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Antiproliferative Properties of Flavone Acetic Acid (NSC 347512) (LM 975), a New Anticancer Agent

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Abstract—*The antiproliferative activity of flavone acetic acid (LM 975) was investigated on human adenocarcinoma cell lines (HCC-P2998, HCC-M1544, HCC-M1410, HT 29, LoVo), on a murine colon adenocarcinoma cell line (Colon 26), on murine pancreatic adenocarcinoma cells growing in primary culture (Pan 03) and on human normal fibroblasts (N1).*

No cytotoxic effects were found against human normal fibroblasts. LM 975 was active against murine adenocarcinoma Pan 03 and Colon 26, known to be sensitive in vivo too and, to variable extents, on human adenocarcinoma cell lines.

LM 975 in vitro cytotoxic potency was relatively low. The high concentrations (1.0–1.4 mM) required to obtain a cytotoxic effect are, however, pharmacologically reasonable since they are comparable with drug plasma levels in mice or in patients treated with tolerable doses.

After a relatively short LM 975 treatment (2 h) DNA, RNA and protein synthesis were inhibited in different proportions. In more sensitive cells LM 975 appeared to inhibit RNA synthesis more than DNA and protein synthesis. Inhibition of macromolecule synthesis after 2 h exposure was completely reversed in 24 h recovery. After 2 h treatment no detectable DNA breakage was found by the alkaline elution method, thus corroborating the idea that this compound does not act by causing DNA damage.

INTRODUCTION

FLAVONE ACETIC ACID (LM 975) is a new anticancer agent which has shown activity in some experimental tumors such as the murine adenocarcinomas Colon 38 [1], Colon 07/A, Colon 51, Colon 10/A and the murine pancreatic ductal adenocarcinomas Pan 02 and Pan 03 [2, 3].

It has been selected for clinical investigation in Europe and in the U.S.A.

No information is yet available on the mechanism of action of this drug, which may act by completely different mechanisms from those described for the anticancer agents identified so far. This hypothesis is suggested by the following considerations: (i) the chemical structure of LM 975 does not resemble that of any known anticancer agent; (ii) LM 975 shows a peculiar pattern of antitumor activity in mice, solid, slow growing tumors being more susceptible than rapidly growing leukemias; (iii) the drug shows a pattern of toxicity different from that of most anticancer agents (i.e. no bone marrow toxicity). As a first step to mechanistic studies on LM 975 we investigated its antiproliferative activity on human colon adenocarcinoma cell lines (HCC-P 2998, HCC-M 1544, HCC-M 1410, HT 29 and LoVo), on a murine colon adenocarcinoma cell line (Colon 26), on mouse pancreatic adenocarcinoma cells growing in primary culture (Pan 03) and on human normal fibroblasts (N1).

We present here the results of these studies and report on the effects of these drugs on DNA, RNA and protein synthesis, on the cell cycle and on DNA integrity.

MATERIALS AND METHODS

Cell cultures

Five human colorectal adenocarcinoma cell lines (HCC-P 2998, HCC-M 1544, HCC-M 1410, HT 29 and LoVo); a murine adenocarcinoma cell line (Colon 26); cells in primary culture derived from a mouse pancreatic adenocarcinoma (Pan 03) and

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normal human fibroblasts (N1), were studied.

HCC-P 2998 was obtained from a primary colorectal carcinoma, and HCC-M 1410 and HCC-M 1544 from liver metastasis of two different patients with primary colorectal carcinoma. HCC-M1410 and HCC-M 1544 were established *in vitro* after growth of the original tumor s.c. in nude mice [4]. The HCC lines were maintained in Ham's F-12 medium (Flow Lab., Irvine, U.K.) supplemented with 10% fetal calf serum (FCS, Gibco Europe, Glasgow, U.K.), 5 μg/ml epidermal growth factor, 5 μg/ml insulin and 2 μg/ml transferrin (Sigma, St. Louis, U.S.A.).

