UKRAIN, A PLANT DERIVED SEMI-SYNTHETIC COMPOUND, EXERTS ANTITUMOR EFFECTS AGAINST MURINE AND HUMAN BREAST CANCER AND INDUCE PROTECTIVE ANTITUMOR IMMUNITY IN MICE

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Despite the recent advances in anti-cancer therapies, breast cancer accounts for the highest percentage of estimated new cases among female cancer patients. The anti-cancer drug Ukrain, a plant-derived semi-synthetic compound, has been shown to be effective in a variety of tumor models including colon, brain, ovarian, melanoma and lymphoma. However, the direct cytotoxic effects of Ukrain have yet to be investigated in breast cancer models. *Aim:* Herein, we investigated the *in vitro* and *in vivo* cytotoxicity of Ukrain using murine (4T07 and TUBO) and human (SKBR-3) breast cancer cell lines. *Methods*: Cells were treated with varying concentrations of Ukrain for up to 72 h and analyzed for viability by trypan blue exclusion, apoptosis by intracellular caspase 3 and Annexin V staining, and proliferative potential by a clonogenic assay. Female BALB/c mice were challenged subcutaneously (s.c.) with 4T07-RG cells and administered 5 mg/kg or 12.5 mg/kg body weight Ukrain intravenously (i.v.) on the same day and 3 days later. Protective immune responses were determined following re-challenge of tumor-free mice 35 days post primary challenge. *Results*: Ukrain exposure induced apoptosis in a dose and time-dependent manner with 50 µg/mL Ukrain leading to >50% cell death after 48 h exposure for all three breast cancer cell lines. Ukrain administration (12.5 mg/kg) led to significant inhibition of 4T07 tumor growth *in vivo* and sustained protective anti-tumor immunity following secondary challenge. *Conclusion*: Our findings demonstrate the *in vitro* and *in vivo* cytotoxic effects of Ukrain on breast cancer cells and may provide insight into designing Ukrain-based therapies for breast cancer patients.
Key Words: Ukrain, breast cancer, anticancer drug, cytotoxicity, protective immunity.

Cancer is among the leading causes of mortality in the United States, second only to heart diseases [1]. While the mortality rates associated with many other diseases have seen a significant decline in the past 50–60 years, a similar reduction has yet to be seen in the cancer field [2]. These statistics speak to the overwhelming necessity to develop more effective treatment options for cancer patients. Closer examination of these statistics contribute to the high mortality reveals that breast cancer is the second leading cause of cancer-related deaths among women and accounts for the highest percentage of estimated new cancers among female cancer patients [3]. While several treatment options are currently available to cancer patients such as surgery, chemotherapy and radiation therapy either alone or in an adjuvant setting, each approach is associated with a wide range of clinical challenges. The most significant drawback to chemotherapeutic drugs, the most widely used anti-cancer treatment option, is their toxicity towards non-malignant cells [4].

The anticancer drug Ukrain (NSC 631570), a semi-synthetic compound derived from the extract of the plant *Chelidonium majus* L., has been shown to exert selective cytotoxic effects towards a variety of malignant cells including colon, brain, ovarian, melanoma and lymphoma without harmful side effects on healthy human cells [5, 6]. The cytotoxic selectivity of Ukrain is thought to be based on the differential membrane potentials of malignant cells and normal cells. It has been reported that the positive charge of the celandine alkaloids comprising the Ukrain allows malignant cells to absorb more Ukrain than healthy cells [7].

To date several clinical trials have assessed the efficacy of Ukrain in patients with an assortment of malignancies such as colorectal, pancreatic, bladder and breast cancers [8]. In each of these trials, patients treated with Ukrain had a more favorable clinical response compared to control groups (placebo or other therapies) which included either longer survival [9], extended periods of non-progression [10] and/or symptomatic improvements [11]. Previously, two groups [12, 13] have assessed the potential effects of Ukrain in breast cancer models citing that Ukrain fails to enhance the radiosensitivity of MDA-MB-231 human breast cancer cells and Ukrain treatment can enhance the cytolytic activity of peritoneal exudate macrophages towards DA-3 mammary tumor cells. However, to the best of our knowledge, a thorough investigation of the direct cytotoxic effects of Ukrain on a variety of breast cancer cells has yet to be reported.

Herein, we demonstrate the *in vitro* effectiveness of Ukrain to selectively kill both mouse and human breast cancer cells. Additionally *in vivo* experiments indicate that Ukrain can induce tumor inhibition in a breast cancer model and for the first time that Ukrain can induce protective antitumor immunity. Taken together, our findings provide evidence of the effectiveness of Ukrain in breast cancer models.

