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In Vitro Chemosensitivity Testing of Flavone Acetic Acid (LM975; NSC 347512) and its Diethylaminoethyl Ester Derivative (LM985; NSC 293015)*

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Abstract—*The antitumor effect of flavone acetic acid, LM975, and its diethylaminoethyl ester derivative, LM985, was studied in four human malignant cell lines [WiDr, a colon carcinoma; LICR (LON) HN-3, a tongue carcinoma; MCF7, a breast carcinoma; K-562, a leukemia] using a colorimetric assay based on the reduction of dimethylthiazol-2-yl-diphenyltetrazolium. The cell lines were exposed continuously for 4–6 days to drug concentrations ranging between 0.1 and 500 μg/ml. For LM975, the concentrations inhibiting the growth of the various cell lines by 50% were 200 ± 10, 97 ± 7, 171 ± 16 and > 500 μg/ml for LICR (LON) HN-3, WiDr, MCF-7, and K-562, respectively. The corresponding concentrations for LM985 were 151 ± 3, 36 ± 4, 86 ± 3 and 140 ± 18 μg/ml, respectively. The difference between LM985 and LM975 was statistically significant for the WiDr and LICR (LON) HN-3 lines. We also evaluated the cytotoxic activity of the two agents on normal human marrow myeloid progenitor cells in a colony-forming assay. Continuous exposure to the drugs gave a dose-dependent inhibition. The concentrations inhibiting the growth by 50% were 76 ± 31 μg/ml for LM975 and 134 ± 41 μg/ml for LM985. One hour incubation with either compound had no toxic effect on the myeloid progenitor cells. In conclusion, LM975 and LM985 do not appear to have a specific cytotoxicity for tumor cells. Our results indicate that, in vitro, toxicity on bone marrow myeloid progenitor cells is concentration dependent. Considering the low plasma concentration found in man after i.v. administration of LM985, our observations correlate well with the absence of drug-induced myelosuppression in patients.*

INTRODUCTION

FLAVONE ACETIC ACID (LM975, NSC 347512) and its diethylaminoethyl ester derivative (LM985, NSC 293015) (Fig. 1) are two chromone derivatives whose antitumor activity was discovered by the National Cancer Institute Screening Program [1]. The mode of action of these compounds has yet to be clarified. However, their pattern of activity in experimental tumors suggests a unique mechanism of action. The two compounds have a limited activity against the murine P388 leukemia and the murine $CD8F_1$ mammary tumor, and are totally inactive against the L1210 leukemia the B16 melanoma and the lung LX-1 and mammary MX-1

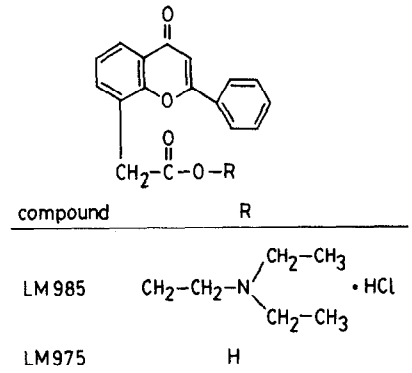

Fig. 1. Comparative structures of flavone acetic acid and its diethylaminoethyl ester derivative.

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human xenografts [1, 2]. However, their activity on the colon 38 tumor was of particular interest. Against this tumor, LM985 and LM975 were highly active [3]. At a dose of 200 mg/kg given intraperitoneally 1 day after subcutaneous implantation of the tumor, there was a 78% inhibition of tumor growth and a 213% increase in life span. With two injections 1 week apart, tumor growth was totally inhibited at a dose of 200 mg/kg and there were 10 long-term (20 days) survivors among 10 animals [2]. More recently, LM975 was shown to be active against a very broad spectrum of slow-growing tumors [4].

In clinical phase I trials, the dose-limiting toxicity of LM985 was sedation and hypotension [5, 6]. No other toxicities were encountered and it was noteworthy that the drug did not induce any hematologic toxicity. LM975 is presently under clinical investigation in European and American institutions [6]. Phase II studies with LM975 will be conducted in the near future.

