS. The change in relative fluorescence intensity (as a percentage of the initial fluorescence intensity) that occurred after 6.5 min (at 20°) of tenidap exposure is indicated. The number (N) of individual determinations also is indicated. Valinomycin-pretreated cells were incubated for 50–60 min with 20 μM valinomycin in αMEM medium containing 1% FBS prior to loading with BCECF; valinomycin was maintained in the medium during tenidap treatment. A similar enhancement in tenidap's acidification activity was observed when BCECF-loaded fibroblasts were exposed to valinomycin just prior to tenidap. Standard deviation of the mean is indicated where appropriate.

the entire volume of a neutrophil. In our standard pHi-lowering assay, 5×10^6 cells were employed and, therefore, a total of approximately 8 nmol of acid equivalents would be required to produce a uniform 0.1 unit reduction of pHi. At a concentration of 50 μM, the total number of tenidap equivalents added extracellularly was 75 nmol. Thus, the amount of tenidap added was sufficient to provide the acid equivalents required to produce a pHi change of the magnitude observed experimentally, but by comparison to the behavior of propionic acid the extracellular concentration did not appear sufficient to drive entry of the acid equivalents into the cells. Tenidap, however, possesses distinct physicochemical features that may allow this acidification to occur.

First, tenidap is a lipophilic acid. Based on partitioning between octanol and water, the partition coefficient of tenidap was determined to be 8931, resulting in a log P value of 3.95*. This partition coefficient is >4000-fold that of propionic acid, which is reported to be 2.09 (log P = 0.32) [40]. Thus, any free acid species of tenidap that exists at a given extracellular pH will preferentially partition into the cell membrane. Once in the membrane, an equilibrium will be established between tenidap in the membrane and that in the cytosol; the force driving the cellular association is the high energy required to solvate the non-ionized free acid species. Attachment of additional halogen atoms to the tenidap core structure enhanced its acidification activity. Analogs possessing 2 or 3 halogen atoms, CP-72,133 and CP-100,829, respectively, produced larger pHi changes than did tenidap. Halogenation is expected to increase the lipophilicity of the molecule and, in turn, to favor the membrane partitioning of the conjugate acid; log P values for CP-72,133 and CP-100,829 were determined to be 4.1 and 5.6, respectively*. Thus, the driving force provided by a high

* The pK_a of tenidap was determined by a reversed-phase HPLC method, and log P values were determined based on

membrane partition coefficient may help to explain how concentrations of tenidap below those of less lipophilic weak acids (such as propionate) achieve intracellular concentrations sufficient to produce a cytoplasmic pH change.

A second feature that distinguishes tenidap from a simple weak acid is that tenidap's export is not the result of a simple passive process. Evidence indicating that the movements of tenidap into and/or out of cells are facilitated was obtained by examining the behavior of [^{14}C]tenidap. In the presence of valinomycin, the net accumulation of tenidap by fibroblasts was increased greatly. The location within the cell where this radiolabeled tenidap accumulated is unknown. Tenidap that was accumulated in the presence of valinomycin remained cell-associated when these cells subsequently were placed in a tenidap-free medium. Valinomycin, therefore, increased the level of cell-associated tenidap and prevented its efflux, indicating that tenidap's entry and/or exit from a cell is not a simple passive process. The mechanism by which valinomycin acted to promote this accumulation is unknown. In the presence of the ionophore, accumulation of [^{14}C]tenidap increased in magnitude >7-fold but less than a 2-fold increase in pHi-lowering activity occurred. The enhanced tenidap accumulation by valinomycin-treated cells required treatment times in excess of 5 min, whereas the valinomycin effect on tenidap's pHi-lowering activity was observed immediately after ionophore addition. As a result of its rapid hyperpolarization of the membrane potential, valinomycin may favor entry of acid equivalents, thus accounting for the enhanced acidification response. On the other hand, hyperpolarization may stimulate a slower accumulation of the tenidap anion by impairing a voltage-dependent export mechanism or by promoting sequestration of tenidap within an intracellular organelle. Alternatively, valinomycin is expected to impair mitochondrial function as a result of its ionophoretic activity [32], resulting in a time-dependent depletion of cytosolic ATP. If an ATP-dependent (either direct or indirect) membrane transporter is involved in export of the tenidap anion, then the effect of valinomycin on the cellular accumulation of tenidap may result from mitochondrial dysfunction. The inability of two other mitochondrial uncouplers (DNP and CCCP) to cause a similar accumulation of [^{14}C]tenidap, however, suggests that disruption of mitochondrial function is not sufficient to promote this accumulation.

