CANCER THERAPY AND PREVENTION

Bladder cancer therapy without toxicity—A dose-escalation study of alpha1-oleate

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Abstract

Potent chemotherapeutic agents are required to counteract the aggressive behavior of cancer cells and patients often experience severe side effects, due to tissue toxicity. Our study addresses if a better balance between efficacy and toxicity can be attained using the tumoricidal complex alpha1-oleate, formed by a synthetic, alpha-helical peptide comprising the N-terminal 39 amino acids of alpha-lactalbumin and the fatty acid oleic acid. Bladder cancer was established, by intravesical instillation of MB49 cells on day 0 and the treatment group received five instillations of alpha1-oleate (1.7-17 mM) on days 3 to 11. A dose-dependent reduction in tumor size, bladder size and bladder weight was recorded in the alpha1-oleate treated group, compared to sham-treated mice. Tumor markers Ki-67, Cyclin D1 and VEGF were inhibited in a dose-dependent manner, as was the expression of cancer-related genes. Remarkably, toxicity for healthy tissue was not detected in alpha1-oleate-treated, tumor-bearing mice or healthy mice or rabbits, challenged with increasing doses of the active complex. The results define a dose-dependent therapeutic effect of alpha1-oleate in a murine bladder cancer model.

KEYWORDS
Alpha1-oleate, bladder cancer therapy, dose escalation, lack of toxicity

1 | INTRODUCTION

Cancer patients have reasons for optimism, as they are being offered a number of novel therapeutic options that prolong survival. Examples include targeted therapies with improved tumor specificity and immunotherapies that stimulate the immune system to attack and sometimes eradicate the tumor.[1,2] Important advances are also being made using combination therapies, where the dose of each toxic drug may be reduced or the order varied, to minimize toxicity.[3,4] Still, virtually all cancer therapies are accompanied by severe side effects and significant mortality.

Bladder cancer is common worldwide and among the most costly cancer forms, due to its high recurrence rate and a lack of curative therapies.[5] Approximately 550 000 new cases were diagnosed in 2018 and about 200 000 deaths were estimated.[6,7] Survival depends on the recurrence rate and the risk for de-differentiation, and invasive tumors may require cystectomy and systemic chemotherapy.[8,9] Superficial papillary tumors, in contrast, are restricted to the mucosa and lamina propria and the short-term prognosis is excellent. The current standard treatment for nonmuscle invasive bladder cancer

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Abbreviations: HAMLET, human alpha-lactalbumin made lethal to tumor cells; Ki-67, MKI67; NF-κB, nuclear factor kappa B; NMIBC, nonmuscle invasive bladder cancer; PCA, principal component analysis; VEGF, vascular endothelial growth factor.

(NMIBC) is transurethral resection of the bladder tumor. Intravesical chemotherapy is generally used as adjuvant therapy after resection and has been shown to reduce recurrence rates, but not disease progression in patients with low-risk NMIBC.[10,11] Intravesical immunotherapy with bacillus Calmette-Guérin (BCG) is recommended after surgery and is superior to intravesical chemotherapy for preventing tumor recurrence.[12] Mitomycin C, which is widely used for intravesical chemotherapy of newly diagnosed superficial bladder cancer, reduces tumor recurrences and prolongs disease free intervals.[13] These therapies are accompanied by significant side effects, however, and a significant risk of tumor recurrence. A less toxic therapeutic option would therefore be a welcome addition in this patient group.

The human milk protein alpha-lactalbumin is essential for lactating mammals to feed their offspring. The native protein acts as a substrate specifier in the lactose synthase complex and is therefore crucial for the expression of milk and for its nutritional value. In addition, alpha-lactalbumins are structurally flexible and transition from the native folded state to partially unfolded, molten globule-like states.[14] We have shown that this structural flexibility enables alpha-lactalbumins to form stable, protein-fatty acid complexes, most prominently with oleic acid and related, 16 to 18 carbon unsaturated fatty acids. These complexes acquire tumoricidal activity and have been shown to kill a large number of different tumor cells in vitro, by an apoptosis-like mechanism. The mechanism of action includes extensive plasma membrane remodeling, internalization of the complex and chromatin interaction, resulting in inhibition of cell proliferation and repair.[15]

Therapeutic effects of the human alpha-lactalbumin and oleic acid complex HAMLET were previously demonstrated in the MB49 bladder cancer model.[16] In a clinical study, HAMLET was shown to trigger tumor cell shedding and reduce tumor size in some patients with NMIBC. Recent molecular analyses identified the N-terminal alpha-helical peptide domain of alpha-lactalbumin as a potent tumoricidal entity, which can be synthetically produced to form complexes with oleic acid. Our study addressed the therapeutic efficacy of the alpha1-oleate complex, using a dose-escalation approach. Potent, dose-dependent, alpha1-oleate therapeutic effects were detected in the murine MB49 bladder cancer model. The increase in therapeutic efficacy was not accompanied by a parallel increase in side effects, and despite extensive analysis, toxicity for healthy tissue was not detected in mice or rabbits. The results suggest that the alpha1-oleate complex should be further explored in efforts to achieve a more specific approach to bladder cancer therapy.

2 | MATERIALS AND METHODS

2.1 | Chemicals and antibodies

Oleic acid (Croda, batch number: 0001120439), poly-L-lysine solution (Sigma, St. Louis, Missouri, Cat#RNBF4239), Alexa-Fluor 568 protein labeling kit (Thermo Scientific, Waltham, Massachusetts, Cat# A10238), ECL Plus detection reagent (GE Healthcare, Chicago, Illinois, Cat# RPN2132), Richard-Allan Scientific Signature Series Hematoxylin and

What's new?

Tumor resection is a standard treatment for bladder cancer and often is followed by adjuvant intravesicle chemotherapy or immunotherapy. These treatments, however, frequently are accompanied by significant side effects. Here, the authors explore the effects of the peptide-fatty acid complex alpha1-oleate, previously reported to possess tumoricidal activity and a promising agent for the treatment of non-muscle invasive bladder cancer. Compared to untreated mice, animals treated with alpha1-oleate experienced a dose-dependent reduction in tumor size, without evidence of local or systemic toxicity. The findings provide essential background for clinical trials examining the effects of alpha1-oleate in humans.

Eosin-Y (Thermo Scientific, Cat# 7211 and 7111), DAPI (4′,6-diamidine-2′-phenylindole, Sigma, Cat# D9542), anti-alpha lactalbumin (MyBioSource, San Diego, California, Cat# MBS175270), monoclonal mouse anti-β-actin (Sigma-Aldrich, St. Louis, Missouri Cat# A2228), polyclonal rabbit anti-mouse IgG-HRP (Dako, Santa Clara, California, Cat# P0260), polyclonal goat anti-rabbit IgG-HRP (Cell Signaling, Danvers, Massachusetts, Cat# 7074), rabbit polyclonal anti-VEGF (Abcam, Cambridge, Massachusetts, Cat# ab46154), mouse monoclonal anti-Ki-67 (BD Biosciences, Franklin Lakes, New Jersey, Cat# 556003), rabbit monoclonal anti-Cyclin D1 (Thermo Fisher, Cat# SC8396), rat monoclonal anti- NIMP-R14 (neutrophil; Abcam, Cat# ab2557, rabbit polyclone anti-IL-6 (Abcam, Cat# ab6672), mouse monoclonal anti-IL-1beta (Abcam, Cat# ab9722), goat anti-rabbit IgG Alexa-Fluor 488 (Thermo Fisher, Cat# A-11034), goat anti-mouse IgG Alexa 568 (Thermo Fisher, Cat# A-11004), DRAQ5 (Abcam, Cat# ab108410). Epirubicin (Sigma-Aldrich, Cat# E9406) and Mitomycin Sigma-Aldrich, Cat# M0440).

2.2 | Peptide synthesis and complex generation

We have identified the N-terminal, alpha-helical domain of alpha-lactalbumin as a tumoricidal entity, which forms a complex with oleic acid.[17] For this study, we synthesized the 39 amino acid peptide (aa 1-39, Ac-KQFTKAELSQLLKDIDGYGGIALPELIATMFHTSGYDTQ-OH), using Fmoc solid phase chemistry with the purity of >95% (Polypeptide group, France). To form the alpha1-oleate complex, the alpha1 peptide was mixed with sodium oleate at a 1:5 M ratio. and solubilized in PBS at concentration of 17 mM. The stock solution was further diluted in PBS to the appropriate concentration for each experiment.

