The Effect of Nifedipine Alone or Combined with Cytotoxic Chemotherapy on the Mouse NC Carcinoma In-vitro and In-vivo

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Abstract—Effects of the calcium antagonist nifedipine on the response of the murine NC carcinoma has been examined alone and together with cytotoxic chemotherapy in-vitro and in-vivo. The cytotoxic drug combination of methotrexate and melphalan, or nifedipine alone (0·2–25 μg mL^{-1}), caused a concentration-related reduction of NC cell growth in culture. At the lower concentrations, combination of the cytotoxic drugs with nifedipine resulted in an addition of the separate drug effects, but with drug concentrations that on their own approached maximal effectiveness the combined response was less than additive. NC tumours were excised from mice 14 days after inoculation s.c. with NC cells, weighed, and extracted for prostanoids. Mouse survival was determined up to day 121, and cancer spread was recorded postmortem. Nifedipine 1, 5 or 10 mg kg^{-1} had little or no effect on the tumour weight, tumour prostanoid content, metastasis to the lymph nodes or lungs, or on the increase of mouse longevity by the cytotoxic drugs.

We have previously shown that indomethacin increases the survival time of mice with NC carcinoma when given with the chemotherapeutic drugs methotrexate and melphalan (Bennett et al 1982). Indomethacin also potentiates the effect of methotrexate on NC cells in culture. This effect does not seem to involve prostaglandins or cAMP phosphodiesterase (Bennett et al 1987a,b), and in an attempt to explain the mechanism we have looked at other properties of the drug.

Indomethacin and various other non-steroidal anti-inflammatory compounds can inhibit calcium uptake by cell membranes from some tissues (Northover 1973). This might affect the calcium that is important for numerous functions of normal and malignant cells, including arachidonate release and metabolism (Metcalfe et al 1986; Balazsovits et al 1988). In addition, calcium channel blockers inhibit platelet aggregation, and this may explain their antimetastatic effects in some tumour models (Honn et al 1985). We have therefore studied the calcium antagonist nifedipine together with the cytotoxic drug combination methotrexate/melphalan used in patients and in our previous experiments on the NC carcinoma. This malignancy arose spontaneously in the mammary region of a WHT/Ht mouse (Hewitt et al 1976) and has been passaged in the same strain. Following tumour excision there is often spread to the lymphatics, metastasis mainly to the lungs and mediastinum, and recurrence in the scar. We have used the same in-vitro and in-vivo methods as previously, so that the results can be compared directly with our earlier findings.

Materials and Methods

Mouse NC tumour cells in culture
NC carcinoma cells from WHT/Ht mice with peritoneal ascites following i.p. injection of tumour were grown in

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minimum essential medium (MEM; Flow Laboratories) containing 10% newborn bovine serum, penicillin and streptomycin (50 units mL^{-1} each), L-glutamine (292 μg mL^{-1}), and 1% non-essential amino acids prepared as directed by Flow Laboratories. NC cells grown in suspension were pelleted by centrifugation, resuspended in 1 mL trypsin/EDTA solution (0·05% : 0·02% w/v) and incubated for 30 s at 37°C. After rapid addition of 9 mL medium, the cells were gently disaggregated by repeated pipetting, counted (Coulter Counter), and adjusted to 250 cells μL^{-1}.

Each of the 96 flat-bottomed wells of a microtest plate received 100 μL of cell suspension plus 100 μL of medium containing drugs at twice the desired final concentration (medium only for the controls). After incubation (37°C, 5% CO_2 in humidified air, 4 days), the transmission of 600 nm light in each test well was determined with a Dynatech microplate reader which expresses the readings in absorbance units (Gaffen et al 1985; Gaffen 1988). Concentration-growth curves were obtained for nifedipine (1 μg mL^{-1}) with or without methotrexate (5–30 ng mL^{-1}/melphalan 3·5–21 ng mL^{-1}) in a ratio of 2 : 1·4 (as in previous experiments, e.g. Bennett et al 1982) and for nifedipine 0·2–25 μg mL^{-1} with or without methotrexate/melphalan 10 and 7 ng mL^{-1}, respectively. These results were analysed using Student's *t*-test for paired data (2-tailed).