The HT 29 colon adenocarcinoma cell line [5] was maintained in Eagle's MEM with Hanks' balanced salt solution with 2% MEM non essential amino acid solution, 1% MEM vitamin solution (Gibco) and 10% FCS; the LoVo line [6] was maintained in Ham's F-12 with 10% FCS.

The murine Colon 26 cell line was obtained in our laboratory by excising the Colon 26 tumor 18 days after s.c. implantation in female BALB/c mice (Charles River, Italy). A tissue/cell suspension was obtained by enzymatic digestion with 0.3% collagenase (Sigma Chemical Co. St Louis, MO) and seeded into tissue culture flasks. Culture medium used was RPMI 1640 (Gibco, Paisley, U.K.) plus 10% FCS. At the third passage cells were used.

The mouse pancreatic adenocarcinoma, Pan 03, was excised from female C57 B1/6 mice 28 days after s.c. implantation and processed as described for Colon 26 but colony forming cells were tested in primary culture.

Normal human fibroblasts N 1 (gift of Dr. L. Witte, University College of Physicians and Surgeons, New York, U.S.A.) were grown in Dulbecco's MEM (Gibco) supplemented with 10% FCS.

All cell types grew as monolayers in 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson and Co., Oxnard, CA) at 37°C in a humidified 5% CO_2 atmosphere.

Cells were harvested using 0.125% trypsin (Eurobio, Paris, France) in Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco), containing 0.5 mM EDTA. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added during treatment.

Drugs

LM 975 was kindly provided by Dr. P. Briet, Lipha, Lyon, France. The drug was dissolved in 1% $NaHCO_3$ and diluted in culture medium (1 : 20) immediately before each experiment.

Growth inhibition

Cells were plated at $1 \times 10^4/cm^2$ in 16-mm wells of a 24-well plate (Falcon). Seventy-two hours later, cells were treated with a freshly prepared solution of LM 975 for 2 or 24 h. At the end of treatment cultures were washed with prewarmed PBS and replenished with drug-free medium. After a 72 h recovery period, cells were harvested and the growth inhibition was evaluated by counting the cells in a Coulter Counter (Coulter Electronics Ltd, Harpenden, Herts, U.K.).

Data are the average of six replications for each experiment and are representative of at least two experiments.

Colony assay

Cells (1×10^3) were plated in each 60 × 15 mm tissue culture dish 24 h before treatment. After 24 h of treatment the dishes were washed with 5 ml of PBS and replenished with fresh drug-free medium. Eight to ten days after the initial plating, colonies were stained with 1% crystal violet in 20% ethanol. Colony growth inhibition was evaluated by counting colonies with more than 50 cells, using a dissecting microscope. Data were expressed as a percentage of controls.

Each experimental point was determined in quadruplicate. Experiments were repeated twice.

Inhibition of DNA, RNA and protein synthesis

Cells were seeded with 3×10^4 cells/cm^2 in 35-mm wells of a 6-multiwell tissue culture plate (Falcon) and incubated in a humidified 5% CO_2 atmosphere at 37°C for about 20 h. The medium was then replaced with either 2 ml of fresh medium (controls) or 2 ml of medium containing 1.4 mM LM 975 for 2 h. DNA, RNA and protein synthesis was determined by adding radiolabeled precursors methyl-[^{3}H]thymidine (sp. act. 70–90 Ci/mmol); 5-[^{3}H]uridine (sp. act. 25–30 Ci/mmol); L-4,5-[^{3}H]leucine (sp. act. 120–190 Ci/mmol), respectively, at three different times: between the 1st and 2nd hour of treatment, between the 7th and 8th hour of recovery in drug free medium and between the 23rd and 24th hour of recovery time. The final concentration of each radiolabeled precursor was 1 μCi/ml.

At the end of radioisotope incubation cells were washed once, harvested and the cell suspension (2 ml) was mixed with 2 ml of cold 10% (v/v) trichloroacetic acid. The precipitate was collected on a 2.5 cm diameter glass microfiber filter (Whatman, GF/C) and washed three times with 2 ml of cold 5% trichloroacetic acid and twice with 2 ml of ethanol.

