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pH-dependent toxicity of sulphur mustard *in vitro*

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Abstract

The dependence of sulphur mustard (HD) toxicity on intracellular (pH_i) and extracellular pH was examined in CHO-K1 cells. HD produced an immediate and significant concentration-dependent decline in cytosolic pH, and also inhibited the mechanisms responsible for restoring pH_i to physiological values. The concentration–response of HD-induced cytosolic acidification, closely paralleled the acidification of the extracellular buffer through HD hydrolysis. A viability study was carried out in order to assess the importance of HD-induced cytosolic acidification. Cultures were exposed to HD for 1 h in media that were adjusted through a pH range (pH 5.0–10), and the 24 h LC_{50} values were assessed using the viability indicator dye alamarBlue™. The toxicity of HD was found to be dependent on extracellular pH, with a greater than eight-fold increase in LD_{50} obtained in cultures treated with HD at pH 9.5, compared to those treated at pH 5.0. Assays of apoptotic cell death, including morphology, soluble DNA, caspase-3 activity and TUNEL also showed that as pH was increased, much greater HD concentrations were required to cause cell death. The modest decline in HD half-life measured in buffers of increasing pH, did not account for the protective effects of basic pH. The early event(s) that HD initiates to eventually culminate in cell death are not known. However, based on the data obtained in this study, we propose that HD causes an extracellular acidification through chemical hydrolysis and that this, in both a concentration and temporally related fashion, results in cytosolic acidification. Furthermore, HD also acts to poison the antiporter systems responsible for maintaining physiological pH_i, so that the cells are unable to recover from this insult. It is this irreversible decline in pH_i that initiates the cascade of events that results in HD-induced cell death.

Introduction

The chemical warfare (CW) agent sulphur mustard (bis (2-chloroethyl) sulphide, NATO Standard Agreed designation; HD) was extensively used in the First World War and most recently during the 1984/1985 Iran/Iraq War. It exerts direct deleterious effects on a variety of tissues including skin, eyes and the respiratory tract and can also cause profound systemic intoxication (Dacre and Goldman, 1996; National Academy Press, 1993; Papirmeister et al., 1991; Smith and Dunn, 1991; Warthin and Weller, 1919). Although HD has been well documented as having potent cytotoxic (Papirmeister et al., 1991), mutagenic (Auerbach, 1949; Auerbach and Robson, 1946) and vesicant properties (Warthin and Weller, 1919; Willems, 1989), its mechanism of vesicant action is not well understood and treatment of the mustard casualty is entirely symptomatic (Dacre and Goldman, 1996; Kehe and Szinicz, 2005; National Academy Press, 1993; Papirmeister et al., 1991; Smith and Dunn, 1991; Willems, 1989).

A large proportion of recent studies have gravitated towards *in vitro* model systems to study HD and the cytotoxic effects of this compound have been well documented in a wide variety of cell types and tissues. This has led to hypotheses implicating a variety of HD-induced effects as being the cause of toxicity. Notable among these efforts has been investigations of HD-induced DNA damage and PARP activation (Papirmeister et al., 1985), calcium deregulation (Hamilton et al., 1998; Hua et al., 1993; Ray et al., 1995; Rosenthal et al., 1998; Sawyer and Hamilton, 2000) and apoptosis (Dabrowska et al., 1996; Michaelson, 2000; Rosenthal et al., 1998, 2001;

Abbreviations: CW, chemical warfare; HD, sulphur mustard, bis (2-chloroethyl) sulphide; EIPA, ethyl isopropyl amiloride; LC_{50}, median lethal concentration; NHE, sodium hydrogen exchanger; pH_i, intracellular pH; SNARF-1, carboxyseminaphthorhodafluor-1.

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Sourdeval et al., 2006; Sun et al., 1998). Although these studies have well characterized these aspects of HD toxicity, it is unclear that they are directly responsible for the toxicity of this CW agent, and/or that interfering with these pathways will result in substantial cellular protection against it.

The regulation of intracellular pH (pH_i) within a closely defined physiological range is vital for a wide spectrum of cellular processes, including cell metabolism, calcium homeostasis, gene expression, cell motility and contractility, cell adhesion and cell death (Lagadic-Gossmann et al., 2004; Pucéat, 1999). In order to maintain constant cytosolic pH, eukaryotic cells employ a variety of mechanisms that include plasma membrane proton pumps and channels, as well as ion transporters (Pucéat, 1999). Perturbation of these processes, particularly of the sodium hydrogen exchanger (NHE) has been implicated as one of the mechanisms in the cytotoxicity of a variety of toxicants (Huc et al., 2004; Lagadic-Gossmann et al., 1999; Mahnensmith and Aronson, 1985).

In this study, the effect of HD on the pH_i of CHO-K1 cells was investigated using the pH sensitive fluorophore SNARF-1. The results of this work also led us to examine the effects of extracellular pH on HD-induced cell death, including different aspects of apoptosis.

Methods

CHO-K1 cell culture. Seed cultures were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in 10% FCS in F-12 culture medium supplemented with streptomycin (100 μg/ml) and penicillin (100 IU/ml), with the medium being changed as required. Stock cultures were closely monitored and not allowed to grow to confluency prior to subculture. Test cultures were seeded so that cells were used just prior to, or at confluency (2–3 days).

Chemical treatment and cytotoxicity studies. All studies involving HD were carried out under DRDC safety guidelines for the handling of chemicals and CW agents. On the day of experimental treatment, cultures were aspirated and re-fed with test medium adjusted to the desired test pH. The cells were then immediately treated with freshly prepared HD treatment medium (at the same test pH) so that the final HD concentration was reached at 0.25% solvent vehicle (ethanol, v/v). The cultures were incubated for 1 h at 37 °C and then the treatment medium was aspirated and replaced with routine culture medium, pH 7.4. Twenty-three hours later, cell viability was assessed using the alamarBlue™ (AccuMed International Inc., Westlake, OH) cytotoxicity assay. The dye was added (10%, v/v) to the cultures and incubated for the last 2–3 h of treatment. The absorbences (570 nm–600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point. All experiments were carried out at least 3 times. Sulphur mustard was prepared at DRDC Suffield at greater than 99% purity.

In additional studies, the potential protective effects of ethyl isopropyl amiloride (EIPA, Sigma Chemical Co., St. Louis, MO) against HD toxicity were examined. Cultures were incubated with varying concentrations of EIPA (5–25 μM) for 30 min in routine culture medium prior to HD exposure. The 24 h viability of the treated cultures was assessed as described above.

Apoptosis studies. In all studies that examined the effect of pH on HD-induced apoptotic endpoints, cells were treated with varying concentrations of the toxicant at the desired test pH for 1 h. The cultures were then refed with fresh medium and apoptotic cell death was assessed 5 h after the initiation of HD exposure.