2.3 | Bladder cancer model

MB49 (RRID:CVCL_7076) cells were provided by Sara Mangsbo, Uppsala University, Sweden. MB49 bladder cancer was established as

previously described.[16] C57BL/6 female mice were bred at the Department of Laboratory Medicine, Lund University and used at ages from 7 to 12 weeks. For procedures, mice were anesthetized by intraperitoneal injection of a cocktail of ketamine (1.48 mg in 100 μL of 0.9% NaCl solution, Intervet) and xylazine (0.22 mg in 100 μL of 0.9% NaCl solution, Vetmedic). On day 0, the bladder was emptied and preconditioned by intravesical instillation of 100 μL of poly-L-lysine solution (0.1 mg/mL) through a soft polyethylene catheter (Clay Adams) with an outer diameter of 0.61 mm. After 30 minutes, MB49 mouse bladder carcinoma cells (2×10^5 cells in 50 μL media) were instilled. On day 3, mice were randomly assigned to the alpha1-oleate treatment or sham treatment group. On days 3, 5, 7, 9 and 11, 100 μL of alpha1-oleate (1.7 mM, 8.5 mM or 17 mM) or PBS were then instilled. The catheter was left in place for about 1 minute, but as the mice remained under anesthesia, the time to voiding (dwelling time) of the substance was 2 to 3 hours. Groups of 5 to 6 mice for each treatment and control were sacrificed at day 12, and bladders were imaged and processed for histology or RNA extraction. Two independent experiments were performed. All experiments were performed with mycoplasma-free cells.

2.4 | Toxicity of alpha1-oleate

A. Female NMRI mice (8-10 weeks old) were divided into two groups of 15 animals to receive the alpha1-oleate treatment or the vehicle PBS (controls) twice weekly for 4 weeks (Figure S1). A concentration of 1.7 mM of alpha1-oleate peptide was used for instillation. One day after the last dose, blood was collected from the orbital plexus of 10 animals from each group for hematological and clinical biochemistry analyses and thereafter euthanized to harvest the organs. The organs were put into 4% formaldehyde solution or Davidson's fixative when applicable. The remaining animals (n = 5 per group) were euthanized and subjected to blood and organ sampling 14 days thereafter, as described above.

B. Female NZW rabbits were divided into two groups of seven animals to receive the alpha1-oleate test item or the vehicle (PBS) twice weekly for 4 weeks. A solution with 2.45 mM concentration of complex corresponding to 2.45 mM of alpha1 peptide and 12.25 mM of oleic acid was used for instillation. Blood samples for hematological and clinical biochemistry analyses were taken before the start of instillations and 1 day after the last instillation from five animals of each group, after which they were euthanized to harvest the organs. The remaining animals were euthanized and subjected to blood and organ sampling 14 days thereafter.

C. C57BL/6 female mice were bred at the Department of Laboratory Medicine, Lund University and used at ages from 7 to 12 weeks. On day 0, the bladder was emptied and preconditioned by intravesical instillation of 100 μL of alpha1-oleate (17 mM) or PBS (controls). Groups of three mice for each treatment and control were sacrificed at each time point (after 24 hours and 7 days). Organs were collected and processed for histological analysis. Two independent experiments were performed.

D. Thirty female NMRI mice were divided into two groups of 15 animals to receive 18 mM of alpha1-oleate test item or the vehicle (PBS controls) twice weekly for 4 weeks. One day after the last dose, 10 animals from each group were subjected to blood sampling for hematological and clinical biochemistry analyses and thereafter euthanized to harvest organs. The remaining animals were euthanized and subjected to blood and organ sampling 14 days thereafter.

2.5 | Histology

Bladders were embedded in O.C.T. compound (VWR), and successive 5 μm sections were collected from the center of each bladder and placed on positively-charged microscope slides (Superfrost/Plus; Thermo Fisher Scientific). For hematoxylin-eosin (H&E) staining, Richard-Allan Scientific Signature Series Hematoxylin 7211 was used followed by Eosin-Y 7111 for counterstaining. Images were captured using the AX10 microscope (Carl Zeiss). The tumor circumferences were measured for analysis of the tumor area using ImageJ software.

2.6 | Immunohistochemistry

For immunohistochemistry analysis, cryosections were permeabilized with 0.25% Triton X-100, 5% fetal calf serum in PBS and incubated with primary anti-VEGF antibody (1:100), anti-Ki-67 antibody (1:100), anti-Cyclin D1 (1:100), IL-6(1:100), IL-1beta (1:100) or neutrophil (1:100) antibodies overnight at 4°C, followed by Alexa-Fluor 488- or 568-labeled secondary antibodies (1:200) incubation for 1 hour at room temperature. Tissues were counterstained with DAPI (4′,6-diamidine-2′-phenylindole, 0.05 mM) and examined by AX10 fluorescence microscopy (Carl Zeiss). Fluorescence quantification was done using ImageJ software.

2.7 | Alpha1-oleate uptake by tumor tissue

Alpha1-oleate peptide was labeled by Alex-Fluor 568 (A10238, ThermoFisher Scientific) and instilled into the bladders of tumor-bearing mice (day 11). The mice were sacrificed after 6 hours and the bladders were harvested as described above. Frozen bladder sections were imaged using an AX10 fluorescence microscope (Carl Zeiss) after nuclear counterstaining with DAPI.

2.8 | Western blot and dot blot analysis

Tumor markers were further quantified by dot blot and Western blot analysis. Frozen bladders from tumor bearing mice were pulverized using liquid nitrogen and lysed in a NP-40 buffer (20 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Protein concentrations were measured by Nano drop using the Pierce 660 nm protein assay reagent (Thermo Fisher, Cat# 22660) according to the

manufacturer's instructions, using bovine serum albumin (BSA) as standard. Total protein extracts (20 μg in 20 μL extraction buffer) were separated on SDS-PAGE using a 4% to 15% Tris-glycine gradient gel (Bio-Rad) and transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk, incubated overnight at 4°C with primary rabbit anti-Ki-67, anti-Cyclin D1, anti-VEGF, anti-alpha-lactalbumin antibodies (all, 1:1000) or a mouse anti-β-actin antibody (1:5000). Membranes were washed with PBS-T and then probed with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:4000) at room temperature (60 minutes) and visualized using ECL detection reagent.

2.9 | Gene expression analysis

Frozen bladder tissue was pulverized using liquid nitrogen.[18] Total RNA was extracted (RNeasy Mini kit, Qiagen). Then, 100 ng of total RNA was amplified using the GeneChip 3′IVT Express Kit and then fragmented. Next, labeled aRNA was hybridized onto Mouse Genome 430 PM array strips (Affymetrix) for 16 hours at 45°C, washed, stained (Applied Biosystems, ThermoFisher Scientific) and scanned using the GeneAtlas system (Affymetrix). All samples passed the internal quality controls included in the array strips (signal intensity by signal to noise ratio; hybridization and labeling controls; sample quality by GAPDH signal and 3′-5′ ratio < 3). Transcriptomic data was normalized using Robust Multi Average implemented in the Transcriptome Analysis Console software (v.4.0.1.36, Applied Biosystems, Thermo-Fisher Scientific). Fold change was calculated by comparing tumor bearing bladders or treated healthy bladders to untreated healthy bladder control tissue. Relative expression levels were analyzed and genes with an absolute fold change >2.0 were considered as differentially expressed. Heat-maps were constructed using the Gitools 2.1.1 software. Differentially expressed genes were functionally characterized using the ingenuity pathway analysis (IPA, Qiagen) software.

2.10 | Statistical analysis

Results are presented as means ± SEMs. P values were calculated by Student's t test or one-way ANOVA followed by Bonferroni's post hoc testing using Prism version 7 (GraphPad Software Inc., La Jolla, California). $P < .05$ was considered statistically significant. *$P < .05$; **$P < .01$; ***$P < .001$.

2.11 | Study approval

Experiments were approved by the Malmö/Lund Animal Experimental Ethics Committee at the Lund District Court, Sweden (#M118-16) or the regional animal experimental ethics committee in Stockholm (#N156/16). Animal care and protocols followed institutional, national and European Union guidelines and were governed by the European Parliament and Council Directive (2016/63, EU), the Swedish Animal Welfare Act (Djurskyddslagen 1988:534), the Swedish Welfare Ordinance (Djurskydssförordningen 1988:539) and Institutional Animal Care and Use Committee (IACUC) Guidelines.

3 | RESULTS

3.1 | Dose escalation in tumor-bearing mice

We performed a dose escalation study of alpha1-oleate treatment in the murine MB49 bladder cancer model (Figure 1A). Mice in the treatment group received five intravesical instillations of alpha1-oleate (1.7, 8.5 or 17 mM) on days 3, 5, 7, 9 and 11 as sham-treated mice received PBS (Figure 1A). The tumor mass gradually filled the bladder lumen, replacing functional bladder tissue. Bladders were harvested at sacrifice on day 12. Macroscopic variables were recorded, and bladder size and weight were quantified (Figure 1B, C and Figure S2A). The tumor size was further evaluated by histopathology (Figures 1C and 2).

