



# The effect of folate on the methotrexate/indomethacin interaction in a murine cancer cell line

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- 1 The effect of folate on the interaction between methotrexate (a folate analogue) and indomethacin has been examined in murine NC carcinoma cells.
- 2 Conditioning of NC cells to a physiological (20 nM) folate concentration after culture in a high folate concentration increased the response to methotrexate. The sensitivity of these conditioned cells to methotrexate related inversely to the folate concentration.
- 3 At 20 nM and 2  $\mu$ M folate, indomethacin 1  $\mu$ g ml<sup>-1</sup> potentiated the cytotoxicity of methotrexate 4 and 8 ng ml<sup>-1</sup> (both  $P < 0.03$ ).
- 4 When NC cells were incubated with [<sup>3</sup>H]-methotrexate at 20 nM and 2  $\mu$ M folate, there was a trend for increased tritium accumulation with indomethacin 0.36  $\mu$ g ml<sup>-1</sup> (1  $\mu$ M;  $P < 0.01$ ).
- 5 We conclude that the folate concentration can affect the sensitivity of NC cells to methotrexate, although the degree of potentiation of cytotoxicity by indomethacin remains similar.

**Keywords:** Folate; indomethacin; methotrexate; murine cancer cells

## Introduction

Increasing the effectiveness of cytotoxic drugs may enhance anticancer therapy. Indomethacin (Indo) potentiates the cytotoxicity of the folate analogue methotrexate (MTX) to the murine NC carcinoma *in vivo* and *in vitro* (Gaffen *et al.*, 1985; Bennett *et al.*, 1987) and, of particular clinical relevance, to two human breast cancer cell lines (T47D and DU4475; Bennett *et al.*, 1989). This may be due to increased MTX uptake and/or retention (Bennett *et al.*, 1989; Bennett & Gaffen, 1989). Indo does not appear to potentiate MTX by blocking cyclo-oxygenase since the effect was not mimicked by the selective inhibitors flurbiprofen or piroxicam, or counteracted by adding prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Bennett *et al.*, 1987; 1989). Displacement of MTX bound to serum proteins, or inhibition of cyclic AMP phosphodiesterase, similarly do not appear to be involved (Bennett *et al.*, 1987; 1989). In contrast, Indo did not increase the killing of normal epithelial-like cells from human embryonic intestine, or their accumulation of tritium during incubation with [<sup>3</sup>H]-MTX (Bennett *et al.*, 1987). We also have similar preliminary data showing no potentiation in two other epithelial-like cell lines from normal human gut (FHs 74 Int cells and HCMC cells; Bennett *et al.*, 1989 and unpublished). However, all of these studies have used culture medium containing a greatly supraphysiological level of folate. Furthermore, in the earlier studies of Bennett *et al.* (1987), the enhancement by Indo of the early accumulation of tritium in NC cells incubated with [<sup>3</sup>H]-MTX occurred with a concentration of MTX at least 100 times greater than that required to kill NC cells during 4 days in culture (Bennett *et al.*, 1987; Hollingsworth *et al.*, 1992; Hollingsworth, 1993). We have now used a lower (physiological) folate level in studying both the MTX/Indo interaction in NC cells, and the effect of Indo on <sup>3</sup>H accumulation using therapeutic concentrations of MTX.

## Methods

### Cells and cell culture

The NC carcinoma arose spontaneously in the mammary region of a WHT/Ht mouse (Hewitt *et al.*, 1976) and has been passaged subsequently in this inbred strain. Primary cultures of NC cells were obtained from the ascitic fluid of a female WHT/Ht mouse 10–14 days following intraperitoneal injection of disaggregated tumour.

The medium used for NC cells, Eagle's modification of Minimal Essential Medium (EMEM), contains 2  $\mu$ M folic acid which is typical of the supraphysiological folate levels in standard culture media, and is 100 times that in normal blood (20 nM; Kamen & Capdevila, 1986). For the present studies we used standard medium containing 2  $\mu$ M folic acid, and medium formulated without folic acid (Flow Laboratories) to which we subsequently added folic acid to a physiological level of 20 nM.

