Altered methional homoeostasis is associated with decreased apoptosis in BAF₃ bcl₂ murine lymphoid cells

Anne-Marie ROCH*, Gerard QUASH*§, Yvonne MICHAL*, Jacqueline CHANTEPIE*, Bernard CHANTEGREL†, Christian DESHAYES†, Alain DOUTHEAU† and Jacqueline MARVEL‡

* Laboratoire d'Immunochimie, Faculté de Médecine Lyon-Sud, Chemin du Petit Revoyet, B.P. 12, 69921 Oullins Cédex, France, † Laboratoire de Chimie Organique, Institut National des Sciences Appliquées, 20 avenue A. Einstein, 69621 Villeurbanne Cédex, France, and ‡ Laboratoire de Biologie Moléculaire et Cellulaire, ENS, 46 allée d'Italie, 69364 Lyon Cédex 07, France

Methional is a potent inducer of apoptosis in an interleukin 3-dependent murine lymphoid cell line BAF₃ b₀ when it is added to the culture medium. In these cells transfected with the bcl₂ gene, BAF₃ bcl₂, the apoptotic-inducing activity of methional is dramatically reduced. The addition of disulfiram (an inhibitor of aldehyde dehydrogenase) in order to reduce methional oxidation brought about an increase in apoptosis in BAF₃ b₀ but not in BAF₃ bcl₂ cells. In contrast, the addition of quercetin (an inhibitor of aldehyde reductase) in an attempt to diminish methional reduction increased apoptosis in both BAF₃ b₀ and BAF₃ bcl₂ cells. The extent of DNA fragmentation in BAF₃ bcl₂ cells approached that in BAF₃ b₀ cells in the presence of quercetin and exogenous methional, suggesting a defect in methional biosynthesis in BAF₃ bcl₂ cells. Direct evidence for this was obtained by measuring labelled methional in cells incubated with

the sodium salt of [U-¹⁴C]4-methylthio-2-oxobutanoic acid (MTOB), the precursor of methional. The 80% decrease in labelled methional in BAF₃ bcl₂ compared with BAF₃ b₀ cells was accompanied by a concomitant rise in the transamination of [¹⁴C]MTOB to [¹⁴C]methionine in BAF₃ bcl₂ cells. Inhibition of the transaminase, however, by a synthetic transition-state-type compound, pyridoxal-L-methionine ethyl ester, induced apoptosis in BAF₃ b₀ but not in BAF₃ bcl₂ cells, confirming that the defect in BAF₃ bcl₂ cells was not in the transaminase itself but rather in the oxidative decarboxylation step MTOB → methional. In addition, no evidence was obtained for the synthesis of [¹⁴C]malondialdehyde from [¹⁴C]methional in BAF₃ bcl₂ cells. As these cells show no deficiency in their content of reactive oxygen species compared with that of BAF₃ b₀ cells, they may possess some other defect in the β-hydroxylase enzyme system itself.

INTRODUCTION

We have previously shown that the sodium salt of 4-methylthio-2-oxobutanoic acid (MTOB), an intermediate in the methionine-salvage pathway, is a metabolite that is situated at the crossroads of two important cellular phenomena. On the one hand, it can alleviate the methionine-dependence characteristic of some transformed cells [1] and on the other it is the precursor of methional, which is a direct inducer of apoptosis in BAF₃ lymphoid cells [2]. This induction of apoptosis takes place as early as 8 h after methional addition and has been found by flow-cytometric analysis to involve 40% of the cell population compared with a basal level of 0.3% in non-methional-treated cells. In BAF₃ cells deprived of interleukin 3 (IL-3) for a similar 8 h period the percentage of apoptotic cells was about 2% rising to 80% of the population at 16 h [3].

Because BAF₃ cells transfected with bcl₂ exhibit no signs of apoptosis of IL-3 deprivation even at 16 h [4], the question arose whether the apoptosis-inducing activity of methional would also be affected by the presence of bcl₂. This question is important for both theoretical and practical reasons: on the theoretical side, in spite of the numerous reports in the literature on the localization of the bcl₂ gene product, the 26 kDa protein, in the mitochondrial membrane [5], nuclear membrane [6] and endoplasmic reticulum [7], on its homo- and hetero-dimerization with other bcl₂-related gene products such as bax [8], bcl_x [9], Bak [10,11] and on its

capacity to protect enucleated cells from staurosporin-induced apoptosis [12], its precise function is not known. For the moment, the only function that has been ascribed to bcl₂ is its capacity to inhibit apoptosis induced by reactive oxygen species (ROS) in neural [13] and lymphoid cells [5].

On the practical side, the findings that cells transfected with bcl₂ [14] as well as bcl₂-positive lymphocytes from patients with lymphomas [15] show resistance to antiproliferative agents as diverse as methotrexate, etoposide, vincristine, cisplatin and hydroperoxycyclophosphamide argue in favour of bcl₂ acting on a pathway at which all these antiproliferative agents abut. This so-called 'final common pathway' in apoptosis, although often invoked [16,17], has, to our knowledge, not yet been defined.

One likely candidate could be the MTOB–methional–malondialdehyde (MDA) pathway [2]. Indeed MDA generated *in situ* in the liver and testis of rats injected with propane 1,3-diol induces the formation of DNA–DNA and DNA–protein cross-links [18,19]. If the MDA derived from methional has a similar role, then it may be responsible for the chromatin condensation that is the hallmark of all apoptosis [20] regardless of the apoptosis-inducing stimulus: addition of glucocorticoids [21] or chemotherapeutic agents [22] or withdrawal of trophic factors [23]. Hence in bcl₂-positive cells refractory to apoptosis inducible by the stimuli cited above, the enzymes and intermediates of the MTOB–methional–MDA pathway could be potential targets of the 26 kDa product of bcl₂.

Abbreviations used: MTOB, sodium salt of 4-methylthio-2-oxobutanoic acid; MTPA, methylthiopropionic acid; ALDH, aldehyde dehydrogenase; ALDR, aldehyde reductase; MDA, malondialdehyde; PI, propidium iodide; 2,4-DNPH, 2,4 dinitrophenylhydrazine; TdT, terminal deoxynucleotidyl-transferase; ROS, reactive oxygen species; HPTLC, high-performance thin-layer chromatography; TS, transition state; IL-3, interleukin 3; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate.

