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Selective inhibition by bis(2-chloroethyl)methylamine (nitrogen mustard) of the $Na^+/K^+/Cl^-$ cotransporter of murine L1210 leukemia cells

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Incubation of L1210 murine leukemia cells in vitro with 10 μM of the bifunctional alkylating agent bis(2-chloroethyl)methylamine (nitrogen mustard, HN2) for 10 min brought about a fall of more than 99.9% in their ability to form colonies when the cells were suspended in 0.5% nutrient agar. Incubation with HN2 also inhibited the influx of the potassium congener $^{86}Rb^+$ to exponentially proliferating L1210 cells in a concentration-dependent manner. This inhibition was specific and was accounted for by a reduction of a diuretic-sensitive component of $^{86}Rb^+$ influx, identified in the preceding paper (Wilcock, C. and Hickman, J.A. (1988) Biochim. Biophys. Acta 946, 359–367) as being mediated by a $Na^+/K^+/Cl^-$ cotransporter. Inhibition by 10 μM HN2 was complete after a 3-h incubation. There was no inhibition at this time of the ouabain-sensitive component of $^{86}Rb^+$ influx, mediated by Na^+/K^+-ATPase. After 3 h of incubation with 10 μM HN2 there was also no change in the membrane potential of the treated cells as measured by the distribution of the [^{3}H]TPMP$^+$, no decrease in cellular ATP concentration and no change in intracellular pH, and the ability of the cells to exclude the vital dye Trypan blue was not significantly different from control values. These effects of HN2, therefore, appeared to follow lethal damage, but precede cell death. In the stationary phase of L1210 cell growth, the component of HN2 and diuretic-sensitive K^+ influx to L1210 cells was reduced, whilst the component constituting the HN2-insensitive ouabain-sensitive sodium pump was increased. The monofunctional alkylating agent MeHN1 (2-chloroethyldimethylamine) which cannot cross-link cellular targets and has no antitumour activity, did not inhibit $^{86}Rb^+$ influx to L1210 cells when incubated at equimolar or equitoxic concentrations to HN2. Intracellular potassium concentration was maintained close to control values of 138 ± 10 mM in HN2-treated cells because of an approx. 35% fall in cell volume. The results suggest that the $Na^+/K^+/Cl^-$ cotransporter is a selectively inhibitable target for HN2, and the lesion is discussed with reference to the cytotoxic effects of this agent.

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Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; DMO, dimethyloxazolidine-2,4-dione; DTNB, 5-dithiobis(2-nitrobenzoic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MeHN1, (2-chloroethyl)dimethylamine; HN2, bis(2-chloroethyl)methylamine (nitrogen mustard); KR, Krebs-Ringer bicarbonate buffer; $TPMP^+$, triphenylmethylphosphonium ion.

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Introduction

The inherent chemical reactivity of the cytotoxic, antineoplastic alkylating agents necessarily makes them promiscuous in their interactions with cellular components. Irrespective of what these cellular targets are, for the expression of antineoplastic activity two or more alkylating groups are required which are capable of the formation of covalent bonds with target molecules [1], so that it appears that reactions are necessary which lead to the chemical cross-linking of vital components of the cell. From this it follows that there are biological targets which, when modified in some way by cross-links, induce cell death. These targets appear to be, specifically, of importance to cells which are committed to replication, since the alkylating agents are more cytotoxic to dividing cells than they are to quiescent cells, a property which provides them with their utility in the treatment of cancer [2].

Despite four decades of studies on the mechanism of action of the alkylating agents [3–5], definitive statements cannot be made regarding the nature of the biochemical events by which they bring about cell death. Generally, the interstrand cross-linking of DNA has been considered to be an important event which prevents cell replication [6–8], and it has been suggested that the disablement of the DNA template inhibits replication of the genome to slow the passage of cells through the cell cycle [9–11]. Despite this, alkylating agent-treated cells may, apparently, complete the duplication of their DNA and go on to die in the G_2 phase of the cell cycle [11,12]. As yet, no unequivocal explanation has been presented of why an inhibition of replication should selectively bring about the death of cells which are committed to division. Ill-defined concepts of the imposition of 'unbalanced growth', presumed to arise from genetic damage, have been promulgated, but little evidence has been provided in support of such hypotheses.

The biochemistry of quiescent and dividing cells differs not only with respect to the synthesis and replication of DNA. On the contrary, a mitogenic stimulus sets in motion a complex cascade of events which results in the replication of the genome and cytokinesis. The earliest of these events takes place at the level of the plasma membrane, where the release of second messengers and changes in ion flux appear to play a pivotal role in the response to growth factors (reviewed in Ref. 13). Subversion of these cascades by the products of certain oncogenes is considered to be partly responsible for the malignant phenotype of some cells (reviewed in Ref. 14). In addition to providing elements of the second messenger signalling systems which regulate cell growth, changes of ion flux and of intracellular ion concentrations also play important roles in the regulation of cell volume [15], the transport of nutrients across the cell membrane [16] and the maintenance of cell viability. The support of a 10000-fold gradient of Ca^{2+} between the cytoplasm (approx. 0.1 μM) and the extracellular milieu (1 mM) is essential for the maintenance of cell viability, and a collapse of this gradient, such that intracellular concentrations of Ca^{2+} rise above approx. 10 μM, has been suggested to be an important element of the onset of cell death [17–19]. It is, therefore, possible to envisage a scenario whereby the inhibition of a discrete, proliferation-associated membrane event, such as a change in ion flux, might lead to a cascade of events which are ultimately lethal to a proliferating cell.

