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# THE MEASUREMENT OF POLYGLUTAMATE METABOLITES OF THE THYMIDYLATE SYNTHASE INHIBITOR, ICI D1694, IN MOUSE AND HUMAN CULTURED CELLS

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**Abstract**—A method is described for the measurement of the polyglutamates of the quinazoline thymidylate synthase inhibitor, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-theonyl)-L-glutamic acid (ICI D1694). This involved incubation of cells with [5-$^3$H]ICI D1694, extraction of the polyglutamates and their analysis by HPLC using an ion-pairing method. Co-chromatography with ICI D1694 and its synthetic di-hexaglutamate standards (UV detection) aided identification of the [$^3$H]polyglutamates in the fractions recovered from the HPLC. Recovery of the polyglutamates at each stage of extraction and analysis was very good (77–84% overall recovery). Polyglutamates readily accumulated as the tri-, tetra and penta forms and occasionally a small amount of hexaglutamate was found. After mouse L1210 leukemia or human W1L2 lymphoblastoid cells were incubated for 30 min with 0.1 μM [$^3$H]ICI D1694 there was a ~6-fold concentration effect intracellularly with most of the $^3$H associated with polyglutamate forms (~75% and 96% for the L1210 and W1L2, respectively). Even some of the higher chain length tetra- and pentaglutamates could be detected at this time. After 4 hr incubation the total level of intracellular $^3$H had risen to 2–3 μM, greater than 96% of which was associated with polyglutamates (mainly tetra- and pentaglutamates). Four other human cell lines, two ovarian (CH1 and 41M), the MCF-7 breast and the HT-29 colon, were examined for their ability to form intracellular polyglutamates. A 4 hr incubation with 0.1 μM [$^3$H]ICI D1694 resulted in a substantial intracellular accumulation of the drug (20–100-fold) in its polyglutamate forms with only 2–20% remaining as the parent monoglutamate, depending on the cell line. The major polyglutamate was again cell line dependent, ranging from the tri to the penta form. Prolonging the incubation time to 24 hr allowed a further accumulation of drug with a larger percentage appearing as tri- to hexaglutamates. Although cell lines differed in the total level of polyglutamates formed and the pattern of chain length observed, rapid and extensive polyglutamation of ICI D1694 occurred in all the cell types examined.

Antifolate drugs mediate their cytotoxic effects through inhibition of one or more key folate-dependent enzymes involved in the *de novo* synthesis of purines and thymidylate. These enzymes include dihydrofolate reductase (DHFR‡), thymidylate synthase (TS), glycinamide ribonucleotide transformylase (GARTF; EC 2.1.2.1) and aminoimidazole carboxamide ribonucleotide transformylase (AICARTF; EC 2.1.2.3). Another enzyme, central to the activity of many antifolates, is the folate-metabolizing enzyme, folylpolyglutamate synthetase (FPGS) which is responsible for the γ-addition of extra glutamate residues to the natural intracellular reduced-folate cofactors [1, 2]. These polyglutamates are generally the preferred substrates for the folate-dependent enzymes and their poly-anionic nature serves to conserve the folate pool by reducing drug efflux. Antifolates may also be substrates for FPGS and the result of polyglutamation may be elicited as (1) an increase or change in specificity for a target enzyme(s); (2) increased cytotoxic potency; (3) a mechanism for drug retention [3–20]. For example, the increased cellular accumulation and drug retention of methotrexate (MTX) through polyglutamation [10, 11, 17, 18] serves to enhance cytotoxicity particularly after only brief exposure times [13]. The polyglutamates of MTX do not inhibit isolated DHFR to a greater extent than the parent drug but do have significantly enhanced inhibitory activity towards other folate-dependent enzymes such as TS, AICARTF and GARTF which may, in some cell types, be additional cellular targets [8, 14, 19, 20].

A class of quinazoline TS inhibitors that include

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‡ Abbreviations: TS, thymidylate synthase (EC 2.1.1.45); DHFR, dihydrofolate reductase (EC 1.5.1.3); FPGS, folypolyglutamate synthetase; CB3717, $N^{10}$-propargyl-5,8-dideazafolic acid; ICI 198583,2-desamino-2-methyl-$N^{10}$-propargyl-5,8-dideazafolic acid; ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; glu-(*n*), glutamates where *n* = total number of glutamates; di–hexaglutamates, ICI D1694 plus 1–5 extra glutamates; MTX, methotrexate; dThd, thymidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PBS, phosphate-buffered saline.