MATERIALS AND METHODS

Cell lines. The 4T07 cell line, a non-metastatic clone derived from a spontaneous adenocarcinoma in BALB/cfC3H mice (410.4), was a kind gift from Fred Miller (Wayne State University) [14]. While cells can be recovered from the lungs of mammary pad inoculated mice, 4T07 fails to colonize the lungs and form metastatic nodules [15]. TUBO cells, derived from a spontaneous carcinoma in BALB-neuT mice [16], are also non-metastatic and were provided by John C. Morris (National Institutes of Health). The 4T07, TUBO and NIH 3T3 (mouse embryonic fibroblasts) cell lines were cultured in DMEM F12 media, supplemented with 10% cosmic calf serum (Hyclone, Logan, Utah) and 1% penicillin streptomycin (Mediatech, Manassas, VA). The human breast cancer cell line, SKBR-3, was cultured in RPMI 1640 media supplemented with 10% cosmic calf serum and 1% penicillin streptomycin and was obtained from American Type Culture Collection (ATCC, Manassas, VA). The TUBO and SKBR-3 cell lines constitutively express the HER-2/neu oncogene, which is overexpressed in 30% of human breast cancer patients. All cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Mouse model. Female BALB/c mice (8–12 week old) were purchased from Jackson Laboratories (Bar Harbor, Maine) and used for all *in vivo* studies. Mice were housed and maintained according to institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) for Emory University in Atlanta, GA.

Reagents. Ukrain was kindly provided by Dr. Wassil J. Nowicky and Stephen Karoly from Nowicky Pharma (Vienna, Austria). The stock solution was supplied at a concentration of 1 mg/mL. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO), BD Cytofix/Cytoperm Fixation and Permeabilization solution and FITC rabbit anti-active caspase-3 antibody (BD Pharmingen, La Jolla, CA). Antibodies used to phenotype isolated splenocytes by flow cytometry included anti-CD4-FITC, anti-CD8-FITC, anti-B220-PE, anti-CD11b-FITC, anti-Gr-1-PE and CD16/32 Fc-block (eBioscience, San Diego, CA).

In vitro drug treatment assay. Murine and human breast cancer cells were seeded at a density of 5x10⁴ cells per well in 24-well plates (in duplicate) in appropriate culture media. After being allowed to adhere, tumor cells were treated with 0; 25; 75; 100 µg/mL Ukrain for 24; 48 and 72 h. At the specified time point, cells were analyzed for viability, apoptosis, and clonogenic potential. As specificity controls, concanavalin A (ConA)-stimulated

mononuclear cells, isolated from the spleens of naive BALB/c mice and recovered by Ficoll-Hypaque density centrifugation, and NIH 3T3 cells were treated with Ukrain at the aforementioned concentrations for 24, 48, and 72 h and cellular viability was subsequently assessed by the trypan-blue exclusion method.

Detection of apoptosis using Annexin V, PI staining. Ukrain-treated cells were washed once with PBS, re-suspended in 1X Binding Buffer and Annexin V-FITC (5 µL) and Propidium Iodide-PE (10 µL) (Sigma, St. Louis, MO) was added to each cell suspension. Cells were incubated for 10 minutes at room temperature in the dark. Staurosporine, 2.5 µM, (Sigma) was used as a positive control for apoptosis induction. Stained cells were analyzed immediately using a FACSCaliber (Beckman Coutler, Fullterton, CA) and Flowjo software.

Quantification of active caspase-3. Following Ukrain treatment, cells were washed, fixed and permeabilized for 20 min at 4 °C using BD cytofix/cytoperm solution (BD Pharmingen). Permeabilized cells were washed and stained for intracellular caspase-3 using the FITC rabbit anti-active caspase-3 antibody (clone C92–605) for 30 min at 4 °C in the dark (BD Pharmingen). Cells were then analyzed using flow cytometry as described previously.

Clonogenic assay. The long-term effects of Ukrain treatment on murine and human breast cancer cells were assessed using a clonogenic assay. Following 24; 48 and 72 h treatment with Ukrain, cells were plated in 60 mm tissue culture plates at 100 cells/plate. Cells were allowed to form colonies for two weeks. The colonies were then gently washed with PBS, fixed and stained with crystal violet (0.5% w/v in 90% ethanol) and counted by light microscopy. The surviving fraction of colonies was calculated as previously described [17].

Tumor growth inhibition studies. 4T07 cells were passaged 4 times *in vivo* to obtain reproducible tumor growth with palpable tumor development within 6 days (4T07RG). To assess the *in vivo* cytotoxicity of Ukrain in a mouse model, female BALB/c mice were challenged subcutaneously (s.c.) with 5x10⁴ 4T07RG cells on the shaved right hind flank. Mice were then administered 5mg/kg or 12.5 mg/kg body weight Ukrain via tail vein injection (i.v.) on the same day. The mice were then administered an additional dose of Ukrain 3 days later. Tumors were measured 2–3 times a week using a Vernier caliper. Mice were sacrificed once the tumors became ulcerated or exceeded 1 cm² in diameter. At the conclusion of the experiment (day 35), tumor-free mice were re-challenged with 5x10⁴ 4T07RG cells and monitored for tumor growth for an additional 35 days to determine whether Ukrain is capable of inducing protective antitumor immunity.

