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Methional derived from 4-methylthio-2-oxobutanoate is a cellular mediator of apoptosis in BAF_3 lymphoid cells

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4-Methylthio-2-oxobutanoic acid is the direct precursor of methional, which is a potent inducer of apoptosis in a BAF_3 murine lymphoid cell line which is interleukin-3 (IL_3)-dependent. Cultures treated for 8 h with methional in the presence of IL_3 show extensive DNA double-strand breaks on flow cytometric analysis, increases in DNA fragmentation as measured by the amount of non-sedimentable DNA present in the 30000 *g* supernatant of cell lysates and the typical laddering pattern of multiples of 180 bp seen upon agarose gel electrophoresis. No such features of apoptosis were found in cells treated with 4-methylthio 2-oxobutanoic acid or propanal, suggesting that the simultaneous presence of the methylthio group on the propanal moiety is essential for apoptosis to take place. Methional is further metabolized in cells by two reactions: oxidation via aldehyde dehydrogenase to (methylthio)propionic acid or β-hydroxylation to malondialdehyde. The formation of malondialdehyde from methional *in vitro* by chemical hydroxylation under the conditions of the Fenton reaction provides a mechanism for the β-hydroxylation which takes place *in vivo*. During apoptosis induced by IL_3 deprivation, the ratio of 2,4-DNPH MDA to 2,4-DNPH methional is 0.94 in cells in IL_3^- medium compared with 0.54 in cells in IL_3^+ medium. These results support a role of cellular methional and malondialdehyde in apoptosis.

INTRODUCTION

Previous work from this laboratory has provided evidence to show that the inhibition of the glutamine-dependent cellular transaminase responsible for the conversion of 4-methylthio-2-oxobutanoate (MTOB) to methionine (Met) is accompanied by a concomitant decrease in the growth of transformed but *not* that of normal cells in culture [1]. This selective growth inhibition was observed with transition-state (TS)-type inhibitors containing pyridoxal and esters either of L-Met or of its structural analogues, such as ethionine or seleno-Met. It was not seen with TS compounds composed of pyridoxal and D-Met esters.

As this inhibition of growth could not be reversed after 3 days by removing the medium with the TS inhibitor and replacing it with fresh medium containing $\leqslant$ 1 mM Met, it appeared that growth arrest was caused not by the depletion of cellular Met but rather by excess MTOB, which had accumulated when the MTOB transaminase was blocked. MTOB, which is formed in cells in the Met salvage pathway from methylthioadenosine [2–4] arising from decarboxylated *S*-adenosyl-L-Met during spermidine and spermine synthesis [5], might act as a growth inhibitor as it is the precursor of (methylthio)propionic acid (MTPA) from which toxic metabolites such as methanethiol and hydrogen disulphide have been shown to arise [6].

The physiological importance of Met degradation via the reversal of the MTOB transaminase pathway is still controversial [7–9]. It has nevertheless been clearly established that MTOB is metabolized *in vivo* by the branched chain oxo-acid dehydrogenase complex (BCOADC) present in liver [9], heart [10] and skeletal muscle [11] via methional to (methylthio)propionyl coenzyme A (MTPCoA).

In view of the apparent tissue specificity of BCOADC, two questions can be raised. What is the identity of the MTOB-derived metabolite responsible for inhibiting the growth of the epithelial cells (HeLa) used in our experiments? Is growth inhibition attributable to the toxic effects of methanethiol and hydrogen disulphide formed from MTPA or is it attributable to the methional formed?

This latter question is all the more important when one notes that both MTOB and methional are free-radical scavengers [12] and ˙OH has recently been shown to be involved in apoptotic cell death [13]. Moreover, there is a paucity of information concerning the identity of the cellular metabolites which are involved in apoptosis.

To investigate MTOB metabolism and, in particular, its role as a precursor of growth inhibitory metabolites, we drew up a metabolic scheme (Scheme 1) from established data [12,14] and hypothetical considerations.

According to this scheme, MTOB is oxidatively decarboxylated by BCOADC to methional, which can then undergo several metabolic conversions: *oxidation* directly to MTPA by the action of aldehyde dehydrogenase (ALDH); or transacylation to MTPCoA, which is then cleaved to yield MTPA. The oxidation of methional to MTPA and its putative action as a growth inhibitor needs therefore to be assessed.

The *reduction* of methional to methionol is a theoretical possibility and could be mediated by aldehyde reductase (ALDR) using NADH or NADPH as a cofactor. However, to our knowledge, no mention has been made so far of the presence of this metabolite in biological samples. Thus the action of methionol as a growth inhibitory agent needs also to be verified.

Concerning the hypothetical β-hydroxylation pathway for

Abbreviations used: ALDH, aldehyde dehydrogenase; ALDR, aldehyde reductase; BCOADC, branched-chain oxo-acid dehydrogenase complex; CoA, coenzyme A; 2,4-DNPH, 2,4-dinitrophenylhydrazine; e.i., electron impact; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; h.p., high-performance; IL_3, interleukin-3; kb, kilobase; MDA, malondialdehyde; MEM, Eagle's minimum essential medium; Met, methionine; MTOB, 4-methylthio-2-oxobutanoate; MTPA, (methylthio)propionic acid; MTPCoA, (methylthio)propionyl CoA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; TBA, thiobarbituric acid; TdT, terminal deoxynucleotidyl transferase; TS, transition-state.

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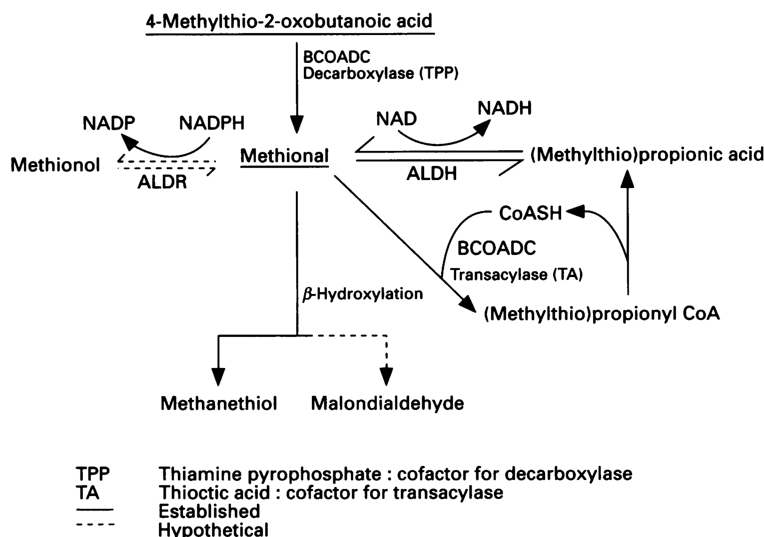


Scheme 1 Scheme of metabolic pathway

methional, reports already exist in the literature showing that products such as ethylene and dimethyl sulphide are formed from it after hydroxy-radical attack [15]. However, the formation of malondialdehyde (MDA) has not been described so far. Recently, MDA has been shown to form DNA cross-links *in vitro* [16] and, more importantly, when it is generated *in situ* from propane-1,3-diol in rat liver and testis *in vivo* [17].