Although not the primary purpose of a phase I clinical trial, no antitumor activity was detected in the above mentioned trials. There may be several explanations for the discrepancy between the outstanding activity of LM975 and LM985 in animal models and the lack of activity in the human studies performed so far. By design, phase I trials include patients with poor performance status, advanced disease and heavy pretreatment. Many of these patients lack evaluable lesions and most receive suboptimal dosages for relatively short periods of time. Pharmacological differences between animals and humans may exist too. When mice are treated at curative dosages, LM985 itself is undetectable in plasma, but LM975 is measured at concentrations of approx. 200 μg/ml. In man, LM985 is rapidly converted to LM975, but the plasma concentrations of the latter compound are almost ten-fold lower than the plasma concentrations of LM975 in the mouse [5–8].

The present work was designed to determine the concentrations of LM985 and LM975 that are required for an *in vitro* cytotoxic effect against human tumors. Additionally, the effect of these compounds on normal human bone marrow progenitor myeloid cells (CFU-GM) was studied.

MATERIAL AND METHODS

Drug supply

LM985 and LM975 were supplied as lyophilized powders by LIPHA (Lyon, France).

Chemosensitivity of human tumor cell lines

Human cell lines. Four human cell lines were used: the human colon WiDr [9], the head and neck LICR (LON) HN-3 [10], the human breast MCF7 [11] and the leukemic K-562 [12] cell lines. The WiDr cell line was developed from a primary adenocarcinoma of the recto-sigmoid colon; it has an epithelial morphology, produces a carcino-embryonic antigen, has been confirmed as human by immunofluorescence and isoenzyme analysis and is highly tumorigenic in nude mice [9]. LICR (LON) HN-3 was derived from a human squamous cell carcinoma of the tongue. The patient had been previously treated with chemotherapy and radiotherapy [10]. The MCF7 cell line was developed from a pleural effusion secondary to an adenocarcinoma of the breast. The patient had not received prior chemotherapy; the time has an epithelial morphology and has been confirmed of human origin by isoenzyme analysis [11]. K-562 was established from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis. He had been treated with chemotherapy. The cell line has been characterized as human multipotential hematopoietic malignant cells [12]. All lines were maintained as monolayers in Falcon plastic culture vessels, using Eagle's minimum essential medium (Gibco, Paisley, U.K.) for WiDr and MCF7, Dulbecco's modified Eagle medium (Flow Laboratories, Irvine, U.K.) for HN-3, and RPMI medium (Gibco) for K-562. Media included 1% penicillin and streptomycin and 1% L-glutamine (Gibco). The culture medium also contained 10% fetal calf serum (Gibco) for WiDr, MCF7 and HN-3, and 15% for K-562.

Drug exposure. Tumor cells were suspended in their respective culture media at a final concentration of 50,000 cells/ml for HN-3 and WiDr, 10,000 cells/ml for MCF7 and 25,000 cells/ml for K-562. Subsequently, 100 μl of cell suspension were plated and treated with 10 μl of LM975 or LM985 at final drug concentrations of 500, 250, 200, 100, 10, 1 and 0.1 μg/ml in 96-well flat-bottomed microtiter plates. The selection of these concentrations was based on the plasma drug levels observed in humans and animals treated with LM975 and LM985 [2, 5–8]. The plates were placed in a humidified 5% CO_2 atmosphere at 37° C for 4 days for HN-3 and 6 days for the three other cell lines. The optimal incubation times and cell concentrations were determined in preliminary experiments. Each experiment was done at least three times in triplicate.

Evaluation and colorimetric assay. Cell survival was measured according to a modification of the technique described by Mosmann [13]. Briefly, at the end of the incubation, 10 μl of 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium (MTT) Janssen, Beerse, Belgium) in phosphate-buffered saline was added to each well and incu-

bated for 4 h at 37°C. The reaction was stopped by adding 100 μl of isopropanol containing 0.04 N chlorhydric acid. The fluid was evaporated and 100 μl of dimethylsulfoxide were added to the wells to dissolve the formazan crystals. After a 30-min shaking, the plates were read on a Dynatech MR 600 Microelisa reader (Guernsey, U.K.) at a wavelength of 490 nm. After subtracting the background absorbance, the relative percentage of surviving cells was calculated by the ratio of absorbance after drug exposure to the absorbance of the controls (concurrently run cultures without drug).

Chemosensitivity of normal human bone marrow myeloid progenitor cells

Marrow samples. Ten to 20 ml bone marrow samples were taken from the sternum or posterior iliac crest in healthy volunteers after having obtained informed consent. The samples were anticoagulated with 10 U/ml heparin (Upjohn Company, Kalamazoo, Michigan). Mononuclear cells were separated from the whole marrow by Ficoll/Hypaque density centrifugation for 20 min at 800 ***g*** at room temperature. The cells at the interface were collected and washed twice in Dulbecco tissue culture medium and resuspended in the same medium enriched with 20% fetal calf serum (Gibco). The yield of this procedure was approximately 3×10^6 mononuclear cells per ml of bone marrow.

