DRUGS EXPTL. CLIN. RES. XXII(Suppl.) 31-41 (1996)

# MODULATION OF IMMUNE EFFECTOR CELL CYTOLYTIC ACTIVITY AND TUMOUR GROWTH INHIBITION *IN VIVO* BY UKRAIN (NSC 631570)

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**Summary**: *Ukrain is a semisynthetic compound consisting of alkaloids from* Chelidonium majus L. *conjugated to thiophosphoric acid, with immunomodulatory and therapeutic properties in cancer patients. The present* in vitro *studies demonstrate that Ukrain is an effective biological response modifier augmenting, by up to 48-fold, the lytic activity of splenic lymphocytes obtained from alloimmunized mice. The lytic activities of interleukin-2 (IL-2) treated spleen cells and peritoneal exudate lymphocytes were also significantly increased by the addition of Ukrain to the cell mediated lysis (CML) assay medium. The highest Ukrain-induced enhancement of splenic lymphocytolytic activity* in vitro *was found to occur at day 18 after alloimmunization, was dose-dependent and specific for the immunizing P815 tumour cells. Since Ukrain was present only during the CML assays, its mode of action is thought to be via direct activation of the effector cells' lytic mechanism(s). The effect of Ukrain on the growth of Balb/c syngenic mammary adenocarcinoma was also evaluated. 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 deleterious 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. Previous studies showed 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 lipopolysaccharide (LPS) plus 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 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.*

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0378-6501/96/0000031 + 11 $2.50/0

## Introduction

The discovery and development of immune response-modifying agents, which could be effective in a clinical setting for the treatment of cancer, have become promising and important goals in immunotherapy. The use of various biological response modifiers (BRMs) which enhance the host immune response with or without *in vitro* activation of immune effector cells, is an active area of investigation (1, 2). The full therapeutic potential of the various BRMs has not been realized due to their undesirable side-effects when used at the concentrations required for maximum biological activity (3-5). Thus, the identification and characterization of new compounds without deleterious side effects would enhance the prospects of immunotherapy as a practical and effective cancer treatment.

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 (6-8) and in cancer patients (9). For example, the indolizidine alkaloid swainsonine has been reported to inhibit experimental lung metastasis of B16-F10 melanoma cells when administered systemically to C57BL/6 mice in a prophylactic setting. The inhibition of pulmonary metastasis was found to be mediated by the host's NK cells. Moreover, swainsonine induced an increase in murine splenocytes and macrophage cytotoxicity, proliferation of bone marrow cells and HLA class I expression on human breast carcinoma cells (7, 8).

Similarly, alkaloids from the plant *Chelidonium majus L.* (10) conjugated to thiophosphoric acid, yield a triaziridide compound known as Ukrain which, in preliminary clinical studies, has been found to have immunomodulatory activity (9, 11). The major immune parameters affected by this alkaloid derivative consist of an increase in total T-lymphocyte number and a normalization of T-helper/T-suppressor cell ratios, without affecting serum immunoglobulin levels, complement components or acute phase proteins (9).

Studies were undertaken to investigate *in vitro* the possible mechanism by which the alkaloid derivative Ukrain might mediate its therapeutic effects in cancer patients (9). The results of the present *in vitro* studies demonstrate that Ukrain significantly increased the lytic activity of splenic lymphocytes obtained from alloimmunized mice. The enhanced lytic activity was dose-dependent and directed against the immunizing H-$2^d$ P815 mastocytoma tumour cells. Furthermore, the time-course after alloimmunization at which maximal *in vitro* enhancement of splenic lymphocytolytic activity could be obtained was found to occur at 18 days, at which time a 48-fold increase of lytic activity could be demonstrated by the addition of Ukrain to the CML assay medium. Lesser but nevertheless significant Ukrain-induced increase in splenic lymphocytolytic activity could also be demonstrated at 6, 12, 26 and 34 days after *in vivo* alloimmunization. The lytic activity of peritoneal exudate lymphocytes could also be augmented by the addition of Ukrain to the CML assay medium. However, in all instances, splenic lymphocytes which had no significant endogenous lytic activity displayed the greatest increase by the addition of Ukrain to the CML assay medium.