NC cells were maintained as suspension cultures in EMEM containing either 2  $\mu$ M or 20 nM folic acid, supplemented with 10% heat-inactivated newborn bovine serum (NBS), 1% non-essential amino acids (Flow Laboratories), 20 mM HEPES, 300  $\mu$ g ml<sup>-1</sup> L-glutamine and 50 units ml<sup>-1</sup> each of penicillin and streptomycin (Gibco) in roller flasks (850 cm<sup>2</sup>, 1 rev min<sup>-1</sup>) at 37°C. The bulk cultures received additions of fresh medium (30–40% by volume) every 48–72 h, and cells in a 10 ml aliquot were counted daily. Experiments were performed on cells from cultures with 0.5 to 1  $\times 10^6$  cells ml<sup>-1</sup>, in the logarithmic phase of growth and with >90% viability (Trypan Blue exclusion).

### Adaptation to physiological folate

The NC cells were adapted to the physiological folate concentration in one of two ways: (1) NC cells originating from primary cultures (ascitic fluid directly *ex vivo*) were subcultured in medium containing the standard folic acid concentration (2  $\mu$ M), pelleted (200 g, 10 min), washed with phosphate buffered saline pH 7.4 (PBS), disaggregated (trypsin/EDTA, 0.05:0.02% w/v) and counted (Coulter counter). Cells were resuspended at a density of 0.1 to 0.3  $\times 10^6$  cells ml<sup>-1</sup> in medium containing the physiological 20 nM folate level, seeded directly into 850 cm<sup>2</sup> roller culture bottles (Falcon), and incubated at 37°C. Culture growth was determined daily

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(Coulter counter). Initially the cells went through a lag phase (4–5 days) whilst adapting to the physiological 20 nM folate medium, after which they entered logarithmic growth. Cultures were maintained as described above and used only when they had undergone at least 15–20 cell cycles. (2) In the second method, cells from peritoneal fluid were washed with PBS to remove erythrocytes, resuspended in medium containing 20 nM folic acid, seeded directly into 850 cm<sup>2</sup> roller culture bottles as a primary culture, incubated at 37°C, and maintained as described above.

### Determination of cell growth

Cell growth was determined by microturbidimetry (Gaffen *et al.*, 1985), by measuring the transmission of 600 nm light through the cell cultures (recorded as absorption units with a Dynatec MR600 microplate reader). This wavelength gave a linear correlation between light 'absorption' and cell numbers ( $10^3$  to  $10^6$  cells in 200  $\mu$ l) at both folate concentrations (20 nM and 2  $\mu$ M; Hollingsworth, 1993).

Cells from bulk suspension cultures were pelleted by centrifugation (200 g, 10 min), disaggregated (trypsin/EDTA, 0.05:0.02% w/v), and counted (Coulter counter). They were diluted in fresh medium to  $5 \times 10^3$ ,  $10^4$ ,  $2.5 \times 10^4$ ,  $5 \times 10^4$ ,  $10^5$ ,  $2.5 \times 10^5$ ,  $5 \times 10^5$  and  $10^6$  cells in 200  $\mu$ l final volume and added to microplate wells. Light absorbance readings were taken immediately, and then on 4 consecutive days, following gassing of the plates for 5 min with CO<sub>2</sub>/O<sub>2</sub> (95:5) in a sealed Perspex box (separate plates for each day). The growth of  $2.5 \times 10^4$  NC cells (in 200  $\mu$ l) was mainly similar over 5 days (1 day more than the drug interaction assays) with 20 nM and 2  $\mu$ M folate (cell doubling time both 1.5–2 days; Hollingsworth, 1993).

In the studies of drug effects, aliquots of 100  $\mu$ l medium containing vehicle alone or drugs at twice the desired concentration were added to each of the 96 flat-bottomed wells of a microtest plate, followed by 100  $\mu$ l of cell suspension ( $2.5 \times 10^4$  cells) or medium alone. After 4 days incubation (37°C, 5% CO<sub>2</sub> in humidified air) the plates were gassed as above with CO<sub>2</sub>/O<sub>2</sub> (95:5), and light 'absorption' was measured at 600 nm.

