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Novel competitive irreversible inhibitors of aldehyde dehydrogenase (ALDH1): restoration of chemosensitivity of L1210 cells overexpressing ALDH1 and induction of apoptosis in BAF_3 cells overexpressing bcl_2

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Abstract

4-Amino-4-methyl-pent-2-ynthioc acid *S*-methyl ester (ampal thiolester: ATE) was used as a lead compound to synthesise new amino-substituted derivatives of α, β acetylenic thiolester compounds as inhibitors of aldehyde dehydrogenase 1, (ALDH1). Of these compounds, the dimethyl derivative (DIMATE) was a competitive irreversible inhibitor (K_i ~280 μM) of baker's yeast ALDH1 *in vitro* showing 80% inhibition at 400 μM when preincubated with the enzyme for 30 min, whereas the trimethyl ammonium and the morpholine derivatives showed only 15% inhibition at 600 μM even after 60 min preincubation. ATE inhibited ALDH1 activity in ALDH1-transfected L1210 T cells resistant to hydroperoxycyclophosphamide (HCPA) and inhibited growth synergistically in the presence of HCPA. In non-transfected L1210 counterparts ATE did not potentiate growth inhibition by HCPA. DIMATE was a 30–100-fold more effective growth inhibitor than ATE. Endogenous ALDH1 activities of BAF_3 cells over-expressing different levels of bcl_2 (0–100%) were similar (16–20 mU/mg protein) and were all inhibited by DIMATE, reaching 20–30% at 4 μM. Up to 4 μM no apoptosis, as measured by DNA-fragmentation was observed, but at 8 and 10 μM DIMATE, DNA-fragmentation increased concomitantly with ALDH1 inhibition. No DNA-fragmentation was observed with ALDH1 irreversible inhibitors devoid of a thiolester group or with thiolesters which were not inhibitors of ALDH1. It was seen only with competitive irreversible inhibitors having the methanethiol and enzyme-inhibitory moieties. The methanethiol putatively released from DIMATE by ALDH1 esterase activity plays a role, albeit undefined, in lowering intramitochondrial glutathione levels which decreased by 47% as DNA-fragmentation increased.

Keywords: ALDH1; Thiolester inhibitors; Chemoresistance; bcl_2; Apoptosis

1. Introduction

ALDH (EC.1.2.1) catalyse the oxidation of various aliphatic and aromatic aldehydes to the corresponding carboxylic acids in the presence of NAD or NADP as cofactor. The enzymes are present in different organs with variations in activity depending on the type of tissue (reviewed in [1]). These enzymes are involved in the detoxification of endogenous aldehydes arising from lipid metabolism [2,3] and evidence has also been provided to show that they play a similar role in the detoxification of xenobiotics [4,5]. Among these, is aldophosphamide, the activated form of cyclophosphamide. In fact, there are reports in the literature showing that resistance to

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Abbreviations: ampal, 4-amino-4-methyl-pent-2-yn-1-al; ATE, ampal thiolester, 4-amino-4-methyl-pent-2-ynthioic acid 6-methylester; DIMATE, dimethyl ampal thiolester; TRIMATE, trimethyl ampal thiolester.

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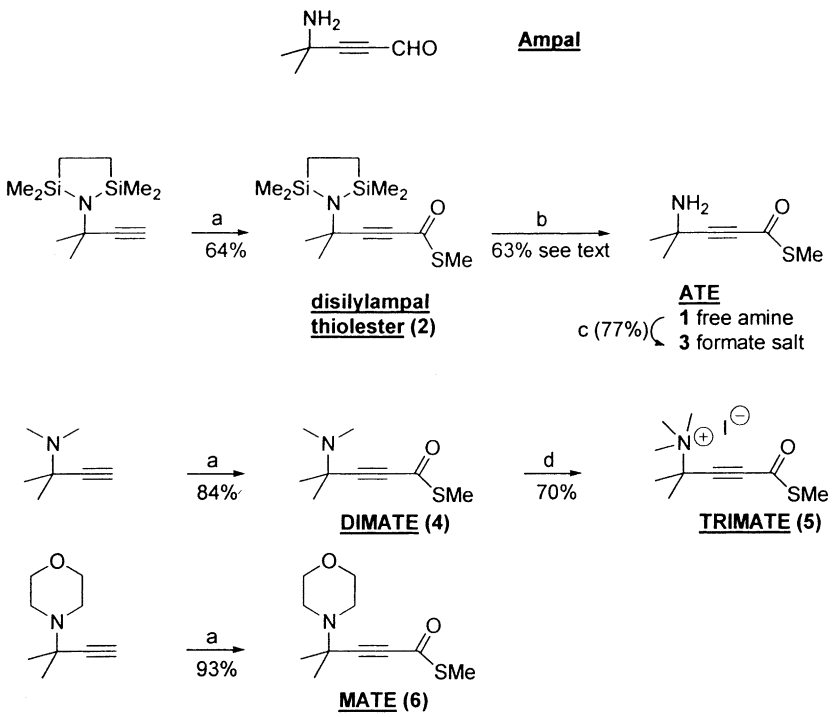
cyclophosphamide is associated with an increase in ALDH activity [6,7].

Of the three major classes of ALDH which have been identified in mammalian cells, it is ALDH3 which appears to be the one most intimately linked to the metabolism of xenobiotics. Indeed, it is this enzyme which is induced in the liver of rats treated with phenobarbital [8] and which in human colon cancer cells oxidises aldophosphamide to carboxyphosphamide [9]. Nevertheless, there is evidence that the oxidation of aldophosphamide can also be accomplished directly by ALDH1 [7,10].

More recently, this conclusion was confirmed by the results of Magni *et al.* [11] using mouse L1210 leukaemia cells transfected with the human ALDH1 gene, in which it was shown that the transfected mouse L1210 leukaemia cells (L1210 T) were more resistant in culture to HCPA, the activated stabilised form of cyclophosphamide, than their mock-transfected counterparts. On this basis, inhibitors of ALDH1 should, at least in theory, be capable of alleviating this chemoresistance. The well-known inhibitors of ALDH1 such as disulfuram cannot be used for this purpose because of its relatively short half life and lack of selectivity in an *in vivo* setting [12,13].

A novel inhibitor of ALDH had been designed and synthesised by us many years ago in the course of a programme devoted to the study of aminoaldehydes arising from polyamine metabolism. The compound so synthesised 4-amino-4-methyl-pent-2-yn-1-al (ampal, see Scheme 1) proved to be a good inhibitor of murine ALDH1 (propanal as substrate) [14] and exhibited some selectivity as a growth inhibitor of transformed compared to normal cells *in vivo* [14] and in culture [15]. It had however, an enormous drawback in that it tended to polymerise due to the simultaneous presence of a primary amine and aldehyde group. In fact, it had to be synthesised as the diethylacetal and cleaved extemporaneously.

This difficulty was subsequently overcome with the synthesis of ATE (**1** see Scheme 1) which, as the water-soluble formate salt **3** (Scheme 1) was found to be a competitive irreversible inhibitor of yeast ALDH1 *in vitro* [16]. During the course of this work we were also able to show that ATE was capable of inducing apoptosis in a mouse lymphoid cell line (BAF_3) overexpressing the human bcl_2 gene (BAF_3 bcl_2 H_{16}). These cells had previously shown no apoptosis in the presence of disulfuram [17] a classical ALDH1 inhibitor, or of methional, a cellular mediator of apoptosis in the non-transfected counterparts BAF_3 bo [18]. Further, ATE inhibited the growth of transformed cells (HeLa, B_{16}) more effectively (IC_{50}, 250 μM) than that of normal MRC5 cells (IC_{50}, 1150 μM) [16]. However, for an eventual application *in vivo* to tumours overexpressing bcl_2 [19,20] the IC_{50} had to be lowered to <10 μM and selectivity also had to be improved. Accordingly, ATE was used as a lead compound for designing



a: nBuLi, THF, -70°C to rt (or 0°C); COS, -70°C to 0°C then MeI 0°C.
b: SiO_2
c: HCO_2H, Et_2O, 0°C
d: MeI, AcOEt, rt.

Scheme 1.

derivatives with different basicity and/or hydrophobicity on the N atom. This led to the synthesis of dimethyl ampal thiolester (DIMATE **4**, see Scheme 1), the trimethyl ammonium salt of ampal thiolester (TRIMATE **5**, see Scheme 1) and morpholino ampal thiolester (MATE **6**, see Scheme 1).