Rapid tumor progression was observed in the sham-treated mice, which developed palpable tumors that altered the macroscopic appearance of the bladders, compared to controls not receiving tumor cells (11/11 mice, $P < .001$, Figures 1B and S2A). Tumor progression was significantly delayed in the alpha1-oleate treatment groups, as shown by a reduction in bladder size, bladder weight and tumor size (Figure 1B,C). A dose-dependent reduction in bladder weight, bladder size and tumor size was recorded in mice receiving 1.7, 8.5 or 17 mM of alpha1-oleate ($P < .001$ compared to sham-treated mice, Figure 1C). At the highest concentration (17 mM), no macroscopically visible tumor tissue remained ($P < .001$ compared to the sham treated group Figure 1C). The bladder weight in these mice did not differ from that in healthy control mice (n.s., Figure 1C).

The reduction in tumor size was confirmed by H&E staining of frozen tissue sections (Figures 2 and S2B). Large tumors were visible in the majority of animals in the sham treated group (7/7), resulting in a virtually complete loss of tissue structure (Figure 2A). With increasing doses of alpha1-oleate, the tumor area gradually became smaller, leaving no visible tumor in mice receiving 17 mM of alpha1-oleate (6/6, Figure 2B-D). At this concentration, bladder tissue structure was comparable to that in healthy control mice (Figure 2D, E). The results identify alpha1-oleate as a tumoricidal complex with therapeutic efficacy that increases with increasing doses of the compound, until most of the tumor is cleared.

To further understand the effect of alpha1-oleate, frozen bladder tissue sections were stained for the tumor proliferation markers Cyclin D1 and Ki-67 and for vascular endothelial growth factor (VEGF), which is essential for tumor neovascularization (Figure 3).[19] The tumor markers were abundantly expressed in the growing tumors of sham-treated mice (5/5) but alpha1-oleate treatment caused a marked, dose-dependent reduction in staining. Cyclin D1 expression was low in mice receiving 17 mM of alpha1-oleate ($P < .01$ and $P < .001$ compared to sham-treated mice, Figures 3A and S3A). Ki-67 and VEGF staining showed a similar pattern ($P < .05$-.001, Figures 3B,C and S3A). The reduction in Ki-67, Cyclin D1 and VEGF protein levels in treated tissues was confirmed by Western blot analysis of whole-bladder tissue extracts (Figure S3B,C).

FIGURE 1 Dose-dependent therapeutic effects of alpha1-oleate in a murine bladder cancer model. A, Schematic of the alpha1-oleate treatment model. Bladder cancer was induced in C57BL/6J female mice by intravesical instillation of MB49 cells (2×10^5 in 100 μL PBS). The treatment group received alpha1-oleate by intravesical instillation (1.7, 8.5 or 17 mM) on days 3, 5, 7, 9 and 11. Sham-treated mice received PBS and all mice were sacrificed on day 12. B, Dose-dependent effect of alpha1-oleate (11/11), deduced from macroscopic inspection of gross bladder pathology. C, Comparison of bladder weight, bladder size and tumor area (see also Figure 2). Data are presented as means ± SEMs of two experiments (n = 6 + 5 mice, *P < .05, **P < .01 and ***P < .001 compared to sham-treated mice). For repeated experiment, see Figure S2
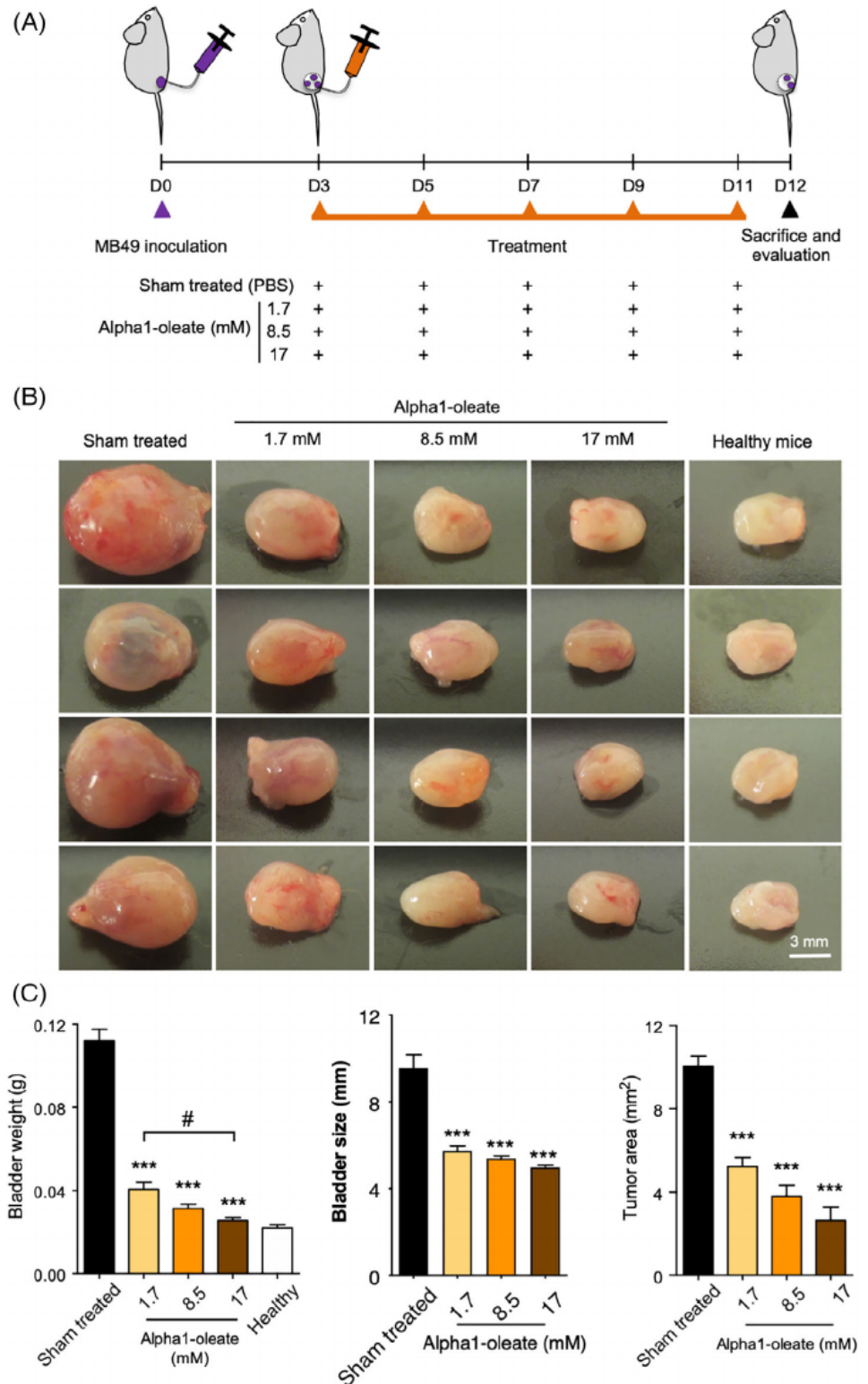
3.2 | Accumulation of alpha1-oleate in tumor tissue