The effect of conditioning NC cells to 20 nM folate was studied by constructing concentration-response curves to MTX 0.1–16 ng ml<sup>-1</sup> (0.22–35 nM). Later experiments examined the effect of Indo 1 and 2  $\mu$ g ml<sup>-1</sup> (2.8 and 5.6  $\mu$ M) alone or with MTX 4 and 8 mg ml<sup>-1</sup> (8.8 and 17.6 nM) on NC cell growth at the 2  $\mu$ M and 20 nM folate levels.

### Methotrexate accumulation studies

The accumulation of [<sup>3</sup>H]-MTX by NC cells was studied previously at 2  $\mu$ M folate (Bennett *et al.*, 1987). We have now re-examined this at the physiological 20 nM folate concentration using the method of Kuonen *et al.* (1992) modified from Henderson *et al.* (1978).

Cells in a 5–10 ml aliquot of bulk suspension culture adapted to 20 nM folate (EMEM, 20 nM folic acid, see above) were disaggregated and counted as above. The remaining cells were harvested by centrifugation (200 g, 10 min), resuspended in 10–20 ml fresh medium, and the large clumps disaggregated by repeated passage into and out of a 10 ml pipette; trypsin was not used for disaggregation as it may damage transport mechanisms. Following centrifugation as above, the cells were resuspended to a final density of  $5 \times 10^6$  cells ml<sup>-1</sup> in cold (1–2°C) EMEM minus folic acid with supplements of 50 units ml<sup>-1</sup> each of penicillin and streptomycin, and 1% non-essential amino acids (but no serum or folic acid).

Triplicate 30 ml universal containers were incubated on ice for 10 min, and then  $5 \times 10^6$  cells in suspension (as above), 6 nM [<sup>3</sup>H]-MTX (2.73 ng ml<sup>-1</sup>; 500,000 d.p.m.), and non-radioactive MTX to final MTX concentrations of 0.45–13.6  $\mu$ g ml<sup>-1</sup> (1–30  $\mu$ M)  $\pm$  Indo 0.36  $\mu$ g ml<sup>-1</sup> (1  $\mu$ M) were added in a final volume of 1.1 ml. They were incubated for 30 min at 37°C in a shaking water bath for total counts, with

triplicate containers on ice for blank controls. The reaction in the test samples was stopped by placing the tubes on ice for 10 min prior to centrifugation (600 g, 5 min). The cell pellets were resuspended in 25 ml ice-cold PBS, centrifuged, transferred to scintillation vials by 3 aliquots of 200  $\mu$ l twice-distilled water, and 3 ml of scintillation cocktail were added (Packard Ultima Gold). Accumulated tritium was determined by standard spectrophotofluorimetry using a Packard Tri-Carb 2200CA scintillation counter (<sup>3</sup>H counting efficiency ~50%), and the difference between total and blank taken as specific accumulation.

### Cell conditioning and the response to methotrexate

Concentration-response curves for MTX 0.1–16 ng ml<sup>-1</sup> were constructed for NC ascites cells cultured in EMEM as follows: (i) with 2  $\mu$ M folate (2  $\mu$ M NC cells), (ii) with 20 nM folate directly from *in vivo* (20 nM folate cells), and (iii) first with 2  $\mu$ M folate followed by adaptation to 20 nM folate (conditioned NC cells).

### Drugs and solvents

Sodium indomethacin trihydrate (Merck Sharp & Dohme) and methotrexate were made up in 154 mM NaCl, adjusted to pH 7.4 with 0.1 M NaOH, filter-sterilised and stored at –20°C. [L-glutamyl-3,4-<sup>3</sup>H]-MTX, 41.0 Ci mmol<sup>-1</sup> (DuPont, U.K.) was diluted 1:9 in sterile double-glass-distilled water and stored at –20°C as a stock solution of 60 nM (~30 ng ml<sup>-1</sup>).

### Calculations

The results are expressed as means  $\pm$  s.e. and analysed by Student's *t* test for paired or unpaired data as appropriate (all tests 2-tailed).

