Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 1695–1705
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Induction of apoptosis without redox catastrophe by thioredoxin-inhibitory compounds

Monica Pallis[a,*], Tracey D. Bradshaw[b], Andrew D. Westwell[b], Martin Grundy[a], Malcolm F.G. Stevens[b], Nigel Russell[a]

[a]*Division of Haematology, Nottingham City Hospital, University of Nottingham, Nottingham NG5 1PB, UK*
[b]*School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG5 1PB, UK*

Received 22 April 2003; accepted 11 June 2003

Abstract

The dithiol-reducing thioredoxin/thioredoxin reductase system normally maintains the reduced state of key enzymes responsible for the cell's anti-oxidant defences. We therefore addressed the question of whether AW 464—a novel thioredoxin inhibitor—as well as broad spectrum dithiol ligands diamide and phenylarsine oxide are able to induce and execute a regular apoptotic sequence of events without overwhelming the cell's ability to detoxify reactive oxygen species. All three agents were found to target the thioredoxin system in a cell-free assay. In HL-60 leukaemia cells, they were also found to induce Bak activation, cytochrome *c* release from mitochondria, $\downarrow\Delta\Psi$m, chromatin condensation, phosphatidyl serine exposure and Tdt-sensitive DNA nicks. At the onset of apoptosis there was no evidence of increases in oxygen free radicals or peroxide in cells treated with AW 464 or diamide. Phenylarsine oxide induced both free radicals and hydrogen peroxide, but this did not appear to interfere with apoptosis. We conclude that pharmacological targeting of thioredoxin can induce a well-orchestrated apoptotic programme.

Keywords: Thioredoxin; AW 464; Diamide; Phenylarsine oxide; Apoptosis

1. Introduction

The first cytotoxic drugs to have their chemical effects on specific molecular receptors described were the arsenicals, and the receptor groups in question were thiols [1]. The search to find the crucial molecular targets of thiol-binding compounds has been going on since the 1920s, and one of the many controversies which has arisen over intervening decades has been over whether particular toxic agents target monothiols or dithiols [1].

In 1997, Marchetti *et al.* described a model using the dithiol oxidant diamide to induce apoptosis in a manner which was dependent on mitochondrial permeability transition [2]. Permeability transition has a key role in the control of apoptotic responses mediated by chemotherapeutic drugs [3]. Monovalent thiol-binding compounds were found to inhibit both permeability transition and apoptosis [2,4]. Moreover, diamide can overcome the apoptosis-inhibitory effect of bcl-2 over-expression [5].

Since protein dithiol oxidation can induce permeability transition and apoptosis, the cell must maintain defences against this oxidation. TX is likely to be a major component of these defences. TX is a ubiquitous 12 kDa cysteine rich molecule [6–8]. It is the cell's principal disulphide reductase with a redox potential of −270 mV [9]. It is normally maintained in a reduced state through the oxidation of NADPH *via* the action of the enzyme thioredoxin reductase [8]. Its many molecular targets include molecules directly engaged in countering oxidative stress, such as peroxiredoxins and glutathione peroxidase [8]. TX over-expression is also associated with resistance to

* Corresponding author. Tel.: +44-115-8404-722; fax: +44-115-8404-721.
E-mail address: monica.pallis@nottingham.ac.uk (M. Pallis).

Abbreviations: 7-AAD, 7-aminoactinomycin D; araC, cytosine arabinoside; ASK1, apoptosis signal-regulating kinase; DHR123, dihydrorhodamine 123; diamide, diazene dicarboxylic acid bis *N*,*N*-dimethylamide; $\downarrow\Delta\Psi$m, mitochondrial membrane depolarisation; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; HE, hydroethidine (dihydroethidium); PAO, phenylarsine oxide; ROS, reactive oxygen species; Tdt, terminal deoxynucleotidyl transferase; TX, thioredoxin.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00471-4

superoxide-generating drugs including doxorubicin and etoposide in prostatic and bladder carcinoma [10]. Moreover, transfection of TX cDNA into WEHI7.2 cancer cells inhibits apoptosis [11] and secreted thioredoxin plays a role as an autocrine growth factor in tumours [12].

Pharmaceutical agents that reduce the cell's ability to deal effectively with oxidative stress may be useful tools for cancer chemotherapy, either as single agents or as chemosensitisers. However, in addition to mediating damage done to cancer cells by drugs and radiation, ROS are natural by-products of redox reactions and electron flow in mitochondria with the potential to cause critical damage both to mitochondria and to other cell components [8,13]. Thus, a potential problem with using anti-oxidant inhibitors as therapeutic agents is that they may interrupt the ordered pattern of apoptosis by allowing oxidative damage to the mitochondria so that the latter can no longer synthesise ATP [14,15]. This in turn would compromise the cell's ability to maintain the integrity of its membranes and to execute downstream apoptotic pathways.

The principal questions addressed by this study were therefore the following: firstly, do pharmacological thioredoxin inhibitors induce a regular apoptotic programme? Secondly, is the apoptotic programme interrupted by redox catastrophe? There is evidence that thioredoxin inhibition might help rather than hinder the apoptotic process. In the resting state TX binds to, and inactivates the signalling intermediary ASK1 [16]. Once TX has become oxidized, it releases ASK1, leading to apoptosis [16]. This indicates that TX might function as a physiological rheostat for the dithiol/disulphide status of cellular proteins, promoting survival or apoptosis in an ordered manner in accordance with its own redox state.

We have made a detailed study of the effects of thioredoxin inhibitors on the mechanism of cell death in leukaemia cells. Thioredoxin was inhibited using the novel heteroaromatic quinol AW 464 [17,18] as well as diamide and phenylarsine oxide. The chemical structures of AW 464, diamide and PAO are shown in Fig. 1. AW 464 acts as an inhibitor of thioredoxin redox cycling presumably through the binding of active site thiol groups in the reduced form of thioredoxin to the electrophilic (thiol-reactive) positions on AW 464 to form an irreversible complex (Fig. 2). PAO and diamide both oxidize thioredoxin and other dithiols [9,19–21].

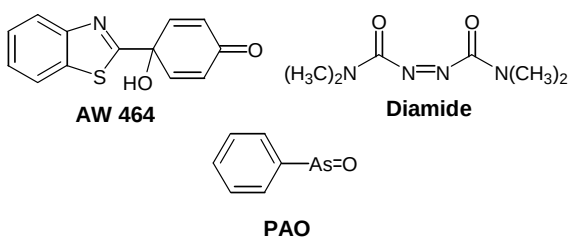


Fig. 1. Chemical structures of AW 464, diamide and PAO.

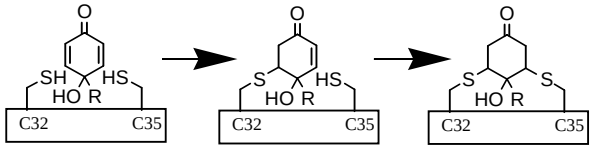


Fig. 2. Proposed cross-linking of quinols in active site of thioredoxin. Thiol groups in the active site of thioredoxin (cysteine residues 32 and 35) are indicated. Addition of the first thiol (nucleophilic) group to the electrophilic (β-carbon) site of AW 464 is thought to be a reversible step; subsequent addition of the second thiol group is however assumed to be irreversible and leads to effective irreversible inhibition of thioredoxin redox cycling.