*In vivo* and *in vitro* studies were further undertaken to investigate the possible mechanism by which Ukrain mediates its therapeutic effects. For this purpose the authors chose the D1-DMBA-3 mammary tumour syngenic to Balb/c mice which in previous studies had been found to cause a profound impairment of various parameters of the immune system (12). *In vivo* studies using this tumour system showed that intravenous (i.v.) 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 (13). The present *in vitro* results demonstrate that Ukrain is able to reverse the defective capacity of PEM from tumour-bearing mice to lyse tumour targets upon LPS stimulation. Furthermore, the restored lytic activity is TNF-$\alpha$ independent, thus indicating that Ukrain activates an alternative lytic mechanism in macrophages from tumour-bearing mice.

## Methods

*Tumour cells*: Mastocytoma P815 and the AKR leukaemia AKIL cell lines (ATCC, Rockville, MD) were maintained in DMEM medium supplemented with 8.0% bovine fetal calf serum (Gibco Labs, Grand Island, NY) containing penicillin and streptomycin, 100 units/ml and 100 μg/ml, respectively. Both cell lines were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$, 95% air.

*Effector cells*: C57BL/6 mice (H-$2^b$) were immunized with a single intraperitoneal (i.p.) injection of 1 x $10^6$ P815 mastocytoma (H-$2^d$) cells. At various times after immunization spleen and peritoneal exudate cells were harvested and used as effector cells. Peritoneal lymphocytes were obtained by peritoneal lavages with 5.0 ml DMEM medium containing 10 units/ml heparin. Peritoneal exudate cells were washed in DMEM medium to remove heparin, transferred to 75 $cm^2$ tissue culture flasks and incubated at 37°C for 2 h to remove adherent cells. Splenic lymphocytes were obtained by mincing the spleens with a scapel followed by pipetting the fragments against the bottom of a Petri dish to disrupt follicles. Red blood cells were lysed by 0.1 M tris-ammonium chloride followed by three washings in DMEM 8% FCS medium. Plastic adherent spleen cells were removed by the same procedure used for peritoneal exudate cells, as described above. Viability of both peritoneal exudate and non-adherent spleen cells was determined by trypan blue exclusion before using the cells as effectors in cell-mediated lysis assays. Both peritoneal exudate and spleen lymphocytes were used as effector cells without secondary *in vitro* stimulation.

*Cell mediated lysis (CML) assays*: Mastocytoma P815 and the AKIL and EL-4 leukaemia cells were labelled with 200 μCi of $Na_2\,^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1 h at 37°C, washed in DMEM-FCS and used as targets in CML assays. CML assays were carried out in 96-well v-bottom plates containing 1 x $10^4$ $^{51}$Cr-labelled target tumour cells and 5 x $10^4$ effector cells per well for an E:T ratio of 5:1. Multiwell plates were centrifuged at 1,000 rpm/5 min and incubated at 37°C for 3.5 h in humidified 95% air, 5% $CO_2$ atmosphere. After this incubation period the specific $^{51}$Cr release was determined by harvesting 150 μl of supernatant per well. Radioactivity counts were measured in a Packard gamma-counter. The percentage of specific $^{51}$Cr release was calculated according to the following formula:

$$\% \text{ specific } ^{51}\text{Cr released} = \frac{(\text{cells + Ukrain}) - \text{spontaneous release} \times 100}{(\text{maximum 51 release})_r - \text{spontaneous release}}$$

*Effects of Ukrain on effector cell mediated lysis of tumour cells*: The possible modulation of effector cell lytic activity by the alkaloid thiophosphoric acid derivative Ukrain was investigated *in vitro* in CML assays. The molecular weight of this alkaloid thiophosphoric acid conjugate, i.e., Ukrain, is 1,470 and is freely soluble in water or tissue culture medium. Serial dilutions of Ukrain were prepared in PBS buffer to yield final concentrations ranging from 37.8 μM down to 0.59 μM after the addition of target and effector cells suspended in DMEM-FCS culture medium. The effects of Ukrain on the spontaneous $^{51}$Cr release by the target tumour cells were also determined and found to be not higher than that of control cells. The percentage of specific $^{51}$Cr release was calculated as described in the preceding section.

