Eur J Cancer Clin Oncol, Vol. 25, No. 2, pp. 263–269, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Comparison of the Effects of Flavone Acetic Acid, Fostriecin, Homoharringtonine and Tumour Necrosis Factor α on Colon 38 Tumours in Mice*

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Abstract—*Advanced subcutaneous Colon 38 tumours in mice were used for the assessment of activity of a number of anticancer drugs. Activity was measured by histological examination of tumours 24 h after a single dose of the drug and in some cases by tumour growth delay. Agents thought to exert their cytotoxic effect by damaging DNA, including Adriamycin, amsacrine and its analogue CI-921, cyclophosphamide, 5-fluorouracil and methotrexate produced no gross histological changes after 24 h, even though some delayed the growth of subcutaneous tumours. In contrast, flavone acetic acid, fostriecin and homoharringtonine caused extensive necrosis of tumours after 24 h, and each delayed the growth of advanced subcutaneous tumours by at least 10 days when administered as a single dose. The histological effects of flavone acetic acid and fostriecin were indistinguishable from those of recombinant human tumour necrosis factor α. It is proposed that histological assay of advanced tumours may provide a useful adjunct to existing methods in screening for antitumour agents with novel mechanisms of action.*

INTRODUCTION

TRANSPLANTABLE MURINE TUMOURS have formed the basis for the screening and selection of most of the currently available clinical antitumour agents [1]. The majority of these agents have been detected primarily on the basis of their activity against transplantable leukaemias. The goal of current drug screening has now turned to the discovery of agents with activity against advanced solid tumours in mice. One such agent, flavone acetic acid (FAA) has pronounced activity against advanced solid murine tumours and xenografts [2–4] and induces histological changes in the tumour (haemorrhagic necrosis) within a few hours of drug administration [3, 4]. FAA is currently undergoing phase II clinical trials [5] but so far has shown negative results. Nevertheless, it is possible that other agents with a similar mode of action may be active against human as well as murine tumours.

Since FAA has activity against a wide spectrum of murine solid tumours but little against transplantable leukaemias, its mode of action is of considerable interest. Evidence has previously been presented that FAA does not act directly on tumour cells [4], and that FAA modulates the activity of some immune cells [6, 7]. Lewis lung cells growing as small tumours in the lungs and Colon 38 cells growing as small tumours in the liver are resistant to FAA, but once the tumours are established the drug becomes effective (Refs. [3, 4] and unpublished results). FAA therefore appears to be quite different to established clinical agents, and its effects show some similarities to those of tumour necrosis factor α (TNFα: reviewed in Ref. [8]).

In previous studies we have described the FAA-induced haemorrhagic necrosis of murine Colon 26, Colon 38 and Lewis lung tumours [3, 4] and of xenografts of several human tumour cell lines growing in athymic mice [4]. We are now using the advanced Colon 38 tumour as a preliminary screening system to search for other compounds with FAA-like activity, using histological assessment as an indication of drug effects. We have tested a number of agents, including TNFα, fostriecin and homoharringtonine, which have diverse modes of antitumour action. Structures of fostriecin, a new phosphate-containing antibiotic scheduled for clinical trial [9], and homoharringtonine, a plant alkaloid which has already undergone some clinical evaluation [10], are shown in Fig. 1. The results show that the rapid induction of haemorrhagic