2. Materials and methods

2.1. Materials

AW 464 was synthesised *via* the addition of 2-lithiobenzothiazole to a protected quinone ketal followed by deprotection [18]. 100 mM stock solutions were prepared in DMSO and aliquots were stored at −20°. Recombinant human thioredoxin and recombinant rat thioredoxin reductase were from IMCO. Diamide, cycloheximide, cytosine arabinoside, insulin (from bovine pancreas), etoposide, the Annexin V kit and 7-AAD were from Sigma. Diamide was stored as a 200 mM stock solution in water at −20°. 7-AAD was stored at 50 μg/mL in PBS at 4°. PAO was from Aldrich and was stored in 10 mM aliquots in DMSO at 4°. HE, DHR123 and $DiOC_6$ were from Molecular Probes. DHR123 and HE were both diluted to 5 mM in DMSO under nitrogen and stored at −20°. 1 mM $DiOC_6$ stock solution in ethanol was stored at room temperature. A 400 nM working solution in PBS was made weekly and stored at 4°. The cell-permeable caspase inhibitor z-VAD-fmk was from Calbiochem: it was prepared as a 10 mM stock solution in DMSO and stored at −20°. The direct method Apoptag TUNEL kit was from Intergen. Anti-cytochrome *c* antibody (Clone 6H2B4) was from BD Pharmingen. Ab-1 anti-Bak antibody was from Oncogene. Isotype control and FITC-conjugated rabbit anti-mouse $f(ab')_2$ antibodies were from Dako. DAPI-containing mounting medium was from Vector Laboratories.

2.2. Assay for the inhibition of the thioredoxin system by AW 464

Thioredoxin/thioredoxin reductase activity was measured using the insulin reduction assay [9], with modifications described by Kunkel *et al.* [22]. Briefly, a range of concentrations of AW 464, PAO and diamide were incubated for 30 min at 37° in HEPES/EDTA buffer containing 1 mM NADPH, 0.16 U/mL thioredoxin reductase, 0.8 μM TX and 2.5 mg/mL insulin. The reaction was stopped by addition of buffer consisting of 6 M guanidine–HCl, 50 mM tris (pH 8) and 10 mM dithionitrobenzoic acid.

The reduction of the latter compound, by transfer of reducing equivalents from NADPH, is measured as an increase in absorbance at 405 nm.

2.3. Cells

The AML cell line HL-60 was maintained in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, and was used in log phase. Cells used in experiments were pelleted and resuspended in fresh medium at 2.5×10^5/mL for each assay. All drug dilutions from stock solutions were made in culture medium, such that the final DMSO concentration never exceeded 0.05%.

2.4. Flow cytometry

Flow cytometry was performed with a FACSCalibur (Becton Dickinson). Fluorescence was stimulated with a 488 nm argon laser. Data was collected using logarithmic amplification. Median fluorescence was measured in relative fluorescence units (RFU) generated by the Instrument's Cellquest software.

2.5. Assays for apoptosis

Viability of cultured cells was initially assessed using flow cytometric 7-AAD/forward scatter measurements. Annexin V was measured with a kit (Sigma) according to manufacturers' instructions.

2.6. Assay for the growth phase specificity of apoptosis

DNA strand breaks in apoptotic cells were measured by the direct (fluorescein-conjugated nucleotide) TUNEL staining method using a kit (Intergen) according to manufacturers' instructions. Cells were counter-stained with propidium iodide to determine DNA content prior to analysis by flow cytometry using a doublet discriminator.

2.7. Measurement of ROS accumulation

All incubations took place at 37° in a CO_2 incubator in sterile FACS tubes. For assay of superoxide, treated and untreated cells were labelled with 2 μM HE for the final 30 min of an 8-hr incubation [23,24]. Flow cytometry was then performed immediately. Ethidium fluorescence was collected in FL3. For hydrogen peroxide assays at timepoints over several hours, treated cells were labelled with 2 μM DHR123 in FACS tubes for the final 10 min of each incubation [25]. R123 fluorescence was collected in FL1.

2.8. Measurement of mitochondrial membrane depolarisation

All incubations took place at 37° in a CO_2 incubator. Mitochondrial membrane potential was measured using $DiOC_6$ at a low concentration (40 nM) for the final 30–60 min of incubations. In a previous study, we demonstrated that under these experimental conditions, $DiOC_6$ fluorescence is independent of KCl (used to induce plasma membrane depolarisation) but dependent on carbonyl cyanide *m*-chlorophenylhydrazone (used to induce mitochondrial membrane depolarisation) [4].

2.9. Measurement of Bak

Based on the assay developed by Griffiths *et al.* [26], treated and untreated cells were fixed in 0.25% paraformaldehyde in PBS for 5 min, rinsed twice in PBS and stored overnight in PBS. The following day they were aliquotted into FACS tubes, resuspended in 0.05% digitonin solution in PBS and incubated with 0.4 μg/mL anti-Bak or isotype-and-concentration-matched control antibodies for 30 min at room temperature. Following three rinses, cells were incubated for a further 30 min with FITC-conjugated rabbit anti-mouse antibodies and then rinsed three times prior to flow cytometric analysis.

2.10. Measurement of cytochrome c release by fluorescence microscopy

Cytospins of treated and untreated cells were air-dried overnight, fixed in 4% paraformaldehyde, rinsed and incubated with a 1:250 dilution of anti-cytochrome *c* antibody in permeabilisation buffer (PBS, 0.1% saponin and 1% albumin) for 1 hr at room temperature. Following rinsing, the slides were incubated for a further hour in a 1:30 dilution of FITC-conjugated rabbit anti-mouse antibodies in permeabilisation buffer. After the final rinses, the cells were mounted in DAPI-containing mounting medium. Images were captured using Photometrics camera and software connected to an Olympus fluorescent microscope (all supplied by VYSIS).

3. Results

3.1. Inhibition of thioredoxin by AW 464

When examined in the NCI Developmental Therapeutics *in vitro* screen (60 human cancer cell lines), the heteroaromatic quinol AW 464 (NSC706704) displayed a highly unusual pattern of selectivity. It was COMPARE negative with standard clinical agents [18]. Some of the compounds in the NCI database with the greatest similarity to AW 464 were found to be known inhibitors of the thioredoxin signalling pathway [18]. Furthermore, thioredoxin reductase was the only gene upregulated by a 24 hr exposure to 1 μM AW 464 in a 10,000-gene–DNA array assay [27]. We confirmed by *in vitro* testing, using the insulin reduction assay (the standard assay for activity of the thioredoxin system), developed by Holmgren [9] that