Cellular phenotyping of splenocytes. Spleens were harvested and weighed from Ukrain treated mice on day 35 following re-challenge with 4T07RG cells. Single cell suspensions were prepared and red blood cells were removed by hypotonic lysis. Splenocytes were then washed in FACS buffer (PBS/1%CCS/1%EDTA), incubated in Fc block (CD16/32, clone 2.4G2) and

stained for 25 minutes at 4 ºC with the appropriate antibodies to detect T cells (CD4 and CD8), B cells, and myeloid derived suppressor cells (MDSCs). Antibodies used for analysis were anti-CD4-FITC, anti-CD8-FITC, anti-B220-PE, anti-CD11b-FITC, anti-Gr-1-PE. Sample readings were collected using a FACSCaliber and analyzed with Flowjo software.

***Statistical analysis*.** All *in vitro* experiments were performed in duplicate or triplicate and were repeated at least twice. Differences between caspase-3 activation following Ukrain treatment as well as tumor growth curves were assessed using ANOVA analysis. For survival curves, *P* values were determined using the Log-rank (Mantel — Cox) test. $P < 0.05$ was considered statistically significant. All graphs and statistical calculations were done using GraphPad Prism software (GraphPad Software Inc., San Diego).

RESULTS

***Ukrain exhibits cytotoxic effects specifically on breast cancer cells not non-malignant cells*.** Human and murine breast cancer cells were treated with 25; 50; 75 and 100 µg/mL Ukrain for 24; 48 and 72 h. At each time-point, viability was determined using the trypan-blue exclusion method. We began to observe morphological changes of all three breast cancer cell lines after 48 hours of Ukrain exposure. The Ukrain-treated cells began to shrink in size, undergo membrane blebbing, loose cell-to-cell contact and adherence to the tissue culture plate (Fig. 1*a*). Additionally, we observed a dose and time-dependent decrease in viability with 50 µg/mL Ukrain leading to 50% cell death after 48 h exposure for all three breast cancer cell lines tested (Fig.1*b*). Following 72 h Ukrain exposure at 100 µg/mL maximal cell death was observed with the viability of 4T07, TUBO and SKBR-3 cells being 12.9%, 17.53% and 30.8% respectively. As specificity controls, mouse fibroblasts (NIH 3T3 cells) and ConA-stimulated mononuclear cells were treated with similar doses of Ukrain. We observed minimal cytotoxic effect of Ukrain on mouse fibroblast cells (Fig.1*c*) and ConA-stimulated lymphocytes (data not shown) even at higher exposure doses with the viability remaining above 90%. These findings indicate that the cytotoxic effects of Ukrain are specific to the breast cancer cells while not affecting non-malignant cells.

***Ukrain treated tumor cells fail to regain proliferative capacity*.** To determine whether Ukrain treated cells have a reduced ability to proliferate, a clonogenic assay was conducted. Following Ukrain treatment for 24; 48 or 72h, cells were washed and replated in fresh culture media lacking Ukrain. After two weeks of culture, colonies were washed, stained with crystal violet and counted by light microscopy. The surviving fraction is defined as the number of colonies derived from pre-treated tumor cells following two week removal of Ukrain expressed in terms of plating efficiency as previously described [17]. The pre-treated cells showed a reduced ability to form colonies relative to untreated cells (Fig. 2). Following 24 h pre-treatment

with 50 µg/mL Ukrain, 4T07, TUBO and SKBR-3 tumor cells exhibited a comparable inability to form colonies with surviving fractions of 0.0024, 0.0039 and 0.0019 respectively. Minimal differences were observed in the surviving fractions of 4T07 and TUBO cells exposed to 50 µg/mL Ukrain for 24, 48 or 72 h. However, 72 h exposure to 50 µg/mL Ukrain led to a 10-fold reduction in the surviving fraction of SKBR-3 cells (0.000135) relative to 24 h and 48 h from exposure from 0.0019 and 0.001315, respectively. Both murine and human breast cancer cells that were pre-treated with Ukrain for 72 h with 100 µg/mL Ukrain, were unable to form any visible colonies (denoted by †). This data illustrates the long-term effects of Ukrain treatment on the clonogenic potential of breast cancer cells and indicates that upon removal of Ukrain, the
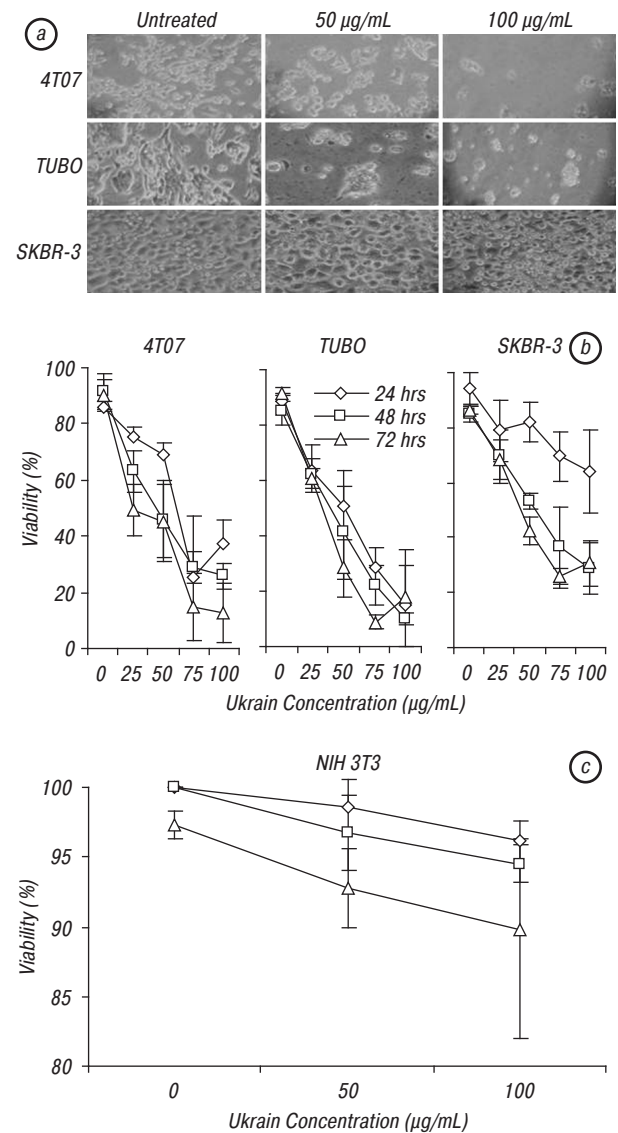