*Mice and tumours*: BALB/c mice are maintained by brother-sister matings in the laboratory of the Department of Microbiology and Immunology, University of Miami. The tumour D1-DMBA-3 is a transplantable mammary adenocarcinoma derived from a nonviral, noncarcinogen-induced preneoplastic alveolar nodule in a BALB/c mouse after treatment with 7,12-dimethyl benzanthracene. D1-DMBA-3 is nonmetastatic and immunogenic to the original host. The tumour is routinely transplanted in BALB/c by s.c. injection of 1 x $10^6$ tumours cells. The tumour becomes apparent 5 days after implantation; by day 30 necrotic areas are evident and the mice begin to die.

In vivo *treatment with Ukrain of tumour-bearing*

*mice*: Ukrain treatment was initiated 5 days after subcutaneous (s.c.) tumour implantation. Three routes of administration were employed: i.v., i.p. and s.c. All three experimental groups, of at least 5 mice each, received 4.0 μg of Ukrain in 0.1 ml PBS. This dose was chosen based on preliminary experiments. The treatment protocol was of 5 consecutive injections at 24 h intervals followed by 72 h without drug. This regimen was repeated three times, i.e., for 3 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.

*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 3- to 4-week tumour-bearers were injected i.p. with 1 ml 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 resuspended in supplemented RPMI 1640 media. The adherent population was obtained following the plastic-adherence technique. 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 cells 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 x $10^3$ labelled target cells were seeded per well. Labelled tumour cells were prepared by incubation 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:

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

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

*Dose-dependent activation of spleen and peritoneal exudate lymphocytolytic activity by Ukrain.* Preliminary experiments were carried out to determine the concentration range within which the alkaloid derivate Ukrain had an effect on the lytic activity of lymphoid effector cells without affecting the spontaneous $^{51}Cr$ release of tumour target

cells. The concentration range of Ukrain found to fulfil these criteria was 0.6 to 38.0 μM.

When freshly isolated splenic lymphocytes from allommunized C57B1/6 mice were used as effector cells without secondary *in vitro* activation, they were found to have no significant endogenous lytic activity. However, the addition of Ukrain to the CML assay medium produced a significant and dose-dependent activation of spleen cell lytic activity (Fig. 1). In the presence of 1.18 μM Ukrain, the lytic activity of spleen lymphocytes increased from 1.35% to 65.0% specific lysis, i.e., a 48-fold increase. Similarly, peritoneal exudate lymphocytes (PEL) harvested from the same animals had an endogenous lytic activity of 7.0%, which in the presence of 2.36 or 4.72 μM Ukrain was increased to 25% specific lysis (Fig. 1). It should be noted that the Ukrain-induced enhancement of lytic activity remained target-specific in that only mastocytoma P815 ($H\text{-}2^d$) were lysed, whereas the non-specific AKR ($H\text{-}2^k$) and EL-4 ($H\text{-}2^d$) leukaemia cells were not, by either effector cell source. All CML assays were carried out simultaneously in triplicate for 3.5 h at the low effector to target cell ratio of 5:1.

*Time course after alloimmunization and Ukrain-inducible activation of spleen cell lytic activity.* Having determined the optimal concentration of Ukrain which induced maximal activation of primed spleen cells lytic activity, i.e., 1.18 μM (Fig. 1), we investigated its effects on spleen cells harvested at various time-intervals after alloimmunization. For this purpose, spleen cells were obtained from C57B1/6 mice at 6, 12, 18, 26 and 34 days after alloimmunization with P815 mastocytoma cells and assayed for lytic activity in the presence and absence of 1.18 μM of Ukrain in the CML assay medium. As illustrated in Fig. 2, Ukrain-induced activation of spleen cell lytic activity could be detected as early as 6 days after alloimmunization, followed by a further increase at day 12, and with the maximal enhancement of lytic activity occurring at day 18. After day 18, the Ukrain-dependent activation of spleen cell lytic activity declined in a time-dependent manner. The Ukrain-induced activation of lytic activity of spleen cells was statistically significant ($p < 0.05$) with respect to controls, at all time intervals after alloimmunization.