Because we were interested in MTOB-derived metabolites which could block cell growth, we decided that, in addition to the putative oxidative and reductive products of methional described above, we would determine whether the formation of MDA from methional does take place chemically *in vitro* and enzymically in intact cells in culture. It is the evidence for the growth inhibitory activity of methional, its production in cells from MTOB and its action as an inducer of apoptosis in BAF_3 lymphoid cells which will be reported here.

MATERIALS AND METHODS

Reagents

L-Met, MTOB, ALDH from baker's yeast, Hoechst 33258 bisbenzimide trihydrochloride, dATP, dCTP, dGTP, avidin-labelled fluorescein isothiocyanate (FITC), propidium iodide (PI), sodium cacodylate, cobalt chloride, BSA, RNAase A, 1,1,3,3-tetraethoxypropane and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.); methional, methionol and thiobarbituric acid (TBA) from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); MTPA from Fluka Chemika-Biochemika (Buchs, Switzerland); biotin-16-dUTP, terminal deoxynucleotidyl transferase (TdT) from calf thymus, and NAD from Boehringer (Mannheim, Germany); and 2,4-dinitrophenylhydrazine (2,4-DNPH) and high-performance (h.p.)-t.l.c. aluminium sheets silica gel 60 from Merck (Darmstad, Germany). Culture media were from Gibco (Grand Island, NY, U.S.A.). Foetal calf serum (FCS) was from ATGC Biochemical Industries (Paris, France). L-[3,4-^{14}C]Met was from Dositek (Orsay, France) and L-[^{35}S]Met was from Amersham (Les Ulis, France).

Cell lines

HeLa cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.). BAF_3 murine bone marrow-derived cell line and Wehi-3B cells were a gift of Dr. J. Marvel (E.N.S., Lyon, France).

Culture media

Cells were maintained at 37 °C in a humid atmosphere of air/CO_2 (95:5). HeLa cells were grown in Eagle's Minimum Essential Medium (MEM) containing 7% (v/v) dialysed FCS.

BAF_3 cells were maintained in Dulbecco's modified MEM (DMEM) containing 6% (v/v) FCS and 5% (v/v) Wehi-3B cells conditioned medium used as a source of interleukin-3 (IL_3) as previously described [18]. They were grown at 37 °C in a humid atmosphere of air/CO_2 (9:1).

Preparation of [^{14}C]MTOB or [^{35}S]MTOB

Oxidative deamination of 2MBq of L-[3,4-^{14}C]Met (2.18 GBq/mmol) or 37 MBq of L-[^{35}S]Met (7.55 GBq/mmol) was carried out by an adaptation of the method described in [19]. After 24 h of oxidation of Met by L-amino acid oxidase (10 units; 1 unit oxidatively deaminates 1.0 μmol of L-phenylalanine/min at pH 6.5 and at 37 °C) in 10 mM sodium/potassium phosphate buffer, pH 7.2, containing 0.14 M NaCl in the presence of catalase (110 units; 1 unit of catalase decomposes 1.0 μmol of H_2O_2/min at pH 7.0 and at 25 °C), the oxoacid formed (MTOB) was isolated by means of cation-exchange chromatography (column of Dowex 50 in the H^+ form). The radiochemical purity of MTOB was controlled by t.l.c. on silica gel 60 (Merck) with butan-ol/acetic acid/water (4:1:2, by vol.) as solvent by radiochromatogram scanning on a System 200 Imaging scanner (Bioscan Inc.).

Effect of metabolites on the growth of HeLa cells

MTOB-derived metabolites

HeLa cells (0.5×10^6/Petri dish) were seeded in 3 ml of MEM containing 10% dialysed FCS. Four hours later, methional (0.01–1 mM), methionol (0.01–20 mM) or MTPA (0.001–20 mM) was added to each of three dishes. After 3 days at 37 °C, the cells were washed twice with 50 mM PBS, pH 7.5, and harvested in this same buffer. Cell growth was routinely assessed by measuring protein [20] or the DNA content in lysates using Hoechst 33258 [21], or by determining lactic dehydrogenase

activity using MTT [22]. Data represent the means of three or four experiments.

2-Oxobutyrate

HeLa cells (0.5×10^6/Petri dish) were seeded in 3 ml of MEM deprived of Met (Met^-) with 10% dialysed FCS. Four hours later, 2-oxobutyrate (10–40 mM) was added in presence of 2 mM MTOB. After 3 days at 37 °C, cell growth was measured as described above.

Effect of metabolites on DNA fragmentation in BAF_3 cells

Cell lysates

BAF_3 cells (3×10^6) cells were seeded in 6 ml of culture media containing IL_3 in Petri dishes in the presence of different concentrations (200–800 μM) of methional, MTOB or propanal. After contact for 8 h, the cells were washed three times in PBS. Pelleted cells were lysed in 2 ml of 0.1% (v/v) Triton X-100, 20 mM EDTA, 5 mM Tris, pH 8, and then centrifuged at 30000 *g* at 4 °C for 30 min. Supernatants were decanted and subjected to two types of analyses.

Quantitative spectrofluorophotometrical analysis (adapted from a method reported previously [21]) was used to measure the concentration of DNA fragments [< 3 kbases (kb)]. This was done by adding, to 2 ml of supernatant, 1 ml of Hoechst 33258 (1 μg/ml) in 3 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8, and monitoring net fluorescence ($\lambda_{max.}$ excitation 365 nm, $\lambda_{max.}$ emission 460 nm). DNA values were calculated against a highly purified DNA standard (0.1–1 μg in 2 ml of lysis buffer) treated as described for the samples and were expressed as nanograms of DNA recovered from 3×10^6 cells.