HN2 has the potential to cross-link proteins as well as nucleic acids, and certain enzymes are the target of cross-linking chemicals [20], including the membrane-bound sodium pump, the Na^+/K^+-ATPase [21]. Proteins which are required to undergo conformational change in order to exert their activity are likely to be inactivated by cross-linking. The inactivation of a single protein which controls a transmembrane ionic gradient, is liable to change intimately related cascades of ion flux, and when these cascades relate to amplification events, for example when sodium concentrations change [22], the inhibitory action of an agent at a single protein locus may be amplified. Such a possibility was not considered when proteins were largely dismissed as potential targets for the alkylating agents [1]. Furthermore, alkylated protein targets were considered to be easily replaced by protein synthesis, through transcription and translation. But, since these processes depend upon the proper maintenance of intracellular ionic homeostasis, for example the maintenance of a sufficient

potassium concentration for protein synthesis [23], replacement of damaged proteins which themselves critically regulate ionic homeostasis may be compromised if an imbalance in ionic homeostasis has been imposed.

We and others have shown that incubation with HN2 inhibits potassium influx to several murine tumours in vivo and in vitro [24–26]. In this paper, we show that in murine L1210 leukemia cells, the inhibition of potassium influx is brought about by a selective inhibition of a $Na^+/K^+/Cl^-$ cotransporter, which was characterised in the preceding paper [27]. The consequences of this inhibition are described and we speculate on their relationship to events which may lead to cell death.

Materials and Methods

Chemicals, radiochemical and reagents

Inorganic salts and general reagents were purchased from BDH Ltd (Poole, U.K.) and were of analytical grade. Dow-Corning 550 silicon oil was obtained from Hopkin and Williams (Romford, U.K.) and corn oil was from local supermarkets. Ouabain, furosemide, fire-fly luciferase-luciferin, ATP, MeHN1 and dibutyryl cyclic 3′,5′-AMP were purchased from Sigma Ltd. (Poole, U.K.). Triphenylmethylphosphonium bromide was purchased from K & K Laboratories (Liverpool, U.K.). Bumetanide was the gift of Leo Laboratories (Aylesbury, U.K.) and nitrogen mustard was the generous gift of Dr V. Holland of the Boots Co. (Nottingham, U.K.). All radiochemicals were purchased from Amersham Plc. or from New England Nuclear (Southampton, U.K.). The liquid scintillant Fisofluor 'mpc' was purchased from Fisons Plc (Loughborough, U.K.).

Cell culture and measurements of growth inhibition

L1210 murine leukemia cell culture was as described in the preceding paper [27]. Short-term cell viability (< 6 h) was determined by the exclusion of a solution of 0.1% Trypan blue. The inhibition of proliferation in suspension culture was estimated by comparison of the increase in cell number in the treated and untreated populations, from an initial cell density of $(2–3) \cdot 10^4$ cells/ml, after 72 h, at which time the population was entering the stationary phase of growth. Alternatively, cells were cloned, after treatment with or without HN2, in RPMI 1640 supplemented with 15%, fetal calf serum and 0.5% Noble agar, essentially according to the method of Chu and Fischer [28].

Measurement of ion flux of L1210 cells

Experiments to determine ion flux were essentially as described in the preceding paper: they were conducted with $5 \cdot 10^6$ cells/ml which, unless otherwise stated, were harvested from cultures in exponential growth. These were washed twice with the appropriate medium (see below) and then suspended either in RPMI 1640 medium (Gibco, Glasgow), or in Krebs-Ringer buffer (pH 7.4): the full buffer consisted of 118 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.17 mM KH_2PO_4 and 5.5 mM glucose with the addition of 1.27 mM $CaCl_2$, saturated with 5% CO_2 in air. For estimations of ion influx, the cell suspensions were preincubated with different agents at 37 °C, with shaking, for various times, while being supplied continuously with 5% CO_2 in air, before the addition of tracer amounts of rubidium chloride (5 μCi/ml), inulin[^{14}C]carboxylic acid (1 μCi/ml) or ^3H_2O (1 μCi/ml). The conditions for the determination of efflux and the method for separating the cells from the extracellular medium were as described in the preceding paper [27].

Estimation of the potassium content of L1210 cells by the use of $^{86}Rb^+$ labelling was performed as follows. $5 \cdot 10^6$ L1210 cells/ml in RPMI 1640 medium were incubated with 5 μCi/ml of $^{86}Rb^+$ under an atmosphere of 5% CO_2 in air at 37 °C for 2 h. Preliminary experiments had shown that by this time the cells had reached isotopic equilibrium. HN2 was then added as appropriate, the incubation was continued for the required time, when 200 μl of the cell suspension was centrifuged through an oil barrier, as described above. The fractional content of $^{86}Rb^+$ was estimated by comparison of the radioactivity associated with the cells and that with the supernatant. The K^+ concentration was estimated by the determination of cellular water, in parallel experiments, with corrections for the entrapment in the intracellular space, and the concentration of intracellular K^+ was calculated on the assumption that Rb^+ was equivalent to K^+, as has been validated in the preceding paper [27].

The volume of the cells was estimated by measurement of the cell-associated tritiated water, with a correction for the extracellular space as estimated by inulin [^{14}C]carboxylic acid.

