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ENHANCEMENT OF MACROPHAGE TUMOURICIDAL ACTIVITY BY THE ALKALOID DERIVATIVE UKRAIN. *IN VITRO* AND *IN VIVO* STUDIES.

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Summary: *Ukrain is a semisynthetic drug with immunomodulatory properties, derived from Chelidonium majus L. alkaloids and thiophosphoric acid. The effect of this compound on the growth of Balb/c syngenic mammary adenocarcinoma was assessed. Intravenous, but not subcutaneous or intraperitoneal, administration of this drug was found to be effective in delaying tumour growth in an actual therapeutic protocol initiated five days after tumour implantation. No untoward side-effects were observed using these* in vivo *treatment modalities. The role of macrophages in the observed retardation of tumour development was investigated using peritoneal exudate macrophages (PEM) in cytotoxicity assays. In previous studies, the authors have found that PEM of mammary tumour bearing mice lose their capacity to kill a variety of tumour target cells including the* in vitro *cultured homologous tumour cells (DA-3). Pretreatment of PEM from normal mice with 2.5 μM Ukrain for 24 h followed by stimulation with either IFN-γ or with LPS + IFN-γ enhanced their cytotoxic activity. Treatment of PEM from tumour bearing mice with 2.5 μM Ukrain and LPS results in a reversal of their defective cytotoxic response against the DA-3 target cells. Furthermore, Ukrain alone, in the absence of a secondary signal, induced the activation of tumouricidal function of PEM from tumour bearing but not from normal mice. These data indicate that Ukrain's* in vivo *effects against the development of mammary tumours may be due, at least in part, to its ability to restore macrophage cytolytic function.*

Introduction

The use of various biological response modifiers (BRMs) which enhance the host immune response, is an active area of investigation due to their possible clinical applications (1–3). The full therapeutic potential of the various existing BRMs has not been fully realized because of their undesirable side effects when used at concentrations required for maximum biological activity (4–6). Thus, the identification and characterization of new compounds that might increase the immunological activity without deleterious side effects, would enhance the prospects of immunotherapy as a practical and effective cancer treatment modality.

A new and promising area of research has evolved from biochemical phytotherapy, where plant alkaloids, as well as their semisynthetic derivatives (phytopharmaceuticals), have been found to be effective immunomodulators in animal models (7–9) and in cancer patients (10, 11). For example, the indolizidine alkaloid swainsonine has been reported to inhibit experimental lung metastasis of B16-F10 melanoma cells when administered systemically

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to C57BL/6 mice in a prophylactic setting. The inhibition of pulmonary metastasis was found to be mediated by the host's NK cells (7, 8). Moreover, swainsonine induced an increase in murine splenocytes and macrophage cytotoxicity and proliferation of bone marrow cells as well as HLA Class I expression on human breast carcinoma cells (9, 12).

Similarly, alkaloids from the plant *Chelidonium majus L.* conjugated to thiophosphoric acid yield a triaziridide compound denoted as Ukrain which, in preliminary clinical studies, has been found to have immunomodulatory activity (10, 11). The major immune parameters affected by this alkaloid derivative consist of an increase in the total T-lymphocyte number and a normalization of the T-helper/T-suppressor cell ratios, without affecting serum immunoglobulin levels, complement components and acute phase proteins (10).

The present studies were undertaken to investigate the possible mechanism by which Ukrain mediates its therapeutic effects (10, 11). For this purpose the authors chose the D1-DMBA-3 mammary tumour syngenic to Balb/c mice which in previous studies has been found to cause a profound impairment of various parameters of the immune system (13–20). *In vivo* studies utilizing this tumour system showed that intravenous administration of Ukrain is effective in reducing the rate of growth of established tumours. It is well known that macrophages (PEM) are important non-specific effector cells with antitumour activity (21, 22). The present *in vitro* results demonstrate that Ukrain is able to reverse the defective capacity of PEM from tumour bearing (TB) mice to lyse tumour targets upon LPS stimulation. Furthermore, the restored lytic activity is TNF-α independent, thus indicating that Ukrain activates an alternate lytic mechanism in macrophages from tumour bearing mice.