Accepted 29 September 1988.
*Supported by the Auckland Division of the Cancer Society of New Zealand; the Medical Research Council of New Zealand.
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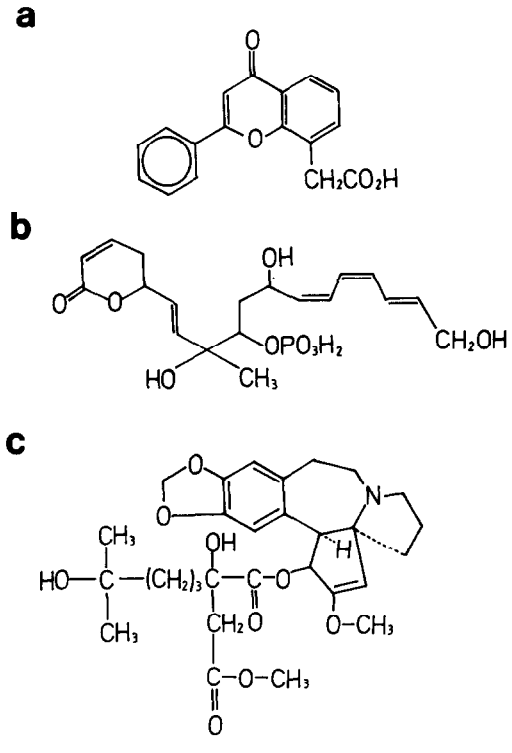

Fig. 1. Structures of FAA (a), fostriecin (b) and homoharringtonine (c).

necrosis is a property of only a few types of antitumour drug.

MATERIALS AND METHODS

Drugs

Fostriecin (NSC 339638 as a stabilized formulation with ascorbic acid in sealed vials), amsacrine isethionate (NSC 156303) and *N*,5-dimethyl-9-((2-methoxy-4-methylsulfonylamino)phenylamino)-4-acridinecarboxamide (CI-921; NSC 343499) were kindly provided by Dr R.C. Jackson, Parke-Davis Division of Warner-Lambert Co., Ann Arbor, Michigan, U.S.A. FAA (NSC 347512), homoharringtonine (NSC 141633) and ellipticine (NSC 71795) were kindly provided by Dr K. Paull, Division of Cancer Treatment, National Cancer Institute, U.S.A. Adriamycin (NSC 123127; Farmitalia), cyclophosphamide (NSC 26271; Mead Johnson), 5-fluorouracil (NSC 19893; Roche) and methotrexate (NSC 740; Eli Lilly) were obtained from Auckland Hospital Pharmacy. Recombinant human TNFα was provided by Professor J.D. Watson, Department of Immunobiology, University of Auckland. *N*-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (acridine carboxamide; NSC 601316) was synthesized in this laboratory [11].

Drugs were dissolved in solvent [FAA in 0.2 ml 5% sodium bicarbonate, cyclophosphamide in saline, amsacrine, CI-921 and acridinecarboxamide in 0.1 ml 30% (v/v) aqueous ethanol, Adriamycin, 5-fluorouracil, methotrexate and recombinant human TNFα in 0.2 ml 5% dextrose] and administered by i.p. injection at 1.5-fold dose intervals.

Colon 38 tumours

Tumour stocks were obtained in 1981 from Mason Research Institute, Worcester, U.S.A. and after *in vivo* passage were stored under standard conditions at −196°C. Tumours were passaged subcutaneously (s.c.) in C57BL/6J mice and grown for experiments s.c. in B6D2F$_1$ mice. C57BL/6J and DBA/2J breeding pairs were originally obtained from Jackson Laboratory, Bar Harbor, Maine, U.S.A. and were bred in the laboratory under conditions of constant temperature, lighting and humidity with sterile bedding, water and food. All experiments were performed according to institutional ethical guidelines. S.c. tumours were grown to a diameter of approx. 10 mm, removed surgically and cut into 1 mm^3 fragments. Recipient mice were anaesthetized by intraperitoneal (i.p.) injection of pentobarbital (90 mg/kg, dissolved in 0.2 ml 0.15 M NaCl). A small incision was made, the tumour fragment placed well under the skin flap and the incision closed with a small Michel wound clip.