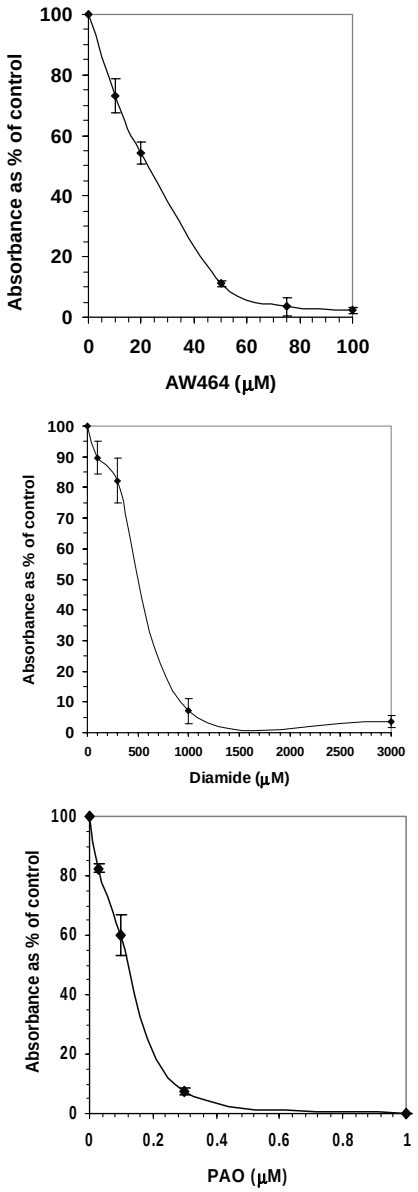


Fig. 3. Insulin reduction assay. Reduction of insulin by thioredoxin/thioredoxin reductase signalling is calculated from dithionitrobenzoic acid absorbance at 405 nm (mean of four experiments, with error bars indicating standard deviations). Baseline absorbance is determined from negative controls lacking either insulin or thioredoxin/thioredoxin reductase.

AW 464 inhibits thioredoxin with an IC_{50} of 23 μM (Fig. 3). In the same assay, the IC_{50} of PAO was 100 nM, and for diamide it was 500 μM (Fig. 3).

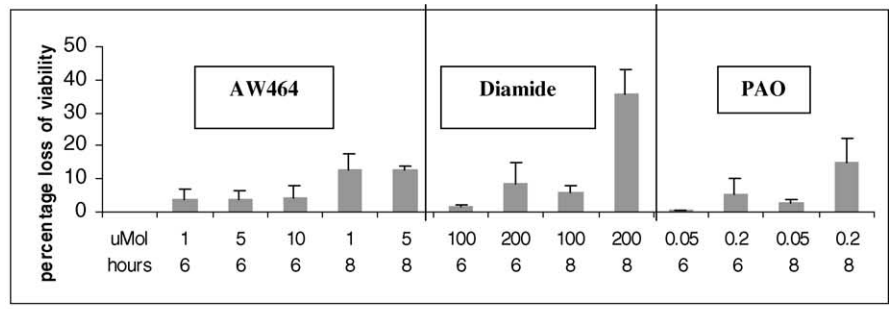
3.2. Delayed apoptosis in AW 464-treated cells

To study cell death induced by thioredoxin-inhibitory agents, we treated HL-60 leukaemia cells with equitoxic doses of AW 464, diamide and PAO. The time at which cell death was first evident was established, using 7-AAD as probe. Approximately 10% loss of viability was evident at 8 hr using 1 μM AW 464, 100 μM diamide or 0.2 μM PAO (Fig. 4A). These doses were strikingly similar to the inhibitory doses for insulin reduction established in the cell-free assay for thioredoxin (Fig. 3). When the cells were examined at 6 hr, no cell death was apparent in the presence of an upto 10-fold increase in AW 464 concentration. However, the effects of PAO and diamide were evident at 6 hr and were dose-dependent, suggesting different mechanisms of activation (Fig. 4A). This lag phase before the induction of death by AW 464 suggested that its toxicity might depend on, or be enhanced by, metabolic transformation and/or the synthesis of new proteins. HL-60 cells were therefore cultured for 8 hr with AW 464 in the presence and absence of the protein synthesis inhibitor cycloheximide. Using Annexin V, we showed firstly that phosphatidyl serine exposure was induced by treatment with all three agents (Fig. 4B) and secondly that this decreased in the presence of cycloheximide (Fig. 4C). The greatest inhibitory effect—80%, SEM 7%—was noted in cells treated with AW 464. However, apoptosis also decreased by 63% (SEM 2.6%) in PAO-treated cells but by only 32% (SEM 12%) in diamide-treated cells.

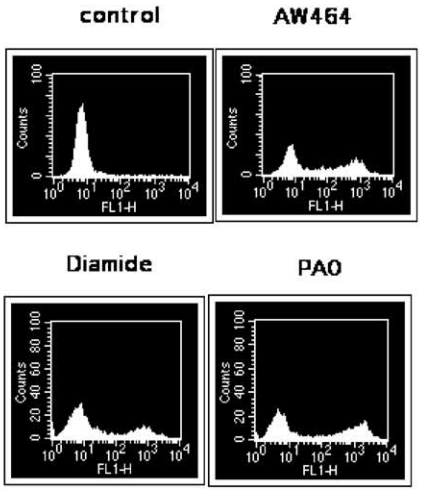
3.3. The mode of death is caspase-dependent and does not depend on overwhelming the cellular anti-oxidant responses

Since all three agents used in our study induce direct or indirect oxidation of molecules involved in cellular responses to oxidative stress [1,8,20], it is possible that classical caspase-mediated pathways of apoptosis are disrupted. As a measure of whether oxidative stress induced by any of our agents would overwhelm the caspase-mediated apoptotic programme, we used the pan-caspase inhibitor z-VAD-fmk with the probe Annexin V. This allowed us to determine whether apoptosis was caspase-dependent. At 8 hr post-treatment, z-VAD inhibited apoptosis induced by AW 464, diamide and PAO by 93.6% (SEM 2.7%), 93.4% (SEM 2.5%) and 92% (SEM 4.4%), respectively, i.e. the inhibition was almost total (Fig. 5A).

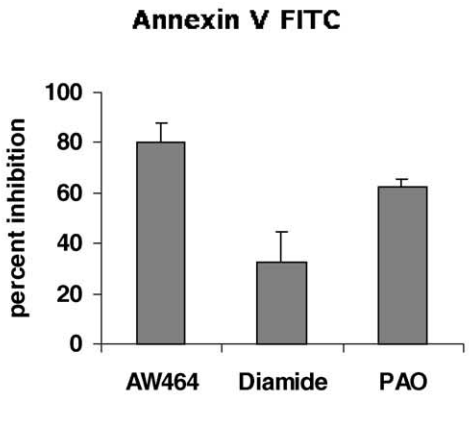
We also reasoned that if cell death occurs as an ordered response to the agents, $\downarrow\Delta\Psi m$ would be noted in the absence of increases in reactive oxygen species, as noted in other systems by Zamzami *et al.* [24]. If, however, thioredoxin inhibition or dithiol oxidants reduce survival by overwhelming anti-oxidant responses, an increase in ROS might precede $\downarrow\Delta\Psi m$. As a probe, we used the compound hydroethidine, which is converted to fluorescent ethidium in the presence of superoxide [23]. When excited at 488 nm, the fluorescence of ethidium can be distinguished from that of the mitochondrial membrane potential-sensitive probe $DiOC_6$ by flow cytometry. As shown in Fig. 5B, subsets of cells treated with AW 464, diamide and PAO had lost mitochondrial membrane potential at 8 hr. Only PAO induced a substantial rise in ethidium



(A)



(B)



(C)