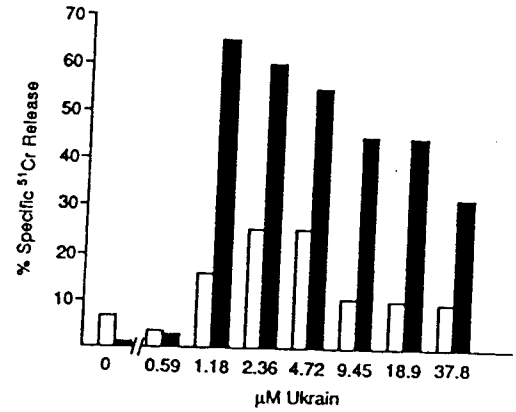


**Fig. 1** Effects of the alkaloid derivative Ukrain on the cytolytic activity of spleen and peritoneal lymphocytes from alloimmunized mice. The lytic activity of both effector cell populations was increased in a dose-dependent manner by the presence of Ukrain in the cell mediated lysis (CML) assay medium. Spleen lymphocytolytic activity was increased from 1.35 ± 1.0%, in the absence of the drug, to 65.0 ± 2.5% in the presence of 1.18 μM of Ukraine, i.e., a 48-fold increase in specific lysis. The peritoneal lymphocytolytic activity was increased from 7.0 ± 2% in the absence of the drug, to 25.0 ± 2.3% in the presence of 2.36 or 4.72 μM of Ukrain, i.e., a 3.75-fold increase in specific lysis (CML assays: E:T = 5:1; 3.5 h).

*Effect of Ukrain on the lytic activity of* in vivo *primed spleen cells followed by* in vitro *culture with or without rIL-2.* To investigate whether Ukrain could enhance the lytic activity of IL-2 treated cells, spleen cells harvested 14 days after *in vivo* alloimmunization were cultured *in vitro* for four days in the presence and absence of 10 units/ml of rIL-2. The lytic activity of spleen cells cultured *in vitro* without IL-2 was 16.3%, whereas that of cells cultured in the presence of IL-2 was 25.0% specific lysis (Fig. 3). The addition of Ukrain to the CML assay medium increased the lytic activity of both effector cell populations in a dose-dependent manner. It is of interest that Ukrain, at the low concentration of 0.59 μM,

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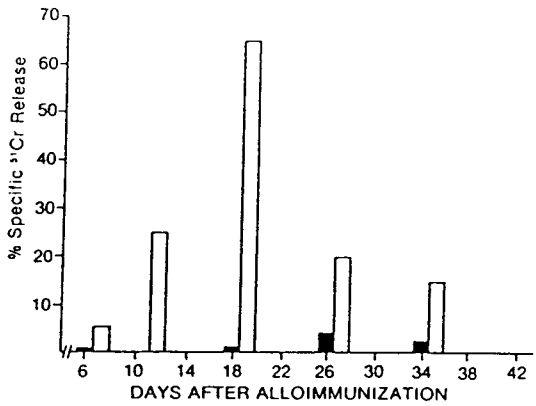


**Fig. 2** Effects of 1.8 μM Ukrain on the cytolytic activity of spleen cells harvested at various time intervals after alloimmunization. Spleen cells were harvested at days 6, 12, 18, 26, 34, and assayed for cytolytic activity in the absence and presence of 1.18 μM Ukrain in the cell mediated lysis (CML) assay medium. The highest Ukrain-induced increase of spleen cell lytic activity was found to occur at day 18 after alloimmunization. The Ukrain-induced increase in spleen cell lytic activity was significant ($p < 0.05$) at all post-alloimmunization times investigated (CML assays: E:T = 1:3; 5 h).

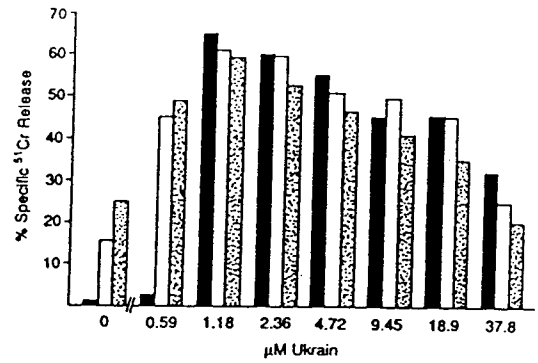


**Fig. 3** Effects of Ukrain on *in vivo* primed (14 days) spleen cells followed by 4 days *in vitro* culture, with and without 10 units/ml rIL-2. The lytic activity of spleen cells cultured *in vitro* without rIL-2 was 16.3 ± 1.5%, which in the presence of 1.8 μM of Ukrain was further increased to 61.2 ± 2.0%, i.e., a 3.75-fold increase in specific lysis. The lytic activity of spleen cells cultured *in vitro* with 10 units/ml or rIL-2 (replenished after 48 h) was 25.0 ± 2%, which in the presence of 1.8 μM of Ukrain was increased to 59.0 ± 1.9%, i.e., 2.36-fold increase in specific lysis (CML assays: E:T = 5:1,3.5 h).