After drying, the filters were transferred to scintillation vials with 10 ml of Lipoluma (Lumac) Soluene (Packard) solution (10 : 1) and radioactivity was determined by a LS 5800 β-counter (Beckman Instrument, Irvine California).

Each point represents the average of six replications.

Alkaline elution

The method of alkaline elution was recently reviewed in detail [7]. Cells were labeled for 24 h using medium supplemented with 0.2 μCi/ml [^{3}H]thymidine (sp. act. 20 Ci/mmol, Amersham) and 10^{-6} M unlabeled thymidine. A post-labeling 18–24 h chasing in medium without [^{3}H]thymidine was performed before drug treatment. Approximately 10^6 cells were resuspended in cold PBS and layered on polycarbonate filters, 0.8 μm pore size and 25 mm diameter (Nucleopore Corp., Pleasanton, CA). Cells were then lysed with a solution containing 2% sodium dodecyl sulfate–0.02 M Na EDTA–0.1 M glycine, pH 10.0 (lysis solution), which was allowed to flow through the filter by gravity.

After connecting the outlet of the filter holders to the pumping system, proteinase K, 2 ml of 0.5 mg/ml (EM Laboratories, Darmstadt, F.R.G.), dissolved in the lysis solution, were added to a reservoir over the polycarbonate filters and pumped for approximately 1 h at a rate of 0.35 ml/min.

DNA was eluted from the filters by pumping 0.02 M EDTA solution adjusted to pH 12.1 with tetrapropylammonium hydroxide (RSA Corp., Elmsford, NY) containing 0.1% sodium dodecyl sulfate through the filters at approx. 2 ml/h. Three-hour fractions were collected, and fractions and filters were processed as described previously [7].

Flow cytometry

For cell cycle studies, aliquots of cells were centrifuged and the pellet was resuspended with 1 ml of staining solution composed of the double-stranded nucleic acid probe propidium iodide (Calbiochem Behring Co., S. Diego, U.S.A.), 50 μg/ml in 0.1% sodium citrate. 15 μl of 1% nonidet P40 (Sigma Chemical Co., St. Louis, U.S.A.) and 15 μl of RNAse (Calbiochem) 0.5 mg/ml stock solution were added to the cell suspension to disrupt the cytoplasm completely and eliminate any disturbances due to double-stranded RNA. After at least 30 min incubation at room temperature, samples were analysed using an Ortho 30L Cytofluorograph (Ortho Instruments, Westwood, U.S.A.) and the DNA frequency histograms were recorded by a HP85 microcomputer fitted to the cytofluorograph.

RESULTS

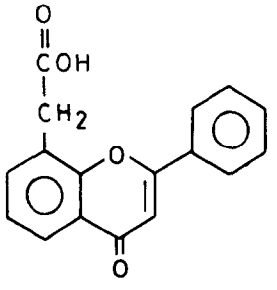
Studies of cytotoxicity

Table 1 shows the growth inhibitory effects of LM 975 in human colonic cell lines and on normal human fibroblasts observed after 2 or 24 h exposure to the drug and after 72 h of post-drug incubation at 37°C in drug-free medium. Two hours treatment only inhibited growth of the HCC-P 2998 cell line:

Table 1. *Antiproliferative effect of LM* 975 *on different cell lines. Data represent the number of surviving cells after* 2 *or* 24 h *of treatment and* 72 h *of post-treatment incubation in drug free medium, expressed as percentages of controls*

Cell lines	Treatment time (h)	0.5 mM LM 975 (%)	1.0 mM LM 975 (%)	1.4 mM LM 975 (%)
HT 29	2	110 ± 6	95 ± 9	90 ± 5
LoVo	2	—	128 ± 6	135 ± 15
HCC-P2998	2	92 ± 17	75 ± 21	42 ± 11**
Colon 26	2	—	—	99 ± 7
N 1	2	—	—	121 ± 8
HT 29	24	113 ± 16	81 ± 9	87 ± 11
HCC-M1410	24	89 ± 31	75 ± 14	65 ± 14
LoVo	24	93 ± 4	79 ± 3*	77 ± 4*
HCC-M1544	24	60 ± 11	51 ± 2*	47 ± 6*
HCC-P2998	24	70 ± 11	46 ± 10*	36 ± 8*
Colon 26	24	—	—	59 ± 2*
N 1	24	116 ± 7	89 ± 6	97 ± 6

*$P < 0.01$, Dunnet's test.



