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CONTRIBUTION OF 4-METHYLTHIO-2-OXOBUTANOATE AND ITS TRANSAMINASE TO THE GROWTH OF METHIONINE-DEPENDENT CELLS IN CULTURE

EFFECT OF TRANSAMINASE INHIBITORS

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Abstract—The growth in culture of methionine-dependent transformed cells of human, rat and mouse origin was arrested in the absence of L-methionine (Met) but took place in the presence of 4-methylthio-2-oxobutanoic acid (MTOB), the keto acid of Met. From 24 hr after seeding, cells grew in 0.1 mM MTOB medium at a rate comparable to that in 0.1 mM Met medium. Using [^{35}S]MTOB, it was found that the Met synthesized was used in normal MRC-5 cells and in transformed HeLa cells to the same extent for protein, adenosylmethionine and adenosylhomocysteine syntheses. However, when the free Met content was examined, it was found to be 3-fold greater in HeLa than in MRC-5 cells. To examine the importance of this free Met for the growth of transformed cells, the transaminase responsible for converting MTOB to Met was chosen as a target enzyme for the synthesis of compounds with potential inhibitory activity. Since this is a multisubstrate enzyme, reduced Schiff bases were prepared containing both pyridoxal or other aromatic groups, as one constituent, and L-Met or other amino-acids in the free acid or ester or amide form, as the other constituent. Only esters containing the pyridoxal moiety and Met or certain of its structural analogues exhibited good selective growth inhibitory activity in that there was little (20%) or no effect on the growth of normal MRC-5 and derm cells, respectively, while that of transformed HeLa, HEp-2 and L1210 cells was strongly inhibited (80%). This inhibition was accompanied by a concomitant decrease in the activity of the MTOB transaminase in both HeLa and MRC-5 cells treated with **3c** the most potent inhibitor. However, using [^{35}S]MTOB it was found that MTOB itself accumulated 48% in HeLa but only 12% in MRC-5 cells treated with **3c**. On the contrary [^{35}S]Met formed from [^{35}S]MTOB increased 3.7-fold in MRC-5 inhibitor-treated cells showing 20% growth inhibition whereas it decreased 38% in HeLa-treated cells showing 80% growth inhibition. This decrease in cellular Met in HeLa is not responsible for growth arrest. Indeed the growth of HeLa cells could not be restored by adding a 10-fold excess of Met. Since MTOB can alleviate Met-dependence, the intracellular homeostasis of this metabolite may play a hitherto unsuspected role in controlling cell growth.

The phenomenon of methionine(Met§)-dependence has been classically described as the inability of cells to grow in a Met-deprived medium (Met^-) supplemented with homocysteine (Hcy^+) and tetrahydrofolate, the cofactor for methionine synthase. Met-dependence has been extensively documented for the majority of transformed cells regardless of their tissue or species origin or of the transforming agent [1]. Normal cells do not exhibit Met-dependence with two exceptions: mouse embryo cells abortively infected by human cytomegalovirus [2] and phyto-haemaglutinin-stimulated human lymphocytes [3].

Met-dependence is not due to the inability of transformed cells to synthesize Met from (Hcy) [4]. However, according to Stern *et al.* [5], the Met synthesized from Hcy is used primarily for protein and not for adenosyl methionine (Ado Met) synthesis. These low Ado Met levels [6] can be increased by the further addition of Met. One reason for the low Ado Met levels could be its increased decarboxylation to decarboxylated Ado Met [7] whose aminopropyl moiety is used for the synthesis of spermidine and spermine [8, 9]. However, Porter and co-workers [10, 11] have reported that inhibitors of Ado Met biosynthesis effectively inhibit growth without affecting polyamine biosynthesis. Therefore, the greater Met requirement of continuously dividing cells such as transformed cells [12] cannot be explained solely on their increased polyamine turnover.

However, even during polyamine synthesis, cells possess a salvage mechanism (Fig. 1) for preserving the methylthio group [13, 14]. Indeed, the by-product of the aminopropyl transfer reaction is 5′-deoxy-5′-methylthioadenosine (MTA) [15, 16] which

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§ Abbreviations: Met, methionine; Hcy, homocysteine; MTA, 5′-deoxy-5′-methylthioadenosine; MTOB, 4-methylthio-2-oxobutanoic acid; Ado Met, adenosylmethionine; Ado Hcy, adenosylhomocysteine; GIE, growth inhibitory effect.

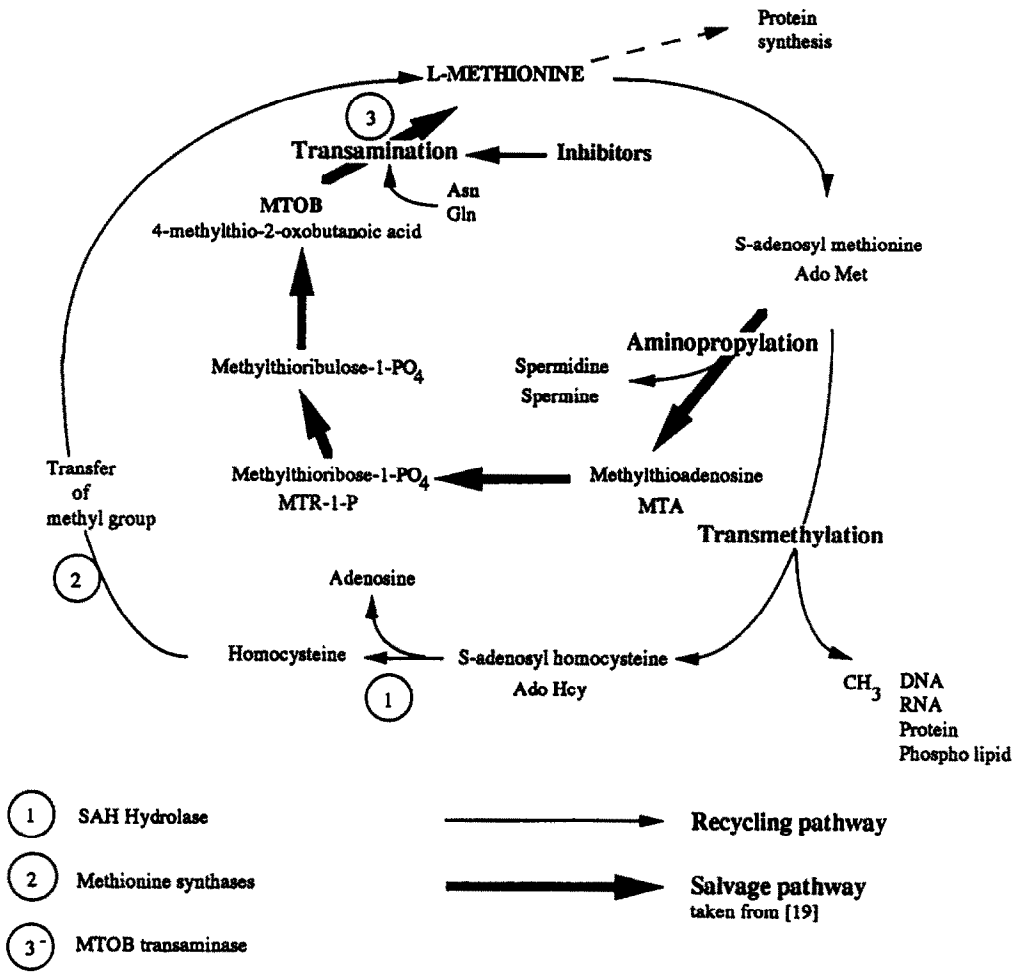


Fig. 1. Metabolism of Met in mammalian cells.

is then phosphorylated by MTA phosphorylase with cleavage of adenine and formation of 5-methylthioribose-1-phosphate [13, 17]. This intermediate is subsequently metabolized via 5-methylthioribulose-1-phosphate [18] to 4-methylthio-2-oxobutanoic acid (MTOB) [14, 18, 19] which can then be transaminated to Met using glutamine or asparagine as amine donors [14].

The contribution of this salvage pathway to cellular Met homeostasis has been the subject of conflicting reports. On one hand, Blom *et al.* [20] have provided evidence to show that less than 10% of cellular Met is degraded via transamination; on the other, Reifsnyder *et al.* [21] have shown in nutrition experiments that animals fed low Met diets but supplemented with 4-methylthio-2-hydroxybutanoic acid (the hydroxy acid of MTOB) gain weight at a rate comparable to those fed diets containing the full complement of Met.

This observation suggesting that the MTOB transamination pathway can compensate *in vivo* under conditions of low Met, was further substantiated by the work of Ghoda *et al.* [22] on Met-dependent cells in culture. They showed that the growth of a clone derived from the DLD-1 human carcinoma cell line is arrested in Met^- medium but can take place in a Met^- MTA^+ medium.

Similar observations have been made by Christa *et al.* [23] on another transformed cell line (Raji) using MTA or MTOB. A defect in MTA phosphorylase has been described for mouse tumour cells including L1210 [24] and for several human malignant cells lines [25]. One interpretation of all these results is that some alteration in the Met salvage pathway between MTA and MTOB may also contribute to Met-dependence. However the capacity of MTOB, the end product of the salvage pathway, to replace systematically Met for the growth of transformed cells has not yet been reported.