Materials and methods

Mice and tumours. BALB/c mice are maintained by brother-sister matings in the laboratory of the Department of Microbiology and Immunology, Miami. The tumour D1 DMBA-3 is a transplantable mammary adenocarcinoma derived from a non viral, non carcinogen-induced preneoplastic alveolar nodule in a BALB/c mouse after treatment with 7,12-dimethylbenzanthracene (23). D1-DMBA-3 is non metastatic and immunogenic to the host of origin. The tumour is routinely transplanted in BALB/c by s.c. injection of 1×10^6 tumour cells. The tumour becomes apparent five days after implantation; by day 30 necrotic areas are evident and the mice begin to die.

Biological response modifier. Ukrain is a semi-synthetic compound consisting of *Chelidonium majus L.* alkaloids conjugated to thiophosphoric acid. This compound is positively charged with m.w. of 1,470 and melting point of 222–223°C. The manufactured product, which complies with the good practice manufacture guidelines for pharmaceutical products, is available at 1.0 mg of Ukrain per 1.0 ml of H_20 (J.W. Nowicky Pharmaceuticals, Margaretenstrasse 7, 1040 Vienna, Austria). In *in vitro* studies the authors used the pertinent tissue culture medium as drug diluent, and PBS in *in vivo* studies.

In vivo *treatment with Ukrain.* Ukrain treatment was initiated five days after subcutaneous tumour implantation. Three routes of administration were employed, i.e., intravenous, intraperitoneal and subcutaneous. All three experimental groups, of at least five mice each, received 4.0 µg Ukrain in 0.1 ml of PBS. This dosage was chosen based on preliminary experiments. The treatment protocol was five consecutive injections at 24 h intervals, followed by 72 h without the drug. This regimen was repeated three times, i.e., three weeks. The mean tumour diameters were determined on days 7, 9, 12, 14, 16, 19, 21 and 23 after tumour implantation. The data, i.e., mean tumour diameters, were statistically analysed by a two-way repeated measures analysis of variance for differences between routes of drug (Ukrain) delivery at eight successive time points. The four averages (three experimental and one control)

at each time point were compared by Tukey's w-procedure at $p < 0.05$. The sample size of five animals for each of four groups re-measured eight times gave sufficient degrees of freedom (= 17 for between groups residual error) for a precise estimation of the between group residual mean square (SD^2) in ANOVA.

Reagents. The culture medium used in these assays was RPMI 1640 supplemented with 100 units of penicillin and 100 μg/ml of streptomycin, 5×10^5 M 2-ME, 2 mM L-glutamine, 1% non-essential amino acids, 1% essential amino acids, 1% sodium pyruvate (all from GIBCO Laboratories, Grand Island, NY), and 10% endotoxin-free fetal bovine serum (Hyclone Laboratories, Logan UT). LPS (*E. coli* 055;B5) from DIFCO Laboratories, Detroit, MI and r-murine INF-γ from Genzyme Corporation, Boston, MA were used as indicated.

Target cells. DA-3 cell line was derived from the *in vivo* D1-DMBA-3 mammary tumour syngenic to BALB/c mice. The DA-3 cell line produces tumours in BALB/c mice with the same growth kinetics and expresses the same tumour associated antigens on its surface as the parent tumour. The DA-3 cell line was maintained *in vitro* as cell suspension in RPMI 1640 FBS supplemented media.

Macrophage cultures. Normal mice and three- to four-week tumour bearers were injected i.p. with 1 ml of thioglycolate (DIFCO Laboratories, Detroit, MI). On day 4, the PEM were obtained by peritoneal lavage with 10 ml ice-cold RPMI 1640/mouse. Peritoneal cells were washed twice and re-suspended in supplemented RPMI 1640 media. The adherent population was obtained following the plastic-adherence technique described by Pennline (24). This procedure provides a population consisting of greater than 95% macrophages as determined by staining with Diff-Quick differential stain (Baxter, McGraw Park, IL) and nonspecific esterase staining (Sigma). Viability of cell was routinely >95% by trypan blue exclusion. PEM were seeded into 96-well, flat bottom microtitre plates (Costar, Cambridge, MA) at cell densities required for the particular assay.