Qualitative analysis of DNA fragmentation was measured by the method described in [21,23]. The supernatants were first treated with ribonuclease A (20 μg/ml) for 1 h at 37 °C (in the presence of 0.5% SDS) and then with proteinase K (100 μg/ml) for 3 h at 50 °C. DNA was purified by two phenol extractions and precipitated with ethanol.

Small-molecular-mass DNA in the final extract was analysed by electrophoresis in 1% (w/v) agarose gels. Following electrophoresis (60 V, 2 h) in 0.045 Tris/boric acid buffer, pH 8, containing 0.001 M EDTA, the gels were stained for 30 min with ethidium bromide (0.5 μg/ml).

Single cells by flow cytometry

DNA fragmentation in single cells was detected by flow cytometry as described in [24]. Following incubation with 600 μM of metabolites for 8 h, 6×10^6 cells were washed in PBS and fixed in 0.5 ml of 1% (v/v) formaldehyde in PBS for 15 min at 4 °C. After two washes in PBS, cells were resuspended in 0.5 ml of 70% ice-cold ethanol and stored at −20 °C for 3 days. After washing in PBS, cells were resuspended in 50 μl of a solution containing 0.1 M sodium cacodylate, pH 7.5, 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 0.05 mg/ml BSA, 10 units of TdT [1 unit of TdT incorporates 1 nmol of dAMP into acid-insoluble products/h at 37 °C using $d(pT)_6$ as primer], 0.5 nmol of biotin-16 dUTP (biotin-16 dUTP is a TTP analogue which carries a biotin molecule linked via a 16 spacer arm to the 5-position of the pyrimidine base), 0.5 nmol of dATP, 0.5 nmol of dCTP and 0.5 nmol of dGTP for 1 h at 37 °C. After 3 washes in PBS, cells were resuspended in 100 μl of 4 × SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) containing 2.5 μg/ml avidin–FITC, 0.1% (w/v) RNAase A, 0.1% Triton X-100 and 5% (w/v) non-fat dry milk for 30 min at room temperature in the dark. Cells were washed in PBS and resuspended in 1 ml of PBS containing 3 μg/ml PI.

Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.); the red (PI) and green (FITC) fluorescence emissions were analysed. The data from 1×10^4 cells were collected and analysed using LYSYS II software.

Preparation of 2,4-DNPH derivatives of reference compounds

The preparation of the 2,4-DNPH derivatives of MTOB, methional and MDA was carried out as described [25], using a solution of 2,4-DNPH made up as follows: 1 g of 2,4-DNPH was dissolved in 5 ml of concentrated H_2SO_4 diluted with 7 ml of water and 25 ml of 95% (v/v) ethanol. MTOB or methional (1 mmol of each) was mixed with 1.05 mmol of 2,4-DNPH present in 5% excess. To prepare 2,4-DNPH pyrazole, the product of the reaction of MDA with 2,4-DNPH, 1 mmol of 1,1,3,3-tetraethoxypropane was added to 2 mmol of 2,4-DNPH and the mixture was heated at 70 °C for 30 min to cleave the protecting tetraethoxy group.

^{1}H-n.m.r. measurements were carried out on a Bruker AM200 in the solvent indicated. Chemical shifts are given in p.p.m. [*J* in Hz relative to tetramethylsilane (0.0 p.p.m.)]. Electron impact (e.i.)-m.s. (70 eV) data, given in m/z (relative %), were measured on a Nermag R10-10S mass spectrometer.

2,4-Dinitrophenylhydrazone of 3-methylthiopropanal

^{1}H-n.m.r.: δ (p.p.m.) [$CDCl_3$] 2.18 (3 H, s, CH_3S), 2.81 (4 H, m, $CH_3SCH_2CH_2$), 7.60 (1 H, m, Ar C*H*), 7.93 (1 H, d, *J* 9.6 Hz, Ar C*H*), 8.31 (1 H, dd, *J* 2.5 and 9.6 Hz, Ar C*H*), 9.12 (1 H, d, *J* 2.5 Hz, Ar C*H*), 11.08 (1 H, broad s, N*H*).

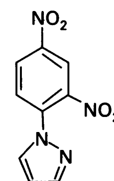
2,4-Dinitrophenylhydrazone of 4-methylthio-2-oxobutanoic acid

^{1}H-n.m.r.: δ (p.p.m.) [$CDCl_3$] (*syn*/*anti* mixture, 1:1, by vol.) 2.19 and 2.26 (3 H, s, CH_3S), 2.9–3.2 (4 H, M,), 8.11 and 8.15 (1 H, d, *J* 9.4 Hz, Ar C*H*), 8.45 (1 H, m, Ar C*H*), 9.15 and 9.19 (1 H, d, *J* 2.4 Hz, Ar C*H*), 11.75 (1 H, broad s, N*H*), 14.2 (1 H, broad s, CO_2H).

1N-(2,4-Dinitrophenyl)pyrazole

^{1}H-n.m.r.: δ (p.p.m.) [acetone-d_6] 6.65 (1 H, m, 1N-CH=C*H*-), 7.81 (1 H, d, *J* 1.6 Hz, 1N-C*H*=), 8.15 (1 H, d, *J* 8.9 Hz, 1N-CH=CH-C*H*=N), 8.38 (1 H, d, *J* 2.5 Hz, Ar C*H*), 8.67 (1 H, dd, *J* 2.5 and 8.9 Hz, Ar C*H*), 8.80 (1 H, d, *J* 2.5 Hz, Ar C*H*).

E.i.-m.s.: m/z 234 (M^+, 38), 217(19), 204(40), 177(21), 158(83), 115(68), 103(49), 88(67), 75(75) and 63(100) (the peak at m/z 63 was taken as 100%).



Analysis of radiolabelled carbonyl products derived from [3,4-^{14}C]MTOB in BAF_3 cells

[3,4-^{14}C]MTOB (5×10^6 d.p.m. corresponding to 38.2 nmol) was added to a 100 ml BAF_3 cell suspension (2.4×10^5 cells/ml) in DMEM containing 6% (v/v) FCS and 5% (w/v) IL_3. After 39 h, the medium was removed, the cells washed twice with PBS

and resuspended at 9×10^5 cells/ml in 100 ml of DMEM supplemented with 6% (v/v) FCS and 5% IL_3 (IL_3^+) or in this same medium but without IL_3 (IL_3^-). To both suspensions (10^8 cells) was added [3,4-^{14}C]MTOB (1.9×10^6 d.p.m., corresponding to 14.5 nmol), and 8 h later, cells were harvested and centrifuged at 400 ***g*** for 5 min. The cell pellets were solubilized with 1.5 ml of 0.5 N NaOH for 30 min at 60 °C, after which was added PCA (to a final concentration of 0.5 M) and 200 μmol of 2,4-DNPH, and the mixture was heated at 70 °C for 30 min. After alkalinization with NaOH, labelled metabolites were extracted into ethyl acetate.