Measurement of cellular ATP content

L1210 cells, harvested in the exponential phase of cell growth, were washed twice and suspended in RPMI 1640 medium at $5 \cdot 10^6$ cells/ml. The cells were incubated with or without 10 μM HN2 for the required times under an atmosphere of 5% CO_2 in air at 37°C with shaking. A 1-ml aliquot of cells was removed for determination of cell volume, as described above, and a 2-ml aliquot resuspended in ice-cold normal saline and centrifuged at 11 600 × g in a Beckman microfuge. The supernatant was removed, and ATP content was determined essentially by the method of Cole et al. [29]. Briefly, the cells were treated with 0.5 ml of ice-cold 12% perchloric acid which was then neutralised, centrifuged and a 50-μl portion of the supernatant was diluted to 5 ml with 45 mM glycylglycine buffer (pH 7.4). 10 μl of this solution was further diluted with 980 μl of buffer and mixed with 10 μl of luciferase-luciferin reagent (20 mg/ml). After 15 s, the luminescence was measured in an LKB-Wallac 1250-001 luminometer. The ATP content was established from a standard linear plot of the luminescence provided by between 0 and 800 ng/ml of ATP.

Measurement of membrane potential and intracellular pH of L1210 cells

The membrane potential of L1210 cells was determined by the equilibrium distribution of [^{3}H]TPMP$^+$ bromide and was performed as described by us elsewhere [30].

Intracellular pH was determined by the estimation of the equilibrium distribution of the radiolabelled weak acids [^{14}C]DMO [31] or [^{14}C]benzoic acid [32]. Briefly, $5 \cdot 10^5$ L1210 cells/ml were incubated with or without HN2 at 37°C in a bicarbonate-free buffer consisting of 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 20 mM Hepes (pH 7.4) for 2 h. Cell volume was determined, as described above, in parallel experiments and the intracellular and extracellular concentrations of the agents were determined at equilibrium, in control cells or those which had been treated with HN2 for various times. When DMO was used, intracellular pH was calculated from the Henderson-Hasselbach equation, as described previously [31] using a pK_a of 6.1. The intracellular pH (pH_i) using radiolabelled benzoic acid was determined according to the method of L'Allemain et al. [32], by substituting into the equation:

$$pH_i = pH_o + \log \frac{[\text{benzoic acid}]^{in}}{[\text{benzoic acid}]^{out}} \quad (1)$$

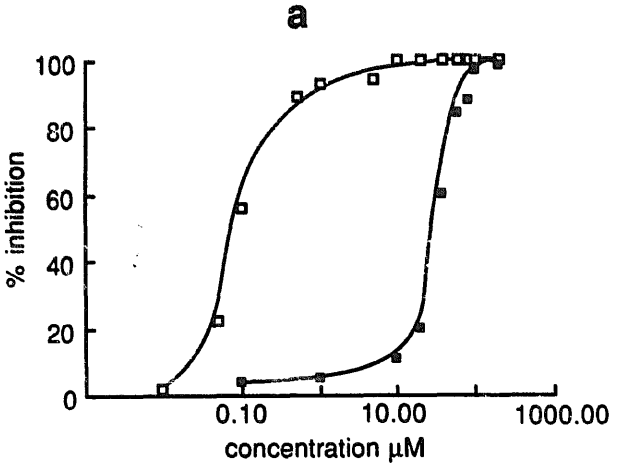
Results

Cytotoxicity of MeHN1, HN2 and loop diuretics to L1210 leukemia cells

The effects of continuous exposure to the monofunctional and bifunctional alkylating agents MeHN1 and HN2 on the growth of L1210 cells in suspension culture over 72 h are shown in Fig. 1a. The concentrations which inhibited proliferation by 50% were $6 \cdot 10^{-5}$ M MeHN1 and $8 \cdot 10^{-8}$ M HN2. In order to determine the minimum concentration of each agent which caused more than 99% of the L1210 cells to die, the cells were incubated with HN2 or MeHN1 for 1 h and then cloned in nutrient agar; Fig. 1b shows the surviving fractions in relation to the concentration of each agent. From this data, the effects of 10 μM HN2 and 100 μM MeHN1 were chosen for the investigation of their effects on ionic homeostasis; these were concentrations which inhibited the proliferative potential of greater than 99.99% of the cells, and would allow no significant level of repair. In addition, these concentrations were chosen because they brought about changes in ion flux in a timescale compatible with the maintenance of reasonable levels of short-term viability in the buffers used: when L1210 cells were incubated with 10 μM HN2 for 3 h in either RPMI 1640 medium or in Krebs-Ringer buffer and their short-term viability was assessed by estimation of their ability to exclude the vital dye Trypan blue, there was only a marginal loss in short-term viability: from 94 ± 4% to 88 ± 6% in RPMI 1640 ($P > 0.1$, $n = 4$), and from 86 ± 6% to 76 ± 6% in Krebs-Ringer buffer ($P > 0.05$, $n = 4$). Changes in viability were accounted for in all estimates of ion

flux measurements. In other experiments, it was determined that incubation of L1210 cells with 10 μM HN2 for 10 min in RPMI 1640 medium, followed by washing in the same medium, produced the same fall decrease in proliferative potential as continuous incubation (data not shown).