Fig. 4. (A) Effects of dose and time on toxicity. HL60 cells were cultured with various doses of AW 464, diamide and PAO, for 6 and 8 hr, as indicated. Loss of viability was assessed using flow cytometric analysis of 7-AAD uptake. (B) AW 464, diamide and PAO induce Annexin V binding. Cells were cultured with 1 μM AW 464, 100 μM diamide or 0.1 μM PAO for 8 hr, prior to analysis of Annexin V binding. Flow cytometric histograms show Annexin V-FITC fluorescence. Results are representative of at least three experiments. (C) Inhibition of Annexin V binding by cycloheximide. 1 μg/mL cycloheximide was added to HL60 hours 30 min before addition of 1 μM AW 464, 100 μM diamide or 0.1 μM PAO and the cells were cultured for a further 8 hr. The chart shows the percentage of specific inhibition of Annexin V binding (mean + SD of three experiments). As cycloheximide alone induces caspase activation and apoptosis [40], specific inhibition was calculated after subtraction of control cell values. Control cell apoptosis in the presence of cycloheximide was 5.7% (SEM 1.1) at 8 hr.

fluorescence at this time point. The presence of some cells in the lower left section of the PAO dotplots, coupled with the absence of events in the upper right section, indicate that mitochondria of cells treated with this agent started to depolarise before ROS began to accumulate, or at the same time, rather than afterwards.

Unexpectedly, the data illustrated in Fig. 5B revealed that a subset of cells cultured with AW 464, diamide and PAO had lower ethidium fluorescence than control cells (cells in the lower right sections of the dotplots). As this phenomenon has not, to our knowledge, previously been described, we sought to discover whether it was particular to our group of agents or a general feature of the response to cytotoxic drugs. HL-60 cells were cultured with cytosine arabinoside or with etoposide. A subset of cells with low superoxide was found following

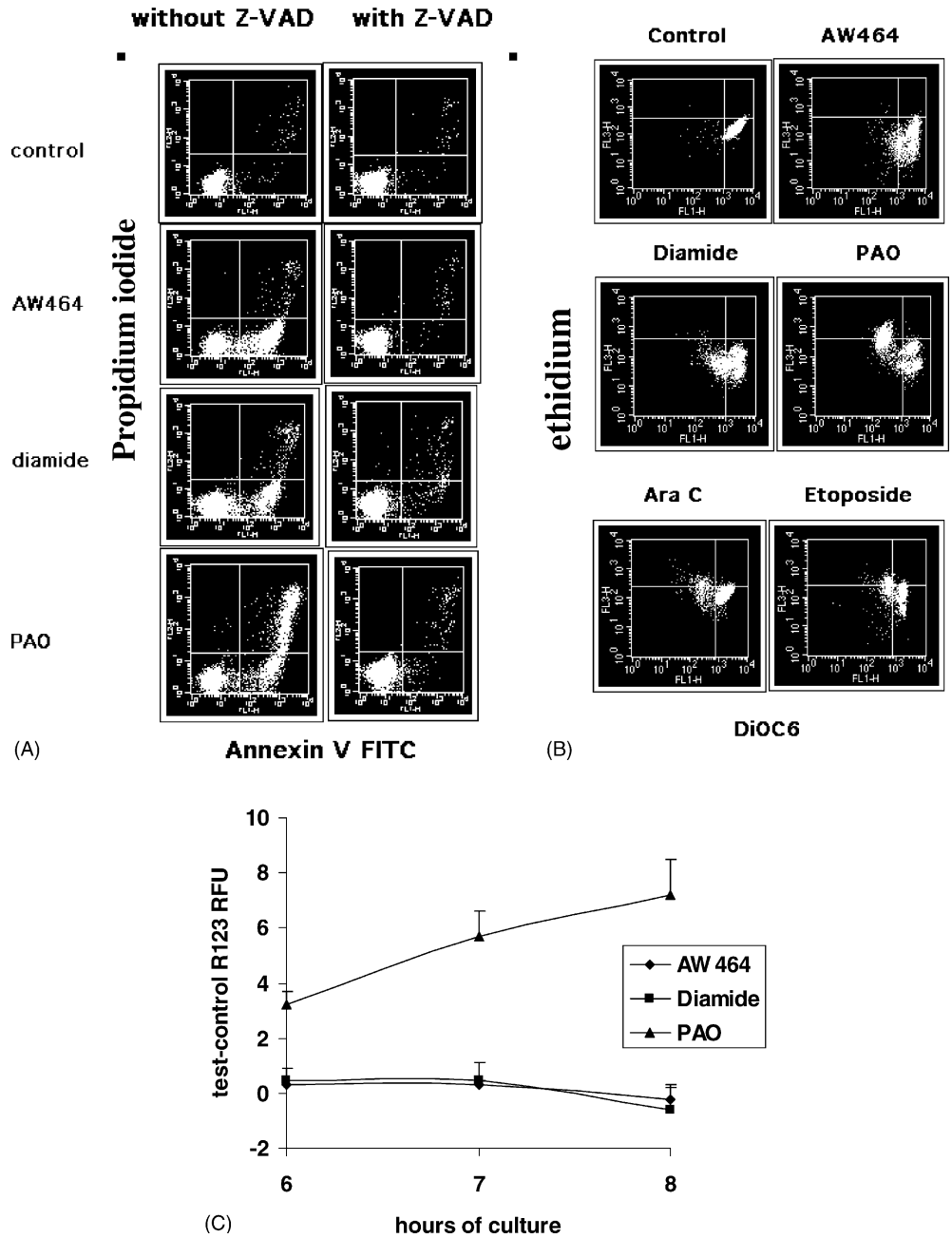


Fig. 5. (A) Blocking of apoptosis by z-VAD-fmk. 50 μM z-VAD was added to HL60 cells 30 min before addition of 1 μM AW 464, 100 μM diamide or 0.1 μM PAO and the cells were cultured for a further 8 hr, before harvesting for Annexin V assay. The flow cytometric dotplots illustrate the >90% loss of Annexin V fluorescence in the lower right quadrants after culture of cells with z-VAD-fmk. (B) Relationship between loss of mitochondrial membrane depolarisation and free radical accumulation. HL60 cells were cultured for 8 hr with 1 μM AW 464, 100 μM diamide or 0.1 μM PAO. In additional experiments cells were cultured for 6 hr with 5 μM araC or 10 μM etoposide. 40 nM $DiOC_6$ and 2 μM HE were added for the final 30 min of the incubation and flow cytometry was then performed immediately. It was important to establish the temporal order of reactivity with the two probes, and since there was considerable spectral overlap between these probes, a tube of cells probed with either HE alone or $DiOC_6$ alone was used to optimise the compensation settings on the flow cytometer for each condition. (Representative of three independent experiments using AW 464, diamide and PAO and two additional experiments using araC and etoposide). (C) Delayed oxidation of dihydrorhodamine 123 by PAO, but not AW 464 or diamide. Treated and untreated cells were incubated for 6, 7, or 8 hr. Separate aliquots were used for each timepoint, and 2 μM DHR123 was added for the final 10 min of each incubation. The fluorescence of untreated cells at each timepoint was subtracted from treated cell fluorescence.

treatment with etoposide, but not with cytosine arabinoside (Fig. 5B).