Soluble DNA. Soluble DNA was measured by the method described by Cui et al. (1994) with modifications. Cells were grown in 24 well plates and log phase growth cultures were radiolabeled by incubating with [^{3}H]thymidine (1 μCi/ml, Amersham Canada Ltd., Oakville, Ontario, Canada) overnight at 37 °C in a humidified 5% CO_2 incubator. The medium containing [^{3}H]thymidine was then removed and the cells rinsed once with PBS. After drug treatment, aliquots of the culture medium (1 ml/well, part A) were saved for radioactivity measurement and the cells were lysed in 0.5 ml TET (10 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.2% Triton X-100) at 4 °C for 30 min. The cell-lysate was then centrifuged (22 min, 14,000×*g*) and the resulting supernatant (part B) was removed and counted. The lysate pellet (part C) was solubilized with 1 N NaOH (0.3 ml/microfuge tube) and counted. The experiments were performed in triplicate. Soluble DNA (percentage) was calculated according to the following formula: (Part A (dpm)+Part B (dpm))/(Part A (dpm)+Part B (dpm)+Part C (dpm))×100.

TUNEL staining. Cells were cultured on Lab Tek II chamber slides (Nalge Nunc International, Naperville, IL). Cells on chamber slides were fixed in 10% buffered formalin before being permeablized in 0.01 M citrate buffer pH 6.0 for 5 min at 90 °C. The TUNEL reaction was carried out using recombinant terminal transferase and Biotin-16-dUTP in TdT buffer (Roche Diagnostics, Basel, Switzerland) for 90 min at 37 °C and stopped with 300 mM NaCl, 30 mM Na citrate. TUNEL positive cells were fluorescently labeled with Texas Red-avidin DCS (Vector Laboratories, Burlingame, CA) and counterstained with Vectashield DAPI (Vector Laboratories). Cells were visualized on an Olympus BH1 microscope (Carl Zeiss, Inc.) with Texas Red/DAPI filter set (Chroma Technology Corp., Rockingham, VT) and images were captured with a SPOT camera and analyzed using Metamorph Imaging software. At least 4 fields and 500 cells per well were counted.

Morphological observations. Cell cultures were grown to subconfluency in 35 mm petri dishes prior to experimentation. After HD treatment, the cells were washed with PBS and then stained with 10 μl of a dye mix (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) as previously described (Duke and Cohen, 1992). The cells were then visualized and scored using fluorescence microscopy. A minimum of 200 cells were visualized and the incidence of each of the following four cellular states was recorded; (i) viable cells with normal nuclei (VN; bright green chromatin with organized structure), (ii) viable cells with apoptotic nuclei (VA: bright green chromatin which is highly condensed or fragmented), (iii) nonviable cells with normal nuclei, considered to be necrotic (NVN; bright orange chromatin with organized structure) and (iv) nonviable cells with apoptotic nuclei (NVA; bright orange chromatin which is highly condensed or fragmented). The percentages of apoptotic and necrotic cells were then calculated according to the formula:

$$\% \text{ apoptotic cells} = (VA + NVA)/(VN + VA + NVN + NVA) \times 100$$

$$\% \text{ necrotic cells} = NVN/(VN + VA + NVN + NVA) \times 100.$$

Caspase-3 activity. Caspase-3 activity induced by HD was measured using the Caspase-3 Colorimetric Activity Assay system (Chemicon International, Temecula, CA). The cells were harvested after 5 h of HD exposure by centrifugation at 1000×*g* for 5 min at 4 °C. The pellet was washed once with ice cold PBS and resuspended in chilled lysis buffer. Cells were incubated on ice for 10 min with gentle shaking. The cell lysates were centrifuged (10,000×*g* for 10 min) and the supernatant was transferred to a fresh tube and kept on ice. Protein concentration for each sample was determined by the Pierce BCA protein assay. Caspase-3 activity in a cell extract containing 100 μg of protein was measured according to the product manual. Briefly, caspase-3 activity in the cell extract cleaves the DEVD *p*-nitroaniline (pNA); the substrate used in the assay. The free chromophore pNA is detected and quantified using a microtiter plate reader at 405 nm wavelength. By comparing the absorbance of pNA from a HD-induced cell death extract with that of a control extract, the effect of treatment on caspase-3 activity was determined.

Intracellular pH. Suspended CHO-K1 cells (5×10^5) were incubated with 0.02% Pluronic-F127 and 5 μM carboxyseminapthorhodafluor-1 (SNARF-1 AM, Molecular Probes, Eugene, OR) in 1 ml assay buffer (Hanks Balanced Salt Solution, Invitrogen, Carlsbad, CA) with 10 mM HEPES, 1 mM $CaCl_2$ and 250 μM sulfinpyrazone at the pH being investigated for 30 min at 37 °C. Cells

were then rinsed with 1 ml fresh assay buffer twice and incubated for at least 20 min on ice prior to analysis. Intracellular pH was monitored at 37 °C using 514 nm excitation of SNARF-1, and ratiometric analysis of 580 nm and 640 nm emission using a Deltascan fluorometer and Felix 32 software (Photon Technology International, Trenton, NJ). The fluorescence ratio of 580/640 was converted to pH units using a calibration curve. pH calibration was performed on SNARF-1 loaded cells in depolarizing buffer (135 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 10 mM HEPES) using 0.5 pH unit steps between pH 6.0 and pH 9.0. Cells were treated with 10 μM nigericin (Molecular Probes) to equilibrate intracellular and extracellular H^+. The effect of 25 μM EIPA on HD-induced cytosolic acidification was assessed by adding this NHE (sodium hydrogen exchanger) inhibitor immediately prior to HD treatment.

Extracellular pH. For the determination of HD-induced changes in extracellular pH, suspensions of CHO-K1 cells were treated in an identical fashion to those being prepared for intracellular pH determination (above). At time zero, HD was added and extracellular pH was measured at 37 °C using a VWR Symphony SB70P pH meter.

Ammonium chloride cytosolic acidification. Cells (5×10^5) were incubated with 0.02% Pluronic-F127 and 5 μM SNARF-1 AM in 1 ml of assay buffer (Hanks Balanced Salt Solution (HBSS), 10 mM HEPES, 1 mM $CaCl_2$ and 250 μM sulfinpyrazone, pH 7.0) for 30 min at 37 °C. The cells were then centrifuged and the cell pellet rinsed once with, and then resuspended in the ammonium chloride loading buffer (HBSS with 20 mM NaCl replaced by 20 mM NH_4Cl, 10 mM HEPES, 1 mM $CaCl_2$ and 250 μM sulfinpyrazone, pH 7.0). After a 30 min incubation, the cell suspension was centrifuged and the cell pellet was then resuspended in 2 ml of assay buffer and immediately monitored for changes in pH_i.

Rate of HD hydrolysis. Test buffer solutions (130 mM NaCl, 40 mM sucrose, 10 mM HEPES, 10 mM glucose) at pH 5.0, 7.0 and 9.5 were incubated at 20 °C in a Haake re-circulating water bath for at least 20 min prior to each sample being spiked with HD (200 μM HD in 0.5% absolute ethanol final concentration). The solutions were vigorously hand shaken for 30 s and incubated at 20 °C in a circulating water bath. Two milliliter samples were taken at 3, 10, 15, 20, 30, 40, 50 and 60 min and transferred into one dram glass vials, containing 0.6 ml dichloromethane. The vials were vigorously hand shaken for 30 s. The dichloromethane layer was allowed to settle and 150 μl sample aliquots were transferred into sample vials for GC analysis.