To examine if alpha1-oleate reaches tumor tissue, tumor-bearing mice were inoculated with Alexa-Fluor 568 labeled alpha1-oleate on day 11, when the tumors have reached a significant size, and bladders were harvested 6 hours after instillation (n = 2, Figure 4A). By direct fluorescence imaging of frozen tissue sections, the Alexa-Fluor labeled complex was shown to accumulate in tumor tissue, in a dose-dependent manner (Figure 4B, C). In contrast, the complex was not retained in bladder tissue of healthy mice subjected to Alexa-Fluor 568-labeled alpha1-oleate instillations (Figure 4B, C). Tissue accumulation of the complex was confirmed by dot blot and Western blot analysis of whole bladder extracts from tumor-bearing mice treated
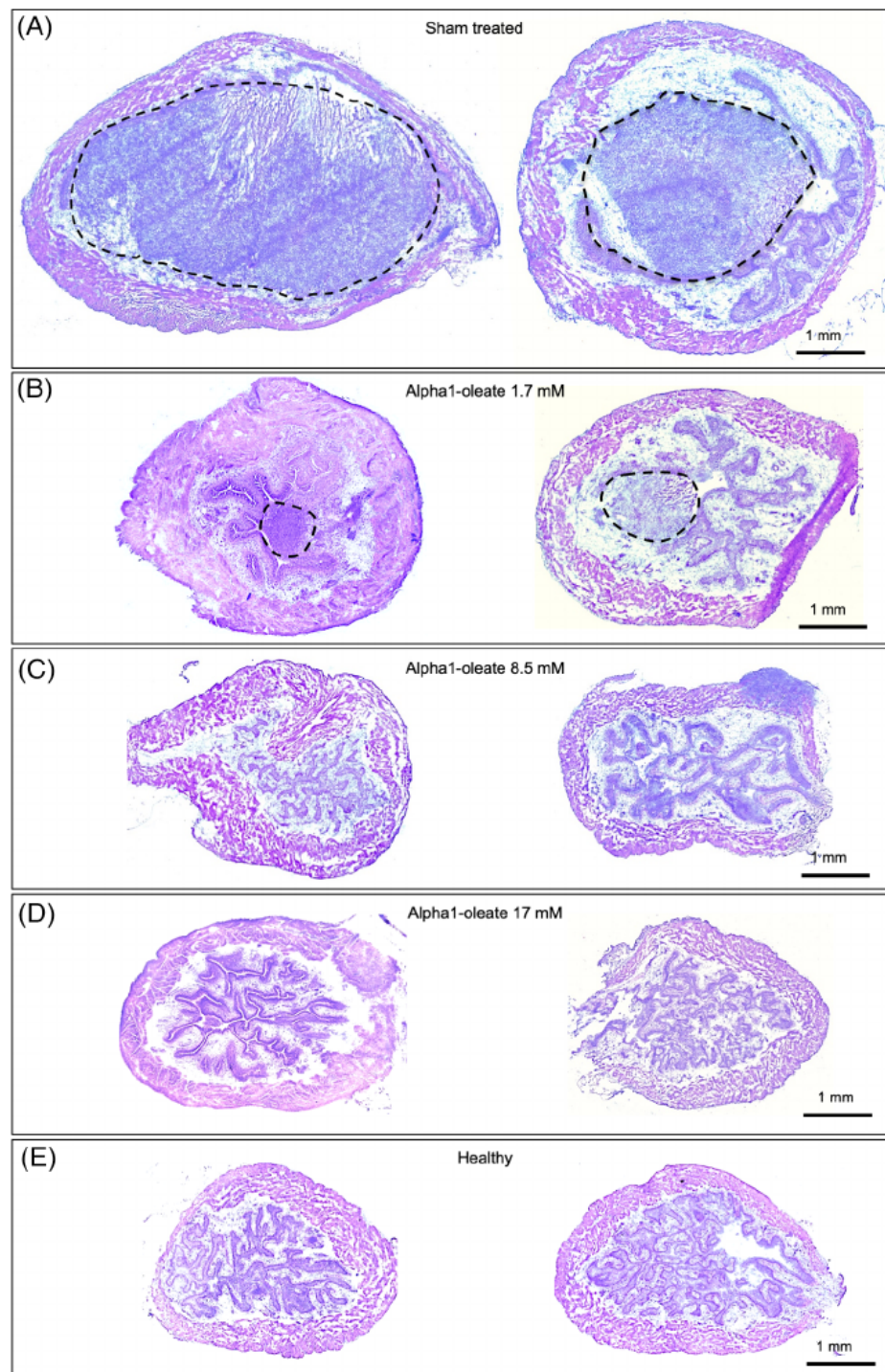
FIGURE 2 Dose-dependent reduction in tumor size. The tumor area was identified in H&E-stained whole bladder sections and quantified using ImageJ. Tumor areas were compared between sham-treated mice and mice receiving 1.7, 8.5 or 17 mM of alpha1-oleate. A, Sham-treated mice show large tumors filling the bladder lumen (4/4, dotted line). B-D, Alpha1-oleate treated mice show a dose-dependent reduction in tumor size. For quantification, see Figure 1C. Data are presented as means ± SEMs of two experiments (n = 6 + 5 mice, *P < .05, **P < .01 and ***P < .001 compared to sham-treated mice). E, Healthy bladder controls. Representative images, n = 5 mice per group, two experiments

with alpha1-oleate (Figures 4D, E and S4). Tissues from sham treated, tumor-bearing mice were used as negative controls (Figures 4D,E and S4).

3.3 | Dose-dependent gene regulation in tumor-bearing mice

To further characterize the tumor response to alpha1-oleate, total bladder RNA was subjected to whole-genome transcriptomic profiling.

Gene expression profiles were compared between the sham treated group and mice receiving increasing doses of alpha1-oleate. Healthy mice bladder tissue served as controls.

The transcriptomic analysis revealed major differences between sham-treated, tumor bearing mice and healthy controls (Figure 5). In tumor tissue, 2365 genes were upregulated and 2391 were suppressed (Figure 5A,B), including classical genes known to regulate cancer development (Figure 5C,D). Top regulated genes included *Cxcl3*, *C15orf48*, *S100a8*, and *Il2rg* (Figure 5E). In the alpha1-oleate treated groups, dose-dependent reduction in the number of
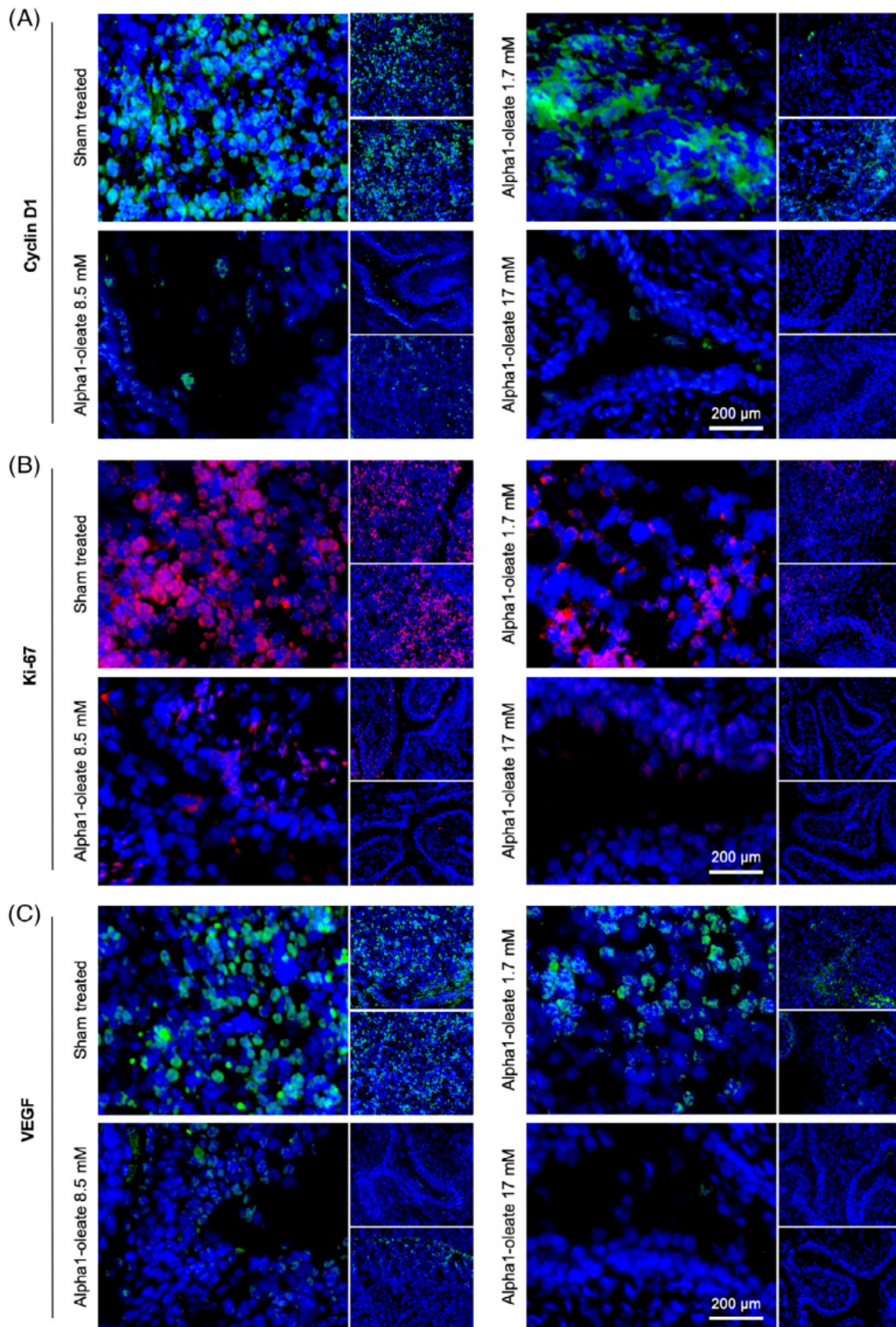
FIGURE 3 Dose-dependent reduction in tumor markers. Frozen bladder tissue sections from sham-treated or alpha1-oleate treated mice were examined by immunohistochemistry, after staining with specific antibodies. A, Dose-dependent reduction in Cyclin D1, B, Ki-67 and C, VEGF staining was observed in the tumor area of alpha1-oleate treated mice, compared to sham-treated controls (1.7, 8.5 or 17 mM). For quantification of Ki-67, Cyclin D1 or VEGF expression, see Figure S3. Three representative images, n = 5 mice per group, two experiments. Data are presented as means ± SEMs of two experiments (n = 6 + 5 mice, *P < .05, **P < .01 and ***P < .001 compared to sham-treated mice)