Histological assessment

Groups of B6D2F$_1$ mice with s.c. tumours 4–12 mm in diameter (approx. 8 days after tumour implantation) were injected with a single i.p. drug dose. At least three experiments were carried out on different occasions using a dose range which included the highest non-toxic dose. Mice were killed by cervical dislocation 24 h after drug injection and tumours were removed and fixed immediately in 10% formalin. Fixed tumours were embedded in paraffin wax and sections taken as close as possible to the centre of the tumour. Sections were stained with haematoxylin and eosin by standard methods. A grid marked at 0.4 mm intervals and printed on acetate sheet was placed over the slide and the intersections of the grid were scored as either 'undamaged' tumour or 'necrotic' (pyknotic and fragmented nuclei, altered staining pattern). Grid intersections on blood vessels or obvious stromal elements were not counted. Both the total number of grid intersections (related to the cross-sectional area) and the percentage of these intersections scored as 'undamaged' were calculated. The percentage of 'undamaged' tumour was divided by the corresponding percentage scored in sections of untreated tumours of the same cross-sectional area to produce a fraction. If this fraction was >0.5, ≤ 0.5 or ≤ 0.1 (average of three of more experiments) the drug was scored as negative, '+' or '++' respectively.

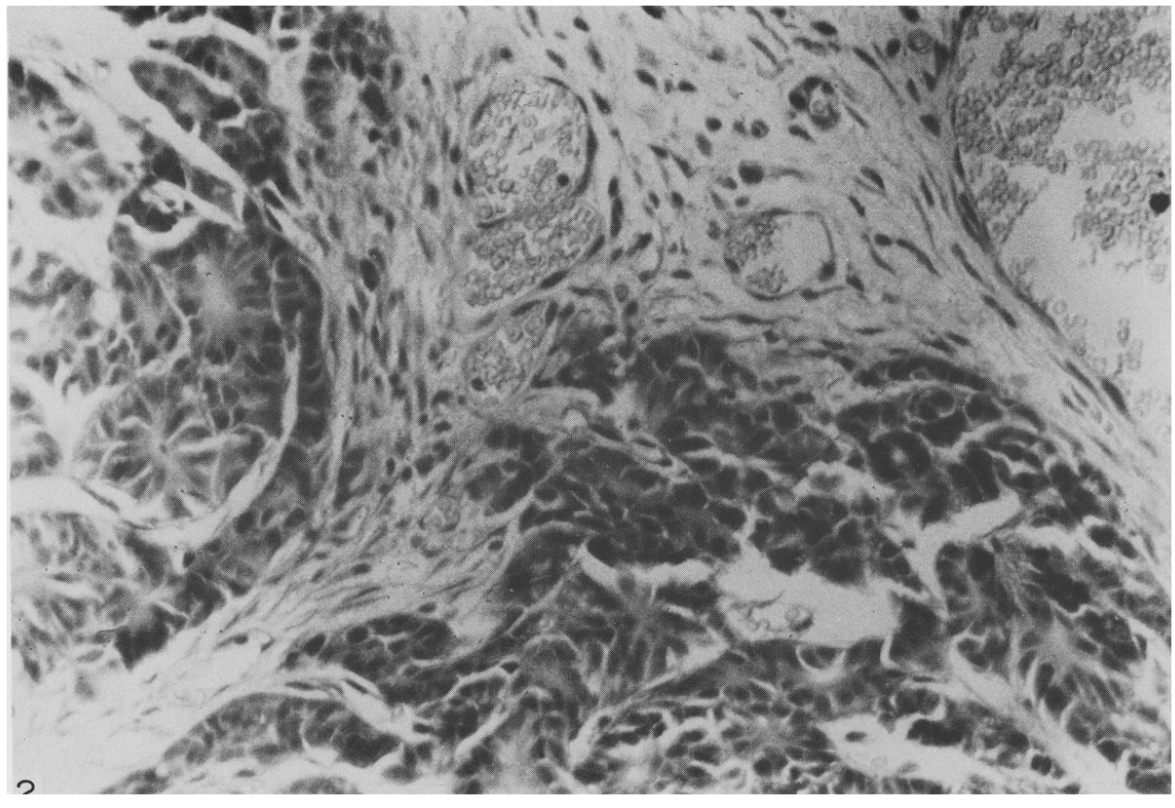

Fig. 2. Histological appearance of part of an untreated Colon 38 tumour. The section demonstrates the presence of stromal elements and blood vessels (upper right quadrant) as well as tumour acini. The latter comprise the majority of the tumour (× 180).