GC analyses. A Varian 3800 gas chromatograph equipped with an 8400 autosampler was used to determine the HD concentration in all dichloromethane extracts. The chromatograph was equipped with a flame ionization detector (FID) for quantitative HD analyses and a pulsed flame photometric detector (PFPD) for sample peak identification. A 15 m DB-1701 column (JW Scientific, Folsom, CA) with an internal diameter of 0.32 mm and a stationary phase film thickness of 0.25 μm was used. The carrier gas was helium at a column flow rate of 10 ml/min. The column flow was equally split and directed to both the FID and PFPD detectors using a standard glass column Y-connector (Chromatographic Specialties, Brockville, Ont., Canada). A Varian 1079 liquid injector was used in these analyses with a standard glass 3.4 mm internal diameter split/splitless injector liner. The injection port was maintained isothermally at 220 °C and a split ratio of 2:1. A 1.0 μl sample was injected for each analysis. Typically three replicate injections were carried out per sample. The FID was set to a constant temperature of 250 °C while the PFPD was maintained at 200 °C. The gas flows in each detector were optimized and calibrated using external HD standards. Initially, the GC column oven was isothermally maintained at 50 °C for 4.9 min after injection, to allow the solvent in the sample to volatilize and clear the column while depositing the HD analyte at the head of the column. The oven temperature was then increased to 250 °C at a rate of 25 °C/min. The temperature of the oven was held at 250 °C for 3.4 min, to drive off any remaining high molecular mass compounds, and then reset to 50 °C. Under these conditions HD eluted as a single well resolved, reproducible and quantifiable peak at 6.95 min via the FID. In these analyses the PFPD was optimized to detect sulphur-containing compounds.

Statistical methods. Data were analyzed by analysis of variance (ANOVA) statistical methods using SAS JMP software (Release 5.1, SAS Institute, Inc., Cary, NC). Subsequent to the ANOVA, groups were compared using either Dunnett's Method Comparisons (Dunnett, 1955), Tukey's HSD Multiple Comparisons (Tukey, 1953; Kramer, 1956) or Student's *t* Means Comparisons. Significant differences between groups were assumed if *p*-values were less than 0.05.

Results

The effect of HD on intracellular pH (pH_i) was examined using the acetyoxymethyl ester form of the fluorescent dye SNARF-1, which diffuses into cells. This dye is then hydrolyzed by cellular esterases, leaving the SNARF-1 trapped within the cell cytoplasm. Figs. 1A–C shows the change in pH_i

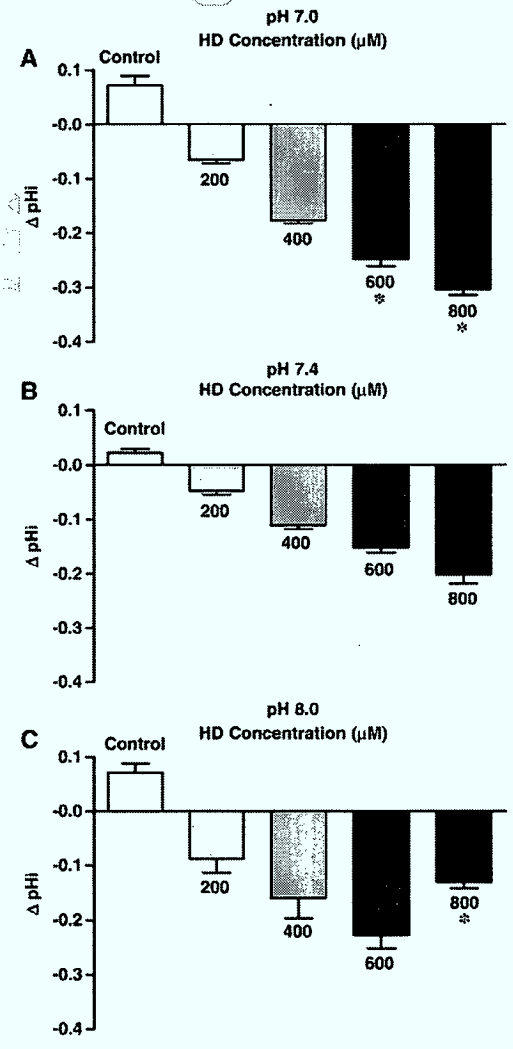

Fig. 1. Effect of pH on HD-induced changes on pH_i in CHO-K1 cells. Cells were loaded with the intracellular pH dye indicator SNARF-1 prior to HD exposure at pH 7.0 (A), pH 7.4 (B) or pH 8.0 (C). Results represent the mean±standard deviation of three separate experiments. Data were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing. All HD treatment groups were different compared to controls at the same pH ($p<0.05$, $^*p<0.05$ vs. corresponding HD concentration at pH 7.4).

(ΔpH_i) of cells incubated in buffers of pH 7.0 (A), pH 7.4 (B) and pH 8.0 (C) and exposed to HD (200–800 μM). A similar concentration–response was observed in all three cases at 30 min, with a maximal decline in pH_i of ~0.25–0.3 pH units, when compared to control values. Figs. 2A–D shows representative measurements of tracings of HD-induced cytosolic acidification. At pH 7.0 and pH 7.4, maximal acidification occurred by ~15 min and then plateaued (Figs. 2A ,B). In contrast, although similar concentration–responses were observed at pH 8.0 (Fig. 2C), a significant and reproducible recovery in pH_i was observed in cells incubated in pH 8.0 buffer and treated with 800 μM HD after ~15 min (see also Fig. 1C). Incubation of the cells with the NHE inhibitor EIPA at pH 7.0 (Fig. 2D), did not alter ΔpH_i compared to control (800 μM HD: $\Delta pH_i = 0.30 \pm 0.02$; 800 μM HD/24 μM EIPA: $\Delta pH_i = 0.31 \pm 0.02$, mean±SD, $n=3$).

The effects of ammonium chloride induced intracellular acidification are depicted in Fig. 3. Acidification of pH_i to ~pH 6.6 (from pH 7.0) was obtained within seconds, after which the cells rapidly recovered to a pH_i of ~6.9. Incubation with 24 μM EIPA did not alter this trend. However, HD not only prevented pH_i recovery, but also caused further acidification. No elevation of pH_i towards physiological levels was observed during the 30 min test period.

Incubation of HD in cell suspensions incubated at 37 °C, caused a concentration-dependent decrease in extracellular pH (Fig. 4A), that very closely paralleled the concentration response of pH_i caused by this agent. The decline in extracellular pH caused by 800 M HD was only slightly larger than the decline in pH_i (Fig. 4B).