We also examined the oxidation of dihydrorhodamine 123, a measure of hydrogen peroxide accumulation. Diamide induces a transient elevation of R123 in the first 2 hr, which probably reflects transient glutathione depletion (data not shown, as reported by others [28]). At 6–8 hr, PAO, but not AW 464 or diamide, induces DHR123 oxidation (Fig. 5C).

3.4. Inhibition of apoptosis by z-VAD occurs upstream of mitochondrial membrane depolarisation, but downstream of Bak activation

Zamzami *et al.* [5] have described the apoptosis induced by diamide as dependent on mitochondrial membrane depolarisation in a T cell hybridoma line. In their system, loss of $DiOC_6$ fluorescence characteristic of depolarisation is independent of caspase inhibition by the pan-caspase inhibitor z-VAD-fmk. We measured mitochondrial membrane depolarisation using $DiOC_6$ in the presence and absence of 50 μM z-VAD-fmk, i.e. under the conditions which we had shown to induce and inhibit apoptosis in Fig. 5A. In our system, z-VAD-fmk inhibited ↓ΔΨm in the majority of AW 464- and PAO-treated cells, suggesting involvement of an initiator caspase upstream of ↓ΔΨm (Fig. 6). Diamide-mediated ↓ΔΨm was considerably less affected by z-VAD (Fig. 6).

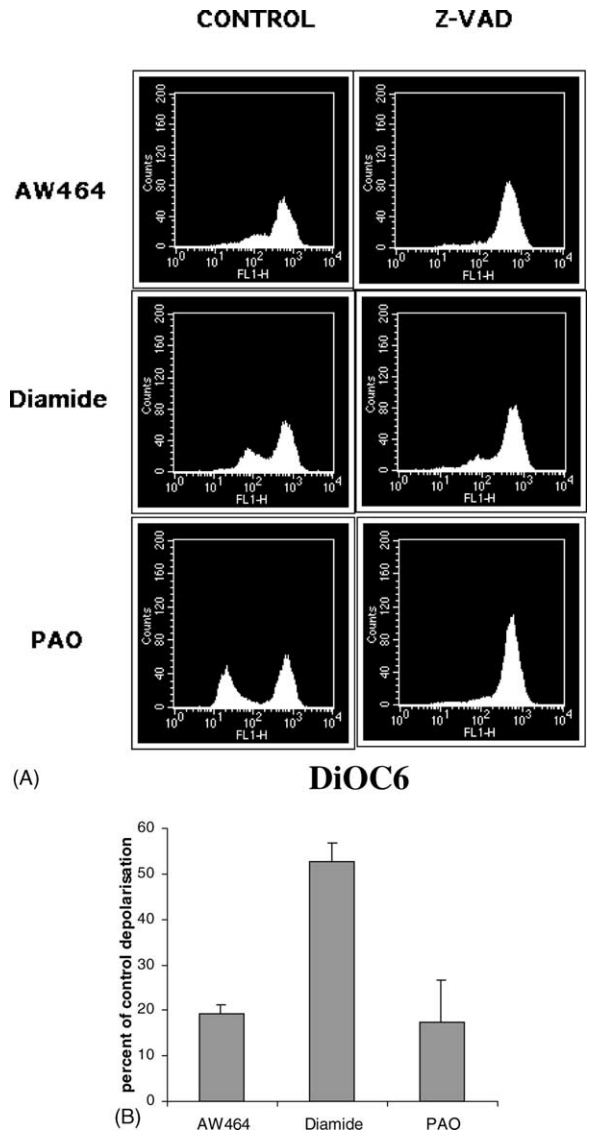


Fig. 6. Inhibition of mitochondrial membrane depolarisation by caspase inhibitors. z-VAD-fmk was added to HL60 cells 30 min before addition of 1 μM AW 464, 100 μM diamide or 0.1 μM PAO and the cells were cultured for a further 8 hr. $DiOC_6$ was added for the final hour. (A) The flow cytometric histograms illustrate mitochondrial membrane potential after preculture with and without 50 μM z-VAD-fmk. (B) Chart summarising mean + SD of three experiments.

Descriptions of apoptosis have been published, in which a conformational change in Bak, permeabilisation of the outer mitochondrial membrane and cytochrome *c* release occur upstream of caspase activation and independently of ↓ΔΨm [26,29,30]. We tested whether either Bak activation or cytochrome *c* release precedes the caspase activation induced by our agents. Bak activation was measured using the flow cytometric method described by Griffiths *et al.*, which makes use of an antibody raised against an epitope of Bak which is cryptic in the resting state, but which becomes accessible at the onset of apoptosis [26]. For cytochrome *c* release, treated cells were double labelled with anti-cytochrome *c* antibody and with DAPI to detect chromatin, and analysed by fluorescence microscopy. z-VAD was found not to have an inhibitory effect on either Bak activation or cytochrome *c* release in cells treated with AW 464 and PAO. However, chromatin condensation was considerably impaired (Fig. 7). These results indicated that caspase inhibition delays or prevents apoptosis at a stage downstream of cytochrome *c* release but upstream of chromatin condensation. Diamide, which was the least sensitive of the agents to z-VAD inhibition of ↓ΔΨm, was comparatively insensitive to z-VAD-mediated inhibition of cytochrome *c* release also.

3.5. AW 464, PAO and diamide induce apoptosis in cycling and non-cycling cells

Cytotoxic drugs can trigger cell cycle checkpoint-dependent and/or stress-dependent death pathways. The TUNEL assay, which can be used to measure apoptotic DNA strand breaks and cellular DNA content simultaneously, enabled us to determine whether the death induced by dithiol-modulating agents occurred at a specific phase of the cell cycle, and whether this death involved DNA strand breaks characteristic of apoptosis. As illustrated in Fig. 8, DNA strand breaks were measurable after treatment with each agent. Furthermore, all the agents killed both resting and cycling cells. At 8 hr, there was a tendency for both AW 464 and diamide to be killing more resting than cycling cells, but the proportion of cycling cells killed by both agents increased by 24 hr. PAO was killing both resting and cycling cells at 8 hr. At 24 hr PAO appeared to be selectively toxic to resting cells, but the low propidium iodide staining could also be due to the rapid degradation of DNA in damaged cells.