CP-100,356 also increased levels of cell-associated tenidap. Unlike valinomycin, this agent did not possess potassium ionophoretic activity but it did lower intracellular pH; it is unknown whether CP-100,356 caused a change in the membrane potential or affected intracellular ATP levels. The increased tenidap accumulation observed in the presence of CP-100,356 did not correlate with an effect on P-glycoprotein function because other P-glycoprotein inhibitors (verapamil and vincristine) did not produce a similar response. CP-100,356 inhibited efflux of [^{14}C]tenidap, indicating that this agent affected tenidap's release from cells; current data do not allow determination of whether CP-100,356 also affected tenidap's entry. Importantly, tenidap and structural analogs that promoted a sustained cytoplasmic acidification also were found to inhibit [^{14}C]tenidap efflux, suggesting that these oxindoles competed for a common cellular target involved in the transport mechanism. In addition, structurally distinct agents that lowered pHi and inhibited anion transport (e.g. UK5099 and meclofenamic acid) blocked [^{14}C]tenidap release. Two non-acidifying anion transport inhibitors, ethacrynic acid and DIDS, on the other hand, did not inhibit [^{14}C]tenidap efflux. Thus, agents that blocked the efflux of tenidap shared an ability to lower pHi. Lowering pHi, however, was not in itself sufficient to impair [^{14}C]tenidap efflux; CCCP and nigericin are expected to cause a cytoplasmic acidification due to their ionophoretic activities, yet these two agents did not impede the efflux of tenidap from cells. Perhaps, therefore, agents that impaired tenidap's efflux entered the cell and competed for binding to a transporter; the identity of this transporter(s) remains unknown.

This postulated role for a membrane transporter in tenidap's acidification response is supported by previous observations concerning several structurally dissimilar agents that also alter pHi. Members of the fenamate family of NSAIDs are reported to interact with chloride channels and Cl^-/HCO_3^- exchangers [33, 37, 41] and, like tenidap, fenamates can lower intracellular pH [19]. Similarly, UK5099 lowers intracellular pH and inhibits several types of anion transporters [18, 35, 42]; this agent is an analog of α-cyano-4-hydroxycinnamate and is a weak acid. Thus, these agents share an ability to produce a sustained intracellular acidification at micromolar concentrations and to inhibit various anion transport processes. Fenamates also are expected to possess high membrane partition coefficients; the estimated log P value for meclofenamate, for example, is 5.26.* The log P value of UK5099 is unknown. In contrast, DIDS inhibits anion transport processes but does not produce a rapid change in pHi. Importantly, DIDS is impermeant and does not readily enter a cell [43]. Therefore, the sustained cytoplasmic acidification produced by tenidap may result not from inhibition of an anion transporter, but rather from tenidap's entry into a cell as a conjugate acid followed by the facilitated efflux of its anion via an anion transporter; as a substrate, tenidap may impair the transporter's normal function. Facilitated efflux of tenidap's anion may result in its rapid recycling to the medium where it enters the pool of extracellular anions and, by mass action, promotes formation of additional membrane permeant free acid. This "recycling" action thus may provide an additional driving force to overcome the internal buffering capacity of the cell and a mechanism for sustaining the acidification response.

The ability of tenidap to maintain a sustained intracellular acidification is not shared by other weak organic acids [18] and is not displayed by all of its analogs. CP-64,912, for example, produced an initial acidification from which neutrophils recovered; this recovery, which achieved a final value greater than the resting pH,

partitioning between octanol and aqueous 50 mM sodium monobasic phosphate buffer at pH 4.9; Dr. E. Fiese (Pfizer Central Research), unpublished data. Cited with permission.