LM 975 (NSC-347512)

Fig. 1. *Structure of LM* 975 (*NSC* 347512).

1.0 mM (269 μg/ml) and 1.4 mM (403 μg/ml) LM 975 inhibited growth by 25 and 58%, respectively.

The growth of normal human fibroblasts (N 1) was not apparently affected by LM 975. After 24 h treatment and 72 h post-incubation time LoVo, HCC-M 1544 and HCC-P 2998 cells were already significantly sensitive at a concentration of 1.0 mM whereas HT 29, HCC-M 1410 were not even at 1.4 mM. HCC-P 2998, whose growth was already affected after only 2 h exposure, was the most sensitive of the cell lines investigated. Even after 24 h exposure, normal fibroblasts (N 1) were not sensitive to LM 975.

Subsequent studies were aimed at investigating whether LM 975 inhibited the clonogenicity of adenocarcinoma cells. Figure 2 illustrates the inhibition of colony formation of LM 975 on four human adenocarcinoma cell lines, previously tested in a growth inhibition assay. LoVo and HT 29 showed minimal sensitivity, their clonogenicity being inhibited only by approx. 20% after 24 h

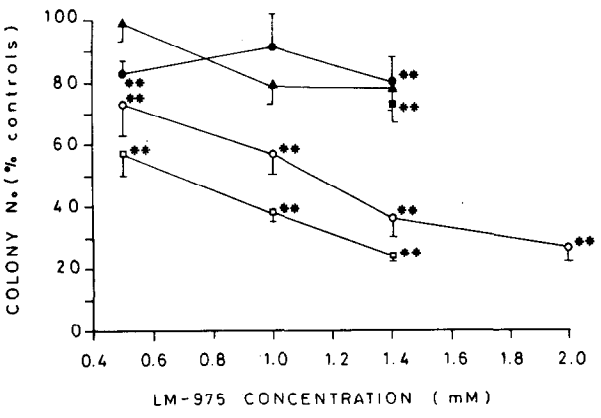


*Fig. 2. Inhibition of colony formation by LM 975. Four human adenocarcinoma cell lines and cells derived from murine pancreatic adenocarcinoma were plated 24 h before treatment as described in Materials and Methods. After 24 h of treatment with different doses, cells were washed with PBS and then maintained in drug-free medium for 8–10 days. Colonies were stained and counted as described in Materials and Methods. Each experimental point was determined in quadruplicate. Bars, S.E. **$P < 0.01$, Dunnett's test. ▲ LoVo; ● HT 29; ■ HCC-M 1544; ○ HCC-P 2998; □ Pan 03.*

exposure to 1.4 mM LM 975. HCC-M 1544 was only slightly more sensitive, whereas HCC-P 2998 colony numbers were already markedly reduced at a drug concentration of 0.5 mM, and progressively more at higher concentrations.

Since murine pancreatic adenocarcinoma Pan 03 is reported as extremely sensitive to LM 975 *in vivo* [2] and we have found that LM 975 at a dose of 220 mg/kg i.v. given on days 3, 7 and 11 after s.c. transplant cured six out of 10 Pan 03 bearing mice (unpublished data), it appeared of interest to investigate whether cells derived from Pan 03 showed susceptibility to LM 975 *in vitro* too.

We failed to obtain a continuous cell line from Pan 03 adenocarcinoma but did manage to set up optimal culture procedures to grow Pan 03 in primary culture and for a few passages. Using these cells growing at the second passage, we tested LM 975 in a colony assay. LM 975 cause very significant inhibition of Pan 03 colony formation (Fig. 2). At 1.4 mM the inhibition was approx. 75%.