Methional, a substrate of ALDH

The oxidation of methional (0.018–2.3 mM) by the action of ALDH (8.5 m-units; 1 m-unit of ALDH oxidizes 1.0 nmol of acetaldehyde/min at pH 8 and 26 °C) from baker's yeast was carried out in 0.2 M Tris, pH 8, in the presence of 0.2 mM NAD as cofactor, 0.1 M KCl, 1.5 mM EDTA and 1.5 mM 2-mercaptoethanol in a total volume of 0.2 ml, in a microtiter plate. Control incubations lacked the enzyme. A_{340} measurements were made after adding ALDH. The kinetic parameters of NADH formation were measured at 25 °C for 20 min at 2.5 min intervals. All experiments were performed in duplicate.

Chemical conversion of methional into MDA

The chemical hydroxylation of methional by the Fenton reaction was carried out in 1.0 ml of PBS containing 0.2 mM methional, 0.02 mM $FeSO_4$, 0.02 mM ascorbate, 0.02 mM EDTA and 0.2 mM H_2O_2. In one set of controls, all the reagents were present except $FeSO_4$, ascorbate and EDTA. In another, only H_2O_2 was missing. After incubation for 2 h at 37 °C, 0.5 ml of concentrated acetic acid and 1.5 ml of 10 mM TBA, dissolved in water and adjusted to pH 8 with NaOH, were added and the mixture was heated for 1 h at 90 °C. The aqueous phase was extracted twice with butanol pre-saturated with HCl.

After centrifugation at 400 ***g*** for 5 min, the fluorescence of the TBA derivatives in the butanol extract was recorded on a fluorimeter ($\lambda_{max.}$ excitation 532 nm, $\lambda_{max.}$ emission 553 nm). The product was identified by comparing its R_f in two different solvent systems with those of tetraethoxypropane which had been submitted to the same TBA derivatization procedure. The solvent systems used were: chloroform/methanol/water (65:25:4, by vol.) and chloroform/methanol/acetic acid (60:20:10, by vol.).

RESULTS

Growth inhibition by MTOB-derived metabolites

To determine which of the MTOB-derived metabolites might be responsible for the growth arrest of transformed cells, methional, methionol and MTPA were added individually to the medium of HeLa cells in culture. The results (Table 1) show that the concentrations of MTPA, methionol and Met (used for comparison) necessary for obtaining 50% inhibition of growth (IC_{50}) are greater than the 1.6 mM found for MTOB, whereas it was 100 μM for methional. Because methionol and MTPA are growth inhibitory only at concentrations which are at least 6-fold *greater* than that of MTOB, they cannot be responsible for the growth arrest seen with 1.6 mM MTOB. This left methional as the sole possible candidate. MDA, a hypothetical metabolite, was not assessed for growth inhibitory activity because of the ease with which this molecule would react with nucleophiles present both in the culture medium and in cells. Hence, any results obtained would have been difficult to interpret.

Table 1 Growth inhibitory activity of MTOB-derived metabolites

HeLa cells were harvested 3 days after adding various concentrations of the different MTOB-derived metabolites. The IC_{50} was measured and cell growth was assessed by measuring the protein or DNA content, or the LDH activity (MTT) of adherent cells. Only data for protein content are given.

Product	IC_{50} (mM)
L-Methionine	> 20.0
MTOB	1.6
Methional	0.1
Methionol	> 20.0
MTPA	10.0

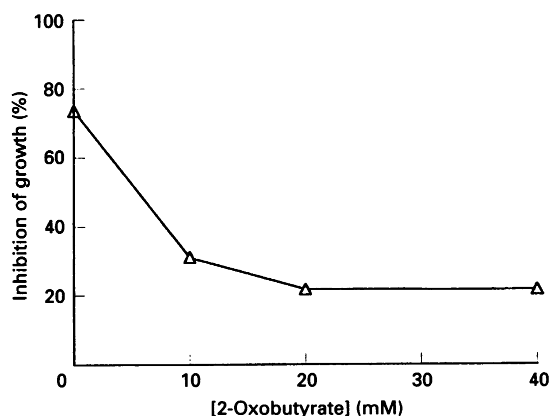


Figure 1 Reduction by 2-oxobutyrate of the growth inhibition induced by MTOB

2-Oxobutyrate (10–40 mM) was added to HeLa cells in the presence of 2 mM MTOB for 3 days. Cell growth was assessed by measuring the protein content of adherent cells. Data are means ($n = 2$).

MTOB decarboxylation is essential for growth inhibition

To try to obtain additional evidence that growth inhibition is effectively caused by the oxidative decarboxylation of MTOB to methional, the capacity of 2-oxobutyrate, a well known substrate of BCOADC (K_m 18 μM) [11] to reduce the 72% growth inhibition induced by 2 mM MTOB was assessed (Figure 1). It is apparent that in the presence of $\geqslant$ 10 mM 2-oxobutyrate, growth inhibition is reduced three-fold. Thus the shunting of BCOADC activity away from MTOB towards a non-methional-forming precursor such as 2-oxobutyrate decreases growth inhibition. It would therefore appear that methional is at least one of the growth inhibitory metabolites that can be derived from MTOB.

Methional as a substrate for ALDH

This experiment was carried out as described in the Materials and methods section. It is apparent from the increase in NADH (Figure 2) that methional is indeed a substrate (K_m 1.1 mM). Further, this increased formation of NADH is accompanied by a concomitant decrease in the concentration of substrate methional measured as the methyl benzolone thiazolone hydrazone derivative in the presence of Fe^{3+} (results not shown).

