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THE EFFECT OF THE METHIONINE ANTAGONIST L-2-AMINO-4-METHOXY-*TRANS*-3-BUTENOIC ACID ON THE GROWTH AND METABOLISM OF WALKER CARCINOSARCOMA *IN VITRO*

MICHAEL J. TISDALE

Department of Biochemistry, St. Thomas's Hospital Medical School, London, SE1 7EH, U.K.

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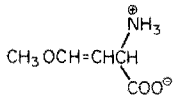
Abstract—L-2-Amino-4-methoxy-*trans*-3-butenoic acid (Ro07-7957) is a structural analogue of methionine with a potent tumour growth inhibitory activity *in vitro*. This agent is transported by the methionine carrier system in Walker carcinoma and causes an initial dose-related depression of the acid-soluble pool of methionine. The depression of the incorporation of L-[methyl-^{3}H]methionine into acid-insoluble material in the presence of Ro07-7957 is greater than that of L-[2-^{3}H]methionine and L-[4,5(*n*)-^{3}H]lysine, suggesting an inhibition of the methylation of macromolecules as well as an inhibition of protein synthesis. There is no effect of the drug on the incorporation of [^{3}H]thymidine into acid-insoluble material during the first 24 hr after treatment, while the incorporation of [5-^{3}H]uridine is stimulated 40 per cent. The ratio of incorporation of L-[2-^{3}H]methionine to L-[methyl-^{14}C]methionine into proteins increases with increasing drug concentration, suggesting an inhibition of protein methylation. This effect is more prevalent at 24 hr than after 8 hr of treatment. The specific activity of tRNA methylase using *E. coli* MRE 600 tRNA as substrate is elevated more than two-fold within 24 hr after treatment, as is also the intracellular level of *S*-adenosyl-L-methionine (SAM). The effects of this agent on macromolecular metabolism is in some respects similar to that observed with the carcinogen ethionine, and suggests the initial formation of an inhibitor of methylation, which is followed by a later attempt by the cells to maintain homeostasis by production of increased amounts of tRNA methylating enzymes.

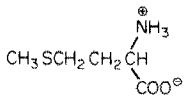
The growth of a number of tumour and transformed cell lines appears to be highly dependent on the presence of L-methionine. Dietary depletion of L-methionine has an inhibitory effect on the growth of Walker carcinosarcoma 256 in rats [1], as does treatment of animals with the enzyme L-methioninase which degrades L-methionine to methanethiol, ammonia and α-ketobutyric acid [2]. Furthermore, several tumour cell lines have demonstrated a marked inability to grow in media in which methionine has been replaced by homocysteine, folate and vitamin B_{12} [3–5], conditions which had little effect on the growth of normal cell lines. Although this concept is also applicable to some human malignant cells, a general discrimination between benign and malignant tissues on the grounds of their methionine requirement is not possible for human cells [6]. Such methionine auxotrophs display high levels of endogenous methionine biosynthesis [5]. The inability of SV40-transformed BHK-21 cells to proliferate in methionine deficient media appears to be due to a lack of 5-methyltetrahydrofolate due to a low level of 5,10-methylenetetrahydrofolate reductase (EC1.1.1.68) [4]. This defect does not seem to be responsible for the inability of Walker carcinoma to proliferate in media depleted of methionine, but supplemented with homocysteine, folic acid and vitamin B_{12} since neither 5-methyltetrahydrofolate or serine, a source of methyl groups for reduced folate, had any effect on growth rate under these conditions [7]. Furthermore, two revertants isolated from SV40-transformed cells, which had regained the ability to grow like normal cells in homocysteine supplemented medium, had no substantial changes in methionine synthetase, methylenetetrahydrofolate reductase or in the uptake of methyltetrahydrofolate by intact cells [8].

The inability of some tumour cells to grow in media of low methionine content may be due to their increased demand for this amino acid. L-Methionine is converted into *S*-adenosyl-L-methionine (SAM) by the enzyme ATP : L-methionine *S*-adenosyl transferase (EC2.4.2.13) and SAM is the sole methyl donor for all mammalian transmethylases except those involved in the biosynthesis of methionine [9], as well as being a precursor of the polyamines spermine and spermidine. These polyamines are linked to cellular growth processes and have been shown to stimulate DNA [10], RNA [11] and protein synthesis [12] and show a high correlation with cell replication [13]. The level of polyamines in the serum of a majority of patients with cancer was found to be augmented [14]. An increased tRNA methylase activity, which catalyses the transfer of a methyl group from SAM to acceptor sites in tRNA, has been found in several experimental and human tumours [15]. Also a correlation appears to exist between the growth rate of tumours and the increased tRNA methylase activity [16]. Methylthio groups may also be required for cell division, since several malignant haematopoietic cells which require sulphydryl compounds and serum for proliferation *in vitro* grow well only if methylthio compounds are provided at appropriate concentrations [17].