Drug testing. One-hour incubation. The drugs were dissolved in Iscove modified medium to obtain the appropriate concentrations (10, 100, 250, 500 μg/ml); 700,000 mononuclear marrow cells were incubated for 60 min at 37°C in a 1 ml final volume of culture medium containing 20% fetal calf serum and various drug concentrations in a controlled atmosphere containing 7.5% CO_2 and 100% H_2O. The incubation was terminated by the addition of a ten-fold excess of Iscove tissue culture medium at 4°C. Cells were then centrifuged twice at 800 ***g*** for 10 min. The cell pellets were resuspended in culture medium and cultured for 7 days as previously described [14, 15].

Continuous exposure One hundred thousand cells in 1-ml Iscove modified medium supplemented with 20% fetal calf serum plus the appropriate drug concentration were directly plated in agar and cultured for 7 days as previously described [14, 15].

Each experiment was done in triplicate.

Data analysis

Colonies (aggregates of more than 40 cells) and clusters (5–39 cells) were counted with an Olympus inverted microscope at 40-fold magnification. Colony counts of the three plates for a particular drug concentration and incubation time were averaged to obtain one data point. To determine cell sensitivity to a particular drug, the percentage of surviving colonies relative to the number of control colonies was plotted vs. drug concentration. The MLAB program [16] was used for semi-logarithmic linear regression analyses. The drug concentrations inhibiting growth by 50 and 90% were calculated for each individual experiment.

Table 1. *Cytotoxic effect of a continuous exposure of LM*975 *and LM*985 *on human tumor cell lines and human normal bone marrow myeloid progenitor cells**

Cell types	Drug	IC_{50}†	IC_{90}†
HN-3	LM975	200 ± 10	> 500
	LM985	151 ± 3	500 ± 56
WiDr	LM975	97 ± 5	344 ± 20
	LM985	36 ± 4	137 ± 10
MCF7	LM975	171 ± 16	> 500
	LM985	86 ± 3	264 ± 3
K-562	LM975	> 500	> 500
	LM985	140 ± 18	> 500
CFU-GM	LM975	76 ± 31	262 ± 69
	LM985	134 ± 41	418 ± 112

*Abbreviations: LM975: flavone acetic acid; LM985: diethylaminoethyl ester of flavone acetic acid; IC_{50}, IC_{90}: concentrations (μg/ml) inhibiting cell growth by 50% and 90% respectively.
†Mean ± S.E.M.

Statistical analyses

Statistical comparisons were done with Student's *t* test.

RESULTS

Cytotoxicity against tumor lines

Both LM985 and LM975 exhibited antitumor activity against the four lines (Table 1). A linear relationship was observed between the logarithm of cell survival and concentration for the two drugs. Generally, LM985 was more potent than LM975; a statistically significant difference was observed with HN-3 at 100 and 500 μg/ml ($P \leq 0.005$ at both concentrations) and with WiDr at concentrations ranging between 10 and 250 μg/ml ($P \leq 0.005$ for all comparisons).

Cytotoxicity against normal bone marrow myeloid progenitor cells

No significant inhibition occurred with the 1-h exposure (Fig. 2). With the continuous exposure, a dose-dependent inhibition was observed. A linear relationship was observed between the logarithm of cell survival and drug concentration. Although LM975 was slightly more potent than LM985, the difference did not achieve statistical significance.

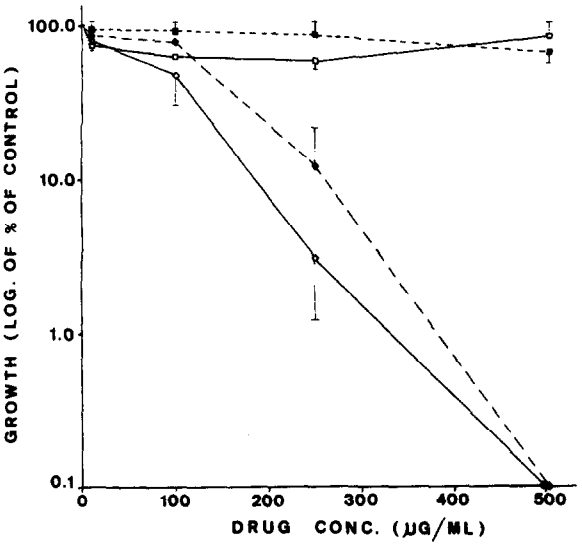

Fig. 2. Growth inhibition of normal human bone marrow myeloid progenitor cells by LM975 (——) and LM985 (---). Bone marrow cells from healthy volunteers were exposed to specified drug concentrations either for 1 h (□——□, ■---■) or continuously (◊——◊, ♦---♦). After a 1-week culture in soft agar, colonies were counted. Points represent the mean of four experiments except for the continuous exposure to LM985 where they represent the mean of three experiments; bars represent the S.E.M.