differentially expressed genes was observed, consistent with the drastic reduction in tumor size in these mice (heat map in Figure 5A). The number of differentially expressed transcripts in sham treated and alpha1-oleate treated mice, is also illustrated in the Venn diagram (Figure 5B). The molecular mechanisms of cancer pathway genes were deactivated in a dose-dependent manner, including *Cdk1*, *Casp3*, *E2f*
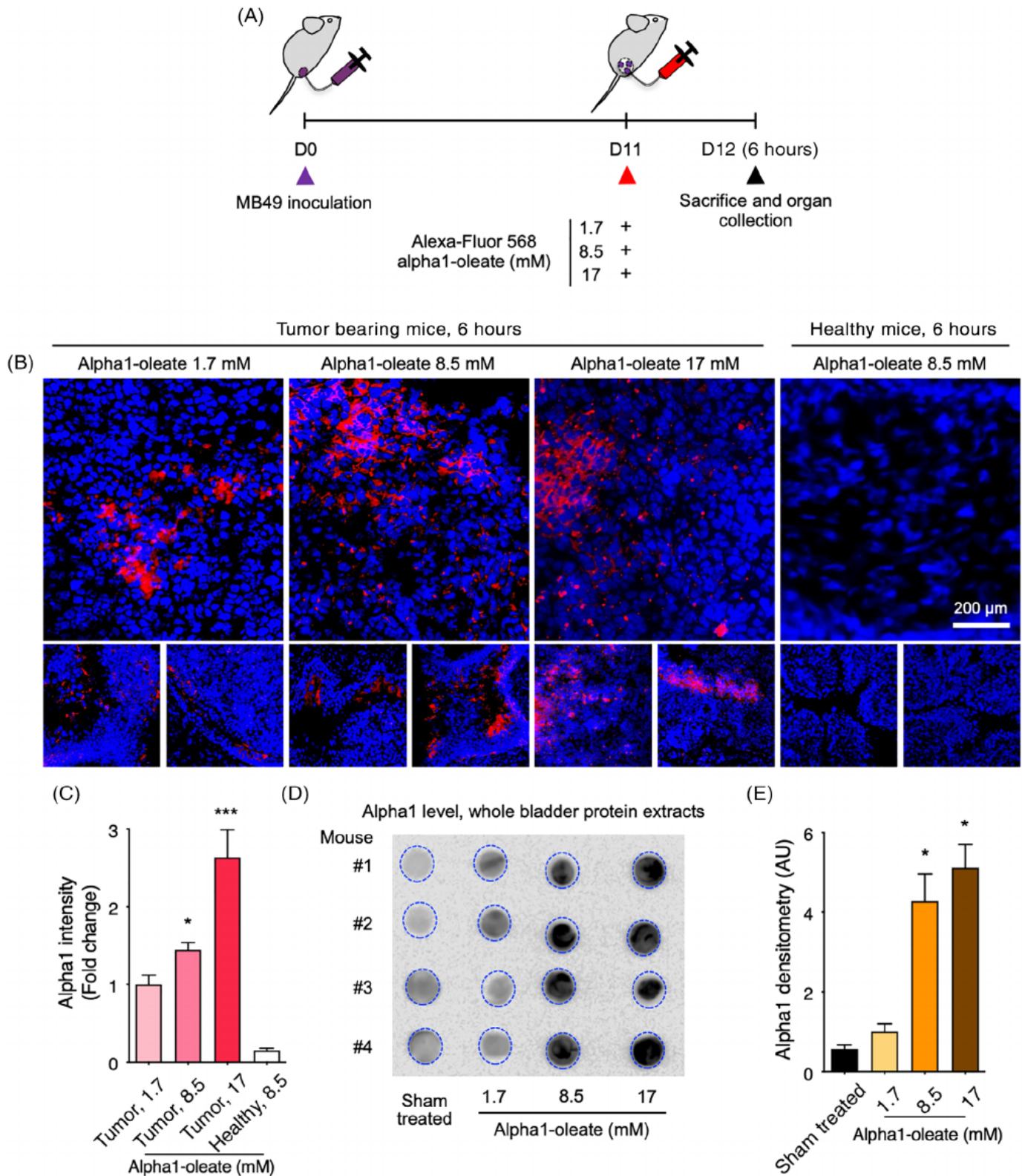
FIGURE 4 Accumulation of alpha1-oleate in tumor tissue. Alexa-Fluor 568-labeled alpha1-oleate was used to challenge tumor-bearing mice and to track the molecule in tumor tissue. A, Scheme of intravesical alpha1-oleate challenge. Intravesical instillations were performed on day 11, the tissues were harvested after 6 hours and healthy mice challenged with Alexa-Fluor 568-labeled alpha1-oleate were used as controls. B, Alexa-Fluor 568-labeled alpha1-oleate was visualized in frozen tissue sections by confocal imaging. C, Quantification of fluorescence intensity in B. Means ± SEMs, 5 images per dose (*$P < .05$ and *** $P < .001$). D, Quantification alpha1-oleate levels in bladder tissue. Dot blot analysis of whole bladder extracts. Filters were stained with anti-alpha1 antibodies. Elevated levels were detected in alpha1-oleate treated tumor-bearing mice compared to sham-treated mice (*$P < .05$). E, Quantification of alpha1-oleate levels in D, means ± SEMs, n = 4
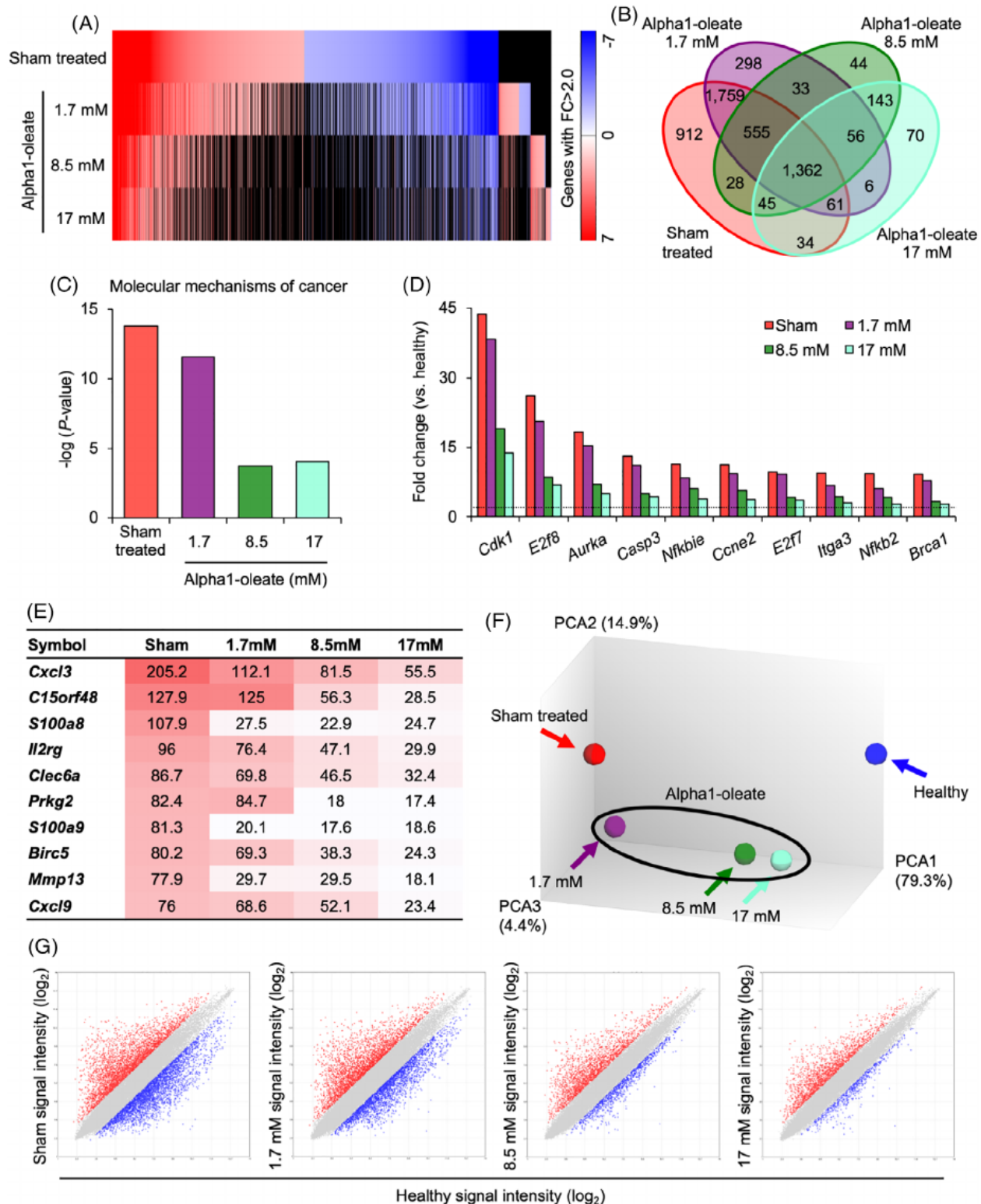
FIGURE 5 Dose-dependent changes in gene expression in tumor-bearing mice. A, Heat map showing a dose-dependent reduction in the number of regulated genes in treated mice. Red: upregulated genes, blue: downregulated genes, black: not regulated genes, cut-off fold change ≥2.0 compared to healthy