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The effects of dipyridamole and indomethacin on methotrexate cytotoxicity in LoVo human colon cancer cells

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Abstract—Dipyridamole and indomethacin were studied for their effects in the in-vitro response of LoVo colon cancer cells to methotrexate (MTX) using a dye elution method. Dipyridamole 0·5–5 $\mu g\ mL^{-1}$ or indomethacin 1 $\mu g\ mL^{-1}$ alone had little or no effect on cell growth. The tumour cells were refractory to even high concentrations of MTX (2·5–10 $\mu g\ mL^{-1}$) alone or with indomethacin 1 $\mu g\ mL^{-1}$. In contrast, dipyridamole 0·5–5 $\mu g\ mL^{-1}$ sensitized the cells to MTX 5 $\mu g\ mL^{-1}$ (their growth was reduced by 25 to 69%), possibly by inhibiting thymidine salvage.

We have previously reported that indomethacin increases the response of the mouse NC carcinoma to methotrexate (MTX) both in-vitro and in-vivo (Gaffen et al 1985; Bennett et al 1987). Furthermore, indomethacin increased the accumulation of tritium in NC carcinoma cells incubated with [^{3}H]MTX. A beneficial interaction between indomethacin and MTX may have therapeutic potential in human breast cancer, since indomethacin increased MTX cytotoxicity to human breast cancer cells in culture (Bennett & Gaffen 1987). In contrast, indomethacin did not affect the response of normal epithelium-like cells from human embryonic intestine to MTX, or their accumulation of tritium after incubation with [^{3}H]MTX, indicating that potentiation of cytotoxicity may be selective for the breast cancer cells.

Because current chemotherapy is of little value in the treatment of human gastrointestinal cancers, we wished to see whether indomethacin could increase the effect of MTX on LoVo human colon cancer cells. We also used these cells to study dipyridamole which enhances the cytotoxicity of MTX in various cell lines (Cabral et al 1984; Nelson & Drake 1984; Kennedy et al 1986); when we started the experiments no work had been reported on human colon cancer cells, although since then one report has appeared (Van Mouwerik et al 1987). We now show that dipyridamole increased the sensitivity of the LoVo cells to MTX, whereas indomethacin had little or no effect on the response to MTX or the potentiation by dipyridamole.

Materials and methods

LoVo cells originated from pieces of a metastatic nodule from the left supraclavicular region of a man with colonic adenocarcinoma (Drewinko et al 1976). A cell sample, obtained from the American Type Culture Collection, was grown and subcultured in Ham's F-12 medium (Flow Laboratories) plus 15% newborn bovine serum (NBS), penicillin and streptomycin (50 units mL^{-1} each), and L-glutamine 146 mg L^{-1}.

Methotrexate (Lederle) 5 mg mL^{-1} was made up in 154 mM NaCl adjusted to pH 8·4 with 0·1 M NaOH solution. Indomethacin (a gift from Merck, Sharp & Dohme) was dissolved in 154 mM NaCl adjusted to pH 7·8 with 0·1 M NaOH to give a solution of 0·5 mg mL^{-1}. Dipyridamole (Sigma Chemical Co.) was made up to 0·5 mg mL^{-1} in methanol. All water used for drug preparation was double-distilled in glass, and the solutions were sterilised by filtration. Volumes added to the culture medium did not exceed 4 $\mu L\ mL^{-1}$.

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Cell growth was measured by a dye elution method. Each well of 96-well microtest plates received 100 μL medium plus drugs at twice the desired final concentration or vehicle. LoVo cells, grown as monolayers, were detached by treatment with trypsin:EDTA solution (0·05%:0·02% w/v for about 10 min), counted (Coulter counter model DN), and diluted in Ham's F-12 medium plus 15% NBS, antibiotics and glutamine as above, so that 100 μL contained 6000 cells. Aliquots of 100 μL cell suspension (or medium only for blanks) were added to each well, and the plates were incubated at 37°C in humidified air/CO_2 (95:5). The cells attached to the plastic within 5 h, and continued to proliferate. After four days the medium was removed from the plates, and the cells were fixed for 15 min by adding 100 μL of 10% formol saline to each well. The fixative was replaced with 100 μL stain (crystal violet 0·5% in 154 mM NaCl) which was removed after 15 min. The plates were washed twice with distilled water, dried in air (5–6 h), and the stain in the cells eluted with 100 μL acidified methanol (5 drops HCl to 100 mL methanol). The amount of dye, which correlates with the number of cells (Barer et al 1986), was estimated by measuring the absorbance through each well at 600 nm using a Dynatech microplate reader as described by the latter authors.