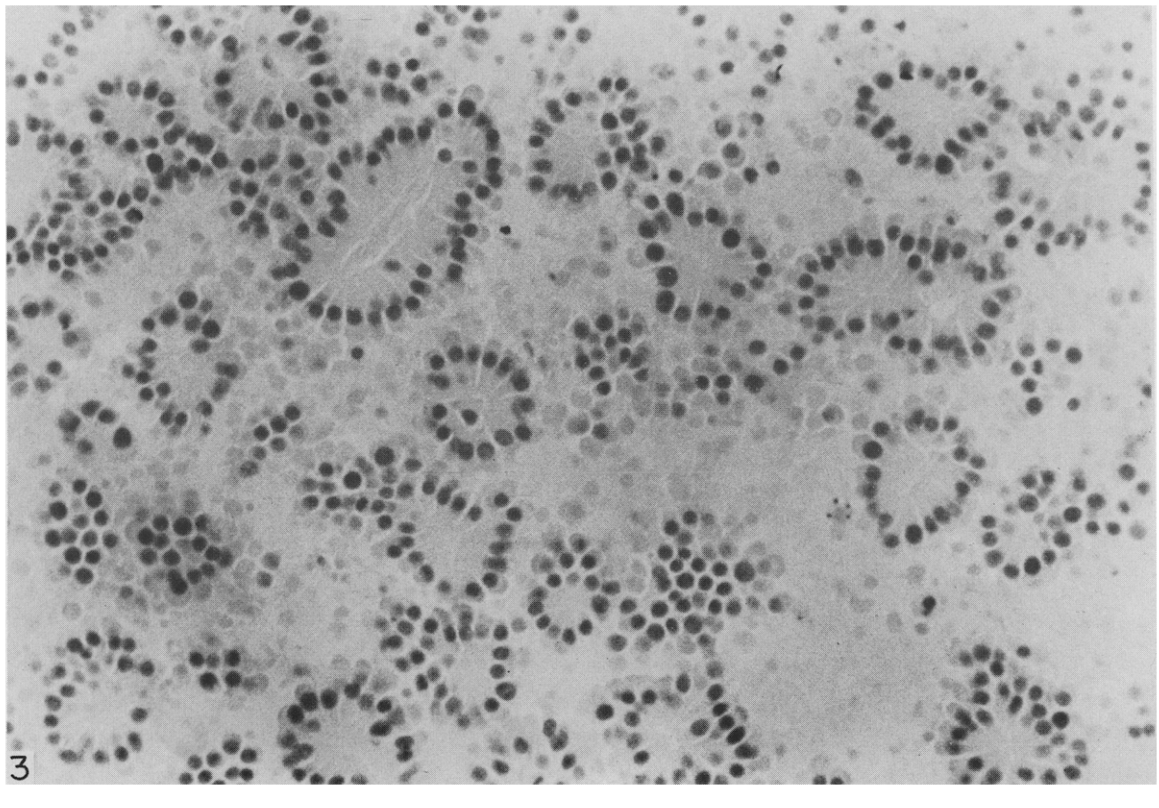

Fig. 3. Histological appearance of an area of 'ghost acini' induced 24 h after treatment with FAA (330 mg/kg), *and also observed following treatment with fostriecin* (65 mg/kg) *and recombinant human TNFα* (300,000 *units*) (× 180).

