Original article

The effect of undecanones and their derivatives on tumor angiogenesis and VEGF content

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Abstract

The *in vivo* effects of some derivatives of aliphatic ketones (2-undecanone, 3-undecanone, 4-undecanone and their derivatives) on L-1 sarcoma tumor angiogenesis and VEGF content were studied in Balb/c mice. Mice that inhaled 10% solution of 3-undecanone(3-on) or 1% solution of 2-undecanone propylene acetal (Acpr2) for 3 days after tumor cells implantation, presented lower neovascular response measured by tumor-induced cutaneous angiogenesis test (TIA) and lower tumor VEGF content in 5-days tumors, than non-inhaled controls. Other substances presented various effects on tumor VEGF concentration and angiogenesis. Histological examination of lesions collected from mice inhaled Acpr2, or non-inhaled controls, revealed small diffused areas of necrosis in the former group. In both groups, slight to moderate inflammatory infiltrations were seen at the tumor's margin. In Acpr2 group, there were less small blood vessels at tumor's margin than in the control group.

Key words: undecan-x-ones, tumors, mice, angiogenesis, VEGF

Introduction

Aliphatic methyl ketones are products of metabolic transformation of fatty acids.

2-undecanone(methyl nonyl ketone) and its derivative undecan-2-ol, are often used as flavoring agents in cosmetics and food industries, according to decisions of FAO/WHO Expert Committee on Food Additives and the EU/Animal food regulations. 2-undecanone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included methyl nonyl ketone at a level of 3 ppm in the list of artificial flavoring substances that may be added to

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foodstuffs without hazard to public health. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg. The acute oral LD_{50} for white mice was found to be 3.88 g/kg (Srepel and Akacic 1962). Some undecanones are present in plants (2-undecanone, 3-undecanone, 2-undecanol), others have not been found in nature. Substantial amounts of 2-undecanone and 2-undecanol are present in essential oils of herbs belonging to the Rutacea family, but also in rhizome and essential oil of ginger (*Zingiber officinale* Roscoe) and other plants. Dextrorotatory undecan 2-ol was extracted from the cacao butter.

We have described previously (Gibka, Gliński 2008) olfactory properties of straight-chain undecan-x-ones, undecan-x-ols(x=2-5) and their derivatives. Most of them have pleasant odors. The most valuable were ketones and their acetals, with intensive, fruity, vegetable, spicy, or herbaceous odors.

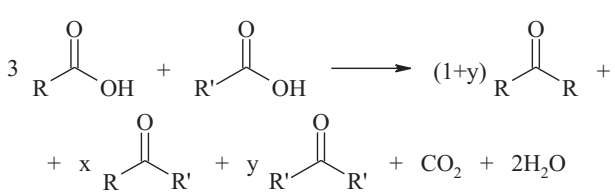
2-undecanone has been recognized as an antifungal, anti-bacterial, anti-viral, antihelmintic and cytotoxic substance. Its antiviral activity was observed against HSV-1, against influenza virus, and against HIV-1. Anti-viral concentrations were lower than concentrations cytotoxic for human and animal tumor cell lines (Hayashi et al 1995).

Recently, we have reported some data on the immunotropic effects of undecan-2-one , undecan 3-one and their derivatives in mice. Some of these substances, introduced to mice by inhalation, influenced cellular and humoral immunity (Skopińska-Różewska et al. 2006, Gibka et al. 2008a,b).

The aim of the present study was to evaluate the *in vivo* effect of 2-undecanone, 3-undecanone, 4-undecanone, and their derivatives, on the early events accompanying development of Sarcoma L-1 tumor in mice skin: neovascular response and vascular-endothelial growth factor (VEGF) production. Previously, we have used this model for evaluating anti-tumor and anti-angiogenic activity of various other substances of synthetic and natural origin (Skopińska-Różewska et al. 1999, Sommer et al. 2001, Skopińska-Różewska et al. 2003, Bany et al. 2004, Siwicki et al. 2004, Wasiutyński et al. 2005, Nartowska et al. 2005, Wasiutyński et al. 2006, Skopińska-Różewska et al. 2007, Skopińska-Różewska et al. 2008a,b).

