was important. The distance from the site of metabolism to the site of action has been claimed to be critical for the genetic activity of chloroform [11], and it is possible that such a factor might have influenced our results as well.

An inhibition of γ-glutamyl-cysteine synthetase fully explains the previously described inhibition of GSH synthesis [2, 3]. The possible *in vivo* significance of these findings was indicated by the loss of activity prior to morphological cell damage. Leakage of enzymes from the liver might have influenced the results, but the activity of lactate dehydrogenase in peripheral plasma is not markedly increased 4 hr after chloroform treatment [12]. Furthermore, the observation that GSH synthesis was equally affected at 4 and 6 hr indicates that leakage was of minor importance.

We conclude that chloroform metabolism may lead to γ-glutamyl-cysteine synthetase inhibition, while GSH synthetase activity is unaffected. This inhibition might be of importance for chloroform-induced liver necrosis.

Acknowledgement—These studies were financially supported by a grant from the Swedish Environment Fund.

Department of Forensic Medicine
Karolinska Institutet
S-104 01 Stockholm
Sweden

TOMAS EKSTRÖM
JOHAN HÖGBERG
BENGT JERNSTRÖM

REFERENCES

1. B. R. Brown, Jr., I. G. Sipes and A. M. Sagalyn, *Anesthesiology* **41**, 554 (1974).
2. T. Ekström and J. Högberg, *Biochem. Pharmac.* **29**, 3059 (1980).
3. J. Högberg, T. Ekström, I. Anundi and A. Kristoferson, *Toxicology* **17**, 113 (1980).
4. A. Meister and S. S. Tate, *A. Rev. Biochem.* **45**, 559 (1976).
5. J. Högberg and A. Kristoferson, *Eur. J. Biochem.* **74**, 77 (1977).
6. P. Gällerfors and B. D. Nelson, *Analyt. Biochem.* **93**, 200 (1979).
7. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
8. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W.W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
9. K. Ormstad, D. P. Jones and S. Orrenius, *J. Biol. Chem.* **255**, 175 (1980).
10. L. R. Pohl, J. L. Martin and J. W. George, *Biochem. Pharmac.* **29**, 3271 (1980).
11. D. F. Callen, C. R. Wolf and R. M. Philpot, *Mutat. Res.* **77**, 55 (1980).
12. J. L. Stevens and M. W. Anders, *Chem. Biol. Interact.* **37**, 207 (1981).

Biochemical Pharmacology, Vol. 31, No. 21, pp. 3513–3517, 1982.
Printed in Great Britain.

0006-2952/82/213513-05 $03.00/0

Differential toxicity of carrier-bound methotrexate against tumor/bone marrow cells *in vivo*

(*Received* 11 *January* 1982; *accepted* 26 *April* 1982)

Chemotherapeutic agents attached to carrier molecules have been shown recently to be effective in overcoming drug resistance *in vivo* [1, 2] and in enhancing toxicity to specific tumor cells. Drugs linked either to antibodies against specific tumor cell-associated antigens [3, 4] or to polypeptide hormones with affinities for tumor cell receptors [5] were shown to be more toxic to these specific cells than the parent compound.

In a previous paper [6], we have shown that methotrexate (MTX)*, when covalently linked to a non-specific molecule, poly-*l*-lysine mol. wt 3,000 or 40,000–60,000 (MTX-PLL 3K or 40–60K), was more toxic *in vitro* to cells derived from human solid tumors than to human lymphocytes or bone marrow cells. Under the same conditions, free MTX or MTX linked to human serum albumin (MTX-HSA) showed no differential toxicity between the two cell types. The MTX-PLL derivatives were more readily taken up by tumor cells than by lymphocytes/bone marrow cells, and inhibition of both cell growth and [^{3}H]deoxyuridine (dUrd) incorporation into DNA by the MTX-PLL derivatives in tumor cells, but not lymphocytes, could be reversed by inhibitors of lysosomal proteases. This indicated that breakdown of the MTX-PLL derivatives by lysosomal proteases occurred more readily in the tumor cells, and that enhanced metabolism may be responsible for their increased toxicity to tumor cells.

In the studies reported below, we have determined whether the differential toxicity of MTX-PLL toward tumor cells *in vitro* could also be observed *in vivo*. We have studied the disposition of MTX-HSA and the MTX-PLL derivatives into tissues when injected *in vivo*, their therapeutic effects on human tumor cells transplanted in nude athymic mice, and their relative toxicities to tumors versus bone marrow cells *in vivo*.