induced a significant increase in lytic activity of these effector cells. A further increase in this activity occurred at 1.18 and 2.36 μM of Ukrain, followed by a gradual decline at higher concentrations (Fig. 3). It is worth noting that the Ukrain-induced increase in lytic activity of both effector cell populations reached its maximum at 1.18 μM, i.e., 61.2 and 59.0%, respectively. This increase in lytic activity represents 3.7- and 2.4-fold increases for spleen cells cultured in DMEM and in DMEM + 10 units/ml of IL-2, respectively. Thus, the *in vitro* culture of *in vivo* primed spleen cells produced an increase in their lytic activity which was further enhanced by the presence of Ukrain in the CML assay medium (Fig. 3).

*Effects of Ukrain on* in vivo *growth of syngenic tumours.* To understand the underlying mechanism by which this compound exerts its anti-tumour effect, we performed *in vivo* and *in vitro* studies using a well defined mouse mammary adenocarcinoma model system.

*In vivo* studies were performed to investigate whether Ukrain would have an effect on the development of the D1-DMBA-3 mammary tumour. We tested three different routes of drug delivery in order to evaluate possible differences in therapeutic efficacy. As shown in Fig. 4, neither s.c. nor i.p. injections 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 i.v. to tumour-bearing mice. As shown in Fig. 5, 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 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

have shown 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 drug dose has developed a tumour at 15 days, while all five control mice have tumours and begin to show signs of cachexia.

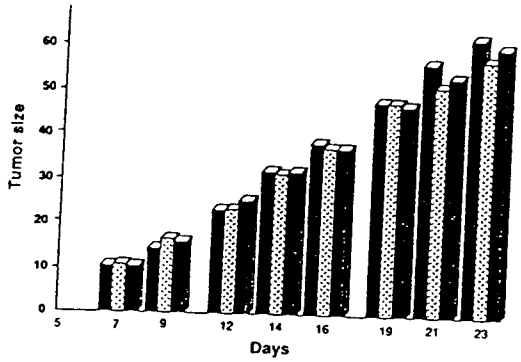


Fig. 4 Effects of Ukrain (4.0 µg/mouse) administered subcutaneously or intraperitoneally on the *in vivo* growth of DA-3 mammary adenocarcinoma tumours in Balb/c mice. These routes of drug administration had no inhibitory effect on the tumour growth rate when compared to controls. At day 21 mice began to die due to tumour burden, hence the apparent differences in tumour size are not statistically significant.

In vitro *studies designed to investigate whether Ukrain has a similar effect on the macrophage population in normal mice and in tumour-bearing animals.* Initial studies were performed using PEM from normal mice, which were treated 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

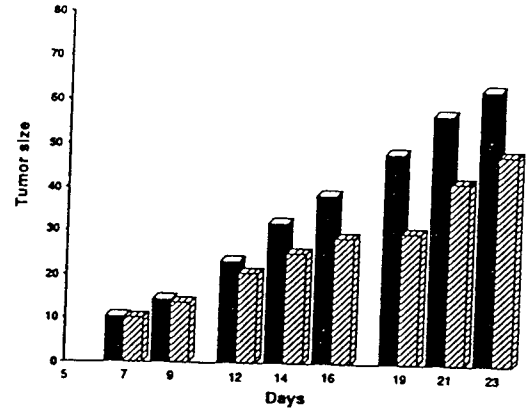


Fig. 5 Effects of Ukrain (4 µg/mouse) administered intravenously on the *in vivo* growth of established DA-3 mammary adenocarcinoma tumours in Balb/c mice. This route of drug administration produced significant ($p < 0.05$) inhibition of tumour growth at day 14 and thereafter, as compared to control mice. All mice in the experiment received five consecutive injections at 24 h intervals, followed by 72 h without drug. This regimen was repeated three times. None of the treated mice, regardless of route of drug administration, showed any adverse effects.