Macrophage cytotoxicity assay. Cytotoxicity was determined by the release of ^{51}Cr from labelled tumour target cells. Briefly, purified PEM from normal mice and tumour bearers were first incubated in either medium alone or with different concentrations of LPS and/or IFN-γ. After 24 h, macrophage monolayers were washed twice with warm RPMI 1640, and 5×10^3 labelled target cells were seeded/well. Labelled tumour cells were prepared by incubating them with 0.1 mCi ^{51}Cr (New England Nuclear, Boston, MA) for 1 h, after which they were washed three times and resuspended in supplemented RPMI 1640 medium. The cultures were further incubated for 16 h and then 0.1 ml of culture medium was removed from each well, and the amount of radioactivity determined in an automatic gamma counter (Packard Auto-Gamma 500, Santa Clara, CA.). Percent specific lysis was calculated by the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

where experimental cpm = counts released in wells containing macrophages and tumour cells; spontaneous cpm = counts released by target cells cultured alone; maximum cpm = the amount of ^{51}Cr released by totally lysed target cells.

Results and discussion

Ukrain has previously been found to have therapeutic potential (10, 11); however, its mode of action in tumour bearing subjects if largely unknown. To understand the underlying mechanism by which this compound exerts its anti-tumour effect, *in vivo* and *in vitro* studies were performed using a well defined mouse mammary adenocarcinoma model system (23).

In vivo studies were performed to investigate whether Ukrain would have an effect on the development of the D1-DMBA-3 mammary tumour. Three different routes of drug delivery were tested in order

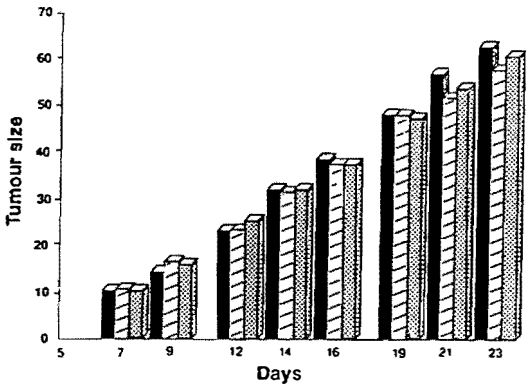

Fig.1 Effects of Ukrain (4.0 μg/mouse) administered subcutaneously (▨) or intraperitoneally (□), on the *in vivo* growth of DA-3 mammary adenocarcinoma tumours in Balb/c mice These routes of drug administration had no inhibitory effect on the tumour growth rate when compared to controls (■). At day 21 mice began to die due to tumour burden, hence the apparent differences in tumour size are not statistically significant.

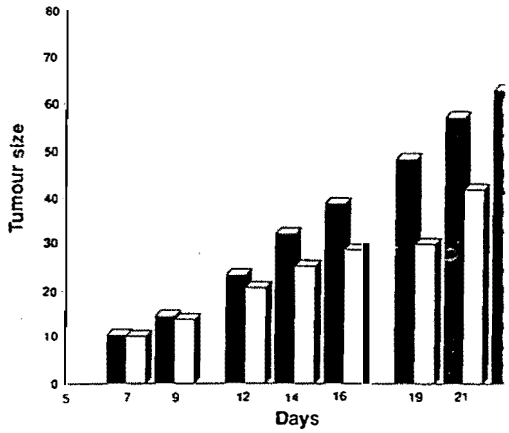

Fig.2 Effects of Ukrain (4.0 μg/mouse) administered i venously (□) on the *in vivo* growth of established DA-3 mam adenocarcinoma tumours in Balb/c mice. This route of druc ministration produced significant ($p < 0.05$) inhibition of tur growth at day 14 and thereafter as compared to control mice (■ mice in the experiment received five consecutive injections at intervals, followed by 72 h without drug. This regimen was repe three times. None of the treated mice, regardless of route of administration, showed any adverse effects.