Materials and methods

The following were obtained from commercial sources: MTX, sodium salt, Lederle Laboratories, Pearl River, NY; human serum albumin and poly-*l*-lysine, Sigma Chemical Co., St. Louis, MO; [3′,5′,7-^{3}H]MTX (20 Ci/mmole), Amersham Radiochemicals, Chicago, IL; and [6-^{3}H]dUrd (24.2 Ci/mmole), New England Nuclear Corp., Boston, MA. MTX-HSA and MTX-PLL derivatives (75–105 mg MTX/g) and the ^{3}H-derivatives (4–14 mCi/mmole) were synthesized as previously described [6].† The T24A astrocytoma and T17 ovarian carcinoma tumors carried in nude mice and non-tumor bearing mice were obtained from the

* Abbreviations: MTX, methotrexate; MTX-HSA, MTX-human serum albumin; MTX-PLL 3K and 40–60K, MTX-poly-*l*-lysine mol. wt 3,000 and 40,000–60,000; [^{3}H]dUrd, [^{3}H]deoxyuridine; and PBS, phosphate-buffered saline.

† All concentrations reported for MTX-HSA and MTX-PLL refer to concentrations of bound MTX.

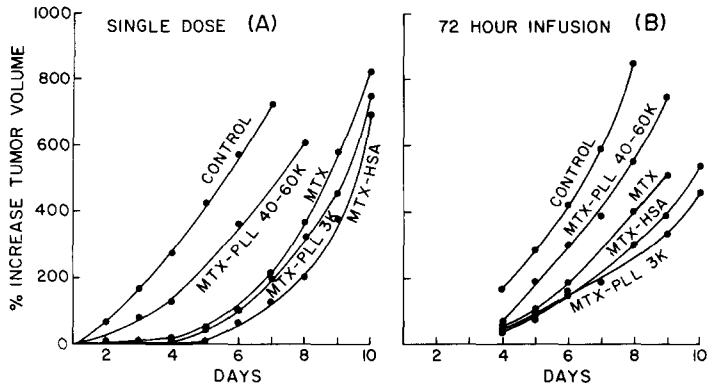

Fig. 1. Growth inhibition of human T24A astrocytoma when MTX, MTX-HSA, MTX-PLL 3K and MTX-PLL 40–60K were administered at maximally tolerated single i.p. doses (95 mg/kg MTX, 35 mg/kg MTX-HSA, 10 mg/kg MTX-PLL 3K, and 3.5 mg/kg MTX-PLL 40–60K) (A) or 72-hr constant infusion (1 mg/kg MTX, 5 mg/kg MTX-HSA, 7.5 mg/kg MTX-PLL 3K, or 13 mg/kg MTX-PLL 40–60K) (B). Nude athymic (BALB/c) mice were transplanted s.c. with 15 mg tumor cell suspensions and the drugs were administered when tumor volume had reached 0.2 to 0.5 cm^3 (approximately 7 days after tumor transplant). Tumor measurements were made daily with calipers. Each point represents an average of at least three experiments (five mice each experiment). Standard deviations ranged between 40 and 60% of indicated points. Values for MTX, MTX-HSA and MTX-PLL 3K were statistically significant, $P < 0.005$ to 0.05, Student's t-test; $P = 0.1$ for MTX-PLL 40–60K.

Nude Mouse Research Center at the University of California, La Jolla, CA. Both tumors were originally established from primary human tumor biopsies.

Drugs were injected in 0.1 to 0.2 ml phosphate-buffered saline (pH 7.4) (PBS) or infused subcutaneously via a Harvard infusion pump in 5 ml PBS over 24 or 72 hr at 3.4 or 0.6 μl/min respectively. MTX serum concentrations were measured by infusing the ^{3}H-compounds and removing blood samples from the retro-orbital sinus during the infusion. The cells were then centrifuged, and 10–25 μl plasma samples were mixed with 10 ml Aquasol (New England Nuclear) and counted in a Beckman LS-100C counter. The MTX concentrations were calculated on the assumption that the radioactivity in the serum existed as undegraded MTX and, therefore, may not be an accurate measure of the real MTX concentration [7]. Tumor measurements were made with calipers daily, using the formula $ab^2\pi/8$, where a and b = larger and smaller tumor diameters, respectively, to calculate tumor volume. Radioactivity in tissues was measured by combustion of 200 mg tissue samples in a Packard 360 Oxidizer. Toxicity to bone marrow cells was determined by decrease in bone marrow cellularity of drug-treated animals compared to untreated control animals. Marrow cells were quantitatively removed from both femurs by aspiration of cells with 3 ml PBS, and the number of cells in each femur was counted with a Coulter Counter model ZBI. For studies determining inhibition of [^{3}H]dUrd incorporation into DNA, mice were infused *in vivo* with predetermined drug doses for 24 hr. They were then killed; marrow cells were removed from femurs, and single suspensions of tumor cells were obtained by mincing and slicing of tumor tissues. Cells were then centrifuged, resuspended in 1 ml of Autopow media (Flow Laboratories, Inglewood, CA) at 10^6 cells/ml, and incubated with