4. Discussion

Cells are generally well protected from oxidative stress through an array of anti-oxidant systems including the thioredoxin, glutathione, peroxiredoxin, superoxide

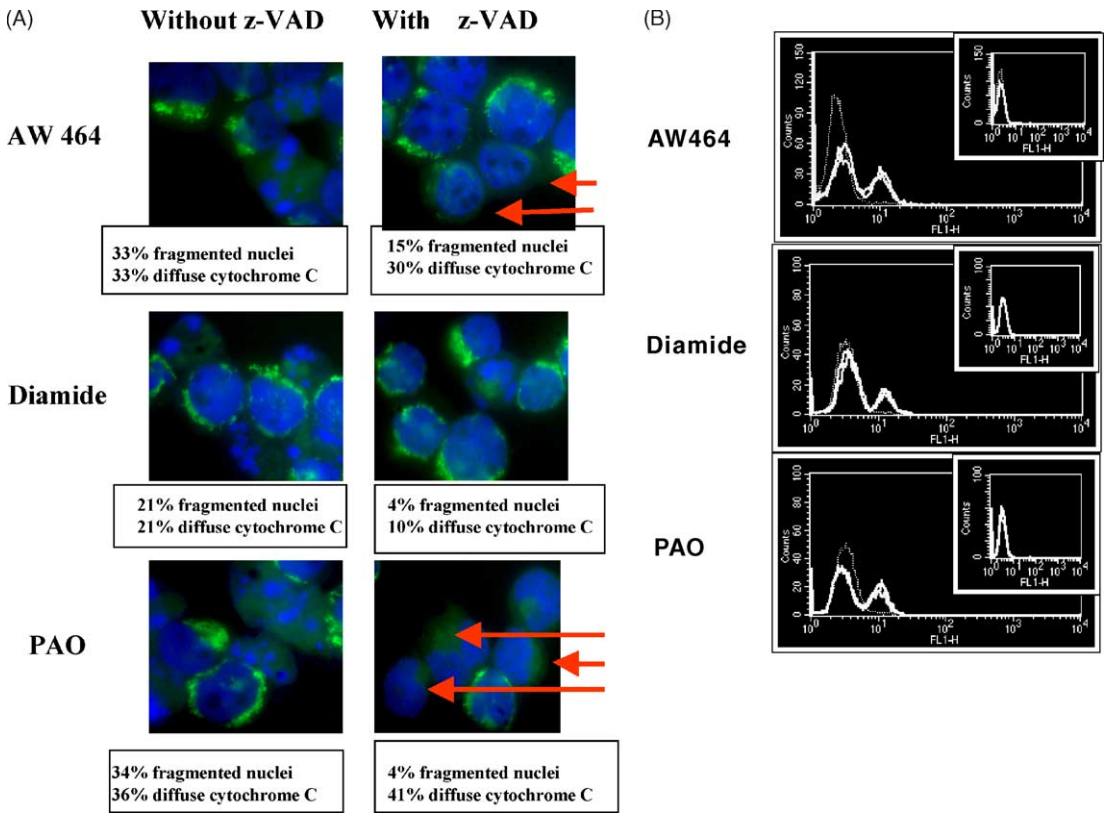


Fig. 7. Effect of z-VAD-fmk on cytochrome *c* release and Bak activation. z-VAD-fmk was added to HL60 cells 30 min before addition of 1 μM AW 464, 100 μM diamide or 0.1 μM PAO and the cells were cultured for a further 8 hr. (A) Fluorescence micrographs of cells double labelled with anti-cytochrome *c* antibody and with DAPI to detect chromatin. Treatment with z-VAD led to a decrease in the number of cells with fragmented nuclei, but in AW 464-treated and PAO-treated cells, cytochrome *c* release was unimpaired. In diamide-treated cells both cytochrome *c* release and nuclear fragmentation were inhibited. Mitochondrion-bound cytochrome *c* appears in a bright green punctate pattern. Released cytochrome *c* appears as a diffuse, paler green. Arrows indicate released cytochrome *c* in cells with nuclei of normal appearance. (B) Flow cytometric dotplots of cells stained with Bak1 (main picture) or control IgG2a (inset) antibody. Dotted lines = untreated cells. White lines = treated cells without z-VAD pre-incubation. Grey lines = treated cells following z-VAD pre-incubation.

dismutase and catalase systems. On the one hand, disruption of anti-oxidant defences in a cancer cell seems an attractive therapeutic option. On the other hand, pharmacological agents which target elements of the cell's system of redox homeostasis may kill cells by grossly overwhelming anti-oxidant defences and inducing necrosis [14,15]. In this study, we have shown that the thioredoxin-inhibitory compounds AW 464, diamide and PAO induce apoptosis in leukaemia cells. Apoptosis was demonstrated by cytochrome *c* release, changes in Bak conformation and Annexin V binding as well as by chromatin condensation and DNA nick-end labelling. Phosphatidyl serine exposure (recognised by Annexin V) is arguably one of the most crucial components of apoptosis *in vivo*, as it allows dying cells to be recognised and removed by phagocytes before they have a chance to discharge their contents and provoke inflammation.

Neither diamide nor AW 464 induced oxidative stress at the onset of apoptosis (Fig. 5). PAO induced both super-oxide and hydrogen peroxide production. Nevertheless, many features of apoptosis (see above paragraph) were apparent in PAO-treated cells. Thioredoxin is a redox-sensitive molecule which may allow the damaged cell to follow an apoptotic rather than a necrotic pathway. Thior-edoxin plays a direct role in the orchestration of apoptosis through ASK1 release [16] and possibly also through control of NFkB [31]. However, thioredoxin inhibition could alternatively lead to its failure to reduce other important redox-related proteins such as peroxiredoxins and glutathione peroxidase, which could then lead to a build up of ROS in the cells [8]. In our system, ROS had not built up before the onset of apoptosis in cells treated with AW 464 and diamide, although there was evidence for ROS build-up in PAO-treated cells.

One unexpected observation in this study is that, 8 hr post-treatment, a subset of cells treated with AW 464, PAO or diamide appeared to be producing fewer oxygen free radicals than untreated cells, as indicated by a drop in ethidium fluorescence seen in Fig. 5B. A decrease in ROS was also noted with etoposide, although not with araC. Either a drop in the synthesis of ROS or an increase in their dismutation could account for this.

In contrast to the other two agents, PAO induced high levels of ROS (Fig. 5B). However, the flow cytometric double labelling indicated that superoxide production did not precede $\downarrow\Delta\Psi m$. ROS production following $\downarrow\Delta\Psi m$ has also been noted in the apoptosis induced in leukaemia cells by cytosine arabinoside, and our results with the same drug support this finding [32]. On a technical note, DHR123 was