Fig. 1. Dose and time dependent cytotoxic effects of Ukrain on breast cancer cells. *a*) Morphological changes are observed for murine and human breast cancer cells following 48 hr exposure to Ukrain by light microscopy (10X magnification). *b*) Following 24; 48; 72 hr Ukrain exposure, the viability of *b*) murine (4T07 and TUBO) and human (SKBR-3) breast cancer cells and *c*) mouse fibroblast cells were assessed for viability by trypan blue exclusion. Data shown is mean ± SD of three individual experiments
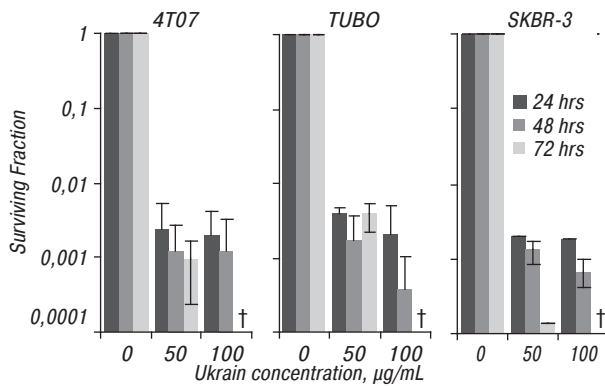
Fig. 2. Survival Fractions following Ukrain treatment. Following 24; 48 and 72 h exposure, Ukrain treated cells were plated for two weeks to allow for colony formation. Colonies were washed, fixed and stained with crystal violet and counted by light microscopy. The surviving fraction was then calculated as the ratio of the plating efficiency of drug-treated cells to the plating efficiency of untreated cells. (†) denotes a surviving fraction of zero. Data shown is mean ± SD of two individual experiments

treated tumor cells are unable to recover from the drug's cytotoxic effects.

Ukrain exerts anti-tumor activity through apoptosis induction. Ukrain has previously been shown to induce apoptosis of Jurkat T cell lymphoma through mitochondrial membrane depolarization and caspase activation [18] and HeLa cervical cancer cells through the activation of the intrinsic pathway (caspase-9 cleavage) [19]. Therefore to investigate whether the observed cytotoxic effects of Ukrain on breast cancer cells are due to the induction of apoptosis we carried out an Annexin-V binding assay as well as intracellular staining for active caspase-3. As shown in Fig. 3*a*, there was a 10–15 fold increase in activated caspase-3 among 4T07 and TUBO cells and a 2–3 fold increase among SKBR-3 within 48 h of drug exposure. Similarly, 80–90% of the Ukrain-treated tumor cells were apoptotic as indicated by AnnexinV/PI dual staining after 48h (Fig. 4*a*). The kinetics of apoptosis induction indicates a dose and time dependent increase in activated caspase 3 and Annexin V/PI staining (Fig. 3*b* and 4*b*). Our findings indicate apoptosis as the cytotoxic mechanism of action which is consistent with previous reports in other tumor model systems [18–21].