A possible interaction between MTX and Indo was examined by subtracting the sum of the separate mean percentage decreases in NC cell growth obtained with MTX and Indo alone, from the mean percentage decrease with the drugs in combination (Hollingsworth, 1993). This can be summarised as follows:

$$\begin{aligned} \% \text{ interaction} = \\ \% \text{ mean effect obtained with (MTX + Indo)} - (\text{MTX}) - (\text{Indo}) \end{aligned}$$

A value above zero indicates potentiation, whilst a negative value indicates antagonism. The raw results were analysed statistically.

## Results

### Cell conditioning and the response to methotrexate

In all cases MTX 0.1–16 ng ml<sup>-1</sup> reduced NC cell growth in a concentration-dependent manner (Figure 1). Sensitivity to MTX was inversely related to the folate concentration; overall reduction of growth by MTX in conditioned cells was (i)  $22.4 \pm 4.6\%$  greater than in 20 nM cells ( $P < 0.002$ ), and (ii)  $24.3 \pm 4.8\%$  greater than in 2  $\mu$ M cells ( $P < 0.001$ ).

### Methotrexate/indomethacin interaction at 2 $\mu$ M folate

MTX 4 and 8 ng ml<sup>-1</sup> alone reduced NC cell growth by  $24.0 \pm 5.1\%$  and  $56.7 \pm 2.5\%$  respectively compared to vehicle controls ( $P < 0.002$  and  $< 0.0001$ ).

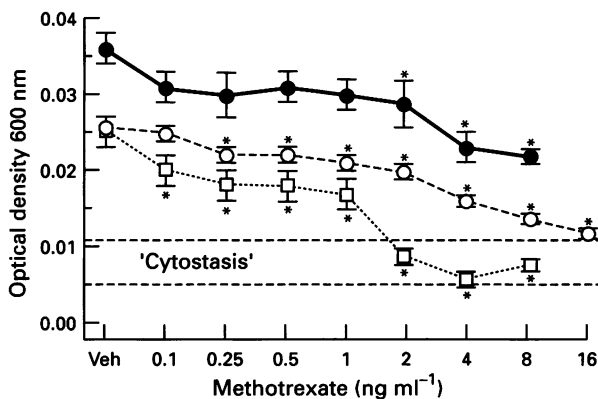
With Indo 1  $\mu$ g ml<sup>-1</sup> alone there was a trend for more NC cell growth ( $12.1 \pm 3.3\%$  compared to controls,  $P < 0.1$ ), and the cytotoxicity of MTX 4 and 8 ng ml<sup>-1</sup> was potentiated by  $33.3 \pm 4.0\%$  and  $15.2 \pm 5.6\%$  respectively (calculated as described in Methods, both  $P < 0.03$ , Figure 2). With Indo 2  $\mu$ g ml<sup>-1</sup> there was a greater mean cytotoxicity of MTX 4 and 8 ng ml<sup>-1</sup> (by  $19.7 \pm 4.8\%$  and  $11.1 \pm 4.2\%$  respectively, both  $P < 0.1$ , Figure 2).

### Methotrexate/indomethacin interaction at 20 nM folate (cells grown directly from *in vivo*)

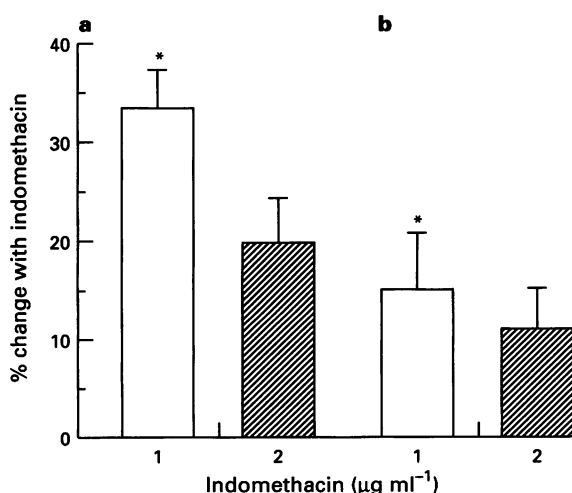
Indo 1 or 2  $\mu\text{g ml}^{-1}$  alone had no significant effect on NC cell growth (respectively  $10.9 \pm 5.2\%$  higher and  $6.2 \pm 5.8\%$  lower than the vehicle controls, both  $P < 0.2$ ).