With these new compounds which were obtained in good yields (>80%), the following questions were asked:

> Are the amine modified derivatives of ATE (a) competitive irreversible inhibitors of ALDH1? (b) selective inhibitors of the growth of cells in culture?
> Can ATE or its derivatives alleviate the resistance to HCPA of mouse L1210 leukaemia cells overexpressing the human ALDH1 gene?
> Is the alleviation of resistance restricted to cells overexpressing ALDH1 or can it be extended to other cell lines which also exhibit chemoresistance due to the overexpression of bcl_2 [19,20].
> What is the mechanism of the proapoptotic action of ATE and its derivatives ? Is it due solely to their effect as inhibitors of ALDH1 or are they also affecting cellular glutathione (GSH) homeostasis? This possibility had to be envisaged as it is well established that the bcl_2 induced resistance to apoptosis is associated with increased cellular GSH levels [21].

We will describe here full details of the synthesis of ATE and its derivatives since only an outline of the synthetic procedure for ATE alone has been presented so far [16] and present evidence to show that like ATE itself, DIMATE is a competitive irreversible inhibitor of ALDH1. ATE effectively abrogates the resistance to HCPA which is manifest in L1210 cells transfected with the human ALDH1 gene; DIMATE induces apoptosis in four mouse lymphoid cell lines overexpressing the bcl_2 protein *via* a mechanism involving, in at least the one lymphoid cell line examined in detail, the inhibition of ALDH1 activity and the reduction of intramitochondrial GSH levels.

2. Material and methods

2.1. Reagents

ALDH from baker's yeast, Hoechst 33258, diaminobenzidine (DAB), bovine serum albumin (BSA), *N*-acetyl cysteine, tri-*n*-butylphosphine, 7-fluoro-2.1,3-benzoxadiazole-4-sulphonamide (ABDF), propanal diethylacetal sodium dodecyl sulphate (SDS), NP40, sodium deoxychlolate (DOC), Triton X-100, glutathione (GSH) and polyacrylamide were from Sigma. Phenyl methane sulfonyl fluoride (PMSF) was from Merck, nitrocellulose from Sartorius, nicotinamide adenine dinucleotide (NAD) was from Boehringer, glutathione diethyl ester was from Bachem. Rabbit anti human bcl_2 from Chemicon, Euromedex, chemiluminescent reagents were from CovalAb. [^{3}H]Thymidine 2368 GBq/mmol was from Isotopchim. Culture media were from Life Technologies. Foetal calf serum (FCS) was from ATGC Biochemical Industries.

2.2. Cell lines

MRC5, normal human embryonic lung fibroblasts were obtained from Institut Merieux.

B_{16} murine melanoma cells were a kind gift of Dr. J. Vila, INSERM U218. BAF_3 bo a murine bone-marrow-derived cell line dependent on IL-3, BAF_3 cells transfected with a 1.9 kb human bcl_2 gene at different levels, Wehi-3B cells were a gift of Dr. J. Marvel, Ecole Normale Supérieure, (Lyon, France). L1210 a mouse lymphocytic leukaemia and L1210 T obtained by infecting L1210 cells with a kat-based retroviral vector carrying the human ALDH1 cDNA (L1210 Kat ALDH 29/5) were provided by Dr. Magni (Milan, Italy). DU145 a brain metastasis of a human prostate carcinoma was a kind gift of Dr. J. André INSERM 329 (Lyon, France).

2.3. Culture media

MRC5 cells, were grown in Eagle's Minimum Essential Medium (MEM), B_{16} cells and DU145 in RPMI 1640 each containing 10% FCS. They were maintained at 37° in a humid atmosphere of air/CO_2 (95:5). BAF_3 bo and BAF_3 bcl_2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 6% FCS and 5% Wehi-3B cell-conditioned medium used as a source of IL_3. They were grown at 37° in a humid atmosphere of air/CO_2 (90:10).

L1210 and L1210 T cells were grown in RPMI 1640 containing 10% FCS in a humid atmosphere of air/CO_2 (90:10).

2.4. Chemistry

Compounds **1–5** were obtained as depicted in Scheme 1.

2.4.1. General remarks

All procedures were conducted in oven dried glassware under positive nitrogen pressure and using syringe-needle transfer techniques. DMF and iPr$_2$NH were distilled from CaH_2, THF from sodium-benzophenone. ^{1}H NMR (200 or 300 MHz) and ^{13}C NMR (50 or 75 MHz) spectra were recorded on a Brucker AC 200 or AM 300. Chemical shifts are reported as d value relative to the solvent peak of $CHCl_3$ set to 7.26 for ^{1}H and 77.16 for ^{13}C. IR spectra were carried out on a Perkin-Elmer 298 spectrophotometer. Melting points were determined in open capillaries and are uncorrected. Flash chromatography preparations were performed using Merck 60 (40–63 μm) silica. Mass spectra were recorded on a Nermag R-10-10S (70 eV). Elemental analyses were performed at the SCA Solaize (France).

*n*Butyllithium solutions in hexanes were titrated by *N*-benzylbenzamide [22].

2.5. 4-Amino-4-methyl-pent-2-ynthioic acid S-methyl ester—tetramethyl disilylazacyclopentane derivative (2)

To 1-(1,1-dimethyl-prop-2-ynyl)-2,2,5,5-tetramethyl-[1,2,5]-azadisilolidine prepared as described [23], (4.10 g, 18.2 mmol) in THF (91 mL) was added within 5 min 8.2 mL (21.9 mmol) of *n*BuLi (2.66 M solution in hexanes) at −70°. Reaction mixture was allowed to rise to room temperature by removing the acetone–CO_2 cooling bath. After which stirring was continued for 1.5 hr. A total of 8 mL of condensed COS were introduced at −70° *via* cannula and stirring was continued for 30 min at this temperature and 30 min at 0°. MeI (1.36 mL, 21.9 mmol) was added and after 2 hr at 0° the reaction mixture was diluted by ether (400 mL), washed with brine (3 × 50 mL) and the organic layer dried (Na_2SO_4). After concentration, the crude silyl-amine-thiolester was purified by Kugelrohr distillation (oven temperature: 150°; 10^{-2} T), 3.5 g (64%) of pure **2** (oil) were isolated. IR (film): $\nu = 2205, 1650, 1170, 1090, 1030, 930, 890, 880, 845, 780$. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.23$ (s, 12H, CH_3Si), 0.68 (s, 4H, CH_2Si), 1.58, (s, 6H, $(CH_3)_2C$), 2.38 (s, 3H, CH_3S).

2.6. ATE [free amine]: 4-amino-4-methyl-pent-2-ynthioic acid S-methyl ester (1)

The crude silyl-amine-thiolester **2** prepared as described above (scale 2 mmol) was deprotected on silica gel as previously described [23] eluting first with ether then a gradient ether/methanol = 90/10 to 80/20. Yield: 63% (oil). Note: some degradation may occur at this stage. IR (film): $\nu = 3360, 3290, 2220, 1645$. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.35$ (s, 6H, CH_3), 2.05 (s, 2H, NH_2), 2.30, (s, 3H, CH_3S). EI-MS: $m/z = 142$ (M-15, 40), 114 (16), 110 (4), 125 (7), 58 (100).

2.7. ATE [formate salt]: 4-amino-4-methyl-pent-2-ynthioic acid S-methyl ester, formate salt (3)

To the free amine **1** (0.139 g, 0.88 mmol) in anhydrous ether (5 mL) was added formic acid (0.041 g, 0.88 mmol) in ether (0.63 mL) at 0°. The mixture was stirred for 5 min and warmed to room temperature. After concentration *in vacuo*, the residue was dispersed in anhydrous ether by trituration. Filtration and drying *in vacuo* provided formate **3** (0.138 g, 77% hygroscopic solid). IR (KBr): $\nu = 2596, 2239, 1652$. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.63$ (s, 6H, CH_3), 2.40 (s, 3H, CH_3S), 7.58 (s, 3H, NH_3), 8.34 (s, 1H, HCO_2). ^{13}C NMR (50 MHz, D_2O): $\delta = 16.4, 34.4, 52.0, 84.7, 94.1, 174.7, 183.7$. Anal.: calc. for $C_8H_{13}NO_3S$: %C = 47.27, %H = 6.45; found: %C = 47.00, %H = 6.00.

