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EXTRACELLULAR pH, TRANSMEMBRANE DISTRIBUTION AND CYTOTOXICITY OF CHLORAMBUCIL

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Abstract—The effects of extracellular pH (6.2 to 7.3) on uptake and cytotoxicity of the weak acid anti-tumor drug chlorambucil were investigated. Decreasing extracellular pH from 7.3 to 6.5 had a negligible effect on the intracellular pH of Chinese hamster V79 fibroblasts, thus resulting in the formation of a transmembrane pH gradient (intracellular alkaline). Addition of high concentrations of acetate or bicarbonate partially collapsed the pH gradient. Chlorambucil ($pK_a = 5.8$) behaved as a weak acid with enhanced accumulation and cytotoxicity at extracellular pH < 7.0. As predicted for a weak acid, partial collapse of the transmembrane pH gradient decreased both uptake and cell killing. Since the interstitial pH of micrometasteses and solid tumors of many cancers is low relative to normal tissues, these results have potential implications for both *in vitro* drug testing and *in vivo* therapy.

Some anti-tumor drugs (e.g. melphalan) are transported by energy-dependent processes and, as a consequence, their intracellular:extracellular concentration distribution ratios can be greater than one (reviewed in Ref. 1). In contrast, drugs accumulated by passive diffusion processes without the input of cellular energy usually exhibit transmembrane distribution ratios equal to one. However, within this latter category of drugs are included lipophilic weak acids (e.g. chlorambucil) and bases (e.g. adriamycin) whose respective distribution ratios may be governed by transmembrane pH gradients [2, 3]. For neoplastic cells that depend extensively on aerobic glycolysis and, as a consequence, acidify the surrounding interstitial fluids, the relative cytotoxicities of weak acid and weak base drugs may therefore depend on transmembrane pH gradients formed as a consequence of this acidification. Cells can maintain an intracellular pH greater than 7 at high extracellular lactate or bicarbonate levels in spite of a substantial extracellular acid load [4]. Furthermore, several studies indicate that the interstitial pH of many solid tumors is acidic relative to normal tissues and can be further acidified either by hyperthermia [5] or glucose infusion [6].

Lipophilic weak acids and bases have been used extensively for the measurement of intracellular pH and transmembrane pH gradients (reviewed in Ref. 7). Their transmembrane distributions are described by the Henderson–Hasselbalch equation with weak acids concentrating in alkaline compartments and weak bases accumulating in acidic compartments. How faithfully these distributions reflect the magnitude of a pH gradient is, in part, dependent on the relative permeabilities of the ionic and non-ionic species. Ideally, the charged species is impermeant and the measured weak acid or base distributions reflect the ratios of dissociated to non-dissociated acid or base in the intracellular and extracellular spaces. With respect to weak acid/base anti-tumor drugs, Skovsgaard [8] with Ehrlich ascites tumor cells and Dalmark and Storm [9] with erythrocytes have shown that uptake of adriamycin is pH sensitive and the drug, in part, behaves as a weak base. A preliminary study by us indicated that the accumulation of chlorambucil by SV40 transformed lymphocytes and Chinese hamster fibroblasts is also pH sensitive [2]. Other investigators have demonstrated recently that chlorambucil exhibits enhanced cytotoxicity upon shifting extracellular pH from 7.8 to 7.2 [10].

In the present report, we examine the relationship between cellular pH gradients and the transmembrane distribution and cytotoxicity of the weak acid chlorambucil. Chlorambucil (NSC 3088) is an alkylating agent with a phenylbutyric acid moiety characterized by a pK_a of approximately 5.8 [11].

EXPERIMENTAL PROCEDURES

The radiochemicals [^{14}C]MA† (60 mCi/mmole), [^{14}C]DMO (60 mCi/mmole), ^3H_2O (1.0 mCi/mg), and [^{3}H]polyethylene glycol (4000 daltons, average molecular weight, 2.0 mCi/g) were products of the New England Nuclear Corp. (Boston, MA). [^{14}C]-Ethylene-chlorambucil (10.8 mCi/mmole) was provided through the auspices of Dr. Robert Engle of the National Cancer Institute and synthesized by Dr. Charles Lauffer of the Stanford Research Institute (Palo Alto, CA). Other reagents and their suppliers are: SF1250 silicone oil (Harwick Chemical Co., Wilmington, MA) and chlorambucil (Sigma Chemical Co., St. Louis, MO).