The reductions in growth by MTX 4 and 8  $\text{ng ml}^{-1}$  alone ( $55.1 \pm 4.1$  and  $68.8 \pm 3.4\%$  respectively, both  $P < 0.001$ ) were greater than at 2  $\mu\text{M}$  folate (by 130 and 21.3%,  $P < 0.0001$  and  $> 0.0003$  respectively).

As in 2  $\mu\text{M}$  folate, Indo 1  $\mu\text{g ml}^{-1}$  potentiated the cytotoxicity of MTX 4 and 8  $\text{ng ml}^{-1}$  (by  $25.8 \pm 4.8$  and  $22.6 \pm 5.5\%$  respectively, both  $P < 0.03$ , Figure 3). However, with Indo 2  $\mu\text{g ml}^{-1}$  there was only a trend for increased MTX cytotoxicity ( $14.6 \pm 6.4\%$  and  $5.2 \pm 6.5\%$  respectively for MTX 4 and 8  $\text{ng ml}^{-1}$ , both  $P < 0.1$ , Figure 3).



**Figure 1** The effect of conditioning NC cells on their response to methotrexate (MTX). Cytostasis (also when growth=death) is marked by dashed lines as the mean optical density  $\pm 2$  s.e. of cells seeded at time zero; (●) cells cultured in medium containing 2  $\mu\text{M}$  folate; (○) cells cultured directly from *in vivo* in medium containing 20 nM folate; (□), cells cultured first in medium containing 2  $\mu\text{M}$  folate and then conditioned to 20 nM folate. Each point is the mean  $\pm$  s.e. of the separate means of 4–18 experiments each with 4–16 replicates. \* $P < 0.01$  MTX vs vehicle controls, Student's *t* test for paired data on the raw results. Veh=vehicle.



**Figure 2** The change caused by indomethacin (Indo) 1 and 2  $\mu\text{g ml}^{-1}$  (open and hatched columns respectively) on the cytotoxicity of methotrexate (a) 4 and (b) 8  $\text{ng ml}^{-1}$  to NC adenocarcinoma cells at 2  $\mu\text{M}$  folate. Each column represents the percentage effect of Indo (calculated as described in Methods) expressed as means  $\pm$  s.e. from 12 experiments each with 8–16 replicates. \* $P < 0.05$ , Student's *t* test for paired data compared to vehicle controls.

### Methotrexate accumulation studies

In earlier studies using NC cells at 2  $\mu\text{M}$  folate, Bennett *et al.* (1987) found that Indo 1  $\mu\text{g ml}^{-1}$  increased the active accumulation of tritium by cells incubated for 1 h with [ $^3\text{H}$ ]-MTX ( $P < 0.001$ ). However, the specific radioactivity of the [ $^3\text{H}$ ]-MTX was rather low, and the amount needed (2  $\mu\text{M}$ ; 909  $\text{ng ml}^{-1}$ ) to obtain adequate label accumulation was at least 100 times the amount required to kill NC cells cultured for 4 days (Hollingsworth, 1993). We have now examined this accumulation using concentrations of labelled MTX similar to the lowest amount required to kill NC cells in culture, and NC cells adapted to 20 nM folate.

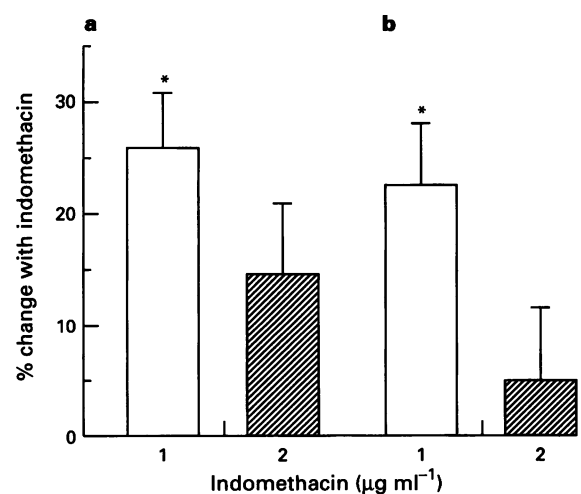
Active accumulation of label (2.73  $\text{ng ml}^{-1}$  [ $^3\text{H}$ ]-MTX) at 37°C was linear with increasing cell density and with time, whereas the readings in the 0°C blanks remained constantly low.

