3-Aminopropanal, formed during cerebral ischaemia, is a potent lysosomotropic neurotoxin

Wei LI*[1], Xi-Ming YUAN*, Svetlana IVANOVA†, Kevin J. TRACEY†, John W. EATON*† and Ulf T. BRUNK*

*Division of Pathology II, Linköping University Hospital, S-581 85 Linköping, Sweden, †Laboratory of Biomedical Science, North Shore-LIE Research Institute, Manhasset, NY 11030, U.S.A., and ‡James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202, U.S.A.

Cytotoxic polyamine-derived amino aldehydes, formed during cerebral ischaemia, damage adjacent tissue (the so-called 'penumbra') not subject to the initial ischaemic insult. One such product is 3-aminopropanal (3-AP), a potent cytotoxin that accumulates in ischaemic brain, although the precise mechanisms responsible for its formation are still unclear. More relevant to the present investigations, the mechanisms by which such a small aldehydic compound might be cytotoxic are also not known, but we hypothesized that 3-AP, having the structure of a weak lysosomotropic base, might concentrate within lysosomes, making these organelles a probable focus of initial toxicity. Indeed, 3-AP leads to lysosomal rupture of D384 glioma cells, a process which clearly precedes caspase activation and apoptotic cell death. Immunohistochemistry reveals that 3-AP concentrates in the lysosomal compartment and prevention of this accumulation by the lysosomotropic base ammonia, NH_3, protects against 3-AP cytotoxicity by increasing lysosomal pH. A thiol compound, *N*-(2-mercaptopropionyl)glycine, reacts with and neutralizes 3-AP and significantly inhibits cytoxocity. Both amino and aldehyde functions of 3-AP are necessary for toxicity: the amino group confers lysosomotropism and the aldehyde is important for additional, presently unknown, reactions. We conclude that 3-AP exerts its toxic effects by accumulating intralysosomally, causing rupture of these organelles and releasing lysosomal enzymes which initiate caspase activation and apoptosis (or necrosis if the lysosomal rupture is extensive). These results may have implications for the development of new therapeutics designed to lessen secondary damage arising from focal cerebral ischaemia.

Key words: amine oxidase, 3-aminopropanal, apoptosis, cerebral ischaemia, lysosome.

INTRODUCTION

A variety of degenerative and traumatic insults to the central nervous system (CNS), including ischaemia, lead to the apoptotic death of neurons and glial cells [1,2]. In cerebral trauma and ischaemia, substantial cell death occurs adjacent to the ischaemic area (the 'penumbra') and is thought to account for a major portion of the resultant damage. The formation of toxic amino aldehydes, especially 3-aminopropanal (3-AP), is considered to play a significant role in this process [3]. Although it seems probable that such toxic amino aldehydes are formed from the breakdown of polyamines, the enzyme(s) and the biochemical pathways responsible for their formation are not yet clearly identified. More important to the present study, the mechanisms by which amino aldehydes such as 3-AP exert cytotoxicity are similarly unclear [4,5]. In brains of experimental animals, 3-AP begins to accumulate shortly after the onset of cerebral ischaemia and similar elevations of 3-AP have also been found in the cerebrospinal fluid of humans with CNS ischaemia, in concentrations directly related to the extent of the ischaemic lesions [3]. *In vitro*, 3-AP causes apoptotic and necrotic death of both neurons and glial cells, with neurons being the more sensitive cell type. 3-AP will also damage other types of mammalian cells, suggesting that this small aldehyde is generally cytotoxic [6,7].

It is probable that, as a reactive aldehyde, 3-AP will react with amino and thiol groups on cellular proteins, possibly impairing their normal functions. Previous observations, cited above, indicated that these or other cytotoxic reactions of 3-AP might participate in the mediation of cell death in the zone of potentially viable tissue surrounding the ischaemic area. The present study was aimed at further elucidation of the molecular bases of the cytotoxic actions of 3-AP, particularly focusing on its possible effects on lysosomal stability. During the last few years, lysosomal destabilization with release of hydrolytic enzymes to the cytosol has been raised as an upstream event preceding apoptosis triggered by a variety of different agonists [8–17]. The initiation of apoptosis may involve direct activation of procaspases by released lysosomal proteases, especially cathepsins B, L and D. Alternatively, cytoplasmic lysosomal enzymes may activate other cytosolic proenzymes, activate BH3 interacting domain death agonist or proteolytically attack mitochondrial membranes, releasing cytochrome *c* [8,11–16,18,19].

We observed previously that lysosomotropic detergents, which are concentrated inside lysosomes by the mechanism of proton trapping, cause lysosomal rupture and ensuing apoptosis [12,15]. 3-AP is an amino-containing aldehyde with a relatively high pK_a value (approx. 9.3), increasing the possibility that its toxicity might be due to accumulation within the lysosomal compartment. The resultant high intralysosomal concentration of this reactive aldehyde might lead to site-specific reactions with critical thiol groups or to protein cross-linking with ensuing damage to lysosomal integrity. To test this general concept, we have assessed the lysosomotropic behaviour of 3-AP, its effects on lysosomal stability and cellular concentrations of GSH, and the relationship between these effects and apoptosis.

EXPERIMENTAL

Cells and experimental conditions

The human astrocytoma cell line, D384, was grown in Dulbecco's modified Eagle's minimal medium containing 10% (v/v) foetal

Abbreviations used: AO, Acridine Orange; 3-AP, 3-aminopropanal; CNS, central nervous system; CHX, cycloheximide; MPG, *N*-(2-mercaptopropionyl)glycine; ss, single-stranded.

[1] To whom correspondence should be addressed (e-mail weili@pat.liu.se).

calf serum (Invitrogen, Paisley, U.K.). The cells were subcultivated twice a week and used for experiments within 24 h of the previous passage. All experiments were performed in 35 mm culture dishes containing approx. 3×10^5 cells with 1 ml of serum-free medium (Opti-MEM I; Invitrogen) to prevent non-specific interaction of 3-AP or propionaldehyde with serum proteins and to mimic the very low protein levels typical of cerebrospinal fluid.