We therefore undertook a survey of different transformed cell lines for their Met-dependence and the ability of MTOB to replace Met. Then, once this was established, the transaminase responsible for converting MTOB to Met was chosen as a target enzyme for the synthesis of inhibitors which were investigated for their growth inhibitory activity. It is the results of these investigations which are reported here.

MATERIALS AND METHODS

Cell culture and tests

Reagents. L-Met, Ado Met, MTA and MTOB were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Ado Hcy came from Boehringer (Mannheim, F.R.G.).

Cell lines. Two normal human fibroblastic diploid cells were used. MRC-5 (lung) were obtained from Institut Merieux (Lyon, France) and derm cells were a gift from Dr Y. Chardonnet (INSERM U 346, Lyon). The tumour cell lines chosen were of human and mouse origin. Human cells derived from carcinoma of cervix (HeLa) and larynx (HEp-2) were obtained from ATCC. Mouse cell lines derived from melanoma (B16) and lymphocytic leukemia (L1210) were gifts of Dr J. Vila (INSERM U 218, Lyon, France) and mouse erythroleukemic cells (MEL clone DS-19) were a gift of Dr S. Sassa (Rockefeller University, New York, U.S.A.).

Culture media. Human and mouse cells were grown in Eagle's minimum essential medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal bovine serum (Flow Laboratories, Irvine, U.K.).

Effect of metabolites and of inhibitors on cell growth. Cells (0.5×10^6 per 6 cm Petri dish) were seeded in 3 mL of medium without Met (Met^-) plus 10% dialysed foetal bovine serum. Four hours later either 100 μM of different metabolites (Hcy, MTA, MTOB or Met) or both 100 μM MTOB and different concentrations of inhibitor (20–200 μM) were added to each of three dishes. After 4 days at 37°, the cells were washed twice with 0.14 M NaCl and scraped off the dishes. Cell growth was assessed by measuring protein by the method of Lowry *et al.* [26]. Each value is the average of three or four experiments. Cells treated with 2.5–30 μM inhibitor were disrupted for 3×5 sec in a sonic oscillator and used as the source of enzyme in assays for MTOB transaminase activity.

Enzymatic preparation of [^{35}S] *or* [^{14}C]*MTOB.* Oxidative deamination of [^{35}S]L-Met (3.7 GBq/mmol; Dositek) or [3,4-^{14}C]L-Met (2.18 GBq/mmol; Dositek) was carried out by an adaptation of the method of Meister [27]. After 24 hr oxidation of Met (10 MBq) by L-amino acid oxidase (10 U) in 0.2 M Tris pH 7.5 in the presence of catalase (110 U) and dithiothreitol (0.14 mM), the keto acid 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 TLC on silica gel 60 (Merck) with 1-butanol–acetic acid–water (4:1:2) as solvent.

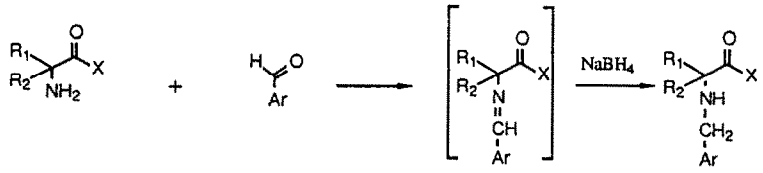
MTOB transaminase activity. The transaminase activity was determined by measurement of the [^{14}C]-Met formed from [^{14}C]MTOB. A standard reaction mixture contained in a total volume of 0.7 mL: 100 mM sodium borate buffer (pH 8.5), 20 mM L-glutamine, 500 μM unlabelled MTOB, 0.54 μM [^{14}C]-MTOB and 1 mg of enzyme. In the blanks, the enzyme was heated for 20 min at 90°. The reaction mixtures were incubated for 1 hr at 37°. After cooling at 4°, the mixtures were passed through Dowex 50 (in the H^+ form) mini columns which retain the Met formed. Then the columns were washed with 15 mL of 5 mM NH_4OH. The Met was eluted with 3 mL of 5 M NH_4OH and the [^{14}C]Met was measured by counting aliquots in pico-fluor 30 scintillation solution (Packard). Specific activities are expressed in nmol of Met formed per mg protein per hr at 37° and are the average of two experiments each performed in duplicate.

Separation of labelled metabolites. [^{35}S]MTOB (0.4 μM) was added to cells 4 hr after seeding in Met^- medium. Unlabelled MTOB (100 μM) was added 24 hr later. Cells were harvested 4 days after the addition of [^{35}S]MTOB. The metabolites derived from [^{35}S]MTOB, i.e. Met, Ado Met and Ado Hcy, were separated by TLC using unlabelled authentic compounds as standards in two systems: silica gel 60 (Merck, Darmstadt, F.R.G.) with 1-butanol–acetate acid–water (4:1:2) and cellulose F (Merck) with 1-butanol–formic acid–water (75:15:10). The R_f values for Ado Met, Ado Hcy, Met and MTOB were respectively 0.05, 0.2, 0.32 and 0.43 on silica gel and 0, 0.24, 0.44 and 0.81 on cellulose. The radioactivity of each metabolite was measured by liquid scintillation counting. The amounts of nmol/mg protein were determined from the specific radioactivity of [^{35}S]MTOB.

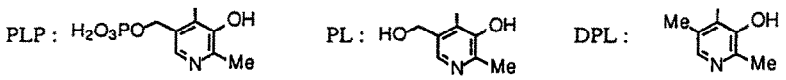
Synthesis

Reagents. Pyridoxal 5-phosphate monohydrate, pyridoxal hydrochloride and salicylaldehyde were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 5-Deoxypyridoxal was prepared according to the method of Iwata [28]. L-Met, L-norleucine, L-Met cycloleucine, L-Met methyl ester hydrochloride, L-Met ethyl ester hydrochloride, L-alanine ethyl ester hydrochloride, L-phenylalanine ethyl ester hydrochloride and L-glutamic acid diethyl ester hydrochloride were purchased from Aldrich. Seleno-L-Met and D-Met methyl ester hydrochloride were obtained from Sigma. L-Met isopropyl ester was prepared as described [29]. Seleno-L-Met ethyl ester, L-ethionine ethyl ester, L-norleucine ethyl ester and cycloleucine ethyl ester were prepared from the corresponding acid according to Wieland *et al.* [30].

Preparation of compounds **1e** *and* **1f.** Oxalyl chloride (5 mL, 57.3 mmol) was added to a solution of *N*-phthaloyl-L-Met [31] (5g, 17.9 mmol) and pyridine (two drops) in anhydrous ether (100 mL). The mixture was stirred at room temperature for 48 hr. The solvent and the excess of oxalyl chloride were evaporated *in vacuo*. The residue was dissolved in anhydrous ether (150 mL) and added to a solution of ethylamine (3 mL of a 70% wt aqueous solution, 37.3 mmol) or morpholine (3.12 g, 35.8 mmol) in ether (150 mL). The mixture was stirred at room temperature for 2 hr. Water (60 mL) was added; the organic layer was separated, washed with saturated aqueous sodium hydrogencarbonate and then with water. The organic solution was dried over anhydrous sodium sulphate and evaporated *in vacuo*. The residue was dissolved in ethanol (50 mL) and hydrazine hydrate (1 mL) was added. The mixture was refluxed for 1 hr. The solvent was evaporated and a 5 M acetic acid solution (40 mL) was added. The solution was filtered, neutralized with 5 M aqueous sodium hydroxide and extracted with ethyl



1a	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OH$	**2a**	Ar = PL	**3a**
1b	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OCH_3$	**2a**	Ar = PL	**3b**
1c	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OC_2H_5$	**2a**	Ar = PL	**3c**
1d	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$- $X = OCH(CH_3)_2$	**2a**	Ar = PL	**3d**
1e	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = NHC_2H_5$	**2a**	Ar = PL	**3e**
1f	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; X = morpholino	**2a**	Ar = PL	**3f**
1c	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PLP	**3g**
1a	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OH$	**2b**	Ar = PLP	**3h**
1c	$R_1 = CH_3\text{-}S\text{-}CH_2\text{-}CH_2$; $R_2 = H$; $X = OC_2H_5$	**2c**	Ar = DPL	**3i**
1b	$R_1 = CH_3\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OCH_3$	**2d**	$Ar = 2\text{-}HO\text{-}C_6H_4\text{-}$	**3j**
1g	$R_1 = CH3$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3k**
1h	$R_1 = C_2H_5\text{-}O_2C\text{-}CH_2\text{-}CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3l**
1i	$R_1 = C_6H_5\text{-}CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3m**
1j	$R_1 = CH_3\text{-}CH_2CH_2\text{-}CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3n**
1k	$R_1 = CH_3\text{-}Se\text{-}CH_2\text{-}CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3o**
1l	$R_1 = C_2H_5\text{-}S\text{-}CH_2CH_2$; $R_2 = H$; $X = OC_2H_5$	**2b**	Ar = PL	**3p**
1n	$R_1, R_2 = -(CH_2)_4-$; $X = OC_2H_5$	**2b**	Ar = PL	**3q**



Scheme 1.

acetate (3 × 30 mL). The organic extracts were dried and the solvent was evaporated *in vacuo* to give crude **1e** (2.78 g, 88%) or **1f** (2.53 g, 54%) which was used in the following step without further purification.