Studies on DNA, RNA and protein synthesis

Figure 3 shows the effects of LM 975 on the incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine in DNA, RNA and protein. In Colon 26 (panel A) and HT 29 (panel B), which were only moderately sensitive to LM 975, between the 1st and 2nd hour of treatment there was 50 and 65% inhibition of incorporation of [^{3}H]dThd, 10 and 15% of [^{3}H]leucine and approx. 65% of [^{3}H]dUrd in both cell lines.

In the HCC-P 2998 cell line (panel D), which in the cytotoxicity studies was very sensitive to LM 975, neither [^{3}H]dThd nor [^{3}H]leucine incorporation was inhibited whereas [^{3}H]dUrd incorporation was reduced 58%.

In Pan 03 adenocarcinoma cells (panel C) LM 975 caused marked inhibition of [^{3}H]dUrd incorporation, corresponding to approx. 75%; it also reduced [^{3}H]leucine incorporation by 40% and [^{3}H]dThd by 28%. In all these adenocarcinoma cells the inhibition of macromolecule synthesis was rapidly reversed upon drug removal, being in most cases no longer observable at 8 h and totally undetectable by 24 h.

Evaluation of DNA damage

LM 975 1.4 mM for 2 h did not appear to cause DNA breakage in HT 29, HCC-P 2998, Colon 26 cell lines or in Pan 03 adenocarcinoma cells growing in primary culture as assessed by the method of alkaline elution. Figure 4 shows that elution profile of DNA of LM 975 treated cells overlapping that of control cells.

Flow cytometry studies

Flow cytometry studies on HCC-P 2998, HT 29 and Colon 26 cells did not show any marked cell cycle perturbation induced by LM 975. In HCC-P 2998, 2 h treatment with 1.4 mM, which significantly inhibited cell proliferation, only slightly raised the percentage of cells in early S phase. This mild effect was reversed in the next 24 h. In HT 29 exposed to 1.4 mM LM 975 for 24 h we saw a 15% increase in cells in G_1 but this was back to control values in 24 h of recovery. In Colon 26 cells no cell cycle perturbation was observed after 24 h exposure to 1.4 mM LM 975 (data not shown).

DISCUSSION

LM 975 is a new anticancer agent which has shown antitumoral activity against some slow-growing murine adenocarcinomas of the colon and pancreas. In the present study we found that LM 975 inhibited proliferation of some cell lines derived from human colon adenocarcinoma.

Its *in vitro* cytotoxic potency was relatively low, the concentration required to achieve a significant effect being around 1 mM, which is unusually high for an anticancer agent. However, this compound has low toxicity in mice to which doses up to 267 mg/kg i.v. [1, 8] can be given without causing any toxic deaths, and in man in whom the maximum tolerated doses were 6.4 g/m^2 (administered as a 1 h i.v. infusion) in a phase I clinical trial [9]. At this dose peak plasma level was 6.9 mM (1936 μg/ml) (Kaye SB, personal communication).

Therefore the concentrations found to be active in the present study against some human adenocarcinoma cell lines do appear realistically attainable in plasma of patients. This suggests a potential