2.8. DIMATE: 4-dimethylamino-4-methyl-pent-2-ynthioic acid S-methyl ester (4)

To 3-dimethylamino-3-methyl-but-1-yne prepared as described [24] (1.33 g, 12 mmol) in THF (60 mL) was added dropwise 5.7 mL (13.2 mmol) of 2.3 M *n*BuLi solution at −70°. After 5 min the cooling bath was removed and the reaction mixture was warmed to 0° and stirred for 30 min. A total of 6 mL of condensed COS were introduced at –70° *via* cannula. The mixture was stirred for 30 min at –70° and 30 min at 0°. Then, 0.82 mL (13.2 mmol) of MeI was added and the yellow solution was kept at 0° for 2 hr. The reaction mixture was diluted with ether (300 mL) and washed with brine (3 × 30 mL). The organic layer was dried (Na_2SO_4); its concentration *in vacuo* and purification of the residue by flash chromatography (petroleum ether/ethyl acetate = 70/30) provided compound **4** (1.87 g, 84%-oil). IR (film): $\nu = 2870, 2820, 2780, 2200, 1650, 1080, 990, 915, 810, 730$. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (s, 6H, $(CH_3)_2$), 2.31 (s, 6H, $N(CH_3)_2$), 2.39 (s, 3H, CH_3S). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 12.1, 27.5, 40.0, 54.7, 81.5, 94.7, 175.8$. EI-MS: $m/z = 185$ (M^+, 7), 170(100), 149(27), 142(75), 125(21), 111(35), 97(52), 86(73), 71(72), 69(74). Anal.: calc. for $C_9H_{15}NOS$: %C = 58.34, %H = 8.16; found: %C = 57.83, %H = 8.30.

2.9. TRIMATE: 4-tetramethylammonium-4-methyl-pent-2-ynthioic acid S-methyl ester (5)

To a solution of the dimethylamine **4** (0.184 g, 0.99 mmol) in ethyl acetate (10 mL) at room temperature was added MeI (0.25 mL, 4.02 mmol). The reaction mixture was kept in the dark for 4 days. After concentration *in vacuo* the purification of the residue by flash chromatography (petroleum ether/ethyl acetate = 70/30) provided the triethylammonium salt **5** as a white solid (0.228 g, 70%). Fus.: 160°; decomp. IR (KBr): $\nu = 2230, 1640, 1080, 925, 810, 680$. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.96$ (s, 6H, $(CH_3)_2$), 2.46 (s, 3H, CH_3S), 3.62 (s, 9H, $N(CH_3)_3$). Anal.: calc. for $C_{10}H_{18}INOS$: %C = 36.70, %H = 5.54, %N = 4.28; found: %C = 36.83, %H = 5.61, %N = 4.36.

2.10. MATE: 4-methyl-4-morpholin-4-yl-pent-2-ynthioic acid S-methyl ester

Prepared as described for DIMATE by using the 4-(1,1-dimethyl-prop-2-ynyl)-morpholine [25] instead of the (1,1-dimethyl-prop-2-ynyl)-dimethylamine. Scale: 13 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 60/40), yield: 93%. White solid, Fus.: 52° (petroleum ether). IR (KBr): $\nu = 2200, 1630, 970, 900, 850, 790, 730$. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (s, 6H, $(CH_3)_2C$), 2.39 (s, 3H, CH_3S), 2.64 (m, 4H, CH_2O), 3.74 (m, 4H, CH_2N). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 12.4, 26.8, 47.5, 54.4, 67.1, 81.7, 94.8, 176.1$. Anal.: calc. for $C_{11}H_{17}NO_2S$: %C = 58.12, %H = 7.54,

%N = 6.16, %O = 14.08; found: %C = 57.83, %H = 7.60, %N = 6.11, %O = 14.30.

2.11. Effect of compounds on cell growth

All cells were seeded at a density of 10^5 in 1 mL culture medium per well of 24-well plates. At 4 hr after seeding MRC5, B_{16} and DU145 adherent cells, compounds were added to the wells at different concentrations. For L1210 and BAF_3 cells which grow in suspension, compounds were added immediately after seeding. Plates were left for 72 hr, after which time, the cell sheet of adherent cells was washed twice with phosphate buffered saline (PBS) and harvested directly with 0.1 M NaOH. Growth was determined by measuring protein content by the Lowry method [26] and DNA by the Hoechst assay [27]. For L1210 and BAF_3 cells, growth was measured by counting the number of viable cells in the presence of 0.1% trypan blue.

2.12. Measurement of ALDH1 activity

To a reaction mixture containing 1 mM EDTA, 100 mM KCl, 2.35 mM NAD, in 60 mM sodium phosphate buffer pH 8.5 was added 206 mU baker's yeast ALDH. The reaction was started by the addition of 2 mM propanal in a final volume of 1 mL and the OD was read at 340 nm at time 0 (just after the addition of the substrate) and at intervals of 5 and 10 min. The reaction was linear over 2 hr and enzyme activity is expressed as ΔOD/min or as units where 1 U refers to 1 μmol NADH formed/min at 37° at pH 8. Results are the average of two experiments done in duplicate.

2.13. Temperature-dependent inhibition

This was carried out by preincubating the enzyme at pH 6 instead of pH 8.5 to avoid the spontaneous cleavage of thiolesters which occurs at pH 8.5. Accordingly, 206 mU of ALDH (baker's yeast) were incubated at 37° or at 0° in 60 mM sodium phosphate buffer pH 6 containing 1 mM EDTA, 100 mM KCl in a final volume of 200 μL. DIMATE at a final concentration of 400 μM was added to each of a series of tubes incubated at 0 and 37°. To the control series no DIMATE was added. Incubation was carried out at both temperatures for periods of time ranging from 5, 10, 20 and 30 min. At the end of each incubation period 100 μg of BSA was added as a carrier to each tube followed by acetone at −20° to a final concentration of 80% to precipitate proteins (ALDH1 and BSA). After standing for 1 hr at −20° the tubes were centrifuged at 9000 *g* for 10 min, washed once with 80% acetone to eliminate any residual DIMATE. The final precipitate was dissolved in 358 μL water and immediately added to the complete enzymatic reaction mixture containing NAD, EDTA, KCl, propanal, phosphate buffer pH 8.5 at the concentrations described above. Activity was measured as described for the baker's yeast enzyme.

2.14. Competitive inhibition

To examine this with excess substrate, glyceraldehyde at 12 and 40 mM was used instead of propanal because of the inhibition of the enzyme with this latter substrate at such high concentrations. To a reaction mixture containing 1 mM EDTA, 100 mM KCl, 2.35 mM NAD in 60 mM sodium phosphate buffer pH 7 was added 260 mU baker's yeast ALDH1 and DIMATE at the concentrations shown. The reaction was started by the addition of glyceraldehyde and performed at pH 7 instead of pH 8.5 to reduce the spontaneous hydrolysis of DIMATE which occurs at alkaline pH. The OD was read at 340 nm at 0 time (on adding the substrate) and at intervals of 5 min thereafter.

2.15. Irreversible inhibition

The method previously described [16] was used with incubation for 20 min in the presence of 100–500 μM DIMATE. After the acetone precipitation, centrifugation and washing steps, the precipitate was dissolved in 10 mM phosphate buffer pH 6 containing 0.14 M NaCl, dialysed against 500 mL of this same buffer with two changes, and finally against 60 mM phosphate buffer pH 8.5, 1 mM EDTA and 100 mM KCl. Activity was measured as described for the baker's yeast enzyme.

2.16. Cell lysates

Cells growing in suspension were harvested by centrifugation and the cell pellets (3×10^6 cells) were incubated directly in 500 μL of 60 mM sodium phosphate buffer pH 7 containing 1% (v/v) Triton X-100, 1 mM EDTA for 30 min at 4°. Lysates were centrifuged at 48,000 *g* for 30 min and the supernatants used for measuring ALDH1 activity. The composition of the reaction mixture was identical to that described above for baker's yeast ALDH1 but with 1 mg of lysate protein as source of enzyme. The reaction was linear over 2 hr and OD at 340 nm was read at 30 and 60 min. Activity is calculated as ΔOD/min/mg protein and expressed in tables and figures as mU/mg protein or as the percentage inhibition of treated vs. non treated cells. All experiments were done in duplicate and results are the average of two experiments.