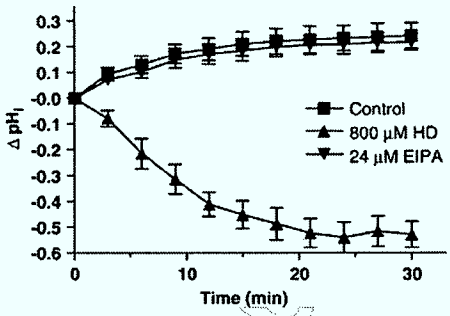

Fig. 3. Ammonium chloride cytosolic acidification of CHO-K1 cells at 7.0. The cytosolic pH of CHO-K1 cells was acidified using the ammonium chloride prepulse technique, and the effect of 800 μM HD or 24 μM EIPA treatment on the recovery of pH_i was monitored. Results represent the mean±standard deviation of 3 separate experiments.

Fig. 5 shows the effect of pH on HD toxicity in CHO-K1 cells as assessed using the indicator dye alamarBlue™. At pH 7.5, cell viability rapidly declined at lower HD concentrations and then only gradually decreased at HD concentrations greater than 200 μM. The concentration–response curve shifted to the left when HD exposure was carried out at pH 5.5, with 300 μM HD producing almost 100% cell death. In contrast, when cultures were exposed to HD in a medium of pH 9.5, just over 50% cell death was observed at 800 μM HD

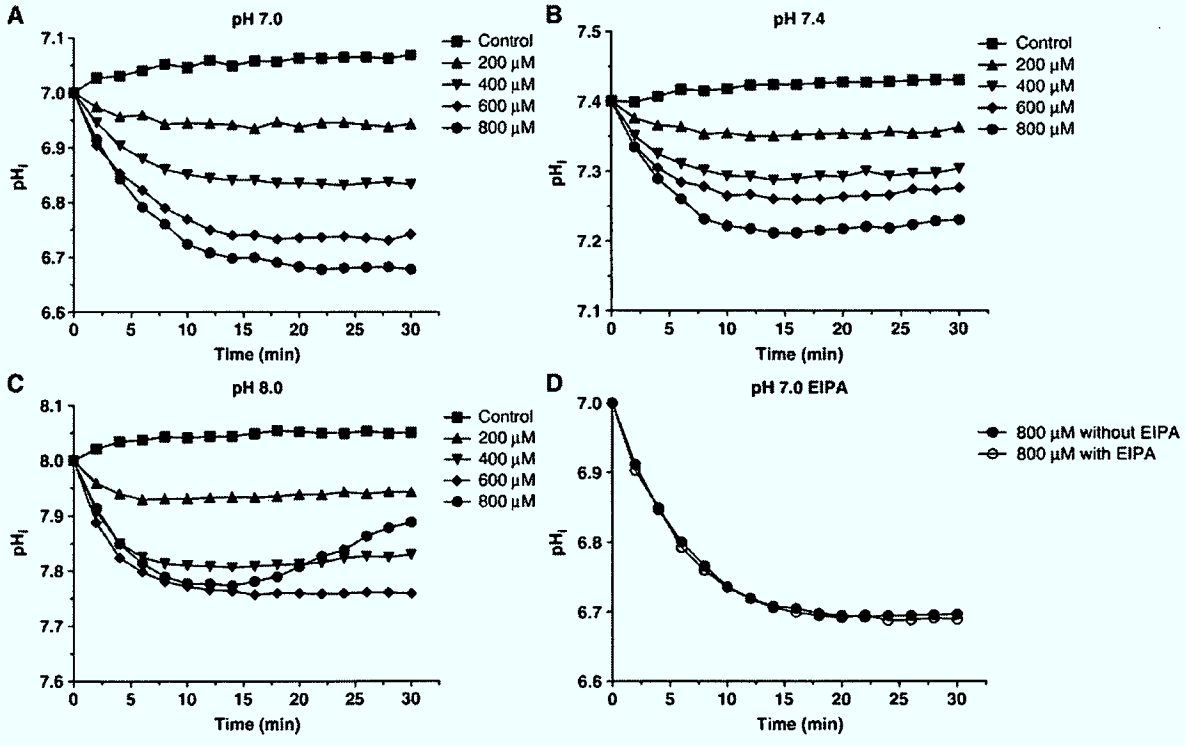

Fig. 2. Time and concentration dependence of HD-induced pH_i acidification in CHO-K1 cells. Representative tracings of HD-induced cytosolic acidification at pH 7.0 (A), pH 7.4 (B) and pH 8.0 (C), and lack of effect of 24 μM EIPA pre-incubation on 800 μM HD-induced cytosolic acidification (D).

(Fig. 5A). This trend is more fully illustrated in Fig. 5B, where the LC_{50} of HD is clearly increased as the exposure is carried out in an increasingly basic environment. The viability of vehicle-treated control cultures incubated in medium at pH 5.0 to pH 10.0 was unaffected. However, incubation of cultures in medium outside of this pH range resulted in toxicity (data not shown).

The effect of HD on apoptotic DNA fragmentation was quantitated using two assays; soluble DNA and the TUNEL reaction. Fig. 6 depicts the concentration-dependent HD-induced DNA fragmentation as measured by soluble DNA. At pH 7.5 fragmentation increased with concentration up to 600 μM, and then decreased. At pH 9.5, the percent fragmentation for control cultures was similar to those of cells incubated in pH 7.5 medium. In contrast to those cells incubated in pH 7.5 medium, HD did not induce any further DNA fragmentation over control values. Very similar trends were obtained when DNA fragmentation was measured using the TUNEL assay (Fig. 7).

The effect of HD on apoptotic and necrotic cell death was assessed using morphological criteria (Fig. 8). While necrotic cells were detected, in no case was there a statistically significant HD-induced phenomenon, and therefore their quantitation was not included in Fig. 8. At both pH 7.5 and 9.5, a concentration-dependent increase in apoptotic cells was induced by HD. This trend was much reduced at pH 9.5, with 800 μM HD inducing a little over 10% apoptosis, as compared to ~70% when cells were exposed to HD in pH 7.5 culture medium.

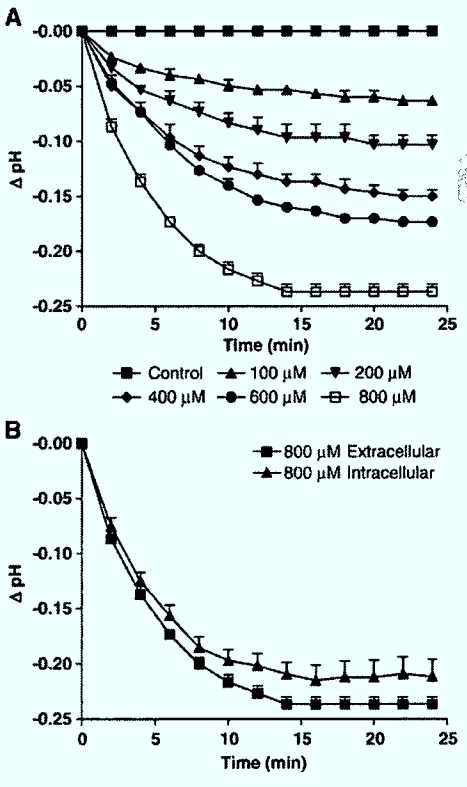

Fig. 4. Effect of HD hydrolysis on extracellular acidification. Cell suspensions (pH 7.4) were treated identically to those that were prepared for pH_i determinations. The suspensions were then monitored immediately after HD treatment for changes in extracellular pH (A). Sulphur mustard (800 μM) induced a extracellular acidification that very closely paralleled its effects on pH_i (B). Results represent the mean±standard deviation of 3 separate experiments.