to evaluate possible differences in therapeutic efficacy. As shown in Fig.1, neither subcutaneous nor intraperitonealinjections of Ukrain (4 μg/mouse) had a tumour growth inhibitory activity. In sharp contrast were the results obtained when Ukrain (4 μg < mouse) was given intravenously to tumour bearing mice. As shown in Fig. 2, significant ($p < 0.05$) cessation of tumour growth was first detected on the fifth day after the start of periodic chemotherapy, i.e., fourteen days after tumour inoculation. Tumour growth continued to progress in the control mice and in treated mice; however, the rate of growth was significantly diminished in the latter. It should be emphasized that in these studies an actual therapeutic protocol was used, i.e., the administration of the drug was not initiated until four days after tumour implantation. Furthermore, neither the experimental nor control mice receiving Ukrain showed any deleterious drug related side effects. In preliminary and ongoing studies using higher doses of Ukrain (8 μg/mouse) and following the same protocol described above, a striking therapeutic effect has been observed in that only one of five mice treated with this dosage de oped tumours at day 15, while all five control m had tumours and began to show signs of cache

Previous *in vitro* studies with Ukrain showed t this compound is capable of activating the l function of lymphocytes obtained from the sple of alloimmunized mice in a dose dependent man (25). At 1.2 μM this drug was found to increase several fold the cytolytic activity of the spl lymphocytes obtained from C57BL/6 (H-2^b) m immunized with mastocytoma P815 (H-2^d) cells i 3.5 h ^{51}Cr release assay at an E/T ratio of 5:1. enhanced lytic activity was dose dependent a remained specific for the cells used as immuno (25). Hence, the present studies were designe investigate whether Ukrain has a similar effect on macrophage population in normal mice and in mour bearing animals. Initial studies were perform using PEM from normal mice, which were trea

Table I *Effects of Ukrain on the capacity of PEM from normal mice to lyse tumour cells in response to LPS and/or INF-γ*

Pretreatment (24 h) Ukrain	% Specific Cytotoxicity			
	Activation (24 h)			
	None	LPS[1]	IFN-γ[2]	LPS + IFN-γ[3]
0	0	10.5 ± 1.8	2.9 ± 2.0	14.0 ± 4.1
25.0 μM	0	0	0	0
5.0 μM	5.0 ± 0.7	0	0	3.1 ± 0.9
2.5 μM	2.4 ± 1.4	12.0 ± 2.6	7.0 ± 2.6	20.1 ± 1.9

E/T ratio: 60:1
[1]LPS: 10 μg/ml
[2]INF-γ: 5.0 U/ml
[3]LPS 10 μg/ml + IFN 5.0 U/ml

Table II *Effects of Ukrain on the cytolytic activity of PEM from normal and tumour bearing mice*

Treatment	Normal PEM % lysis	PEM from tumour-bearing mice % lysis
None	0	0
Ukrain 2.5 μM	4.8 ± 1.7	13.0 ± 3.1
LPS 10 μg/ml	29.0 ± 2.0	12.0 ± 1.7
LPS 10 μg/ml - Ukrain	25.0 ± 3.7	24.0 ± 3.0
INF-γ	0	0
INF-γ + Ukrain	4.5 + 0.9	11.0 ± 0.8
LPS + IFN-γ	31.0 ± 3.0	37.0 ± 1.2
LPS + IFN-γ - Ukrain	39.0 ± 3.0	39.0 ± 2.0

with various concentrations of Ukrain for 24 h, followed by stimulation with LPS and/or INF-γ for an additional 24 h. As shown in Table I, Ukrain alone, when used at low concentrations, was able to trigger a nominal macrophage cytotoxicity against syngenic DA-3 mammary tumour cells. However, Ukrain at 25 μM and 5 μM did not prime PEM from normal animals to become more responsive to subsequent stimulation with either LPS, INF-γ, or combination thereof. In sharp contrast were the results obtained with Ukrain used at 2.5 μM. This dose was able to prime macrophages to display enhanced cytotoxic activities when they were further stimulated with either 5 U/ml of INF-γ (2.9 ± 2. *vs* 7.0 ± 2.6% killing) or a combination of LPS + INF-γ (14.0 ± 4.1 *vs* 20.1 ± 1.9% killing). Thus, 2.5 μM of the drug seemed to be the optimal concentration needed to achieve an enhancing effect on macrophage tumouricidal activity. Based on these results, this concentration of drug was used in subsequent experiments.