Mouse NC tumour in-vivo
Female WHT/Ht mice, 2–4 months old, were fed on SDS No. 1 modified diet, and had free access to water. They were weighed twice weekly starting at least 2 weeks before the experiment until death or day 121. There were 6 separate experiments with 5–15 mice/group. On day 0, 10^6 NC tumour cells (prepared as described by Bennett et al 1979) were injected s.c. into the left flank. Methotrexate 2·0 mg kg^{-1}/melphalan 1·4 mg kg^{-1} and/or nifedipine 1, 5 or 10 mg kg^{-1} were given orally in 0·1 mL 50% syrup BP. The cytotoxic compounds were administered on days 15–17, 22–24 and 29–31. Nifedipine or vehicle (50% syrup) was given daily either

from day 1 (the day of inoculation with NC cells, 4 experiments) or day 13 (2 experiments, nifedipine 1 mg kg^{-1} only) for 5 days/week (Monday to Friday) until death or the end of the experiment (day 121). All tumours were excised on day14. Those from mice treated from day 1 were weighed, homogenized in acidified ethanol (Bennett et al 1973), and extracted for prostanoids.

Mice with advanced carcinomatosis, or those surviving to day 121, were killed humanely to prevent suffering (Bennett et al 1982). At postmortem, the incidences of scar recurrence, lymph node involvement and distant metastasis were noted. Survival time was measured from the day of tumour inoculation and analysed by the method of Lee & Desu (1972). Metastasis was analysed by the Mann-Whitney U-test or, where specified, by Fisher's exact test (both 2-tailed).

Drugs

Methotrexate (Lederle) was dissolved in 154 mM NaCl adjusted to pH 8·4 with 0·1 M NaOH to give 1 mg mL^{-1}. Melphalan (Burroughs Wellcome) was dissolved in 70% ethanol to give 0·7 mg mL^{-1}. The solutions were sterilized by filtration. Nifedipine (Sigma) was dissolved in 100% ethanol to give 5 and 25 mg mL^{-1}. Volumes of drugs or solvents added to the culture medium did not exceed 0·4%.

Radioimmunoassay

Radioimmunoassay measurements (Hennam et al 1974) for the initial experiments using Miles PGE antibody; Jaffe & Behrman (1974) for the later experiments using the ICN PGE antibody) were made of PGE (N.B., the antibodies used do not distinguish between PGE$_1$ and PGE$_2$), 6-keto-PGF$_{1\alpha}$ and thromboxane B$_2$ (TxB$_2$). The percent cross-reactivities of the antisera were as follows: PGE antiserum (Miles Scientific; first two experiments, nifedipine 1 mg kg^{-1}): PGE$_2$ 100; PGE$_1$ 53; PGF$_{2\alpha}$ 10; PGA$_1$ 2·7; PGF$_{1\alpha}$ 2·6; PGB$_2$ 1·5; PGA$_2$ 1·4; PGB$_1$ 0·9. PGE antiserum (ICN Biomedicals, experiments with nifedipine 5 and 10 mg kg^{-1}): PGE$_2$ 100; PGE$_1$ 240; PGF$_{2\alpha}$ 0·5; PGF$_{1\alpha}$ 0·35; 6-keto-PGF$_{1\alpha}$ 0·6; TxB$_2$ <0·04. 6-keto-PGF$_{1\alpha}$ antiserum (Wellcome Research Laboratories): 6-keto-PGF$_{1\alpha}$ 100; PGF$_{2\alpha}$ 3·0; PGE$_2$ 0·1; TxB$_2$ 0·02. TxB$_2$ antiserum (Wellcome Research Laboratories): TxB$_2$ 100; PGF$_{2\alpha}$ 0·11; 6-keto-PGF$_{1\alpha}$ 0·01; PGE$_2$ <0·01. Intra- and inter-assay coefficients of variation were respectively 10–11% and 15–21%, and the lower limits of detection were (pg/100 μL): PGE 15·6; 6-keto-PGF$_{1\alpha}$ 12·5; TxB$_2$ 7·8. Tritiated compounds, obtained from Amersham International, had the following specific activities (TBq mmol^{-1}): PGE$_2$ 5·92; 6-keto-PGF$_{1\alpha}$ 5·55; TxB$_2$ 6·66. The bound and unbound compounds were separated by adding 1 mL of cold (4°C) ammonium sulphate/calcium sulphate (65% saturated ammonium sulphate solution pH 7·6 and calcium sulphate 1 g/25 mL, maintained as an even suspension with a magnetic stirrer).