L-2-Amino-4-methoxy-*trans*-3-butenoic acid (I) is an antibiotic produced by *Pseudomonas aeruginosa*



I



II

growing on *n*-paraffins as the sole source of carbon and energy [18]. The structure of I closely resembles that of the amino acid methionine (II), and in *E. coli* K12 it appears to inhibit the biosynthetic pathway of methionine at a step prior to formation of cystathionine by mimicking methionine in regulating the formation and activity of homoserine *O*-transsuccinylase [18]. In view of the increased methionine requirement of tumour cells, the effect of I on tumour cell growth and methionine metabolism was investigated with Walker rat mammary carcinosarcoma cells in tissue culture.

MATERIALS AND METHODS

L-[Methyl-^{3}H]methionine (sp.act. 12.0 Ci/mmole), L-[methyl-^{14}C]methionine (sp.act. 57.2 mCi/mmole), L-[2-^{3}H]methionine (sp.act. 6.5 Ci/mmole), L-[4,5(*n*)-^{3}H]lysine (sp.act. 97.5 Ci/mmole), [5-^{3}H-methyl]thymidine (sp.act. 5.0 Ci/mmole), [5-^{3}H]uridine (sp.act. 28 Ci/mmole), *S*-adenosyl-L-[methyl-^{14}C]methionine (sp.act. 59 mCi/mmole), *S*-adenosyl-L-[methyl-^{3}H]methionine (sp.act. 12 Ci/mmole) and 3,4-dihydroxy[ring-G-^{3}H]phenylethylamine hydrochloride (sp.act. 5.0 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, England. Dulbecco's modified Eagle's medium lacking methionine and folic acid and virus and mycoplasma screened foetal calf serum were from GIBCO Bio-cult, London, England. L-2-Amino-4-methoxy-*trans*-3-butenoic acid (Ro07–7957) was a kind gift from Dr. P. G. Philpott, Roche Products Ltd., Welwyn Garden City, Herts, England. *S*-Adenosyl-L-methionine and *E. coli* MRE 600 tRNA were from Boehringer Corp. London, England.

Cell culture. Walker carcinoma and TLX5 lymphoma were routinely grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum under an atmosphere of 10% CO_2 in air. For growth experiments cells were grown in duplicate wells (3.5 ml) of a 24-well plastic plate (Flow Laboratories, Scotland). Cell number was enumerated using a Coulter counter, model F_B. Ro07-7957 was dissolved in culture medium for growth experiments. The mouse bladder carcinoma was treated with 0.025% trypsin, 10 mM EDTA prior to counting.

Nucleic acid and protein synthesis. Walker cells were suspended at 2×10^5 cells per ml in Dulbecco's modified Eagle's medium containing 2.0 μCi per ml of either L-[methyl-^{3}H]methionine, L-[2-^{3}H]methionine or L-[4,5(*n*)-^{3}H]lysine and incubated with the indicated concentrations of drug. At time intervals of 1 ml portions of the cell suspension were removed and placed on glass-fibre filter discs (Whatman GF/C 2.5 cm) wetted with saline. The cells were washed with 10 ml of 0.9% NaCl, 10 ml cold 5% trichloroacetic acid, and 5 ml of absolute ethanol. After drying at 70° for 2 hr, the radioactivity on the filters was determined using a toluene, PPO, POPOP scintillation mixture. For the assay of DNA and RNA synthesis 1 ml, portions of treated and control cultures were withdrawn periodically and incubated at 37° for 1 hr with [methyl-^{3}H]thymidine or [5-^{3}H]uridine at an isotope concentration of 5 μCi/ml. The radioactivity incorporated into acid-insoluble material was determined as above.

When the incorporation of radioactivity into nucleic acids and proteins was determined simultaneously, cells (1.5×10^5 per ml) were incubated with the indicated concentrations of drug in the presence of 20 mM sodium formate and at an isotope concentration of 0.5 μCi/ml. For the simultaneous assay of incorporation of L-[methyl-^{14}C] and L-[2-^{3}H]methionine, the isotope concentrations were 0.5 and 2.0 μCi/ml, respectively. Periodically, portions (10 ml) of the cell suspension were removed and sedimented by centrifugation at 300 *g* for 3 min, followed by washing in 0.9% NaCl and recentrifugation. The cell pellet was treated with 1 ml of ice-cold 0.5 M perchloric acid, and the precipitate was washed four times by resuspension and centrifugation in 1 ml of 0.5 M perchloric acid. An aliquot of the acid supernatant after neutralization with 5N KOH was counted in PCS scintillation fluid (Hopkin & Williams) to determine the acid-soluble radioactivity. A nucleic acid-soluble fraction (DNA + RNA) was prepared by heating the acid precipitate at 70° for 20 min in 1 ml of 1.0 M perchloric acid, cooling rapidly on ice and centrifuging at 600 *g* for 10 min at 4°. The 70° perchlorate hydrolysis was repeated on the remaining residue and after neutralization of a portion (1.6 ml) of the combined supernatant, the radioactivity was determined as above. The concentration of protein in the residue was determined by the method of Lowry *et al.* [19] using bovine serum albumin as a standard.