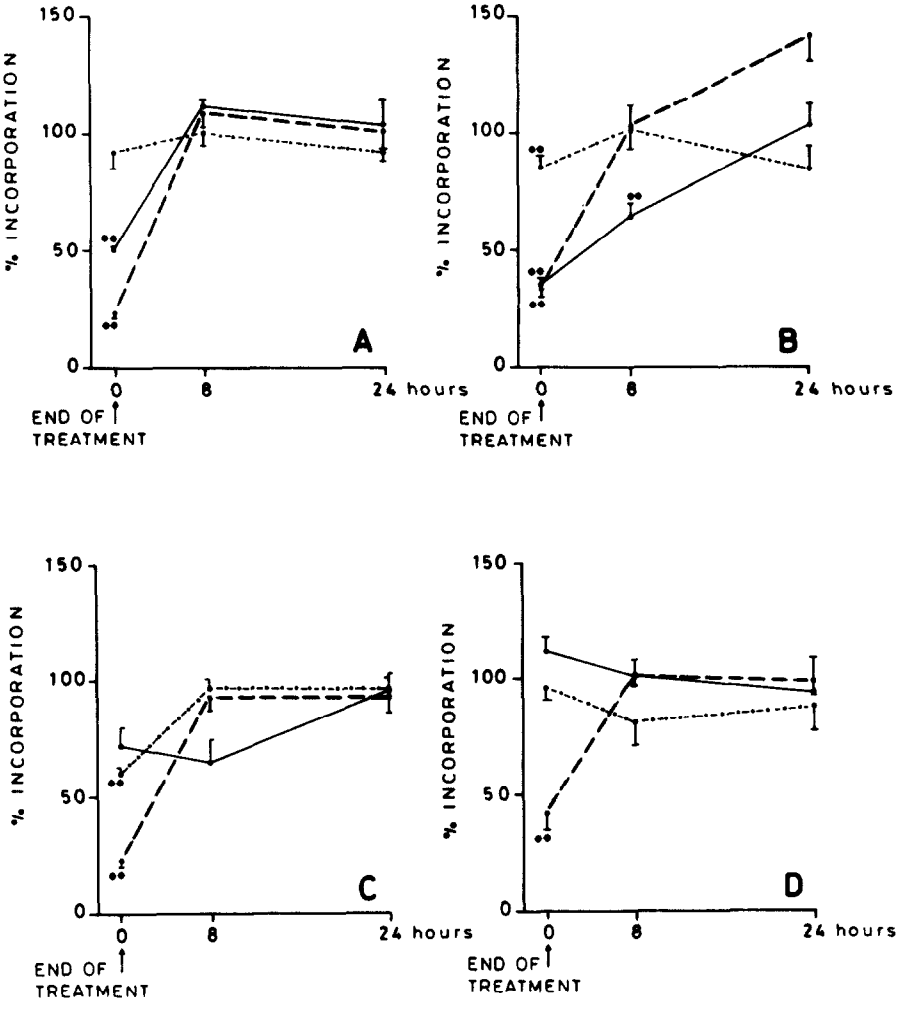


*Fig. 3. Inhibition of DNA, RNA and protein synthesis by LM 975 in Colon 26 (panel A); HT 29 (panel B); Pan 03 (panel C) and HCC-P 2998 (panel D). Cells were seeded and treated with 1.4 mM LM 975 for 2 h as described in Materials and Methods. DNA, RNA and protein synthesis was determined adding radiolabeled precursors methyl-[^{3}H]thymidine; 5-[^{3}H] uridine; L-4,5-[^{3}H] leucine, respectively, at three different times: between the 1st and 2nd hour of treatment, between the 7th and 8th hour of recovery time in drug-free medium and between the 23rd and 24th hour of recovery. The final concentration of each radiolabeled precursor was 1 μCi/ml. At the end of radioisotope incubation cells were washed once, harvested and the cell suspension was mixed with cold 10% (v/v) trichloroacetic acid. The precipitate was collected on a glass microfiber filter and processed as described in Materials and Methods for the determination of macromolecule-bound radioactivity. At time 0 (end of treatment) and 8 or 24 h recovery time each point represents the percentage of controls of six replications. Symbols: (——) methyl-[^{3}H]thymidine, (---) 5-[^{3}H]uridine; (-·-) L-4,5-[^{3}H]leucine. Bars, S.E. **$P < 0.01$, Dunnett's test.*

therapeutic efficacy of LM 975 against human colon cancer.