The concentration-dependent increase in  $^3\text{H}$  accumulation obtained in the presence and absence of 0.36  $\mu\text{g ml}^{-1}$  (1  $\mu\text{M}$ ) Indo (Figure 4a) did not appear to be maximal with the highest concentration of MTX examined (13.6  $\mu\text{g ml}^{-1}$ ; 30  $\mu\text{M}$ ). Results, expressed as  $\log_{10}$  pmol  $^3\text{H}$  accumulated, gave a linear relationship that facilitated a statistical evaluation of the Indo effect (Figure 4b). Linear regression analysis was performed and the slopes of the lines (*m*) and y-intercept (*c*) values in the control and Indo-treated groups were compared by Student's *t* test for paired data.

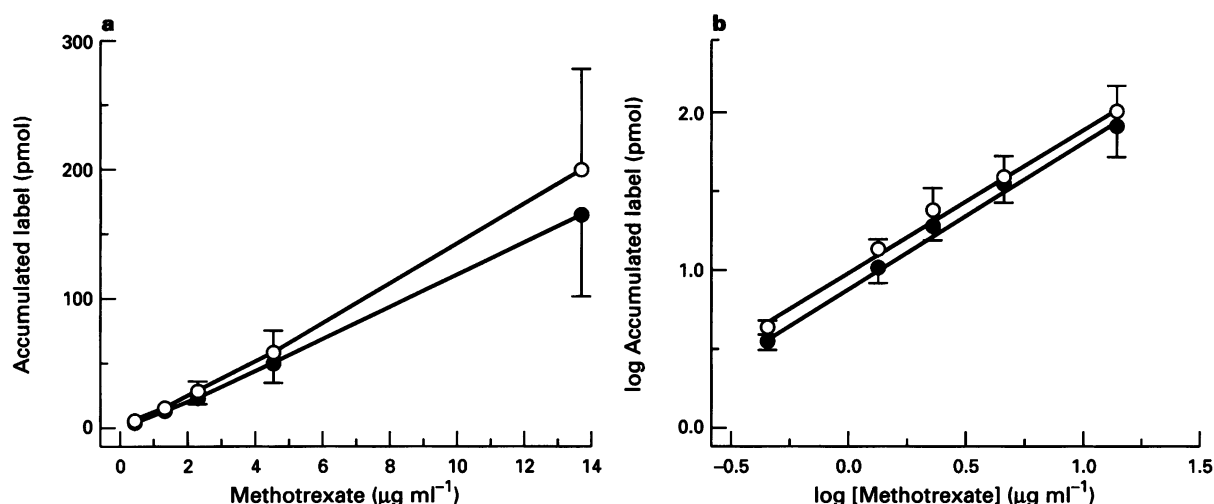
With Indo 0.36  $\mu\text{g ml}^{-1}$  there was no significant effect on the slope of the line ( $P < 0.04$ ), but there was a trend for a greater y-intercept ( $P < 0.1$ ), indicating more  $^3\text{H}$  accumulation at all concentrations of MTX examined (by  $23.68 \pm 5.49$ ,  $30.48 \pm 6.25$ ,  $29.70 \pm 7.20$ ,  $14.46 \pm 5.37$  and  $24.68 \pm 5.80\%$  respectively for MTX 1, 3, 5, 10 and 30  $\mu\text{M}$  [0.45, 1.36, 2.27, 4.55 and 13.6  $\mu\text{g ml}^{-1}$ ]).