Materials and Methods

A series of undecan-x-ones (x=2-4) was prepared by vapour phase catalytic ketonization of mixtures of aliphatic monocarboxylic acids according to the equation:



R, R' = CH_3, C_2H_5, n-C_3H_7, n-C_4H_9, n-C_5H_{11}; R + R' = $C_{10}H_{21}$; x +2y = 1

Three ketones were formed – an unsymmetric ketone and two symmetric ones when various acids were used as substrates.

Syntheses were carried out in a tubular quartz reactor filled with 20wt% MnO_2/Al_2O_3 catalyst heated by an electric furnace to the reaction temperature (673-698 K). A liquid mixture of acids was dropped to the reactor chamber, evaporated there and mixed with a stream of nitrogen which had been passed through the catalyst bed. Liquid reaction products were condensed, washed with a $NaHCO_3$ solution and dried over anhydrous $MgSO_4$. The final separation of ketones was achieved by fractional distillation through a 50 cm long vacuum jacketed glass Vigreux column. Typical yields were in the range of 61-70%. The details of the method used, physical properties and spectral data of the obtained ketones have already been described (Gliński, Gibka 2004, Gibka, Gliński 2006).

All derivatives of undecan-x-ones were prepared in a 2-5 g scale and were purified by double distillation under reduced pressure. The details of the synthesis are summarized briefly for each group of compounds in three general procedures given below. Ethylene and propylene acetals of undecan-x-ones, undecan-x-ols and their acetates were formed according to the following schemes:

Ethylene and propylene acetals of undecan-x-ones

A mixture of a ketone (4.26 g, 25 mmole), ethylene glycol (2.33 g, 37.5 mmole) or propylene glycol (2.85 g, 37.5 mmole), 1 g of sulfo- salicylic acid and 70 cm^3 of toluene was heated under reflux for 1.5 h. Water was separated as a heteroazeotrope with toluene in a Dean-Stark trap. After cooling, water (100 cm^3) was added to the reaction mixture and the layers were separated. The water layer was extracted (3 x 20 cm^3) with toluene and organic layers were collected, washed with brine and with 5% Na_2CO_3 solution and dried over $MgSO_4$. After evaporation of toluene in a rotatory evaporator, the crude product was distilled under reduced pressure.

Undecan-x-ols

To the stirred solution of an undecan-x-one (4.26 g, 25 mmole) in 100 cm^3 of propan-2-ol a solution of $NaBH_4$ (1.00 g, 26 mmole) in 30 cm^3 50/50 v/v, water-propan-2-ol was added dropwise at room temperature. The mixture was stirred for 10 h, 150 cm^3 of water were added and 2-propanol was distilled off in

a rotatory evaporator. The resulted mixture was separated, the water layer extracted with hexane (3x20 cm^3) and extracts were collected. The organic layer was washed with brine, then with water until neutral and dried over $MgSO_4$. After evaporation of toluene in a rotatory evaporator, the crude product was distilled under reduced pressure.

Undec-x-ol acetates

A mixture of an undecan-x-ol (2.58 g, 15 mmole), acetic anhydride (1.60 g, 16 mmole) and anhydrous sodium acetate (0.50 g, 6 mmole) was heated under reflux for 6 h. To the cold mixture 50 cm^3 of water was added and heated under reflux for 15 min. After cooling, the layers were separated, the water layer was extracted with toluene (3x30 cm^3). The organic layers were collected, washed with brine and with 5% $NaHCO_3$ solution until neutral and dried over $MgSO_4$. After evaporation of toluene in a rotatory evaporator, the crude product was distilled under reduced pressure.

Synthesis of enantiomers

Enantiomerically pure undecan-2-ols and undecan-2-ol acetates were prepared by biocatalytic enantioselective synthesis. Products of these reactions were separated on the column chromatography. Column chromatography was performed on silica gel (Kieselgel 230-400 mesh) and mixture of hexan, etyl acetate. Specific rotations of enantiomers were measured in etanol with an Autopol (Rudolf) polarimeter.

The purity of compounds: was analyzed by GC. Analyses were performed using chromatograph HRGC 5300 Carlo Erba, FID, DB-1701 capillary column. The identity of compounds was confirmed by GC/MS, IR, ^1H NMR and the measurements of refractive index. The ^1H NMR spectra were recorded on a Bruker 250 DPX spectrometer, in $CDCl_3$ using TMS as the internal standard. The IR spectra were measured using a Shimadzu IR 408 spectrometer (film).