§ To whom correspondence should be addressed.

Scheme 1 MTOB–methional–MDA pathway

BCOADC, branched-chain oxoacid dehydrogenase complex; ALDH, aldehyde dehydrogenase; ALDR, aldehyde reductase; TPP, thiamine pyrophosphate (cofactor for decarboxylase); TA, thioctic acid (cofactor for transacylase); PP, pyridoxal phosphate (cofactor for MTOB transaminase). —, Established; —*, established but active only with excess methionine in the diet; ———, hypothetical.

Is there evidence in the literature linking any of the enzymes and metabolites of the MTOB–methional–MDA pathway to apoptosis? One enzyme for which unequivocal evidence exists is aldehyde dehydrogenase (ALDH). It has been shown that the activity of one of its isoenzymes is increased in preneoplastic liver nodules in rats injected with phenobarbital [24]. When the injection of phenobarbital was stopped, the preneoplastic cells died by apoptosis but on resumption of the phenobarbital treatment apoptosis was inhibited [25,26]. Therefore increased ALDH activity could inhibit apoptosis by decreasing intracellular methional which would be converted into 3-methylthiopropionic acid (MTPA) which is devoid of growth-inhibitory activity [2].

As far as metabolites are concerned, both MTOB and methional are targets of the hydroxyl radical ($\cdot\text{OH}$) [27] and we have recently shown that MDA is produced from methional both *in vitro* by the Fenton reaction and in $\text{BAF}_3 \text{ bcl}_2$ cells in culture undergoing apoptosis on trophic factor (IL-3) withdrawal [2]. Hence the reduction in apoptosis reported for neural cells transfected with bcl_2 [13] which show a reduction in levels of ROS such as $\text{H}_2\text{O}_2/\cdot\text{OH}$ could also be explained by an impairment of the conversion of intracellular methional into MDA.

To try to identify the putative targets of bcl_2 in the MTOB–methional–MDA pathway (Scheme 1) we decided to investigate whether apoptosis could be induced in $\text{BAF}_3 \text{ bcl}_2$ cells by adding to the culture medium on the one hand exogenous methional and on the other known inhibitors of the enzymes involved in methional degradation so as to increase its endogenous levels. Disulfiram was used to inhibit ALDH [28,29], which oxidizes

methional to MTPA [2], and quercetin to inhibit ALDR [30], which could reduce methional to methionol. It was considered, however, that this approach might be ineffective if $\text{BAF}_3 \text{ bcl}_2$ cells, in addition to the putative defects in ALDH and ALDR, possessed a reduced capacity to synthesize methional from MTOB. Therefore the inhibition of ALDH and ALDR was also undertaken in the presence of exogenous methional. As MTOB itself can be transaminated to methionine (Scheme 1), there was the possibility that, if increased transamination took place, there would be less MTOB available for methional biosynthesis. To examine this, a proven transition-state (TS)-type inhibitor of MTOB transaminase [1] was used. Further, measurement of $\text{H}_2\text{O}_2/\cdot\text{OH}$ levels was undertaken to ascertain the contribution, if any, of $\text{H}_2\text{O}_2/\cdot\text{OH}$ to any decrease in apoptosis in $\text{BAF}_3 \text{ bcl}_2$ cells. Finally, in order to obtain direct evidence for a perturbation of methional metabolism, the formation, of [^{14}C]methional and [^{14}C]MDA from [^{14}C]MTOB was also measured in $\text{BAF}_3 \text{ bcl}_2$ cells.

MATERIALS AND METHODS

Reagents

Culture media were from Gibco (Grand Island, NY, U.S.A.). Foetal calf serum came from ATGC Biochemical Industries (Paris, France), and methional and quercetin dihydrate from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [^3H]Thymidine was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). NaB^3H_4 in 0.01 M NaOH solution (25 mCi/ml;

specific radioactivity 12.6 Ci/mmol) was purchased from DuPont/NEN (Les Ulis, France). dATP, dCTP, dGTP, avidin-labelled fluorescein isothiocyanate (FITC), propidium iodide (PI), sodium cacodylate, cobalt chloride, BSA, RNase A, Triton X-100, ethidium bromide and disulfiram were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Biotin-16-dUTP and terminal DNA deoxynucleotidyl transferase (TdT) from calf thymus, glycogen and proteinase K came from Boehringer (Mannheim, Germany). Pico-fluor 30 scintillation solution was from Packard (Meriden, CT, U.S.A.). 2,4-Dinitrophenylhydrazine (2,4-DNPH) and HPTLC plastic sheets coated with silica-gel 60 were purchased from Merck (Darmstadt, Germany). 2',7'-dichlorofluorescein diacetate was from Molecular Probes (Eugene, OR, U.S.A.).

Cell lines

BAF₃ b₀, a murine bone-marrow-derived cell line dependent on IL-3, and BAF₃ cells transfected with a 1.9 kb human bcl₂ cDNA (BAF₃ bcl₂) were obtained as described [4].

Culture media

BAF₃ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 6% foetal calf serum and 5% Wehi-3B cell-conditioned medium used as a source of IL-3 as previously described [4]. They were grown at 37 °C in a humid atmosphere of air/CO₂ (90:/10 v/v). For further experiments involving the addition or not of compounds to the culture medium, cells were grown in the presence of IL-3.

Synthesis of the TS inhibitor of MTOB transaminase

Pyridoxal-L-methionine ethyl ester was synthesized as previously described [1] on a 0.7 mmol scale. For the preparation of the ³H-labelled compound the following modifications were made: reduction of the intermediate imine was carried out first with 1 ml of NaB³H₄ in 0.01 M NaOH and then completed with NaBH₄ (37 mg). Final specific radioactivity was 10 mCi/mmol.