### Discussion

Both folate levels produced a concentration-dependent reduction by MTX in NC cell growth, as seen previously with 2  $\mu\text{M}$  folate (Gaffen, 1988; Hollingsworth *et al.*, 1992; Hollingsworth, 1993). Conditioning of NC cells from 2  $\mu\text{M}$  folate to 20 nM folate increased the sensitivity to MTX, as in human epidermoid carcinoma KB cells (Kane *et al.*, 1986) and the



**Figure 3** The change caused by indomethacin (Indo) 1 and 2  $\mu\text{g ml}^{-1}$  (open and hatched columns respectively) on the cytotoxicity of methotrexate (a) 4 and (b) 8  $\text{ng ml}^{-1}$  to NC cells at 20 nM folate. Each column represents the percentage effect of Indo expressed as means  $\pm$  s.e. from 12 experiments each with 8–16 replicates. \* $P < 0.05$ , Student's *t* test for paired data compared to vehicle controls.



**Figure 4** (a, b) The change caused by indomethacin on tritium accumulation by  $5 \times 10^6$  NC cells incubated for 30 min with [ $^3\text{H}$ ]-methotrexate and indomethacin ( $0.36 \mu\text{g ml}^{-1}$ ,  $\circ$ ) or vehicle ( $\bullet$ ). Each point represents the mean  $\pm$  s.e. of the separate means from 8 experiments each with 3 replicates.

CEM-7A variant of CCRF-CEM human leukaemia cells (Jansen *et al.*, 1990). This may be due to reduced competition between folate and MTX for dihydrofolate reductase (DHFR). Up-regulation of reduced folate/MTX receptors at low intracellular folate concentrations facilitates MTX influx (Sirotnak *et al.*, 1984; Yang *et al.*, 1988; Jansen *et al.*, 1990), and subsequent polyglutamation would further increase the total intracellular MTX. Cells grown *ex vivo* in  $20 \mu\text{M}$  folate were less sensitive than conditioned NC cells to MTX, perhaps because they use predominantly folic acid uptake sites (Sirotnak, 1985) or salvage pathways for preformed purines and pyrimidines (relatively insensitive to MTX) rather than the normal reduced folate uptake sites (sensitive to MTX). Altered responses of NC cells to MTX at different folate concentrations might reflect changed folate uptake or metabolism. However, at both folate concentrations NC cells showed mainly similar growth characteristics over 5 days (1 day more than the assay incubation period).

MTX and reduced folates enter mammalian cells via the same simple ion-exchange transport system (Huennekens *et al.*, 1978). Cells possess high-affinity folate receptors which are very specific for MTX and 5-methyltetrahydrofolate (5-MTHF). In a monkey kidney cell line (MA104) adapted for growth in low folate medium, binding of tritiated 5-MTHF depended on the cellular folate level (Kamen & Capdevila, 1986). Furthermore, the length of exposure to folate seemed to regulate the receptor number and binding of 5-MTHF. Cells adapted to low folate over-produce the carrier protein for reduced folates and MTX (Sirotnak *et al.*, 1984; Yang *et al.*, 1988; Jansen *et al.*, 1990). However, there is a separate uptake system for folic acid (present in culture media, unlike reduced folates). Various investigators have identified a further carrier-mediated transport system for the influx of MTX and reduced folates distinct from the 'classical' system, which depends strongly on pH and can be inhibited by micromolar folate concentrations (Sirotnak *et al.*, 1987; Henderson & Strauss, 1990). The folate status of the cell may also regulate MTX polyglutamation (Galivan *et al.*, 1983; Nimec & Galivan, 1983). Folate deficiency increases MTX polyglutamation (H35 cells, Galivan *et al.*, 1983; KB cells, Kane *et al.*, 1986), perhaps by altering folypolyglutamyl synthase activity (Johnson *et al.*, 1988).