Concentration-growth curves were obtained with MTX 2·5–10 $\mu g\ mL^{-1} \pm$ indomethacin 1 $\mu g\ mL^{-1}$, and with MTX 5 $\mu g\ mL^{-1} \pm$ dipyridamole 0·5–5 $\mu g\ mL^{-1}$. In another series of experiments, the growth of LoVo cells was examined after exposure to either MTX 5 $\mu g\ mL^{-1}$, indomethacin 1 $\mu g\ mL^{-1}$ or dipyridamole 1 $\mu g\ mL^{-1}$ alone, MTX 5 $\mu g\ mL^{-1} \pm$ dipyridamole 1 $\mu g\ mL^{-1}$, or all three drugs combined. About 1 h elapsed between trypsinization and the addition of drugs. The results are given as means ± s.e.m. and analysed statistically by Student's *t*-test (2-tailed) for paired data with Bonferroni's correction, using the mean result from a series of 8–16 wells as one datum point.

Results

The LoVo cells were refractory to MTX 2·5–10 $\mu g\ mL^{-1}$. The combined results from the three separate studies, each with 6–9 experiments, show that with MTX 5 $\mu g\ mL^{-1}$ the mean cell growth, as judged by the amount of dye eluted, was 2 ± 4% higher than controls ($P < 0·8$). With indomethacin 1 $\mu g\ mL^{-1}$ alone, the cell growth was 4 ± 4% less than control ($P < 0·3$). Overall, indomethacin 1 $\mu g\ mL^{-1}$ had little or no effect on the response of the LoVo cells to MTX except for a reduction of 8 ± 2% ($P < 0·05$) at the highest concentration of MTX (10 $\mu g\ mL^{-1}$; Fig. 1).

With dipyridamole 0·5 $\mu g\ mL^{-1}$, cell growth was slightly greater (8 ± 2·5%, $P < 0·05$) but with 2 $\mu g\ mL^{-1}$ there was little or no change, whilst with 5 $\mu g\ mL^{-1}$ the growth tended to be less (7·2 ± 8·5%, $P < 0·1$; Fig. 2). Thus there might be a small concentration-related difference of effect.

Unlike indomethacin, dipyridamole, 0·5–5 $\mu g\ mL^{-1}$ caused a marked concentration-related decrease in cell growth (by 25 ± 3% to 69 ± 4%) when combined with MTX 5 $\mu g\ mL^{-1}$ (Fig. 2). Microscopic examination of the LoVo cells after 4 days incubation in the microplate wells showed that dipyridamole greatly increased the number of cells killed by MTX.

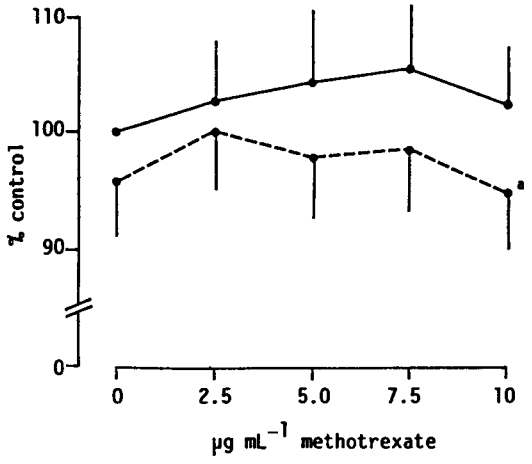

FIG. 1. The LoVo cells were refractory to MTX (2·5–10 μg mL^{-1}; solid line). There was little or no effect of indomethacin 1 μg mL^{-1} alone on cell growth as measured by dye elution (4 ± 4% less, $P < 0.3$), or on the response to MTX (broken line) except for a reduction of 8 ± 2% at the highest concentration of MTX (10 μg mL^{-1}). The results are the means ± s.e.m. of the separate means from 6 experiments, each with 8 replicates (16 replicates for vehicle controls). a, $P < 0.05$; Student's *t*-test for paired data with Bonferroni's correction on the raw results.