bladder tissue. B, Venn diagram of significantly regulated genes in the sham- or alpha1-oleate-treated mice. C, A dose-dependent reduction in the molecular mechanisms of cancer pathway genes was observed. D, Effects on individual genes in this pathway. E, Top genes upregulated in the sham-treated bladders. F, Principal component analysis of mRNA profiles in whole bladder tissue. Increasing doses of alpha1-oleate shifted the transcriptomic profiles from the tumor-bearing, sham-treated cluster toward healthy bladder tissue cluster. G, Scatter plot of probes comparing transcriptomic profiles in bladders from tumor-bearing mice to healthy mice (log$_2$ signal intensity values; cut-off fold change ≥2.0 compared to healthy bladder tissue)
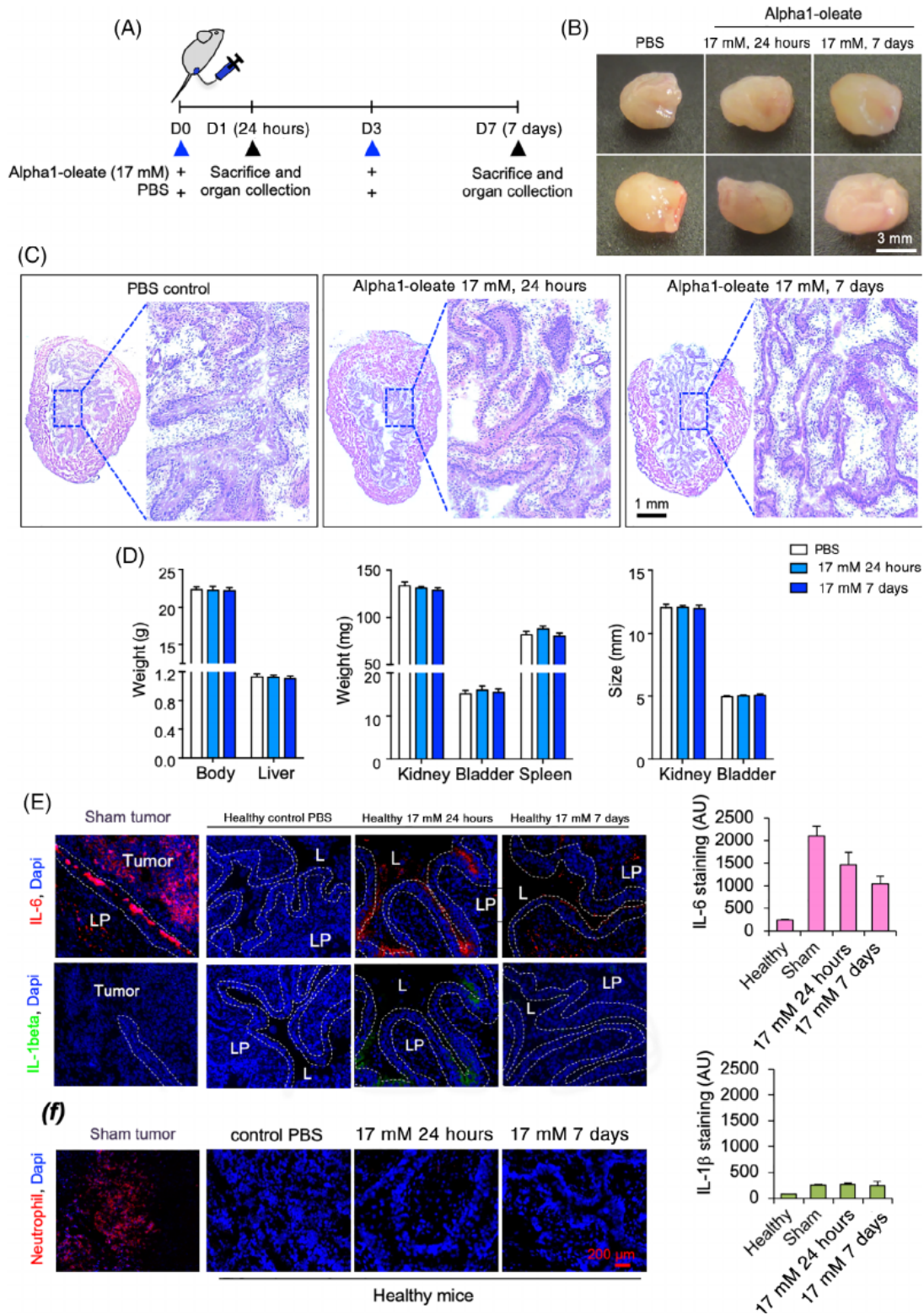
FIGURE 6 Lack of toxicity of alpha1-oleate (17 mM) in C57BL/6J mice. A, Schematic model for toxicity testing in healthy mice. Alpha1-oleate was instilled intravesically in C57BL/6J female mice (17 mM). The acute group was sacrificed after 24 hours (n = 3) and the second group after 7 days (n = 3). Controls received PBS (n = 6). B, Alpha1-oleate did not alter the macroscopic appearance of the bladders. C, No change was detected in tissue structure or organ weight and size, bladder weight, bladder size, kidney weight, kidney size, body weight, liver weight and spleen weight, D, in healthy mice receiving 17 mM alpha1-oleate. Representative H&E stained bladder tissue sections, n = 2-4 mice per group. E,F, Frozen bladder tissue sections from sham-treated tumor-bearing mice, healthy mice and healthy mice receiving 17 mM alpha1-oleate 24 hours and 7 days were examined by immunohistochemistry. Bladder tissue sections were stained with specific antibodies IL-6, IL-1β and neutrophil