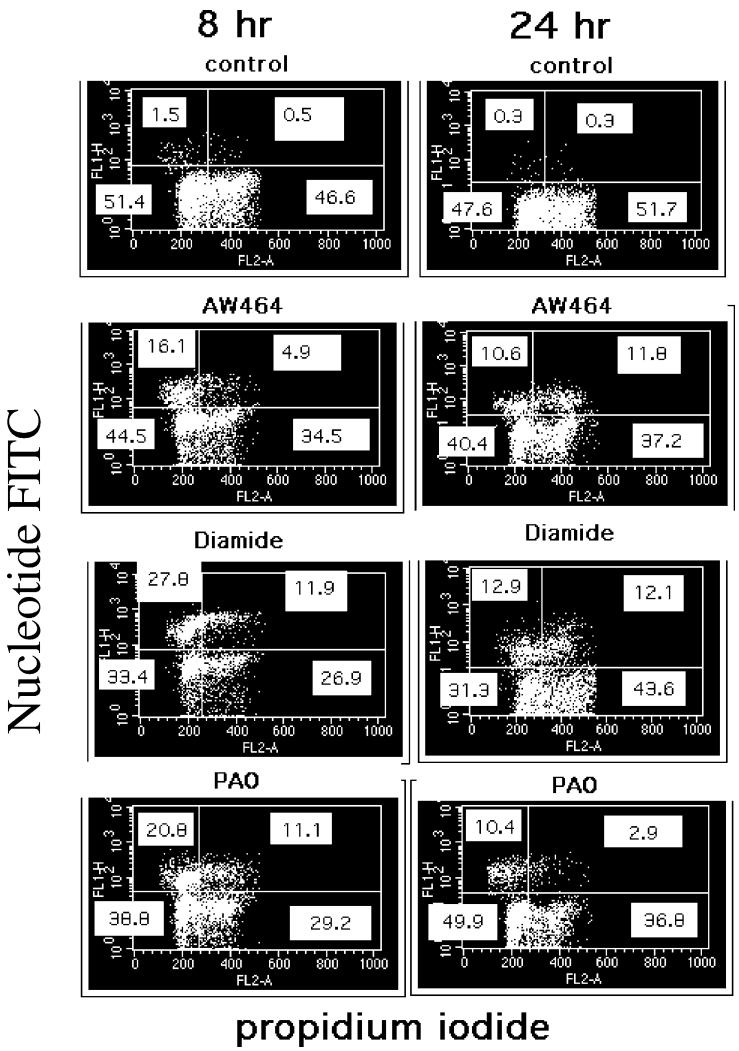


Fig. 8. Determination of free 3′OH DNA termini and DNA content using the TUNEL assay. Cells were cultured with AW 464, diamide or PAO for 8 and 24 hr. The doses used for the 8 hr experiment were 1 μM AW 464, 100 μM diamide and 0.1 μM PAO. These doses were too toxic for a clear picture at 24 hr, therefore 0.75 μM AW 464, 75 μM diamide and 75 nM PAO were used for the latter timepoint. The percentage of cells at each timepoint is indicated. (Representative of at least three experiments).

chosen as probe for peroxide after extensive preliminary studies, during which it was established that retention of the more commonly used dichlorodihydrofluorescein diacetate was enhanced by anion pump inhibitors, and that this probe therefore may interfere with oxidative stress pathways.

In our hands the pan-caspase inhibitor z-VAD-fmk did not inhibit Bak activation or cytochrome *c* release induced by AW 464 or PAO but did inhibit $\downarrow\Delta\Psi$m, placing the former two events upstream of $\downarrow\Delta\Psi$m in the apoptotic process (Fig. 9). A slightly different pattern was given by diamide: z-VAD did not inhibit Bak activation, but did inhibit cytochrome *c* release as well as $\downarrow\Delta\Psi$m. A pattern of apoptosis has been described in which apoptosis-initiating events affecting the mitochondrial transmembrane potential occur before caspase activation, and therefore the pan-caspase inhibitor z-VAD has no effect on $\downarrow\Delta\Psi$m [24]. However, other patterns have been described in which z-VAD does inhibit loss of $\downarrow\Delta\Psi$m [33,34]. Cytochrome *c* release can occur upstream of caspase activation but independently of $\downarrow\Delta\Psi$m [29,35]. A conformational change in the N-terminal portion of Bak, which occurs in response to cell damage and appears to be independent of increases in Bak protein expression, electrophoretic mobility or subcellular localisation, has also been described [26]. Moreover, Bak activation can occur early in apoptosis, before cytochrome *c* is released [30].

The onset of apoptosis is delayed in cells treated with AW 464 as compared to cells treated by diamide and PAO (Fig. 4). Furthermore, the protein synthesis inhibitor cycloheximide greatly inhibited the apoptosis induced by AW 464, but had an intermediate effect on PAO and a minor effect on diamide. The chemistry by which AW 464 is thought to inhibit thioredoxin signalling (Fig. 2) compared to the mechanism of action of the thiol oxidants might underpin these different behaviours. Our data also suggest the action of AW 464 is enhanced by metabolic transformation, and this possibility is currently under investigation in our laboratories. PAO and diamide oxidize a range of

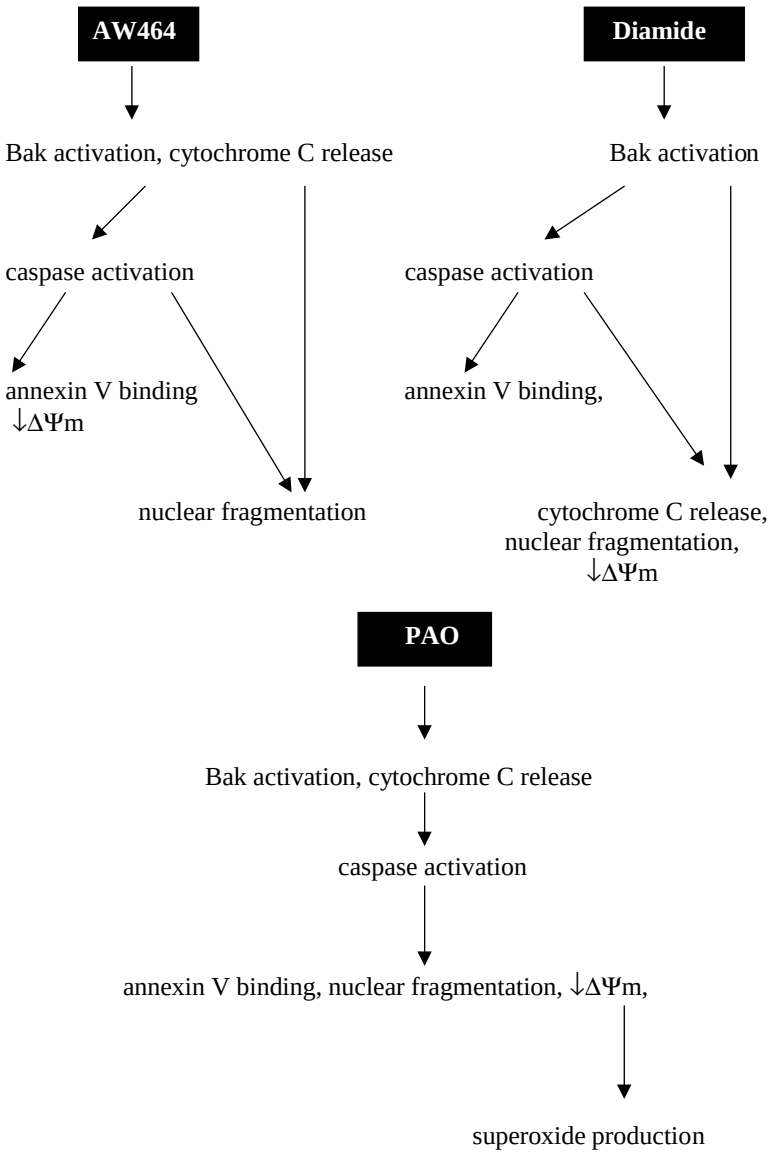


Fig. 9. Models for apoptosis induced by AW 464, PAO and diamide. The order of the apoptotic programme induced by each agent has been derived from the experiments described in this study.

thiols in addition to thioredoxin [1,36], and moreover a direct role for both these compounds in mitochondrial permeability transition has been described [2,37,38]. These additional roles will help account for the minor differences in apoptotic programme induced by the three agents.

We have characterised early (8-hr) apoptosis in cells treated with AW 464, diamide and PAO and shown it to occur largely in quiescent cells (Fig. 8). However, the mode of cell death can change during prolonged cell culture [39], and our TUNEL data indicated changes in cell cycle-stage-specific apoptosis over time. The finding that the proportion of AW 464-induced apoptosis in G_2/M phase cells increased between 8 and 24 hr suggests a delay in the activation of apoptotic pathway(s) based on the G_2/M cell cycle checkpoint.