Cells were either exposed directly to various concentrations of 3-AP, prepared as described previously [3], or pretreated with 10 mM NH_4Cl for 30 min (to alkalinize the acidic vacuolar compartments) and then exposed to 3-AP in the continued presence of NH_4Cl for different periods of time. In some experiments, cells were exposed for 24 h to various concentrations of the corresponding lysosomotropic alcohol (3-aminopropanol) or the related non-lysosomotropic aldehyde (propionaldehyde). In additional experiments, cells were exposed to 3-AP in combination with *N*-(2-mercaptopropionyl)glycine (MPG; Sigma, St. Louis, MO, U.S.A.), which reacts with 3-AP to yield a non-toxic thioacetal adduct [20]. MPG was added either together with 3-AP or up to 5 h after the addition of 3-AP.

To determine whether *de novo* protein synthesis is required for 3-AP-induced apoptosis, some cells were pretreated for 3 h with 10–100 μM cycloheximide (CHX) and then exposed to 3-AP for another 5 h in the continued presence of CHX.

Generation of antibodies against 3-AP-modified proteins

Antibodies were raised against 3-AP-modified proteins as described previously [20]. In brief, a preparation of keyhole limpet haemocyanin (weak haptens, such as 3-AP, require powerful immunogenic carriers such as keyhole limpet haemocyanin) was incubated with 200 μM (final concentration) of 3-AP in the presence of 200 μM cyanoborohydride (to reduce the Schiff base) for 16 h at 22 °C. The modified protein was purified by ultrafiltration and three subsequent washes in PBS using Centricon-10 membranes (Millipore, Bedford, MA, U.S.A.). The antigen was inoculated into rabbits at Bio-synthesis Inc. (Lewisville, TX, U.S.A.). Antibodies reactive against 3-AP-modified protein were screened by dot blotting against standards of 3-AP-modified BSA (Invitrogen). The rationale for making antibodies which recognize 3-AP adducts was that *in vivo* 3-AP will react rapidly with both thiols and amine groups and most of this material will therefore be present as protein adducts. The resultant antibodies were reactive with 3-AP-modified BSA. The antibody was unreactive against acrolein- and malonyldialdehyde-modified BSA.

Immunocytochemistry of 3-AP-modified proteins

Intracellular localization of 3-AP in cells pre-exposed to 3-AP for 3 h was detected immunocytochemically using 3-AP antibodies. In brief, cells were fixed for 20 min at 4 °C in 4% (w/v) paraformaldehyde with 0.05% glutaraldehyde in Dulbecco's PBS. The fixed cells were rinsed with Tris-buffered saline (50 mM Tris base/150 mM NaCl; pH 7.4) and permeabilized with 0.1% saponin in PBS buffer. They were incubated with rabbit polyclonal antibodies (4 °C, overnight), which recognize 3-AP modified proteins (see the methods described above), then with secondary antibodies (alkaline phosphatase-conjugated goat anti-rabbit) at room temperature (22 °C) for 1 h. The antibody reaction was visualized by demonstration of the conjugated alkaline phosphatase, using 4-benzoylamino-2,5-diethoxybenzene diazonium chloride as a substrate.

Assessment of apoptosis

For morphological assessment, cultured cells were either directly viewed by phase-contrast light microscopy or fixed in 4% (w/v) formaldehyde and stained with Wright-Giemsa, and then examined by light microscopy. By morphologic criteria, shrunken cells with pycnotic or fragmented nuclei were considered apoptotic. Digitized images of the cells were obtained using a Nikon Microphot-SA microscope (Nikon, Tokyo, Japan) equipped with a Hamamatsu (Bridgewater, NJ, U.S.A.) C4742-95 digital camera.

Apoptotic cell death was determined by detection of single-stranded DNA (ssDNA) using a specific anti-ssDNA monoclonal antibody (Alexis, San Diego, CA, U.S.A.). The cells were fixed overnight in methanol, incubated in formamide for 20 min at 56 °C and transferred to ice-cold Tris-buffered saline for another 10 min. The anti-ssDNA monoclonal antibody was applied for 30 min at room temperature and followed by peroxidase-conjugated anti-mouse IgM for 30 min. The antibody binding was detected with 3,3′-diaminobenzidine tetrahydrochloride. Non-specific immunoreactions were blocked with 3% (w/v) non-fat dry milk before exposure to the primary antibody. The cells were examined and digitized images were obtained by light microscopy as above. Apoptotic cells showed brown nuclei.

Caspase activation was assessed using an FITC conjugate of the cell-permeant pan caspase inhibitor, VAD-FMK (caspACE™ FITC-VAD-FMK *In Situ* Marker; Promega Biosciences, San Luis Obispo, CA, U.S.A.), which irreversibly binds to activated caspases. Cells with activated caspases show an intense green fluorescence. Digitized micrographs (see above) were taken in fluorescence mode using blue light excitation, and fluorescence intensity/cell was estimated using the Adobe Photoshop (5.5) computer program.

Determination of lysosomal stability by Acridine Orange (AO) staining

Lysosomal integrity was assessed by the AO uptake technique as described previously [8,12,21]. After exposure to 3-AP, cells were exposed to AO (5 μg/ml) for 15 min at 37 °C, rinsed in complete culture medium and examined by either confocal laser scanning microscopy or by static cytofluorometry. AO is a lysosomotropic base and a metachromatic fluorophore that accumulates in the acidic lysosomal compartment due to proton trapping. After exposure to low concentrations of AO, normal cells exhibit a strong red granular (lysosomal) fluorescence on excitation with either blue or green light.

Immunocytochemistry of lysosomal cathepsin B

Intracellular release of the lysosomal cysteine protease, cathepsin B, was detected immunocytochemically. Normal and treated cells were fixed and permeabilized as described above. Polyclonal goat anti-human cathepsin B (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used as the primary antibody (4 °C, overnight). Non-specific immunoreactions were blocked with swine serum before addition of the primary antibody. Cells were then treated with an alkaline phosphatase-conjugated swine anti-goat antibody for 1 h at room temperature. The antibody reaction was visualized by 4-benzoylamino-2,5-diethoxybenzene diazonium chloride. As a negative control, the primary antibody was omitted. The samples were examined by light microscopy and images were obtained as described above.

Measurement of intracellular GSH

Cells exposed to 3-AP, propionaldehyde or to 3-AP and NH_4Cl in combination were scraped in 0.5 M $HClO_4$, containing 1 mM EDTA and centrifuged at 500 ***g*** for 10 min at 4 °C. GSH in the supernatant was assayed by HPLC using electrochemical detection as described previously [22,23]. GSH concentration was calculated with reference to a standard curve.