The evidence presented so far for the direct inhibition of growth by 100 μM methional, the dependence of growth inhibition on MTOB decarboxylation, the reports in the literature

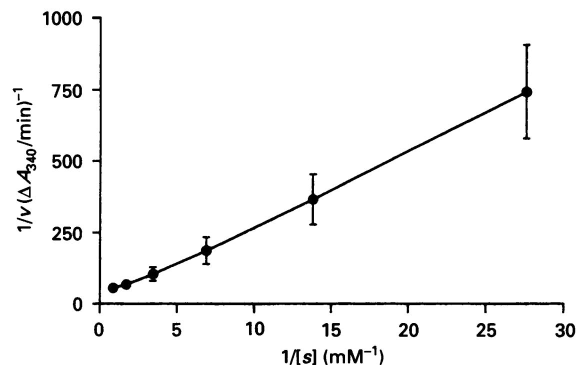


Figure 2 Double-reciprocal of 1/[v] against 1/[s] for baker's yeast ALDH with methional as a substrate

The reaction mixture contained various concentrations of methional (0.018–2.3 mM), 0.2 mM NAD and 8.5 m-units of baker's yeast ALDH in a total volume of 200 μl. ALDH activity was measured by the increase in A_{340} of the NADH formed and is expressed as ΔA_{340}/min. Data are means ± S.E.M. ($n = 3$–4).

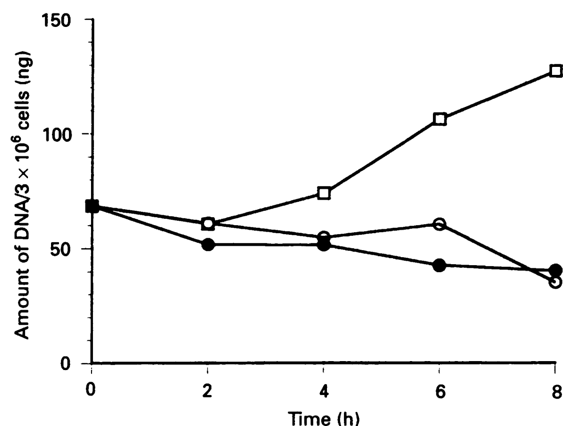


Figure 3 Time-dependent fragmentation of DNA in BAF_3 cells according to the metabolite added

Time course of accumulation of DNA in the non-sedimentable fraction of BAF_3 cells treated with 600 μM methional (□), MTOB (●) or propanal (○) up to 8 h. DNA was measured by its fluorescence in the presence of Hoechst 33258. Data represent means ($n = 2$).

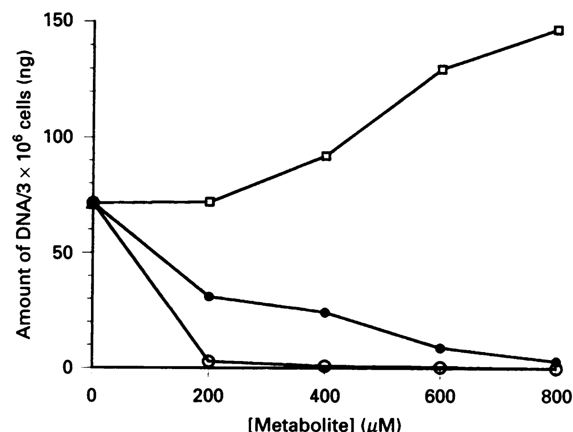


Figure 4 Dose-dependent fragmentation of DNA in BAF_3 cells incubated with different concentrations (200–800 μM) of metabolite

Methional (□), MTOB (●) or propanal (○) was incubated with 3×10^6 BAF_3 cells for 8 h. Non-sedimentable DNA in lysates was quantified by fluorescence with Hoechst 33258. Results are expressed as ng of DNA/3×10^6 cells. Data represent means ($n = 2$).

Table 2 Comparison of DNA fragmentation in IL_3^+ and IL_3^- samples and in IL_3^+ sample treated with 600 μM methional

BAF_3 cells (3×10^6) were harvested after 8 h treatment and non-sedimentable DNA in lysates was measured by fluorescence with Hoechst 33258.

Samples	Amount of DNA/3×10^6 cells (ng)
$+IL_3$	63
$+IL_3$ + methional	127
$-IL_3$	150

showing that MTOB and methional are efficient hydroxy-radical scavengers [12,15], and the recently reported involvement of hydroxy radicals in apoptosis [13] raised the question whether the growth arrest we had previously seen with MTOB and methional was caused by apoptosis.

Effect of MTOB and methional as inducers of apoptosis

To determine whether MTOB and methional were involved in apoptosis we used as a model system the BAF_3 lymphocyte cell line, which requires IL_3 for growth and in which more than 80 % of the cells undergo apoptosis in the absence of IL_3 during 16 h [18]. DNA fragmentation was measured quantitatively using two methods: flow cytometry of whole cells, and Hoechst 33258 assay of cell lysates.

The degree of fragmentation was assessed qualitatively according to the fragmentation pattern of the non-sedimentable DNA present in lysates after electrophoresis on 1 % agarose gels. As the quantitative assay using Hoechst 33258 could be carried out rapidly, we used it to determine first the contact time and the concentration of methional necessary to induce apoptosis. It is apparent (Figure 3) that the amount of non-sedimentable DNA in lysates from cells treated with 600 μM methional increases with time up to 8 h after treatment. Fragmentation also increased with increasing concentrations of methional but began to reach a plateau at 600–800 μM (Figure 4). Thus for further experimentation cells were treated with 600 μM methional for 8 h. There was no change in cell viability as assessed by Trypan Blue exclusion under these conditions. It should also be noted that the 2–3-fold increase in fragmentation found in cells treated with IL_3 and methional is approximately similar to that found when BAF_3 cells were placed in a medium deprived of IL_3 for the same 8 h period (Table 2). In contrast, in the presence of equimolar concentrations of MTOB, there was no increase compared with controls (Figures 3 and 4).

The induction of apoptosis by methional raised the question whether the effect was caused by methional itself or by methional acting as a substrate for ALDH, thereby sparing some intracellular aldehyde(s), the endogenous mediator(s) of apoptosis. To investigate this, the capacity to induce apoptosis of another three-carbon aldehyde, propanol, well known to be a substrate of ALDH [26], was investigated. As also shown (Figures 3 and 4), apoptosis does not take place in BAF_3 cells treated with propanal