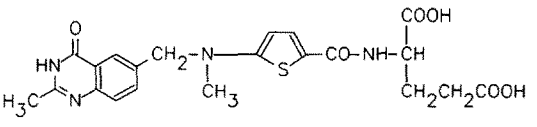

Fig. 1. Structure of ICI D1694.

the clinically evaluated drug, $N^{10}$-propargyl-5,8-dideazafolic acid (CB3717), are also polyglutamated intracellularly. These polyglutamates are substantially better (up to two orders of magnitude) as inhibitors of TS and are generally considered to be the cytotoxic drug species as they are selectively retained intracellularly over the parent drugs [15, 16, 21]. CB3717 caused dose-limiting and life-threatening nephrotoxicity [22] due to poor solubility in the acid pH of the urine and its withdrawal from clinical study precipitated the search for non-nephrotoxic analogues [16, 21 and Refs therein]. The result was the development of the more water-soluble drug, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl-L-glutamic acid (ICI D1694) (Fig. 1), with a significantly improved antitumour activity over CB3717 in mouse models [32–30]. ICI D1694 has completed Phase I evaluation and is now in Phase II study.

ICI D1694 is ~500-fold more active as an inhibitor of cell growth (mouse and human cell lines) than CB3717 despite being 20-fold less effective as an inhibitor of isolated TS ($K_i = 60$ nM) [23]. The excellent substrate activity for isolated FPGS ($K_m = 1.3\ \mu M$; $V_{max}/K_m = 220$ relative to folic acid) suggested that at low intracellular concentrations, ICI D1694 would be metabolized to at least diglutamate ~200-fold faster than CB3717 [24]. The synthetic polyglutamates of ICI D1694 were also tested as inhibitors of isolated TS and DHFR and, as usually seen with quinazoline TS inhibitors, a significant increase in TS inhibition was observed ($K_i$ for the tetraglutamate = 1 nM) although inhibition of DHFR was unchanged ($K_i = \sim 100$ nM) [23]. The cytotoxicity of ICI D1694 is prevented by co-incubation with thymidine indicating that TS is the locus of action [23]. Similarly, ICI D1694 is not active against a human W1L2 TS over-producing cell line [23] but has good activity against an L1210 DHFR-overproducing line (data not shown). The whole cell (*in situ*) TS assay (*in vitro* and *in vivo*) [23, 29] and short-exposure assays [29, 30] all indicate that ICI D1694 is still highly active after extracellular drug removal consistent with extensive polyglutamate formation. Nevertheless unambiguous measurement of their synthesis was required. We now describe a method for the estimation of polyglutamate metabolites of ICI D1694 in cultured cells.

A reverse-phase HPLC method was previously described for the measurement of the polyglutamates of CB3717 [31] and its 2-desamino-2-methyl analogue (ICI 198583) [16]. Although resolution of the polyglutamates (1–4 extra glutamate residues; di-pentaglutamates) was satisfactory it was thought that if ICI D1694 was metabolized further to higher polyglutamates than the pentaglutamate, resolution would be inadequate. Therefore, we have developed an ion-pairing method based on that reported for the measurement of MTX polyglutamates by Pizzorno *et al.* [32]. Aided by use of the synthetic di- to hexapolyglutamate standards we have identified all of these polyglutamates of ICI D1694 in extracts from both mouse and human cells.

## MATERIALS AND METHODS

*Materials.* The synthesis of ICI D1694 and its synthetic polyglutamates (di–hexaglutamate; 1–5 extra glutamate residues) has been described previously [33]. All reagents used were of analytical grade and obtained from standard suppliers unless otherwise stated. Tissue culture media and sera were obtained from Flow Laboratories (Irvine, U.K.) unless otherwise stated.

HPLC was performed on a Waters Associates Chromatograph (Waters Associates, Harrow, U.K.) using a Kontron MT2 data acquisition system (St Albans, U.K.). Liquid scintillation counting was performed using a Tricarb 2000 liquid scintillation counter (Canberra Packard Ltd, Pangbourne, U.K.). Coulter counting was performed using a ZM Coulter Counter (Coulter Electronics Ltd, Luton, U.K.).