Animals

The study was performed on 10-12 weeks old female inbred Balb/c mice delivered from the Polish Academy of Sciences breeding colony. For all experiments the animals were handled according to the Polish law on the protection of animals and National Institute of Health (NIH) standards. All experiments were accepted by the local Ethics Committee (No. 1/N/WDP-1/19.01.2006).

L-1 Sarcoma

Sarcoma cells were delivered from the Warsaw's Cancer Center Collection, and then passaged through several generations of Balb/c mice. Briefly, sarcoma cells were grafted

(10^6 /0.1 ml) subcutaneously into the subscapular region. After 14 days, the tumours were excised, cut to smaller pieces, rubbed through sieve and suspended in 5 ml of PBS. The suspension was left for 10 min at room temperature. After sedimentation of tissue debris, the supernatant was collected and centrifuged for 10 min at 1400 rpm. Obtained sarcoma cells were washed once with PBS for 10 min, then centrifuged at 1500 rpm, and resuspended in Parker medium in concentration of $4x10^6$ /ml (for experiments) or 10^7 /ml(for the next passage).

Angiogenesis induced in the skin of Balb/c mice after grafting of L-1 sarcoma cells

Cutaneous angiogenesis assay was performed as previously described (Skopińska-Różewska et al. 1999, Skopińska-Różewska et al. 2007). Briefly, multiple (4-6) 0.05 ml samples of 200 thousand of cells were injected intradermally into partly shaved, narcotised Balb/c mouse (at least 3 mice per group). In order to facilitate the localization of cell injection sites later on, the suspension was colored with 0.1% of trypan blue. On the day of cells grafting and on the following two days mice were subjected to inhalations, according to the following scheme: 5 mice in one cage, 5 drops of tested compound for 60 minutes, the cage covered by linen during inhalation. The cages with control mice were accordingly covered by linen for 60 minutes. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6x, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels , directed to the point of cells injection, differ from the background vasculature in their tortuosity and divarications. All experiments were performed in anesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

In some experiments, mice were killed 5 days after tumor cells grafting, 6 lesions from each group were harvested, pooled and frozen at -78°C (suspended in PBS in proportion 100 mg per 1 ml) for later angiogenic cytokines measuring. Six lesions from each group were fixed in 10% formalin for histopathology.

Measurement of VEGF concentration

The tumour samples collected on the day five after tumor cells grafting were homogenized with an ultrasonic disrupter VirSonic (Virtis) for 2 minutes, at frequency 22.5 KHz. Cytokine levels were determined using standard ELISA R&D kits for mouse VEGF, according to producer instructions. From each material, 6 repetitions were established. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokine concentration was expressed as pg/ml. Inhibition/stimulation indices were calculated dividing results of tests performed with tumours collected from inhaled mice by mean concentration of cytokines in control tumours.

Morphological examination was done on the cellular level using light – microscopic analysis. Immediately after resection, tumor specimens were fixed in 10% formalin solution. After fixation the specimens were dehydrated in increased concentrations of alcohol and embedded in paraffin. Paraffin tissue block was sectioned on 4 ,m thick sections. The specimens were contrasted by hematoxyline and eosine for first screening light microscopic examination.

Statistical analysis

The results were verified statistically by a one-way ANOVA analysis of variance (GraphPad Prism software package) and the significance of differences between the groups was verified with a Tukey's test.

Results

The following substances were used for inhalations: 2-undecanone(2-on) and its derivatives: undecan-2-ol (2-ol) , enantiomers E1 and E2, S(+)undecan-2-ol acetate(E3), R(-) undecan-2-ol acetate (E4), undecan-2-one ethylene acetal (Acet2) and undecan-2-one propylene acetal (Acpr2); 3-undecanone (3-on) and its derivatives undecan-3-ol(3-ol), undecan-3-ol acetate (OC3); 4-undecanone (4-on) and its derivatives undecan-4-ol (4-ol), undecan-4-one ethylene acetal (Acet4) and undecan-4-one propylene acetal (Acpr4).

The results of tumors VEGF content are presented on Figs 1-3. Generally, most of inhaled compounds did not affect this cytokine concentration or enhanced it. Inhibitory effect was presented by all acetals and by 3-undecanone.

The results of the effect of inhalations on tumor-induced-angiogenesis are presented on Figs 4-6. Inhibitory effect was obtained in mice inhaled with R(-) undecan- 2-ol acetate (E-4), 10% 2-ol, Acpr2, 3-on and 3-ol, 4-on, 4-ol and Acpr4.