Compound **1e**; **i.r.**: $\nu NH_2 = 3300\,cm^{-1}$, $\nu CO = 1650\,cm^{-1}$; ^{1}H-RMN ($CDCl_3$): 1.12 (t, 3H, J = 7 Hz), 1.5–1.9 (m, 4H), 2.12 (s, 3H), 2.62 (pt, 2H, J = 7 Hz), 2.9–3.7 (m, 3H), 7.03–7.83 (broad s, 1H).

Compound **1f**; **i.r.**: $\nu NH_2 = 3350\,cm^{-1}$, $\nu CO = 1645\,cm^{-1}$; ^{1}H-RMN ($CDCl_3$): 1.5–2.3 (m, 7H with a singlet at 2.10), 2.67 (pt, 2H, J = 7 Hz), 3.77–3.97 (m, 9H).

Preparation of compounds **3**: *design of the inhibitors*. Over the past years, mechanism based inhibitors and transition state analogues have become among the most promising enzyme inhibitors. They provide an attractive basis for rational drug design since, unlike other kinds of inhibitors, they tend to be selective for a single enzyme [32]. Several compounds belonging to the former class ("suicide" substrates) have been shown to inhibit enzymes which require pyridoxal phosphate as coenzyme, such as decarboxylases or transaminases [33]. If the second approach has successfully been used to develop very potent inhibitors of single substrate enzymes, to our knowledge, only limited efforts have been made to use it to develop inhibitors for enzymes involving more than one substrate [32]. Some years ago the inhibition of various transaminases [34] and decarboxylases [35, 36] was observed with reduced Schiff bases. These compounds may be seen as topographical anologues of certain transition structures for the enzyme-catalysed reaction. More recently conformationally restricted bicyclic multisubstrate analogues designed to mimic a calculated transition state were synthesized as inhibitors of GABA-transaminase [37] or L-dopa decarboxylase [38]. These results demonstrated the ability of such compounds to inhibit pyridoxal phosphate dependent enzymes. It therefore seemed reasonable to investigate whether similar reduced Schiff bases of the general formula **3** could be inhibitors of our target MTOB transaminase. To accomplish this, several compounds of this type were synthesized (Scheme 1).

Compounds **3a, 3g** and **3h** were prepared in a similar manner as described for the synthesis of *N*-(5′-phosphopyridoxyl)-amino acids [35]. The yields were in the range 80–90% and the ^{1}H-NMR data were fully consistent with the structures (See Table 1).

Compound **3b–3f, 3i–3q**: a solution of the aldehyde **2** (7.5 mmol) in methanol (15 mL) (neutralized with 7.5 mL of 1 M methanolic potassium hydroxide if pyridoxal hydrochloride was used as starting material) was added in one portion to a solution of **1** (7.5 mmol) in methanol (15 mL) (7.5 mL of 1 M methanolic KOH was also added when a hydrochloride derivative of **1** was used as starting material). The mixture was stirred at room temperature for 30 min and then cooled in a water-ice bath. Sodium borohydride (0.4 g) was added in several portions; after stirring for 15 min, the reaction mixture was neutralized to pH 5 with acetic acid. The solvent was evaporated under reduced pressure

Table 1. Pertinent ^{1}H-NMR (80 MHz) data for compounds **3** [δ (ppm); J (Hz)]

	R_1	R_2	X	NH-C$\underline{H}_2$-Ar	Ar
3a*	C$\underline{H}_3$-S: 2.04 (s)	3.34 (t, J = 6.0)	—	4.43 (s)	$C_{6'}$-$\underline{H}$: 7.81 (s) $C_{2'}$-C$\underline{H}_3$: 2.37 (s)
3b†	C$\underline{H}_3$-S: 2.08 (s)	3.49 (pt, J = 6.4)	C$\underline{H}_3$: 3.78 (s)	4.08§ J_{AB} = 14.5	$C_{6'}$-$\underline{H}$: 7.72 (s) $C_{2'}$-C$\underline{H}_3$: 2.42 (s)
3c†	C$\underline{H}_3$-S: 2.08) (s)	3.46 (pt, J = 6.4)	C$\underline{H}_2$: 4.24 (q) C$\underline{H}_3$: 1.30 (t)	4.07§ J_{AB} = 14.6	$C_{6'}$-$\underline{H}$: 7.68 (s) $C_{2'}$-C$\underline{H}_3$: 2.42 (s)
3d†	C$\underline{H}_3$-S: 2.07 (s)	3.42 (pt, J = 6.4)	C$\underline{H}$: 5.09 (sept) C$\underline{H}_3$: 1.29 (d)	4.05§ J_{AB} = 14.8	$C_{6'}$-$\underline{H}$: 7.60 (s) $C_{2'}$-C$\underline{H}_3$: 2.48 (s)
3e†	C$\underline{H}_3$-S: 2.04 (s)	Buried in the m (3H) at 3.1–3.6	C$\underline{H}_2$: 3.1–3.6 (m) C$\underline{H}_3$: 1.14 (t)	3.98 (s large)	$C_{6'}$-$\underline{H}$: 7.62 (s) $C_{2'}$-C$\underline{H}_3$: 2.36 (s)
3f†	C$\underline{H}_3$-S: 2.06 (s)	3.48 (pt, J = 4.4)	C$\underline{H}_2$O and C$\underline{H}_2$N 3.5–3.8 (m)	3.97§ J_{AB} = 14.7	$C_{6'}$-$\underline{H}$: 7.75 (s) $C_{2'}$-C$\underline{H}_3$: 2.44 (s)
3g‡	C$\underline{H}_3$-S: 2.11 (s)	3.99 (t, J = 6.0)	C$\underline{H}_2$: 4.30 (q) C$\underline{H}_3$: 1.30 (t)	4.32 (s)	$C_{6'}$-$\underline{H}$: 7.80 (s) $C_{2'}$-C$\underline{H}_3$: 2.48 (s)
3h‡	C$\underline{H}_3$-S: 2.12 (s)	3.82 (t, J = 5.7)	—	4.40 (s)	$C_{6'}$-$\underline{H}$: 7.71 (2) $C_{2'}$-C$\underline{H}_3$: 2.48 (s)
3i†	C$\underline{H}_3$-S: 2.07 (s)	3.39 (pt, J = 6.5)	C$\underline{H}_2$: 4.20 (q) C$\underline{H}_3$: 1.24 (t)	3.86§ J_{AB} = 14.6	$C_{6'}$-$\underline{H}$: 7.71 (s) $C_{5'}$-C$\underline{H}_3$: 2.02 (s) $C_{2'}$-C$\underline{H}_3$: 2.35 (s)
3j†	C$\underline{H}_3$-S: 2.07 (s)	3.50 (pt, J = 6.5)	C$\underline{H}_3$: 3.77 (s)	3.90§ J_{AB} = 13.6	6.65–7.32 (m)
3k†	C$\underline{H}_3$: 1.30 (d)	3.3 (pt, J = 6.1)	C$\underline{H}_2$: 4.14 (q) C$\underline{H}_3$: 1.21 (t)	4.00§ J_{AB} = 14.7	$C_{6'}$-$\underline{H}$: 7.55 (s) $C_{2'}$-C$\underline{H}_3$: 2.32 (s)
3l†	C$\underline{H}_3$-CH$_2$-O$_2$C-CH$_2$ 1.25 (t)	3.33 (pt, J = 6.5)	C$\underline{H}_2$: 4.18 (q) C$\underline{H}_3$: 1.20 (t)	Buried in the m (5H) at 3.9–4.45	$C_{6'}$-$\underline{H}$: 7.70 (s) $C_{2'}$-C$\underline{H}_3$: 2.37 (s)
3m†	C$_6\underline{H}_5$: 7.03–7.45 (m)	3.48 (pq, J = 6.6)	C$\underline{H}_2$: 4.12 (q) C$\underline{H}_3$: 1.15 (t)	3.95§ J_{AB} = 14.7	$C_{6'}$-$\underline{H}$: 7.57 (s) $C_{2'}$-C$\underline{H}_3$: 2.29 (s)
3n†	C$\underline{H}_3$-(CH$_2$)$_3$: 0.83 (pt)	3.13 (pt, J = 6.1)	C$\underline{H}_2$: 4.15 (q) C$\underline{H}_3$: 1.21 (t)	3.98§ J_{AB} = 15.0	$C_{6'}$-$\underline{H}$: 7.56 (s) $C_{2'}$-C$\underline{H}_3$: 2.33 (s)
3o†	C$\underline{H}_3$-Se: 1.91 (s)	3.39 (pt, J = 6.4)	C$\underline{H}_2$: 4.27 (q) C$\underline{H}_3$: 1.24 (t)	4.02§ J_{AB} = 14.7	$C_{6'}$-$\underline{H}$: 7.68 (s) $C_{2'}$-C$\underline{H}_3$: 2.37 (s)
3p†	CH$_3$-C$\underline{H}_2$-S: 2.48 (q) C$\underline{H}_3$-CH$_2$-S: 1.17 (t)	3.39 (pt, J = 6.6)	C$\underline{H}_2$: 4.16 (q) C$\underline{H}_3$: 1.22 (t)	3.99§ J_{AB} = 14.8	$C_{6'}$-$\underline{H}$: 7.54 (s) $C_{2'}$-C$\underline{H}_3$: 2.31 (s)
3q†	-(CH$_2$)$_4$-: 1.4–2.2 (m)		C$\underline{H}_2$: 4.17 (q) C$\underline{H}_3$: 1.24 (t)	3.92 (s)	$C_{6'}$-$\underline{H}$: 7.61 (s) $C_{2'}$-C$\underline{H}_3$: 2.34 (s)

* in DMSO-d_6.
† in $CDCl_3$.
‡ in D_2O.
§ AB spectrum centered at the indicated chemical shift.

and water was added. After extraction with dichloromethane, the organic extracts were washed with saturated aqueous sodium hydrogencarbonate and then with water. The organic solution was dried over anhydrous sodium sulphate and the solvent was evaporated. The residue was chromatographed on silica gel using ethyl acetate–ethanol (9:1) as eluent to afford pure compounds **3** [the yields were in the range 65–90% except for **3k** (50%) and **3i** (51%)]. The **i.r.** (νCO_2R = 1720–1740 cm^{-1} for **3b–3d, 3i–3q**; νCON = 1650 cm^{-1} for **3e, 3f**) and ^{1}H-NMR data (see Table 1) were fully consistent with the structures. These compounds were converted into crystalline hydrochlorides which were easier to handle, by treatment with methanolic (**3b, 3j**), ethanolic (**3c, 3e, 3f, 3i, 3k–3q**) or ethereal (**3d**) solutions of anhydrous hydrochloric acid at room temperature.