enhanced cytotoxic activities when further stimulated with either 5 U/ml of INF-γ (2.9 ± 2.0 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 on *in vitro* stimulation with LPS. Significantly, the syngenic DA-3 mammary tumour cells are not killed by macrophages from mammary tumour-bearing animals (14). Thus, subsequent experiments were designed to investigate the conditions that may 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

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

| Pretreatment (24 h) Ukrain | Percent Specific Cytotoxicity | | | |
|---|---|---|---|---|
| | Activation (24h) | | | |
| | None | LPS | IFN-γ[2] | LPS ± INF-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 + INF 5.0 U/ml

tumour-bearers with 2.5 μM 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 combined with 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 we 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 macrophage tumouridical activity (12). As shown in Table II, incubation of PEM from normal and tumour-bearers with 5 U/ml of IFN-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 the LPS-inducible mechanism of macrophage cytotoxicity.

**Table II** *Effects of Ukrain on the cytolytic activity of peritoneal exudate macrohages (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 + INF-γ | 31.0 ± 3.0 | 37.0 ± 1.2 |
| LPS + IFN-γ + Ukrain | 39.0 ± 3.0 | 39.0 ± 2.0 |

To evaluate further whether this drug can also reverse the depressed macrophage cytotoxicity found in other experimental conditions, the *in vivo* LPS desensitization model was used. 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 a higher dose (15). This treatment leads to inactivation of macrophageolytic 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 cells in response to a second *in vitro* challenge with LPS, as compared to PEM from untreated mice which received only 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 (8.2% vs 4.8% killing) or in combination with INF-γ (16.2% vs 4.5% killing).

**Table III** *Effects of Ukrain on the cytolytic activity of peritoneal exudate macrophages (PEM) from normal and* in vivo *lipopolysaccharide (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 | 8.1 ± 1.1 |
| LPS 10 μg/ml | 29.0 ± 2.0 | 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 |

## Discussion

Certain plant alkaloids have been found to modulate favourably the host's immune response to tumours in terms of reduced metastasis (6, 7), increased splenocyte and macrophage cytotoxicity as well as an increase in HLA Class I antigen expression in human breast carcinoma cells (8). The immunomodulatory effects so far reported occurred in a prophylactic setting by pretreatment of animals and/or tumour cells with the alkaloids under investigation (7, 16). On the other hand, the alkaloid thiophosphoric acid triaziridide, Ukrain, has been found to modulate cellular immune parameters in cancer patients (9). The major immune parameters affected by Ukrain were found to consist of an increase in total T-lymphocyte and NK cell numbers and normalization of T-helper/T-suppressor cell ratios without affecting serum immunoglobulin, complement and acute phase proteins.

Our present studies were undertaken to determine whether Ukrain would have a direct effect *in vitro* on the lytic activity of *in vivo* primed spleen lymphocytes. The results of our studies demonstrate that this compound had a direct and dose-dependent effect on the cytolytic activity of spleen lymphocytes from alloimmunized mice when assayed *in vitro*. A 48-fold increase in spleen lymphocytolytic activity could be obtained by the presence of 1.18 μM of Ukrain in the CML assay medium. Furthermore, the Ukrain-induced activation of spleen cell lytic activity was specific for mastocytoma P815 tumour cells used for alloimmunization, insofar as AKR (H-$2^d$) leukaemia cells, used as control target cells in all experiments, were not lysed. It is significant that *in vivo* primed spleen cells, harvested 18 days after alloimmunization, had no significant endogenous lytic activity, i.e., 1.3% specific lysis, whereas in the presence of Ukrain their lytic activity increased to 65.0% (c.f. Fig. 1). On the other hand, peritoneal exudate lymphocytes harvested from the same animals had an endogenous lytic activity of 7.0%, and could be increased to 24-25% specific lysis by the addition of 2.3 and 4.7 μM of Ukrain to the CML assay medium. Thus, peritoneal exudate cells were far less susceptible than spleen cells with respect to Ukrain-mediated activation of lytic activity. The reasons for these major differences in the Ukrain-induced activation of specific lysis by these two effector cell populations are not at present understood.