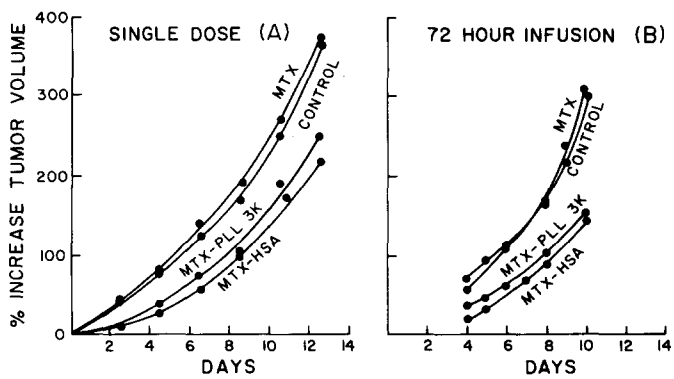

Fig. 2. Growth inhibition of human T17 ovarian carcinoma when MTX, MTX-HSA and MTX-PLL 3K were administered at maximally tolerated single i.p. doses (95 mg/kg MTX, 35 mg/kg MTX-HSA, and 10 mg/kg MTX-PLL 3K) (A) or 72 hr constant infusion (1 mg/kg MTX, 5 mg/kg, MTX-HSA, and 7.5 mg/kg MTX-PLL 3K) (B). Nude athymic mice were transplanted s.c. with 15 mg tumor cell suspensions, and the drugs were administered when tumor volume had reached 0.24 to 0.5 cm^3 (approximately 14 days after tumor transplant). Tumor measurements were made daily with calipers. Each point represents an average of three experiments (five mice each experiment). Standard deviations ranged between 40 and 70% of each point. Values for MTX-PLL 3K and MTX-HSA were statistically significant, $P < 0.05$ for points after 4 days.

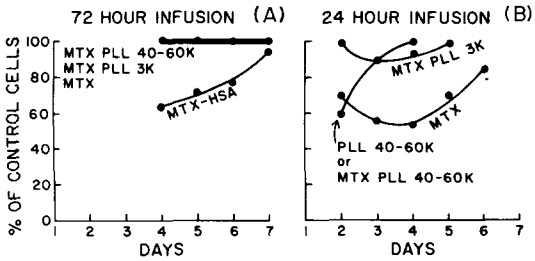

Fig. 3. Decrease in bone marrow cellularity when MTX, MTX-HSA, MTX-PLL 3K and MTX-PLL 40–60K were infused at maximum tolerated doses over 72 hr (1 mg/kg MTX, 5 mg/kg MTX-HSA, 7.5 mg/kg MTX-PLL 3K, and 13 mg/kg MTX-PLL 40–60K) (A) or over 24 hr (5 mg/kg MTX, 12 mg/kg MTX-PLL 3K, and 19 mg/kg MTX-PLL 40–60K) (B). On subsequent days after infusion, animals were killed, and cells were removed from both femurs of each animal and counted. Femurs from control untreated animals contained an average of 9×10^6 cells/femur. Each point represents cell counts from an average of at least three animals per day for each drug used.

2 μCi/ml [^{3}H]dUrd at 37° for 15 min. The cells were then washed and the DNA was precipitated as previously described [8]. Maximum tolerated doses of MTX and the MTX derivatives were determined by treating groups of eight to ten mice with increasing drug doses. The dose just below the LD_{10} was designated the maximum tolerated dose.