Systemic administration of Ukrain significantly reduces breast cancer tumor growth in mice. To assess the *in vivo* efficacy of Ukrain in causing tumor inhibition in a murine breast cancer model, mice were challenged with 5x10⁴ 4T07RG cells (s.c.) and administered varying doses of Ukrain (5 mg/kg or 12.5 mg/kg) on the same day (i.v.). Three days later, mice received an additional dose of Ukrain (i.v.). It has previously been reported that following subcutaneous or intraperitoneal injection of Ukrain, no inhibitory effect on the growth of established DA-3 mammary adenocarcinoma was observed whereas significant inhibition was observed following intravenous Ukrain administration [12]. We observed that treatment with two doses of 12.5 mg/kg Ukrain led to a significant reduction in overall tumor burden by day 31 relative to untreated mice with an average tumor burden of 22.7 mm² com-
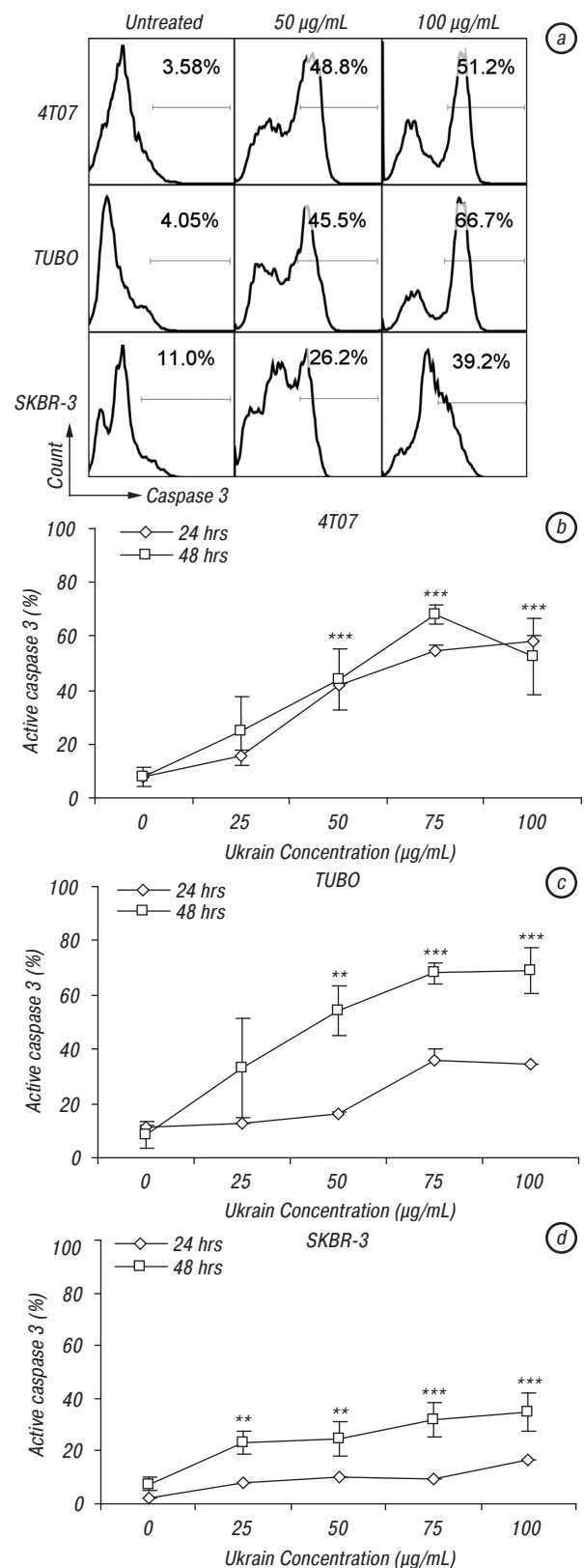

Fig. 3. Ukrain induces the activation of intracellular caspase-3 in a dose and time dependent manner. Following Ukrain treatment, cells were washed, fixed, permeabilized and stained for intracellular caspase 3 with FITC rabbit anti-active caspase-3 antibody *a*) Representative plots of intracellular caspase 3 activation following 48 h Ukrain exposure by flow cytometry. *b*) Dose response and kinetics of intracellular caspase 3 activation following 24 h and 48 h Ukrain treatment. Data shown is mean ± SD of two individual experiments. Statistical analyses of caspase 3 activation P values were calculated using ANOVA. (*) p < 0.05, (**) p<0.01, (***) p< 0.001 relative to untreated
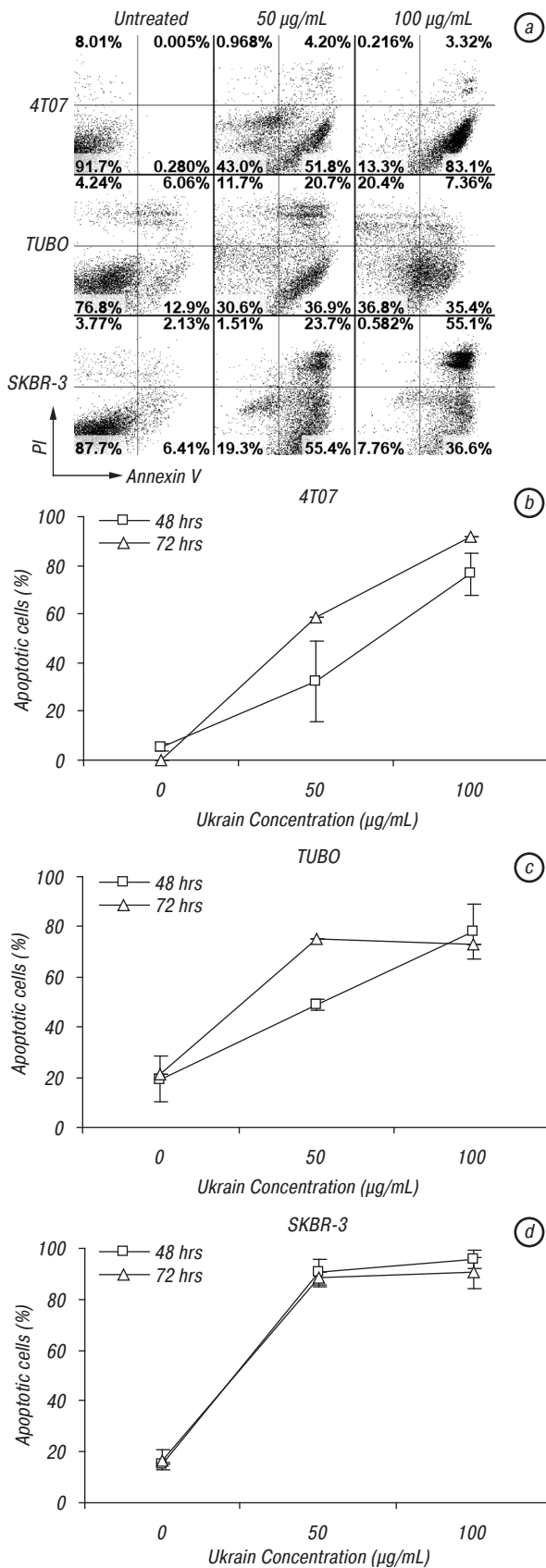
Fig. 4. Cytotoxic effects of Ukrain is mediated by apoptosis. Ukrain treated cells were washed and stained with Annexin-V-FITC and PI-PE and analyzed by flow cytometry. *a*) Representative plots of Annexin V/PI staining following 48 h Ukrain exposure by flow cytometry. *b*) Mean ± SD of two individual experiments. *b*) Dose response and kinetics of Annexin V/PI staining following 48 h and 72 h Ukrain treatment. Mean ± SD of two individual experiments