RESULTS

Cell growth in Met$^-$ medium supplemented with Hcy, MTA, MTOB or Met

To determine whether the cells used were in fact Met-dependent and whether MTOB was capable of substituting for Met during their growth, cells were grown in Met$^-$, Hcy$^-$, MTA$^-$ and MTOB$^-$ medium but supplemented, as shown in Table 2, with 100 μM of either one or another of these four compounds. It is apparent that all the transformed cells examined were either totally or partially Met-dependent. Indeed, L1210 and MEL exhibited no growth whatsoever in Met$^-$ Hcy$^+$ medium whereas HeLa, HEp-2 and B16 were only partly dependent in that there was about 25% residual growth in Met$^-$ Hcy$^+$ medium. When the Met$^-$ medium was supplemented with MTOB, full growth was resumed in all the transformed cells. On the contrary, in the presence of MTA, L1210 cells showed no growth whereas with HeLa and MEL cells a 14% increase was observed over that in Hcy$^+$ medium. Furthermore, as shown in Fig. 2, the transformed cells were able to meet all of their Met requirements for growth, irrespective of concentration of MTOB added over the range 1–200 μM.

In an attempt to investigate these requirements, ^{35}S-labelled MTOB was added to MRC-5 and HeLa

Table 2. Effect of Hcy, MTA, MTOB and Met on the growth of normal human cells and of tumour-derived human and mouse cells

Media	% Growth*						
	MRC-5	Derm	HeLa	HEp-2	B16	L1210	MEL
Met^- Hcy^+ MTA^- $MTOB^-$	100	ND	26	38	23	5	0
Met^- Hcy^- MTA^+ $MTOB^-$	93	ND	40	ND	ND	6	14
Met^- Hcy^- MTA^- $MTOB^+$	100	100	100	98	84	95	83
Met^+ Hcy^- MTA^- $MTOB^-$	100	100	100	100	100	100	100

* Cell growth was measured by protein determination and expressed as a percentage of protein content of cells in Met^+ medium.
ND, not done.

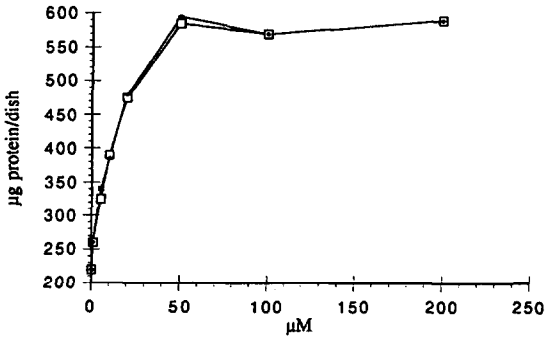


Fig. 2. Comparison of the effect of different concentrations of MTOB and Met on the growth of HeLa cells in Met^- $MTOB^+$ medium (□) and in Met^+ $MTOB^-$ medium (●). Cell growth was assessed by protein measurement after 4 days incubation at 37°. Each point is the mean ± SD of three to four experiments. SD are so small on the ordinate scale that they are undistinguishable.

cells in culture and the distribution of radioactivity in proteins and in Met-derived metabolites was determined after 4 days.

[^{35}S]*MTOB-derived cellular constituents*

Proteins. The specific activity of proteins after hydrolysis of RNA (0.3 N NaOH, 18 hr at 37°) and of DNA (0.5 N PCA, 2 hr at 90°) was found to be 13,000 dpm/mg for MRC-5 cells and 11,700 dpm/mg for HeLa cells. It appeared that Met derived from MTOB was used to the same extent for protein synthesis in normal and transformed cells.

Metabolites. When the [^{35}S]MTOB-derived metabolites were measured, it was found (Table 3) that the amount of metabolites expressed as nmol/mg of cellular proteins was of the same order of magnitude for MTOB, Ado Hcy and Ado Met even though for this last compound there was a 42% increase in MRC-5 cells compared to HeLa cells. On the contrary the amount of free Met was 3-fold greater in HeLa cells than in MRC-5 cells. In view of the apparent preferential conversion of MTOB to Met observed in HeLa cells, the MTOB transaminase was chosen as the target for the synthesis of inhibitors which were screened for their differential effect on the growth of transformed (HeLa) and normal (MRC-5) cells in culture.

Table 3. Amounts of MTOB, Met, Ado Met and Ado Hcy in MRC-5 and HeLa cells

Metabolites	MRC-5	HeLa
MTOB	3.24	3.16
Met	0.57	1.82
Ado Met	1.17	0.82
Ado Hcy	0.95	0.86

Values are nmol/mg protein.
Cells were harvested 4 days after addition of [^{35}S]-MTOB. The metabolites were separated by TLC. The amounts of nmol/mg protein were determined from specific radioactivity of MTOB.
Data are the means of two experiments.

Effect on cell growth of:

1. *The COX functionality*. In analogy with active compounds reported by the groups of Severin [34]

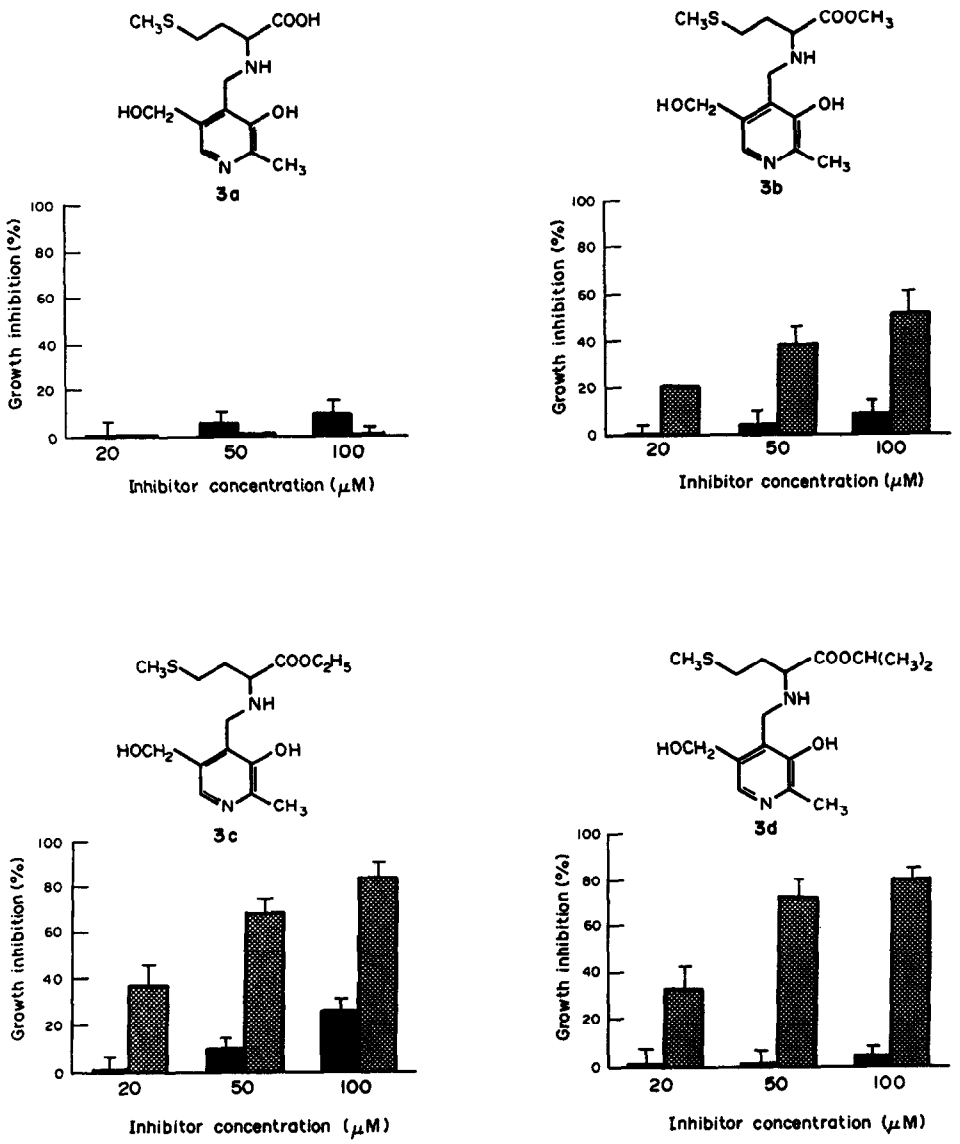


Fig. 3. Effect of compounds 3 with different COX functionalities on the growth of MRC-5 (▩) and HeLa (□) cells. Inhibitors were added with 100 μM MTOB 4 hr after seeding of cells in Met⁻ medium. Growth inhibition measured after 4 days is expressed as a percentage of the difference in protein content between inhibitor-treated cells and non-treated controls. Data represent means ± SD of at least four separate experiments.