Results and discussion

Figure 1 shows the growth inhibition of the T24A astrocytoma when MTX and the MTX–carrier complexes were administered at a maximally tolerated dose either as a single i.p. injection (95 mg/kg MTX, 35 mg/kg MTX-HSA, 10 mg/kg MTX-PLL 3K, and 3.5 mg/kg MTX-PLL 60K) or as a 72-hr s.c. constant infusion (1 mg/kg MTX, 5 mg/kg MTX-HSA, 7.5 mg/kg MTX-PLL 3K, and 13 mg/kg MTX-PLL 40–60K). Figure 2 shows the same experiment with the more slowly growing T17 ovarian carcinoma. The results indicate (1) that free MTX was effective against the rapidly growing astrocytoma, delaying the time needed to increase tumor volume to three times initial volume from 4 to 7 days; MTX was not effective against the slowly growing ovarian carcinoma; (2) that MTX-HSA and MTX-PLL 3K were as effective as MTX in delaying growth of the astrocytoma and more effective than MTX against the ovarian carcinoma; and (3) that MTX-PLL 40–60K, previously found to have the greatest selective toxicity against the astrocytoma cells *in vitro* [6], was the least effective against the same tumor *in vivo*. The ineffectiveness of MTX-PLL 40–60K, when given as a single dose, could be attributed to the small amount of drug that could be tolerated (3.5 mg/kg) due to the toxicity of PLL 40–60K. However, although considerably larger doses could be tolerated when infused slowly over 72 hr (13 mg/kg), this derivative was still not cytotoxic to tumor cells.

Figure 3A shows the simultaneous effects on bone marrow cellularity when the drugs were infused at the same doses over 72 hr, resulting in steady-state serum concentrations of $5–6 \times 10^{-8}$ M MTX, $3–5 \times 10^{-7}$ M MTX-PLL 3K or 40–60K and $1–2 \times 10^{-6}$ M MTX-HSA. No significant toxicity to bone marrow cells was observed with MTX and the MTX-PLL derivatives on this dose schedule, but considerable toxicity was incurred with MTX-HSA. Thus, on a maximally tolerated long infusion schedule, both MTX and MTX-PLL 3K were more toxic to tumor than bone marrow cells (Fig. 1B). If a larger dose of MTX (5 mg/kg) was infused over a shorter period (24 hr), resulting in 5×10^{-7} M serum concentrations over 24 hr, then considerable bone marrow toxicity did occur, but no significant bone marrow depression was incurred when a maximum tolerated MTX-PLL 3K dose was infused over 24 hr, and the bone marrow damage incurred with a near toxic infusion of MTX-PLL 40–60K over 24 hr was due to the damaging effects of the carrier. These experiments thus confirm *in vitro* experiments which indicate that bone marrow cells are relatively resistant to the MTX-PLL derivatives.

The amounts of [^{3}H]MTX (ng) reaching tumor, bone marrow, liver, spleen, kidney and intestine after maximum tolerated single doses of MTX and the MTX–carrier complexes, calculated as ng MTX equivalents per 100 mg tissue, are shown in Table 1. These results indicate that MTX-HSA levels in tumor 24 hr after injection (240 ng/100 mg tumor) were considerably higher than free MTX levels (62 ng/100 mg tumor tissue) or MTX-PLL 3K (16 ng/100 mg tissue) and MTX-PLL 40–60K (6.5 ng/100 mg tissue). However, considerable drug also accumulated in bone marrow, and the great majority of MTX-HSA was taken up in the liver. Drug uptake of MTX-PLL 3K by tumor and bone marrow cells was not significantly different and, although very little uptake of MTX-PLL 40–60K by bone marrow cells was seen, the MTX concentrations in tumor was also considerably lower than the other derivatives, thus accounting for its poor therapeutic effects. Uptake of the MTX-PLL derivatives occurred primarily in kidney, liver and spleen.

Previous *in vitro* experiments had indicated that the MTX-PLL derivatives were able to inhibit [^{3}H]dUrd incorporation into DNA of T24A astrocytoma cells at lower concentrations than those needed to inhibit dUrd incorporation in bone marrow cells. Figure 4 shows the effects