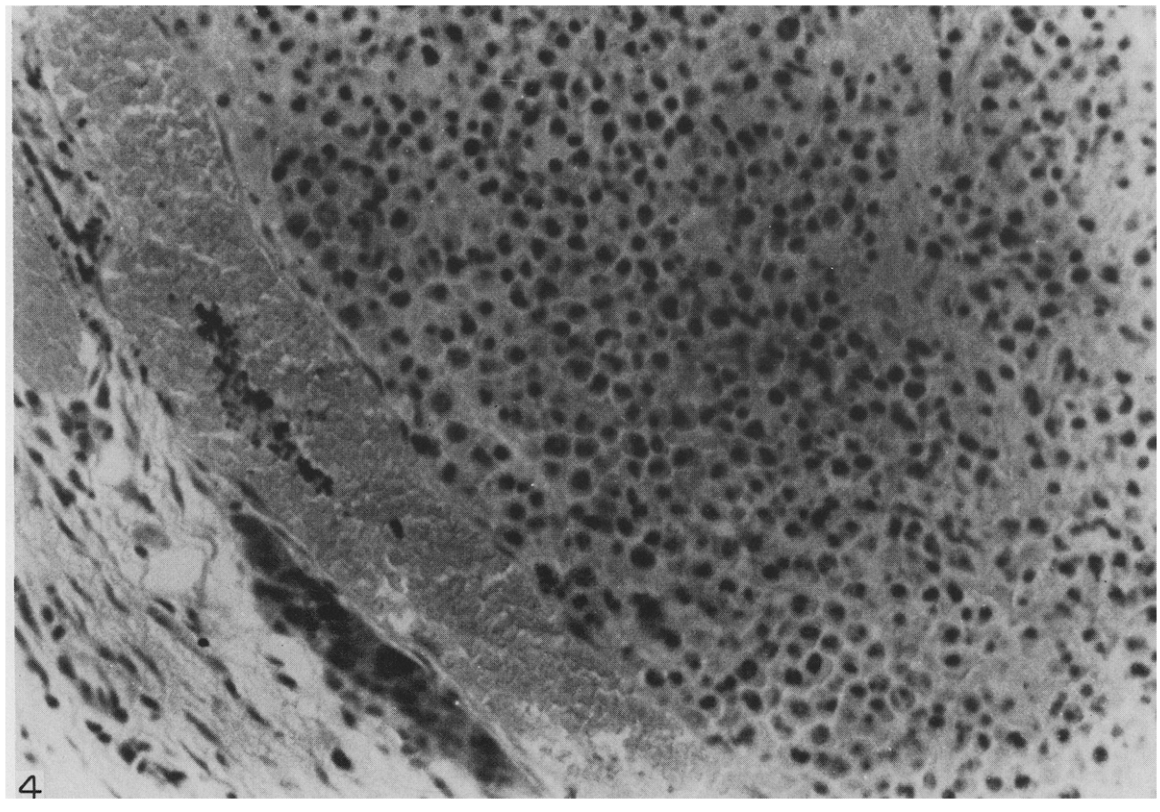

Fig. 4. Histological appearance of area of less structured necrosis induced 24 h *after treatment with fostriecin* (65 mg/kg), *and also observed after treatment with FAA and recombinant human TNF*α (300,000 *units*). *On the left a large blood vessel engorged with erythrocytes can be seen* (× 180).