Effect of methional on DNA fragmentation

Quantitative analysis of DNA fragments in cell lysates and medium using [³H]thymidine-prelabelled cells

BAF₃ cells were labelled by an adaptation of the method of Wright *et al.* [31] by incubating cells (2.5 × 10⁵/ml) with [³H]-thymidine (0.5 μCi/ml) for 35 h at 37 °C. After two washes with culture medium, 3 × 10⁶ cells were seeded in 6 ml of culture medium in the absence or presence of methional (50–800 μM). After contact for 8 h, the cells were harvested by centrifugation at 400 g for 5 min and medium was collected. Pelleted cells were washed twice in 50 mM PBS, pH 7.5, lysed in 2 ml of 0.1% Triton X-100/20 mM EDTA/5 mM Tris/HCl, pH 8, for 16 h at 4 °C and then centrifuged at 30000 g at 4 °C for 30 min [32]. Supernatants were collected and pellets dissolved in 0.3 ml of 0.5 M NaOH. Aliquots of culture medium (1 ml), supernatant (0.5 ml) and solubilized pellet (0.1 ml) were counted in a liquid-scintillation counter. Percentage DNA fragmentation was calculated as follows:

Labelling of DNA-strand breaks in apoptotic cells using the TdT assay

DNA-strand breaks were labelled *in situ* in individual fixed and permeabilized cells with biotinylated dUTP (detected with fluoresceinated avidin) using the TdT assay as described by Gorczyca *et al.* [33]. BAF₃ cells (1.5 × 10⁶) were seeded in 3 ml of culture medium in the presence or absence of methional (600 μM). After incubation, cells were washed in PBS and fixed in formaldehyde and then ethanol and then treated as described previously [2]. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) with an excitation wavelength of 488 nm and an emission wavelength of 520 nm (FITC) or 620 nm (PI). Some 10000 cells were analysed per assay.

Effect on DNA fragmentation of adding the ALDH inhibitor disulfiram

After being labelled with [³H]thymidine as described above, cells were washed twice with culture medium. Cells (3 × 10⁶) were seeded in 6 ml of culture medium in the absence or presence of different concentrations (10–200 μM) of disulfiram. After 8 h they were harvested and treated as described above. For studying DNA fragmentation by flow cytometry, 1.5 × 10⁶ cells were seeded in 3 ml of culture medium in the absence or presence of 25 or 50 μM disulfiram for 8 h.

In other experiments cells were first treated for 30 min with disulfiram (50 μM) and then for 8 h with methional (200–800 μM).

Effect on DNA fragmentation of adding the ALDR inhibitor quercetin

After being labelled with [³H]thymidine as described above, cells were washed twice with culture medium. Then cells (3 × 10⁶) were seeded in 6 ml of culture medium in the absence or presence of increasing concentrations of quercetin (10–60 μM) and harvested after 8 h. In other experiments, after 30 min in the presence of quercetin, 400 μM methional or buffer alone as control was added for 8 h. Cells were harvested and treated as described above.

Qualitative analysis of DNA fragmentation in cells treated with quercetin

BAF₃₀ b₀ (3 × 10⁶) and BAF₃ bcl₂ (5 × 10⁶) cells were seeded in 6 or 10 ml respectively of culture medium containing two concentrations of quercetin (20 or 50 μM) or PBS as control for 30 min and then in the absence or presence of 400 μM methional. After contact for 2, 4 or 8 h, the cells were washed three times in PBS. Pelleted cells were lysed in 2 ml of 0.1% Triton X-100/20 mM EDTA/5 mM Tris/HCl, pH 8, for 16 h at 4 °C and then centrifuged at 30000 g at 4 °C for 30 min. The supernatants were first treated with proteinase K (100 μg/ml) for 3 h at 50 °C in the presence of 0.5% SDS and then with RNase A (20 μg/ml) for 1 h at 37 °C. DNA was purified by two phenol extractions and precipitated with ethanol in the presence of 30 μg of glycogen. It was analysed by electrophoresis in 1% (w/v) agarose gel in 45 mM Tris/boric acid buffer, pH 8, containing 1 mM EDTA. After electrophoresis (60 V; 2 h in the Tris/boric acid/EDTA

$$\% \text{ fragmented DNA} = \frac{\text{d.p.m. in culture medium} + \text{d.p.m. in supernatant}}{\text{d.p.m. in culture medium} + \text{d.p.m. in supernatant} + \text{d.p.m. in solubilized pellet}} \times 100$$

buffer), the gel was stained for 30 min with ethidium bromide (0.5 µg/ml).

Effect on DNA fragmentation of adding the TS inhibitor of MTOB transaminase

After being labelled with [³H]thymidine as described above, cells were washed twice with culture medium. Then 3×10^6 cells were seeded in 6 ml of culture medium in the absence or presence of different concentrations (25–200 µM) of TS inhibitor. After 24 h contact, the culture media were collected, the cells washed and then lysed as described above.

For studying TS inhibitor uptake, 1×10^6 BAF₃ cells in 2 ml of culture medium were treated for 24 h with 100 or 200 µM [³H]TS inhibitor corresponding to 4×10^6 d.p.m. or 8×10^6 d.p.m. respectively. At the end of this period, cells were harvested and washed by centrifugation at 400 g for 5 min. The pellets were solubilized with 0.5 ml of 0.5 M NaOH for 30 min at 37 °C. Protein content was measured in 30 µl [34] and radioactive content in 100 µl.

Analysis of radiolabelled carbonyl products derived from [U-¹⁴C]MTOB in BAF₃ b₀ and BAF₃ bcl₂ cells

[U-¹⁴C]MTOB was prepared by oxidative deamination of [U-¹⁴C]methionine as described previously [1]. [U-¹⁴C]MTOB (3.7×10^6 d.p.m. corresponding to 6.56 nmol) was added to 50 ml of BAF₃ cell suspension (2.4×10^5 cells/ml) in DMEM containing 6% foetal calf serum and 5% IL-3. After 39 h the medium was removed, the cells washed twice with DMEM and resuspended in culture medium. To 100 ml of cell suspension (1.4×10^8 cells) was added [U-¹⁴C]MTOB (2×10^6 d.p.m. corresponding to 3.55 nmol) and after 8 h cells were harvested by centrifugation at 400 g for 5 min. Cell pellets were solubilized with 1.5 ml of 0.5 M NaOH for 30 min at 60 °C. Total radioactivity present in the cells, protein content and DNA content were measured in 10 µl aliquots, after which perchloric acid was added to the alkaline hydrolysate to a final concentration of 0.5 M followed immediately by 200 µmol of 2,4-DNPH, the mixture was heated at 70 °C for 30 min. The reaction mixture was centrifuged at 400 g for 5 min and washed with 0.5 M perchloric acid. The precipitate was solubilized in 1 ml of 0.5 M NaOH, and its radioactive content determined in 50 µl. The supernatant was brought to pH 10 with NaOH. Labelled metabolites were extracted into ethyl acetate, and the organic phase was dried, acidified and extracted into dichloromethane. The labelled metabolites in the organic phase were identified by HPTLC on precoated silica-gel 60 plastic sheets in solvent systems indicated [chloroform/acetone/acetic acid (10:1:0.5, by vol.) or ethyl acetate/light petroleum/acetic acid (15:15:2, by vol.)] by comparison with the 2,4-DNPH derivatives of reference compounds as described previously [2]. Radiochromatogram scanning was achieved on a System 200 Imaging Scanner (Bioscan Inc.).