The effect of continuous incubation with various concentrations of bumetanide and furosemide on L1210 cell growth over a 72-h period is shown in Fig. 2.



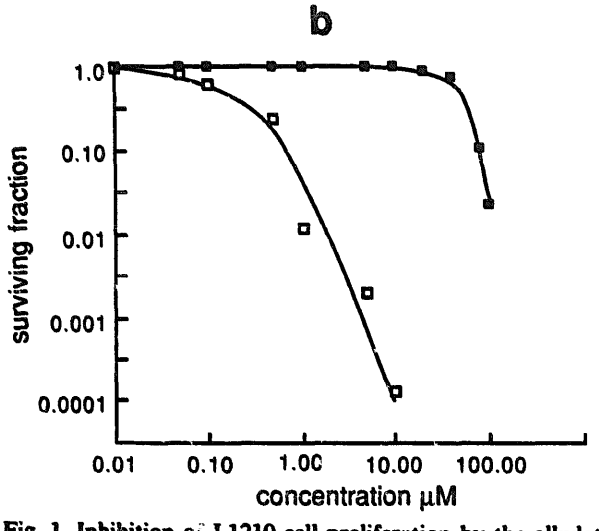


Fig. 1. Inhibition of L1210 cell proliferation by the alkylating agents HN2 (□) and MeHN1 (■). (a) The percentage inhibition of cell growth in suspension culture at various concentrations of each agent. (b) The effects of various concentration of each agent on the colony-forming ability of L1210 cells, expressed as the surviving fraction compared to controls.

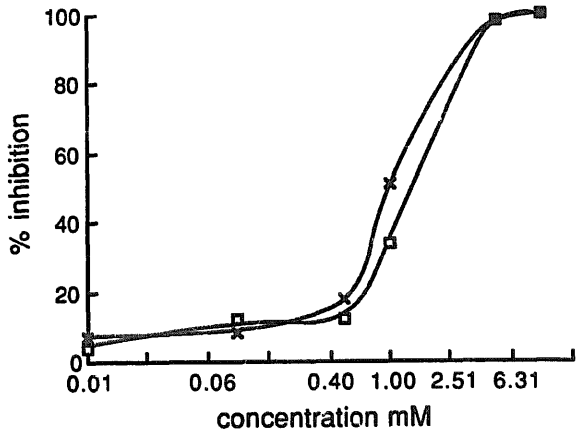


Fig. 2. Inhibition of L1210 cell proliferation in suspension culture by continuous exposure to various concentrations of bumetanide (□) or furosemide (×).

The effects of HN2 and MeHN1 on $^{86}Rb^+$ influx to L1210 cells

The components of potassium influx to L1210 cells, measured by tracer amounts of $^{86}Rb^+$, which was shown to be a complete congener of K^+, have been described in the preceding paper [27], and may be described as an ouabain-sensitive fraction, a loop diuretic-sensitive fraction and what was presumed to be a small component of passive flux. The influx of $^{86}Rb^+$ to L1210 cells was also shown to be linear over the initial 15 min of incubation. The effects of incubation of the cells in a Krebs-Ringer buffer at 37°C with various concentrations of HN2 for 3 h on the initial rate of $^{86}Rb^+$ influx are shown in Fig. 3. Because of decreasing cell viability in Krebs-Ringer buffer (see above) it was considered that estimates of ion flux should not be made after 3 h. However, viability in RPMI 1640 medium was maintained at approx. 90% after 4 h of incubation and Fig. 4a shows the inhibition of $^{86}Rb^+$ influx to L1210 cells incubated in RPMI 1640 medium for 4 h with 10 μM HN2. Fig. 4b shows the immediate inhibition of $^{86}Rb^+$ influx to L1210 cells when 1 or 10 mM of HN2 was added to the cells.

Analysis of the nature of the inhibition of K^+ influx to L1210 cells was performed by measurement of the initial rate of $^{86}Rb^+$ influx, in treated and untreated cells, in the presence of inhibitors of Rb^+ and K^+ flux. Fig. 5 shows the effect of a 3-h incubation with HN2 on the components of loop diuretic- and ouabain-sensitive $^{86}Rb^+$ influx

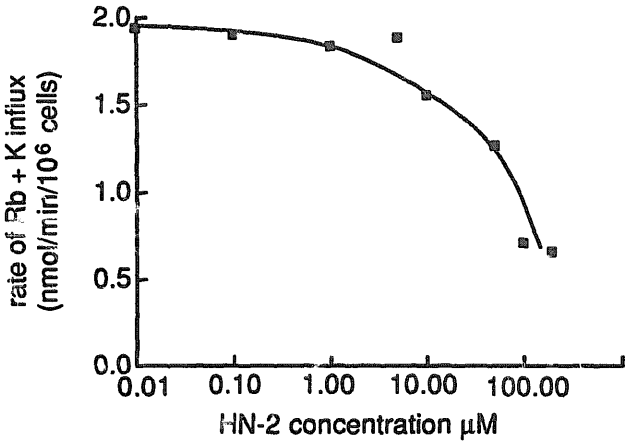


Fig. 3. The effect of incubation for 3 h in Krebs-Ringer buffer of various concentrations of HN2 on the initial rate of $^{86}Rb^+$ plus K^+ influx to L1210 cells.