2.17. Effect of DIMATE and TRIMATE on DNA fragmentation

BAF_3 bcl_2 cells were seeded at 10^5 cells/mL, labelled with [^{3}H] thymidine (4.62 KBq/mL) for 40 hr at 37°. After two washes with culture medium, cells at 5×10^5/mL were reseeded in 5 mL culture medium in the absence or presence of DIMATE (1–8 μM) or TRIMATE (1–600 μM). After contact for 24 or 6 hr, cells were harvested and DNA fragmentation measured as described previously [17]. Results are the average of two experiments done in duplicate.

2.18. Western blot analysis

Cell extracts were prepared by lysing BAF_3 bo and BAF_3 bcl_2 cells (H_{16}, G_{18}, B_{14}, G_{21}) with RIPA (150 mM NaCl, 1% NP40 (v/v), 0.5% DOC, 0.1% SDS, 50 mM Tris–HCl, pH 8) in the presence of 100 μM PMSF. After centrifuging 10 min at 15,000 *g*, the protein content of the supernatants was measured. 100 μg protein from each cell extract were analysed by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose at 100 V for l hr. After 1 hr saturation in TBS (0.14 M NaCl, 20 mM Tris–HCl, pH 7.6) containing 5% skimmed milk, the blot was incubated overnight at 4° with a rabbit anti-human bcl_2 serum diluted in TBS containing 1% skimmed milk. Nitrocellulose was washed in TBS containing 0.1% Tween 20 and incubated for 1 hr with a goat serum anti rabbit IgG coupled to peroxidase at a dilution of 1/2000 in TBS. After four washes in TBS, containing 0.1% Tween 20, the blot was revealed by chemoluminescence according to the manufacturer's instructions. The blot was scanned on a SAMBA™ scanner.

2.19. Isolation of mitochondria

Cells were resuspended in 1 mM EDTA, 250 mM sucrose, 0.1% BSA and 10 mM Hepes, pH 7.4 (buffer A) and homogenised with 10 strokes of a Potter–Thomas homogeniser. Nuclei and unbroken cells were pelleted for 5 min at 1000 *g*. Mitochondria were isolated from the post-nuclear supernatant by a 15 min centrifugation at 10,000 *g*. Mitochondria were washed twice in 250 mM sucrose, 10 mM Hepes, pH 7.4 (buffer B) and purified as previously reported [28]. Briefly, washed mitochondria were layered on a hybrid Percoll/Metrizamide gradient in buffer B centrifuged for 20 min at 50,000 *g*. Mitochondria band at the 17:35% interface. Purified mitochondria were removed from the gradient and washed three times in buffer B.

2.20. Determination of glutathione levels

Concentrations of glutathione were determined by the original technique of Cornwell *et al*. [29] as modified by Jacob *et al*. [30] with further modifications, using HPLC separation of the fluorescent derivatives obtained with ABDF. A total of 300 μL of cell extract were mixed with the same volume of 10% trichloroacetic acid and precipitated proteins removed by centrifugation. Then, a 100 μL aliquot of the supernatant was taken, neutralised with 20 μL of 1.55 M NaOH, and the resulting solution was buffered by adding 250 μL of 125 mM borate buffer pH 8. Thirty microliters of 4.6 mM ABDF, in borate buffer, were added as derivatising agent, left at 50° for 20 min, then rapidly cooled and acidified with 40 μL of 1 M, HCl. *N*-acetylcysteine was used as internal standard. After derivatisation, 50 μL of the resulting solutions were submitted to chromatographic separation; the HPLC apparatus consisted of a Wisp® autoinjector, model 715 (Waters), a C18 μBondapak 3.9, 300 mm, 10 μm (Waters), a fluorimeter model 1050 (Hitachi) operated at 385 and 515 nm wavelength for excitation and emission, respectively. The mobile phase consisted of 0.1 M phosphate buffer/acetonitrile: 9/1(v/v), adjusted after mixing to pH 3.2 with 1 M phosphoric acid; it was delivered at a flow rate of 1 mL/min by a model 510 pump (Waters). The resulting chromatograms were recorded by a Millennium workstation before integration of peak heights. Chromatographic responses were calculated as the ratio of peak height of derivatised glutathione to that of the internal standard. The lower limit of detection was 0.3 nmol GSH. All reagents used throughout these operations were of analytical grade. Results are the average of three experiments done in duplicate.

3. Results

3.1. ALDH1 activity and HCPA sensitivity in L1210 and L1210 T cells

To examine these parameters in a model system with proven differences in ALDH1 activity, we used L1210 T and their non-transfected counterparts L1210 as controls. The characteristics of these experimental and control cells have been previously described [11]. As one of their salient features is the increased resistance of L1210 T to the anti-proliferative action of HCPA, we used susceptibility to HCPA as a criterion for assessing the effect of inhibiting ALDH1 activity by ATE. Accordingly, the IC_{50} of HCPA on L1210 and L1210 T was investigated and found to be 3.9 and 9.0 μM respectively. The ALDH activity of L1210 was 2.1 mU/mg protein and that of L1210 T 3.01 mU/mg protein, an increase of 1.5-fold.

3.2. Effect of ATE on ALDH1 activity and on the growth of L1210 cells in culture

To determine the minimum concentration of ATE to be used for inhibiting cellular ALDH1 activity, L1210 cells were incubated with the compound at doses of 50, 100 and 150 μM just after seeding, and ALDH1 activity and the number of viable cells were measured after 3 days in culture. It is apparent (Table 1) that the inhibition of ALDH activity in L1210 cells increases as ATE concentrations increase. With L1210 T, the inhibition of ALDH1 activity was similar to that of L1210 at 50 and 100 μM but reached only 24% at 150 μM whereas in L1210 it was 44% at an equivalent concentration.

As regards cell growth, no inhibition was seen at 50 μM ATE with either cell type (Table 1) and IC_{50}'s of 90 and 140 μM were obtained for L1210 and L1210 T respectively. A total of 50 μM ATE was therefore used to investigate the effect on the susceptibility to HCPA of sensitive L1210 and resistant L1210 T when ALDH1 activity was partially inhibited.

Table 1
Dose-dependent inhibition of ALDH1 activity and growth of L1210 and L1210 T cells incubated with ATE

ATE (μM)	Inhibition (%)			
	L1210		L1210 T	
	ALDH1	Growth	ALDH1	Growth
50	10	0	5	0
100	17	53	14	3
150	44	91	24	55

To L1210 and L1210 T cells seeded each at a density of 1×10^5 cells/mL were added immediately after seeding vehicle (ethanol to a final concentration of 0.5%) or ATE in vehicle at the concentrations indicated. After 3 days, cell growth was assessed by measuring viable cells in the presence of 0.1% trypan blue. For enzyme determinations cells were centrifuged washed twice with PBS and the pellet lysed directly in 500 μL 60 mM sodium phosphate buffer pH 7 containing 1% (v/v) Triton X-100, 1 mM EDTA for 30 min at 4°. Lysates were centrifuged at 48,000 *g* for 30 min; and the supernatants used for measuring ALDH1 activity by adding 1 mg lysate protein to a reaction mixture containing 60 mM sodium phosphate buffer pH 8.5 1 mM EDTA, 100 mM KCl, 2.35 mM NAD. The reaction was started by the addition of 2 mM propanal and the final volume was 1 mL. ALDH1 activity was measured by the increase in OD_{340} of the NADH formed and inhibition as the % loss of activity of treated vs. that of non-ATE treated cells. Values are means of two determinations each done in duplicate.

3.3. Effect of the combined action of ATE and HCPA on the growth of L1210 and L1210 T

This was investigated as described in Section 2. As can be seen (Table 2) there is a difference in the response of L1210 and L1210 T to the combination of the two compounds.