Uptake of methionine. Cells (2×10^7 per ml) were allowed to incubate at 37° in a calcium-free Krebs–Ringer phosphate solution for 5 min prior to the addition of L-[methyl-^{3}H]methionine (0.2 μCi per μmole; final concentration of methionine 2 mM). The cells were shaken to avoid clumping and aliquots (2 ml) were taken out at time intervals. The samples were added to 3 ml ice-cold Krebs–Ringer solution, centrifuged, the supernatant was removed and the cells were resuspended in a further 5 ml of ice-cold Krebs–Ringer solution. The suspension was centrifuged again, the supernatant removed, and the interior of the tubes was dried with paper tissue. To the washed cells was then added 2 ml 95% ethanol and a minimum period of 30 min was allowed for completion of extraction of alcohol-soluble materials. After centrifugation, 1.5 ml of the supernatant was counted in a toluene/PPO scintillation fluid.

Analysis of SAM levels. Catechol-*O*-methyltransferase (COMT) was purified from rat liver according to the method of Nikodejevic *et al.* [20]. The procedure included homogenization of rat liver in aqueous KCl (1.1%) followed by centrifugation at 17,000 *g*, acid precipitation at pH 5.2, ammonium sulphate precipitation (30–50 per cent saturation), elution from Sephadex G-25, addition of calcium

phosphate gel, concentration by vacuum dialysis and finally centrifugation at 100,000 *g* for 3 hr.

Walker cells (about 10^7) were washed with 0.9% NaCl and sonicated in 0.5 ml ice-cold 1.0 M perchloric acid and [^{14}C]SAM (4nCi) was added. The insoluble precipitate was removed by centrifugation at 2000 *g* for 30 min at 4°, the supernatant was adjusted to neutrality by addition of 5M KOH, and the insoluble potassium perchlorate was removed by centrifugation.

The assay is based on the enzymatic transfer of a methyl group from SAM to an acceptor molecule [^{3}H]dopamine, in the presence of COMT as described by Yu [21]. An aliquot of SAM or tissue extract (225 μl) was added to a COMT reaction mixture (100 μl) containing 50 μmol of Tris–HCl (pH 8.6), 3 μmol of dithiothreitol, 1.25 μmol of $MgCl_2$, 0.25 μmol (1 μCi) of [^{3}H]dopamine and enzyme. After incubation at 37° for 40 min the reaction was terminated by adding 600 μl of 0.5 M borate buffer (pH 10). The labelled 3-methoxytyramine was extracted from the incubation mixture by shaking with 1 ml of toluene : isoamylalcohol (3 : 2 v/v). After centrifugation, 800 μl of the organic phase was transferred to a clean tube containing 600 μl of 0.4 N HCl, vortex mixed and centrifuged. The radioactivity in the aqueous HCl phase (500 μl) was determined in 5 ml of PCS scintillation fluid. A standard curve was performed for each experiment.

Assay of protein methylation in vivo. Carboxy-*O*-methylation was measured by the method of Pike *et al.* [22] with modifications. Cells (2.5×10^5 per ml; 40 ml per treatment) after incubation with varying concentrations of Ro07-7957 were sedimented by centrifugation at 300 *g* for 3 min, washed once with 0.9% NaCl and recentrifuged. They were then resuspended in 40 ml of Dulbecco's modified Eagle's medium lacking folic acid and methionine, together with 80 μCi of L-[methyl-^{3}H]methionine and reincubated for 3 hr at 37°. After this time the cells were again sedimented by centrifugation, washed with 0.9% NaCl and the pellet treated with 100 μl of 1.0 M perchloric acid, and the precipitate was pelleted once more by centrifugation and washed once with 70% ethanol. The precipitated, washed protein was dissolved in 0.4 ml of 1.0 M sodium borate (pH 11), containing 0.7% methanol and incubated at 23° for 45 min to hydrolyse methyl esters to methanol. The solution was then extracted with 6 ml of toluene : isoamylalcohol (3 : 2 v/v) and 2.5 ml portions of the organic phase were transferred to each of two scintillation vials. Any [^{3}H]methanol present was removed from one vial by evaporation at 70°, after which the radiolabel was measured in both vials after dissolution in 5 ml of PCS scintillation fluid. The difference in radiolabel before and after evaporation was taken as a measure of carboxy-*O*-methylation.