Statistical analysis

Since data showed evidence of skewing, non-parametric tests, the Mann–Whitney *U* test and the Kruskal–Wallis analysis, were used for comparisons between groups and multiple groups respectively. Differences were considered significant at values of $P \leqslant 0.05$. Results are given as means ± S.E.M. Unless otherwise stated, $n \geqslant 3$.

RESULTS

3-AP-induced cytotoxicity

Preliminary experiments were performed to establish the toxicity of 3-AP against D384 cells. We used Trypan Blue exclusion in combination with Giemsa staining to estimate cell viability, apoptosis (pycnotic nuclei within cells excluding Trypan Blue) and necrosis (swollen nuclei within cells stained blue by Trypan Blue). The results showed that 3-AP caused a dose- and time-dependent cytotoxicity (results not shown), with 3-AP concentrations of $< 50\ \mu$M causing little or no toxicity even after prolonged exposure (> 8 h), whereas 100 μM induced necrosis within 3–5 h (approx. 70 and 90 % necrotic cell death respectively). At a dose of 75 μM, some cell shrinkage and nuclear condensation was observed at 3 h and most cells became morphologically apoptotic within 5 h (cellular shrinkage, membrane budding and nuclear pycnosis with intact plasma membranes). Thus to track more precisely the time course of the various cellular effects, 75 μM 3-AP was used in most of the following experiments. It was reported previously [20] that endogenous brain levels of 3-AP after damage could be up to approx. 350 μM, which is higher than in the present study on astrocytoma cells *in vitro*. *In vivo* conditions are more complicated and not directly comparable with a simple cell culture situation.

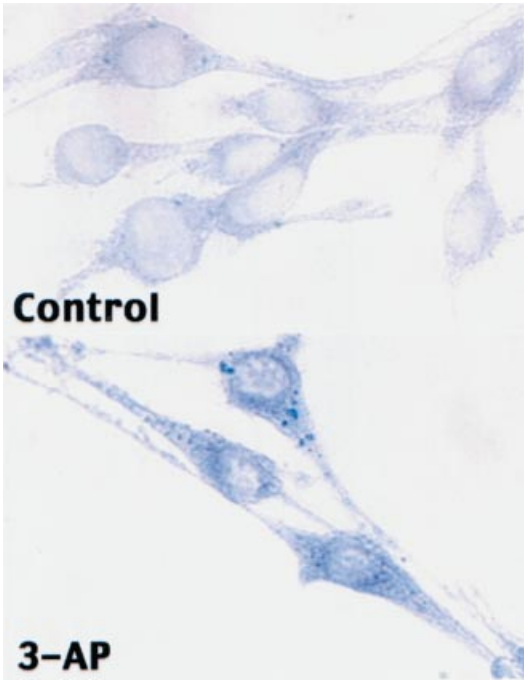

Figure 1 Immunocytochemical detection of 3-AP-modified proteins using polyclonal antibodies prepared against 3-AP-modified keyhole limpet haemocyanin

Note granular reaction product with a typical lysosomal size and distribution pattern (cf. also Figure 3) in 3-AP-exposed cells, whereas control cells show few such granular structures. Original magnification × 1000.

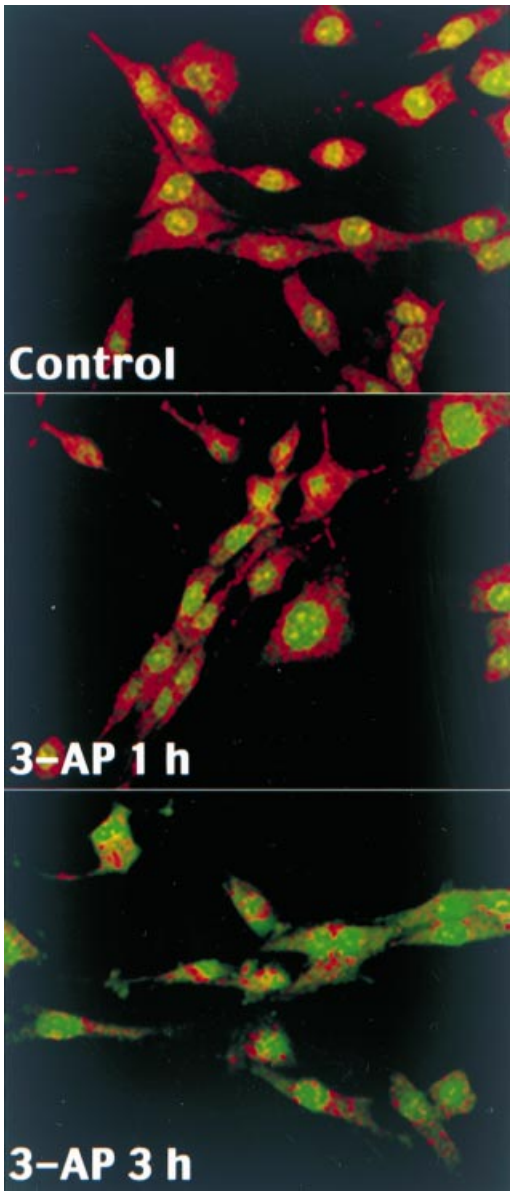

Figure 2 3-AP-induced lysosomal destabilization

Control and 3-AP-treated (75 μM) cells were stained with AO and examined by confocal laser-scanning microscopy using blue light excitation. Note the decreasing numbers of intact (red) lysosomes, especially after 3 h exposure to 3-AP, as well as enhanced nuclear and cytoplasmic green fluorescence (reflecting decreased pH perhaps arising from lysosomal rupture). Original magnification × 600.