Indeed, in L1210 T the addition of 50 μM ATE alone or of 2.5 μM HCPA alone has no effect on cell growth, but

Table 2
Effect of ATE and HCPA on the growth of L1210 and L1210 T cells

Compounds	Inhibition (%)	
	Cells	
	L1210	L1210 T
ATE (50 μM)	0	0
HCPA (2.5 μM)	35	0
ATE + HCPA (50 + 2.5 μM)	35	27

To L1210 and L1210 T cells seeded each at a density of 1×10^5 cells/mL were added immediately after seeding vehicle alone (ethanol to a final concentration 0.5%), ATE alone, HCPA alone at the concentrations shown or ATE followed by HCPA. After 3 days, cell growth was measured by determining the number of viable cells in the presence of 0.1% trypan blue. Value are the means of two determinations each done in duplicate.

brings about 27% inhibition when added sequentially. Thus a neat synergy is observed. On the contrary, in L1210 no synergistic effect is observed when the cells are treated with 50 μM ATE and 2.5 μM HCPA. The 35% inhibition of cell growth seen in the presence of HCPA was not potentiated by the addition of ATE. From these results it would appear that in this model system, it is the transfected ALDH1 rather than the endogenous ALDH's which plays the major role in the resistance of L1210 T cells to HCPA. In view of this result, the newly synthesised amino-modified derivatives were screened for ALDH1 inhibitory activity first *in vitro* and then on cells in culture.

3.4. Effect of DIMATE, TRIMATE and MATE on the activity of baker's yeast ALDH1 in vitro

When this was investigated as described in Section 2, it was found that DIMATE (Fig. 1) was an inhibitor of

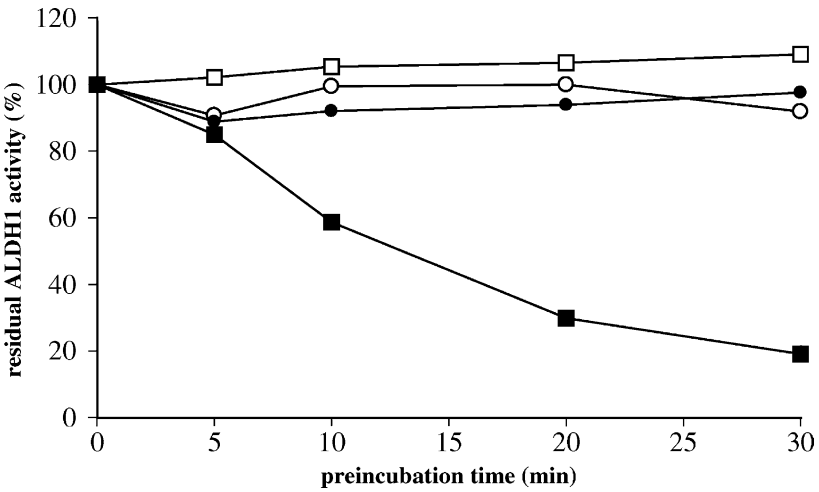


Fig. 1. Time course of inactivation of ALDH1 by DIMATE. The reaction mixture contained 1 mM EDTA, 100 mM KCl, in 60 mM sodium phosphate buffer pH 6, 206 mU baker's yeast ALDH in a final volume of 200 μL. DIMATE at a final concentration of 400 μM was added to each of a series of tubes incubated at 0° and at 37°. At intervals shown 100 μg BSA was added as a carrier to each tube and enzyme + BSA were removed from the incubation mixture by precipitation with acetone at −20° (80% final concentration). After standing for 1 hr at −20° the contents were centrifuged and washed once with 80% acetone. The precipitate was dissolved in 358 μL water and added to the reaction mixture described above buffered to pH 8.5 instead of pH 6 containing 2.35 mM NAD. The reaction was started by the addition of 2 mM propanal in a final volume of 1 mL. (○) Without DIMATE at 0°; (●) with DIMATE at 0°. (□) Without DIMATE at 37°; (■) with DIMATE at 37°. ALDH1 activity was measured by the increase in OD_{340} of the NADH formed and is expressed as ΔOD_{340}/min.

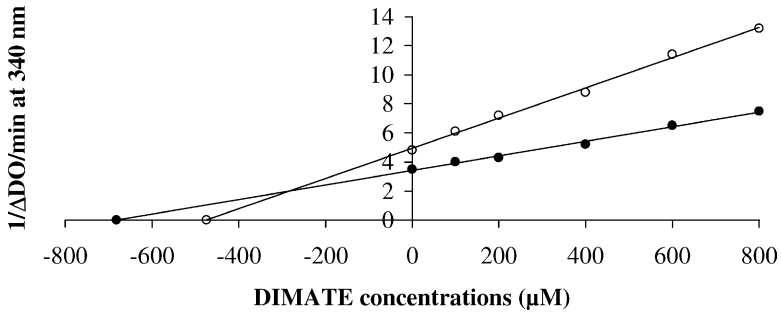


Fig. 2. Dixon plot of enzyme activity vs. inhibitor concentration. This was measured with glyceraldehyde 12 and 40 mM as substrate. To a reaction mixture containing 1 mM EDTA, 100 mM KCl, 2.35 mM NAD in 60 mM sodium phosphate buffer pH 7 was added 260 mU baker's yeast ALDH1 and DIMATE at the concentrations shown. The reaction was started by the addition of glyceraldehyde. The OD was read at 340 nm at 0 time (on adding the substrate) and at intervals of 5 min thereafter. (○) 12 mM glyceraldehyde. (●) 40 mM glyceraldehyde.

ALDH1 when the enzyme was preincubated with inhibitor at 37° but not at 4°. On the contrary, with TRIMATE and MATE only 15% inhibition was observed at even higher concentrations (400 and 600 μM). In view of these results, further investigations were pursued with DIMATE. Possible reasons for the comparably low activity of TRIMATE and MATE will be discussed later. As regards the type of inhibition, when it was examined using glyceraldehyde as substrate for reasons outlined in Section 2, it was found to be competitive as shown by the Dixon plot (Fig. 2) with an apparent K_i of 280 μM.

In the work-up of the incubation mixture to measure residual activity, enzyme was separated from free inhibitor by precipitation with acetone at −20° in the presence of 100 μg BSA. The precipitate was then dissolved and diluted 100-fold in the reaction mixture to measure enzyme activity. As inhibition was still observed with the enzyme preincubated with 400 μM DIMATE at 37° but not with that preincubated at 4° (Fig. 1) it indicated that inhibition was irreversible. To verify this, the acetone precipitates of enzyme–DIMATE mixtures at 37 and 4° were dialysed against a phosphate buffer pH 6 and the activity measured at the end of dialysis. It was found that the enzyme coming from the 37° incubation was still inhibited while that from the 4° had retained its activity. These results provide confirmatory evidence for irreversible inhibition.

3.5. Comparison of the growth inhibitory activity of ATE and DIMATE

This was investigated on the L1210 and L1210 T cell lines used so far, on other transformed cell lines: mouse melanoma B_{16}, human prostate carcinoma DU145, and on normal human diploid cells: embryonic lung fibroblasts MRC5 in order to determine the spectrum and eventual selectivity of any growth inhibitory activity. It is apparent (Table 3) that of the two derivatives DIMATE and ATE, DIMATE showed a 30–100-fold decrease in IC_{50} compared to ATE, in growth inhibitory efficacy towards all cell types, while still maintaining some preferential inhibition for the transformed cells compared to normal MRC5 cells. On the criteria of inhibitory activity to ALDH1 and efficacy in inhibiting growth, DIMATE was chosen for further experimentation. Now the overexpression of ALDH1 is not the only mechanism responsible for the resistance to chemotherapy. Indeed, it is well established that tumour cells become resistant to chemotherapeutic agents with chemical structures as diverse as those of cyclosphosphamide, bischloronitrosourea and camptothecin when they overexpress the bcl_2 gene (bcl_2^+) [19,20]. In as much as we had already shown that ATE itself was capable of inducing one bcl_2 overexpressing cell line: BAF_3 bcl_2 H_{16} into apoptosis [16] we now had to determine whether this proapoptotic effect was also manifest by DIMATE not only on this one cell line BAF_3 bcl_2 H_{16} but also on other mouse lymphoid bcl_2^+cell lines expressing different levels of bcl_2.