Summarizing, mice that inhaled 10% solution of 3-undecanone (3-on) or 1% solution of 2-undecanone propylene acetal (Acpr2) for 3 days after tumor cells implantation, presented both lower neovascular response measured by tumor-induced cutaneous angiogenesis test (TIA) and lower tumor VEGF content in 5-days tumors, than non-inhaled controls. Other substances presented various effects on tumor VEGF concentration and angiogenesis.

Histological examination of lesions collected from mice inhaled Acpr2, and from non-inhaled controls, revealed small diffused areas of necrosis in the former group. (Fig. 7). In both groups slight to moderate inflammatory infiltrations were observed at the tumor's margin (Fig. 8 and Fig. 9). In Acpr2 group there were less small blood vessels at the tumor's margin than in the control group (Figs 10-11).

Discussion

In this paper we present the evidence of modulatory activity of 2-, 3-, and 4-undecanones and their derivatives on tumor angiogenesis. We observed various effects of various compounds – stimulation, inhibition, or no effect. Only two from the group of 15 tested substances inhibited both tumor-induced neovascular reaction and VEGF production. They were 3-undecanone and 2- undecanone propylene acetal.

In our previous studies, 3-undecanone, contrary to its derivative undecan-3-ol, did not stimulate specific and nonspecific cellular immunity in mice, and slightly suppressed antibody production (Gibka et al. 2008a). Undecan-2-one and its derivatives stimulated immunological reactions in mice *in vitro* and *in vivo* (Skopińska-Różewska et al. 2006, Gibka et al. 2008b).

Undecan-2-one is a component of a few essential oils and the extracts obtained from many exotic plants. High concentrations of it are observed in the essential oils derived from plants belonging to the *Rutaceae* family.

Similarly to undecan 2-one, undecan-2-ol has been found in nature too. It exists in many plants, as one of the major components of essential oils from *Ruta graveolens* and *Philodendron acutatum,* and from *Eucalyptus risdoni,* from *Curcuma aeruginosa* Roxb. and *Curcuma heyneana* Val. R (-) – undecan -2-ol was detected in the essential oil from *Listea*

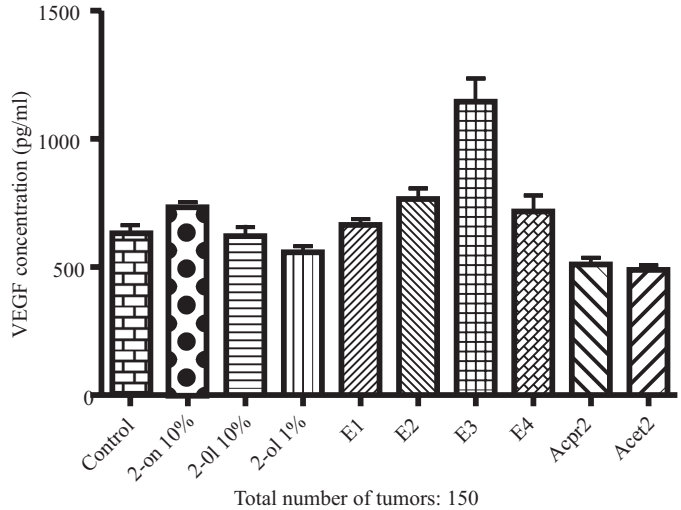


Fig. 1. VEGF In tumors collected from mice 5 days after grafting of L-1 sarcoma cells and inhaling for 3 days 2-undecanone or its derivatives (mean+/-SD).

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?
Control vs 2-on 10%	-107.4	7.301	Yes
Control vs 2-ol 10 %	11.25	0.7646	No
Control vs 2-ol 1%	74.75	5.081	Yes
Control vs E1	-31.75	2.158	No
Control vs E2	-132.8	9.023	Yes
Control vs E3	-513.7	34.92	Yes
Control vs E4	-84.42	5,.38	Yes
Control vs Acpr2	122.4	8.321	Yes
Control vs Acet2	142.9	9.714	Yes