The cytotoxicity studies showed variable sensitivity among the five human adenocarcinoma cell lines investigated. HCC-P 2998, recently isolated from a biopsy of a primary human colorectal carcinoma, was susceptible even when exposed to 1.0 mM LM 975 for only 2 h. The least sensitive cell line, HT 29, was only slightly affected when 1.4 mM drug concentrations were maintained for 24 h. The identification of some cell lines partially sensitive to this drug is a first step towards studies aimed at elucidating the mode of action of LM 975. With this in mind, we chose a relatively short duration of treatment (2 h) to avoid unspecific effects due to cell damage induced by drug cytotoxicity. Indeed already between the 1st and 2nd hour of treatment DNA, RNA and protein synthesis was inhibited by LM 975. However, the synthesis of macromolecules was affected to a different extent and proportion in the different cell lines. In sensitive cell lines LM 975 caused stronger inhibition of RNA synthesis than DNA synthesis. For example, in HCC-P 2998, which was the most sensitive

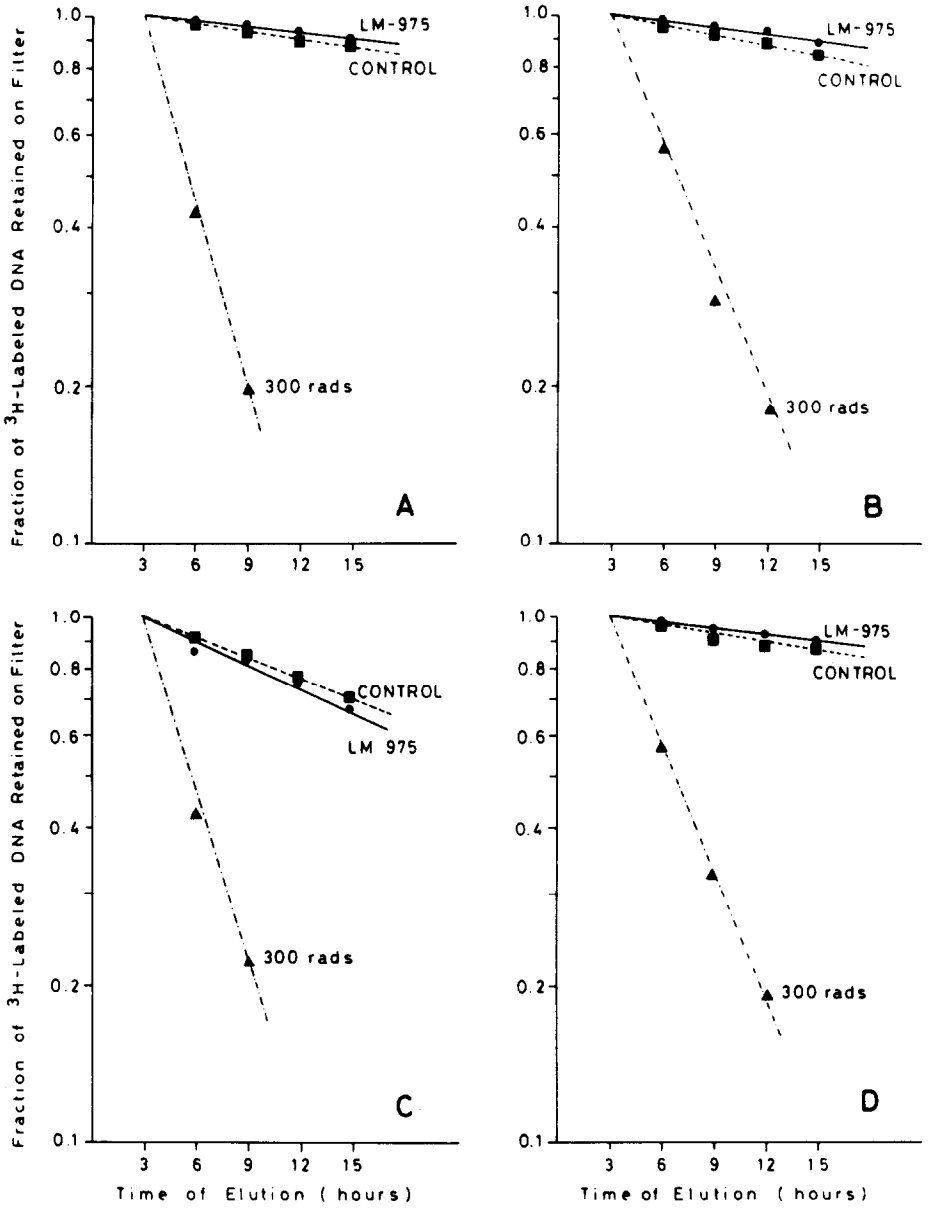


Fig. 4. Evaluation of DNA damage. Alkaline elution profiles of DNA from Colon 26 *(panel A); HT* 29 *(panel B); Pan* 03 *(panel C) and HCC-P* 2998 *(panel D). Cells were treated with* 1.4 mM *LM* 975 *for* 2 h. *Alkaline elution was carried out as described in Materials and Methods. Each point is the mean of two values. The dotted lines represent the elution of [^{3}H]DNA from untreated cells after irradiation with* 300 rads *in the cold.*

human cell among those investigated, RNA synthesis was cut by 58% whereas DNA synthesis was unaffected. The rapid reversion of inhibition of macromolecule synthesis upon drug removal may argue for an advantage of prolonged drug exposure if this inhibition is related to the drug's cytotoxic effects.

Again, using 2 h exposure, which significantly inhibited macromolecule synthesis, we investigated whether flavone acetic acid caused DNA damage and whether this effect was related to its antiproliferative effects. Using the alkaline elution method, which is sufficiently sensitive to detect even a small number of DNA breaks, we could find no changes in DNA integrity.

These data are apparently in conflict with those recently obtained by M. Bissery (personal communication). They found that LM 975 caused DNA breakage in cells taken from sensitive murine tumors treated *in vivo* with the drug. We believe that these *in vivo* findings should be evaluated with caution since it is difficult to know whether the observed DNA damage is the result rather than the cause of cell cytotoxicity.