The purity of chlorambucil was assessed by silica gel chromatography with the following solvent system: chloroform–methanol (9:1, v/v). With this solvent system, an $R_f = 0.5$ was obtained for chlo-

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† Abbreviations: MA, methylamine; and DMO, 5,5-dimethyloxazolidine-2,4-dione.

rambucil. All solutions of this drug were prepared in methanol immediately before use, a necessary precaution suggested by other investigators [12]. Incubation under the experimental conditions described below did not result in degradation of chlorambucil as monitored by chromatography.

Chinese hamster fetal lung fibroblasts (V79) were grown in roller bottles, as previously described, in RPMI 1640 with 10% fetal calf serum [13], and drug cytotoxicity was assessed by plating treated cells in 60 mm diameter dishes in quadruplicate and incubating for 7 days at 37°. After staining with Giemsa, colonies were scored. The plating efficiency was 55–60% and did not vary with the experimental conditions employed except at very low pH (pH 6.2) where in some experiments it was reduced slightly.

Cytotoxicity and drug uptake experiments were performed under identical incubation conditions. The final methanol concentration contributed by the drug solution did not exceed 0.2%. Cells were harvested and washed twice with phosphate-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 5 mM sodium phosphate) titrated to the appropriate pH. Where indicated, acetate or bicarbonate was added as their sodium salts and replaced an equimolar amount of NaCl in the solution. For cytotoxicity studies, cells ($2–5 \times 10^6$ cells/ml) were treated with drugs at the concentrations indicated in the text for 1 hr at 37°. After incubation, cells were diluted 10-fold with culture medium and washed once before dilution and plating.

For transport experiments, cells were split into three aliquots and incubated at 37° at times indicated in Results. Two aliquots contained either 1.0 μCi/ml ^3H_2O to determine total pellet volume or 1.0 μCi/ml [^{3}H]polyethylene glycol to measure extracellular space. The difference between these two measurements provided an estimate of intracellular water space [14]. In the third aliquot, [^{14}C]chlorambucil (1.0 μCi/ml; 1–200 μg/ml), or 1.0 μCi/ml [^{14}C]MA, or [^{14}C]DMO was added after a 5-min equilibration at 37°. At designated times during incubation, 200-μl samples were removed and layered over 500 μl silicone oil and centrifuged for 30 sec at 10,000 *g* min in a Beckman microfuge to separate cells from their suspending medium. The supernatant fraction and silicone oil were aspirated off, and the cell pellet at the tip of the microfuge tube was cut off into a scintillation vial. An aliquot of each supernatant fraction was used to determine total pellet water, polyethylene glycol and drug concentrations. In some experiments, 10% ice-cold trichloroacetic acid was added to cells incubated with [^{14}C]chlorambucil to distinguish covalently bound from free intracellular drug. Intracellular pH and chlorambucil concentrations were calculated as described in Ref. 7.

Some additional experimental features require noting. Chlorambucil at the concentrations employed did not alter intracellular or extracellular water spaces. The extracellular pH values given in the text represent an average of initial and end of incubation pH since V79 cells at pH > 7.0 can slightly acidify their incubation buffer (0.2 pH units after 1 hr of incubation at 37° and initial pH = 7.5).

RESULTS AND DISCUSSION

Intracellular pH and transmembrane pH gradients are calculated as a function of extracellular pH from measurements of the transmembrane distributions of the weak acid, DMO, and the weak base, MA (Table 1). The uptake of both probes reached equilibrium within 10 min and remained unchanged for up to 60 min. Experiments with both pH probes indicated that V79 cells, as do other cells (e.g. Ref. 4), can maintain an intracellular pH > 7.0 at values of extracellular pH > 6.5. The resulting transmembrane pH gradient is constant for at least 1 hr of incubation at the experimental conditions employed. With MA as probe, estimates of intracellular pH are lower in comparison to values obtained with DMO but, as noted by others [7], this probably reflects the differential permeabilities of the respective charged species of MA and DMO or intracellular organelle accumulation (mitochondria with DMO and lysosomes with MA).