in (a) cells harvested in the logarithmic phase of cell growth, and (b) cells harvested from the stationary (plateau) phase of growth. The data show that HN2 inhibited the loop diuretic-sensitive component of the (Rb^+/K^+) influx, identified in the preceding paper as being mediated by the $Na^+/K^+/Cl^-$ cotransporter [27]. Additional experiments to test this hypothesis showed that there was no inhibition of the $^{86}Rb^+$ influx to L1210 cells incubated in a chloride-free or sodium-free modified Krebs-Ringer buffer, as described in the preceding paper [27]. It can be seen from Fig. 5 that the drug was without effect on the ouabain-sensitive sodium pump. Incubation of L1210 cells

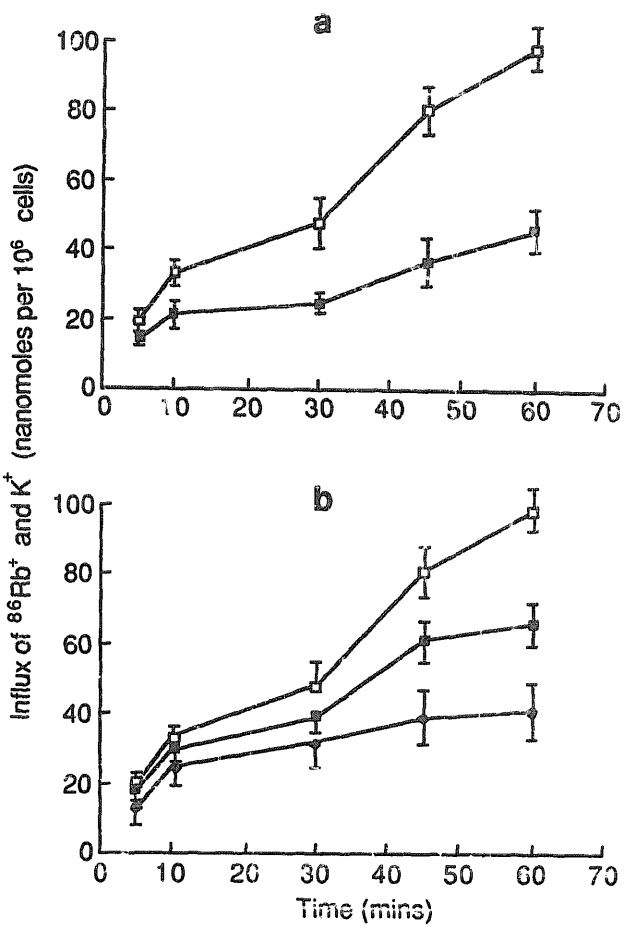


Fig. 4. The effects of various concentrations of HN2 on the influx kinetics of $^{86}Rb^+$ plus K^+ to L1210 cells incubated in serum-free RPMI 1640 medium. (a) Influx kinetics for control (□) cells or those treated with 10 μM HN2 for 4 h (■). (b) Influx kinetics for the immediate addition of (■) 1 mM or (◆) 10 mM HN2.

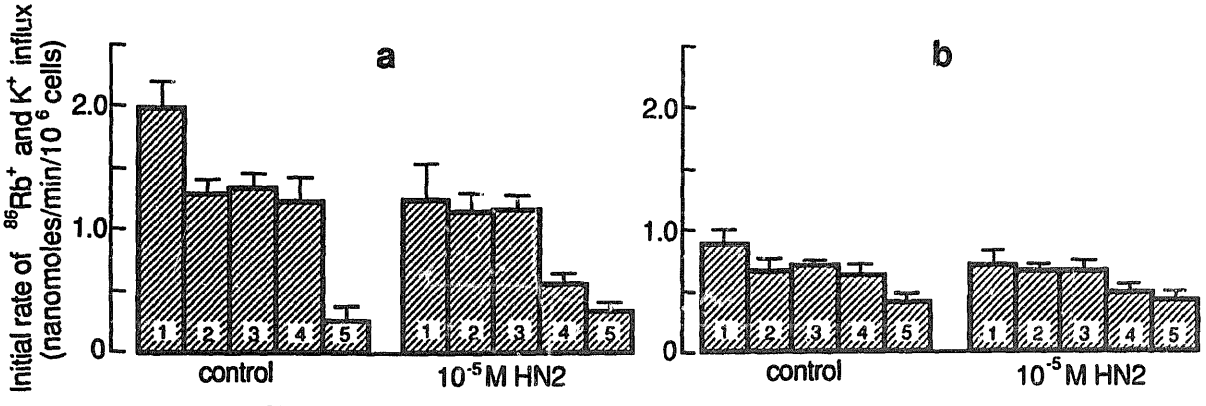


Fig. 5. Histograms of the rates of $^{86}Rb^+$ influx to L1210 cells preincubated in Krebs-Ringer buffer for 3 h in the presence or absence of 10 μM HN2. The cells had been harvested from (a) the exponential phase of cell growth or (b) the stationary phase of cell growth. After the incubation period with HN2, they were incubated with certain inhibitors: 1, controls; 2, 1 mM furosemide; 3, 100 μM bumetanide; 4, 1 mM ouabain; 5, 1 mM ouabain plus 1 mM furosemide.

in Krebs-Ringer buffer with 10 or 100 μM of the monofunctional alkylating agent MeHN1 for 3 h was without any significant effect on the initial rate of $^{86}Rb^+$ influx (data not shown).