Methylation of tRNA. After incubation with various concentrations of Ro07-7957, the cells were sedimented by centrifugation at 300 *g* for 3 min, washed with 0.9% NaCl and sonicated in 10 mM Tris–HCl, pH 7.4, containing 10 mM NaCl, 1.5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The supernatant fraction obtained after centrifugation at 2000 *g* for 1 hr was used as a source of methylase. The reaction mixture (final volume 125 μl) contained 50 mM Tris–HCl, pH 7.8, 0.2 M NaCl, 4 mM dithiothreitol, 100 μg *E. coli* tRNA, 83 pmole *S*-adenosyl-L-[methyl-^{3}H]methionine and enzyme extract. After incubation at 37° for varying times, the reaction was stopped by the addition of an equal volume of 10% tricholoroacetic acid. The insoluble material was collected by filtration through glass fibre filters (Whatman GF/C 2.5 cm) after 10 min at 4°. The filters were washed with ethanol and dried at 70° for 2 hr and the radioactivity was determined in a toluene, PPO scintillation mixture.

RESULTS

Toxicity. The LD_{50}'s for inhibition of growth of Walker carcinoma, TLX5 lymphoma and a mouse bladder carcinoma by Ro07-7957 were 8.5, 9.5 and 19 μg/ml, respectively. Thus the potency of this agent for inhibition of cell growth *in vitro* is similar to that of the alkylating agents and antimetabolites. Although no *in vivo* tumour growth inhibition experiments have been carried out so far, the LD_{50} for mice (354 mg/kg i.p.; determined by Roche Products Ltd.) is much higher than that for tumour cells *in vitro*, and thus this agent might be expected to be an effective antitumour agent. There is no correlation between the LD_{50}'s to Ro07-7957 and the methionine requirements of the cell lines. Surprisingly, in view of the structural similarity to methionine, there is a direct relationship between growth inhibition of the TLX5 lymphoma by Ro07-7957 and the methionine concentration in the medium (Fig. 1). Concentrations of methionine up to 100 μg/ml had no adverse effect on the growth of the TLX5 lymphoma. It is also impossible to reverse the growth inhibitory effect of Ro07-7957 by methionine or homocysteine.

Uptake. The sensitivity of the methionine transport system in Walker carcinoma to Ro07-7957 was investigated by its addition to cells previously equilibrated with methionine. There was a rapid efflux of methionine from the alcohol-soluble pool of the cells and the level of methionine fell by over 60 per cent (Fig. 2). Since two amino acids which are transported by the same system should be capable of

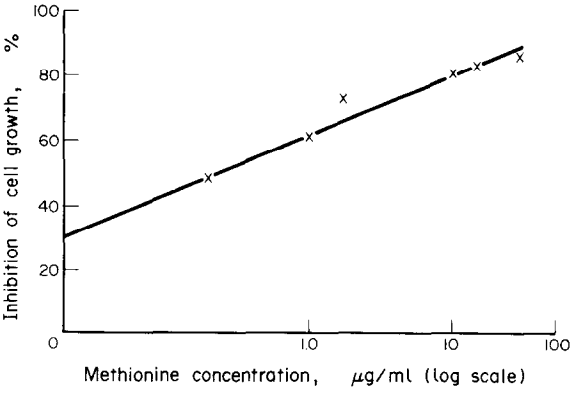


Fig. 1. The effect of methionine concentration on the growth of TLX5 lymphoma in the presence of 8 μg/ml of Ro07-7957.

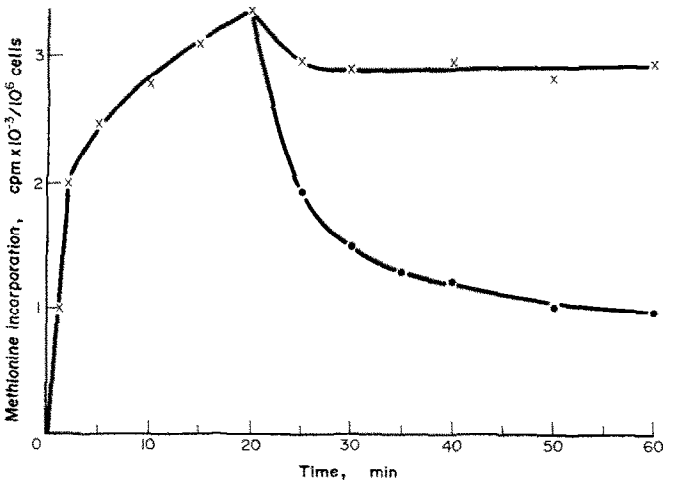


Fig. 2. The effect of Ro07-7957 on the steady state level of methionine in Walker carcinoma. Cells were incubated with 2 mM L-[methyl-^{3}H]methionine (×——×) in Krebs–Ringer phosphate solution at 37° and after 20 min Ro07-7957 (1 mM) (●——●) was added directly to the incubation medium. Samples were removed at intervals throughout and the alcohol-soluble radioactivity was assayed as described in Materials and Methods.