pared to 78.9 mm^2 for untreated mice (Fig. 5*a*). While all mice had palpable tumors by day 7 post challenge, we began to observe the effects of the Ukrain treatment regimes on day 14 post challenge (11 days after the last Ukrain administration). The mice that received 5 mg/kg Ukrain, on average, also had smaller tumors that developed more slowly than the untreated mice with a mean tumor burden of 47.7 mm^2. By the end of the experiment, tumors in several mice treated with 5 mg/kg and 12.5 mg/kg had completely regressed with tumor incidences being 60 and 40%, respectively (Fig. 5*b*). Importantly, we observed no visible signs of morbidity such as weight loss among the Ukrain treated groups (Fig. 5*c*). Interestingly, when the Ukrain dose was increased to 20 mg/kg, the treated mice failed to be protected and developed progressive tumor growth similar to the untreated mice (data not shown). While 25 mg/kg Ukrain has been reported to effectively inhibit metastases of murine Lewis lung carcinoma [22], our observation suggests that similarly high doses of Ukrain may not be effective *in vivo* in breast cancer models. Taken together, these findings indicate that moderate doses of Ukrain can inhibit tumor progression and induce tumor regression in a highly tumorigenic mouse breast cancer model.

Ukrain treatment induces protective immunity. Next, to determine whether Ukrain administration can provide protection against a secondary tumor challenge, thirty-five days post initial challenge, tumor-free mice from each Ukrain-treated group were re-challenged with 5x10^4 4T07RG cells. We observed that all mice remained tumor free up to day 35 post re-challenge indicating that protective immunity was induced following Ukrain treatment (Fig. 6*a*).