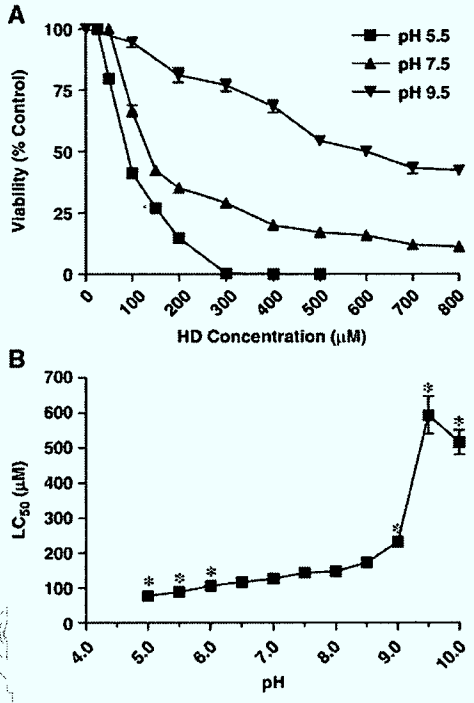

Fig. 5. Effect of pH on the cytotoxicity of HD in CHO-K1 cultures. (A) Just-confluent CHO-K1 cultures were treated with varying HD concentrations in culture medium of different pH. After 1 h, the test medium was removed and replaced with routine culture medium (pH 7.4). Cytotoxicity was determined at 24 h using the alamarBlue™ cytotoxicity assay. (B) LC_{50} values were obtained graphically. Results represent the mean±standard deviation of 3 separate experiments. Data were analyzed by ANOVA and *post hoc* Dunnett's Method Comparison test using pH 7.5 as the control group (*$p<0.05$ vs. pH 7.5).

The effect of HD on caspase-3 activity is depicted in Fig. 9. At both pH 7.5 and 9.5, HD induced a concentration-dependent increase in activity, although maximal levels were significantly reduced at pH 9.5, as compared to those obtained at pH 7.5. At pH 7.5, induced activity was maximal at 400 μM HD and then declined, compared to cells exposed to HD at pH 9.5, where the maximal induction occurred at 600 μM HD.

A 30 min pre-incubation with EIPA (5–25 μM) had no effect on the toxicity of HD in CHO-K1 cells as measured by the alamarBlue cytotoxicity assay (Fig. 10).

Table 1 shows the LC_{50} of HD on CHO-K1 cells exposed in medium of various pH. In addition, it depicts the half-life of HD when incubated at 37 °C in physiological buffer at these pHs, and the calculated 1 h concentration × time integral of these half-lives. Cells incubated in pH 9.5 medium were almost eight times more resistant to the toxicity of HD than when they were incubated at 5.0. The half-life of HD slightly, but significantly decreased as the pH was increased. This was also reflected in

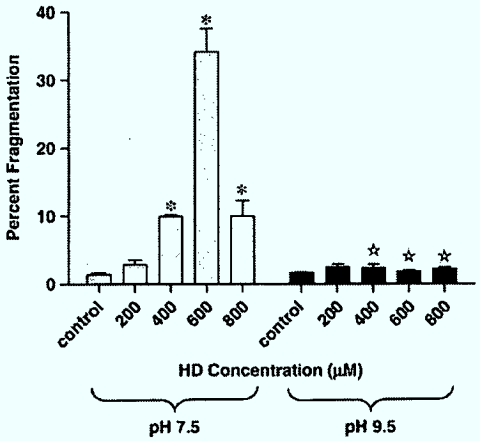

Fig. 6. Effect of pH on HD-induced DNA fragmentation (soluble DNA) in CHO-K1 cultures. Cells were grown in 24 well multiplates and log growth cultures were labeled with ^{3}H-thymidine (1 μCi/ml) for 24 h prior to HD treatment. The cultures were then treated with varying HD concentrations in culture medium of different pH for 1 h. Following the 1 h incubation, the test medium was removed and replaced with routine culture medium (pH 7.4). DNA fragmentation was assessed as soluble DNA, 5 h post-treatment as described in "Methods". Results represent the mean±standard deviation of 3 separate experiments. Data were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing (*$p<0.05$ vs. respective controls; ☆$p<0.05$ vs. corresponding HD concentration at pH 7.5).

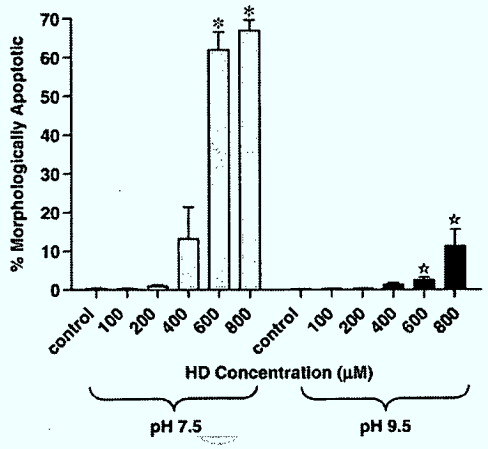

Fig. 8. Effect of pH on HD-induced morphology in CHO-K1 cultures. First passage cells were grown on four well slides. Just-confluent cultures were treated with varying HD concentrations in culture medium of different pH for 1 h. Following the incubation period, the test medium was removed and replaced with routine culture medium (pH 7.4). Morphology was assessed 5 h post-treatment as described in "Methods". Results represent the mean±standard deviation of 3 separate experiments. Data were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing (*$p<0.05$ vs. respective controls; ☆$p<0.05$ vs. corresponding HD concentration at pH 7.5).

the 1 h concentration × time integral, which only decreased by a factor of 1.2 from pH 5.0 to pH 9.5.