and Heller [35], we first prepared **3a** with a free carboxylic acid function. It is apparent (Fig. 3) that it was without any effect whereas the corresponding methyl ester **3b**, ethyl ester **3c** and isopropyl ester **3d** all showed significant growth inhibitory activity. It is noteworthy that these inhibitors are selective in that there was little or no effect on the growth of normal MRC-5 cells. To determine whether the activity of the esters was linked to the absence of a charged acidic function or to the presence of the ester group itself, two amides were prepared; the ethylamide derivative **3e** and the morpholino derivative **3f**. Neither of those compounds had any effect on cell growth. There are two possible reasons for this result: (a) the amides may have been cleaved in the culture medium thus releasing the inhibitor in its free acid form which is itself inactive, (b) for inhibitory activity, the ester group is necessary.

2. *The* Ar *substitution*. Compounds incorporating pyridoxal, and not pyridoxal phosphate, were initially prepared because they were easier to obtain synthetically plus the fact that pyridoxal is itself phosphorylated intracellularly. Nevertheless, in the course of studying the influence of the *Ar* moiety on the activity of compounds **3**, the phosphorylated analogue of **3c**, **3g**, was tested and appeared to be as effective and selective as **3c** (Fig. 4). Here again it should be noted that the replacement of the ethyl

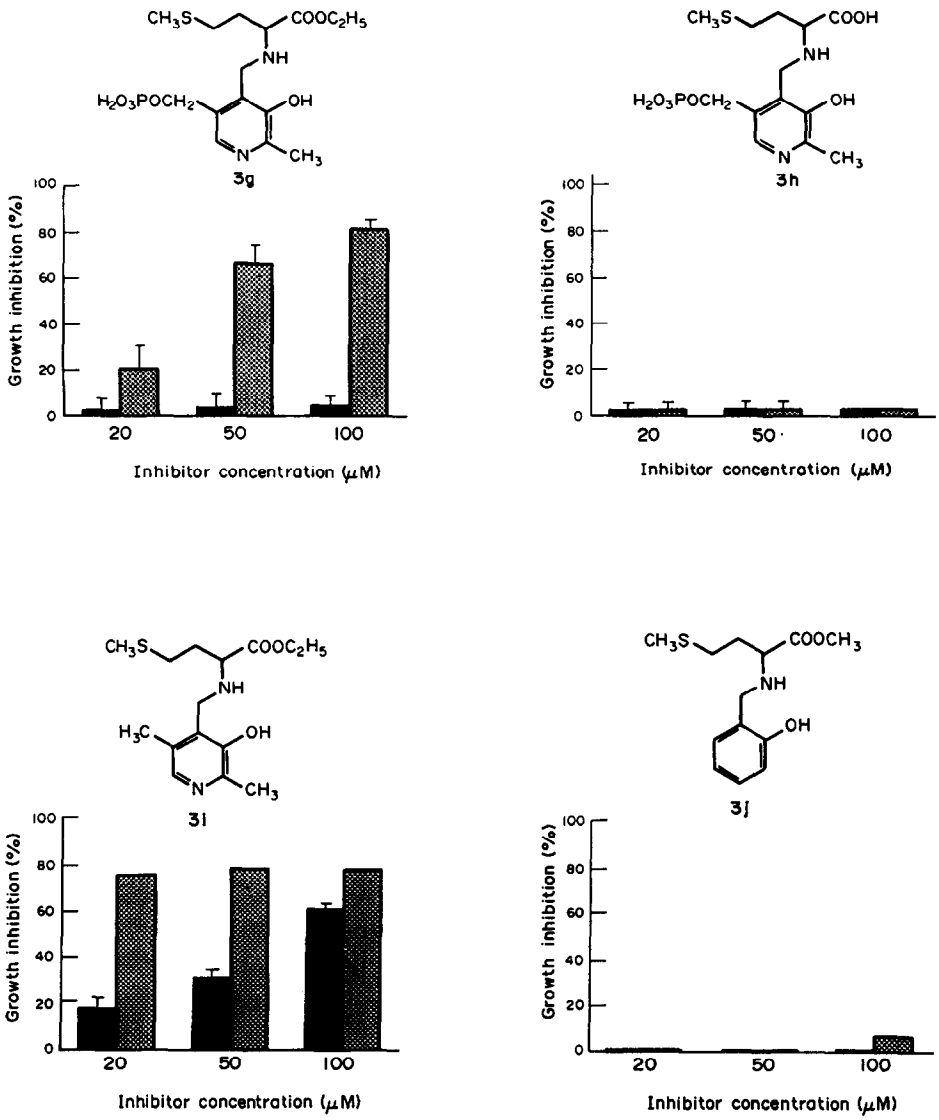


Fig. 4. Effect of compounds 3 with different *Ar* moieties on the growth of MRC-5 (▩) and HeLa (▨) cells. Experimental conditions were the same as in Fig. 3.

group by a free carboxylic acid function renders the molecule **3h** totally inactive. Then, the compound **3i** incorporating deoxypyridoxal, known to function as well as pyridoxal as a cofactor of certain transaminases [39], was prepared. The activity of **3i** was slightly enhanced compared to **3c** but the selectivity was partially lost since the growth of both HeLa and MRC-5 cells was inhibited. Finally we observed that when pyridoxal was replaced by salicaldehyde, the compound **3j** was inactive. If these results permitted us to define the substituents COX and *Ar* necessary for activity, they left open the question of the specificity of R_1 and R_2 to the growth inhibitory effect.

3. *The nature of R_1 and R_2.* Since Met was the amino acid resulting from the transaminase we wished to inhibit, we first prepared compounds **3** incorporating its methylthio carbon chain. Then several reduced Schiff bases were synthesized from esters of different amino acids. When the ethyl esters of non-essential amino acids such as alanine (**3k**) and glutamic acid (**3l**) were components of the inhibitors, no inhibition was observed. When they were replaced by the ethyl esters of phenylalanine (**3m**), an essential amino acid, the growth of both normal and transformed cells was affected (Fig. 5). A good selective inhibitory activity was retained only with amino acids structurally related to Met such as norleucine (**3n**), selenomethionine (**3o**), ethionine (**3p**) and with cycloleucine (**3q**), a well-documented inhibitor of transmethylation [10, 40]. In addition, the enantiomer of compound **3c**, prepared from the ethyl ester of D-Met, had no effect whatsoever on the growth of either normal or