Measurement of intracellular levels of ROS

BAF₃ b₀ or BAF₃ bcl₂ cells (1.5×10^6), seeded in 3 ml of culture medium, were treated with 10 µM 2',7'-dichlorofluorescein diacetate. After 1 h at 37 °C, the cells were washed twice with PBS. PI (3 µg/ml) was added 10 min before the fluorescence analysis of dichlorofluorescein by flow cytometry. Some 10 000 cells were analysed per assay at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Figure 1 Dose-dependent fragmentation of DNA in BAF₃ b₀ and BAF₃ bcl₂ cells incubated with methional

BAF₃ b₀ (○) or BAF₃ bcl₂ (●) cells (3×10^6) were prelabelled with [³H]thymidine and incubated with different concentrations (50–800 µM) of methional. After 8 h, they were harvested, lysed and centrifuged at 30 000 g for 30 min. The radioactivity present in the non-sedimentable DNA, the pellet and the culture medium was measured. Results are expressed as the percentage of fragmented DNA, calculated as described in the Materials and methods section, and are the means ± S.D. of four experiments.

Table 1 Flow-cytometric analysis of DNA-strand breaks in BAF₃ cells treated with methional

BAF₃ cells (1.5×10^5) were treated for 8 h in the absence or presence of 600 µM methional. Fixed cells were incubated in the presence of biotin-dUTP and TdT. The amount of biotinylated dUTP incorporated was assessed with avidin-FITC and the cellular DNA content by PI staining. Stained cells (10 000 per assay) were analysed by flow cytometry.

Samples	Percentage of cycling cells with DNA-strand breaks	
	BAF ₃ b ₀	BAF ₃ bcl ₂
Control	1.8 ± 1	2 ± 1
+ Methional	27 ± 9	9 ± 4

RESULTS

The Effect of adding exogenous methional on apoptosis

It is apparent from Figure 1 that the addition of increasing concentrations of methional to BAF₃ b₀ cells increases DNA fragmentation to a maximum of 26% at 8 h compared with the 7% seen in non-methional-treated control cells. In BAF₃ bcl₂ cells treated with equivalent concentrations of methional, DNA fragmentation reaches a maximum of 7% and in non-methional-treated controls it is 3%.

An examination of the number of double-strand breaks in these cells by flow cytometry after TdT assay confirmed the results obtained on measuring [³H]thymidine-labelled DNA fragments in BAF₃ b₀ and BAF₃ bcl₂ cells (Table 1). Indeed the percentage of fluorescent-positive cells increased from 1.8 ± 1 to 27 ± 9 in BAF₃ b₀ cells, whereas in BAF₃ bcl₂ cells the percentage positivity increased from 2 ± 1 to 9 ± 4 .

It would therefore appear that the presence of bcl₂ prevents the apoptosis-inducing capacity of exogenous methional to be manifested in BAF₃ bcl₂ cells compared with its expression in BAF₃ b₀ cells. This reduction could have been due to decreased methional availability, resulting from increased ALDH and/or increased

Figure 2 Effect of an inhibitor of ALDH (disulfiram) on DNA fragmentation

Top, in the absence of exogenous methional, BAF₃ b₀ (○) or BAF₃ bcl₂ (●) cells (3×10^6) prelabelled with [³H]thymidine were harvested after 8 h treatment with disulfiram (12.5–200 μM). DNA fragmentation was measured as described in the legend to Figure 1. Bottom, BAF₃ b₀ (○, □) or BAF₃ bcl₂ (●, ■) cells (3×10^6) prelabelled with [³H]thymidine were incubated with 25 (○, ●) or 50 (□, ■) μM disulfiram in the presence of methional (200–800 μM) and harvested after 8 h. DNA fragmentation was measured as described in the legend to Figure 1. Values represent means $\pm$ S.D. from duplicate samples in three experiments. Some standard deviations are so small that they are within the symbols.

ALDR activity (see Scheme 1). Decreased uptake of exogenous methional into BAF₃ bcl₂ cells could also have been responsible. We therefore undertook the verification of each of these steps in BAF₃ b₀ and BAF₃ bcl₂ cells.

Effect of adding an ALDH inhibitor on apoptosis

In the absence of exogenous methional

As shown in Figure 2 (top), the addition of disulfiram, a well-established inhibitor of ALDH, to the medium of BAF₃ b₀ and BAF₃ bcl₂ cells brought about a dose-dependent increase in DNA fragmentation in BAF₃ b₀ cells up to 50 μM after which there was a sharp decrease. In BAF₃ bcl₂ cells a small increase in fragmentation was observed over the same concentration range reaching a maximum of 5% at 75 μM and decreasing thereafter.

Confirmation of these results was obtained by flow cytometry in which the number of double-strand breaks was measured by TdT assay. It is apparent from Table 2 that the fluorescence of BAF₃ b₀ cells was increased 5-fold at 25 μM and 17-fold at 50 μM compared with that in controls. In BAF₃ bcl₂ no increases were observed.

If the DNA fragmentation seen here in BAF₃ b₀ cells is due to

Table 2 Flow-cytometric analysis of DNA-strand breaks in BAF₃ cells treated with disulfiram

Cells (3×10^6) were treated with disulfiram for 8 h. DNA strand breaks were determined by the TdT assay as described in the legend to Table 1.

Samples	Percentage of cycling cells with DNA-strand breaks	
	BAF ₃ b ₀	BAF ₃ bcl ₂
Control	1.9 $\pm$ 0.7	2.6 $\pm$ 0.5
Disulfiram 25 μM	9.5 $\pm$ 1.5	3.3 $\pm$ 0.8
Disulfiram 50 μM	32.3 $\pm$ 1.4	3.7 $\pm$ 0.9

Figure 3 Effect of an inhibitor of ALDR (quercetin) on DNA fragmentation

BAF₃ b₀ (○, □) or BAF₃ bcl₂ (●, ■) cells (3×10^6) prelabelled with [³H]thymidine were incubated with quercetin (10–60 μM) in the absence (top) or presence (bottom) of methional (400 μM) and harvested after 8 h. DNA fragmentation was measured as described in the legend to Figure 1. Values represent means $\pm$ S.D. of duplicate samples from three experiments.

the accumulation of endogenous methional, then it would suggest that BAF₃ bcl₂ cells are deficient in methional. In order to overcome this, exogenous methional was added.