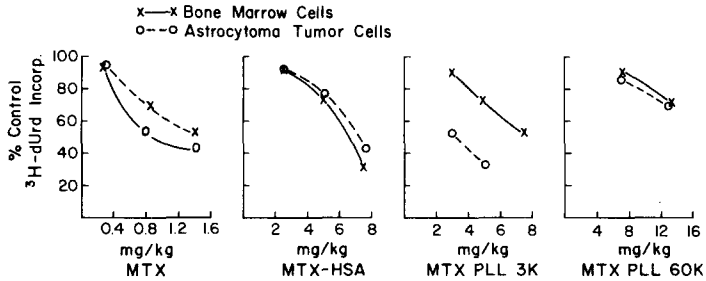

Fig. 4. Inhibition of [^{3}H]dUrd incorporation into DNA of T24A astrocytoma tumor and bone marrow cells by MTX, MTX-HSA, MTX-PLL 3K and MTX-PLL 40–60K. Mice bearing astrocytoma tumors (0.07 to 0.15 cm^3) were infused with PBS or the drugs at the doses indicated for 24 hr. Animals were then killed. Bone marrow and tumor cells were isolated and incubated at 37° for 15 min with 2 μCi/ml [^{3}H]dUrd. Cells were then washed free of radioactivity and the DNA was precipitated with 10% trichloroacetic acid. Control cells incorporated an average of 20,000 cpm/10^6 marrow cells or 12,000 cpm/10^6 tumor cells. Each point represents an average of at least three animals.

Table 1. [^{3}H]MTX associated radioactivity in astrocytoma and other tissues at various times after injections of [^{3}H]MTX, [^{3}H]MTX-PLL 3K or 40–60K, and [^{3}H]MTX-HSA*

	MTX tissue content (ng/100 mg tissue† and percentage of total measured drug in 100 mg tissue)													
	MTX				MTX-HSA		MTX-PLL 3K				MTX-PLL 40–60K			
	3 hr		24 hr		24 hr		3 hr		24 hr		3 hr		24 hr	
	ng	%	ng	%	ng	%	ng	%	ng	%	ng	%	ng	%
Tumor	97 ± 47	2 ± 0.8	62 ± 31	12 ± 3	240 ± 70	3 ± 0.5	34 ± 25	1.3 ± 1	16 ± 4	3 ± 1	16 ± 3	2 ± 0.2	6.5 ± 3	1 ± 0.3
Liver	845 ± 162	25 ± 8	132 ± 111	23 ± 6	4570 ± 1032	72 ± 4	365 ± 134	12 ± 2	77 ± 25	15 ± 3	200 ± 54	26 ± 8	178 ± 83	39 ± 4
Spleen	160 ± 43	3 ± 1	92 ± 39	24 ± 5	726 ± 76	12 ± 2	255 ± 133	10 ± 3	35 ± 7	7 ± 1	92 ± 35	5 ± 2	108 ± 25	25 ± 4
Kidney	563 ± 450	12 ± 4	100 ± 37	21 ± 6	308 ± 46	5 ± 1	1840 ± 86	66 ± 5	410 ± 155	69 ± 8	410 ± 150	56 ± 9	122 ± 59	25 ± 3
Intestine	960 ± 600	55 ± 15	97 ± 72	17 ± 4	300 ± 170	3 ± 1	260 ± 50	9 ± 1	30 ± 10	5 ± 1	80 ± 31	10 ± 3	45 ± 7	10 ± 2
Bone marrow†	130 ± 32	2 ± 1	37 ± 11	3 ± 1	575 ± 233	4 ± 1	49 ± 9	2 ± 1	20 ± 4	1 ± 1	5 ± 3	1 ± 1	2 ± 1	0

* Nude athymic mice bearing astrocytoma tumors (0.03 to 0.15 cm^3) were injected i.p. with maximum tolerated doses of [^{3}H]MTX (95 mg/kg), [^{3}H]MTX-HSA (35 mg/kg), [^{3}H]MTX-PLL 3K (10 mg/kg) and [^{3}H]MTX-PLL 40–60K (3.5 mg/kg). Tissues were removed when radioactivity in blood had reached undetectable levels (3 hr after injection of MTX and the MTX-PLL derivatives and 24 hr after MTX-HSA injection). Tissue samples (200 mg) were processed for radioactivity in a Packard 360 Oxidizer. Each value is the mean value (±S.D.) derived from three animals. The percentage of total measured drug that was found in each tissue represents the average percentage of drug in 100 mg tissue samples from three individual animals.

† Bone marrow results are indicated as ng MTX per femur (the average weight of one femur was 50 mg). However, because of the low moisture content, this constitutes an average of 10^7 cells versus the average 4–5 × 10^6 cells contained in the average 100 mg tumor sample.

of infusing increasing doses of MTX, MTX-HSA, MTX-PLL 3K and MTX-PLL 40–60K over 24 hr on dUrd incorporation into DNA of bone marrow and tumor cells.