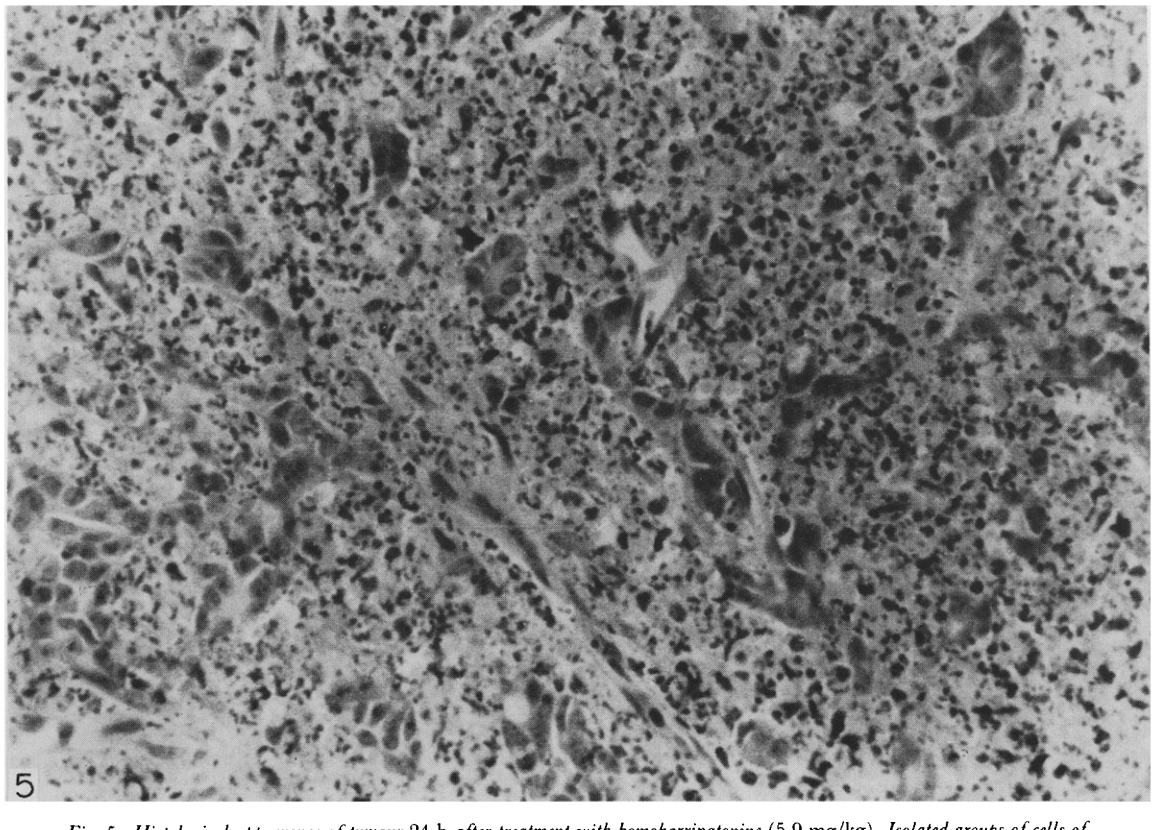

Fig. 5. Histological appearance of tumour 24 h *after treatment with homoharringtonine* (5.9 mg/kg). *Isolated groups of cells of normal appearance are arranged in irregular and loose clumps and strands among areas of cell destruction containing ill-defined cells with eosinophilic cytoplasm and fragmented nuclei. Areas of haemorrhage are evident in zones of necrosis, and cell death is most evident in the central part of the tumour* (× 180).

Growth delay experiments

Treated and control groups of five to six $B6D2F_1$ mice with s.c. tumours (5–10 mm diameter) were used. Mice were randomized with respect to tumour size, ear-tagged and injected with the appropriate drug. Tumours were measured thereafter three times weekly with digital calipers and tumour volumes calculated as $0.52a^2b$, where a and b are the minor and major axes of the tumour. Means and standard errors were calculated on the basis of the logarithms of tumour volume.

RESULTS

Histological appearance of untreated tumours

Sections of 27 s.c. Colon 38 tumours from untreated mice were examined to provide a baseline for the assessment of drug effects. Tumour maximal diameters ranged from 3 to 12 mm, and the areas of (maximal area) cross sections ranged from 11 to 120 mm^2. The appearance of an untreated tumour is shown in Fig. 2. Areas of necrosis were found in sections of all but six of the tumours, and the percentage of the total section area was estimated using a grid scoring system. A significant relationship ($r = 0.76$) between the estimated percentage area of necrosis and total cross-sectional areas of tumours was found. The percentage of necrosis was found to correspond approximately to 0.26 times the cross-sectional area in mm^2.

Screening of clinical and experimental agents

Tumours were generally removed from mice 24 h after drug treatment. Tumour sections were scored for areas of necrosis using a grid scoring system and compared to tumours of the same cross-sectional area as described in Materials and Methods. Treatment with FAA (330 mg/kg) resulted in '++' rating (less than 10% undamaged tumour cells) in all tumours examined. Tumours were also examined at various times after FAA treatment. Extensive changes were apparent over the whole histological section after 4 h although complete necrosis had not occurred. The necrotic appearance of the tumour section was achieved after 12 h and was preserved with little change up to 9 days after treatment.