variants and nuclear factor kappa B (NF-κB variants, Figure 5C,D). By principal component analysis (PCA, Figure 5F), tumor-bearing, sham-treated mice formed a distinct cluster, which was clearly separated from the healthy controls and PCA1 (79.3% of the variation) was dominated by differences between sham-treated mice and healthy bladders. Among the alpha1-oleate treated mice, their gene expression profile shifted toward that in healthy mice, in a dose-dependent manner. Scatter plot analysis of probe signal intensities confirmed the approximation of the transcriptomic profiles in the treated mice to that in healthy tissue (Figure 5G). This dose-dependent separation of the alpha1-oleate treated from the sham-treated cluster and approximation to the healthy control cluster, suggests that the gene expression profiles of the treated bladders gradually become more similar to healthy tissue.

3.4 | Evaluation of acute toxicity in healthy mice

Intravesical instillations of alpha1-oleate (17 mM) were performed in healthy mice to investigate if alpha1-oleate is toxic for bladder tissue. One group was sacrificed after 24 hours (1 dose, n = 3 mice) and a second group on day 7 (3 dose, n = 3, Figure 6A). By macroscopic inspection, the bladders remained normal and the bladder weight was not significantly altered (Figure 6B-D). Furthermore, the body weight and weight of other organs was unchanged (kidneys, livers, spleens)
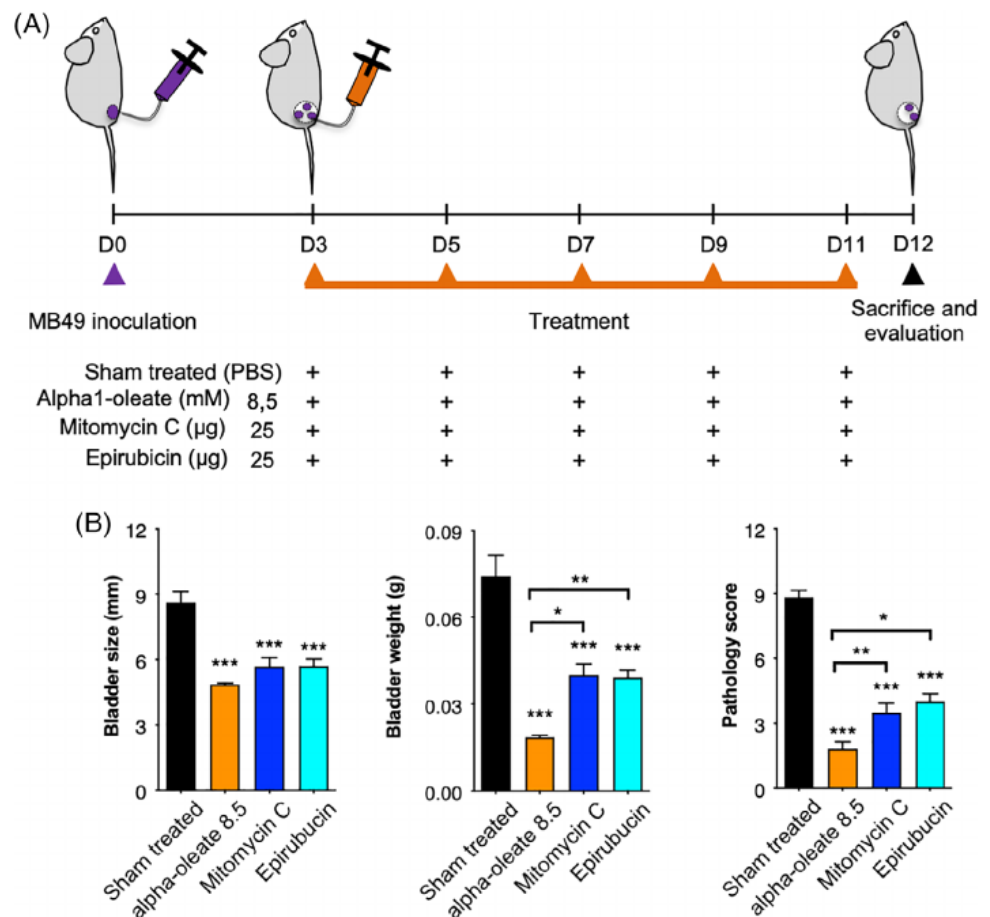
compared to healthy controls receiving PBS (Figure 6D). Bladder tissue integrity was retained as defined by histopathology of frozen tissue sections (Figure 6C), suggesting that alpha1-oleate is not toxic for healthy bladder tissue during the first week after instillation.

The tissue response to alpha1-oleate was further examined by staining of frozen tissue sections from mice inoculated with 17 mM of alpha1-oleate, compared to sham-treated mice and healthy controls not receiving alpha1-oleate. Significant Interleukin-6 (IL-6) staining was detected in the sham-treated mice, mainly in the tumor area. The IL-6 response probably reflected the biology of the disease rather than "inflammation" in general, as there was no IL-1 response and no neutrophil infiltration in these mice.

Healthy bladder tissue did not show detectable levels of IL-6 or IL-1 but in healthy mice treated with alpha1-oleate (17 mM), weak IL-6 staining was detected after 24 hours. The IL-6 response had subsided after 7 days despite repeated inoculations. There was no IL-1 response after 24 hours or 7 days and there was no evidence of neutrophil infiltration, as would be expected if this was an inflammatory response (Figure 6E,F).

3.5 | Evaluation of long-term toxicity

Toxicity was further investigated in healthy NMRI mice subjected to repeated intravesical instillations of 1.7 or 18 mM alpha1-oleate or

FIGURE 7 Therapeutic efficacy of alpha1-oleate compared to Mitomycin C or Epirubicin. A, Schematic of the comparative treatment protocol. Bladder cancer was induced as described in Figure 1 and the treatment groups received alpha1-oleate (8.5 mM), Mitomycin C (25 μg) or Epirubicin (25 μg) on days 3, 5, 7, 9 and 11. Sham-treated mice received PBS and all mice were sacrificed on day 12. B, Comparison of bladder weight, bladder size and pathology score between sham-treated mice, mice receiving 8.5 mM alpha1-oleated, 25 μg Mitomycin or 25 μg Epirubicin (n = 6, *P < .05, **P < .01 and ***P < .001 compared to sham-treated mice)

PBS twice weekly for 4 weeks (Figure S5). At necropsy, no macroscopic signs of toxicity or change in organ weights were noted. Furthermore, the health status remained normal. By histopathology, there were no findings related to alpha1-oleate treatment in urinary bladders and no variation by hematological or clinical chemistry analyses, related to alpha1-oleate (Tables S1 and S2). In mice subjected to repeated intravesical instillations of 18 mM of alpha1-oleate, slight inflammatory changes were seen in two mice animals treated with alpha1-oleate and a slight to moderate submucosal infiltration of inflammatory cells was detected by histopathology, mainly of neutrophils and lymphocytes. This response was not seen in the remaining mice or in the recovery groups.

The local tolerance to alpha1-oleate was further evaluated in female NZW rabbits after repeated intravesical instillations of alpha1-oleate (2.45 mM), twice weekly for 4 weeks (Figure S6). The health status of the rabbits remained normal and no signs of toxicity or changes in organ weights were noted. Variation in hematology and clinical chemistry parameters was within the normal range (Tables S3 and S4). The results suggest that repeated instillations of alpha1-oleate over a 23-day period are well tolerated and cause no local or systemic toxicity.

3.6 | Comparative analysis of therapeutic efficacy

The therapeutic efficacy of alpha1-oleate (8.5 mM) was subsequently compared to conventional bladder cancer chemotherapy (Figure 7A). MB49 tumors were established as described (Figure 7A). Mice were subjected to repeated intravesical instillations of Mitomycin C (25 μg/dose) or Epirubucin (25 μg/dose), using the same protocol as for alpha1-oleate. At sacrifice on day 12, macroscopic variables were quantified to assign a pathology score and bladder size and weight were measured (Figure 7B). The results show significant treatment effects of Mitomycin C, Epirubucin and alpha1-oleate (Figure 7B).

4 | DISCUSSION

The limitations of current bladder cancer therapies are a major concern. While surgical and pharmacological interventions have been optimized, recurrence rates remain high and progression to invasive disease occurs quite frequently. Immunotherapy and chemotherapy is useful but limited by the risk of serious side effects.[20] This study investigated the therapeutic efficacy of the peptide-fatty acid complex alpha1-oleate, using a dose-escalation protocol. Bladder cancer development was effectively delayed and at the highest dose, tumor tissue was virtually absent. Furthermore, toxicity for healthy tissue was not detected in treated, tumor bearing mice or healthy control mice challenged with alpha1-oleate. The findings identify alpha1-oleate as a drug candidate with therapeutic efficacy against bladder cancer without a parallel increase in tissue toxicity.

Alpha-lactalbumins are structurally flexible and form stable, protein-fatty acid complexes, most prominently with oleic acid.

Comparisons with the native proteins, which lack tumoricidal activity, have emphasized the importance of structural flexibility for the mechanism of action in tumor cells.[14] In this study, the N-terminal alpha-helical domain of alpha-lactalbumin was produced synthetically, and the oleic acid complex was formed under standardized conditions. By NMR spectroscopy and molecular modeling, the alpha1 peptide has been shown to retain structural flexibility when bound to oleic acid; features linked to potent membrane effects in tumor cells that initiate the cell death response.[17] In addition to plasma membrane remodeling, the mechanism of action includes internalization of the complex, nuclear translocation and chromatin interactions, inhibiting cell proliferation and preventing repair.[15] Furthermore, broad inhibitory effects on oncogenes such as the Ras GTPases, cancer related signaling pathways and kinase activity and have been identified.[15]

The rapidly growing MB49 murine bladder cancer cell line establishes a significant tumor mass within 2 weeks of instillation. The treatment window used here (day 3-11) was selected to efficiently limit tumor progression. The prominent, dose-dependent therapeutic effect of alpha1-oleate was documented using several end points. By macroscopic inspection, visible tumor mass was reduced in all treated mice and at the highest concentration of alpha1-oleate, the increase in bladder size caused by tumor growth was virtually abolished. The dose-dependent reduction in tumor area was documented by histopathology, suggesting that the malignant process is inhibited in a dose-dependent manner. This was further supported by a dose-dependent decrease in tumor markers to levels seen in healthy bladder tissue. The reduction in Cyclin D1, Ki-67 and VEGF levels in the treated tumors, suggested anti-proliferative and anti-angiogenetic effects. A gradual loss of tumor mass was clearly accompanied by a change in gene expression, with increasing doses of alpha1-oleate, indicating a shift toward a healthy phenotype in the treated bladder tissue. The results suggest that alpha1-oleate treatment prevents tumor progression by depleting the tissues of tumor cells, leaving behind healthy tissue stroma.