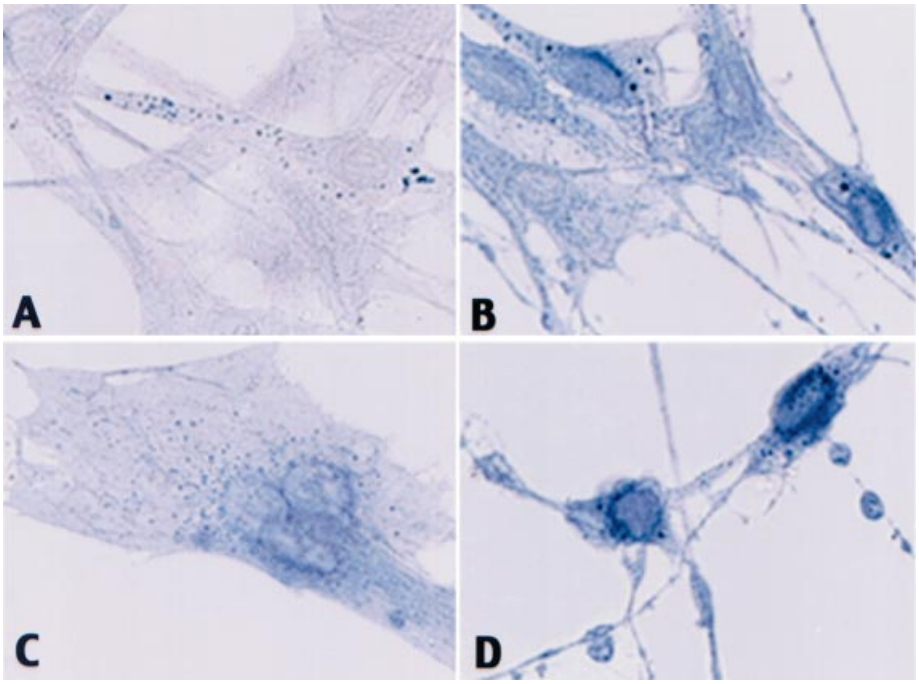

Figure 3 Pattern of cathepsin B immunoreactivity in control and 3-AP-exposed cells

Most control cells (**A**) displayed a few cathepsin B-containing granules (lysosomes). Exposure to 3-AP for 3 h (**B**–**D**) caused an enhanced granular cathepsin B immunoreactivity and resulted in free cytosolic and nuclear relocation of the lysosomal enzyme in both morphologically normal and apoptotic cells. Diffuse cathepsin B immunoreactivity of the cytosol and nuclei was a consistent finding in apoptotic cells (**D**). Original magnification $\times 1000$.

Immunocytochemical localization of intracellular 3-AP

To determine whether 3-AP might selectively localize within particular intracellular compartments, we performed immunocytochemical localization of 3-AP-modified proteins. As shown in Figure 1, antibodies recognizing 3-AP-modified protein exhibited a typical lysosomal pattern of the reaction product. In contrast, control cells showed only a weak, generally diffuse pattern of reaction, providing support for the idea that 3-AP might act as a lysosomotropic toxin.

Destabilization of lysosomal membranes and relocation of lysosomal cathepsin B by 3-AP

To determine whether 3-AP might affect lysosomal stability, lysosomal integrity was assayed by the AO-uptake test using both static cytofluorometry and confocal microscopy. The results (Figure 2) show that 3-AP does cause lysosomal rupture and this occurs well before the onset of detectable apoptosis.

Lysosomal integrity was assessed by immunocytochemical studies of cathepsin B. Most untreated control cells displayed a faint granular immunoreactivity to anti-cathepsin B antibodies, mainly in the periphery of the cell, reflecting the normal lysosomal localization of this protease (Figure 3A). In contrast, exposure to 3-AP caused diffuse cytosolic cathepsin B immunoreactivity in almost all cells (morphologically normal and apoptotic cells alike; Figures 3B–3D). Interestingly, in apoptotic cells, immunoreactive cathepsin B also appeared within the condensed nuclei (Figure 3D).

Overall, these findings suggest that 3-AP causes early lysosomal destabilization and release of lysosomal enzymes, such as cathepsin B, followed by more extensive lysosomal rupture and finally apoptosis. The increased cathepsin B immunoreactivity, and its relocation to nuclei, also has been described in apoptosis induced by 7-oxysterols [21] and may play a presently unknown role in the apoptotic process.

Apoptosis induced by 3-AP and protection by NH_4Cl

3-AP caused typical apoptotic cell death, detectable after 3 h and marked after 5 h of continuous exposure to this cytotoxin. Most of this cell death was apoptotic as indicated by caspase activation (Figures 4A and 4B), cleavage of ssDNA (Figure 4C), and formation of pycnotic nuclei in Giemsa-stained specimens (Figure 4D).

To assess further the possible importance of 3-AP accumulation within the acidic lysosomal environment and the role of lysosomal destabilization in apoptotic and necrotic cell death, some cultures were pretreated with 10 mM NH_4Cl for 30 min before being exposed to 75 μM (apoptosis) or 100 μM (necrosis) 3-AP in the continued presence of NH_4Cl. Under such circumstances, NH_3 ($pK_a = 9.25$) is formed and diffuses into the cells and the lysosomes. In the latter compartment, the base becomes protonated and trapped as NH_4^+, substantially increasing the lysosomal pH [24,25] and preventing the accumulation of other lysosomotropic substances, such as 3-AP. In support of the requirement for lysosomal concentration of 3-AP in the causation of cellular toxicity, NH_4Cl significantly protected cells against apoptosis (Figures 4A–4D), and almost fully preserved cell morphology, even after exposure to 100 μM 3-AP (results not shown). It appears that the entry of 3-AP into cells (and, therefore, its cytotoxicity) is not instantaneous. In some experiments, cells were treated with 3-AP with/without NH_4Cl for 1–3 h, washed and returned to standard culture conditions. Cell morphology was analysed by light microscopy on Giemsa-stained