Discussion

An early theory of HD toxicity postulated that cell death was due to the direct toxic action of hydrochloric acid, produced through the hydrolysis of HD in an aqueous environment. Although this hypothesis was rapidly dismissed at the time (Papirmeister et al., 1991), it is now known that xenobiotic-induced alterations in pH_i, particularly acidification, can have

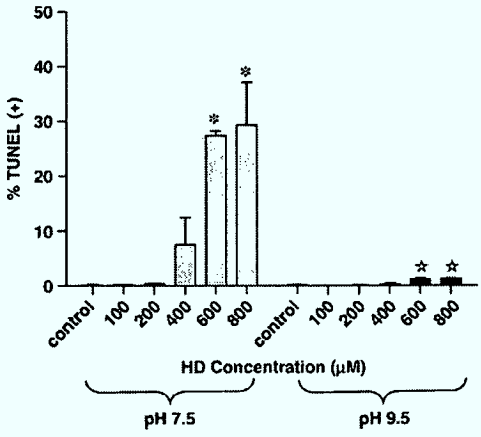

Fig. 7. Effect of pH on HD-induced DNA fragmention (TUNEL assay) in CHO-K1 cultures. Cells were grown on coverslips (13 mm diameter) in 24 well multiplates. Just-confluent cultures were treated with varying HD concentrations in culture medium of different pH for 1 h. Following the 1 h incubation, the test medium was removed and replaced with routine culture medium (pH 7.4). DNA fragmentation was assessed using the TUNEL assay, 5 h post-treatment as described in "Methods". Results represent the mean±standard deviation of 3 separate experiments. Data were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing (*$p<0.05$ vs. respective controls; ☆$p<0.05$ vs. corresponding HD concentration at pH 7.5).

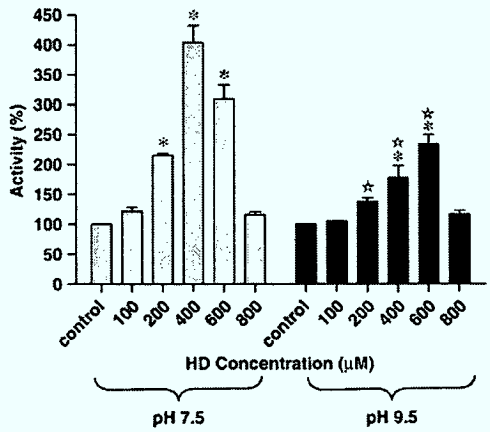

Fig. 9. Effect of pH on HD-induced caspase-3 activity in CHO-K1 cultures. First passage cells were grown in 35 mm Petri dishes. Just-confluent cultures were treated with varying HD concentrations in culture medium of different pH for 1 h. Following the 1 h incubation period, the test medium was removed and replaced with routine culture medium (pH 7.4). Caspase activity was assessed 5 h post-treatment as described in "Methods". Results represent the mean±standard deviation of 3 experiments. Data were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing (*$p<0.05$ vs. respective controls; ☆$p<0.05$ vs. corresponding HD concentration at pH 7.5).

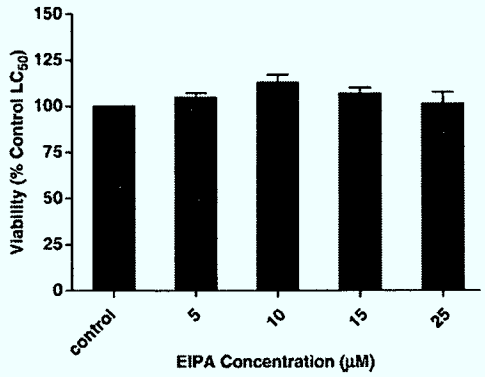

Fig. 10. Effect of EIPA on HD-induced cytotoxicity in CHO-K1 cultures. Just-confluent CHO-K1 cultures were pretreated with varying concentrations of EIPA. After 30 min the cells were treated with varying HD concentrations and cytotoxicity was determined at 24 h using the alamarBlue™ cytotoxicity assay. Results represent the mean±standard deviation of 3 experiments. Data were analyzed by ANOVA and *post hoc* Dunnett's Method Comparison test using control (0 μM HD) as the control group (*$p<0.05$ vs. control).

profound implications with respect to the regulation of diverse biochemical pathways including calcium metabolism, apoptosis, cell growth, lymphocyte activation and neoplastic transformation (Benedetti et al., 2001; Grinstein et al., 1988; Lagadic-Gossmann et al., 2004; Li and Eastman, 1995; Pucéat, 1999; Putney et al., 2002; Reshkin et al., 2000; Vaughan-Jones et al., 1983). In order to examine the possibility that HD exerts its toxicity by interfering with intracellular pH regulation, we investigated the effect of HD on the pH_i of CHO-K1 cells using the pH sensitive dye, SNARF-1.

In cells incubated in buffer at physiological pH, HD induced a significant and rapid concentration-dependent decline in pH_i. The acidification induced by 800 μM HD was ~0.25 pH units compared to controls, was complete in ~15 min and then plateaued within the 30 min test period. Cytosolic pH is maintained by intracellular buffers such as organic acids, bases and proteins, as well as by several plasma membrane-bound antiporters. It is possible that, at higher concentrations of HD, these intracellular buffering systems were overcome, eventually resulting in toxicity due to intracellular acidification. The NHE has been described as playing a major role in cytotoxic and apoptotic processes where alterations in pH_i are involved (Boyle et al., 1997; Huc et al., 2004; Karmazyn and Moffat, 1993; Lagadic-Gossmann et al., 2004). This antiporter system is activated by acidic pH_i, and serves to pump H^+ ions out of the cytoplasm in exchange for Na^+ ions. For this reason we tested the effect of EIPA, a potent and specific inhibitor of this exchanger (Vigne et al., 1983). If the NHE was involved, then EIPA may potentiate HD-induced cytosolic acidification. Our results showed that EIPA had no effect on either this endpoint, or on the cytotoxicity of HD, suggesting that the NHE is unlikely to participate in HD-induced pH_i changes.

We further examined the effect of both HD and EIPA on pH_i using the ammonium chloride prepulse technique. Pulsing the cells with ammonium chloride rapidly produced a decline in pH_i of ~0.4 units. In control cells, the antiporter systems responsible for maintaining physiological pH_i, rapidly returned the cells back to physiological levels. Unexpectedly, incubation of the cell suspensions with 24 μM (or 40 μM, data not shown) EIPA failed to have any effect on pH_i recovery, seeming to rule out a role for NHE in maintaining pH_i in this cell culture system. Although this is consistent with our results that showed that this inhibitor did not have any effect on HD toxicity, this finding warrants further investigation. Possibly the NHE in this cell system requires higher EIPA concentrations than most cell types for inhibition. Whereas EIPA had no effect on pH_i recovery, the effects of HD were dramatic. In addition to causing a further and very significant decline in cytosolic pH, HD also prevented any recovery from the combined ammonium chloride/HD acidification. Thus, although this cell culture system has robust mechanisms with which to protect itself against acidic pH_i, HD not only causes cytosolic acidification, but also poisons the very mechanisms that are in place to counteract this kind of insult.

The hydrolysis of HD has been shown to result in acidic degradation products (Brimfield et al., 2006) and we therefore examined the effect of varying HD concentrations in cell suspensions incubated at 37 °C. Sulphur mustard induced a concentration-dependent decline in pH that very closely paralleled the acidification in pH_i that it causes in this same cell type. It must therefore be concluded that HD-induced pH_i acidification is a direct result of the extracellular acidification caused by HD hydrolysis.