Even though with Indo  $1 \mu\text{g ml}^{-1}$  alone there was a trend for increased NC cell growth, it potentiated the cytotoxicity of MTX 4 and  $8 \text{ ng ml}^{-1}$  to similar extents at both folate concentrations (Figures 2 and 3). Our findings confirm those of Gaffen (1988) using  $2 \mu\text{M}$  folate. Indo increased the cytotoxicity

of MTX in murine Lewis lung carcinoma cells, and of etoposide in various cultured murine and human tumour cells (Maca, 1991), including CCRF-CEM and YAC-1 cells in which production of eicosanoids (Goldyne, 1988) was below the limit of detection. As with the potentiation of tritium accumulation by NC cells incubated with Indo/[ $^3\text{H}$ ]-MTX (Bennett *et al.*, 1987), exogenous prostanoids did not change the effect of Indo. Increased formation by Indo of 5-lipoxygenase arachidonate products (Docherty & Wilson, 1991), or inhibition of cyclic AMP-dependent protein kinases (Kantor & Hampton, 1978) do not seem to explain the potentiation by Indo (Maca, 1991).

CEM-7A cells, produced by step-wise adaptation of the human CCRF-CEM leukaemia cell line to decreasing concentrations of 5-formyltetrahydrofolate, over-produced the reduced folate/MTX carrier protein, showed 5 fold greater sensitivity to MTX than the parent cells, and exhibited a 95 fold greater  $V_{\text{max}}$  for [ $^3\text{H}$ ]-MTX influx with an approximately 30 fold greater level of carrier protein (Jansen *et al.*, 1990). However, the percentage potentiation of MTX cytotoxicity to NC cells by Indo was mainly similar at both folate concentrations, despite the greater sensitivity to MTX at  $20 \text{ nM}$  folate.

The fatty acid composition affects membrane viscosity, and this can affect MTX transport (Burns *et al.*, 1979; Clarke *et al.*, 1990). Indo can increase membrane viscosity (Abramson *et al.*, 1990), but it potentiated the changes induced by MTX in NC cell membrane fatty acids only after incubation for 48 h; no effect was seen within 1–3 h (Yazici *et al.*, 1992; Hollingsworth, 1993). The lack of an early effect argues against alteration of membrane structure and fluidity in the potentiation of tritium accumulation during Indo/[ $^3\text{H}$ ]-MTX incubation for up to 1 h (Yazici *et al.*, 1992; Hollingsworth, 1993). The importance of an initial increase of MTX accumulation by Indo in long-term cytotoxicity is unclear.

Reduced cellular efflux of MTX and/or its metabolites might be involved. MTX, like natural folates, is extensively polyglutamated intracellularly (Baugh *et al.*, 1973). These polyglutamates inhibit DHFR more than does the parent compound (Kumar *et al.*, 1986) and are retained to cause prolonged cytotoxicity (Rosenblatt *et al.*, 1978; Pizzorno *et al.*, 1989; Mini *et al.*, 1991).

Vincristine and the epidophyllotoxins increase MTX polyglutamation and cytotoxicity by inhibiting its efflux (Fry *et al.*, 1982a,b), as does insulin (human breast cancer cell line MDA.MB.436; Kennedy *et al.*, 1983) and probenecid (L1210 murine leukaemia cells; Sirotnak *et al.*, 1981; Henderson &

Zeverly, 1985). Indo  $1 \mu\text{g ml}^{-1}$  reduced  $^3\text{H}$  efflux from NC cells preloaded with tritiated MTX (E.M. Anderson, S.J. Hollingsworth & A. Bennett, unpublished).

MTX polyglutamates were up to 300 fold more potent than MTX as inhibitors of thymidylate synthase (TS) (Allegra *et al.*, 1985), and this might help to explain the potentiation of MTX by Indo.  $\alpha$ - and  $\gamma$ -interferon enhanced MTX cytotoxicity against the human promyelocytic cell line HL-60 by lowering intracellular thymidine levels (Sur *et al.*, 1991). Azidothymidine (AZT), which competitively inhibits thymidine kinase, synergized with MTX (Weber *et al.*, 1990; 1991). Inhibition of nucleoside transport by dipyridamole (Jarvis, 1986) might help to explain the potentiation of MTX (see Hollingsworth, 1993).

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The MTX/Indo interaction may therefore involve various pathways. Regardless of the mechanism, if Indo selectively potentiates the cytotoxicity of MTX to human malignant cells *in vivo* this could increase the effectiveness of cytotoxic chemotherapy in cancer.

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