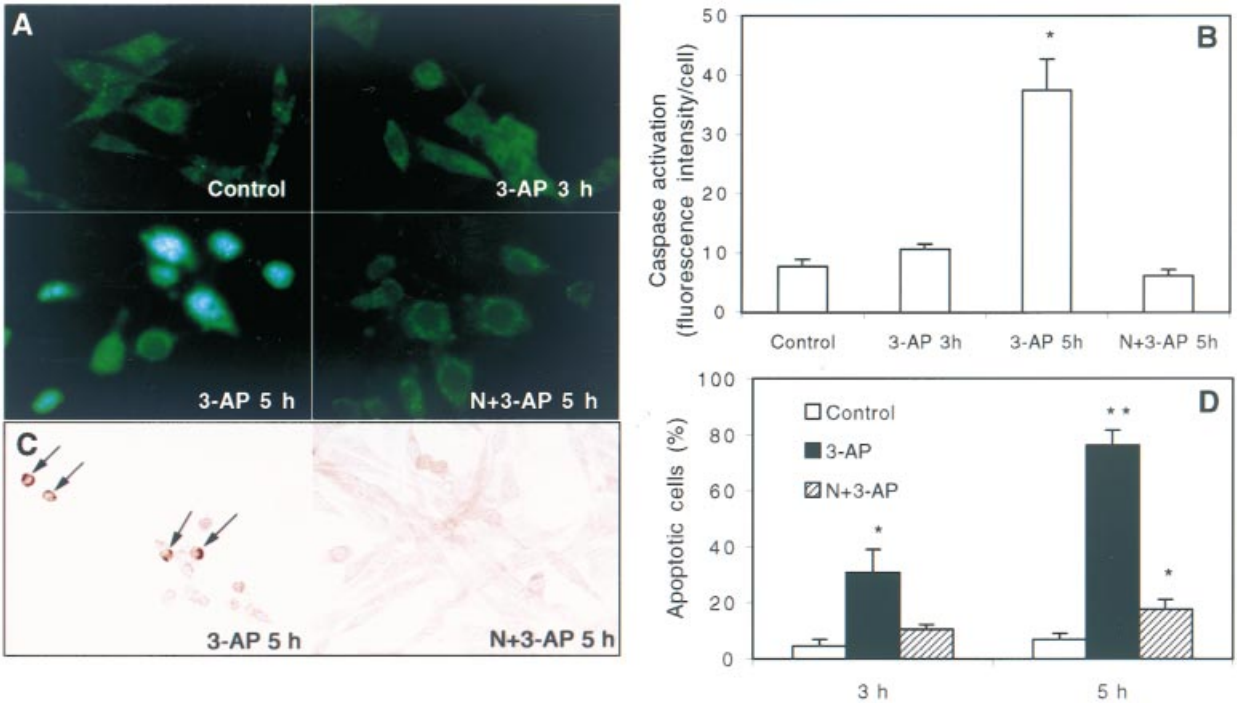

Figure 4 Caspase activation, DNA fragmentation, apoptotic morphology and protective effects of NH_4Cl in cells exposed to 3-AP for 3–5 h

(**A**) Caspase activation was detected using FITC-VAD-FMK. Original magnification $\times 600$. (**B**) Fluorescence intensity was also quantified from digitized micrographs using the Adobe Photoshop® computer program. N, NH_4Cl treatment. (**C**) DNA fragmentation was assayed using an antibody to ssDNA (positive cells indicated by arrows). Original magnification $\times 400$. (**D**) Quantification of apoptotic cells in Giemsa-stained preparations using light microscopy and morphological criteria. Significant differences from control groups are indicated ($^*P < 0.05$, $^{**}P < 0.01$).

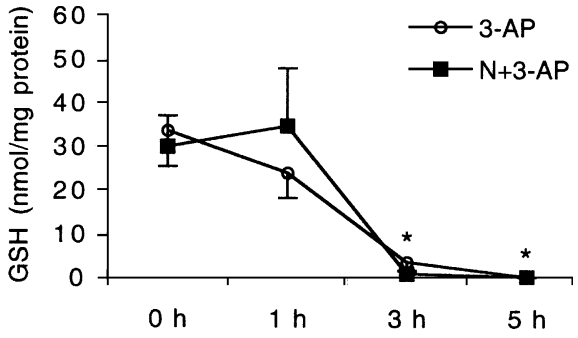

Figure 5 Depletion of intracellular GSH by 3-AP

D384 cells were exposed to 3-AP for up to 5 h with or without NH_4Cl, lysed as described in the Experimental section and GSH was analysed by HPLC. Note similar GSH depletion between groups, indicating that NH_3/NH_4^+ does not directly interfere with 3-AP and that temporary GSH depletion is not cytocidal. N, NH_4Cl treatment; $^*P < 0.05$ versus 0 h controls.

cultures 15 h later. No morphologically significant alterations were observed in cells exposed for 2 h to 3-AP, whereas an exposure for 3 h gave rise to pronounced apoptotic morphology that was prevented by NH_4Cl (results not shown).

In apoptosis of CNS cells caused by certain other agonists, *de novo* protein synthesis appears to be required [26]. However, in the present instance, cells pretreated with CHX (which by itself had no toxic effect after up to 8 h incubation) were not protected against 3-AP (75 μM); rather they showed a somewhat higher frequency of necrosis (results not shown). The lack of protective effect by CHX suggests that no protein synthesis is needed for apoptosis in this model, which indicates that preformed proteases, such as caspases and/or cathepsins, are sufficient to perform the apoptotic process.

Overall, these results indicate that (i) cells are capable of surviving limited release of lysosomal enzymes (such as that released within 2–3 h of exposure to 3-AP), (ii) more extensive lysosomal rupture leads inexorably to apoptosis and (iii) increasing the lysosomal pH prevents cell death due to decreased intra-lysosomal accumulation of 3-AP and consenquent reduced interaction with protein thiol groups.

GSH depletion by 3-AP and propionaldehyde

In considering plausible direct reactions that might explain the toxicity of 3-AP, we hypothesized that, as an aldehyde, 3-AP might undergo nucleophilic addition reactions with thiols such as GSH. If so, one possible explanation for the cytotoxicity of 3-AP would be simple depletion of cellular GSH. Indeed, as shown in Figure 5, cells exposed to 3-AP do show a profound decrease in GSH. However, these large decrements in GSH may not be causally related to either lysosomal destabilization or ensuing apoptosis, because pre-exposure of the cells to NH_4Cl, which prevents apoptosis, did not suppress 3-AP-induced GSH depletion. This indicates that GSH depletion is not involved in the early stages of apoptotic cell death and is in accord with a previous study that C6 glioma cells remain viable after glutamate-induced GSH depletion [27]. This result also excludes the unlikely possibility that the added ammonium might somehow directly neutralize the 3-AP. Furthermore, a dramatic (approx. 90%) and more rapid depletion of GSH occurred after 30 min of exposure to propionaldehyde. However, on further incubation,