Intracellular ion concentrations and volume of L1210 cells treated with HN2 and MeHN1

The inhibition by HN2 of K^+ influx to L1210 cells via the $Na^+/K^+/Cl^-$ cotransporter, raised the possibility that a decrease may have occurred in the intracellular concentrations of K^+ ($[K^+]_i$) and, potentially, of associated ions. Measurement of the $^{86}Rb^+$ content of L1210 cells, labelled to isotopic equilibrium in Krebs-Ringer buffer and treated with HN2 or ouabain, showed that the steady-state level of K^+ under isosmotic conditions, expressed as the fraction of the $^{86}Rb^+$ associated with the cells, had apparently decreased (Fig. 6). Inhibition of the sodium pump by ouabain led to a rapid decrease in the fraction of $^{86}Rb^+$ loaded into the cells and, apparently, the influx mediated by the cotransporter was unable to compensate for this loss. However, when the total cell water was estimated in these experiments, the intracellular concentration of Rb^+ (K^+) of HN2 treated cells was not significantly different from the controls ($P > 0.5$, $n = 3$). Measurement of $[K^+]_i$ of cells incubated in RPMI 1640 medium, estimated by measurement of the steady state concentration of $^{86}Rb^+$, and the assumption, validated in the previous paper [27], that Rb^+ was a complete K^+ congener, showed that $[K^+]_i$ remained constant after treatment with 10 μM HN2: after 3 h, the controls contained 120 ± 10 mM K^+ and the treated cells contained 138 ± 10 mM (n = 8, $P > 0.1$). Similar results were obtained by atomic absorption spectroscopy (data not shown).

In the preceding paper [27], we reported that incubation with furosemide or bumetanide for 3 h reduced the volume of L1210 cells harvested in the exponential phase of growth, but was without effect on cells in the plateau phase of their growth; measurements of cell volume, using tritiated water, after 3 h of treatment of exponentially dividing cells with 10 μM nitrogen mustard showed that the volume was reduced from 0.883 ± 0.131 μl/10^6 cells to 0.630 ± 0.174 μl/10^6 cells ($n = 6$, $P < 0.02$). The same treatment for 3 h with the monofunctional analogue of nitrogen mustard (MeHN1)

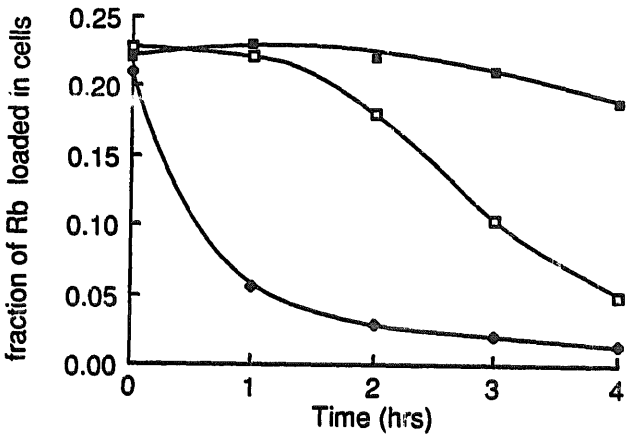


Fig. 6. The fraction of $^{86}Rb^+$ which remained loaded into L1210 cells after incubation for 4 h with various agents. (■) Controls; (□) 10 μM HN2; (◆) 1 mM ouabain.

showed the exponentially dividing cells to have a volume of 0.855 ± 0.197 μl/10^6 cells. No volume change was observed when quiescent cells, harvested from a stationary phase of growth and which already had a reduced volume, were treated with HN2.

Effect of HN2 on intracellular ATP concentrations of L1210 cells

The maintenance of transmembrane gradients of ions, and their potential to perform work, depends upon the provision of cellular energy provided by ATP. Although it is claimed that the $Na^+/K^+/Cl^-$ cotransporter does not require metabolic energy in the way that the sodium pump does, there is, nevertheless, evidence that ATP may regulate its activity [31–35] and it was possible that the inhibition of the cotransporter was the result of a decrease in intracellular ATP, although this hypothesis is not supported by the observation that the ATP-dependent sodium pump was unaffected by HN2 (Fig. 5). L1210 cells harvested from exponential growth in RPMI 1640 medium had an ATP concentration, measured by the firefly-luciferase method, of 9.8 ± 5.6 mM ($n = 13$), and after incubation in RPMI for 3 h this decreased to 3.6 ± 2.6 mM ($n = 20$, $P < 0.05$). Treatment for 3 h with 10 μM HN2 left the ATP concentration at 5.5 ± 2.8 mM, which was not significantly reduced from the controls.

Effects of HN2 on the membrane potential and intracellular pH of L1210 cells

The membrane potential of L1210 cells, determined by the equilibrium distribution of [^{3}H]TPMP$^+$ after 1 h of incubation, as described previously by us [30], was -58 ± 11 mV and, after a 3-h incubation with HN2 in Krebs-Ringer buffer, was -67 ± 10 mV ($P > 0.05$, $n = 11$).