* Calculated log P value for meclofenamate was determined using Pomona Med Chem Project software for partitioning of the neutral carboxylic acid between *n*-octanol and water; Dr. Chris Lipinski (Pfizer Central Research), unpublished data. Cited with permission.

appeared to be mediated via a Na^+/H^+ antiporter since it was blocked by 5-*N*,*N*-diethylamiloride. Likewise, tenidap's cellular partitioning is not characteristic of all weak organic acids. The antitumor agent *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea, for example, is reported to accumulate within adenocarcinoma cells [44, 45], but its accumulation is not sensitive to the same effectors as tenidap. Thus, valinomycin did not increase cell-associated levels of *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea, while nigericin decreased accumulation of this agent [46]. Therefore, although both tenidap and *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea are weak organic acids, their association with cells in culture is modulated by distinct agents. This differential behavior is consistent with the notion that the cellular entry and/or exit of tenidap is not a simple passive process.

Despite uncertainty as to the identity of the transporter(s) involved in tenidap's efflux, the data provide evidence with which to propose a model for tenidap's acidification activity (Fig. 8). In this model, the net effect of tenidap is dependent upon three key physicochemical parameters: (1) pK_a, (2) partition coefficient of the free acid species (log P), and (3) the affinity of the anion for an anion transporter. The ability of tenidap to display its pH-lowering activity *in vivo* will depend on the local environment within which a cell is exposed to this agent. Levels of tenidap in patients achieve peak plasma concentrations of 60 μM [9–11]; the avid binding of tenidap to serum proteins, however, reduces the effective free concentration to levels that will not support the pH-lowering activity. At local tissue sites, however, extracellular pH can become acidic; for example, inflammatory effusions from rheumatoid arthritis patients [46], the pericellular environment of a chondrocyte [47], and the lumenal content of the proximal tubule [48] all are reported to possess acidic pH values. Moreover, cells may change their membrane potential and/or intracellular pH during different states of activation [49, 50]. Likewise, binding of tenidap to serum proteins is affected by environmental factors including the concentration of fatty acids that compete for albumin binding sites and the types of counter ions present.* Therefore, local "privileged" environments may favor the pH-lowering activity by increasing the free concentration of tenidap, by increasing the amount of the membrane permeant conjugate acid through acidification, and, based on analogy to a valinomycin-treated cell, by changing the physiological state of the cell to enhance the acidification response.

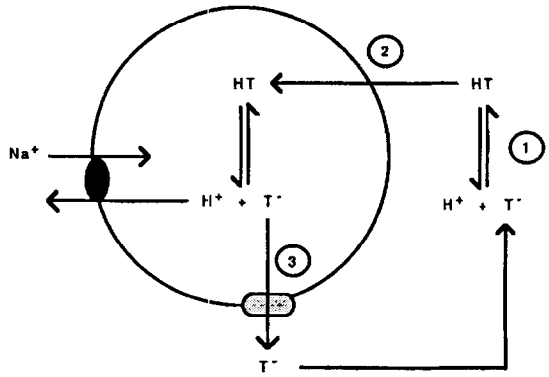

Fig. 8. Model to explain the acidification activity of tenidap. First, the conjugate acid of tenidap enters a cell via diffusion (Step 2); the abundance of the protonated species is determined by the pK_a of the acid (Step 1). In the cell, an equilibrium is reestablished between the protonated and unprotonated forms. The Na^+/H^+ antiporter functions in an attempt to counteract the resulting acid load. The tenidap anion, however, is exported from the cell via an anion transporter (Step 3). Unlike the case with impermeant anions (such as propionate), export of the tenidap anion prevents its buildup intracellularly, which, in turn, permits additional influx of tenidap's conjugate acid. This "recycling" action sustains the acidified state of the cell in the face of regulatory elements (such as the Na^+/H^+ antiporter) working to restore the original pHi and the internal buffering capacity of the cytosol.

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