In conclusion, we have investigated the mode of cell death in leukaemia cells treated with agents which inhibit thioredoxin. The results of our experiments lead us to conclude that thioredoxin inhibitory compounds induce a well-orchestrated apoptotic programme.

Acknowledgments

This work was supported in part by Cancer Research UK and in part by the Nottinghamshire Leukaemia Appeal.

References

[1] Webb J. Enzyme and metabolic inhibitors, vol. III. New York: Academic press, 1966.
[2] Marchetti P, Decaudin D, Macho A, Zamzami N, Hirsch T, Susin SA, Kroemer G. Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. Eur J Immunol 1997;27:289–96.

[3] Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000; 92:1042–53.
[4] Pallis M, Grundy M, Turzanski J, Kofler R, Russell N. Mitochondrial membane sensitivity to depolarisation in acute myeloblastic leukemia is associated with spontaneous *in vitro* apoptosis, wild-type TP53 and vicinal thiol/disulfide status. Blood 2001;98:405–13.
[5] Zamzami N, Marzo I, Susin SA, Brenner C, Larochette N, Marchetti P, Reed J, Kofler R, Kroemer G. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. Oncogene 1998;16:1055–63.
[6] Nakamura H, Nakamura K, Yodoi J. Redox regulation of cellular activation. Annu Rev Immunol 1997;15:351–69.
[7] Powis G, Mustacich D, Coon A. The role of the redox protein thioredoxin in cell growth and cancer. Free Radic Biol Med 2000;29:312–22.
[8] Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system (1). Free Radic Biol Med 2001; 31:1287–312.
[9] Holmgren A. Reduction of disulfides by thioredoxin. Exceptional reactivity of insulin and suggested functions of thioredoxin in mechanism of hormone action. J Biol Chem 1979;254:9113–9.
[10] Yokomizo A, Ono M, Nanri H, Makino Y, Ohga T, Wada M, Okamoto T, Yodoi J, Kuwano M, Kohno K. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. Cancer Res 1995;55:4293–6.
[11] Baker A, Payne CM, Briehl MM, Powis G. Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis *in vitro* and *in vivo*. Cancer Res 1997;57:5162–7.
[12] Becker K, Gromer S, Schirmer RH, Muller S. Thioredoxin reductase as a pathophysiological factor and drug target. Eur J Biochem 2000;267:6118–25.
[13] Lenaz G. Role of mitochondria in oxidative stress and ageing. Biochim Biophys Acta 1998;1366:53–67.
[14] Nicotera P, Leist M, Ferrando-May E. Intracellular ATP, a switch in the decision between apoptosis and necrosis. Toxicol Lett 1998;102/ 103:139–42.
[15] Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J 1999;341:233–49.
[16] Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. Embo J 1998;17:2596–606.
[17] Wells G, Bradshaw TD, Diana P, Seaton A, Shi DF, Westwell AD, Stevens MFG. Antitumour benzothiazoles. Part 10. The synthesis and antitumour activity of benzothiazole substituted quinol derivatives. Bioorg Med Chem Lett 2000;10:513–5.
[18] Wells G, Berry JM, Bradshaw TD, Burger AM, Seaton A, Wang B, Westwell AD, Stevens MFG. 4-Substituted 4-hydroxycyclohexa-2-5-dien-1-ones with selective activities against colon and renal cancer cell lines. J Med Chem 2003;46:532–41.
[19] Park EM, Thomas JA. The mechanisms of reduction of protein mixed disulfides (dethiolation) in cardiac tissue. Arch Biochem Biophys 1989;274:47–54.
[20] Sato N, Iwata S, Nakamura K, Hori T, Mori K, Yodoi J. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. J Immunol 1995;154:3194–203.
[21] Bogumil R, Ullrich V. Phenylarsine oxide affinity chromatography to identify proteins involved in redox regulation: dithiol–disulfide equilibrium in serine/threonine phosphatase calcineurin. Methods Enzymol 2002;348:271–80.
[22] Kunkel M, Kirkpatrick D, Johnson J, Powis G. Cell-line-directed screening assay for inhibitors of thioredoxin reductase signaling as potential anti-cancer drugs. Anti Cancer Drug Design 1997;12:659–70.
[23] Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2′,7′-dichlorofluorescin. J Leukoc Biol 1990;47:440–8.
[24] Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 1995;182:367–77.
[25] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993; 302:348–55.
[26] Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, Dive C, Hickman JA. Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis. J Cell Biol 1999;144:903–14.
[27] Westwell AD, Wells G, Berry JM, Bradshaw TD, Burger AM, Matthews CS, Monks A, Stevens MFG. Synthesis of heteroaromatic quinols, novel agents with selective activity clustered in colon and renal cell lines. Proc Am Assoc Cancer Res 2002;43:1034.
[28] Collison MW, Beidler D, Grimm LM, Thomas JA. A comparison of protein *S*-thiolation (protein mixed-disulfide formation) in heart cells treated with *t*-butyl hydroperoxide or diamide. Biochim Biophys Acta 1986;885:58–67.
[29] Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, Green DR. Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. J Cell Biol 2001;153:319–28.
[30] Wang GQ, Gastman BR, Wieckowski E, Goldstein LA, Gambotto A, Kim TH, Fang B, Rabinovitz A, Yin XM, Rabinowich H. A role for mitochondrial Bak in apoptotic response to anticancer drugs. J Biol Chem 2001;276:34307–17.
[31] Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. J Biol Chem 1999;274:27891–7.
[32] Backway KL, McCulloch EA, Chow S, Hedley DW. Relationships between the mitochondrial permeability transition and oxidative stress during ara-C toxicity. Cancer Res 1997;57:2446–51.
[33] Gamen S, Anel A, Perez-Galan P, Lasierra P, Johnson D, Pineiro A, Naval J. Doxorubicin treatment activates, and apoptosis in Jurkat cells a Z-VAD-sensitive caspase, which causes deltapsim loss, caspase-9 activity. Exp Cell Res 2000;258:223–35.
[34] Liu FT, Kelsey SM, Newland AC, Jia L. Generation of reactive oxygen species is not involved in idarubicin-induced apoptosis in human leukaemic cells. Br J Haematol 2001;115:817–25.
[35] Kantrow SP, Piantadosi CA. Release of cytochrome *c* from liver mitochondria during permeability transition. Biochem Biophys Res Commun 1997;232:669–71.
[36] Freeman ML, Meredith MJ. Modulation of diamide toxicity in thermotolerant cells by inhibition of protein synthesis. Cancer Res 1989;49:4493–8.
[37] Halestrap AP, Woodfield KY, Connern CP. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J Biol Chem 1997;272:3346–54.
[38] Costantini P, Belzacq AS, Vieira HL, Larochette N, de Pablo MA, Zamzami N, Susin SA, Brenner C, Kroemer G. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene 2000;19:307–14.
[39] Hirsch T, Marchetti P, Susin SA, Dallaporta B, Zamzami N, Marzo I, Geuskens M, Kroemer G. The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. Oncogene 1997;15:1573–81.
[40] Gurtu V, Kain SR, Zhang G. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Anal Biochem 1997; 251:98–102.