In order to gain additional understanding of the importance of HD-induced cytosolic acidification, we examined the effect of extracellular pH on HD toxicity, since it has been shown to modulate pH_i (Prigent et al., 1997; Vaughan-Jones and Wu, 1990). Studies utilizing cell culture are constrained by the rigorous conditions required for the successful cultivation of cells in an artificial environment. However, since HD hydrolyzes quickly in an aqueous environment and initiates its deleterious effects maximally over a very short time frame, a certain degree of flexibility was available to enable us to examine the effect of pH on HD toxicity. These studies were carried out so that cultures were incubated for 1 h with HD in medium of the test pH. The culture medium was then changed to one of routine pH 7.4, and the cultures left for the remainder of the 24 h test period. The short 1 h incubation time ensured that the viability of the cells was not compromised due to non-physiological pH conditions, and at the same time allowed maximal HD toxicity

Table 1
Effect of pH on HD LC_{50} values, half-life and CT exposure[a]

pH	LC_{50} (μM) (x±SD)	Half-life (x±SD, min)	CT (1 h) (AUC, ng-min ml) (x±SD)
5.0	76.9±2.6[b]	25.3±0.7	29.5±0.5
7.0	126.1±5.0	22.4±0.4	27.3±0.3
9.5	593.7±53.7	19.1±0.5	24.4±0.5

[a] Half-life values were determined using 200 μM HD in buffer at 20 °C. Concentration±time (CT) values were calculated based on a 60 min exposure. Results represent the mean±SD of three experiments and were analyzed by ANOVA and *post hoc* Tukey HSD Multiple Comparison testing.

[b] Not significantly different from value obtained at pH 7.0 ($p>0.05$). All other values within columns were significantly different from each other ($p>0.05$).

to take place. We were thus able to examine the effect of pH on the very early, and possibly initiating event(s) that HD induces to eventually cause cell death. The range tested was pH 5.0–10. In this range, control cultures showed no measurable loss in viability. We observed a very gradual decrease in HD toxicity from pH 5.0 to pH 8.5, a slightly steeper decrease at pH 9.0 and then a dramatic increase in LC_{50} at pH 9.5 and 10.0. With the exception of the protective effects of L-thiocitrulline (Sawyer et al., 1998), whose mechanism of action has yet to be elucidated, the extent of the protective effects obtained *in vitro* at basic pH was unprecedented.

Sulphur mustard has been well characterized as causing apoptosis in a number of different cell types (Dabrowska et al., 1996; Michaelson, 2000; Rosenthal et al., 1998, 2001; Sourdeval et al., 2006; Sun et al., 1998). We therefore examined the effects of altering the pH of the treatment medium on HD-induced apoptosis in CHO-K1 cells. Not surprisingly, under routine culture conditions, HD induced a concentration-dependent increase in a number of different apoptotic endpoints, including DNA fragmentation, morphology and caspase-3 induction. The effects of altering the pH of the treatment medium very closely mirrored the cytotoxicity results; at pH 9.5 a dramatic decrease in apoptotic cell death was observed, compared to that obtained at pH 7.5. Intracellular acidification has been shown to be a relatively common pre-condition for apoptotic cell death (Lagadic-Gossmann et al., 2004) and these results are consistent with this. We were not able to carry out these former studies (using SNARF-1) at pH 9.5, due to viability issues surrounding the time it took for the cells to equilibrate. However, in experiments utilizing buffers of pH 7.0–8.0, HD induced a similar concentration-dependent decrease in pH_i, regardless of external pH. In our cell death experiments, the cultures were changed into the pH test medium immediately before HD treatment, and incubation of the cultures at the test pH (with HD) was only carried out for 1 h. The treatment was then removed and the cultures refed with routine culture medium. It is likely that at basic pH, this treatment regimen resulted in a dynamic situation where a continual buffering of HD-induced acidification was taking place, prior to the cells regaining physiological pH_i. In those test regimens where pH_i did not enter into apoptosis-permissive conditions, cell death was prevented.

Although HD hydrolysis has been reported not to be pH-dependent (Papirmeister et al., 1991), we nevertheless determined the half-lives of HD in buffer at pH 5.0, 7.0 and 9.5 to rule out the possibility that our cytotoxicity findings were simply the result of pH-induced alterations in HD exposure. We found that HD half-life decreased slightly as the buffer became increasingly basic. Integration of the decay curves over the 1 h time period that we treated our cultures, did not explain the almost eight-fold decrease in HD toxicity from pH 5.0 to pH 9.5. It appears that the effect of pH on HD-induced toxicity must be biochemical/pharmacological in nature and not an effect on the rate of HD hydrolysis.

The data obtained in this study support the hypothesis that HD causes an extracellular acidification through chemical hydrolysis and that this, in both a concentration and temporally related fashion, results in cytosolic acidification. Sulphur mustard also acts to poison the antiporter systems responsible for maintaining physiological pH_i, so that the cells are unable to recover from this insult. It is this irreversible decline in pH_i that initiates the cascade of events which results in HD-induced cell death. Our work shows that the NHE is unlikely to be a target for HD. However, the role of several other membrane-bound ion exchange systems remains to be investigated. These antiporter systems, which may be the targets at which HD initiates its toxicity, are currently being investigated.

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References

Auerbach, C., 1949. Chemical mutagenesis. Biol. Rev. 24, 355–391.

Auerbach, C., Robson, J.M., 1946. Chemical production of mutation. Nature 157, 302.

Benedetti, A., Di Sario, A., Casini, A., Ridolfi, F., Bendia, E., Pigini, P., Tonnini, C., D'Ambrosio, L., Feliciangeli, G., Macarri, G., Svegliati-Baroni, G., 2001. Inhibition of the Na(+)/H(+) exchanger reduces rat hepatic stellate cell activity and liver fibrosis: an in vitro and in vivo study. Gastroenterology 120, 545–556.

Boyle, K.M., Irwin, J.P., Humes, B.R., Runge, S.W., 1997. Apoptosis in C3H-10T1/2 cells: roles of intracellular pH, protein kinase C, and the Na^+/H^+ antiporter. J. Cell. Biochem. 67, 231–240.

Brimfield, A.A., Novak, M.J., Hodgen, E., 2006. Thiodiglycol, the hydrolysis product of sulfur mustard: analysis of in vitro biotransformation by mammalian alcohol dehydrogenases using nuclear resonance. Toxicol. Appl. Pharmacol. 213, 207–215.

Cui, S., Reichner, J.S., Mateo, R.B., Albina, J.E., 1994. Activated murine macrophage induced apoptosis in tumor cells through nitric oxide-dependent mechanisms. Cancer Res. 54, 2462–2467.

Dabrowska, M.I., Becks, L., Lelli, J.L., Levee, M.G., Hinshaw, D.B., 1996. Sulfur mustard induces apoptosis and necrosis in endothelial cells. Toxicol. Appl. Pharmacol. 141, 568–583.

Dacre, J.C., Goldman, M., 1996. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. Pharmacol. Rev. 48, 289–325.

Duke, R.C., Cohen, J.J., 1992. Morphological and biochemical assays of apoptosis. Current Protocols in Immunology, Suppl. 3.17, pp. 1–16.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1096–1121.