These results indicate that after 24 hr of exposure to increasing concentrations of MTX or MTX-HSA, the concentrations of the drugs needed to inhibit dUrd incorporation into DNA of bone marrow or tumor cells were similar. For MTX-PLL 40–60K, little inhibition of dUrd incorporation was seen in bone marrow or tumor cells at the maximally tolerated dose, indicating that the limiting toxicity for this agent was another unknown tissue. Only MTX-PLL 3K inhibited [^{3}H]dUrd incorporation in the T24A astrocytoma cells at lower doses than bone marrow cells. Bone marrow cells, after infusion with MTX and the MTX derivatives at the maximum dose indicated in Fig. 4, had recovered from [^{3}H]dUrd incorporation inhibition 48 hr after the start of drug infusion, whereas [^{3}H]dUrd incorporation into DNA of tumor cells remained inhibited at this time, and recovery was observed 4 days after the start of infusion (data not shown).

We conclude from these results, first, that there was evidence of different toxicities to astrocytoma tumor and bone marrow cells *in vivo* when MTX-PLL 3K was injected or infused into mice bearing the astrocytoma tumor. The above data indicate tumor growth inhibition with eventual recovery to control growth rates, compared with low depression of marrow cellularity over the same time interval. This confirms the *in vitro* evidence indicating its preferential toxicity to astrocytoma cells versus bone marrow cells [6]. However, MTX-PLL 40–60K did not demonstrate the same differential toxicity toward tumor cells *in vivo* as it had *in vitro*, since it was not cytotoxic for either bone marrow or tumor cells *in vivo*. Its ineffectiveness against tumor cells *in vivo* may have been due to its poor penetration between cells into the interior of the tumor mass. Second, the limiting factor in the use of either MTX-PLL 3K or 40–60K given as a single dose was the toxicity of the carrier PLL, which is believed to cause leakage of ions from the cells and perturbation of cell surfaces [9]. The derivative was considerably less toxic when given as a constant infusion, but was taken up primarily by kidney, liver and spleen cells. Third, MTX-HSA was taken up by the astrocytoma tumor *in vivo* better than MTX or the MTX-PLL derivatives; this contrasts with the poor uptake of this derivative by the same tumor cells *in vitro* [6]. The high drug levels at the tumor site may also account for the ability of MTX-HSA to inhibit growth of the T17 ovarian carcinoma whereas the parent free drug was ineffective. However, MTX-HSA also had high affinity for bone marrow cells *in vivo*, indicating a lack of selectivity of this agent. Fourth, MTX-HSA and MTX-PLL 3K, but not the parent compound MTX, inhibited growth of the T17 ovarian carcinoma; doxorubicin was shown previously to be ineffective against this tumor [10]. Fifth, although the toxicity of the PLL molecule limits its usefulness as a carrier for chemotherapeutic agents, the demonstration that a carrier molecule can reduce the toxicity of a chemotherapeutic agent to bone marrow cells while not affecting its cytotoxicity to tumor cells, both *in vitro* and *in vivo*, indicates the potential of using carrier molecules to induce differential toxicity to various cell types and to increase overall therapeutic effectiveness.

Acknowledgements—We wish to thank Jan Goodsell for skilled technical assistance and the Allergan Pharmaceutical Co., Irvine, CA, for the use of the Packard 360 Oxidizer. This investigation was supported by Grant CA 26152 awarded by the National Cancer Institute, DHEW.

T-006 Department of Medicine and Cancer Center
University of California
La Jolla, CA 92093, U.S.A.

BARBARA C. F. CHU*
STEPHEN B. HOWELL

* Author to whom correspondence should be directed.

REFERENCES

1. H. J-P. Ryser, D. Pritchard and W. C. Shen, *Proc. Am. Ass. Cancer Res.* **20**, 820 (1979).
2. B. C. F. Chu, C. C. Fan and S. B. Howell, *J. natn. Cancer Inst.* **66**, 121 (1981).
3. P. N. Kulkarni, A. H. Blair and T. I. Ghose, *Cancer Res.* **41**, 2700 (1981).
4. M. B. Davis and J. F. Preston III, *Science* **213**, 1385 (1981).
5. J. M. Varga, N. Asato, S. Lande and A. B. Lerner, *Nature, Lond.* **267**, 56 (1977).
6. B. C. F. Chu and S. B. Howell, *Biochem. Pharmac.* **30**, 2545 (1981).
7. D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmac.* **19**, 2923 (1970).
8. A Krishan, K. D. Paika and E. Frei III, *Cancer Res.* **36**, 138 (1976).
9. S. E. Kornguth and M. A. Stahmann, *Cancer Res.* **21**, 907 (1961).
10. F. C. Giuliani, K. A. Zirvi and N. O. Kaplan, *Cancer Res.* **41**, 325 (1981).