A number of 'DNA damaging' drugs were tested and found to be negative in the Colon 38 24 h histology assay, as shown in Table 1. In contrast, fostriecin (65 mg/kg) and recombinant human TNFα (300,000 units) were scored as '++' in the assay. Homoharringtonine (5.9 mg/kg) also induced extensive tumour necrosis, but because cells with an undamaged appearance were scattered through the tumour section it was not possible to score the section reliably with the grid system. The histological appearance of some of the treated tumours is shown in Figs. 2–5.

Approximately 50% of the necrotic tissue in tumours treated with FAA, fostriecin and TNFα was composed of 'ghost acini' (Fig. 3) with intervening exudate, haemorrhage and cellular debris. The remainder was composed of relatively structureless areas of disintegrating cells with pyknotic nuclei (Fig. 4). Blood vessels were congested and there were extensive areas of haemorrhage into the tumour tissue. The areas of necrotic cells were qualitatively similar to necrotic areas in sections of untreated tumours. The effects of homoharringtonine (5.9 mg/kg) contrasted with those of the other agents (Fig. 5).

Growth delay experiments

FAA, fostriecin and homoharringtonine all induced significant delays in the growth of advanced (5–10 mm) Colon 38 tumours (Fig. 6). In contrast to the other two drugs, homoharringtonine induced a marked reduction of tumour volume. Growth delay experiments were also carried out for some of the compounds inactive in the histology assay (Table 1). 5-Fluorouracil was the most active of these compounds, inducing a delay of beween 10 and 15 days over a number of experiments.

DISCUSSION

One of the aims of this study was to determine whether the 24 h histology assay detects agents other than FAA. The clinical 'DNA damaging' agents tested induced a DNA alkylating agent (cyclophosphamide), two antimetabolites (methotrexate and 5-fluorouracil) and two agents which induce DNA breakage by interaction with the enzyme topoisomerase II [12] (Adriamycin and amsacrine). Other topoisomerase II-directed agents tested included CI-921, an analogue of amsacrine currently undergoing clinical trial [13], acridinecarboxamide, another acridine derivative with high solid tumour activity [11] and ellipticine, a compound with high activity against early Colon 38 tumours [1]. Some of these agents were found to delay the growth of advanced Colon 38 tumours (Table 1), although none were active in the 24 h histology assay. All three classes of compounds are capable of causing arrest of cycling cells in the G2 phase of the cell division cycle, and death of cells follows after 2–4 days by a process of cell enlargement and eventual breakdown [14]. Damage appears not to become evident histologically by 24 h.

Compounds showing activity in the histology assay (Fig. 2) appear not to share the same mechanism of action as the above agents. FAA has broad activity against murine solid tumours and human tumour xenografts [4] but little activity against cultured tumour cells [4, 15]. Fostriecin has, in contrast to FAA, good antileukaemia activity [9]

Table 1. *Compounds inactive in the* 24 h *histology assay*

Compound	Dose in histology assay (mg/kg)	Colon 38 growth delay assay	
		Dose (mg/kg)	Growth delay (days)
Acridinecarboxamide	220	100 × 3*	3.0
Adriamycin	45		
Amsacrine	45	13.3 × 3	2.0
CI-921	100	30 × 3	7.8
Cyclophosphamide	220	220	6.5
Ellipticine	750		
5-Fluorouracil	500	65 × 3	12.5
Methotrexate	750		

*Multiple doses, where indicated, were administered at intervals of 4 days.