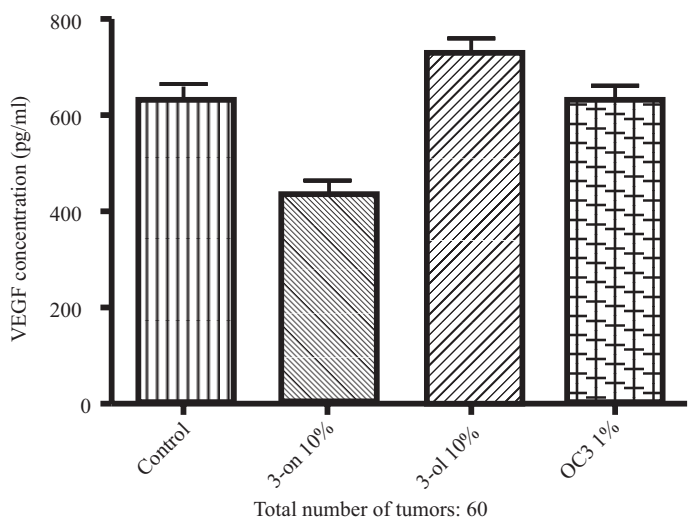


Fig. 2. VEGF In tumors collected from mice 5 days after grafting of L-1 sarcoma cells and inhaling for 3 days 3-undecanone or its derivatives (mean+/-SD).

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs 3-on 10 %	206.6	18.10	Yes	***
Control vs 3-ol 10%	-101.9	8.928	Yes	***
Control vs OC3 1%	-1.083	0.09489	No	ns

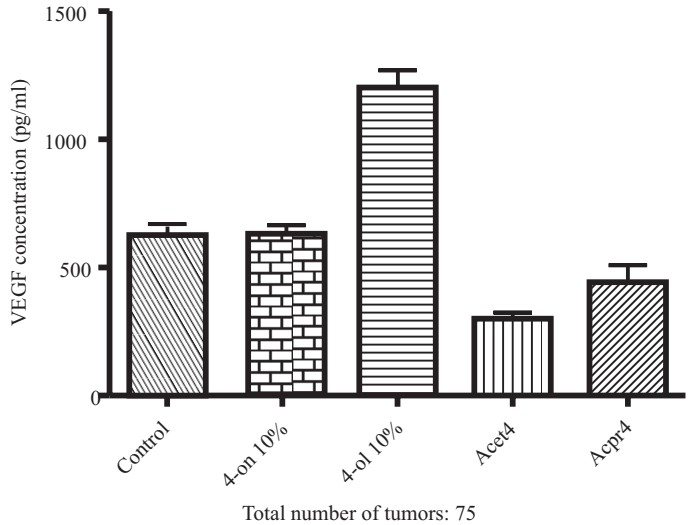


Fig. 3. VEGF In tumors collected from mice 5 days after grafting of L-1 sarcoma cells and inhaling for 3 days 4-undecanone or its derivatives (mean+/-SD).

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	I of diff
Control vs 4-on 10%	6.667	0.3889	No	ns	76.88
Control vs 4-ol 10%	-567.7	33.11	Yes	***	
Control vs Acet4	330.3	19.27	Yes	***	
Control vs Acpr4	188.0	10.97	Yes	***	

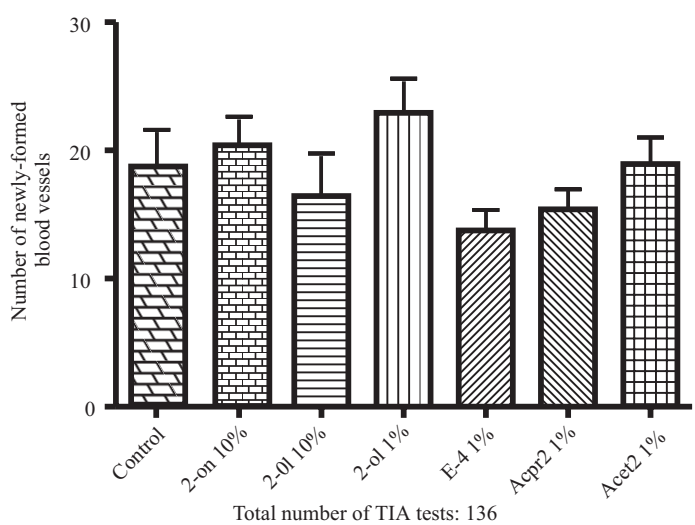


Fig. 4. Neovascular reaction in mice grafted with L-1 sarcoma cells and inhaled 2-undecanone or its derivatives for 3 days (mean+/-SD).