Grinstein, S., Smith, J.D., Onizuka, R., Cheung, R.K., Gelfand, E.W., Benedict, S., 1988. Activation of Na^+/H^+ exchange and the expression of cellular proto-oncogenes in mitogen- and phorbol ester-treated lymphocytes. J. Biol. Chem. 263, 8658–8665.

Hamilton, M.G., Dorandeu, F.M., McCaffery, M., Lundy, P.M., Sawyer, T.W., 1998. Modification of cytosolic free calcium concentrations in human keratinocytes after sulphur mustard exposure. Toxicol. In Vitro 12, 365–372.

Hua, A., Daniel, R., Jasseron, M.P., Thiriot, C., 1993. Early cytotoxic effects induced by bis-chloroethyl sulphide (sulphur mustard): $[Ca^{2+}]I$ rise and time-dependent inhibition of B77 fibroblast serum response. J. Appl. Toxicol. 13, 161–168.

Huc, L., Sparfel, L., Rissel, M., Dimanche-Boitrel, M-T., Guillouzo, A., Fardel, O., Lagadic-Gossmann, D., 2004. Identification of Na^+/H^+ exchange as a new target for toxic polycyclic aromatic hydrocarbons in liver cells. FASEB J. 18, 344–346.

Karmazyn, M., Moffat, M.P., 1993. Role of Na+/H+ exchange in cardiac physiology and pathophysiology: mediation of myocardial reperfusion injury by the pH paradox. Cardiovas. Res. 27, 915–924.

Kehe, K., Szinicz, L., 2005. Medical aspects of sulphur mustard poisoning. Toxicology 214, 198–209.

Kramer, C.Y., 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrica 12, 309–310.

Lagadic-Gossmann, D., Rissel, M., Galisteo, M., Guillouzo, A., 1999. Intracellular pH alterations induced by tacrine in a rat liver biliary epithelial cell line. Br. J. Pharmacol. 128, 1673–1682.

Lagadic-Gossmann, D., Huc, L., Lecureur, V., 2004. Alterations of intracellular pH homeostasis in apoptosis: origins and roles. Cell Death Differ. 11, 953–961.

Li, J., Eastman, A., 1995. Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na(+)/H(+) antiport. J. Biol. Chem. 270, 3203–3211.

Mahnensmith, R.L., Aronson, P.S., 1985. The plasma membrane sodium–hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. 56, 773–788.

Michaelson, S., 2000. DNA fragmentation pattern induced in thymocytes by sulphur mustard. Chemico-Biol. Interact. 125, 1–15.

National Academy Press, 1993. In: Pechura, C.M., Rall, D.P. (Eds.), Veterans at Risk: The Health Effects of Mustard Gas and Lewisite. Institute of Medicine, Washington, DC.

Papirmeister, B., Feister, A.J., Robinson, S.I., Ford, R.D., 1991. Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications. CRC Press, Boca Raton, FL.

Papirmeister, B., Gross, C.L., Meier, H.L., Petrali, J.P., Johnson, J.B., 1985. Molecular basis of mustard-induced vesication. Fundam. Appl. Toxicol. 5, S134–S149.

Prigent, K.L., Lagadic-Gossmann, D., Feuvray, D., 1997. Modulation by pH_o and intracellular Ca^{2+} of Na^+–H^+ exchange in diabetic rat isolated ventricular myocytes. Circ. Res. 80, 253–260.

Pucéat, M., 1999. pHi regulatory ion transporters: an update on structure, regulation and cell function. Cell. Mol. Life Sci. 55, 1216–1229.

Putney, L.K., Denker, S.P., Barber, D.L., 2002. The changing face of the Na^+/H^+ exchanger, NHE1: structure, regulation, and cellular actions. Annu. Rev. Pharmacol. Toxicol. 42, 527–552.

Ray, R., Legere, R.H., Majerus, B.J., Petrali, J.P., 1995. Sulfur mustard-induced increase in intracellular free calcium level and arachidonic acid release from cell membrane. Toxicol. Appl. Pharmacol. 131, 44–52.

Reshkin, S.J., Bellizzi, A., Caldeira, S., Albarani, V., Malanchi, I., Poignee, M., Alunni-Fabbroni, M., Casavola, V., Tommasino, M., 2000. Na^+/H^+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. FASEB J. 14, 2185–2197.

Rosenthal, D.S., Simbulan-Rosenthal, C.M.G., Iyer, S., Spoonde, A., Smith, W., Ray, R., Smulson, M.E., 1998. Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and caspase dependent pathway. J. Invest. Dermatol. 111, 57–70.

Rosenthal, D.S., Simbulan-Rosenthal, C.M., Liu, W.F., Velena, A., Anderson, D., Benton, B., Wang, Z.Q., Smith, W., Ray, R., Smulson, M.E., 2001. PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. J. Invest. Dermatol. 117, 1566–1573.

Sawyer, T.W., Hamilton, M.G., 2000. Effect of intracellular calcium modulation on sulfur mustard cytotoxicity in cultured human neonatal keratinocytes. Toxicol. In Vitro 14, 149–157.

Sawyer, T.W., Hancock, J.R., D'Agostino, P.A., 1998. L-Thiocitrulline: a potent protective agent against the toxicity of sulphur mustard in vitro. Toxicol. Appl. Pharmacol. 151, 340–346.

Smith, W., Dunn, M.A., 1991. Medical defence against blistering chemical warfare agents. Arch. Dermatol. 127, 1207–1213.

Sourdeval, M., Lemaire, C., Deniaud, A., Taysee, L., Daulon, S., Breton, P., Brenner, C., Boisvieux-Ulrich, E., Marano, F., 2006. Inhibition of Caspase-Dependent Mitochondrial Permeability Transition Protects Airway Epithelial Cells Against Mustard Induced Apoptosis. Apoptosis 11, 1545–1559.

Sun, J., Wang, Y.-X., Sun, M.-J., 1998. Apoptosis and necrosis induced by sulfur mustard in HeLa cells. Acta Pharmacol. Sin. 20, 445–448.

Tukey, J., 1953. A Problem of Multiple Comparisons. Princeton Univ. Press, Princeton, NJ.

Vaughan-Jones, R.D., Wu, M.-L., 1990. Extracellular H^+ inactivation of Na^+–H^+ exchange in the sheep cardiac purkinje fibre. J. Physiol. 428, 441–466.

Vaughan-Jones, R.D., Lederer, W.J., Eisner, D.A., 1983. Ca^{2+} ions can affect intracellular pH in mammalian cardiac cells. Nature 301, 522–524.

Vigne, P., Frelin, C., Cragoe Jr., E.J., Lazdunzki, M., 1983. Ethylisopropyl-amiloride: a new and highly potent derivative of amiloride for the inhibition of the Na^+/H^+ exchange system in various cell types. Biochem. Biophys. Res. Commun. 116, 86–90.

Warthin, S.W., Weller, C.V., 1919. The Medical Aspects of Mustard Gas Poisoning. Mosby, St. Louis, MO, USA.

Willems, J.L., 1989. Clinical management of mustard gas casualties. Ann. Med. Mil. Belg. (Suppl. 3), 1–61.