Interestingly, we observed that untreated, tumor-bearing mice developed significant splenomegaly (1.16 g) relative to the Ukrain treated mice (0.1 g) whose spleens were comparable in size and weight to naïve, unchallenged mice (Fig. 6*b*, photograph). To gain insight into potential immune modulatory effects of Ukrain that could be responsible for the observed protective immunity, we conducted cellular phenotyping of cells that were found within the spleens of Ukrain treated mice. Previous reports have shown that splenomegaly in the 4T1 mouse breast cancer model, derived from the same parental clone as 4T07, to be associated with an increased infiltration of myeloid derived suppressor cells (MDSCs) [23]. Our observations indicate that in the 4T07 tumor model system, wild-type challenged mice develop a similar splenomegaly which correlates with a 12–15 fold increase in the percentage of CD11b$^+$Gr-1$^+$ MDSCs relative to the Ukrain treated, tumor-free mice (Fig. 6*c*). Interestingly, when we compared the percentage of MDSCs in naïve mice to the Ukrain-treated, tumor-free mice, we noticed a 2–3 fold decrease in the splenic population of these cells. Taken together, these studies demonstrate that Ukrain treatment results in persistent protective immunity in a highly immunosuppressive and tumorigenic breast cancer model.
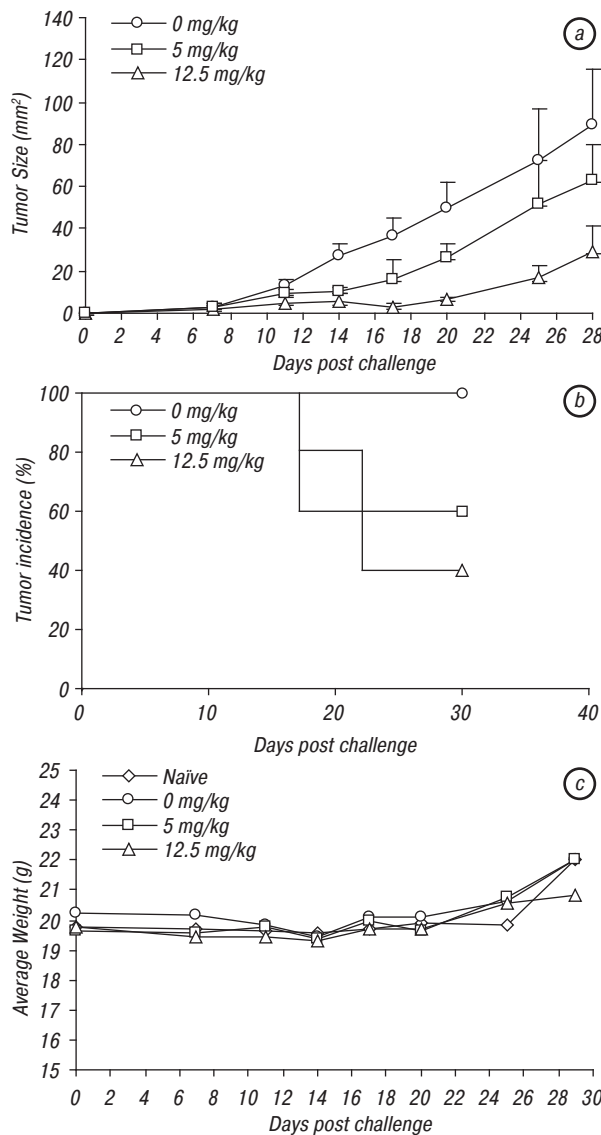
Fig. 5. Ukrain administration led to significant tumor inhibition. Mice (n=10) were challenged with 5x10⁴ 4T07RG cells. Ukrain was administered (i.v.) on the same day and day 3 post challenge. *a*) Average tumor size of mice treated with Ukrain. *b*) Tumor incidence *c*) Average Body Weight. Statistical analyses of tumor growth P values were calculated using one-way ANOVA. (*) p < 0.05 relative to untreated. Data shown is mean ± SEM. Representative of 2 independent experiments

Fig. 6. Ukrain administration induced protective anti-tumor immunity. On day 35 post primary challenge, Ukrain treated, tumor-free mice (n=10) were re-challenged with 5x10⁴ 4T07RG cells and monitored for an additional 35 days for tumor development (*a*). On day 35 post re-challenge, spleens were harvested from Ukrain treated mice and analyzed for MDSCs (CD11b⁺, Gr-1⁺). Protective immunity induced by Ukrain correlates with a reduced spleen weight (*b*) and reduced prevalence of splenic MDSCs (*c*)

DISCUSSION

The anticancer drug Ukrain has been assessed for potential clinical efficacy in Europe in patients suffering from colorectal, pancreatic, bladder and breast cancers as reviewed in [8]. *In vitro* studies have demonstrated the anti-proliferative and cytotoxic effects of Ukrain in a wider variety of tumor models including colon, brain, ovarian, Ewing, melanoma, lymphoma and glioblastoma cell lines [5, 24–27]. The property that distinguishes Ukrain from typical therapies, such as radiation therapy and chemotherapy, lies in its unique ability to specifically target and kill malignant cells while leaving healthy cells unharmed [5]. However, the direct anti-tumor effects of Ukrain in breast cancer models remain obscure. Therefore, in this report we investigated the *in vitro* and *in vivo* efficacy of Uk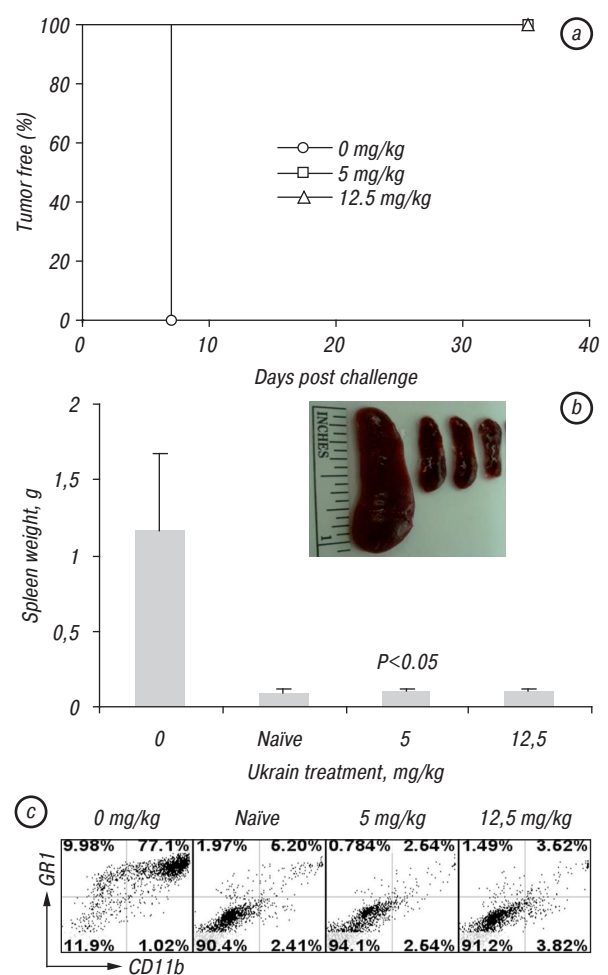rain in murine and human breast cancer models. Consistent with previous reports in other tumor models, we observed that Ukrain induces apoptosis of breast cancer cells through caspase 3 activation. Additionally, after a short exposure time, 24 h, Ukrain treated cells have reduced clonogenic potential in the absence of the drug and are unable to form colonies following longer exposure (72h) at 100 µg/mL Ukrain. Most importantly, *in vitro* cytotoxicity is translatable *in vivo* in the form of significant tumor inhibition and regression. Interestingly, we observed that Ukrain is also capable of inducing protective anti-tumor immune responses which is consistent with clinical observations of patients achieving sustained/long-term remissions for years following Ukrain treatment [28–30].