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs 2-on 10%	-1.123	1.801	No	ns
Control vs 2-ol 10%	2.251	4.60	Yes	*
Control vs 2-ol 1%	-4.242	7.869	Yes	***
Control vs E-4 1%	4.946	8.214	Yes	***
Control vs Acpr2 1%	3.284	6.229	Yes	***
Control vs Acet2 1%	-0.2418	0.4486	No	ns

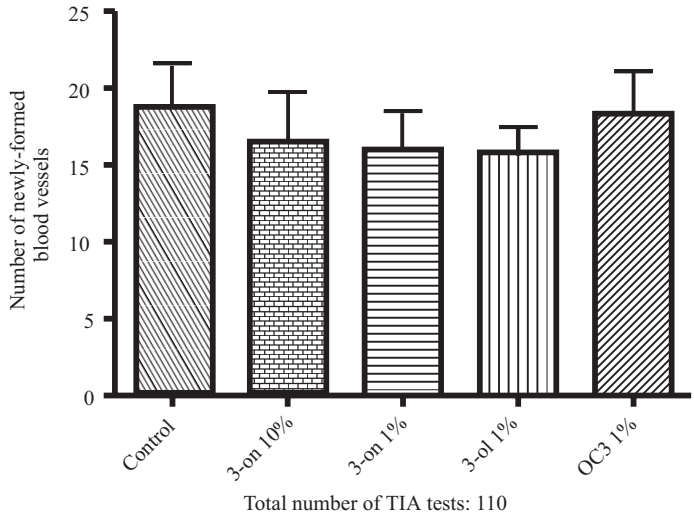


Fig. 5. Neovascular reaction in mice grafted with L-1 sarcoma cells and inhaled 3-undecanone or its derivatives for 3 days (mean +/-SD).

One-way analysis of variance				
P value	0.0005			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs 3-on 10%	2.251	4.093	Yes	*
Control vs 3-on 1%	2.696	4.801	Yes	**
Control vs 3-ol 1%	2.862	4.464	Yes	*
Control vs OC3 1%	0.3427	0.6104	No	ns

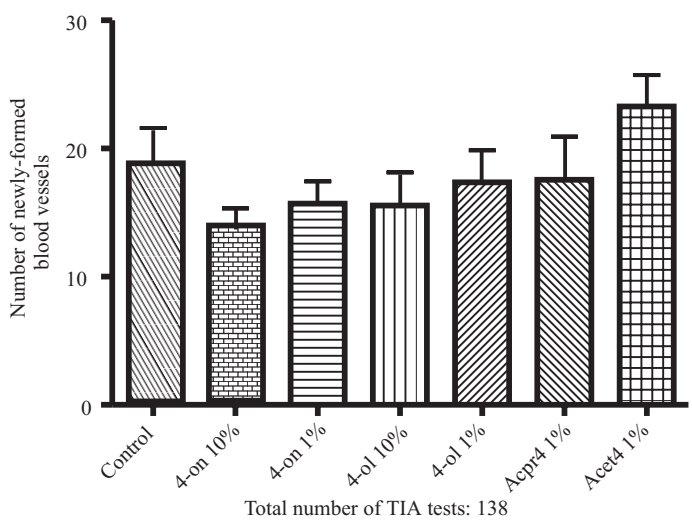


Fig. 6. Neovascular reaction in mice grafted with L-1 sarcoma cells and inhaled 4-undecanone or its derivatives for 3 days (mean+/-SD).

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs 4-on 10%	4.807	9.421	Yes	***
Control vs 4-on 1%	2.990	5.740	Yes	**
Control vs 4-ol 10%	3.133	5.882	Yes	**
Control vs 4-ol 1%	1.332	2.163	No	ns
Control vs Acpr4 1%	1.133	2.127	No	ns
Control vs Acet4 1%	-4.590	8.195	Yes	***