Results

In-vitro studies

In cell culture, methotrexate/melphalan (respectively 5–30 and 3·5–21 ng mL^{-1}; ratio 2:1·4), caused a concentration-related reduction of NC cell growth as judged by microturbidimetry (Fig. 1). Nifedipine 1 μg mL^{-1} alone reduced the cell
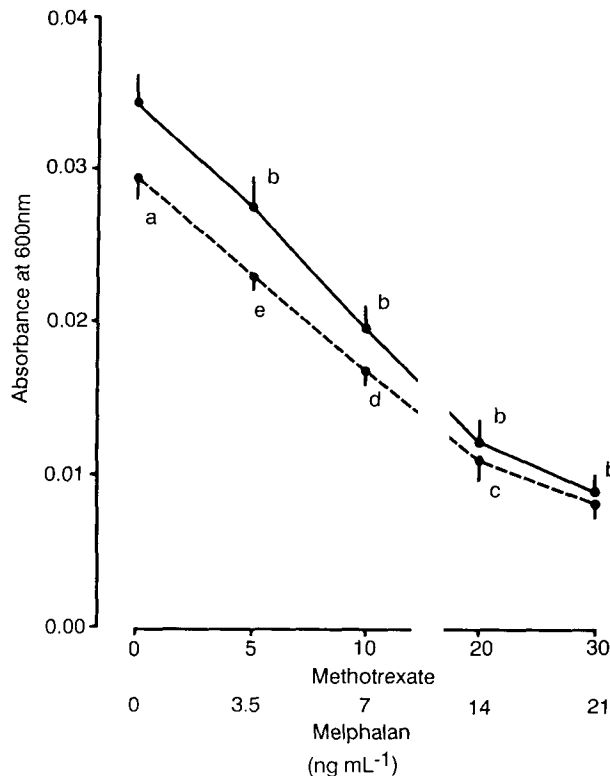

FIG. 1. Methotrexate/melphalan (solid line) caused a concentration-related decrease in NC cell growth. Methotrexate/melphalan 5:3·5, 10:7, 20:14 and 30:21 ng mL^{-1} decreased the absorbance readings (indicating decreased growth) by respectively 19·6 ± 2·7, 41·4 ± 3·2, 64·1 ± 3·3, and 74·5 ± 2·4%. Nifedipine 1 μg mL^{-1} alone (the first point on the broken line) decreased cell growth by 13·2 ± 3·4% ($P < 0.02$), and increased the response of the cells to methotrexate/melphalan mainly by an additive effect ($P < 0.001$ for the overall mean difference). Compared with the cytotoxic drugs alone, when nifedipine 1 μg mL^{-1} was also present with methotrexate/melphalan 5:3·5, 10:7, 20:14 and 30:21 ng mL^{-1} the cell growth was smaller still by 15·5 ± 3·2, 13·3 ± 3·7, 8·9 ± 6·2 and 6·6 ± 7·2%, respectively ($P < 0.02$, $P < 0.05$, $P < 0.2$ and $P < 0.3$ compared with the response to methotrexate/melphalan alone). Each point is the mean ± s.e.m. of separate means from 6 experiments each with 8 replicates (16 replicates for vehicle controls). P values: a < 0·02; b < 0·001 compared with vehicle controls; Student's t-test for paired data on the raw results; c < 0·02; d < 0·05; e < 0·02 compared with methotrexate/melphalan.

growth by 13·2% (Fig. 1). In combination with nifedipine the cytotoxic drugs appear to act mainly additively at their lower concentrations, but less than additively at concentrations approaching maximum inhibitory activity.