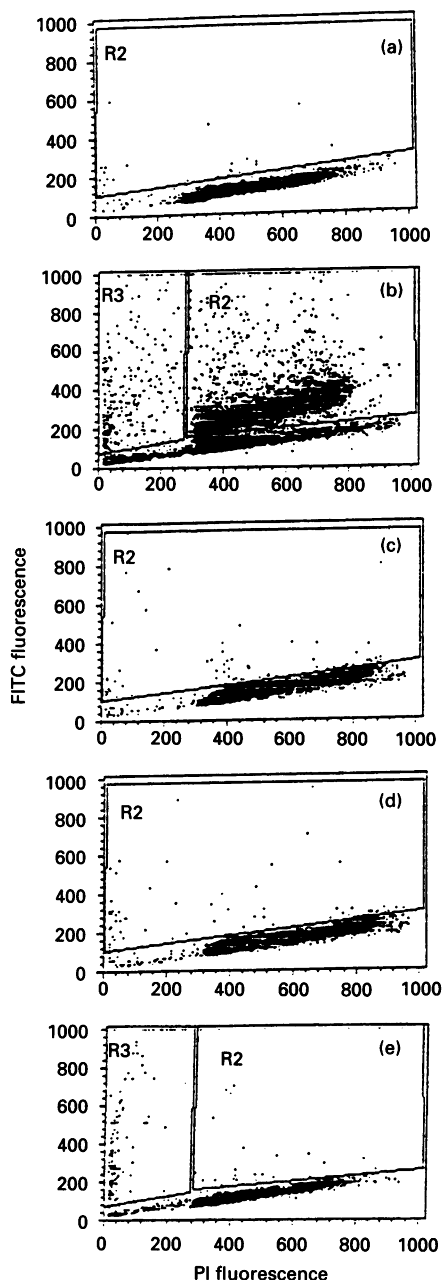


Figure 5 Effect of different metabolites on the induction of DNA double-strand breaks, revealed by the action of TdT in the presence of biotinylated dUTP

BAF_3 cells (3×10^6) were treated for 8 h with IL_3 alone (**a**), or with IL_3 in the presence of 600 μM methional (**b**), MTOB (**c**) or propanal (**d**). Fixed cells were incubated in the presence of biotin–d-UTP and TdT except in (**e**) where enzyme was omitted [as a control for (**b**)]. Bivariate analysis of the incorporated biotinylated dUTP assessed with avidin–FITC and cellular DNA content by PI staining was obtained by flow cytometry.

at concentrations equimolar to those of methional. Thus, the apoptosis induced by methional would appear to be linked to the presence of the methylthio moiety on propanal.

The results of the flow cytometric analyses of TdT-treated cells counterstained with PI (Figure 5) show that the DNA of cells treated with methional has extensive double-strand breaks after 8 h treatment, as the green fluorescence typical of bound avidin–FITC is increased in a population of cells distributed throughout the cell cycle, as judged by the red fluorescence attributable to PI. This is clearly distinguishable from controls (Figure 5a). These breaks were found in 40% of the entire population of cells which were still in the G_1/S and G_2/M phases (Figure 5b; R2) before the appearance of final stage apoptotic cells in sub-G_1 phase (Figure 5b; R3). Cells treated with MTOB (Figure 5c), or with propanal (Figure 5d) show no differences compared with controls.

The characteristic ladder pattern of non-sedimentable DNA (Figure 6) further confirmed that, compared with control cells (lane 1), apoptosis was effectively taking place in methional-treated (lane 2) cells but not MTOB-treated (lane 3) or propanal-treated (lane 4) cells.

We therefore sought to determine whether the metabolism of intracellular methional was altered in BAF_3 cells induced into apoptosis by IL_3 deprivation. Before investigating this, the hypothetical β-hydroxylation pathway of methional was examined *in vitro*.

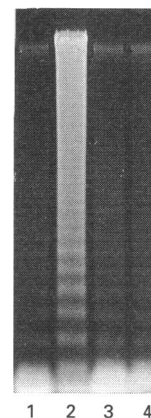


Figure 6 Electrophoretic analysis of non-sedimentable DNA from lysated cells

BAF_3 cells (3×10^6) were lysed after 8 h treatment with or without different metabolites. Non-sedimentable DNA was analysed by electrophoresis on 1% (w/v) agarose gel. Lane 1, control; lane 2, methional (600 μM); lane 3, MTOB (600 μM); lane 4, propanal (600 μM).

Table 3 Formation of MDA from methional by the Fenton reaction

The Fenton reaction was carried out in tubes containing 0.2 mM methional, and where indicated 0.2 mM H_2O_2, 0.02 mM $FeSO_4$, 0.02 mM ascorbate and 0.02 mM EDTA in a final volume of 1 ml. MDA formed was measured by the fluorescence of the derivative obtained on adding TBA.

Reaction system	Amount of MDA formed (nmol)
Methional + H_2O_2 + $FeSO_4$ + ascorbate + EDTA	3.00
Methional + $FeSO_4$ + ascorbate + EDTA	0.17
Methional + H_2O_2	0.24
Methional	0.03

Chemical conversion of methional into MDA

Methional was tested as a substrate for chemical hydroxylation by the Fenton reaction. It is apparent (Table 3) that MDA can be formed from methional when the reaction medium contains $FeSO_4$, ascorbic acid, EDTA and H_2O_2. The identity of the product formed was assessed by comparison of the R_f values of the TBA derivative with those of the TBA derivative of authentic

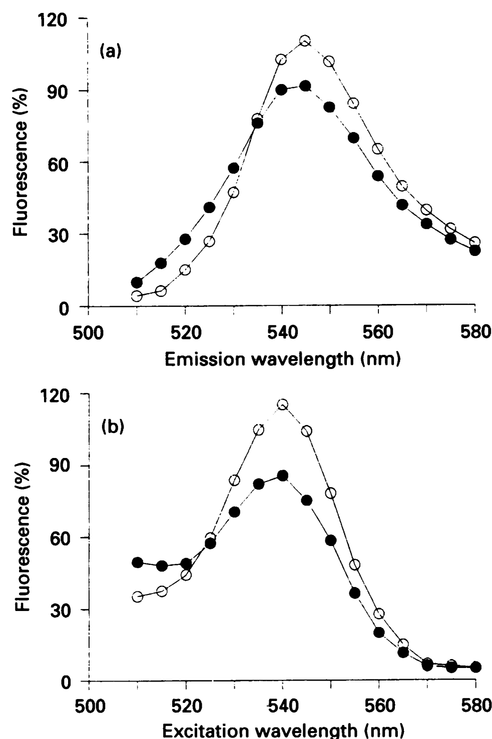


Figure 7 Fluorescence spectrum of MDA-TBA derivative formed from methional by the Fenton reaction (●) compared with that of authentic MDA (○)

(**a**) Excitation wavelength fixed at 532 nm. (**b**) Emission wavelength fixed at 553 nm. 100% fluorescence corresponds to 1.5 nmol of MDA-TBA.