The fact that DNA damage could not be seen in HCC-P 2998 exposed for 2 h, a duration of

treatment which in this cell line did inhibit cell growth, supports the idea that LM 975 does not act by causing DNA breakage. In addition, preliminary experiments indicate that LM 975 does not bind markedly to DNA as assessed by equilibrium dialysis.

To our knowledge all drugs which act by causing DNA damage produce an accumulation of cells in S phase and G_2 [10, 11]; the finding that LM 975 did not cause this cell cycle perturbation also argues against the hypothesis that the drug acts through DNA damage. A further argument against DNA damage being at the basis of LM 975 activity derives from observations that the drug does not cause bone marrow toxicity; this would be uncommon for a drug that acts by damaging DNA.

In conclusion the present study provides evidence that LM 975 has a moderate antiproliferative activity on some adenocarcinoma cells at concentrations which are pharmacologically achievable in plasma of patients receiving tolerable doses.

The mechanism of action of this drug should be further investigated but studies so far suggest that it does not act by producing DNA damage. A finding that requires confirmation is whether RNA synthesis inhibition by LM 975 is a general feature in all drug-sensitive cells and what is its molecular basis.

The cytotoxic effect of LM 975 is not very high. Even on Pan 03 cells derived from a mouse tumor which can be cured by LM 975 [2], less than a log cell kill was observed. The greater *in vivo* activity can be tentatively explained in two ways:

(i) LM 975 could be converted *in vivo* to more effective metabolites which are not formed when the drug is tested against cells growing in culture.

(ii) LM 975 could stimulate some immunological response which in turn may be partially responsible for the *in vivo* antitumoral activity.

We are inclined to exclude the former possibility since we could not identify any major LM 975 bioproduct in plasma, urine or tissues of mice treated with therapeutic doses of the drug or by incubating LM 975 with fortified mouse liver microsomes (Damia G *et al.*, manuscript in preparation). The second possibility which is under investigation in this laboratory has recently been suggested (Wiltrout RH, personal communication).

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