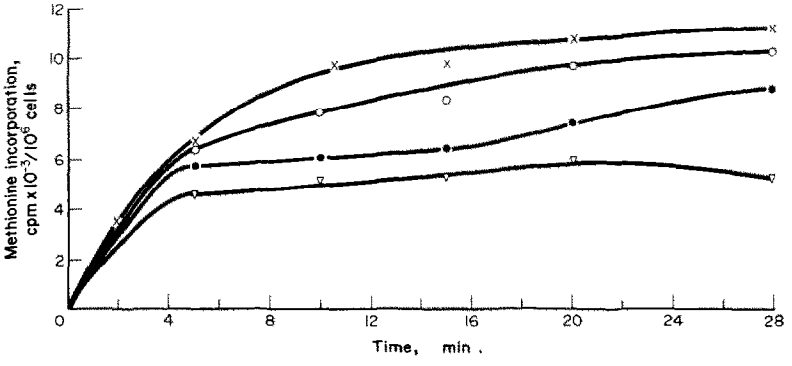


Fig. 3. The effect of Ro07-7957 on the initial uptake of methionine into Walker carcinoma. Cells were preincubated for 5 min in Krebs–Ringer solution at 37° before addition of 50 (○——○), 100 (●——●) or 200 (△——△) μg/ml of Ro07-7957. After a further 10 min, 1 mM L-methionine was added and the uptake into the alcohol-soluble pool was compared with cells receiving no drug (×——×).

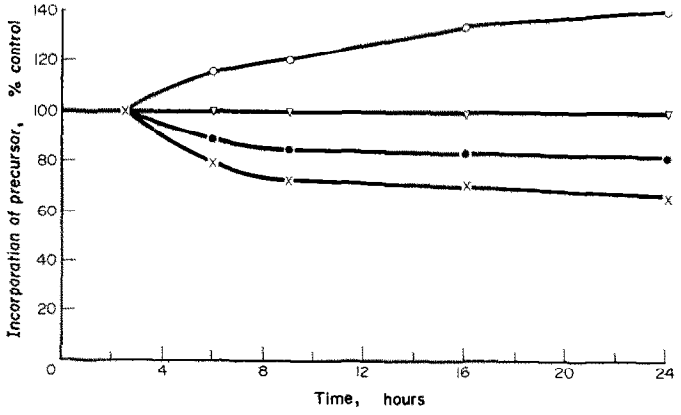


Fig. 4. The effect of Ro07-7957 (25 μg/ml) on precursor incorporation into acid-insoluble material. Cells were incubated with drug in media containing L-[methyl-^{3}H]methionine (×——×), L-[2-^{3}H]methionine or L-[4,5(*n*)-^{3}H]lysine (●——●) and the incorporation of radioactivity into acid-insoluble material was determined as in Materials and Methods. The incorporation of [methyl-^{3}H]thymidine (▽——▽) or [5-^{3}H]uridine (○——○) into acid-insoluble material was determined by a 1 hr pulse label.

exchange diffusion [23], this shows that Ro07-7957 is transported by the methionine carrier system in Walker cells. This is also confirmed by the results in Fig. 3 which show that this amino acid prevents initial uptake of methionine into cells by an amount which is proportional to the extracellular concentration.

Effect on nucleic acid and protein synthesis. The results presented in Fig. 4 show the effect of Ro07-7957 on the incorporation of precursors into proteins and nucleic acids of Walker cells. The effect on precursor incorporation is fairly rapid and apart from [5-^{3}H]uridine, no effect was observed after the first 9 hr of incubation with drug. The most significant depressive effect is seen on the incorporation of L-[methyl-^{3}H]methionine into acid-insoluble material. Since the incorporation of L-[2-^{3}H]methionine and L-[4,5(n)^{3}H]lysine into acid-insoluble material are depressed to the same extent, which is less than the extent of suppression of L-[methyl-^{3}H]methionine, which can serve both as a precursor for proteins as well as incorporation of the methyl group into proteins and nucleic acids, this suggests that Ro07-7957 exerts a suppressive effect on the methylation of macromolecules. There is no significant effect on the incorporation of [^{3}H]thymidine into DNA up to 24 hr after treatment, suggesting that the drug has little effect on [^{3}H]methyl group incorporation into the 'one carbon' pool. The extent of RNA synthesis as measured by the incorporation of [5-^{3}H]uridine into acid-insoluble material is increased after treatment of Walker carcinoma with Ro07-7957 and reaches a maximal 140 per cent of the control 24 hr after drug addition. The stimulation of RNA synthesis by Ro07-7957 increased with increasing drug concentration (Fig. 5). The most pronounced depression of L-[methyl-^{3}H]methionine incorporation into acid-insoluble material occurred at concentrations of Ro07-7957 near the ID_{50} value.