These results indicate that, over the limited pH range tested, intracellular pH was relatively constant and the magnitude of ΔpH was proportional to extracellular pH. At pH < 6.5, there was a progressive decrease in intracellular pH although a substantial ΔpH was still observed. Inclusion of 20 mM sodium acetate (or bicarbonate, data not shown) in the incubation medium to collapse the pH gradient was only partially effective with an approximate 50% reduction in ΔpH at pH = 6.6 (Table 1). Other investigators have also demonstrated that high weak acid concentrations do not completely collapse ΔpH [4].

The time course for transport of chlorambucil by V79 cells is shown in Fig. 1. At extracellular pH values of 7.3 and 6.5, the time course for uptake was qualitatively the same. An initial rapid uptake phase was essentially complete by 2 min, and this was followed by a phase of lower rate of accumulation. The latter stage was mostly due to covalent binding of the drug since subtraction of trichloroacetic acid

Table 1. Intracellular pH as a function of extracellular pH

Extracellular pH	Intracellular pH		ΔpH	
	DMO	MA	DMO	MA
pH = 7.3	7.36 ± 0.05	7.28 ± 0.06	0.06	−0.02*
pH = 7.3 (+20 mM acetate)	7.39 ± 0.08	7.29 ± 0.02	0.09	−0.01
pH = 6.6	7.04 ± 0.04	7.07 ± 0.04	0.44	0.47
pH = 6.6 (+20 mM acetate)	6.81 ± 0.09	6.75 ± 0.07	0.21	0.21

* Negative sign indicates an acidic pH inside relative to extracellular pH.

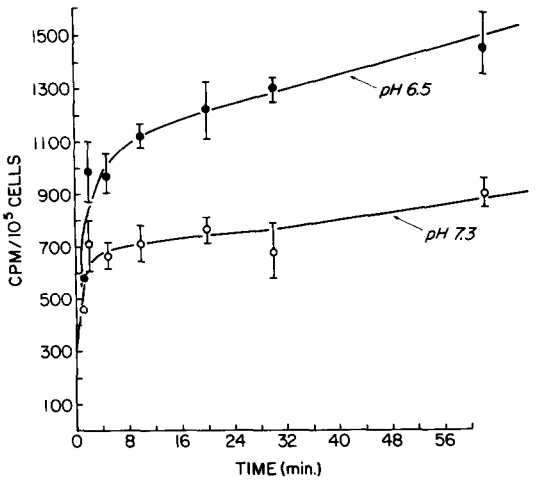

Fig. 1. Chlorambucil uptake as a function of extracellular pH. Uptake was measured as described in Experimental Procedures and is uncorrected for extracellular space or covalent binding of drug. Data points are the average of triplicate samples ± S.E.M. Drug concentration was 1.0 μg/ml. Key: (○—○) pH 7.3; and (●—●) pH 6.5.

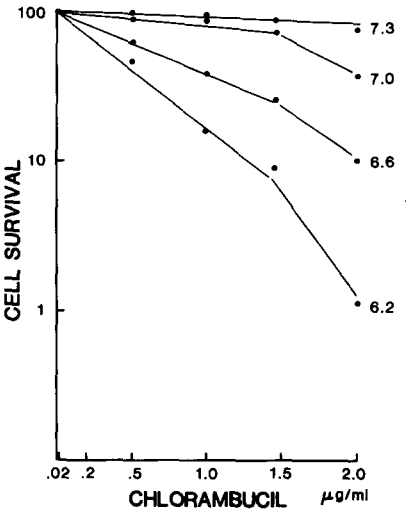

Fig. 2. Chlorambucil cytotoxicity as a function of extracellular pH. Cells were incubated for 1 hr at 37° at designated pH and drug concentration. The results represent the average of quadruplicate samples with a cell count S.E.M. < 10%.

precipitable counts eliminated this uptake. Both the initial rate of uptake and the steady state levels were greater at the lower extracellular pH. However, the amount of covalently bound drug as a percentage of total cell associated drug (approximately 10% at 30 min of incubation) did not differ over the pH range tested, i.e. the alkylating activity of the drug was pH insensitive.