In the presence of exogenous methional

Using disulfiram at 25 and 50 μM and methional at 200, 400 and 800 μM it was found (Figure 2, bottom) that the 5% increase shown by BAF₃ bcl₂ cells at 50 μM disulfiram and 400 μM methional was not much greater than that observed with 50 μM

disulfiram alone. In contrast, in BAF₃ b₀ cells, it was found that the addition of increasing concentrations of methional provoked a concentration-dependent decrease in DNA fragmentation similar to that observed earlier (Figure 2, top) with 75 and 100 μ M disulfiram alone. One explanation for this reduction in DNA fragmentation was that there was an optimal apoptosis-inducing concentration for methional and that when this concentration was exceeded, by inhibiting its oxidation, the compound became toxic. This hypothesis can be refuted by the finding that there was no decrease in cell viability as assessed by the Trypan Blue-exclusion test. It remained at 96% over the concentration range of methional used in the presence of 25 or 50 μ M disulfiram. Possible reasons for this decrease in DNA fragmentation in disulfiram- and methional-treated BAF₃ b₀ cells will be discussed below. At any rate, the addition of disulfiram followed by methional did not increase maximum DNA fragmentation in BAF₃ bcl₂ cells to the level seen in BAF₃ b₀ cells.

Effect of adding an ALDR inhibitor on apoptosis

In the absence of exogenous methional

ALDR inhibition was achieved by adding quercetin to the culture medium. As can be seen in Figure 3 (top) the addition of quercetin brought about a dose-dependent increase in DNA fragmentation in BAF₃ b₀ cells with a maximum of 34% at 20 and 30 μ M and a decrease at 40, 50 and 60 μ M. This decrease is not due to any loss in cell viability which remained constant at around 92%. In BAF₃ bcl₂ cells (Figure 3, top) there was also a concentration-dependent increase which reached a maximum of 15% but at a higher quercetin concentration (50 μ M). This suggested that ALDR activity was involved in controlling DNA fragmentation in both BAF₃ b₀ and BAF₃ bcl₂ cells, but that, in the latter, either ALDR activity was higher because of the higher concentration (50 μ M) of quercetin needed and/or the level of endogenous methional was lower. In order to overcome any putative limitation in endogenous methional concentrations, experiments were repeated with methional added to the culture medium.

In the presence of exogenous methional

Because the optimal quercetin concentrations were different for BAF₃ b₀ and BAF₃ bcl₂ cells, 20 and 50 μ M respectively, the effect of methional was investigated at one concentration (400 μ M) by adding it to cells that had been pretreated for 30 min with quercetin at concentrations ranging from 10 to 60 μ M. From the results presented, it is apparent that the addition of methional maintained maximum DNA fragmentation in BAF₃ b₀ cells (Figure 3, bottom) at about 34% between 30 and 60 μ M quercetin. The decrease previously observed in BAF₃ b₀ cells at quercetin concentrations of 40, 50 and 60 μ M was no longer observed in the presence of methional. Possible reasons for this observation will also be discussed below. In the case of BAF₃ bcl₂ cells treated similarly, there was a steady increase in DNA fragmentation also, with a maximum of 24% at 50 μ M quercetin (Figure 3, bottom). This result provides evidence that the addition of exogenous methional can indeed increase apoptosis in BAF₃ bcl₂ cells, eliminating thereby the possibility that methional uptake is defective in BAF₃ bcl₂ cells. Because quercetin has been reported to induce DNA fragmentation [35] and also apoptosis [36], it was imperative to determine whether the fragmentation seen here was random or showed the 180 bp laddering pattern typical of apoptosis. Preliminary experiments were performed at 2, 4 and 8 h to determine the minimum incubation time necessary for laddering to be observed. From

Figure 4 Electrophoretic analysis of non-sedimentable DNA from cell lysates

BAF₃ b₀ (3×10^6) and BAF₃ bcl₂ (5×10^6) cells were lysed after 4 h treatment with or without 400 μ M methional and 20 or 50 μ M quercetin. Non-sedimentable DNA was prepared from the 30 000 g supernatant and analysed by electrophoresis on 1% (w/v) agarose gel. (a) BAF₃ b₀ cells; (b) BAF₃ bcl₂ cells. Lane 1, control; lane 2, methional; lane 3, 20 μ M quercetin; lane 4, 20 μ M quercetin + methional; lane 5, 50 μ M quercetin; lane 6, 50 μ M quercetin + methional.

Figure 5 Effect of the TS inhibitor of MTOB transaminase on DNA fragmentation

Different concentrations (25–200 μ M) of TS inhibitor were added to the culture media of BAF₃ b₀ (○) or BAF₃ bcl₂ (●) cells for 24 h. Percentage of fragmented DNA was measured as described in the legend to Figure 1. Values represent means $\pm$ S.D. of duplicate samples in three experiments. The error bars are within the symbols.

these results the 4 h time point was chosen and the electrophoretic profile is presented in Figure 4. It is apparent that the pattern is indeed typical of apoptosis and in addition there are more high-molecular-mass fragments (see position of arrow in lanes 3 and 4 of Figure 4b) in methional-treated than untreated cells.

Thus DNA fragmentation in BAF₃ bcl₂ cells can attain the

Table 3 Comparison of the uptake of ³H-labelled TS inhibitor in BAF₃ cells

Cells were harvested by centrifugation 24 h after the addition of ³H-labelled TS inhibitor to the culture medium. After being washed, pellets were solubilized in 0.5 ml of 0.5 M NaOH and aliquots were taken for determination of radioactivity and protein content as described in the Materials and Methods section.