3.6. Determination of the level of expression of the bcl_2 protein in cells transfected with the bcl_2 gene

Before doing this, we first had to measure the level of expression of the bcl_2 protein in the different BAF_3 cell

Table 3
Effect of ATE and DIMATE on the growth of transformed and normal cells in culture

Compounds	Cells; IC_{50} (μM)				
	L1210	L1210 T	B_{16}	MRC5	DU145
ATE	90	140	25	1150	650
DIMATE	1.2	1.6	0.8	11.2	5.4

All cells were seeded at a density of 1×10^5 cells in 1 mL culture medium per well of 24-well plates. Compounds at different concentrations were added to the wells either immediately in the case of cells growing in suspension (L1210 and L1210 T) or 4 hr after seeding in the case of adhering cells MRC5, B_{16} and DU145. After 3 days in culture, the cell sheet of adhering cells was washed twice with PBS and harvested directly with 0.1 M NaOH. Cell growth was measured by determining both the protein and DNA content. For cells growing in suspension growth was measured by viable cell count in the presence of 0.1% trypan blue. The IC_{50} corresponds to the concentration of compound which inhibits growth by 50%. Values are means of two determinations for (L1210, L1210 T, B_{16}); three determinations for (MRC5) and five determinations for (DU145) each done in duplicate.

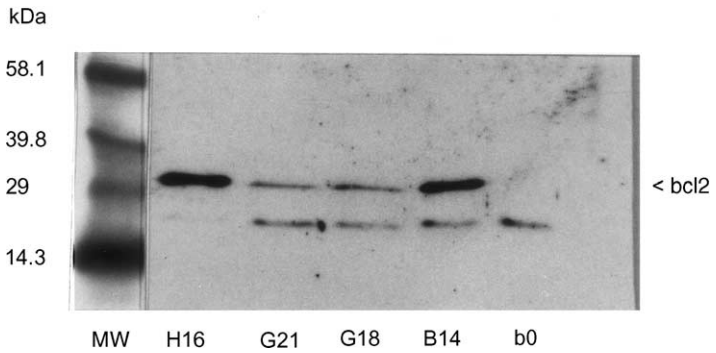


Fig. 3. Western blot analysis of mouse lymphoid cells H_{16}, G_{21}, G_{18}, B_{14} overexpressing the bcl_2 protein and on BAF_3 bo not expressing bcl_2. An amount of 100 μg protein lysate from each cell type was separated by electrophoresis on 10% PAGE. After transfer to a nitrocellulose membrane, the presence of the bcl_2 protein was revealed using as primary antibody a rabbit serum anti human bcl_2 and as secondary antibody a peroxidase labelled goat anti rabbit IgG POD activity was assessed by chemiluminescence and the intensity of the bands determined on a SAMBA™ scanner.

lines. This was investigated by Western blots, as described in Section 2, on lysates of the different cell lines. It is apparent (Fig. 3) that the bcl_2 protein is present at varying levels in all the bcl_2 transfected cell lines but not in the non-transfected control cells BAF_3 bo. From a scan of the blots, the degree of expression on an arbitrary scale was as shown in Table 4.

3.7. DNA fragmentation in four bcl_2^+ lymphoid cell lines treated with DIMATE for 24 hr

The addition of increasing concentrations of DIMATE (1–8 μM) induced DNA fragmentation in all four BAF_3 bcl_2^+ overexpressing cell lines and also in the control cells BAF_3 bo (Fig. 4). Therefore the induction of apoptosis in bcl_2 overexpressing cells by DIMATE would appear to be a generalised phenomenon. After 24 hr incubation however, loss of cell viability and cell lysis were too extensive to undertake meaningful enzymatic assays for ALDH1 on cell homogenates. The contact time with DIMATE was therefore reduced to 6 hr and DIMATE concentrations varied from 1 to 10 μM.

Table 4
Effect of DIMATE on ALDH1 activity of BAF_3 cells

Cells	bcl_2 expression (%)	DIMATE (μM)			
		0	1	2	4
		ALDH1 activity (mU/mg protein)	Inhibition (%)		
BAF_3 bo	0	20.4	8	20	21
BAF_3 bcl_2 G_{21}	8	19.5	7	16	23
BAF_3 bcl_2 G_{18}	31	16.3	0	8	20
BAF_3 bcl_2 B_{14}	71	19.1	16	19	32
BAF_3 bcl_2 H_{16}	100	17.7	20	17	23

Cells were seeded at 5×10^5 cells/mL in a total volume of 100 mL. DIMATE was added to the final concentrations shown. After 6 hr incubation, cells were harvested by centrifugation, washed twice with PBS and the pellets frozen at −20° for ALDH1 determinations. Pellets were lysed and lysates used for measuring ALDH1 activity as described in the legend to Table 1. Values are means of two determinations each done in duplicate.

3.8. ALDH1 activity in bcl_2^+ cells treated with DIMATE for 6 hr

As a first step, the endogenous ALDH1 activity of each of the strains was measured and as shown in Table 4 their ALDH1 activities were similar (16–20 mU/mg protein) even though bcl_2 expression varied from 0 to 100%. When treated with DIMATE, ALDH1 activity was inhibited in all the strains and no decrease in cell viability was observed. ALDH1 inhibition at 4 μM was between 20 and 30% for all the strains (Table 4). There was no apparent direct or inverse correlation between the degree of expression of

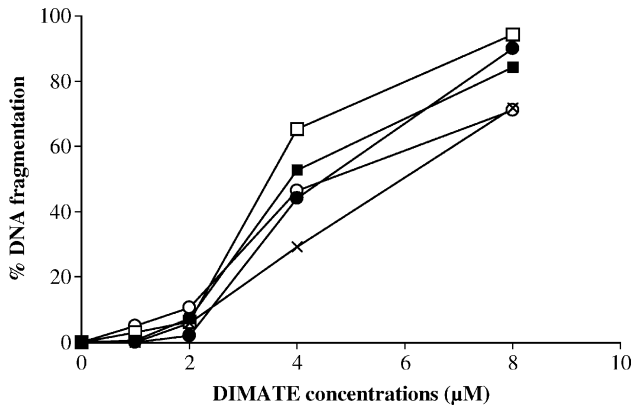


Fig. 4. Dose-dependent fragmentation of DNA in BAF_3 bo and BAF_3 bcl_2 cells incubated with DIMATE. BAF_3 bo (○) or BAF_3 bcl_2 (H_{16}, □); (B_{14}, ●); (G_{18}, ■); (G_{21}, +) at a density of 1×10^5 cells/mL were prelabelled with [^{3}H]thymidine for 40 hr at 37°. After harvesting and washing, 2.5×10^6 cells were resuspended in 5 mL culture medium in the absence or presence of DIMATE (1–8 μM). After 24 hr, cells were harvested, washed twice in PBS, lysed in 2 mL of 0.1% Triton X-100, 20 mM EDTA, 5 mM Tris–HCl, pH 8 for 16 hr at 4°, then centrifuged at 30,000 *g* for 30 min. 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 solubilised pellet (0.1 mL) were counted in a liquid scintillation counter. Percentage DNA fragmentation was calculated as follows:

$$\%\ \text{fragmented DNA} = \frac{\text{dpm in culture medium} + \text{dpm in supernatant}}{\text{dpm in culture medium} + \text{dpm in supernatant} + \text{dpm in solubilized pellet}} \times 100$$

bcl_2 and the susceptibility of ALDH1 to inhibition by DIMATE.

3.9. ALDH1 activity and DNA fragmentation in bcl_2 H_{16} cells treated with DIMATE for 6 hr

To determine whether ALDH1 inhibition was concomitant with DNA fragmentation, the H_{16} strain which expresses the greatest amount of bcl_2 was used as a typical model of bcl_2 overexpressing cells. H_{16} cells were incubated with 1–10 μM DIMATE for 6 hr, at which time ALDH1 activity and DNA fragmentation were measured. It is apparent (Fig. 5) that 20% inhibition of ALDH1 took place at concentrations of DIMATE from 1 to 4 μM at which no DNA fragmentation was seen. At higher concentrations of DIMATE 8 and 10 μM there was a steep rise in DNA fragmentation of 72 and 93%, respectively and an increase in the inhibition of ALDH1 activity which reached 38% at 8 μM and 54% at 10 μM DIMATE. This observation showing that at 6 hr ALDH1 is inhibited before DNA fragmentation takes place and increases further as DNA fragmentation itself increases, suggests that some type of relationship may exist between the endogenous aldehydes which accumulate when ALDH1 is inhibited and DNA fragmentation in bcl_2^+ cells. This will be discussed later. But we had previously shown that the mere inhibition of ALDH1 by disulfiram, a well-known irreversible inhibitor of the enzyme, did not induce BAF_3 bcl_2 H_{16} cells into apoptosis [17] whereas ATE was capable of doing so [16]. The question therefore arose whether the apoptogenic activity of DIMATE like that of ATE was due simply to the presence of a conjugated thiolester.