Estimation of the intracellular pH of L1210 cells, and the effects of a 3-h incubation with 10 μM HN2 upon intracellular pH as estimated by the distribution of either [^{14}C]DMO or [^{14}C]benzoic acid showed that the fractional uptake of these substances into L1210 cells incubated in bicarbonate-free saline was initially rapid and reached equilibrium within 15 min (data not shown). After a 3-h incubation, control cell pH, estimated by the distribution of [^{14}C]DMO, was 7.45 ± 0.31 and with HN2 was 7.3 ± 0.06 ($n = 3$, $P > 0.1$). The intracellular pH of untreated cells measured by the distribution of [^{14}C]benzoic acid was 7.73 $\pm$ 0.15, and of cells treated with 10 μM HN2 for 3 h was 7.81 ± 0.14 ($n = 3$, $P > 0.1$).

L1210 cells were found to contain negligible amounts of non-specific esterase activity, and attempts to load and release from the ester proform the fluorescent probe of pH, 2′,7′-bis(carboxyethyl)5,6-carboxyfluorescein, or the probe of intracellular calcium concentration, quin2/AM, were consequently, without success.

Discussion

The highly chemically reactive alkylating agent HN2 might be expected to present itself at its highest concentration at the surface of a cell, where it may alkylate elements of the plasma membrane and cytoskeleton. Indeed, the early studies of Peters and colleagues on the pharmacology of the mustards led him to suggest that the cell surface was an important target organelle for the expression of alkylating agent toxicity [3]. The high potential for the covalent reaction of HN2 with the membrane might also be considered to be non-specific in nature, but although incubation of L1210 cells with 10 μM HN2 rapidly reduced their proliferative potential, so that more than 99.99% of cells failed to form colonies in soft agar (Fig. 1), membrane integrity was maintained. This was characterised by the exclusion of the so-called vital dye Trypan blue, by the maintenance of control-level ATP concentrations and a membrane potential of almost -70 mV (see results). This suggests that cell death does not occur by a process of early cellular necrosis, characterised by a collapse of the ionic gradients of the cell, or by a fall in intracellular ATP concentration [36]. Examples of necrotic cell death have been described by Orrenius and colleagues in their studies of the generation of free radicals by certain drugs [37–39]. The process was characterised by rapid and gross disruption of the membrane, observed as blebbing and by early (< 1 h) increases in intracellular free calcium, possibly as the result of damage to the calcium pump.

In contrast to the circumstances whereby reactive free radicals rapidly and massively damage the cell membrane, HN2 showed a surprisingly subtle inhibition of membrane function, characterised by a selective and progressive decrease in K^+ influx mediated by the $Na^+/K^+/Cl^-$ cotransporter (Fig. 5). A hypothesis that this lesion may only be representative of a common, lethal response of L1210 cells to a variety of toxins, is contradicted by our previous findings that the potent cytotoxin adriamycin was without any effect on K^+ influx to L1210 cells [30], and by unpublished work which showed, under conditions identical to those described here, that the cytotoxic concentrations of the alkylating imidazotetrazinone mitozolomide, which chloroethylates and ultimately cross-links biological targets with a two-carbon cross-link [40], was without effect on K^+ transport into L1210 cells. This suggests that elements of biochemical selectivity exist at the level of the cell membrane in respect to alkylating agents which covalently attach either a five-atom (-CH_2-CH_2-N(Me)-CH_2CH_2-) cross-linking arm which is provided by the mustards such as HN2, or a two-atom cross-link [-CH_2-CH_2-], like that provided by the imidazotetrazinones, and again showed that the inhibition of the $Na^+/K^+/Cl^-$ cotransporter was not an event which simply reflected the imposition of drug-mediated cytotoxicity.

Cross-linking agents, such as the copper phenanthrolines, have been shown to interact with the sulphydryl groups of the Na^+/K^+-ATPase to

inactivate it [21] and we were initially surprised that HN2 had no effect on the activity of the sodium pump (Fig. 5). Doppler et al. [26] have shown that HN2 inactivated the $Na^+/K^+/Cl^-$ cotransporter of Ehrlich ascites cells and that the sodium pump was only inhibited at high concentrations of the drug (> 10 μM), results which support the proposal that HN2 is a specific inhibitor of the cotransporter at pharmacologically relevant concentrations. Moreover, the failure of HN2 to reduce cellular levels of ATP (see Results) allows the continued activity of active transport processes. This in turn suggests that the selective inhibition of the cotransporter is not the consequence of a collapse of ion gradients, such as that of Na^+, but is of a more direct nature.

The inhibition of K^+ influx to L1210 cells harvested from cells in exponential growth, measured by the flux of tracer $^{86}Rb^+$, was not accompanied by an inhibition of K^+ efflux from cells loaded to equilibrium with $^{86}Rb^+$ (data not shown). In the preceding paper [27], we reported that the activity of the $Na^+/K^+/Cl^-$ of L1210 cells was apparently asymmetric, and the failure of HN2 to inhibit efflux parallelled the activity of the loop diuretics in this respect. Although the HN2-induced changes in the net flux of K^+ led to a decrease in intracellular K^+ as measured by the steady-state level of $^{86}Rb^+$ (Fig. 6), this did not result in a reduction of the intracellular concentration of K^+, since water was lost from the cell and the volume fell (see Results). Treatment of L1210 cells for 3 h with HN2 resulted in a reduction of cell volume to a value (0.630 $\mu l/10^6$ cells) similar to that of quiescent cells (0.622 $\mu l/10^6$ cells). Our results contrast with those of Doppler et al. [26], who found that in Ehrlich ascites cells K^+ influx was inhibited by HN2 without an accompanying decrease in cell volume. The difference may be reconciled by the observations of Levinson, who recently reported that the volume of Ehrlich ascites cells under isosmotic conditions was unaffected by the loop diuretics [41], and it might, therefore, be similarly insensitive to HN2.