With a fixed amount of the cytotoxic drug mixture, in no case was the combined effect as great as the sum of the separate effects, and indeed nifedipine 25 μg mL^{-1} produced almost the same inhibition as when methotrexate and melphalan were also present (Fig. 2).

In-vivo studies

Treatment of the mice from day 1 with nifedipine 1, 5 or 10 mg kg^{-1} (approximately 1–10 times the therapeutic dose for man) had no significant effect on the weights of the excised tumours. There were two separate experiments with nifedipine 1 mg kg^{-1} given from day 1, and later there were two separate experiments combining both of the higher doses. Median and interquartile ranges of tumour weights (mg)

Table 1. Effect of nifedipine on mouse tumour prostanoids.

Nifedipine (mg kg^{-1})	PGE (ng g^{-1})	n	6-keto-PGF$_{1\alpha}$ (ng g^{-1})	n	TxB$_2$ (ng g^{-1})	n
Controls	124 (76–150)	20	64 (48–90)	17	32 (19–44)	20
1	146 (58–167)	20	85 (58–167)	19	34 (22–52)	20
Controls	90 (66–119)	20	17 (12–23)	20	16 (6–23)	20
5	85 (20–109)	20	16 (7–22)	20	13 (3–21)	20
10	107 (80–130)	20	18 (9–25)	20	13 (5–20)	20
Pooled controls	109 (66–137)	40	25 (15–63)	37	21 (12–33)	40

Nifedipine had no effect on the amounts of tumour prostanoids (shown as medians with interquartile ranges in parentheses). Comparisons between the prostanoids in the 40 control mice were: PGE vs 6-keto-PGF$_{1\alpha}$ $P<0.0001$; PGE vs TxB$_2$ $P<0.0001$; 6-keto-PGF$_{1\alpha}$ vs TxB$_2$ $P=0.095$.

were: nifedipine 1 mg kg^{-1}, 100 (70–200), n = 40 compared with the control group values of 140 (100–210), n = 39, $P>0.2$; nifedipine 5 and 10 mg kg^{-1}, 280 (190–430) and 290 (190–370) mg, respectively, compared with the control group tumour weights of 280 (310–330) mg, all n = 20/group, both comparisons with control $P>0.6$.
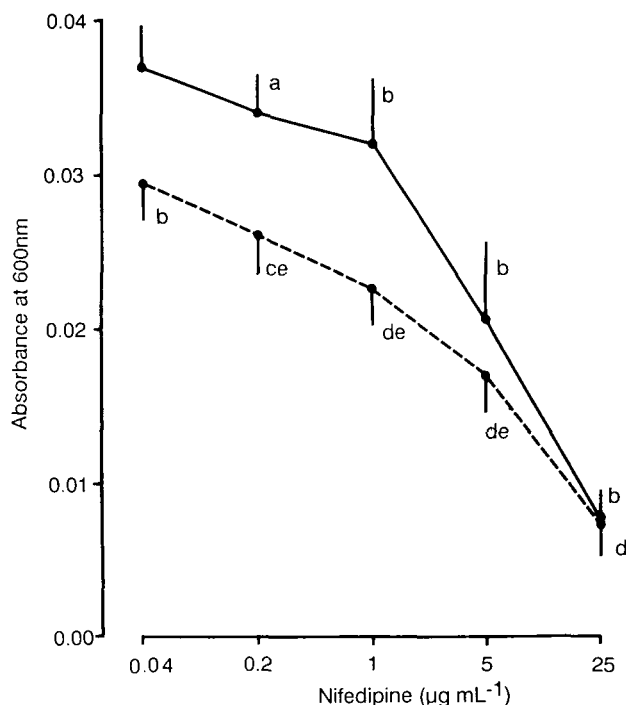