At present, the mechanism(s) responsible for these protective anti-tumor responses induced by Ukrain remain unclear. However, it is possible that Ukrain could potentially be working through two related mechanisms: 1) direct cytotoxic effects on tumor cells leading to a significant reduction in overall tumor burden and 2) as a result of the direct killing of tumor cells,

antigen-presenting cells such as dendritic cells (DCs) could endocytose tumor-derived apoptotic bodies and in turn elicit a robust tumor-specific CTL response as reported in other tumor models [31]. The induction of "immunogenic cell death" has been demonstrated following treatment with chemotherapeutic agents such as doxorubicin and cyclophosphamide such that due to their enhanced antigenicity, apoptotic tumor cells are more efficiently phagocytosed by DCs [32, 33]. The characteristics of immunogenic apoptosis of cancer cells include: phosphatidylserine (PS) exposure, caspase activation, and mitochondrial depolarization [34]. Additionally, this type of cell death is associated with the surface expression of certain molecules including calreticulin and HSP90 as well as the ability to elicit a protective immune response against tumor cells [35, 36]. As we observed in our studies, Ukrain treatment led to the activation of effector caspase 3 and PS surface expression as detected by positive Annexin-V staining in all three breast cancer models tested *in vitro*. Further, Ukrain has been shown to induce mitochondrial deplorization in Jurkat cells [18]. Our study also demonstrated the *in vivo* efficacy of 5 mg/kg and 12.5 mg/kg Ukrain administration in the form of tumor inhibition and protective immunity. However, at a higher dose, 25 mg/kg, these responses were not observed which suggest the induction of a less immunogenic, potentially tolerogenic, form of cell death as reported following treatment with other anticancer drugs such as alkylating agents and cisplatin [36, 37]. Taken together our findings, along with others, are in support of the potential induction of immunogenic tumor cell death following "low dose" Ukrain treatment.

Alternatively, Ukrain could also act directly on immune cells and thus mediate the induction of anti-tumor immune responses. It has been reported that Ukrain can enhance the cytolytic activity of macrophages and lymphocytes *in vitro* [12, 38]. We observed that following secondary challenge, Ukrain treated mice remained tumor free, failed to develop splenomegaly and correspondingly had a significantly lower prevalence of splenic MDSCs relative to untreated mice. Additionally, the Ukrain treated mice had increased percentages of CD4$^+$, CD8$^+$ and B220$^+$ cells in the spleen relative to the untreated, tumor-bearing mice (data not shown). Interestingly, we also observed a 2–3 fold decrease in the percentage of MDSCs in the spleens of Ukrain treated mice when compared to naïve mice. These findings suggest that Ukrain could potentially inhibit the expansion of splenic CD11b$^+$Gr-1$^+$ MDSCs thus reducing the overall level of immune suppression present within the treated mice. This reduction in immune suppression could ultimately lead to protection against subsequent challenge and the splenomegaly observed in untreated, tumor-bearing mice. While chemotherapeutic drugs, namely gemcitabine and 5-fluorouracil, have been shown to effectively deplete MDSCs [39, 40], additional studies would be needed to determine whether Ukrain has any direct effects on this immune suppressive cell population *in vivo* particularly in tumor-bearing mice.

Taken together, our findings provide insight into the direct cytotoxic effects of Ukrain on breast cancer cells and the *in vivo* efficacy of Ukrain administration on the reduction of overall tumor burden and the induction of protective antitumor immunity. Based on these observations, it is likely that the immune modulatory effects following Ukrain administration *in vivo* are a result of enhanced phagocytosis of apoptotic tumor cells either alone or in combination with direct effects on the relative abundance/prevalence of effector and suppressive immune cell populations.

CONFLICTS OF INTEREST
None declared.

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