Analysis of chlorambucil transport as a function of drug concentration (0.5 to 50 μg/ml) over the extracellular pH range 6.6 to 7.3 demonstrated that drug uptake was exclusively by passive diffusion. This confirms results obtained in studies with Yoshida ascites sarcoma cells and L5178Y lymphoblasts (e.g. Refs. 12 and 15].

The transmembrane distribution ratios for chlorambucil over the pH range 6.6 to 7.3 indicate that this drug distributes across the plasma membrane of V79 cells as a weak acid (Table 2). As with DMO, increased drug uptake was observed with decreasing extracellular pH. At pH 6.6 for example, total cell associated chlorambucil was approximately five times greater than at pH 7.3. In terms of free drug concentrations, the transmembrane distribution at pH 6.6 was 3.5-fold greater. Uptake at extracellular pH < 7.0 was also partially inhibited upon addition of 20 mM sodium acetate. Acetate had no measurable effect on drug accumulation at pH > 7.0.

Figure 2 is a survival curve for V79 cells as a function of drug concentration and extracellular pH. As predicted from the results in Table 2, enhanced cell killing was observed at pH values less than 7.0 over the entire drug concentration tested. Furthermore, the enhanced cell killing was observed throughout treatment (15–60 min, unpublished).

The effect of sodium acetate on cell survival is shown in Fig. 3. At extracellular pH = 6.6 or 6.2, cell survival in the presence of 1.0 μg/ml chlorambucil was increased markedly by co-incubation with acetate. At pH 6.2 without drug (Column 3), acetate reduced clonogenicity possibly by a process involving intracellular acidification during the 1-hr incubation. Acetate had no effect at pH = 7.3 with or without drug.

The above experiments demonstrate that, at an extracellular pH < 7.0 and with formation of a transmembrane ΔpH (intracellular alkaline), increased cell killing is observed with weak acid drugs. These results and work describing the weak base behavior of adriamycin [8, 9] indicate that extra-

Table 2. Chlorambucil distribution as a function of extracellular pH*

Extracellular pH	Intracellular [chlorambucil] / Extracellular [chlorambucil]
pH = 7.3	1.2
pH = 6.6	4.0
pH = 7.3 (+20 mM acetate)	1.1
pH = 6.6 (+20 mM acetate)	2.3

* Chlorambucil concentration was 0.6 μg/ml, and uptake was terminated after a 30-min incubation at 37°. Concentration ratios were corrected for covalently bound drug and extracellular space.

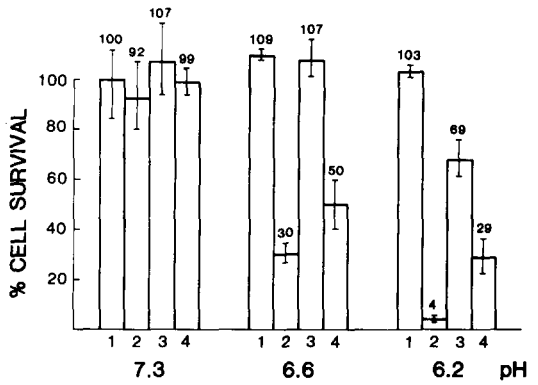

Fig. 3. Effect of sodium acetate on chlorambucil cytotoxicity as a function of extracellular pH. Cells were incubated for 1 hr at 37° at designated pH and at a chlorambucil concentration of 1.0 μg/ml. Percent survival was calculated from the average of quadruplicate samples (cell count S.E.M. < 10%) relative to column 1 (pH 7.3). Key: column 1, no drug; column 2, 1.0 μg/ml drug; column 3, 20 mM sodium acetate; and column 4, 1.0 μg/ml drug plus 20 mM sodium acetate.

cellular pH can be a critical factor in determining the effectiveness *in vitro* of anti-tumor drugs that exhibit weak acid/base properties. These findings may also be of potential clinical significance. *In vivo*, the interstitial pH of some solid tumors is low, and additional acidification can be achieved with hyperthermia [5] and/or glucose perfusion [6]. Normal tissues on the other hand maintain an interstitial pH > 7.0. Thus chlorambucil and other weak acid drugs may be candidates for thermochemotherapy studies. Furthermore, these results present one rationale for multi-drug therapy combining weak acid and weak base drugs.

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