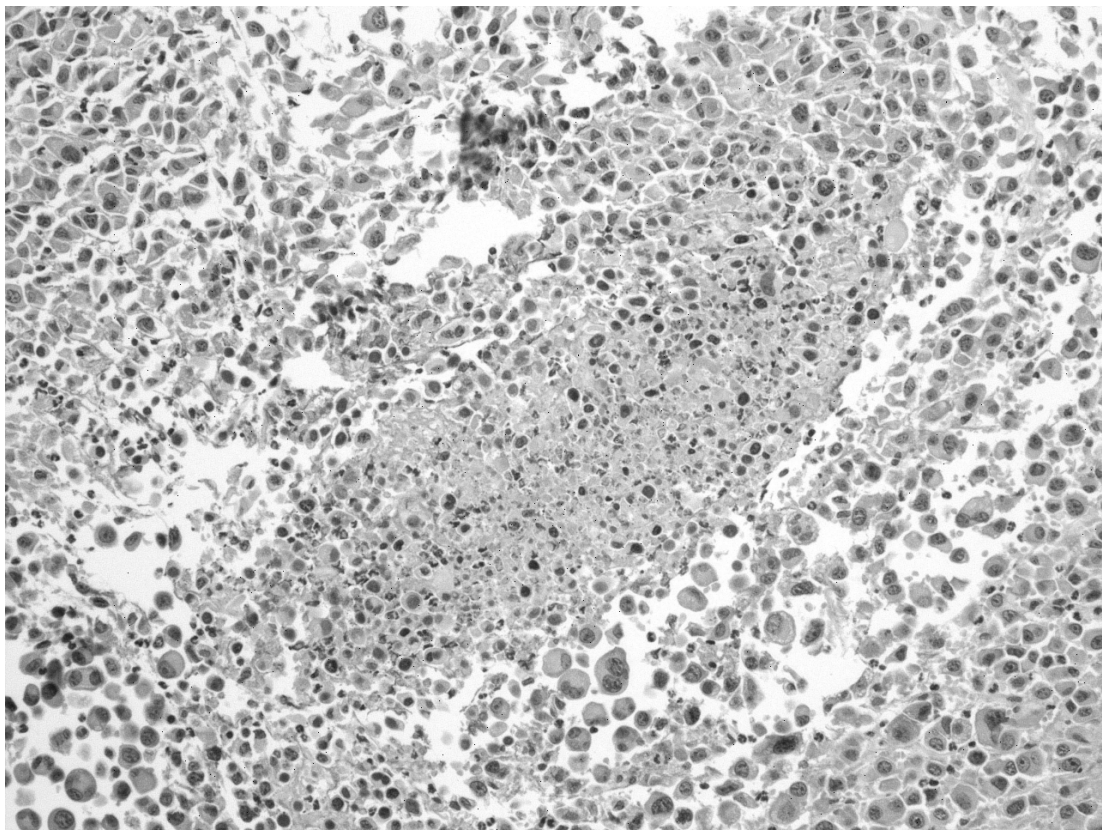


Fig. 7. Focus of necrosis inside the tumor. Photo taken under 20 x lens. Experimental group.