As most immune effector mechanisms to antigens are not constant, we investigated the Ukrain inducible activation of spleen cell lytic activity at various time intervals after alloimmunization. A significant Ukrain-induced enhancement of spleen cell lytic activity could be detected as early as 6 days after alloimmunization, with a further increase at 12 days and maximal activation occurring at 18 days, followed by a decrease at 26 and 34 days (Fig. 2). These results indicate, albeit indirectly, that Ukrain acts on the spleen lyphocytes used as effector cells and not on the tumour target cells. This interpretation is substantiated by the finding that peritoneal exudate lymphocytes, harvested 12-14 days after alloimmunization, which had an endogenous lytic activity of 70.0% specific lysis in our standardized

CML assays (E:T = 5:1; 3.5 h), could not be further increased by addition of Ukrain to the CML assay medium (data not shown).

Other alkaloids such as swainsonine, when systematically administered to nude mice in conjunction with oestradiol 17-β before implantation of subcutaneous MCF-7 cell xenografts, were found to increase the spleen cell lytic activity against murine lymphoma YAC targets. However, no direct activation of spleen cell lytic activity was reported *in vitro* in those studies (8). It is of interest that the lytic activity of macrophages obtained from thioglycolate-treated mice and preincubated *in vitro* with swainsonine were found to have an enhanced lytic activity against the human breast carcinoma cell line MDA-MB-231. *In vivo* pretreatment of mice with swainsonine was reported to result in enhanced macrophage lytic activity (8).

As *in vitro* activation of antigen-specific effector cell lytic activity may be of clinical relevance, we investigated the effects of Ukrain on *in vivo* primed spleen lymphocytes followed by *in vitro* culture with a low concentration of IL-2. Our results showed that the lytic activity of these cells could be further enhanced by the addition of Ukrain to the CML assay medium (c.f. Fig. 3). However, Ukrain also induced a significant and dose-dependent increase in the lytic activity of control spleen cells maintained *in vitro* in the absence of IL-2. The Ukrain-induced increase in lytic activity of both spleen cell populations was not significantly different one from the other. Thus, regardless of whether or not *in vivo* primed spleen cells were further treated *in vitro* with IL-2, the addition of Ukrain to the CML assays produced maximal activation of specific lytic activity, which was 60.0 ± 5% at 1.18 μM. Taking into consideration that peritoneal exudate lymphocytes, with endogenous lytic activity of 70.0%, could not be further increased by the addition of Ukrain to the CML assays (data not shown) suggests that this alkaloid derivative induces the expression of the maximal lytic activity attainable by the effector cell populations used in these studies. We are not aware of any other BRM capable of direct *in vitro* activation of a latent lytic potential of *in vivo* primed effector cells.

The results from the present studies demonstrate that the alkaloid derivative Ukrain is an effective BRM in that it directly activates the lytic mechanism of immune effector cells. The enhanced lytic activity of spleen and peritoneal exudate lymphocytes retained specificity for the alloimmunizing tumour cells. Significantly, lymphocytes from alloimmunized mice, which had no significant endogenous lytic activity, displayed the greatest increase in target-specific lytic activity, i.e., a 48-fold increase, by the addition of Ukrain to the CML assay medium.

The effects of Ukrain on the growth of Balb/c syngenic mammary adenocarcinoma was also evaluated. Results showed that i.v., but not s.c. or i.p., administration of Ukrain was found to be effective in delaying tumour growth in an actual therapeutic protocol in which treatment was initiated on established tumours five days after implantation. No deleterious side-effects were observed using these *in vivo* treatment modalities.

The role of macrophages in the observed retardation of tumour growth was investigated using peritoneal exudate macrophages (PEM) in *in vitro* cytotoxicity assays. These *in vitro* studies showed that PEM from tumour-bearing mice, which have a decreased cytolytic activity, could be activated by Ukrain. Thus, the anergy of macrophages obtained from tumour bearing mice could be overcome by Ukrain treatment. These results, taken in conjunction that Ukrain also enhances the cytolytic activity of spleen lymphocytes, indicate that the therapeutic effect of Ukrain observed *in vivo* is probably mediated by stimulating immune effector cell cytolytic activity.

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