The present study further showed that the increase in therapeutic efficacy occurred without a parallel increase in side effects. Tissue toxicity was not detected in the treated mice or after intravesical challenge of healthy mice. This included exposure of healthy mice to 18 mM of alpha1-oleate, which exceeds the highest therapeutic concentration in the dose-escalation study (17 mM). Extensive analyses of lower doses performed in mice and rabbits confirmed this lack of toxicity. The findings are unusual, as increasing doses of chemotherapy almost invariably increase the tissue toxicity but the lack of toxicity has been validated in clinical trials. In a recent placebo-controlled study of alpha1-oleate in patients with NMIBC, a rapid tumor response was detected within 2 hours of alpha1-oleate instillation, with tumor cell shedding and release of tumor fragments into the urine and after 1 month, a significant increase in tumor cell apoptosis was accompanied by a reduction in tumor size (manuscript in preparation). The analysis did not detect drug-related side effects, supporting that efficacy of alpha1-oleate treatment can be achieved without side effects of the drug.

Bladder cancer progression was previously shown to be delayed in mice treated with HAMLET; a complex formed by the alpha-lactalbumin holo-protein and oleic acid.[16] Clinical relevance was

demonstrated in patients with bladder cancer, where rapid tumor cell shedding occurred after intravesical instillation of the HAMLET complex.[21] The alpha1-oleate complex has been shown to reproduce essential effects of HAMLET in cancer cells. Like HAMLET, alpha1-oleate perturbs tumor cell membranes and triggers rapid ion fluxes, inducing Na^+ and Ca^{2+} influx and K^+ efflux.[22] HAMLET inhibits Ras and MAPK signaling,[22] which play important roles in tumor cells death and a large number of additional kinases are affected, as well. Like HAMLET, alpha1-oleate accumulates in tumor cell nuclei[15] and perturbs nucleosome formation by binding to histones.[23] Furthermore, HAMLET interacts with the F-ATP synthase and inhibits ATP formation.[24] As cancer cells are dependent on glycolysis HAMLET's effects on F-ATP synthase can reduce cellular energy and cause cell death.[24] HAMLET inhibits 20S proteasomes in tumor cells by disintegration or fragmentation[25] and interacts with mitochondria causing swelling and depolarization of mitochondrial membranes[26] and release of cytochrome c results in caspase activation. These mechanisms have not yet been investigated for the alpha1-oleate complex.

Mitomycin C, which is widely used for intravesical chemotherapy of newly diagnosed and frequently recurring NMIBC, reduces tumor recurrence rates and prolongs disease free intervals, but with significant side effect.[27] Currently, due to a shortage of BCG and Mitomycin C, the onco-urological community resorts to alternatives such as Epirubicin or Doxorubicin.[28] We therefore compared the effects of alpha1-oleate to Mitomycin C and Epirubicin, in the MB49 model. A similar level of efficacy was detected for alpha1-oleate (8.5 mM) and the two chemotherapeutic agents, suggesting that alpha1-oleate might be useful as a standalone therapy, especially in view of the low toxicity advantage. Exploring the effects of combination therapy will also be of significant interest. The dose-escalation study in mice presented here is encouraging, as increased doses clearly improved the outcome. The findings suggest that human dose-escalation studies should be viewed with optimism and such studies are being initiated to define the therapeutic window in upcoming clinical trials.

CONFLICT OF INTEREST
C. S. holds shares in HAMLET Pharma, as a representative of scientists in the HAMLET group.

DATA ACCESSIBILITY
The microarray data for our study have been deposited in the NCBI's Gene Expression Omnibus repository and is accessible through GEO Series accession number GSE148308. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES
1. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12:252-264.
2. Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc Chem Res*. 2007;41:98-107.
3. Hu Q, Sun W, Wang C, Gu Z. Recent advances of cocktail chemotherapy by combination drug delivery systems. *Adv Drug Deliv Rev*. 2016; 98:19-34.
4. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell*. 2015;161: 205-214.
5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65:5-29.
6. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
7. Jalilian H, Ziaei M, Weiderpass E, Rueegg CS, Khosravi Y, Kjaerheim K. Cancer incidence and mortality among firefighters. *Int J Cancer*. 2019;145:2639-2646.
8. Soloway MS, Sofer M, Vaidya A. Contemporary management of stage T1 transitional cell carcinoma of the bladder. *J Urol*. 2002;167:1573-1583.
9. Stein JP, Lieskovsky G, Cote R, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J Clin Oncol*. 2001;19:666-675.
10. Kang M, Jeong CW, Kwak C, et al. Single, immediate postoperative instillation of chemotherapy in non-muscle invasive bladder cancer: a systematic review and network meta-analysis of randomized clinical trials using different drugs. *Oncotarget*. 2016;7:45479.
11. Sylvester RJ, Oosterlinck W, Holmang S, et al. Systematic review and individual patient data meta-analysis of randomized trials comparing a single immediate instillation of chemotherapy after transurethral resection with transurethral resection alone in patients with stage pTa–pT1 urothelial carcinoma of the bladder: which patients benefit from the instillation? *Eur Urol*. 2016;69:231-244.
12. Kamat AM, Hahn NM, Efstathiou JA, et al. Bladder cancer. *Lancet*. 2016;388:2796-2810.
13. Malmström P-U, Sylvester RJ, Crawford DE, et al. An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus bacillus Calmette-Guérin for non–muscle-invasive bladder cancer. *Eur Urol*. 2009;56:247-256.
14. Mok KH, Nagashima T, Day IJ, Hore PJ, Dobson CM. Multiple subsets of side-chain packing in partially folded states of α-lactalbumins. *Proc Natl Acad Sci U S A*. 2005;102:8899-8904.
15. Nadeem A, Sanborn J, Gettel DL, et al. Protein receptor-independent plasma membrane remodeling by HAMLET: a tumoricidal protein-lipid complex. *Sci Rep*. 2015;5:16432.
16. Mossberg A-K, Hou Y, Svensson M, Holmqvist B, Svanborg C. HAMLET treatment delays bladder cancer development. *J Urol*. 2010;183: 1590-1597.
17. Ho J, Rydstrom A, Manimekalai MSS, et al. Low resolution solution structure of HAMLET and the importance of its alpha-domains in tumoricidal activity. *PLoS One*. 2012;7:e53051.
18. Hien TT, Garcia-Vaz E, Stenkula KG, et al. MicroRNA-dependent regulation of KLF4 by glucose in vascular smooth muscle. *J Cell Physiol*. 2018;233:7195-7205.
19. Shariat SF, Youssef RF, Gupta A, et al. Association of angiogenesis related markers with bladder cancer outcomes and other molecular markers. *J Urol*. 2010;183:1744-1750.
20. Dovedi SJ, Davies BR. Emerging targeted therapies for bladder cancer: a disease waiting for a drug. *Cancer Metastasis Rev*. 2009;28:355-367.

21. Mossberg AK, Wullt B, Gustafsson L, Månsson W, Ljunggren E, Svanborg C. Bladder cancers respond to intravesical instillation of (HAMLET human α-lactalbumin made lethal to tumor cells). *Int J Cancer*. 2007;121:1352-1359.

22. Storm P, Klausen TK, Trulsson M, et al. A unifying mechanism for cancer cell death through ion channel activation by HAMLET. *PLoS One*. 2013;8:e58578.

23. Düringer C, Hamiche A, Gustafsson L, Kimura H, Svanborg C. HAMLET interacts with histones and chromatin in tumor cell nuclei. *J Biol Chem*. 2003;278:42131-42135.

24. Ho J, Sielaff H, Nadeem A, Svanborg C, Grüber G. The molecular motor F-ATP synthase is targeted by the tumoricidal protein HAMLET. *J Mol Biol*. 2015;427:1866-1874.

25. Gustafsson L, Aits S, Önnerfjord P, Trulsson M, Storm P, Svanborg C. Changes in proteasome structure and function caused by HAMLET in tumor cells. *PLoS One*. 2009;4:e5229.

26. Köhler C, Gogvadze V, Håkansson A, Svanborg C, Orrenius S, Zhivotovsky B. A folding variant of human α-lactalbumin induces mitochondrial permeability transition in isolated mitochondria. *Eur J Biochem*. 2001;268:186-191.

27. Colomer AG, Martínez RR, Castillo CP, et al. Dermatological side effects of intravesical Mitomycin C: delayed hypersensitivity. *Arch Esp Urol*. 2016;69:89-91.

28. Chou R, Selph S, Buckley DI, et al. Intravesical therapy for the treatment of nonmuscle invasive bladder cancer: a systematic review and meta-analysis. *J Urol*. 2017;197:1189-1199.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.