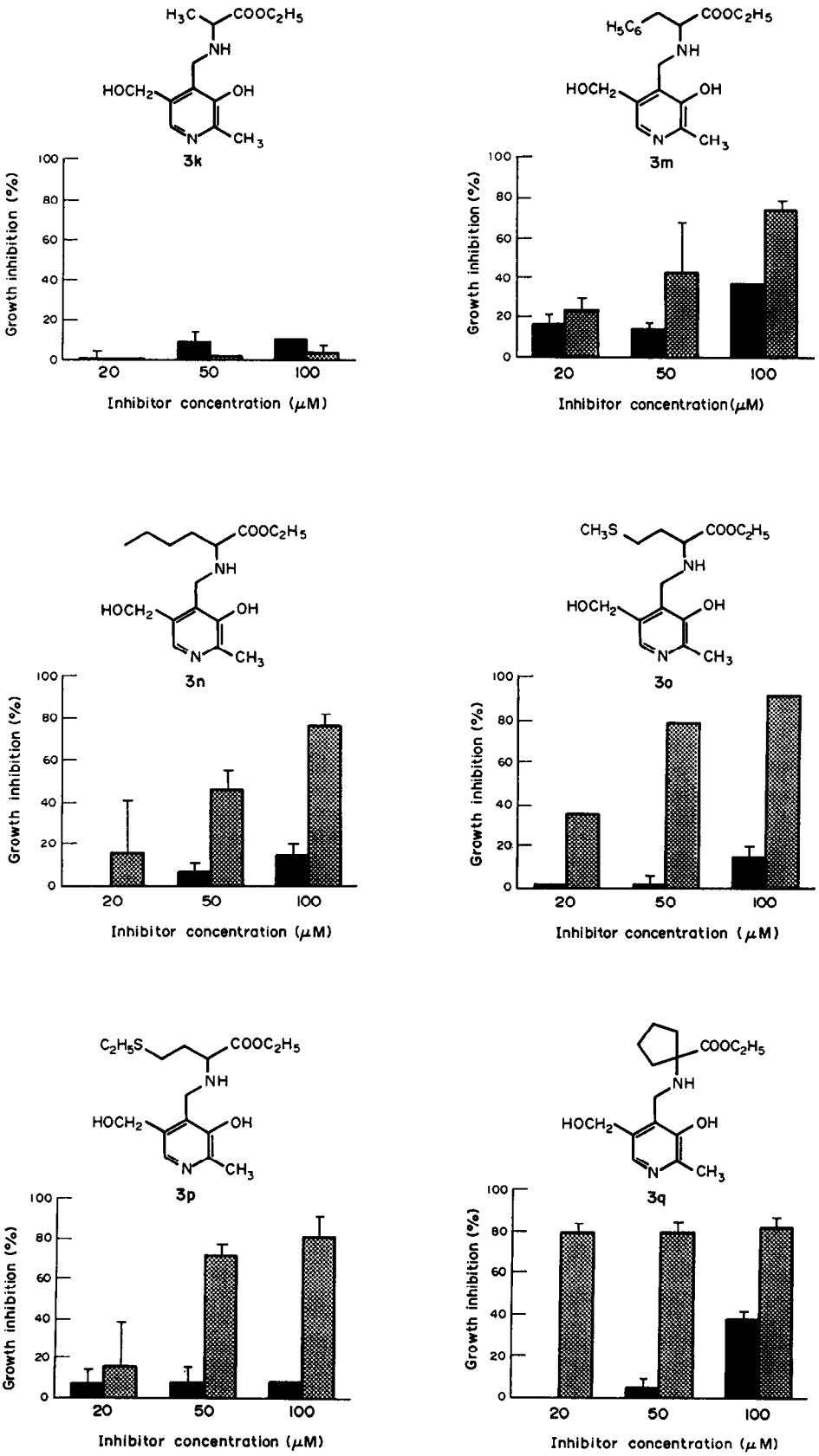


Fig. 5. Effect of compounds 3 with R_1 and R_2 substitution on the growth of MRC-5 (▩) and HeLa (▨) cells. Experimental conditions were the same as in Fig. 3.

transformed cells whereas **3c** is active. This last result showing that the growth inhibitory activity of compounds **3** is dependent on the enantiomeric form of Met, plus the observation that L-Met ethyl ester itself is inactive, provide strong evidence for the specificity of interaction of our putative transition state inhibitor with some target enzyme that reacts with both L-Met and pyridoxal. Before investigating the effect of **3c** on the supposed target enzyme, it was necessary to verify whether the growth of other normal and transformed cells would be influenced by the addition of **3c**. This was investigated on derm cells as an example of normal cells and on HEp-2 and L1210 cells as examples of transformed cells, in addition to the MRC-5 and HeLa cells examined so far. It is apparent from the results presented in Fig. 6, that only the transformed cells were sensitive to the action of **3c** (approximately 80% inhibition at 100 μM). The derm cells were totally refractory and MRC-5 showed only 20% inhibition at this same concentration.

Identification of the target enzyme

Measurement of enzyme activity. To determine whether a decrease in MTOB transaminase activity did take place in cells exhibiting reduced growth in the presence of an inhibitor, the activity of this enzyme was measured in HeLa cells in culture treated with different concentrations of **3c**. It is apparent (Fig. 7) that in one group of experiments, the decrease in enzyme activity was quantitatively similar to the decrease in cell growth, whereas in another group of experiments, with cells at a different passage, enzyme inhibition also increased with growth inhibition but their absolute values were not identical. These results show that the MTOB transaminase is at least one target for **3c** and that its inhibition accompanies the growth inhibitory effect (GIE) of **3c**.

To determine whether this GIE was due to a putative decrease in cellular Met, the effect of Met in reversing growth inhibition was examined. It is apparent (Table 4) that Met, at non-toxic concentrations ranging from 0.1 to 1 mM, was incapable of reversing the GIE observed with 100 μM **3c**.

Measurement of radiolabelled metabolites. To try to obtain further evidence for the impact of this inhibitor, cells which were growing in Met^- medium supplemented with [^{35}S]MTOB were treated or not with **3c**. The amounts of MTOB, Met and Met derived metabolites Ado Met and Ado Hcy were determined and expressed as nmol/mg cellular protein. It is apparent (Table 5) that there was a 38% decrease in [^{35}S]Met formed in **3c** treated HeLa cells compared to controls, whereas in MRC-5 cells, which only showed a 20% inhibition of growth, the Met formed was increased 3.7-fold after **3c** treatment. As regards the MTOB content, this same treatment with **3c** brought about a rise of 12% and 48% in MRC-5 and HeLa cells, respectively. No changes were observed in Ado Met and Ado Hcy in either of the cell types treated with **3c**. An increase in MTOB would appear to be the main feature of growth inhibition induced by **3c** in HeLa cells.

DISCUSSION

The results presented here provide evidence that MTOB can replace Met for the growth of totally or partially Met-dependent transformed cells (Table 2). This observation held true for human cells derived from spontaneously occurring carcinomas of cervix (HeLa) and larynx (Hep-2) and for mouse cells derived either from virus-induced erythroleucemia (MEL) or from chemically induced melanoma (B16) and lymphocytic leukaemia (L1210). They therefore confirm previous reports [23] although they were restricted to cells of lymphoid tissue. This capacity of MTOB to meet the Met requirements of cells in culture under conditions of Met starvation substantiates reports in the literature describing the same phenomenon in farm animals fed on a low diet supplemented with the hydroxy acid precursor of MTOB [21, 41, 42].

However, in cells in culture, the metabolic fate of Met derived from MTOB would appear to be different between normal and transformed cells. Indeed using [^{35}S]MTOB, it was found (Table 3) that in MRC-5 cells, Met accounts for about 10% of the metabolites whereas in HeLa cells, the proportion is 27%. Further, the 3-fold greater content of free Met in HeLa cells compared to MRC-5 cells is in contradistinction to (a) the similarity in uptake in MTOB since there was the same residual MTOB in MRC-5 and HeLa cells, (b) almost the same distribution of Ado Met and Ado Hcy in each kind of cell: 1.17 and 0.95 nmol/mg, respectively, in MRC-5 and 0.82 and 0.86 nmol/mg, respectively in HeLa cells, (c) the similarity in the specific radioactivity of cellular proteins in both cell types. Based on these findings, it would seem reasonable to conclude that Met derived from MTOB accumulates in HeLa cells due to some defect in its utilization in pathways other than protein synthesis and transmethylation. An answer will be forthcoming only when the flux through the transmethylation, aminopropylation and transulfuration pathways is examined using [*methyl*-^{14}C]MTOB, [C_3,C_4-^{14}C]-MTOB and [^{35}S]MTOB, respectively.

One factor which could certainly contribute to the deficiency of MTOB in tumours is the reduced activity of MTA phosphorylase which has been documented for certain human leukaemias and solid tumours [43], human gliomas [44] and also for other human malignant cells [45]. For such tumours, if MTOB in neighbouring normal cells were implicated in the alleviation of Met-dependence, then the transaminase responsible for converting MTOB to Met may constitute a target enzyme for a rational therapeutic approach. Accordingly, this aim was pursued by synthesizing transition state analogues which, unlike other kinds of inhibitor, tend to be selective for a single enzyme [32]. As was expected, it is only compounds incorporating the dual specificity for substrate (Met) and cofactor (pyridoxal) which exhibited a marked GIE on HeLa cells and a low GIE on MRC-5 cells used to determine eventual selectivity. Indeed, when pyridoxal was replaced by salicylaldehyde or deoxypyridoxal, inhibitory activity was lost with the former and selectivity decreased with the latter.