The progressive growth of the D1-DMBA-3 mammary adenocarcinoma in BALB/c mice results in a profound impairment of the capacity of PEM obtained from these animals to kill a variety of tumour target cells upon *in vitro* stimulation with LPS. Significantly, the syngenic DA-3 mammary tumour cells are not killed by macrophages from mammary tumour bearing animals (19). Thus, subsequent experiments were designed to investigate the conditions that might overcome the inability of these PEM from tumour bearing animals to kill tumour cells *in vitro*. As illustrated in Table II, incubation of PEM from normal and tumour bearers with 2.5 μM of Ukrain alone for 24 h resulted in tumouricidal activation of PEM from tumour bearing mice (13%), whereas PEM from normal mice did not display such significant activation (4.8%). Moreover, Ukrain added together to LPS did not alter the cytolytic capacity of normal mice PEM in response to LPS activation. In contrast, this alkaloid was able to overcome the defective response of PEM from tumour bearers to LPS stimulation (from 12% to 24%). In recent experiments the authors have found that this increase in lytic activity is associated with a significant decrease in TNF-α production, i.e., from 50 down to 5.0 U/ml (data not shown). These results suggest that immunostimulation by Ukrain, resulting in reversal of the depressed macrophage cytotoxicity in tumour bearers, involves a TNF-α independent mechanism of action.

Previous studies have shown that INF-γ used in combination with other agents can activate

Table III *Effects of Ukrain on the cytolytic activity of PEM from normal and* in vivo *LPS-treated mice*

In vitro treatment (24 h)	PEM from normal % lysis	PEM from LPS-treated mice % lysis
None	0	0
Ukrain 2.5 μM	4.8 ± 1.7	8.2 ± 1.1
LPS 10 μg/ml	29.0 ± 1.0	0
LPS + Ukrain < 2.5 μM	25.0 ± 3.7	23.5 ± 2.3
INF-γ 5 U/ml	0	0
INF-γ + Ukrain 2.5 μM	4.5 ± 0.9	16.2 ± 2.3

macrophage tumouricidal activity (26). As shown in Table II, incubation of PEM from normal and tumour bearers with 5 U/ml of INF-γ alone, did not result in triggering of macrophage cytotoxicity against DA-3 targets. Moreover, no enhancement (over that induced by Ukrain alone) was noted when this cytokine was used in combination with Ukrain, either in PEM from normal or in PEM from tumour bearing mice. In addition, Ukrain added to the combination of LPS and INF-γ did not result in a significant further enhancement of macrophage tumouricidal activities over those obtained with LPS + IFN-γ alone. These results suggest that Ukrain is exerting its immune enhancing effect at the level of LPS-inducible mechanism of macrophage cytotoxicity.

To evaluate further whether this drug can also reverse the depressed macrophage cytotoxicity found in other experimental conditions, the authors chose the *in vivo* LPS desensitization model for study. LPS desensitization is a well known phenomenon that occurs when normal mice and/or macrophages are treated either *in vivo* or *in vitro* with a small dose of LPS followed by a second challenge with the same agent at higher doses (27). This treatment leads to inactivation of macrophage lytic function and cytokine production. As shown in Table III, PEM from *in vivo* LPS-treated mice (10 μg/mouse 24 h before PEM harvest) could not be activated to kill tumour cells in response to a second *in vitro* challenge with LPS, as compared to PEM from untreated mice which only received the activating agent *in vitro* (0% *vs* 29.0% killing). However, when Ukrain was added together with LPS *in vitro*, this treatment resulted in a reversal of the suppressed cytotoxicity of PEM from LPS-treated mice, thus overcoming the *in vivo* LPS-induced desensitization. Moreover, this drug when used alone, was able to induce higher levels of macrophage cytotoxicity in PEM from LPS-treated mice than in PEM from control untreated mice when it was used alone (8.2 *vs* 4.8% killing) or when used in combination with INF-γ (16.2% *vs* 4.5% killing).

The data available from previous studies (10, 11, 25) taken in conjunction with the results presented herein indicate that Ukrain functions as an up-regulator of the immune system and as an effective antitumour drug when administered i.v. to tumour bearing mice. Not only are the various effector cells functionally enhanced by Ukrain, but in preliminary studies the authors have found a decrease in the *in vitro* TNF-α production by macrophages treated with this drug. Since the authors have observed that the tumour bearing mice die after development of cachexia, it is tempting to speculate that an additional possible benefit of Ukrain treatment could be the amelioration of the cachectic state.

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