Amount of ³ H-labelled TS inhibitor present in 2 ml of culture medium (d.p.m.)	Amount of ³ H-labelled TS inhibitor present in cells (d.p.m./mg of protein)	
	BAF ₃ b ₀	BAF ₃ bcl ₂
4 × 10 ⁶	5350	10290
8 × 10 ⁶	12880	25100

level seen in BAF₃ b₀ cells when ALDR is inhibited and exogenous methional added, whereas in BAF₃ bcl₂ cells inhibition of ALDR alone brings about this maximum increase. This provides some indirect evidence for the existence of a deficiency in endogenous methional levels in BAF₃ bcl₂ cells.

Effect of adding a TS inhibitor of MTOB transaminase on apoptosis

Before undertaking the measurement of radiolabelled methional we had to verify whether the suspected methional deficiency of BAF₃ bcl₂ cells was due to increased conversion of its precursor MTOB into methionine. We therefore decided to inhibit the MTOB transaminase, using a TS inhibitor that had previously been synthesized and verified for its capacity to inhibit MTOB transaminase and cell growth [1].

It is apparent from Figure 5 that, after 24 h incubation, BAF₃ b₀ cells exhibited an increase in DNA fragmentation which reached a maximum of 30 %, whereas no increase whatsoever in DNA fragmentation was seen in BAF₃ bcl₂ cells. The incubation time with the TS inhibitor was increased to 24 h before fragmentation was examined because our preliminary results had shown that 200 μM TS inhibitor had no effect on growth before 8 h.

As the difference in DNA fragmentation could have been due to differences in uptake of the TS inhibitor into BAF₃ b₀ and BAF₃ bcl₂ cells, ³H-labelled TS inhibitor was prepared as described in the Materials and methods section and its uptake by both cell types measured. It is apparent from Table 3 that the uptake of the TS inhibitor by BAF₃ bcl₂ cells was about twice as high as that by BAF₃ b₀ cells. Therefore the lower apoptotic-inducing activity of the TS inhibitor in BAF₃ bcl₂ cells is not due to its decreased uptake.

All these lines of indirect evidence obtained from the inhibition of ALDH, ALDR and MTOB transaminase lead to the same tentative conclusion, i.e. the existence of a deficiency in the production of methional from MTOB in BAF₃ bcl₂ cells. In order to obtain direct evidence for this, the production of [¹⁴C]metabolites from [¹⁴C]MTOB was measured.

Measurement of [¹⁴C]methionine, [¹⁴C]methional and [¹⁴C]MDA derived from [¹⁴C]MTOB in BAF₃ b₀ and BAF₃ bcl₂ cells

[¹⁴C]Methional and [¹⁴C]MDA as their 2,4-DNPH derivatives and [¹⁴C]methionine were separated by HPTLC and quantified by radiochromatogram scanning as described in the Materials and methods section. It is apparent from Table 4 that the total amount of radioactivity on a per mg of DNA basis present in each cell type was similar after 47 h of incubation. In addition,

Table 4 Amount of radiolabelled products derived from [¹⁴C]MTOB in BAF₃ cells

Cells were incubated for 39 h and then for a further 8 h with [¹⁴C]MTOB. Radioactive 2,4-DNPH metabolites were separated by HPTLC on precoated silica-gel 60 aluminium sheets in the solvent system chloroform/acetone/acetic acid (10:1:0.5, by vol.) and radioactivity was determined on a system 200 Imaging scanner. Labelled 2,4-DNPH metabolites were identified by comparison with the 2,4-DNPH derivatives of reference compounds.

	BAF ₃ b ₀	BAF ₃ bcl ₂
Total intracellular radioactivity (d.p.m./mg of DNA)	1252000	1111000
Specific radioactivity of cellular proteins (d.p.m./mg of protein)	23500	20860
Free methionine (d.p.m./mg of DNA)	36820	51440
2,4-DNPH methional (d.p.m./mg of DNA)	14050	7650
2,4-DNPH MDA (2,4-DNPH pyrazole) (d.p.m./mg of DNA)	800	0

the specific radioactivity (presumably in methionine) of cellular proteins was similar in BAF₃ b₀ and BAF₃ bcl₂ cells. As regards free methionine a 40 % increase was observed in BAF₃ bcl₂ cells compared with that in BAF₃ b₀ cells. The radioactivity present in methional was 80 % higher in BAF₃ b₀ than in BAF₃ bcl₂ cells. Taken together these results confirm that in BAF₃ bcl₂ cells the decreased formation of methional from MTOB is accompanied by a concomitant increase in methionine formation. When [¹⁴C]MDA formation was measured, it corresponded to 800 d.p.m./mg of DNA in BAF₃ b₀ cells, whereas no radioactive MDA whatsoever was detected in extracts of BAF₃ bcl₂ cells. It would therefore seem that BAF₃ bcl₂ cells have an additional major defect in forming MDA from methional.

Because the conversion of methional into MDA requires [•]OH [2] the possibility existed that there was a deficiency in the ROS content of BAF₃ bcl₂ cells as shown for other bcl₂-transfected cells [13].

Comparison of H₂O₂/[•]OH levels in BAF₃ b₀ and BAF₃ bcl₂ cells

To investigate this, cellular levels of H₂O₂/[•]OH were measured by flow cytometry using dichlorofluorescein diacetate as described in the Materials and methods section. Although there is a tendency for BAF₃ bcl₂ cells to have more ROS than BAF₃ b₀ cells, (211 ± 52 arbitrary fluorescence units were found for BAF₃ bcl₂ cells compared with 154 ± 32 for BAF₃ b₀ cells; means ± S.D. obtained from ten assays for each cell type), there are no significant differences in H₂O₂/[•]OH levels between the two cell types. This result strongly suggests that the inability of BAF₃ bcl₂ cells to synthesize MDA from methional is not due to any decrease in their ROS content. It could, however, be due to a defect in the β-hydroxylation reaction itself.

DISCUSSION

The results presented provide evidence that the addition of exogenous methional to BAF₃ cells increases DNA fragmentation in BAF₃ b₀ cells to 26 % compared with the 7 % seen in untreated cells. In BAF₃ bcl₂ cells the methional-mediated increase is 7 % compared with 3 % in non-methional-treated controls. In order to determine the reason for this reduced efficacy of methional in BAF₃ bcl₂ cells we investigated three pathways that could influence methional homeostasis: its oxidation, reduction and

decreased formation from its precursor MTOB. In this last case any increased conversion of MTOB by MTOB transaminase into methionine (Scheme 1) would reduce the amount of MTOB available for methionine synthesis.