*Purification of* [5-$^3$H]*ICI D1694.* [5-$^3$H]ICI D1694 was synthesized by ICI Cambridge Research Biochemicals Ltd (Billingham, U.K.) at a specific activity of 8.9–10.8 Ci/mmol (1 mCi/mL in 11% aqueous methanol). This was later diluted to 0.7 mCi/mL with ethanol to prevent freezing at −20°. Prior to use, [$^3$H]ICI D1694 was re-purified because of the appearance of radiolysis products. Two methods have been used for the first stages of the purification procedure. The initial one involved adding ethanol (1 mL) to [$^3$H]ICI D1694 (1 mL) and drying to ~100 μl at 40° under a gentle stream of nitrogen. A second, improved method involved the use of a crude separation using a C-18 6 mL bond Elut cartridge (Analytichem International, Hengoed, Mid Glamorgan, U.K.). Initially the bond Elut was equilibrated using washes of methanol (3 mL), deionized water (3 mL) and 0.1 M sodium acetate pH 4.0 (3 mL), respectively. [$^3$H]ICI D1694, 1.5 mL (~1 mCi) was diluted with 1.5 mL of deionized water and then applied to the column. This was then washed with 2 × 3 mL 0.1 M acetic acid pH 4.0, 1 mL 10% methanol and 1 mL 20% methanol. [$^3$H]-ICI D1694 was eluted in 90% methanol (2 mL), and then reduced to ~100 μL at 40° under a gentle stream of nitrogen. This latter method had a significant advantage in that further radiolysis did not occur throughout the procedure and recovery was improved. The radioactive material was applied to a spherisorb C6 5 μm column, 15 cm × 0.46 cm (Phase Separations, Deeside, Clwyd, U.K.) and eluted using an acetonitrile gradient (5–20% over 15 min in 0.1 M sodium acetate, pH 5.0) at a flow rate of 2 mL/min. Fractions (~1 mL) were collected and the radioactive content determined by liquid scintillation counting in 10 mL of Ultima Gold Scintillant (Canberra Packard). The majority of the

$^3$H had a retention time co-incident with that of ICI D1694. The fraction(s) of interest (1–2 mL) were pooled and then diluted 1:1 with ethanol and stored at −20° for up to 1 month. The concentration was 20–80 μM and the radioactive purity was >99% as assayed using this HPLC procedure.

*Cell culture and extraction of* [5-$^3$H]*ICI D1694 and its polyglutamate metabolites.* L1210 mouse leukemia cells and W1L2 human lymphoblastoid cells were grown by suspension culture in RPMI 1640 medium without sodium bicarbonate but with 20 mM HEPES and with the addition of 10% donor horse serum (L1210) or foetal calf serum (W1L2). Cultures (10 mL each) of exponentially growing cells were used for the experiments (~3–5 × $10^5$/mL). All other human cell lines were grown as monolayers. The human ovarian cell lines (CH1 and 41M) [34] and the human colon HT29 (European Collection of Animal Cell Culture, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K.) were grown in Dulbecco's Modified Eagles Medium containing 10% foetal calf serum (Imperial Laboratories, Andover, U.K.) as described previously [34]. The MCF-7 cells were grown in RPMI 1640 supplemented with 5% foetal calf serum as reported previously [35]. The cells were grown in Nunc T80 plastic tissue culture flasks (Nunclon, Roskilde, Denmark) until the cells were approaching confluence but still in the logarithmic growth phase (~$10^7$ depending on cell type). Unless specified, experiments were performed in duplicate. L1210 cells were incubated at 37° with 0.1 or 0.5 μM [$^3$H] ICI D1694 for the times indicated. The human cell lines were incubated with 0.1 μM [$^3$H]ICI D1694 for 4 and 24 hr (plus 30 min for the W1L2 cells). After incubation L1210 and W1L2 cells were centrifuged at 600 *g* × 5 min at 4°, resuspended in 50 mL ice-cold phosphate-buffered saline (PBS), recentrifuged, and then resuspended in 10 mL PBS and transferred to preweighed 15 mL glass conical test tubes. An aliquot (100 μL) was taken for cell counting and the rest was again re-centrifuged. The pellets were resuspended in 0.7 mL ice-cold de-ionized water and sonicated using an MSE Soniprep 150 ultrasonicator for 30 sec (70 kHz, probe amplitude 70 microns) (MSE, Crawley, U.K.). CH1, 41M, MCF7 and HT29 cells were washed twice with 50 mL of ice-cold PBS and then scraped from the plastic flasks with cell scrapers in 1 mL of water. Cell counts were performed on parallel cultures using an Improved Neubauer Haemacytometer after trypsinization. Total cellular $^3$H-material in each sonicate was estimated by taking 3 × 25 μL aliquots which were hydrolysed overnight at 40° in 500 μL 1 M NaOH, neutralized with 160 μL of glacial acetic acid and radioactivity determined by liquid scintillation counting (Ultima Gold Scintillant). The cellular concentration was calculated taking into account the volume of cell extract (by weighing the glass tubes containing the extract) and the cell volumes ($10^6$ L1210 cells = 0.63 μL; W1L2 cells = 0.89 μL; MCF7 = 2 μL; CH1, 41M and HT29 = 1 μL). The remaining extract was boiled for 15 min and the tubes again re-weighed to estimate any volume change. A mixture of ICI D1694 and its di- to hexaglutamates (60 μL) was added to 600 μL of the boiled samples to give a final concentration of 20 μM each. These samples were then centrifuged through an Amicon Micropartition Filter (molecular cut off 30 kDa; Amicon Ltd, Stonehouse, U.K.) at 1400 *g* × 30 min at 4°. The filtrate was again analysed for radioactivity (2 × 50 μL) to allow assessment of any loss during filtration and subsequent HPLC analysis. The remaining sample was stored at −20° until HPLC analysis (up to 2 weeks).