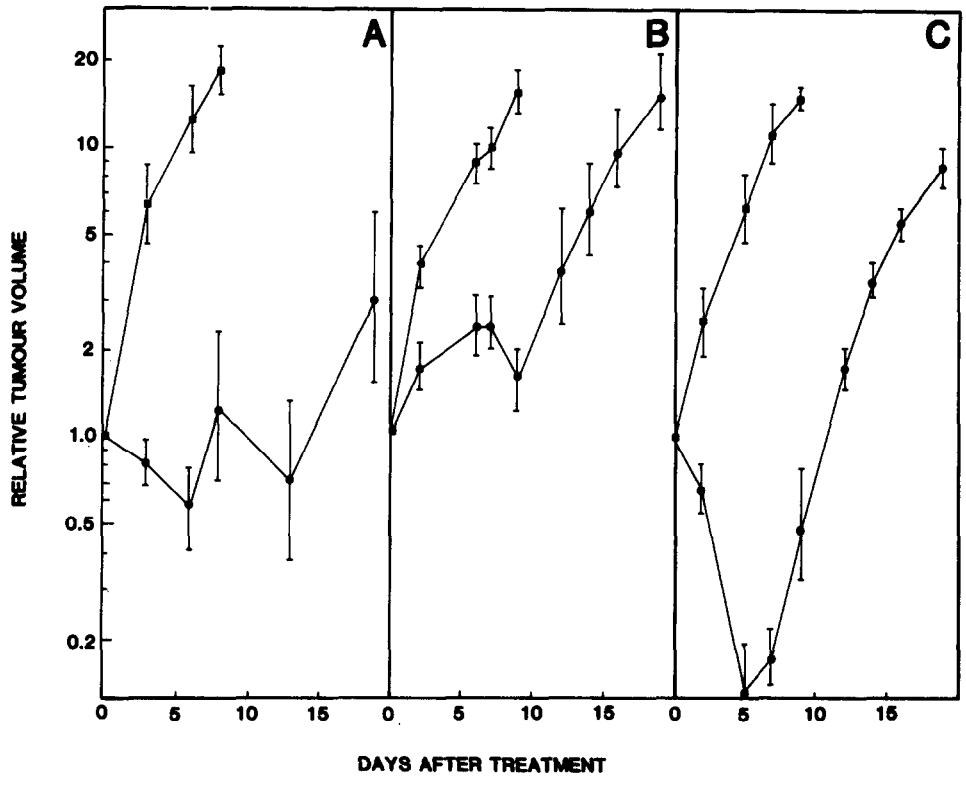

Fig. 6. *Growth delays induced by single doses of* (*A*) *FAA* (330 mg/kg); (*B*) *fostriecin* (65 mg/kg); (*C*) *homoharringtonine* (5.9 mg/kg). *Initial mean tumour diameters were approx.* 8 mm. *Average tumour volumes* (*relative to initial volumes*) *are shown for treated* (*circles*) *and untreated* (*squares*) *groups of mice. Vertical lines indicate standard errors of the mean.*

and inhibits nucleic acid and protein synthesis in cultured L1210 cells. It is a potent inhibitor of topoisomerase II, although it does not induce DNA–protein cross-links in the manner of Adriamycin and amsacrine [16]. Homoharringtonine is active againt leukaemia and Colon 38 *in vivo* and is an inhibitor of protein synthesis [10]. There is little chemical similarity between the compounds except that fostriecin and FAA are both acids, while homoharringtonine is an acid ester.

In conclusion, the 24 h Colon 38 histological assay is simple, economical and humane to perform. Although, like any histological assay, it is difficult to quantitate, good reproducibility has been obtained in routine testing. The assay may be used to complement existing life extension, drug toxicity and tumour growth delay assays in the screening of new compounds for antitumour activity. Other s.c. solid tumours including human tumour xenografts can be used, although in our experience a longer

assay time (48 h) may be preferable for these studies. The assay has demonstrated unexpected similarities in the action of four agents, FAA, fostriecin, homoharringtonine and TNFα, and has led to the demonstration of the activity of fostriecin and homoharringtonine against advanced s.c. Colon 38 tumours in mice. The active compounds detected by the assay have widely divergent structures and further work will be required to determine the mechanistic basis for the similarities in their action. The assay has also identified active FAA analogues in this laboratory [17] and is now being used to identify further active compounds.

Acknowledgements—The authors are grateful to Lorna Allan and Carolyn Hunter-Walker for cutting and staining the histology sections and to Sandy Miller for printing the photographs.

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