Effect on tRNA and histone methylases and SAM pools. The methylation of 'methyl deficient' *E. coli* MRE 600 tRNA by cytosolic extracts of Walker carcinoma was linear with incubation time up to at least 30 min (Fig. 6). The specific activity of tRNA methylase was increased significantly within 24 hr after treatment with Ro07-7957 and remained elevated for a 48 hr period (Fig. 6 and Table 1). There was a concomitant, though smaller, elevation of the specific activity of histone methylase (Table 1), but a decrease in carboxy-*O*-methylation of proteins at low doses and a slight stimulation at higher doses of the drug (Table 2). There was an initial dose related increase in the intracellular level of SAM which was evident within 24 hr of drug treatment (Table 2). At 48 hr post-treatment there was no difference between the level of SAM in Ro07-7957 and control cultures.

Methylation of cellular proteins and nucleic acids in whole cells. The results in Table 3 show the effect of various concentrations of Ro07-7957 on the extent of incorporation of L-[methyl-^{14}C]- and L-[2-^{3}H]methionine into the acid soluble pool, nucleic acids and proteins of Walker carcinoma. The drug causes an initial drop in the acid-soluble pool of methionine in Walker cells, evident within 4 hr of treatment, which is related to the extracellular concentration of the drug. This initial drop in the methionine pool size is probably due to competition of Ro07-7957 for uptake into the cell (Fig. 3) and is followed by an increase in the size of the acid-soluble pool to levels which exceed that of the control. No effect on the incorporation of label into proteins or nucleic acids is evident until 8 hr after treatment. The effect on the incorporation of label into nucleic acids after 8 hr is dependent on the dose of Ro07-7957. Up to 10 μg/ml there is a stimulation of the incorporation of both ^{14}C and ^{3}H into nucleic acids, followed by a decrease to below control values at concentrations of Ro07-7957 above 15 μg/ml. An inhibition of the incorporation of both labels into

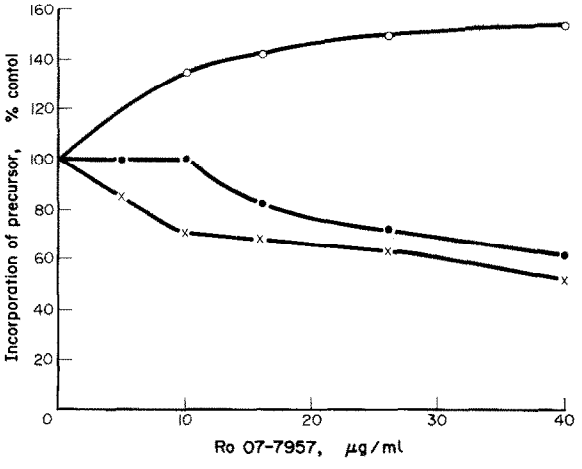


Fig. 5. The effect of Ro07-7957 concentration on the incorporation of L-[methyl-^{3}H]methionine (×——×), L-[2-^{3}H]methionine (●——●) and [5-^{3}H]uridine (○——○) into acid-insoluble material measured 24 hr after drug addition.

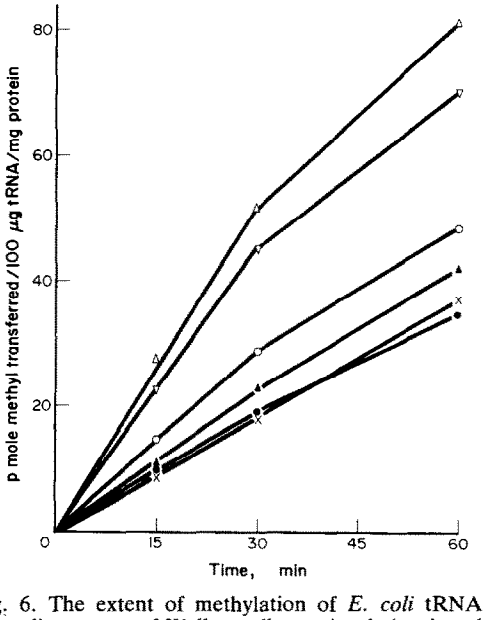


Fig. 6. The extent of methylation of *E. coli* tRNA by cytosolic extracts of Walker cells previously incubated for 48 hr in the absence of drug (×——×) or in the presence of 5 (●——●), 10 (▲——▲), 15 (○——○), 25 (▽——▽) or 40 (△——△) μg/ml Ro07-7957.