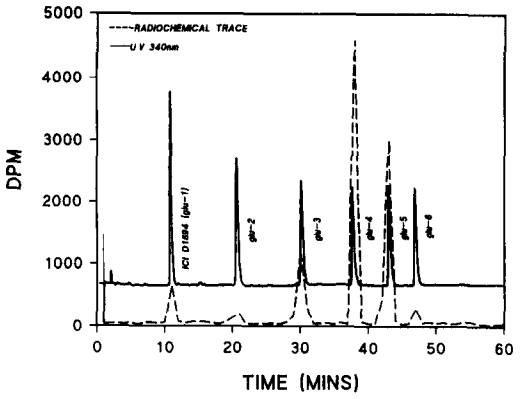


Fig. 2. HPLC separation of synthetic ICI D1694 polyglutamates (solid line). The dotted line is the radiochromatogram of an extract of L1210 cells exposed to 0.1 μM [5-$^3$H]ICI D1694 for 24 hr.

*HPLC analysis.* Aliquots (100 μL) were applied to a C-18 polygosil 15 cm × 0.46 cm 5 μm column (Camlab, Cambridge, U.K.) and eluted using an acetonitrile gradient as follows: 20–45% acetonitrile in 1.5 mM tetrabutylammonium hydroxide buffered to pH 7.0 with $H_3PO_4$, over 60 min, held for 30 min and back to 20% over 10 min at a flow rate of 2 mL/min. The effluent was continually monitored by UV at 280 and 340 nm. Seventy × 1 min fractions were collected and each one was mixed with 10 mL Ultima Gold Scintillant and quantitated for radioactivity. Co-chromatography with synthetic standards allowed identification of ICI D1694 and its polyglutamates (Fig. 2). Data is presented below demonstrating that the small loss of $^3$H during the filtration and HPLC steps was not associated with any preferential loss of one fraction over another or evidence of $^3$H not associated with ICI D1694 or its polyglutamates. Therefore the level of each fraction can be expressed as % total cellular $^3$H recovered from the HPLC and this in turn can allow the calculation of the molar concentration of each metabolite, i.e. (total cellular $^3$H × fraction as a % of the total cellular $^3$H recovered from HPLC)/100.

A second, reverse-phase HPLC system was also employed to verify the results of the ion-pairing method. A spherisorb C6 5 μm 15 × 0.46 cm column was used. Elution was as follows: 5–30% acetonitrile linear gradient in 0.1 M sodium acetate pH 5.0 for 20 min, held for 20 min, followed by a linear gradient back to 5% acetonitrile over 10 min. Flow rate was 2 mL/min. Fractions were collected as 56 × 30 sec fractions and analysed for radioactivity as described above.