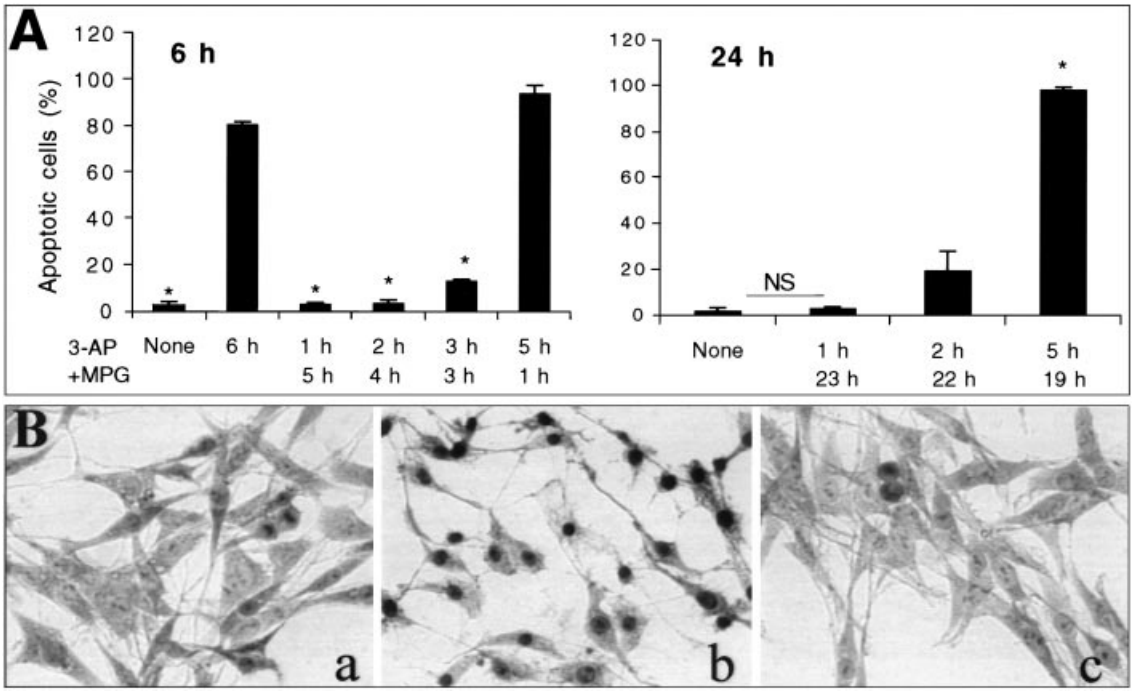

Figure 6 Prevention of 3-AP-induced cell death by MPG

(**A**) D384 cells were treated for different periods of time with 3-AP in the presence or absence of 150 μM MPG and then stained with Giemsa. MPG was added 1–5 h after initiation of 3-AP exposure (75 μM) as indicated, and cells were stained after a total incubation time of either 6 h (left) or 24 h (right). Note that not until cells had been exposed to 3-AP for more than 3 h before MPG addition did MPG fail to protect. Left panel: $^*P < 0.05$ versus 3-AP (6 h). Right panel: $^*P < 0.05$ versus None and 3-AP (1 h) + MPG (23 h). (**B**) After exposure to 100 μM 3-AP for 1 h, cells were treated with MPG for another 5 h. a, Control; b, 3-AP treated cells; c, MPG and 3-AP treated cells. Note well-preserved cells in 'c'.

GSH levels rebounded to 50–70 % after 3 h exposure and to almost normal levels after 5 h, and cell viability was not affected.

3-AP cytotoxicity is dependent on both the lysosomotropic amine and the aldehyde moieties

In an effort to determine the nature of the cytotoxic principle involved, we tested the importance of both the aldehyde and amine functionalities of 3-AP by exposing cells to propionaldehyde (an aldehyde which is non-lysosomotropic) and 3-aminopropanol (a lysosomotropic alcohol). In the concentrations tested (0–200 μM), neither of these compounds showed any cytotoxicity during a 24 h period, suggesting that the toxicity of 3-AP requires both lysosomotropism and an aldehydic moiety. We should, however, note that at concentrations above 300 μM propionaldehyde did have cytotoxic effects within 24 h, perhaps due to long-term depletion of cellular GSH.

The depletion of intracellular GSH caused by 3-AP suggests that other thiol compounds which might similarly react with 3-AP might be cytoprotective. Indeed, the 3-AP toxicity was fully blocked when the cells were either exposed to 3-AP (75 μM), together with MPG or first to 3-AP for less than 3 h before the addition of MPG (Figure 6A). MPG also fully protected cells exposed to 100 μM 3-AP (Figure 6B). However, if the addition of MPG was delayed for more than 3 h, a time-dependent loss of protection was observed. The MPG-induced protection probably reflects the formation of a non-toxic thioacetal adduct between the carbonyl function of 3-AP and the thiol group of MPG [20].

These results provide further support for the conclusion that the toxicity of 3-AP depends on the simultaneous presence of both the lysosomotropic amine and the aldehyde groups. Furthermore, these observations suggest that 3-AP penetrates into lysosomes slowly, which might provide time during which administration of MPG or similar 3-AP neutralizing agents might be therapeutically effective.

DISCUSSION

After ischaemic or traumatic insult to the CNS, death of 'innocent by-stander' cells adjacent to the areas directly affected, the so-called 'penumbra', can occur and this secondary cell death may constitute most of the total damage. This suggests that the cells that die as a result of direct ischaemic damage may produce one or more cytotoxins, which then cause this death in the penumbra. In this regard, we observed previously that, consequent to ischaemia, there is activation of polyamine oxidases and formation of the amino-aldehyde 3-AP, which is a potent cytotoxin [3]. Polyamine oxidase, being a possibly involved amine oxidase, is selectively inactivated by MDL 72527 [28,29], which at moderate concentration has been shown to provide neuroprotection after traumatic brain injury in rat models [5,30]. Interestingly, at high concentration, MDL 72527 may induce apoptosis by lysosomotropic effects [31]. However, the processes leading to the formation of 3-AP and the mechanisms behind 3-AP cytotoxicity are not known in detail [5]. One obvious possibility involves nucleophilic addition reactions with cellular thiols such as protein thiol groups and GSH. In partial support of this, we did observe a profound decrease in the GSH content of cells exposed to 3-AP. However, this alone does not explain the cytotoxic effects of 3-AP, because a similar decrease in GSH occurs both in NH_4Cl pre-exposed cells (which are protected against 3-AP toxicity) and in cells exposed to the closely related, but far less-toxic aldehyde,

propionaldehyde (which lacks only the lysosomotropic, weak base functionality of the amine group).