Table 1. Effect of Ro07-7957 on the specific activity of tRNA and histone methylases*

Treatment	tRNA methylase pmole methyl transferred/100 μg tRNA/mg protein/min ± S.E.M.		Histone methylase pmole methyl transferred/mg histone/min ± S.E.M.
Time	24 hr	48 hr	24 hr
None	0.61 ± 0.03	0.64 ± 0.02	0.95 ± 0.05
Ro07-7959 10 μg/ml	1.39 ± 0.05	0.71 ± 0.03	1.10 ± 0.06
Ro07-7959 15 μg/ml	1.52 ± 0.04	0.97 ± 0.05	1.20 ± 0.04
Ro07-7959 25 μg/ml	1.30 ± 0.06	1.49 ± 0.04	1.67 ± 0.06
Ro07-7959 40 μg/ml	1.25 ± 0.07	1.71 ± 0.06	1.33 ± 0.1

* Histone methylation was determined using 0.4 mg histone as substrate and the incorporation of [methyl-^{3}H] into acid insoluble material was measured as for methylation of tRNA.

nucleic acids is seen at all concentrations of Ro07-7957 24 hr after treatment, and a small increase in the ratio of incorporation of $^3H/^{14}C$. There is a dose-related inhibition of the incorporation of both ^3H and ^{14}C into proteins, which is evident 8 hr after treatment. There is also an increase in the ratio of incorporation of $^3H/^{14}C$ with increasing drug concentrations at 24 hr after treatment, suggesting that Ro07-7957 causes both an inhibition of methylation and biosynthesis of proteins.

DISCUSSION

The structural similarity of Ro07-7957 to methionine and the presence of an unsaturated linkage suggested that it could act as an irreversible antimetabolite of methionine. This agent exerts a strong growth inhibitory activity *in vitro*, indicating that it could possibly be a useful anti-tumour agent. A strong growth inhibitory activity of this amino acid antagonist has previously been shown towards *E. coli* K12 [18]. In this case growth inhibition was reversed by methionine, homocysteine and cystathionine, but the cytostatic effect towards eukaryotic cells shows no such reversal pattern. In fact, increasing methionine concentrations increase the growth inhibitory effect of Ro07-7957. This suggests that Ro07-7957 inhibits growth of tumour cells by a different mechanism from that operating in bacteria, which is probably at a step prior to the formation of cystathionine [18]. Eukaryotic cells do not show *de novo* biosynthesis of methionine. Ro07-7957 inhibits the transport of methionine into Walker carcinoma cells and also undergoes exchange diffusion with this amino acid. This suggests that these two amino acids are transported by the same system, which probably accounts for the initial fall in the acid-soluble pool of methionine when Ro07-7957 is added to cells.

The tRNA methyltransferases, a family of enzymes that modify the structure of preformed tRNA by the insertion of methyl groups, have been shown to be elevated in all malignant neoplasms examined so far, but not in benign ovarian tumours [24]. From a study of the methylation patterns in Morris hepatomas, it has been concluded [25] that the increase in methylase activities in the tumours reflect an increase in the overall synthesis and degradation of RNA rather than an overmethylation of hepatoma tRNA. As a rule, tRNA cannot usually be methylated further by enzymes from the same cell and further methylation can only be achieved on a heterologous tRNA substrate from an organism lower on the evolutionary scale than that from which the methylase preparation originated, since tRNA from lower organisms is less methylated. The substrate used in the studies on Walker carcinoma tRNA methylase was *E. coli* tRNA. Using such a substrate, there was more than a two-fold elevation in the specific activity of tRNA methylase after Ro07-7957 treatment, which was evident within 24 hr of drug addition. A similar, though smaller, elevation of histone methylase was also observed. Histone methylase has been shown to be considerably less sensitive

Table 2. Effect of Ro07-7957 on the intracellular level of SAM and on protein carboxy-*O*-methylation

Treatment	SAM ng/mg protein ± S.E.M.		Carboxy-*O*-methylation (% control) after 48 hr
Time	24 hr	48 hr	
None	216 ± 30	320 ± 42	100
Ro07-7957 10 μg/ml	315 ± 40	329 ± 38	83
Ro07-7957 15 μg/ml	327 ± 38	329 ± 18	70
Ro07-7957 25 μg/ml	393 ± 36	410 ± 30	118
Ro07-7957 40 μg/ml	477 ± 56	270 ± 30	110

Table 3. Extent of incorporation of L[methyl-^{14}C]- and L-[2-^{3}H]methionine into Walker carcinoma in the presence of Ro07-7957 (dpm/0.1 mg protein)*