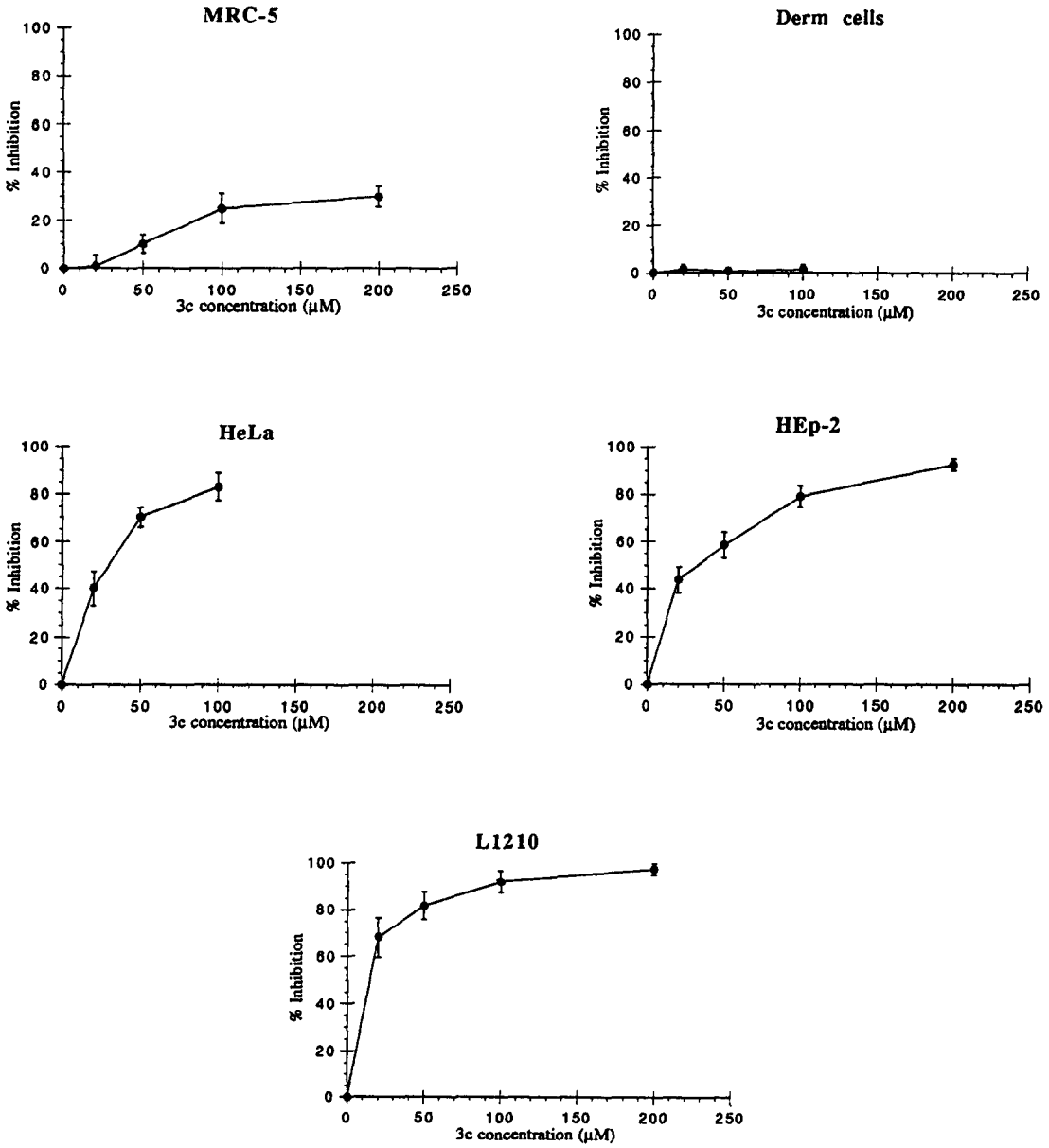


Fig. 6. Effect of inhibitor **3c** on the growth of MRC-5 and normal derm cells, and of HeLa, HEp-2 and L1210 transformed cells. Experimental conditions were the same as in Fig. 3. Values are means ± SD of four experiments.

As regards the importance of the ester group for inhibitory activity, the results of our experiments show that all Met-derived inhibitors with methyl, ethyl and isopropyl esters were active. Since esterified L-Met alone was inactive, it confirms the finding that inhibitory activity requires the simultaneous presence of Met and pyridoxal. As regards the substitution of the COX moiety, other authors have shown that reduced Schiff bases prepared from pyridoxal and amino acids with a free acidic function do inhibit transaminases [34] or decarboxylases [35]. It may therefore be that the GIE of our compounds with an ester function but not an acidic one may be linked to the facility of penetration of the former into cells and their subsequent hydrolysis to the free acid form. In support of this hypothesis is the observation that amides **3e** and **3f** which may be much less sensitive to enzymatic hydrolysis are inactive.

When we examined the importance of the amino acid component of the inhibitors for growth inhibition, it was clear that esterified compounds with non-essential amino acids such as alanine **3k** and glutamic acid ester **3l** were without effect. This indicated that inhibition is not due simply to the presence of pyridoxal in the form of a reduced Schiff base with irrelevant amino acids. On the contrary,

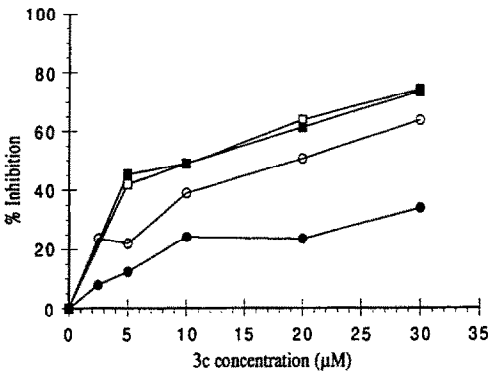


Fig. 7. Effect of inhibitor **3c** on HeLa cells growth and transaminase activity. Cell extracts were used as the source of enzyme. Inhibition of transaminase activity measured after 4 days is expressed as a percentage of the difference in nmol Met/mg protein between **3c**-treated cells and non-treated controls. Each point is the mean of two experiments each performed in duplicate: (1) cell growth (□), enzyme activity (■); (2) cell growth (○), enzyme activity (●).

Table 4. Assay of reversion of the growth inhibitory effect of **3c** (100 μM) with L-Met (0.1–1 mM)

L-Met (mM)	% Inhibition cell growth
0.0	74
0.1	75
0.5	70
1.0	70

Met was added 24 hr after inhibitor. Cells were harvested 3 days later.
Data are the means of two experiments.

Table 5. Effect of the inhibitor **3c** on the amounts of MTOB, Met, Ado Met and Ado Hcy in MRC-5 and HeLa cells

	MRC-5		HeLa	
	Inhibitor concentration (μM)			
Metabolites	0	10	0	10
MTOB	3.24	3.62	3.16	4.69
Met	0.57	2.09	1.82	1.12
Ado Met	1.17	1.11	0.82	0.75
Ado Hcy	0.95	0.76	0.86	0.64

Values are nmol/mg protein.
The legend is the same as in Table 3.

when esterified phenylalanine, an essential amino acid, was a component of the inhibitor, inhibitory activity was retained but selectivity was lost, in that the compound inhibited the growth of both HeLa and MRC-5 cells. Since the effect on HeLa cells was greater than that on MRC-5 cells, and the transaminases which are involved in phenylalanine and Met metabolism are different [46], it would appear that there may also be differences in phenylalanine metabolism between normal and transformed cells. Further work is needed to elucidate this point.

It is only when Met, or amino acids structurally related to Met such as selenomethionine, norleucine, ethionine and cycloleucine, were part of the inhibitor that good growth inhibitory activity and selectivity were observed. Confirmatory evidence was forthcoming with the observation that the compound prepared from esterified D-Met was inactive. This observation finds support in the result of Andrews *et al.* [37] who reported that the inhibitory activity towards L-dopa decarboxylase of D-dopa derivatives was greatly decreased compared to their L-dopa counterparts. Using **3c** as the model compound for selectively inhibiting HeLa cell growth, an attempt was made to determine whether the target enzyme, MTOB transaminase, had in fact been inhibited. Not only did a decrease in activity parallel growth inhibition (Fig. 7), but using [^{35}S]MTOB we were also able to obtain evidence in HeLa cells for a drop in cellular Met and a concomitant rise in MTOB (Table 5). If the rise (48%) in MTOB roughly parallels the drop (38%) in Met in inhibitor-treated HeLa cells, there were no corresponding changes in Ado Met and Ado Hcy. In inhibitor-treated normal MRC-5 cells, the situation is different in that the 12% rise in MTOB was accompanied by a 270% increase in Met. However, this increase in Met was not accompanied, here also, by any corresponding increase in Met-derived products such as Ado Met and Ado Hcy. Further, both cell growth and transaminase activity were inhibited to a maximum of 20% in **3c**-treated MRC-5 cells. It would therefore seem reasonable to conclude that Met derived from MTOB is utilized differently in MRC-5 and HeLa cells. However, our results do not permit us to explain this difference in Met metabolism.

The GIE seen on HeLa cells could therefore have been due either to the drop in Met or to the increase in MTOB. To investigate the former hypothesis, the reversal of growth arrest was attempted with increasing concentrations of Met up to 1 mM. No resumption of growth was observed. It can therefore be inferred that inhibition of growth may be due to the accumulation of MTOB or one of its metabolite(s). In this context it was found that the addition of 2 mM MTOB to the culture medium of both HeLa and MRC-5 cells was cytotoxic whereas the addition of up to 20 mM Met has no effect (results not shown). MTOB would seem to be the precursor of some type of toxic metabolite(s) which could possibly arise by way of oxidative decarboxylation via the action of the branched chain oxo-acid complex to form methylthiopropanal [47]. This compound can itself be converted to methylthiopropionic acid, a known toxic metabolite of Met [48]. Further, a role for keto acids including MTOB in controlling cell growth has been well documented [49, 50]. However, it must be recalled that MTOB alone in the absence of transaminase inhibitors promotes the growth of Met-dependent

cells. It would therefore seem that the intracellular concentration of MTOB or its metabolite(s) is critical in promoting or inhibiting growth. This is presently being investigated.

Regardless of the precise mechanism(s) responsible for the GIE of the synthetic compounds described here, their efficacy and selectivity on cells in culture are sufficiently well established to justify undertaking trials on animals grafted with Met-dependent tumours.