An additional potential mechanism of 3-AP toxicity involves the spontaneous formation of acrolein from 3-AP [32]. In at least three different cell lines, micromolar amounts of acrolein have been found to block DNA synthesis over an incubation period of 48 h. However, we calculated (from data in [32]) that $< 3\ \mu$M acrolein would form during our incubations. In additional experiments (results not shown), we found no cytotoxicity when cells were exposed to acrolein for 6 h at concentrations up to 10 μM. In agreement with this, similar low concentrations of acrolein, while inhibiting cell proliferation, have been reported not to cause cell death [33]. Finally, it is unlikely that inclusion of NH_4Cl in the culture medium, shown here to provide almost full protection from 3-AP toxicity, would prevent acrolein-induced cytotoxicity.

The structure of 3-AP and its pK_a value (approx. 9.3) suggest that it should be a lysosomotropic compound that could become highly concentrated within lysosomes [34]. The resulting elevated concentration of a reactive aldehyde within lysosomes might then focus damage on this particular organelle. Indeed, immunocytochemically a high lysosomal concentration of 3-AP-modified proteins was detected. In support of the importance of lysosomal concentration of the 3-AP, pretreatment of cells with NH_4Cl (10 mM), which increases lysosomal pH to approx. 6.3 [25] and, thus greatly diminishes ion trapping of 3-AP [35,36], does protect cells against cell death, both apoptotic and necrotic. At a lysosomal pH of approx. 6.3, the amount of trapped 3-AP would be approx. 100 times less than that at pH 4.5, according to the Henderson–Hasselbach equation ($pH = pK_a + \log$ [base]/[acid]).

Overall, our experimental results suggest a close association between 3-AP-mediated lysosomal destabilization and cell death. In fact, we have suggested previously that release, into the cytosol, of hydrolytic lysosomal enzymes, in moderate amounts, may be an early upstream event in the apoptotic process [12,14]. The released lysosomal enzymes may then directly activate the caspase cascade, attack mitochondria causing release of cytochrome *c* and other apoptosis-inducing factors, activate BH3 interacting domain death agonist or activate still unidentified cytosolic lytic proenzymes that, in turn, contribute to further mitochondrial and lysosomal rupture [8–19]. In the present study, we show that 3-AP does, indeed, engender lysosomal leakage and rupture, although the precise events involved in this process remain unknown. Certainly, one possibility is that aldehydic attack on thiol-containing proteins, such as the lysosomal membrane proton pump [36], is involved. Inactivation of this proton pump and, therefore, of proton translocation is known to result in lysosomal rupture [37].

The observation that compounds such as MPG can effectively prevent 3-AP toxicity may have therapeutic implications. Administration of this, or related, thiol-containing drugs (especially if made lysosomotropic and with a pK_a value allowing them to penetrate cells comparatively rapidly) soon after ischaemic or traumatic injury to the CNS may be effective in preventing the secondary death of adjacent cells, thereby minimizing CNS damage. Because the pK_a value of 3-AP is approx. 9.3, most of it would be protonated already at neutral pH and, consequently, its uptake into cells and lysosomes in significant amounts would be comparatively slow, especially when it occurs at low concentrations [34] [ammonium, having about the same pK_a value as 3-AP but here added in a much higher concentration (10 mM), would build up a significant intra-lysosomal concentration much quicker than 3-AP at 75 μM]. Another explanation for the observed delayed cytotoxicity of 3-AP by ammonium may be that it reacts only slowly with thiol groups. This delay, however, might be an advantage inasmuch as it may allow time to administer therapeutic agents such as MPG, which, by complexation of 3-AP, may prevent death in the penumbra and lessen the extent of ischaemic damage to the CNS.

We thank Dr Karin Öllinger and Dr Uno Johansson for their assistance with GSH measurement. This work was supported by the Swedish Medical Research Council (grant no. 3490 to X.M.Y.) and the Swedish Cancer Foundation (grant no. 4296 to U.T.B.). J.W.E. was the recipient of a Visiting Professorship from the Linköping University Hospital and was also supported by the Commonwealth of Kentucky Research Challenge Trust Fund.