FIG. 2. Nifedipine (solid line) caused a concentration-related decrease of NC cell growth. Methotrexate/melphalan, used in this experiment only at 10 and 7 ng mL^{-1}, respectively (first point on the broken line), reduced cell growth by $20.9 \pm 1.8\%$ ($P<0.001$). In no case was the combined effect as great as the sum of the separate effects, and indeed nifedipine 25 μg mL^{-1} alone produced almost the same inhibition as when methotrexate/melphalan were also present. The inhibition of cell growth (decreased light absorbance readings compared with the starting value) by nifedipine alone was 6.5 ± 1.9, 14.3 ± 2.8, 46.9 ± 4.9 and $81.8 \pm 4.2\%$, respectively at 0.2, 1, 5 and 25 μg mL^{-1}, compared with 11.9 ± 2.4, 25.0 ± 3.2, 45.2 ± 4.5 and $79.0 \pm 5.6\%$ in the presence of methotrexate/melphalan. Each point is the mean ± s.e.m. of the separate means from 10 experiments each with 8 replicates (16 replicates for vehicle controls). P values: a < 0.02; b < 0.001 compared with vehicle controls; Student's t-test for paired data on the raw results; c < 0.005, d < 0.001 compared with methotrexate/melphalan; e < 0.001 compared with nifedipine alone.

Radioimmunoassays of tumour extracts showed little or no difference between controls and nifedipine-treated mice. The amounts extracted were PGE > 6-keto-PGF$_{1\alpha}$ > TxB$_2$ (Table 1).

As in our previous studies, cytotoxic chemotherapy (methotrexate 2/melphalan 1.4 mg kg^{-1}) improved the survival of mice compared with controls (Table 2). Mice given nifedipine 10 mg kg^{-1} alone had a longer median survival time than controls ($P=0.057$), but 1 or 5 mg kg^{-1} had no effect. In no case, including two other studies with nifedipine 1 mg kg^{-1} started on day 13 (the day before tumour excision) did the drug alter the increase in survival with methotrexate/melphalan (Table 2).

Treatment from day 1 with nifedipine 1 mg kg^{-1} produced no differences in the postmortem findings regarding lymph node involvement (14/20) or lung metastasis (15/20) compared with controls (17/20 and 16/20, respectively). However, the incidence of recurrence in the scar with nifedipine 1 mg kg^{-1} alone or together with methotrexate/melphalan (both 9/20) tended to be greater than in controls (3/20, $P=0.082$, Fisher's exact test) or mice receiving chemotherapy alone (4/19). There were no significant differences in lymph node involvement, lung metastasis or recurrence in the scar with the higher doses of nifedipine or with 1 mg kg^{-1} from day 13 (all $P>0.1$).

Discussion

The lack of potentiation of the effect of methotrexate/melphalan by nifedipine contrasts with the effect of indomethacin in-vivo and in-vitro (Bennett et al 1982, 1987a,b). Thus the enhancement by indomethacin may not involve just a change in cellular calcium. However, before coming to a firm conclusion it would be important to measure calcium changes, and to use various concentrations of other calcium antagonists including those which act intracellularly such as TMB 8 (Malagodi & Chiou 1974). Furthermore, we have not excluded the possibility that the indomethacin effect might be due to simultaneous inhibition of calcium transport and of one or more other pathways such as cyclo-oxygenase, phosphodiesterase or protein kinase.

Arachidonate metabolites can affect cell replication (Bennett 1982; Karmali 1983). Intracellular calcium is required for 5-lipoxygenase and phospholipase A$_2$ activity but not for cyclo-oxygenase (Zor et al 1987). The absence of change in

Table 2. Effect of nifedipine on mouse survival.