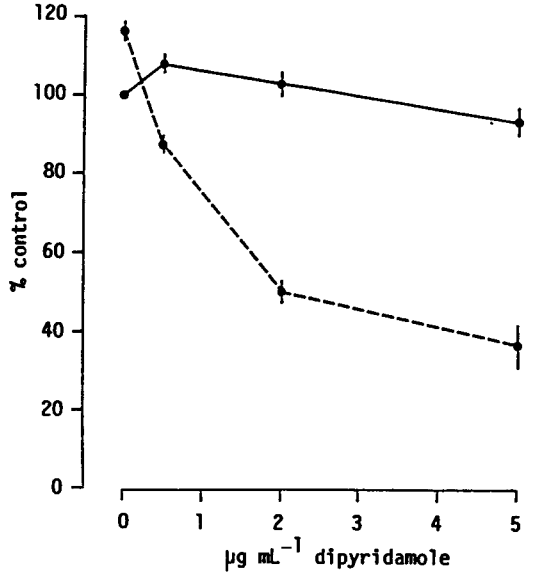

FIG. 2. Dipyridamole 0·5–5 μg mL^{-1} had little or no effect on the growth of LoVo cells (solid line). However, marked cytotoxicity occurred when dipyridamole was combined with MTX 5 μg mL^{-1} (broken line). The results are the means ± s.e.m. of the separate means from 6 experiments, each with 8–12 replicates (12 replicates for the vehicle controls). With dipyridamole alone vs dipyridamole + MTX 5 μg mL^{-1} all *P* values were < 0.05 using Student's *t*-test for paired data with Bonferroni's correction on the raw results.

A third experimental series examined whether indomethacin 1 μg mL^{-1} affected the killing of LoVo cells by MTX plus dipyridamole. As before, indomethacin 1 μg mL^{-1} or dipyridamole 1 μg mL^{-1} alone had little or no effect on cell growth, but dipyridamole 1 μg mL^{-1} again greatly increased the toxicity of MTX 5 μg mL^{-1} (by 39 ± 10%, $P < 0.05$). Indomethacin 1 μg mL^{-1} given in addition did not change the potentiation by dipyridamole (Fig. 3).

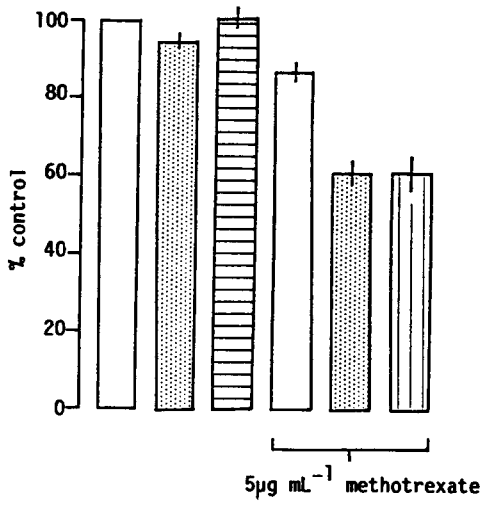

FIG. 3. Dipyridamole 1 μg mL^{-1} (stippled columns) or indomethacin 1 μg mL^{-1} (horizontal hatching) had little or no effect on cell growth compared with controls (open columns). However dipyridamole 1 μg mL^{-1} increased the toxicity of MTX 5 μg mL^{-1} by 39 ± 10% ($P < 0.05$ compared to control, Student's *t*-test for paired data with Bonferroni's correction on the raw results). Indomethacin 1 μg mL^{-1} given in addition (vertical hatching) did not change the potentiation by dipyridamole. The results are the means ± s.e.m. of the separate means from 9 experiments each with 8–16 replicates (20 replicates for vehicle controls).