Before discussing the effect on apoptosis of modulating methionine metabolism, it must be clearly stated that our investigations to date have been restricted to this mouse lymphoma cell line. We can therefore, for the moment, make no case for generalization of the results obtained to other cell lines.

Bearing this restriction in mind it should be noted that on inhibiting ALDH-mediated methionine oxidation by the addition of increasing concentrations of disulfiram to cells, a clear-cut increase in DNA fragmentation, up to 28%, was observed in BAF₃ b₀ cells at 50 μ M disulfiram and declined thereafter. An inverse relationship between ALDH activity and apoptosis, as mentioned above, had been inferred from the results of other authors [24,25] but the mechanism had not been defined. Our results with disulfiram-treated BAF₃ b₀ cells provide an indication that methionine may be one of the cellular aldehydes involved. In BAF₃ bcl₂ cells the maximum increase was 7%, suggesting that BAF₃ b₀ but not BAF₃ bcl₂ cells contained sufficient endogenous methionine to reach its apoptosis-inducing threshold.

An attempt to correct this putative defect by adding increasing concentrations of exogenous methionine (200–800 μ M) to disulfiram-treated BAF₃ bcl₂ cells brought about only a negligible increase in apoptosis above that seen with disulfiram alone. In contrast, BAF₃ b₀ cells treated similarly showed a dose-dependent decrease in DNA fragmentation compared with that seen with disulfiram alone. Our results do not offer any direct explanation for this finding, but one hypothesis that can be put forward is that the methionine that accumulates in BAF₃ b₀ cells when ALDH is blocked may act as a scavenger of intracellular H₂O₂, an inducer of apoptosis at low concentration. Evidence in favour of this explanation has already been provided by the finding that the inhibition of glutathione synthesis involved in cellular H₂O₂ detoxification concomitantly increases apoptosis [13,37]. Regardless of the mechanism responsible for the decreased fragmentation in disulfiram/methionine-treated BAF₃ b₀ cells, the results obtained so far strongly suggest that these cells accumulate more methionine than BAF₃ bcl₂ cells. When this suggestion was subjected to experimental verification using [¹⁴C]MTOB as precursor, it was found that BAF₃ b₀ cells do indeed contain 80% more [¹⁴C]methionine than BAF₃ bcl₂ cells (Table 4).

In contradistinction to the inhibition of ALDH, the inhibition of ALDR by quercetin alone brought about a dose-dependent increase in DNA fragmentation in both BAF₃ b₀ and BAF₃ bcl₂ cells but with an important difference. DNA fragmentation rose steadily with increasing quercetin concentration in BAF₃ bcl₂ cells from 50 μ M onwards whereas it rapidly attained a maximum at 20 and 30 μ M quercetin in BAF₃ b₀ cells and then declined at higher concentrations (40–60 μ M). The combination quercetin/methionine provoked no further increase in apoptosis above that seen with 20 μ M quercetin alone in BAF₃ b₀ cells, but reduced the inhibition of apoptosis seen with concentrations of quercetin at 40, 50 and 60 μ M. It is also noteworthy that even if the quantity of DNA fragmentation in BAF₃ b₀ cells is similar at 20 μ M quercetin in the absence (Figure 3, top) and presence (Figure 3, bottom) of methionine, there are more high-molecular-mass fragments (see position of arrow in lanes 3 and 4 of Figure 4b) in cells treated with either 20 or 50 μ M quercetin in the presence of methionine than in its absence. The maximum fragmentation in BAF₃ bcl₂ cells (15%) seen with quercetin alone rose to 24% in the presence of quercetin and methionine. Taken together these findings provide a strong indication that cellular ALDR is involved in the control of DNA fragmentation

in both cell types and suggest that BAF₃ bcl₂ cells may possess an additional defect in methionine production. Direct evidence for lower methionine levels in BAF₃ bcl₂ cells was subsequently obtained (Table 4). No evidence, however, was obtained for the presence of labelled methionine when cell extracts and culture media from BAF₃ b₀ and BAF₃ bcl₂ cells were examined. On the contrary, a 2-fold increase in [¹⁴C]MTPA in the medium of BAF₃ bcl₂ compared with that in BAF₃ b₀ cells was seen on selective extraction of the acidified but not the alkaline culture medium into dichloromethane and by HPTLC of the dichloromethane extract in two different solvent systems: chloroform/acetone/acetic acid (10:1:0.02, by vol.) and ethyl acetate/light petroleum/acetic acid (15:15:2, by vol.). It may be that any methionine excreted into the medium is rapidly oxidized to methionine and then on to MTPA. This will be verified when labelled methionine becomes available.

Decreased methionine production in BAF₃ bcl₂ cells could have been due to the increased transamination of its precursor MTOB to methionine as shown in Table 4. This pathway, although theoretically reversible, has been shown to be operative in the direction methionine $\rightarrow$ MTOB only under conditions of excess methionine [38,39]. When MTOB transaminase was blocked by the addition of the TS inhibitor, it was only in BAF₃ b₀ cells that DNA fragmentation took place and not in BAF₃ bcl₂ cells.

From the indirect evidence obtained with the enzyme inhibitors, it is clear that the inhibition of each of the three enzymes (Scheme 1) that can deplete cells of their methionine content brings about a concomitant increase in DNA fragmentation in BAF₃ b₀ cells. In BAF₃ bcl₂ cells an increase is seen only when ALDR is inhibited. From the direct measurements of metabolite levels presented in Table 4 it seems clear that the lower DNA fragmentation seen in BAF₃ bcl₂ cells compared with BAF₃ b₀ cells is associated with decreased methionine production from MTOB in BAF₃ bcl₂ cells. Further, no evidence could be obtained, under our experimental conditions, for the presence of labelled MDA in BAF₃ bcl₂ cells which have ROS levels comparable with those of BAF₃ b₀ cells. This finding is in agreement with the recent results of other authors showing that there is no impairment of ROS metabolism in bcl₂-transfected cells [40,41]. It may therefore be that BAF₃ bcl₂ cells have an additional impairment of the β -hydroxylation reaction itself in which methionine is converted by $\cdot$ OH to MDA. Here too the answer will be sought by using two approaches: (1) measuring β -hydroxylase activity in BAF₃ b₀ and in BAF₃ bcl₂ cells in the presence of labelled methionine; (2) measuring methionine levels in cells, other than lymphoid cells, transfected with the bcl₂ gene and expressing different amounts of the 26 kDa protein.