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REFERENCES

1. Mecham JO, Rowitch D, Wallace CD, Stern PH and Hoffman RM, The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. *Biochem Biophys Res Commun* **117**: 429–434, 1983.
2. Koziorowska J, Mazurowa N and Tautt J, Methionine dependence of virus-infected cells. *Exp Cell Res* **190**: 290–293, 1990.
3. Hall CA, Begley JA and Chu RC, Methionine dependency of cultured human lymphocytes. *Proc Soc Exp Biol Med* **182**: 215–220, 1986.
4. Hoffman RM and Erbe RW, High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. *Proc Natl Acad Sci USA* **73**: 1523–1527, 1976.
5. Stern PH, Mecham JO, Wallace CD and Hoffman RM, Reduced free-methionine in methionine-dependent SV40-transformed human fibroblasts synthesizing apparently normal amounts of methionine. *J Cell Physiol* **117**: 9–14, 1983.
6. Coalson DW, Mecham JO, Stern PH and Hoffman RM, Reduced availability of endogenously synthesized methionine for *S*-adenosylmethionine formation in methionine-dependent cancer cells. *Proc Natl Acad Sci USA* **79**: 4248–4251, 1982.
7. Judde JG, Ellis M and Frost P, Biochemical analysis of the role of transmethylation in the methionine dependence of tumor cells. *Cancer Res* **49**: 4859–4865, 1989.
8. Pegg AE and Williams-Ashman HG, Enzymic synthesis of spermine in rat prostate. *Arch Biochem Biophys* **137**: 156–165, 1970.
9. Jänne J, Pösö H and Raina A, Polyamines in rapid growth and cancer. *Biochim Biophys Acta* **473**: 241–293, 1978.
10. Porter CW, Sufrin JR and Keith DD, Growth inhibition by methionine analog inhibitors of *S*-adenosylmethionine biosynthesis in the absence of polyamine depletion. *Biochem Biophys Res Commun* **122**: 350–357, 1984.
11. Porter CW and Sufrin JR, Interference with polyamine biosynthesis and/or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Res* **6**: 525–542, 1986.
12. Tisdale MJ, Utilization of preformed and endogenously synthesized methionine by cells in tissue culture. *Br J Cancer* **49**: 315–320, 1984.
13. Backlund PS Jr and Smith RA, Methionine synthesis from 5′-methylthioadenosine in rat liver. *J Biol Chem* **256**: 1533–1535, 1981.
14. Backlund PS Jr, Chang CP and Smith RA, Identification of 2-keto-4-methylthiobutyrate as an intermediate compound in methionine synthesis from 5′-methylthioadenosine. *J Biol Chem* **257**: 4196–4202, 1982.
15. Tabor H, Rosenthal SM and Tabor CW, The biosynthesis of spermidine and spermine from putrescine and methionine. *J Biol Chem* **233**: 907–914, 1958.
16. Shapiro SK and Mather AN, The enzymatic decomposition of *S*-adenosyl-L-methionine. *J Biol Chem* **233**: 631–633, 1958.
17. Pegg AE and Williams-Ashman HG, Phosphate-stimulated breakdown of 5′-methylthioadenosine by rat ventral prostate. *Biochem J* **115**: 241–247, 1969.
18. Trackman PC and Abeles RH, Methionine synthesis from 5′-*S*-methylthioadenosine. Resolution of enzyme activities and identification of 1-phospho-5-*S*-methylthioribulose. *J Biol Chem* **258**: 6717–6720, 1983.
19. Trackman PC and Abeles RH, The metabolism of 1-phospho-5-methylthioribose. *Biochem Biophys Res Commun* **103**: 1238–1244, 1981.
20. Blom HJ, Boers GHJ, Van Den Elzen JPAM, Gahl WA and Tangerman A, Transamination of methionine in humans. *Clin Science* **76**: 43–49, 1989.
21. Reifsnyder DH, Young CT and Jones EE, The use of low protein liquid diets to determine the methionine requirement and the efficacy of methionine hydroxy analogue for the three-week-old pig. *J Nutr* **114**: 1705–1715, 1984.
22. Ghoda LY, Savarese TM, Dexter DL, Parks RE Jr, Trackman PC and Abeles RH, Characterization of a defect in the pathway for converting 5′-deoxy-5′-methylthioadenosine to methionine in a subline of a cultured heterogeneous human colon carcinoma. *J Biol Chem* **259**: 6715–6719, 1984.
23. Christa L, Kersual J, Auge J and Perignon JL, Salvage of 5′-deoxy-5′-methylthioadenosine and L-homocysteine into methionine in cells cultured in a methionine-free medium: a study of "methionine-dependence". *Biochem Biophys Res Commun* **135**: 131–138, 1986.
24. Toohey JI, Methylthio group cleavage from methylthioadenosine. Description of an enzyme and its relationship to the methylthio requirement of certain cells in culture. *Biochem Biophys Res Commun* **78**: 1273–1280, 1977.
25. Kamatani N, Nelson-Rees WA and Carson DA, Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolic enzyme. *Proc Natl Acad Sci USA* **78**: 1219–1223, 1981.
26. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
27. Meister A, Enzymatic preparation of α-keto acids. *J Biol Chem* **197**: 309–317, 1952.
28. Iwata C, 5-Deoxypyridoxal synthesis. *Biochem Prep* **12**: 117–121, 1968.
29. Brenner M and Kocher V, Eine einfache enzymatische methode zur herstellung von D- und L-methionin. *Helv Chim Acta* **32**: 333–337, 1949.
30. Wieland T, Müller R, Niemann E, Birkofer L, Schöberl A, Wagner A and Söll H, Methoden zur herstellung und umwandlung von aminosäuren und derivaten. In: *Methoden der organischen Chemie* (Ed. Houben-Weyl-Müller), pp. 355–356. Georg Thieme Verlag, Stuttgart, Band XI/2 1958.
31. Guttmann St and Boissonnas RA, Synthèse du *N*-acétyl-L-séryl-L-tyrosyl-L-séryl-L-méthionyl-γ-L-glutamate de benzyle et de peptides apparentés. *Helv Chim Acta* **41**: 1852–1867, 1958.
32. Sandler M and John H, *Design of Enzyme Inhibitors as Drugs*. Oxford Sciences Publications, 1989.
33. Walsh CT, Suicide substrates, mechanism-based

enzyme inactivators: recent developments. *Annu Rev Biochem* **53**: 493–535, 1983.
34. Severin ES, Gulyaev NN, Khurs EN and Khomutov RM, The synthesis and properties of phosphopyridoxyl amino acids. *Biochem Biophys Res Commun* **35**: 318–323, 1969.
35. Heller JS, Canellakis ES, Bussolotti DL and Coward JK, Stable multisubstrate adducts as enzyme inhibitors. *Biochim Biophys Acta* **403**: 197–207, 1975.
36. Rudd EA, Cunningham WC and Thanassi JW, Coenzyme-substrate adducts as inhibitors of mouse liver 3,4 dihydroxyphenylalanine decarboxylase. *J Med Chem* **22**: 233–237, 1979.
37. Andrews PR, Iskander MN, Jones GP and Winkler DA, Design of transition-state analogues for GABA-transaminase. *Int J Quantum Chem: Quantum Biol Symp* **9**: 345–353, 1982.
38. Brinkworth RI, Iles MM, Iskander MN and Andrews PR, Transition-state analogues as inhibitors of L-dopa decarboxylase. *Int J Biochem* **20**: 1273–1279, 1988.
39. Ayling JE and Snell EE, Relation of structure to activity of pyridoxal analogs as substrates for pyridoxamine pyruvate transaminase. *Biochemistry* 7: 1626–1636, 1968.
40. Caboche M and Hatzfeld J, Methionine metabolism in BHK cells: preliminary characterization of the physiological effects of cycloleucine, an inhibitor of *S*-adenosylmethionine biosynthesis. *J Cell Physiol* **97**: 361–370, 1978.
41. Dupuis L, Saunderson CL, Puigserver A and Brachet P, Oxidation of methionine and 2-hydroxy 4-methylthiobutanoic acid stereoisomers in chicken tissues. *Br J Nutr* **62**: 63–75, 1989.
42. Dibner JJ, Durley RC, Kostelc JG and Ivey FJ, 2-hydroxy-4-(methylthio)butanoic acid is a naturally occurring methionine precursor in the chick. *J Nutr* **120**: 553–560, 1990.
43. Fitchen JH, Risco MK, Dana BW, Lawrence HJ and Ferro AJ, Methylthioadenosine phosphorylase deficiency in human leukemias and solid tumours. *Cancer Res* **46**: 5409–5412, 1986.
44. Nobori T, Karras JG, Della Ragione F, Waltz TA, Chen PP and Carson DA, Absence of methylthioadenosine phosphorylase in human gliomas. *Cancer Res* **51**: 3193–3197, 1991.
45. Della Ragione F, Oliva A, Palumbo R, Russo GL, Gragnaniello V and Zappia V, Deficiency of 5′-deoxy-5′-methylthioadenosine phosphorylase activity in malignancy. *Biochem J* **281**: 533–538, 1992.
46. Haüssinger D, Stehle T and Gerok W, Glutamine metabolism in isolated perfused rat liver. The transamination pathway. *Biol Chem Hoppe Seyler* **366**: 527–536, 1985.
47. Jones SMA and Yeaman SJ, Oxidative decarboxylation of 4-methylthio-2-oxobutyrate by branched-chain 2-oxo-acid dehydrogenase complex. *Biochem J* **237**: 621–623, 1986.
48. Steele RD and Benevenga NJ, Identification of 3-methylthiopropionic acid as an intermediate in mammalian methionine metabolism *in vitro*. *J Biol Chem* **253**: 7844–7850, 1978.
49. McKeehan WL and McKeehan KA, Oxocarboxylic acids pyridine nucleotide-linked oxidoreductases and serum factors in regulation of cell proliferation. *J Cell Physiol* **101**: 9–16, 1979.
50. Groelke J and Amos H, Transaminase inhibitors block glycolysis and G_1 to S phase progression in chick embryo fibroblasts: reversal by α-ketoacids. *J Cell Physiol* **119**: 133–136, 1984.