Table 1. The levels of ICI D1694 and its polyglutamate forms after incubation of L1210 cells for 4 hr with 0.5 μM [5-$^3$H]ICI D1694 (five parallel cultures)

| | Cellular concentration, μM (range) Not corrected | Corrected* |
|---|---|---|
| Total cellular [$^3$H]drug-derived material | | |
| before extraction | 3.46–3.64 | — |
| after extraction | 3.15–3.49 | — |
| Recovered from HPLC | 2.81–2.91 | — |
| Monoglutamate (parent drug) | 0.36–0.39 | 0.44–0.49 |
| Diglutamate | 0.12–0.13 | 0.15–0.17 |
| Triglutamate | 0.97–1.03 | 1.26–1.27 |
| Tetraglutamate | 1.28–1.36 | 1.56–1.61 |
| Pentaglutamate | 0.05–0.06 | 0.06–0.07 |
| Hexaglutamate | 0 | 0 |

* Corrected for loss during extraction and HPLC.

## RESULTS

### *Extraction and HPLC analysis of* [5-$^3$H]*ICI D1694 and its polyglutamates from L1210 cells*

An experiment was performed to assess the inter-experimental variation and recovery of ICI D1694 and its polyglutamate metabolites at each stage of the analysis. Five L1210 cultures were treated with 0.5 μM [$^3$H]ICI D1694 for 4 hr and then each was separately taken through the extraction and HPLC procedures described above. Samples were removed for quantitation of radioactive content at every stage of the procedure. Taking into account any loss through boiling (determined by pre and post tube weighings) and through removal of samples for scintillation counting it was found that $^3$H was only significantly lost at two stages, filtration and HPLC analysis. The recovery at the filtration stage was between 83 and 92% and at the HPLC stage was between 88 and 97%. Variability at the HPLC stage is expected (particularly when low levels of radioactivity are applied) since this relies on the addition of all radioactive counts and subtraction of a blank value. The good recovery suggested that ICI D1694 was not being metabolized to anything other than polyglutamate forms. A different reverse-phase HPLC method supported this conclusion as recovery of $^3$H material from the column was 96% and $^3$H was not associated with peaks other than those of ICI D1694 and its polyglutamates (data not shown).

As the purpose of the filtration stage was to remove denatured protein it was important to determine whether the loss of tritium at this stage was associated with the preferential retention of one polyglutamate over another (or the parent drug). We therefore added a mixture of ICI D1694 and its synthetic polyglutamates (final concentration of 50 μM) to five aliquots of an L1210 cell extract. After boiling and filtration the samples were analysed by HPLC and the concentration in each peak determined by UV absorption at 340 nm. A parallel experiment was performed using 0.15 M $NaHCO_3$ instead of cell extract. The results clearly showed that there was the same loss in the presence or absence of cell extract and no preferential loss of any one metabolite (91 ± 3% in extract and 92 ± 4% in $NaHCO_3$).

### *Polyglutamate formation in L1210 (mouse) and W1L2 (human) cells*

The radiochromatogram (Fig. 2) illustrates the distribution of radioactivity from an L1210 culture incubated for 24 hr with 0.1 μM [$^3$H]ICI D1694 and compared with the retention times of the synthetic polyglutamate standards. The majority of the tritiated material was associated with the tri–pentapolyglutamates, with very little unmetabolized ICI D1694 remaining. The results of the five parallel cultures (0.5 μM for 4 hr) are summarized in Table 1. The overall recovery of $^3$H throughout the procedures was 77–84%. The major metabolites were triglutamate (~35%) and tetraglutamate (~46%). Very little di- or pentaglutamate could be detected (~5 and 2%, respectively) and unmetabolized drug accounted for ~13% of the cellular $^3$H. It is interesting that when the incubation time was extended to 24 hr tetraglutamate remained the predominant drug form (65%) although the pentaglutamate now formed 19% of the cellular $^3$H (total cellular $^3$H = 6.6 μM) [23]. Even increasing the extracellular concentration of [$^3$H]ICI D1694 to 1 μM and incubating for 48 hr (total cellular $^3$H = 11.6 μM) did not allow metabolism to alter greatly the pattern of polyglutamation with tetra- and pentaglutamates forming 56 and 29% of the polyglutamate pool, although now some hexaglutamate could be detected (~1%) (data not shown).

Figure 3 compares the formation of ICI D1694 polyglutamates in the mouse L1210 and human W1L2 cell lines (both grown by suspension culture) after 0.5, 4 and 24 hr of exposure to 0.1 μM drug. The absolute cellular levels of drug-derived material were similar for both cell types at 0.5 and 4 hr but slightly higher at 24 hr in the W1L2 cells. At 0.5 hr more unmetabolized drug was found in the L1210 cells with less metabolism to the higher polyglutamates than in the W1L2 cells. The predominant polyglutamate was different for the two cell lines at 4 and 24 hr, being tetraglutamate in the L1210 and pentaglutamate in the W1L2.