We gratefully acknowledge the technical assistance of Monique Bouvier, Mireille Gavot, who provided the radiochromatogram scans, and Geneviève Panaye, who performed the flow cytometric analyses. We also thank Sylvie Gaulupeau for her skilled secretarial help. This study was financially supported in part by Le Conseil Régional Rhône Alpes and La Ligue Nationale Contre le Cancer: Comité de la Drome et Comité du Rhône.

REFERENCES

- Ogier, G., Chantepie, J., Deshayes, C., Chantegrel, B., Charlot, C., Doutheau, A. and Quash, G. (1993) *Biochem. Pharmacol.* **45**, 1631–1644
- Quash, G., Roch, A. M., Chantepie, J., Michal, Y., Fournet, G. and Dumontet, C. (1995) *Biochem. J.* **305**, 1017–1025
- Rodriguez-Tarduchy, G., Collins, M. and Lopez-Rivas, A. (1990) *EMBO J.* **9**, 2997–3002
- Collins, M. K. L., Marvel, J., Malde, P. and Lopez-Rivas, A. (1992) *J. Exp. Med.* **176**, 1043–1051
- Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Millman, C. L. and Korsmeyer, S. J. (1993) *Cell* **75**, 241–251

- 6 Chen-Levy, Z., Nourse, J. and Cleary, M. Z. (1989) *Mol. Cell Biol.* **9**, 701–710
- 7 Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W. and Reed, J. C. (1993) *Cancer Res.* **53**, 4701–4714
- 8 Oltvai, Z. N., Millman, C. L. and Korsmeyer, S. J. (1993) *Cell* **74**, 609–619
- 9 Krajewski, S., Krajewska, M., Shabaik, A., Wang, H.-G., Irie, S., Fong, L. and Reed, J. C. (1994) *Cancer Res.* **54**, 5501–5507
- 10 Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz, R. J., Evan, G. I. and Guild, B. C. (1995) *Nature (London)* **374**, 733–736
- 11 Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D. and Barr, P. J. (1995) *Nature (London)* **374**, 736–739
- 12 Jacobson, M. D., Burne, J. F. and Raff, M. C. (1994) *EMBO J.* **13**, 1899–1910
- 13 Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesen, D. E. (1993) *Science* **262**, 1274–1277
- 14 Miyashita, T. and Reed, J. C. (1993) *Blood* **81**, 151–157
- 15 Campos, L., Rouault, J. P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J. P. and Guyotat, D. (1993) *Blood* **81**, 3091–3096
- 16 Thompson, C. B. (1995) *Science* **267**, 1456–1462
- 17 Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R. and Sikorska, M. (1993) *EMBO J.* **12**, 3679–3684
- 18 Summerfield, F. W. and Tappel, A. L. (1981) *Anal. Biochem.* **111**, 77–82
- 19 Summerfield, F. W. and Tappel, A. L. (1984) *Chem. Biol. Interact.* **50**, 87–96
- 20 Cohen, G. M., Sun, X.-M., Snowden, R. T., Dinsdale, D. and Skilleter, D. N. (1992) *Biochem. J.* **286**, 331–334
- 21 McConkey, D. J., Hartzell, P., Perez, J. F., Orrenius, S. and Jondal, M. (1989) *J. Immunol.* **143**, 1801–1806
- 22 Cotter, T. G., Glynn, J. M., Echeverri, F. and Green, D. R. (1992) *Anticancer Res.* **12**, 773–780
- 23 Arends, M. J. and Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32**, 223–254
- 24 Dietrich, R. A., Bludeau, P., Stock, T. and Roper, M. (1977) *J. Biol. Chem.* **252**, 6169–6176
- 25 Bursch, W., Paffe, S., Putz, B., Barthel, G. and Schulte-Hermann, R. (1990) *Carcinogenesis* **11**, 847–853
- 26 Bursch, W., Oberhammer, F. and Schulte-Hermann, R. (1992) *Pharmacol. Sci. Trends* **13**, 245–251
- 27 Cohen, G. and Cederbaum, A. I. (1980) *Arch. Biochem. Biophys.* **199**, 438–447
- 28 Vallari, R. C. and Pietruszko, R. (1982) *Science* **216**, 637–639
- 29 MacKerell, A. D., Vallari, R. C. and Pietruszko, R. (1985) *FEBS Lett.* **179**, 77–81
- 30 Flynn, T. G. and Cromlish, J. A. (1982) in *Enzymology of Carbonyl Metabolism: Aldehyde Dehydrogenase and Aldo/Keto Reductase* (Weiner, H. and Wermuth, B., eds.), pp. 209–222, Alan R. Liss, New York
- 31 Wright, S. C., Kumar, P., Tam, A. W., Shen, N., Varma, M. and Larrick, J. W. (1992) *J. Cell. Biochem.* **48**, 344–355
- 32 Jarvis, W. D., Kolesnick, R. N., Fornari, F. A., Traylor, R. S., Gewirtz, D. A. and Grant, S. (1994) *Proc. Natl. Acad. Sci., U.S.A.* **91**, 73–77
- 33 Gorczyca, W., Gong, J. and Darzynkiewicz, Z. (1993) *Cancer Res.* **53**, 1945–1951
- 34 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 35 Ahmed, M. S., Ramesh, V., Nagaraja, V., Parish, J. H. and Hadi, S. M. (1994) *Mutagenesis* **9**, 193–197
- 36 Wei, Y. Q., Zhao, X., Kariya, Y., Fukata, H., Teshigawara, K. and Uchida, A. (1994) *Cancer Res.* **54**, 4952–4957
- 37 Harlan, J. M., Levine, J. D., Callahan, K. S., Schwartz, B. R. and Harker, L. A. (1984) *J. Clin. Invest.* **73**, 706–713
- 38 Finkelstein, J. D. and Martin, J. J. (1986) *J. Biol. Chem.* **261**, 1582–1587
- 39 Blom, H. J., Boers, G. H. J., van den Elzen, J. P. A. M., Gahl, W. A. and Tangerman, A. (1989) *Clin. Sci.* **76**, 43–49
- 40 Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1995) *Nature (London)* **274**, 811–813
- 41 Jacobson, M. D. and Raff, M. C. (1995) *Nature (London)* **374**, 814–816