REFERENCES

1 Love, S., Barber, R. and Wilcock, G. K. (1998) Apoptosis and expression of DNA repair proteins in ischemic brain injury in man. Neuroreport **9**, 955–959
2 Robertson, G. S., Crocker, S. J., Nicholson, D. W. and Schulz, J. B. (2000) Neuroprotection by the inhibition of apoptosis. Brain Pathol. **10**, 283–292
3 Ivanova, S., Botchkina, G. I., Al-Abed, Y., Meistrell, III, M., Batliwalla, F., Dubinsky, J. M., Iadecola, C., Wang, H., Gregersen, P. K., Eaton, J. W. et al. (1998) Cerebral ischemia enhances polyamine oxidation: identification of enzymatically formed 3-aminopropanal as an endogenous mediator of neuronal and glial cell death. J. Exp. Med. **188**, 327–340
4 Seiler, N. (1995) Polyamine oxidase, properties and functions. Prog. Brain Res. **106**, 333–344
5 Seiler, N. (2000) Oxidation of polyamines and brain injury. Neurochem. Res. **25**, 471–490
6 Ferrante, A., Rzepczyk, C. M. and Saul, A. J. (1984) Polyamine oxidase-mediated trypanosome killing: the role of hydrogen peroxide and aldehydes. J. Immunol. **133**, 2157–2162
7 Seiler, N., Duranton, B., Gosse, F. and Raul, F. (2000) Spermine cytotoxicity to human colon carcinoma-derived cells (CaCo-2). Cell Biol. Toxicol. **16**, 117–130
8 Brunk, U. T., Dalen, H., Roberg, K. and Hellquist, H. B. (1997) Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. Free Radic. Biol. Med. **23**, 616–626
9 Roberts, L. R., Kurosawa, H., Bronk, S. F., Fesmier, P. J., Agellon, L. B., Leung, W. Y., Mao, F. and Gores, G. J. (1997) Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes. Gastroenterology **113**, 1714–1726
10 Ishisaka, R., Utsumi, T., Kanno, T., Arita, K., Katunuma, N., Akiyama, J. and Utsumi, K. (1999) Participation of a cathepsin L-type protease in the activation of caspase-3. Cell Struct. Funct. **24**, 465–470
11 Roberg, K., Johansson, U. and Ollinger, K. (1999) Lysosomal release of cathepsin D precedes relocation of cytochrome *c* and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. Free Radic. Biol. Med. **27**, 1228–1237
12 Li, W., Yuan, X. M., Nordgren, G., Dalen, H., Dubowchik, G. M., Firestone, R. A. and Brunk, U. T. (2000) Induction of cell death by the lysosomotropic detergent MSDH. FEBS Lett. **470**, 35–39
13 Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H. and Gores, G. J. (2000) Cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c*. J. Clin. Invest. **106**, 1127–1137
14 Brunk, U. T., Neuzil, J. and Eaton, J. W. (2001) Lysosomal involvement in apoptosis. Redox Rep. **6**, 91–97
15 Kagedal, K., Zhao, M., Svensson, I. and Brunk, U. T. (2001) Sphingosine induces apoptosis through lysosomal leakage of proteases. Biochem. J. **359**, 335–343
16 Leist, M. and Jaattela, M. (2001) Triggering apoptosis by cathepsins. Cell Death Differ. **8**, 324–326
17 Zang, Y., Beard, R. L., Chandraratna, R. A. and Kang, J. X. (2001) Evidence of a lysosomal pathway for apoptosis induced by the synthetic retinoid CD437 in human leukemia HL-60 cells. Cell Death Differ. **8**, 477–485
18 Stoka, V., Turk, B., Schendel, S. L., Kim, T. H., Cirman, T., Snipas, S. J., Ellerby, L. M., Bredesen, D., Freeze, H., Abrahamson, M. et al. (2001) Lysosomal protease pathways to apoptosis: cleavage of bid, not pro-caspases, is the most likely route. J. Biol. Chem. **276**, 3149–3157
19 Tsuchiya, K., Kohda, Y., Yoshida, M., Zhao, L., Ueno, T., Yamashita, J., Yoshioka, T., Kominami, E. and Yamashima, T. (1999) Postictal blockade of ischemic hippocampal neuronal death in primates using selective cathepsin inhibitors. Exp. Neurol. **155**, 187–194
20 Ivanova, S., Batliwalla, F., Mocco, J., Kiss, S., Huang, J., Mack, W., Coon, A., Eaton, J. W., Al-Abed, Y., Gregersen, P. K. et al. (2002) Neuroprotection in cerebral ischemia by neutralization of 3-aminopropanal. Proc. Natl. Acad. Sci. U.S.A. **16**, 5579–5584

21 Li, W., Dalen, H., Eaton, J. W. and Yuan, X. M. (2001) Apoptotic death of inflammatory cells in human atheroma. Arterioscler. Thromb. Vasc. Biol. **21**, 1124–1130
22 Honegger, C. G., Langemann, H., Krenger, W. and Kempf, A. (1989) Liquid chromatographic determination of common water-soluble antioxidants in biological samples. J. Chromatogr. **487**, 463–468
23 Thioudellet, C., Oster, T., Leroy, P., Nicolas, A. and Wellman, M. (1995) Influence of sample preparation on cellular glutathione recovery from adherent cells in culture. Cell Biol. Toxicol. **11**, 103–111
24 Seglen, P. O. (1983) Inhibitors of lysosomal function. Methods Enzymol. **96**, 737–764
25 Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. **75**, 3327–3331
26 Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G. and Johnson, Jr, E. M. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol. **106**, 829–844
27 Higuchi, Y. and Matsukawa, S. (1998) Active oxygen-mediated chromosomal 1–2 Mbp giant DNA fragmentation into internucleosomal DNA fragmentation in apoptosis of glioma cells induced by glutamate. Free Radic. Biol. Med. **24**, 418–426
28 Bolkenius, F. N., Bey, P. and Seiler, N. (1985) Specific inhibition of polyamine oxidase *in vivo* is a method for the elucidation of its physiological role. Biochim. Biophys. Acta **838**, 69–76
29 Seiler, N. and Bolkenius, F. N. (1985) Polyamine reutilization and turnover in brain. Neurochem. Res. **10**, 529–544
30 Dogan, A., Rao, A. M., Baskaya, M. K., Hatcher, J., Temiz, C., Rao, V. L. and Dempsey, R. J. (1999) Contribution of polyamine oxidase to brain injury after trauma. J. Neurosurg. **90**, 1078–1082
31 Dai, H., Kramer, D. L., Yang, C., Murti, K. G., Porter, C. W. and Cleveland, J. L. (1999) The polyamine oxidase inhibitor MDL-72,527 selectively induces apoptosis of transformed hematopoietic cells through lysosomotropic effects. Cancer Res. **59**, 4944–4954
32 Sharmin, S., Sakata, K., Kashiwagi, K., Ueda, S., Iwasaki, S., Shirahata, A. and Igarashi, K. (2001) Polyamine cytotoxicity in the presence of bovine serum amine oxidase. Biochem. Biophys. Res. Commun. **282**, 228–235
33 Kehrer, J. P. and Biswal, S. S. (2000) The molecular effects of acrolein. Toxicol. Sci. **57**, 6–15
34 de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1974) Lysosomotropic agents. Biochem. Pharmacol. **23**, 2495–2531
35 Feng, Y. and Forgac, M. (1992) A novel mechanism for regulation of vacuolar acidification. J. Biol. Chem. **267**, 19769–19772
36 Forgac, M. (1989) Structure and function of vacuolar class of ATP-driven proton pumps. Physiol. Rev. **69**, 765–796
37 Yao, J. and Zhang, G. J. (1996) Loss of lysosomal integrity caused by the decrease of proton translocation in methylene blue-mediated photosensitization. Biochim. Biophys. Acta **1284**, 35–40

Received 30 September 2002/29 November 2002; accepted 6 January 2003
Published as BJ Immediate Publication 6 January 2003, DOI 10.1042/BJ20021520