Treatment mg kg^{-1}	Treatment from day 1		Treatment from day 13	
	Survival (days)	n	Survival (days)	n
Controls	44 (40–48)	20	38 (35–42)	25
Nifedipine	46 (40–50)	20	38 (35–39)	24
Methotrexate/melphalan	55 (55–78)[b]	19	53 (47–56)[c]	19
Nifedipine 1 + methotrexate/melphalan	57 (48–65)[a,h]	20	53 (44–57)[b,e]	19
Controls	37 (36–41)	10		
Nifedipine 5	39 (37–41)	10		
Nifedipine 10	42 (41–42)	10		
Methotrexate/melphalan	48 (41–51)[d]	10		
Nifedipine 5 + methotrexate/melphalan	51 (45–56)[f]	10		
Nifedipine 10 + methotrexate/melphalan	49 (35–51)[g]	10		

Days of mouse survival are shown as medians with interquartile ranges in parentheses. Survival was lengthened by chemotherapy (methotrexate 2 mg kg^{-1}/melphalan 1·4 mg kg^{-1}). Nifedipine 10 mg kg^{-1} alone tended to improve survival ($P = 0.057$) but the other doses were ineffective. Nifedipine 1, 5 or 10 mg kg^{-1} had little or no effect on the response to chemotherapy. P values: a = 0·14; b < 0·001; c < 0·0001; d = 0·02 compared with vehicle controls; e = 0·88; f = 0·19; g = 0·73; h = 0·5 compared with methotrexate/melphalan. There were 2 experiments each with nifedipine 1 mg kg^{-1} started on day 1 or 13, and 2 experiments with the higher doses.

NC tumour prostanoids with nifedipine is consistent with our finding that verapamil had little or no effect on unstimulated eicosanoid production in rat isolated intestine (Capasso et al 1985). However, with mouse zymosan-activated peritoneal macrophages in-vitro, nifedipine inhibited phospholipase A_2 activity and leukotriene C_4 and PGE_2 production in a dose-dependent manner (Chang et al 1987). In the same study nifedipine, but not verapamil, inhibited platelet phospholipase A_2 activity by a mechanism apparently independent of calcium-blocking activity. The reason for the different effects of these two calcium antagonists is not known, but they differ both in chemical class and clinical effects (Singh 1986), and they have pharmacological actions not necessarily related to calcium (Chang et al 1987; Nayler & Dillon 1986).

Although nifedipine in concentrations as low as 1 μg mL^{-1} reduced the growth of cultured NC cells, amounts in-vivo similar to or greater than the therapeutic dose in man had little or no effect on tumour size or the response to the cytotoxic drugs. The reason for the discrepancy is not clear, but may relate to plasma protein binding of nifedipine which in human plasma varies from 92–98% with concentrations of 20–0·2 mg L^{-1}. More binding would be expected with the mice in-vivo than in cell culture, since the concentration of newborn bovine serum in the medium was only 10%.

We obtained a similar discrepancy with prednisolone in-vitro and in-vivo; there was concentration-related inhibition of NC cell growth in culture, and increased response to methotrexate (Bennett & Gaffen 1989; Gaffen et al unpublished data), but in-vivo there was little or no effect on survival, tumour growth and spread, response to chemotherapy, or formation of tumour prostanoids (Bennett et al 1987b).

Nifedipine reduced the metastasis of murine amelanotic melanoma B16a cells, possibly by inhibiting tumour-cell-induced platelet aggregation and platelet-enhanced tumour cell adhesion to endothelial cells (Honn et al 1985). The failure of nifedipine to alter NC tumour metastasis may

indicate that platelets are not important for its spread, and is consistent with the lack of effect of the thromboxane synthase inhibitor dazmegrel on NC tumour metastasis (Stamford et al 1986).

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