		Control			Ro07-7957 (μg/ml) 5			10			15			25			40		
th		^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C
4	$HClO_4$ soluble	3378	9460	2.8	3883	9739	2.5	2758	7585	2.8	1738	4685	2.7	1754	4807	2.7	1833	5033	2.7
	Nucleic acids	3287	3626	1.1	3603	4334	1.2	3356	4136	1.2	2696	3239	1.2	3131	3597	1.1	3227	4112	1.3
	Proteins	19,299	31,401	1.6	19,890	32,144	1.6	19,908	37,183	1.8	18,260	35,419	1.9	21,375	39,294	1.8	20,518	32,113	1.6
8	$HClO_4$ soluble	5368	17,532	3.2	5560	20,081	3.6	6266	22,657	3.6	6132	21,592	3.5	7131	21,058	3.0	6117	18,361	3.0
	Nucleic acids	5095	6095	1.2	5242	6373	1.2	6733	8448	1.3	4479	5694	1.3	3097	3297	1.0	2849	3369	1.2
	Proteins	35,243	70,771	2.0	38,956	74,098	1.9	43,240	66,818	1.5	30,577	60,477	2.0	25,437	41,641	1.6	19,222	35,209	1.8
24	$HClO_4$ soluble	22,606	34,718	1.5	32,178	51,767	1.6	26,320	45,756	1.7	29,654	55,490	1.9	22,911	37,477	1.6	17,195	32,305	1.9
	Nucleic acids	18,948	21,835	1.1	18,765	22,653	1.2	15,915	20,320	1.3	14,917	18,631	1.3	13,226	15,979	1.2	11,392	14,582	1.3
	Proteins	48,136	32,099	0.7	44,704	32,622	0.7	35,219	49,089	1.4	29,447	30,432	1.0	28,808	27,244	0.95	27,244	28,304	1.0

* Mean of three experiments. The experimental points did not deviate more than 5 per cent.

than tRNA methylase to the inhibitory action of 3,4-dihydroxyphenylethylamine [26]. Administration of the carcinogens ethionine [27], dimethylnitrosamine [28] and *N*-nitrosomethylurea [29] has been shown to result in a similar elevation of the level of tRNA methylating enzymes. The reason for this elevated methylase activity is unknown, but may reflect the expression of foetal properties.

A similar paradoxical situation exists after treatment of Walker carcinoma with Ro07-7957 as exists in animals treated with ethionine, i.e. although there is an increase in tRNA and histone methylating enzyme activity, there is incompletely methylated nucleic acids and proteins. Wainfan *et al.* [30] showed the presence of a low molecular weight material that inhibited tRNA methylation, which accumulated in rat liver shortly after ethionine administration. Subsequently, in the presence of inhibitor tRNA methylase activity increased. Although the inhibitor has not been characterized it seems likely that *S*-adenosylethionine is one of the components of the low molecular weight inhibitor fraction generated after ethionine administration. A similar formation of the *S*-adenosyl complex of Ro07-7957 seems less likely because of the lowered stability of oxonium ions. An experiment was performed to test such a possibility by incubating a cytosolic fraction of Walker carcinoma with *E. coli* tRNA and various concentrations of Ro07-7957 up to 80 μg/ml in the presence or absence of 6.5 μM ATP. Under such conditions there was no effect of Ro07-7957 on the incorporation of [^{3}H]methyl groups from SAM into tRNA in the presence or absence of ATP. There was also no effect of Ro07-7957 on the formation of SAM from L-[methyl-^{3}H]methionine in an *in vitro* assay using a crude cytosolic fraction of Walker carcinoma. These results argue against the possibility of the formation of an *S*-adenosyl derivative of Ro07-7957.

A possible sequence of events following addition of Ro07-7957 to Walker cells could be (a) an initial efflux of methionine due to exchange diffusion followed by an increase in the size of the acid-soluble pool to levels which exceed that of the control, (b) production of an inhibitor of methylation, (c) reduction in methylation of proteins and possibly nucleic acids, (d) derepression of the locus for tRNA and histone methylase synthesis, (e) nearly normal methylation due to excess enzyme. A similar increase in tRNA methylase activity is observed in Walker cells deprived of exogenous methionine and supplemented with homocysteine, folic acid and vitamin B_{12}, conditions which rapidly lead to growth inhibition and eventual cell death, probably due to insufficient SAM to maintain the levels of methylation required [7]. In the presence of Ro07-7957 the SAM levels are elevated within 24 hr, but not after 48 hr of treatment, suggesting either an inhibition of the conversion of SAM to polyamines by SAM decarboxylase or of methylation reactions. In view of the importance of methylation reactions to tumour cell growth, the effectiveness of Ro07-7957 as an antitumour agent is to be further studied on a xenografted human tumour, the growth requirements of which can be studied *in vitro*.

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