3.10. Requirement for both the conjugated thiol ester and the dimethyl ampal moiety for apoptosis

DNA fragmentation was compared in the cell line BAF_3 bcl_2 H_{16} (100% bcl_2) treated with ampal, the free amino aldehyde which does not have a thiolester group and is an irreversible inhibitor of ALDH1, methional thiolester (CH_3 SCH_2 CH_2 $COSCH_3$), a compound with a thiolester group but without the α, β-acetylenic carbonyl and ATE in which the conjugated carbonyl group is a thiolester. It is apparent (Fig. 6) that neither ampal as the free amino aldehyde nor methional thiolester is capable of inducing BAF_3 bcl_2 H_{16} into apoptosis. On the contrary, ATE does so. These results provide good evidence that the simultaneous presence of the thiolester functionality and enzyme inhibitory (ampal) moiety is essential for the apoptosis inducing activity of ATE on bcl_2^+ cells.

To confirm that the inhibition of ALDH1 activity was necessary for the apoptosis of BAF_3 bcl_2 H_{16} cells, we compared the apoptogenic activity of DIMATE to that of TRIMATE which also contains the thiolester and α, β acetylenic moieties but is a weak inhibitor of ALDH1. With DIMATE 50% fragmentation took place with 6.8 μM as previously shown in (Figs. 4 and 5) whereas with TRIMATE over the same concentration range no DNA

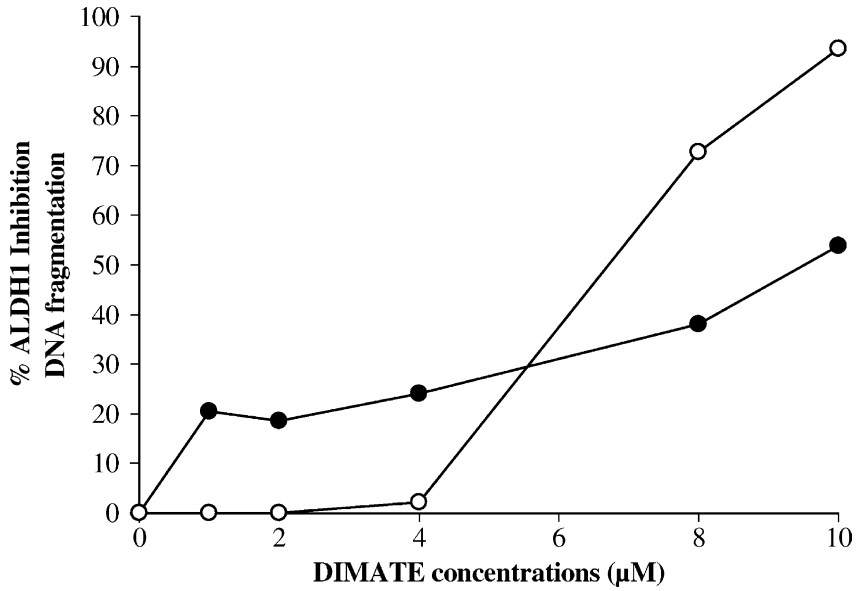


Fig. 5. Dose dependent inhibition of ALDH1 and of DNA fragmentation in BAF_3 bcl_2 H_{16} cells incubated with DIMATE. Cells at a density 5×10^5/mL were incubated for 6 hr with DIMATE at the concentrations indicated. At the end of this period, cells were harvested by centrifugation and washed with PBS. 3×10^6 cells were lysed directly in 500 μL 60 mM sodium phosphate buffer pH 7 containing 1% (v/v) Triton X-100, 1 mM EDTA for 30 min at 4°. Lysates were centrifuged at 48,000 *g* for 30 min and the supernatants used for measuring ALDH activity at pH 8.5 as described for baker's yeast ALDH, but with 1 mg lysate protein as source of enzyme. Activity was calculated as ΔOD/min/mg protein and inhibition as the % loss of activity of treated vs. that of non-treated cells. BAF_3 bcl_2 H_{16} cells at a density of 1×10^5/cells/mL were prelabelled with [^{3}H]thymidine for 40 hr at 37°. After harvesting and washing, cells at a density of 5×10^5/mL were resuspended in 5 mL culture medium for 6 hr in the absence or presence of DIMATE (1–10 μM). DNA fragmentation, (○); ALDH1 activity, (●).

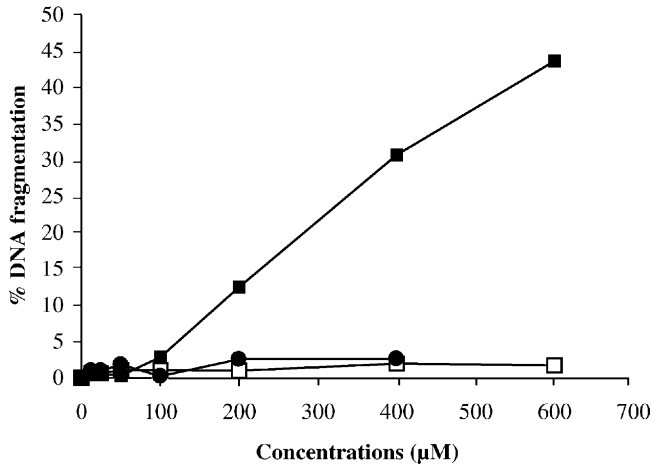


Fig. 6. Dose-dependent DNA fragmentation of BAF_3 bcl_2 H_{16} cells incubated with different concentrations of compounds. BAF_3 bcl_2 H_{16} cells at a density of 1×10^5/mL were prelabelled with [^{3}H]thymidine for 40 hr at 37°. After harvesting and washing, cells at a density of 5×10^5/mL were resuspended in 5 mL culture medium for 6 hr in the presence of different concentrations of compounds (□) ampal, (■) ATE, (●) methional thiolester. At the end of this period DNA fragmentation was measured on lysates as described for Fig. 3.

fragmentation was observed. This confirms that the thiolester and dimethyl ampal moieties are necessary for apoptosis.

3.11. Glutathione levels in DIMATE treated BAF_3 bcl_2 H_{16} cells

One possible role for the thiolester moiety could be in perturbing GSH homeostasis. Indeed, it is well established that cellular GSH levels decrease when cells go into apoptosis [31] and more so those of intramitochondrial GSH (mGSH) (reviewed in [32]). Accordingly mGSH levels were measured in BAF_3 bcl_2 H_{16} cells treated with 0, 6 and 10 μM DIMATE for 6 hr. As shown in Table 5 mGSH decreased by 47% at 6 μM and by 54% at 10 μM. These results provide at least one line of evidence for the perturbation of mGSH homeostasis when DIMATE-induced DNA fragmentation takes place and a possible mechanism will be discussed later.

Table 5
Dose-dependent decrease of mitochondrial GSH in BAF_3 bcl_2 H_{16} cells incubated with DIMATE for 6 hr

Additions DIMATE (μM)	GSH (nmol/100 μg protein)
0	1.67
6	0.89 ± 0.05
10	0.76 ± 0.01

Cells were seeded at 5×10^5/mL in a total volume of 100 mL. DIMATE was added to the final concentrations shown. After 6 hr incubation, cells were harvested by centrifugation, washed twice with PBS and resuspended in 1 mM EDTA, 250 mM sucrose, 0.1% BSA and 10 mM Hepes pH 7.4. After homogenisation and elimination of nuclei and unbroken cells by centrifugation at 1000 *g* for 5 min mitochondria were prepared by centrifugation at 20,000 *g* for 20 min through a Percoll/Metrizamide gradient. A total of 300 μL of mitochondria (about 400 μg protein) were deproteinised with an equal volume of 10% TCA. After removal of precipitated proteins a 100 μL aliquot of the supernatant was neutralised, treated with ABDF and left for 20 min at 50°, then cooled and acidified with 1 M HCl. *N*-acetyl cysteine was used as internal standard. Fifty microliter aliquots were separated by HPLC on a C18 μBondapak column 3.9 × 300 mm, 10 μm (Waters) attached to a fluorimeter, model 1050 (Hitachi) operated at λ ex 385 and λ em 515. Chromatograms were recorded on a Millenium workstation, peak heights integrated and responses calculated as the ratio of peak height of derivatised glutathione to that of internal standard. Values are means of two determinations each made in duplicate.