### *Polyglutamate formation in other human cells*

Two human ovarian (CH1 and 41M), one human

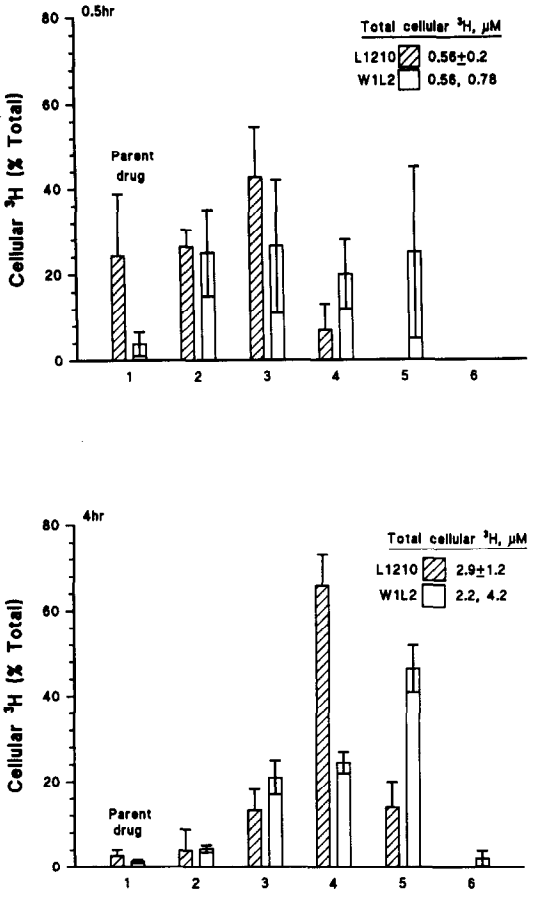


Fig. 3. The metabolism of 0.1 μM [5-$^3$H]ICI D1694 to polyglutamate forms in mouse L1210 and human W1L2 cells at 0.5, 4 and 24 hr. The total cellular level of $^3$H drug-derived material is given as either mean ± SD (N = 3 or 4) or individual results where each is the mean of duplicate cultures. N = one experiment (usually duplicate cultures) performed on separate days. Figures in parentheses refer to a value without an HPLC analysis. Error on bars is either SD (N = 3 or 4 and denoted by single-headed error bars) or range (N = 2 and denoted by double-headed error bars). No error bars = mean of duplicate cultures.

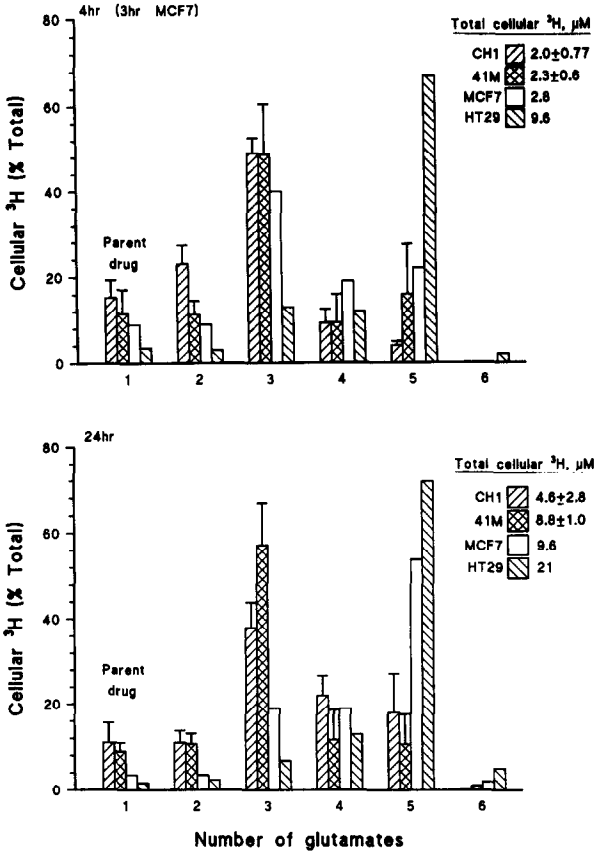


Fig. 4. The metabolism of 0.1 μM [5-$^3$H]ICI D1694 to polyglutamate forms in four human cell lines. Details as in Fig. 3.