MDA on t.l.c. in two different solvent systems [chloroform/methanol/water (65:25:4, by vol.), R_f 0.260; and chloroform/methanol/acetic acid (60:20:10, by vol.), R_f 0.63] and by its

Table 4 R_f values in three different solvent systems of methionine and the 2,4-DNPH derivatives of MTOB, methional and MDA (2,4-DNPH pyrazole)

H.p.-t.l.c. was carried out on pre-coated silica gel 60 aluminium sheets in the solvent systems indicated (ratio of constituents given by vol.) and radioactivity was determined on a System 200 Imaging Scanner. Chl, chloroform; Ace, acetone; AA, acetic acid; EtA, ethyl acetate; LP, light petroleum.

	R_f		
Samples	Chl/Ace (10:1)	Chl/Ace/AA (10:1:0.5)	EtA/LP/AA (15:15:2)
2,4-DNPH MTOB	0.00	0.16	0.2
2,4-DNPH methional	0.80	0.77	0.73
2,4-DNPH MDA (2,4-DNPH pyrazole)	0.64	0.59	0.63
L-Methionine	0.00	0.00	0.00

fluorescence spectrum ($\lambda_{max.}$ excitation 532 nm, $\lambda_{max.}$ emission 553 nm; Figure 7). From the chromatographic and spectral data, it would appear that MDA can be derived from methional by β-hydroxylation.

Metabolic breakdown products of MTOB during apoptosis induced by IL_3 deprivation

[3,4-^{14}C] MTOB was added to BAF_3 cells growing in the presence of IL_3. After 39 h of contact the medium was removed, and the cells were washed twice with PBS and resuspended in IL_3^+ or IL_3^- medium each re-supplemented with [3,4-^{14}C] MTOB. Cells were harvested after 8 h and metabolites analysed as their 2,4-DNPH derivatives as described in the Materials and methods section. The ethyl acetate extracts derived from IL_3^+ and IL_3^- cells were subjected to t.l.c. The radiochromatogram scan of the t.l.c. plate developed in chloroform/acetone/acetic acid (10:1:0.5, by vol.) is shown in Figure 8. It is apparent that three peaks of radioactivity are present in areas in which authentic Met (area 1), 2,4-DNPH derivatives of MDA (area 3) and methional (area 4)

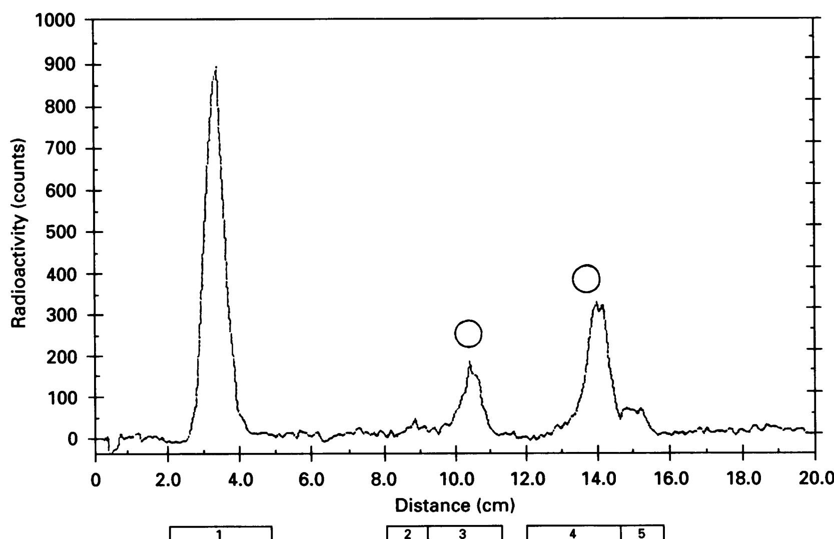


Figure 8 Radiochromatogram scan of 2,4-DNPH derivatives of metabolites derived from [3,4-^{14}C]MTOB added to BAF_3 cells in the presence of IL_3

The position of authentic 2,4-DNPH of MDA (2,4-DNPH pyrazole) (area 3) and of 2,4-DNPH of methional (area 4) are indicated, (area 1) corresponds to methionine formed from [3,4-^{14}C]MTOB.

Table 5 Amounts of [^{14}C]MDA and [^{14}C]methional as their 2,4-DNPH derivatives in BAF$_3$ cells in the presence or absence of IL$_3$

2,4-DNPH methional and 2,4-DNPH MDA were separated by h.p.-t.l.c. as described in Table 4 and the radioactive content of each fraction was measured on a System 200 Imaging Scanner.

Samples	Amount of MDA (pmol)	Amount of methional (pmol)	MDA/methional ratio
$+IL_3$	344	635	0.54
$-IL_3$	388	413	0.94

migrate. In the case of MDA, the derivative formed is really the 2,4-DNPH pyrazole, as shown by ^{1}H-n.m.r. data.

To confirm the identity of the radioactive components, t.l.c. was carried out in two other solvent systems. It is clear (Table 4) from the similarity in R_f with authentic products that 2,4-DNPH methional and 2,4-DNPH pyrazole are formed from [3,4-^{14}C]MTOB in both IL_3^+ and IL_3^- BAF_3 cells.

Further, the conversion of methional into MDA appears to take place more efficiently in IL_3^- cells, as the ratio of the radioactive counts in the 2,4-DNPH derivatives of MDA and methional determined by three t.l.c. analyses in three different solvent systems increased from an average of 0.54 in IL_3^+ cells to 0.94 in IL_3^- cells (Table 5).

Confirmatory evidence for the formation of MDA by cleavage of methional was obtained from the finding that labelled methional but no labelled 2,4-DNPH pyrazole was detected when [^{35}S]MTOB was used as a precursor.

DISCUSSION

The results presented here provide evidence that MTOB is an indirect, and methional a direct, inhibitor of HeLa cell growth in culture. As the other MTOB-derived metabolites such as MTPA and methionol were inhibitory at concentrations 10 times greater than that of MTOB, they cannot be responsible for the growth inhibition seen with 1.6 mM MTOB. Evidence in support of the oxidative decarboxylation of MTOB to methional by BCOADC is already well documented in the literature [9–11] and is confirmed here by two findings: (a) 2-oxobutyrate, another well known substrate of BCOADC with a K_m of 18 μM, reduces 3-fold the growth inhibitory activity of 2 mM MTOB (Figure 1); and (b) [^{14}C]methional was identified in extracts of cells incubated in the presence of [^{14}C]MTOB (Figure 8).