Discussion

The present study was initially designed to see if indomethacin could enhance the killing by MTX of the LoVo cell line obtained from a human colon adenocarcinoma. These cells were refractory even to high concentrations of MTX, and indomethacin did not seem to change their sensitivity.

The function of some tissues (erythrocytes, leucocytes, bone marrow, intestinal epithelium) may depend on the uptake of preformed purines from the circulation, since these cells have limited or no capacity for *de novo* purine synthesis (Howell et al 1981; Jackson & Harkrader 1981; Muller et al 1983). By analogy, the salvage of thymidine (from Ham's F-12 medium which contains 0·727 mg L^{-1} thymidine), might explain the resistance of the LoVo human colon cancer cells to MTX. Dipyridamole potently inhibits the facilitated transport of nucleosides (Jarvis 1986). Chinese hamster ovary cells were resistant to MTX, probably because of the purines and pyrimidines in the undialysed serum (Nelson & Drake 1984). Dipyridamole sensitised the ovary cells to MTX in-vitro, and it enhanced the effect of MTX on human cell lines from two osteosarcomas (Nelson & Drake 1984), a breast cancer (Kennedy et al 1986), and HCT 116 cells from a colon cancer (Van Mouwerik et al 1987). In the latter paper the effect was shown to be mediated through inhibition of thymidine salvage. With VACO 5 human colon cancer cells, small amounts of added cytidine and guanosine triphosphate prevented the cytotoxicity to acivicin, a drug that depletes the intracellular pools of these nucleosides (Fischer et al 1984). It therefore seems likely that the effects of dipyridamole on the response of LoVo cells to MTX at least partly involves inhibition of thymidine uptake. This hypothesis could be examined by growing LoVo cells in the absence of thymidine.

Not all malignant cells show an interaction between dipyridamole and MTX; the chemotherapeutic activity of MTX against Ridgway osteogenic sarcoma or L1210 leukaemia cells in-vivo was at most only slightly improved by dipyridamole, possibly because of factors other than salvage of preformed purines and pyrimidines (Nelson & Drake 1984). Dipyridamole can inhibit

cyclic AMP phosphodiesterase (Harker et al 1983), and it was reported to enhance vascular PGI_2 biosynthesis (Bult et al 1982) although more recent evidence does not support this (Boeynaems et al 1986).

Dipyridamole might help overcome the resistance of human colon cancers to MTX. It is not valid to extrapolate from our experiments to responses in-vivo, since the conditions are different. One aspect is that trypsin has a prolonged effect on cell membranes and cytoskeletal elements (Furcht et al 1978), and might therefore alter the response to dipyridamole and MTX. However, in other studies that we have cited, dipyridamole enhanced the effect of MTX when trypsin had either not been used, or drugs were added 12–24 h after trypsinisation. Furthermore, it would be important for the drug interaction to be selective for the malignant cells, but this may not be so since dipyridamole enhanced MTX toxicity in normal mice (Nelson & Drake 1984). Dipyridamole and indomethacin adversely affect renal function in man (Seideman et al 1987), and may therefore increase the effectiveness of MTX by decreasing its clearance. Indomethacin appears to delay the clearance of MTX from plasma in man (Christophidis et al 1985). Neverthless, our results suggest that it might be worth testing the value of combining MTX with indomethacin or dipyridamole in human cancer subjects, taking care to monitor blood MTX levels. Perhaps the addition of dipyridamole might be best with MTX-resistant tumours able to salvage preformed nucleosides. Although the serum and urine of man, dog and some other mammals seem to contain little or no thymidine (unlike rats and mice; Nottebrock & Then 1977), and therefore potentiation of MTX by dipyridamole in man might be weak, plasma nucleoside levels may be elevated in some human malignancies (Zakaria 1984).

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