colon (HT29) and one human breast (MCF7) cell lines were investigated for their ability to form ICI D1694 polyglutamates (Fig. 4). At an extracellular concentration of 0.1 μM all the cell lines accumulated a high intracellular concentration of $^3$H material by 4 hr (2–10 μM). This represents a 20–100-fold accumulation of the drug intracellularly. Less than 20% of the drug remained as parent monoglutamate and the major metabolite was either tri-, tetra- or pentaglutamate depending on the cell line. Both human ovarian lines had triglutamate (~50%) and the HT29 (and W1L2) had the pentaglutamate (~70%) as the major forms. Although in the MCF7 the largest amount of radioactivity was associated with the triglutamate (~40%) the incubation time was shorter (3 hr). After 24 hr incubation the intracellular $^3$H level had increased a further ~2–5-fold so that now the intracellular concentration was ~45–200-fold higher than the extracellular environment. At this time >90% of the cellular $^3$H was associated with polyglutamates. The two ovarian lines had higher levels of parent drug, di- and triglutamate (major metabolite) than the other cell lines where parent drug and diglutamate were each less than 4% and pentaglutamate (major metabolite) was >55%.

## DISCUSSION

A method is described for the measurement of ICI D1694 and its polyglutamate metabolites in cell extracts using high specific activity [5-$^3$H]ICI D1694 and a reverse-phase ion-pairing HPLC method. Recovery of ICI D1694 and drug-derived material was high at each stage of extraction and after chromatography with no evidence of preferential loss of one polyglutamate (or parent drug) over another or the formation of metabolites other than polyglutamates. This ion-pairing chromatography technique allowed for the separation of the hexaglutamate and if necessary could be used for the detection of higher chain length polyglutamates. Using the cell numbers and extraction method described the limit of detection for each polyglutamate is an intracellular concentration of ~4 nM (~300 dpm above background). Less than this is possible if cell numbers are increased.

Both the mouse L1210 and human W1L2 cell lines readily formed polyglutamates so that even after 30 min incubation with 0.1 $\mu$M [$^3$H]ICI D1694 the majority of the intracellular $^3$H was found as these metabolites. After 4 hr very little parent drug could be detected (<5%) and the concentration of polyglutamates was 2–3 $\mu$M. The predominant polyglutamate species at both 4 and 24 hr was tetraglutamate in the L1210 and pentaglutamate in the W1L2 cell line. Efficient polyglutamation occurred in all the cell lines (human) examined with either tri- or pentaglutamates being the major forms. Three quinazoline TS inhibitors including ICI D1694 have now been evaluated for their ability to form polyglutamate derivatives in L1210 cells. Less metabolism to polyglutamates was seen with CB3717 where incubation with 50 $\mu$M drug for 6 hr led to less than 20% being found as polyglutamates (~0.6 $\mu$M) [15]. At later times the polyglutamate levels rose to ~50% of the total cellular $^3$H (~3 $\mu$M). The 2-desamino-2-methyl analogue of CB3717 (ICI 198583) was metabolized better than CB3717 and 4 hr incubation with 1 $\mu$M ICI 198583 resulted in ~40% of the drug being found as polyglutamates (~0.7 $\mu$M) [16]. All three drugs are predominantly metabolized to the tetraglutamate in L1210 cells. The formation of polyglutamates and particularly the higher species (>triglutamate) has also been shown to be a mechanism of drug retention as they are not readily lost from the cells [15, 16, 23]. The improved ability of ICI D1964 to be metabolized to polyglutamates over the other two compounds is consistent with (a) its superior FPGS substrate activity (1.3 $\mu$M compared with 40 $\mu$M) and (b) its use of the reduced-folate/MTX cell membrane transport carrier which is shared by ICI 198583 but not by CB3717 [23]. In light of the greater TS inhibitory activity of the polyglutamates (up to two orders of magnitude) [23] it is apparent that the high level of ICI D1694 polyglutamation accounts for the high potency of the drug *in vitro*. This study highlights the importance of identifying any possible metabolism of glutamate-containing antifolates to help explain their cytotoxic activity since their potency for isolated TS alone can sometimes be a poor indicator.

ICI D1694 demonstrated activity in the Phase I clinical study and dose-limiting toxicities were gastrointestinal, malaise and haematological ([36] and S. Clarke, personal communication). The current Phase II studies should establish whether a TS inhibitor that undergoes extensive polyglutamation is a viable drug for the treatment of cancer.

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