The maintenance of ATP concentrations in HN2 treated cells and the lack of any inhibition of the sodium pump (Fig. 2) in the presence of a reduced influx of Na^+ via the $Na^+/K^+/Cl^-$, suggested that $[Na^+]_i$ may decrease, because of the continued activity of the Na^+/K^+-ATPase. The reduction of cell volume was presumed to maintain Na^+ homeostasis in part, but it might have been expected that sodium influx may increase in order to balance the continued activity of the sodium pump. However, we were unable to obtain reproducible profiles of Na^+ flux kinetics to investigate this possibility. Measurement of intracellular pH in HCO_3-free medium, showed that there was no activation of the Na^+/H^+ antiport, which would have resulted in an alkalinisation of the cell, and it is presumed that leak flux, together with the decrease in cell volume, was sufficient to maintain Na^+ homeostasis.

The selective inhibition of the $Na^+/K^+/Cl^-$ cotransporter by HN2, and the decrease in cell volume to maintain $[K^+]_i$, raises the question of whether the effects of HN2 are directly upon the $Na^+/K^+/Cl^-$ cotransporter, or whether the activity of the cotransporter is inhibited indirectly. The inhibition of the diuretic-sensitive component of L1210 K^+ influx, accompanied by a fall in cell volume, also raises the question of whether, under steady-state conditions (isosmolar), the maintenance of cell volume is regulated by proteins which directly modulate ion flux, or whether their activity is secondary, although closely associated with changes in cell volume which are regulated, perhaps, by membrane–cytoskeletal interactions. This suggests the possibility of a scenario of an HN2-activated cell volume reduction induced by changes in the cytoskeleton, which then modulates the activity of the cotransporter. Alkylating agents have been shown to react with elements of the erythrocyte cytoskeleton [42–44] and preliminary results from this laboratory show that HN2 inhibits diuretic-sensitive K^+ influx to the red cell. Recently, it has been suggested that the activity of the $Na^+/K^+/Cl^-$ cotransporter may be regulated in the human erythrocyte by association with the cytoskeletal protein band 4.1 [35]. The slow onset of inhibition by 10 μM HN2 of K^+ influx to L1210 cells contrasts with the rapid inhibition exerted by the loop diuretics. This suggests that the inhibition might be indirect, although it does not necessarily discount a slow alkylation of the cotransporter, perhaps in some hydrophobic domain in which the rate of reactivity of the alkylating agent would be diminished, as shown in model

membrane systems [45]. The addition of high concentrations of HN2 to L1210 cells, immediately brought about inhibition of the K^+ influx (Fig. 4b), supporting the idea that a mass action phenomenon is involved, similar to that reported for the slow inactivation by HN2 of the low K_m form of cAMP phosphodiesterase by chlorambucil [46]. The reported inhibition of the low K_m form of cAMP phosphodiesterase by the β-chloroethylamine alkylating agents [47] also raised the possibility that the inhibition of the $Na^+/K^+/Cl^-$ cotransporter may be exerted indirectly by an elevation of intracellular cAMP. However, as reported in the preceding paper [27], incubation of L1210 cells with dibutyryl cAMP had no effect upon the Rb^+ influx. We are currently investigating the role of a number of kinases in the control of the cotransporter, and hope to clarify the mode of action of the inhibition of the cotransporter by HN2 by these and other experiments.

Whatever the precise mechanism whereby HN2 selectively inhibits K^+ influx to L1210 cells, the question remains of whether this constitutes a potentially lethal lesion to a cell which is committed to proliferate. In part, the answer to this question awaits an analysis of the ion homeostasis of HN2-treated cells as they lose viability: in the investigations presented here of the early phase of the action of HN2, it has been shown that the reduction of K^+ influx is accompanied by a reduction of cell volume, so as to maintain $[K^+]_i$, and further work concentrating on stages after treatment with HN2, is required to determine whether this reduction is maintained, to enforce conditions which we consider would prejudice cytokinesis. The results show that cells treated with HN2 attain a volume equivalent to those in quiescence. However, contrary to a hypothesis that the inhibition of the cotransporter constitutes a lethal lesion, is our failure to inhibit cell growth substantially when L1210 cells were incubated with concentrations of bumetanide or furosemide which completely inhibited $^{86}Rb^+$ plus K^+ influx via the cotransporter (Figs. 2 and 5). A similar result has been reported by Doppler et al. in Ehrlich ascites cells [26]. We consider that the possibilities of differential recovery from this inhibition must be investigated, given the different nature of the drugs. We are also open to the possibility that the inhibition of the cotransporter may alone not be a lethal lesion. For example, the inhibition of recovery from sub-lethal radiation-induced DNA damage by a subsequent exposure to a hypertonic medium (a condition analogous to inhibition of the cotransporter) has been reported [48]. In these experiments, the hypertonic conditions were themselves without lethality and caused no DNA damage, but in combination proved lethal. We are interested in determining the effects of cotransporter inhibition on HN2-mediated DNA damage, where it is possible that the imposition of potentially lethal genetic damage is similarly made lethal by concomitant but non-toxic membrane lesions. This would support the hypothesis that membrane damage constitutes a causative, but not independent, lesion which is responsible for cell death. These and other hypotheses await further experimentation.

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