DISCUSSION

A single exponential pattern of CFU-GM and tumor lines growth inhibition was observed for both LM975 and LM985. A single exponential relationship between CFU-GM and drug concentration suggests good homogeneity in terms of drug sensitivity. With the GFU-GM, relatively important individual variations in *in vitro* sensitivity were found. These observations are similar to those reported with other agents [15].

Clinical phase I studies with LM985 showed that the drug does not induce myelosuppression [5, 6]. In the ongoing phase I studies with LM975, no myelosuppression has been observed so far [8]. *In vitro*, LM975 and LM985 did not inhibit significantly the CFU-GM growth after a 1-h exposure even at a very high concentration (500 µg/ml). Cytotoxicity was observed only with the continuous exposure. When LM985 is administered to humans, negligible plasma concentrations of LM985 are measured, whereas concentrations ranging between 10 and 50 µg/ml of LM975 are observed [5, 6, 8]. Phase I and pharmacokinetic studies with LM975 are still ongoing; however, at the currently administered dosage, plasma concentrations of LM975 are ranging between 150 and 600 µg/ml [7]. The disappearance of LM975 and LM985 from plasma is extremely rapid with terminal disposition half-lives of 2–5 h [2, 5–8]. Since *in vitro* cytotoxicity is achieved when the cells are exposed to relatively high concentrations ($\geq$ 250 µg/ml) for prolonged periods of time (7 days), our data are consistent with the absence of drug-induced myelosuppression in man. It will be interesting to see whether myelosuppression will be observed with continuous infusion of LM975. This schedule is presently under clinical investigation; based on our data, myelosuppression should be observed when plasma concentrations of 250 µg/ml are maintained for a sufficient period of time.

Both LM975 and LM985 exhibited cytotoxic activity against the four cell lines used in this study. The use of a single cell line per tumor type precludes any reliable determination of *in vitro* response rates in specific malignancies. This would require larger numbers of cell lines of each tumor type, similar to the number of patients required to perform phase II clinical studies [17].

In our experiments, LM985 was slightly more potent than LM975. Similar results were found by d'Incalci *et al.* [18]; they reported a 20–60% inhibition of several tumor cell lines after a 2-h exposure to LM975 and LM985 at a concentration of 600 µg/ml, with LM985 being more potent than LM975. The DNA, RNA and protein syntheses inhibition was also more pronounced with LM985. The comparison of the potency of LM985 and LM975 against malignant cell lines vs. myeloid progenitor cells indicate that LM985 has a better *in vitro* therapeutic index. This better *in vitro* therapeutic index cannot, however, be transposed *in vivo* since LM985 acts as a prodrug of LM975 [5, 6]. In addition, it has to be remembered that the methodology of chemosensitivity testing of the cell lines and the bone marrow cells was different and that any comparison of the results should be done with caution. The introduction of a culture medium supporting the growth of both malignant cells and normal myeloid stem cells is necessary to overcome this difficulty.

After intravenous administration of LM985 to patients, dose-limiting toxicity is observed at doses producing plasma concentrations of $\leq$ 2.5 µg/ml of the parent drug and $\leq$ 60 µg/ml of LM975 for short periods of time [5, 6]; since, at these concentrations, *in vitro* antitumor activity is not observed even after prolonged exposure, our data would predict that there is little hope for clinically useful antitumor activity with LM985. In contrast, *in vitro* antitumor activity was observed after a continuous exposure to LM975 at concentrations that, after clinical administration of this drug, are attainable in plasma, at least for periods of 1–6 h [7]. Ongoing phase I trials will determine whether such concentrations are tolerable for longer periods of time. On the other side, our laboratory is presently investigating whether the *in vitro* antitumor activity is maintained after shorter exposure. These additional studies should further clarify the potential role of LM975 as a clinically useful anticancer agent.

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