4. Discussion

The results presented provide evidence that of the three amino substituted derivatives of ATE which were synthesised, TRIMATE and MATE at the concentrations used (50–600 μM) were weak inhibitors of baker's yeast ALDH1 and showed only 15% inhibition at 600 μM. On the contrary, DIMATE at 400 μM was a competitive inhibitor of the enzyme *in vitro* (Fig. 1) with an apparent K_i of 280 μM. As regards irreversibility, little or no activity was found in the enzyme pre incubated with 400 μM DIMATE at 37° after separation of the enzyme from excess inhibitor by acetone precipitation. In addition, the dialysed enzyme which had been preincubated with DIMATE at 37° also showed no activity in contrast to that recovered from the preincubation at 4° which retained activity. These observations strongly indicate that inhibition was irreversible. The decrease in enzyme inhibitory activity of MATE and TRIMATE may be due to the bulky nature of the morpholino group, and/or positive charge of the trimethyl ammonium group. It could also have been due to the relatively short preincubation time (0–30 min) between the enzyme and weak inhibitors such as TRIMATE and MATE. However, there was no increase in inhibition when incubation time was prolonged to 60 min.

As ALDH1 can oxidise compounds like retinal [33] it supports the view that the substrate-binding site of the enzyme is capacious and therefore should be able to accommodate MATE. The reason for the decrease in activity of MATE therefore remains to be determined whereas a recent report describing the active site of the enzyme as hydrophobic [34] provides a more plausible explanation for the difficulty of a positively-charged compound like TRIMATE to enter and/or remain in the active site. Computer models of the active site of ALDH1 are being examined to try to obtain the structural parameters for the most effective inhibitors.

DIMATE can therefore be added to the group of inhibitors of ALDH1 which include the methane sulfonate and dimethylcarbamate derivatives of resorufin [35], a reporter group which confers on the enzyme a covalently-bound yellow colour when it reacts with Cys 302 in the active site [35]. DIMATE does not possess an easily identifiable reporter group but as an α, β-unsaturated carbonyl

compound, it could, like propynal [36] and vinyl ketone [37] also interact with active site Cys 302. Cys 302 of ALDH2 has also been shown to be the target of disulfuram but in a recent report it was shown using HPLC/MS that inhibition in this case is due to the formation of a disulfide bond between Cys 302 and one of the other adjacent cysteines at 301 or 303 [38]. Further work is therefore needed to determine the precise mechanism by which DIMATE inhibits ALDH1 activity (propanal as substrate) in murine cells more so as it has been found in rat hepatoma cells to inhibit not only ALDH1 (propanal as substrate) but also ALDH3 with benzaldehyde as substrate [39].

From the cell-culture experiments, it is clear that L1210 T cells which are resistant to the anti-proliferative action of HCPA, reacquire sensitivity when ALDH1 is partially inhibited. In fact, a synergistic effect is observed with HCPA at 2.5 μM and ATE at 50 μM (Table 2). As no synergistic effect is observed when non-transfected L1210 cells are similarly treated, indicates quite clearly that HCPA resistance in these cells is due to the overexpression of the transfected ALDH1 gene. These results do not rule out an effect of ATE on ALDH2 and ALDH3 in L 1210 and L1210 T cells, but even if this were the case, it would seem, from the results obtained in at least this model system, that these two classes of ALDH play no significant role in HCPA resistance.

As regards bcl_2-induced resistance to apoptosis we had previously reported that ATE was an inducer of apoptosis in the lymphoid cell line BAF_3 bcl_2 H_{16} overexpressing the bcl_2 gene [16]. In this present work, not only was DIMATE found to be more apoptogenic than ATE (ED_{50} DIMATE 6.8 μM vs. 400 μM for ATE) but it was also seen with a human epithelial prostate cancer cell line DU145 which overexpresses bcl_2 [40]. Therefore, the action of DIMATE would appear not to be restricted to cells of lymphoid origin only but this observation requires confirmation on many other epithelial cell lines overexpressing bcl_2 before it can be generalised.

However, no correlations were found in the murine cells between the degree of expression of the bcl_2 protein and their endogenous ALDH1 activity, (all around 20 mU/mg protein) or the susceptibility of their ALDH's to DIMATE, or even the concentration of DIMATE required to induce DNA fragmentation. What did emerge from this study though, is that the inhibition of ALDH1 activity at 6 hr took place at DIMATE concentrations (1–4 μM) at which no DNA fragmentation was seen and at 8 and 10 μM increased concomitantly with DNA fragmentation, suggesting therefore some type of causal relationship between the accumulation of endogenous apoptogenic aldehydes and DNA fragmentation. The crucial question which remained concerned the identity of the endogenous aldehydes whose accumulation induces DNA fragmentation.

This question was not addressed in the present work but our previous results had shown that methional $CH_3SCH_2CH_2CHO$, a mitochondrial metabolite of the oxoacid of methionine: 4-methylthio-2-oxo-butanoate, is a powerful apoptogenic agent which loses its growth inhibitory effect when it is oxidised by ALDH1 to methyl thio propionic acid [18]. In fact, this was one of the findings which prompted the design and synthesis of inhibitors of ALDH1. BAF_3 bcl_2 H_{16} cells could not be induced into apoptosis by methional even at 200 μM, but a synergistic apoptotic effect was observed in BAF_3 bcl_2 H_{16} cells when ALDH1 was inhibited by ATE in the presence of 200 μM methional [16]. As DIMATE is even more apoptogenic than ATE (ED_{50} 6.8 μM vs. 400 μM) the same mechanism can be invoked here. The endogenous methional which accumulates when ALDH1 is blocked could then undergo β hydroxylation as shown by its susceptibility to the Fenton reagent [18] to give methanethiol and malondialdehyde, a chromatin cross-linking agent *in vivo* [41]. Chromatin condensation is one of the early hall-marks of apoptosis [42].

But the inhibition of ALDH1 alone is not sufficient to induce apoptosis. Indeed, ATE and DIMATE the two thiolester inhibitors of ALDH1 were apoptogenic whereas no apoptosis of BAF_3 bcl_2 H_{16} cells was observed with non-thiolester inhibitors like disulfuram [17] or ampal (Fig. 5). This strongly suggests that the methanethiol present on the thiolesters ATE and DIMATE also contributes to their apoptogenic activity. One way in which thiolester inhibitors may contribute is by acting as substrates for ALDH1 whose active site exhibits two activities: a dehydrogenase and an esterase [43,44]. The inhibition of ALDH1 dehydrogenase activity which was seen at 37° but not at 4° (Fig. 1) raises the possibility that the methane thiol moiety may have been cleaved at 37°. The CH_3SH so released could then interact with intracellular GSH. In favour of this hypothesis is the observation that a decrease in mGSH accompanies DIMATE-induced apoptosis (Table 5). A similar finding has also been reported for Jurkatt cells induced into apoptosis by treatment with antibodies to CD95 [45]. To try to obtain direct evidence for the interaction of this putative CH_3SH with GSH, the presence of GSH as the mixed disulphide $GSSCH_3$ in DIMATE-treated cells was looked for, but with no success so far. BAF_3 cells treated with DIMATE still continued to express the bcl_2 protein (results not shown) but its subcellular localisation was not determined. We therefore do not know if the mitochondrial permeability transition pore which is reportedly closed in the presence of bcl_2 [32] was opened in the presence of DIMATE.

Regardless of the detailed mechanism whereby DIMATE induces apoptosis in chemoresistant bcl_2^+ cells, this compound, by its combined action on ALDH1 and on mitochondrial GSH merits further investigation to determine, on one hand, the precise mechanism by which it inhibits ALDH1, decreases mGSH levels, perturbs intracellular methional homeostasis and, on the other, its efficacy as an adjuvant in apoptogenic regimens for treating chemoresistant cancer cells grafted in experimental animals.

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