The activity of BCOADC is regulated *in vivo* by the action of specific kinases [27] and phosphatases [28] and specific inhibitors of these phosphatases [29] (for a review, see [14]). In its phosphorylated state BCOADC is inactive but active when dephosphorylated. The activity of the kinase which specifically phosphorylates BCOADC is subject to inhibition by the oxoacid substrates [30,31], which, in the case of MTOB, would be deficient in Met-dependent transformed cells because of the decrease in MTA phosphorylase activity [32]. Hence, with increased kinase activity, BCOADC activity would be correspondingly decreased. Furthermore, the kinase is stimulated by the reaction products such as NADH and MTPA [14]. Consequently, were methional to be converted directly into MTPA by ALDH, BCOADC activity would also be reduced.

We have been able to show that methional is indeed a substrate (K_m 1.1 mM) for baker' s yeast ALDH with NAD as cofactor *in vitro*. When labelled metabolites derived from [^{35}S]MTOB-fed cells were examined, evidence was also obtained for methional and MTPA in both IL_3^+ and IL_3^- cells when the ethyl acetate extraction procedure was carried out on the acid-derivatized fraction instead of on the alkaline fraction. This suggests that the oxidative pathway also contributes to the regulation of intracellular methional levels. In this context it must be recalled that the activity of one of the isoenzymes of cytosolic ALDH has been shown to increase in pre-neoplastic liver nodules in rats injected with diethylnitrosamine [33] or with phenobarbital [34]. Further, when the injection of phenobarbital was stopped, the preneoplastic cells died by apoptosis. On resumption of the phenobarbital treatment apoptosis was inhibited [35,36]. From these examples in the literature, it would appear then that an inverse relationship exists between ALDH activity and apoptosis in epithelial cells, and the simultaneous presence of methional and MTPA in cell extracts examined here provides some evidence for the pertinence of this pathway in lymphoid cells.

The induction of apoptosis, as assessed by TdT labelling, was clearly seen by flow cytometry in cells which had been treated with methional for 8 h. Apoptotic cells were in the G1, S, G2 and M phases of the cell cycle. In the sub-G1 phase characteristic of apoptotic BAF_3 cells, only 5% of cells were present. These findings suggest that the DNA double-strand breaks induced by methional are a relatively precocious event.

Further evidence for DNA fragmentation was obtained on cell lysates by measuring the amount of non-sedimentable DNA, which increased from 0.3% in controls to 60% in methional-treated cells, a 200-fold increase. Confirmatory evidence was obtained from the electrophoretic pattern of non-sedimentable DNA, which revealed typical laddering (Figure 6).

It is noteworthy that both methional ($CH_3SCH_2CH_2CHO$) and MTOB ($CH_3SCH_2CH_2COCOOH$) are very effective free-radical scavengers [12,13] but only methional has been found to induce apoptosis in BAF_3 cells (Figures 4 and 5). As no apoptosis was induced by propanal (CH_3CH_2CHO), it strongly suggests that the simultaneous presence of the methylthio group and the aldehyde moiety in methional is essential for the induction of apoptosis.

Was it methional *per se* or one of its breakdown products which was responsible for inducing apoptosis? In view of the radical-scavenging properties of methional described above, and the findings that $^{\bullet}OH$ radicals are inducers of apoptosis in the murine IL_3-dependent lymphocyte cell line $FL_{5.12}$ [13], we investigated whether methional was a target for $^{\bullet}OH$. We were able to show that MDA is one of the products formed when methional is subjected *in vitro* to $^{\bullet}OH$ attack under the conditions of the Fenton reaction (H_2O_2 and Fe^{2+}) (Table 3). Indeed, according to fluorescence spectroscopy and t.l.c. in two different solvent systems, the characteristics of the TBA-MDA derivative formed from methional were identical to those of the TBA-MDA derivative formed from authentic MDA. Supporting evidence was obtained by comparing these derivatives with the TBA-MDA derivative obtained on treating methional with bleomycin and Fe^{2+}, another system for generating $^{\bullet}OH$ [37]. In addition, bleomycin has been reported to induce apoptosis in mature lymphocytes [38]. It is therefore possible that methional is one of the intracellular targets of $^{\bullet}OH$ during the induction of apoptosis by irradiation [39] or by drugs that influence the homeostasis of reactive oxygen species [40].

This formation of MDA from methional also takes place in cells in culture, as shown by three lines of evidence: (a) two labelled 2,4-DNPH derivatives with R_f values identical to those of the authentic 2,4-DNPH derivatives of methional and MDA (Table 4) are found in cells incubated with [3,4-^{14}C]MTOB; (b) no labelled MDA was found when [^{35}S]MTOB was used; and (c) [^{14}C]methional levels decrease from 635 to 413 pmol/10^8 cells

and MDA levels increase from 344 to 388 pmol/10^8 cells when BAF_3 cells are induced into apoptosis by IL_3deprivation. Or stated another way, the ratio MDA/methional increases from 0.54 to 0.94 (Table 5). Taken together, these findings provide at least one line of evidence in favour of the β-hydroxylation of methional in cells growing in culture.

Is this MDA directly responsible for the induction of apoptosis by initiating chromatin condensation, one of the hallmarks of apoptosis [41]? This we believe to be the case, because work from other authors has shown that MDA generated *in situ* from 1,3-propanediol does bring about DNA–protein cross-links [16,17]. An unequivocal answer is presently being sought by measuring systematically the levels of both free and bound [^{14}C]methional and [^{14}C]MDA in BAF_3 cells incubated with [^{14}C]MTOB at intervals after IL_3withdrawal.

How then do tumour cells differ from normal cells in their ability to undergo apoptosis? One pertinent finding at least in Met-dependent tumours, and 70% of all tumours are Met-dependent [42], is that tumour cells are deficient in MTOB, an intermediate in the Met salvage pathway [1]. The reason for this deficiency, as stated earlier, is a defect in MTA phosphorylase [43], which converts MTA into methylthioribose phosphate the intracellular precursor of MTOB [2–4]. Further, the Met dependency of all the tumour cells which we have examined so far can be alleviated by adding MTOB to the medium [1]. Normal cells show no Met dependence [1]. They therefore have their full load of MTOB and are able to undergo apoptosis in the presence of ·OH, whereas tumour cells with their deficiency in MTOB would be equally deficient in methional and therefore would be unable to undergo apoptosis.

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