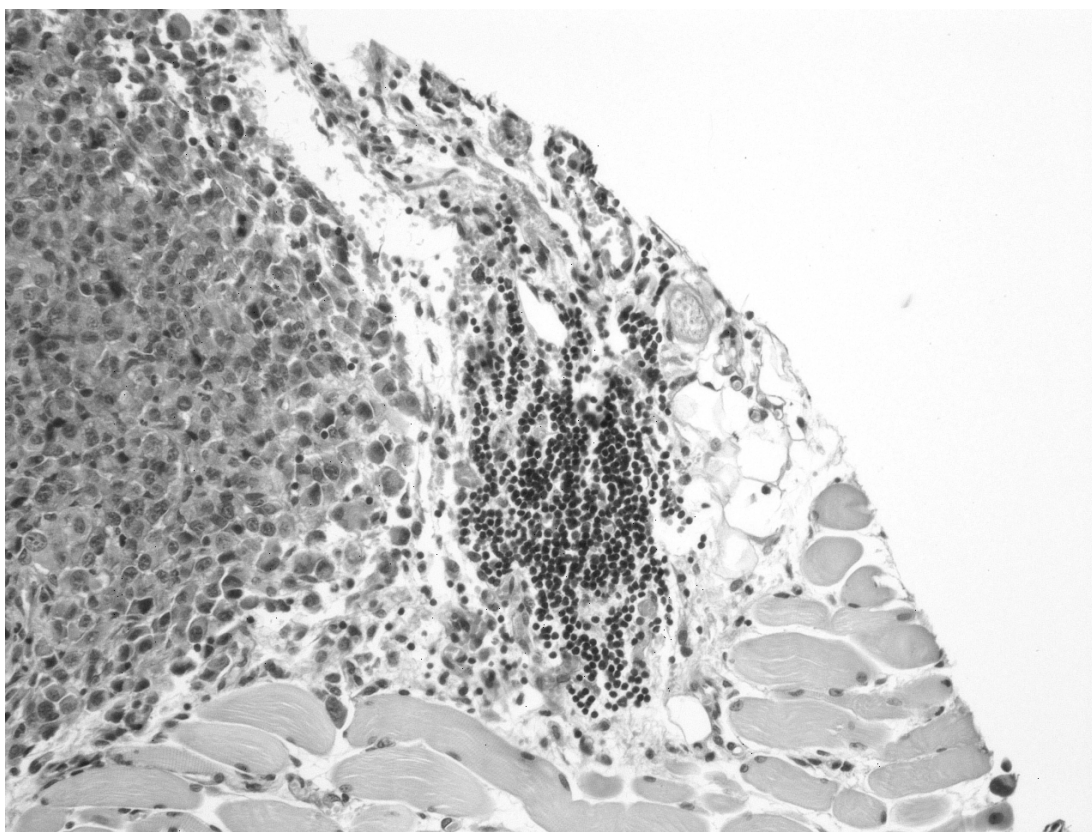


Fig. 8. Inflammatory infiltration at the tumor margin. Photo taken under 20 x lens. Control group.

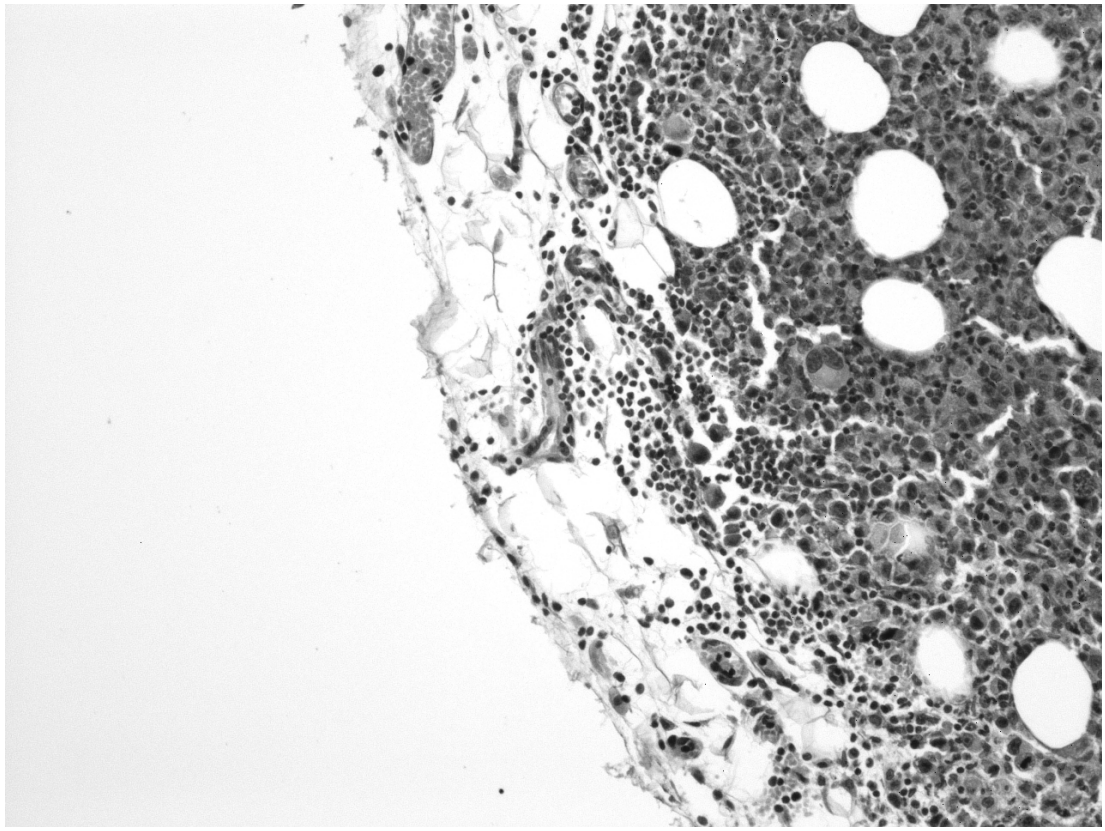


Fig. 9. Inflammatory infiltration at the tumor margin. Photo taken under 20 x lens. Experimental group.

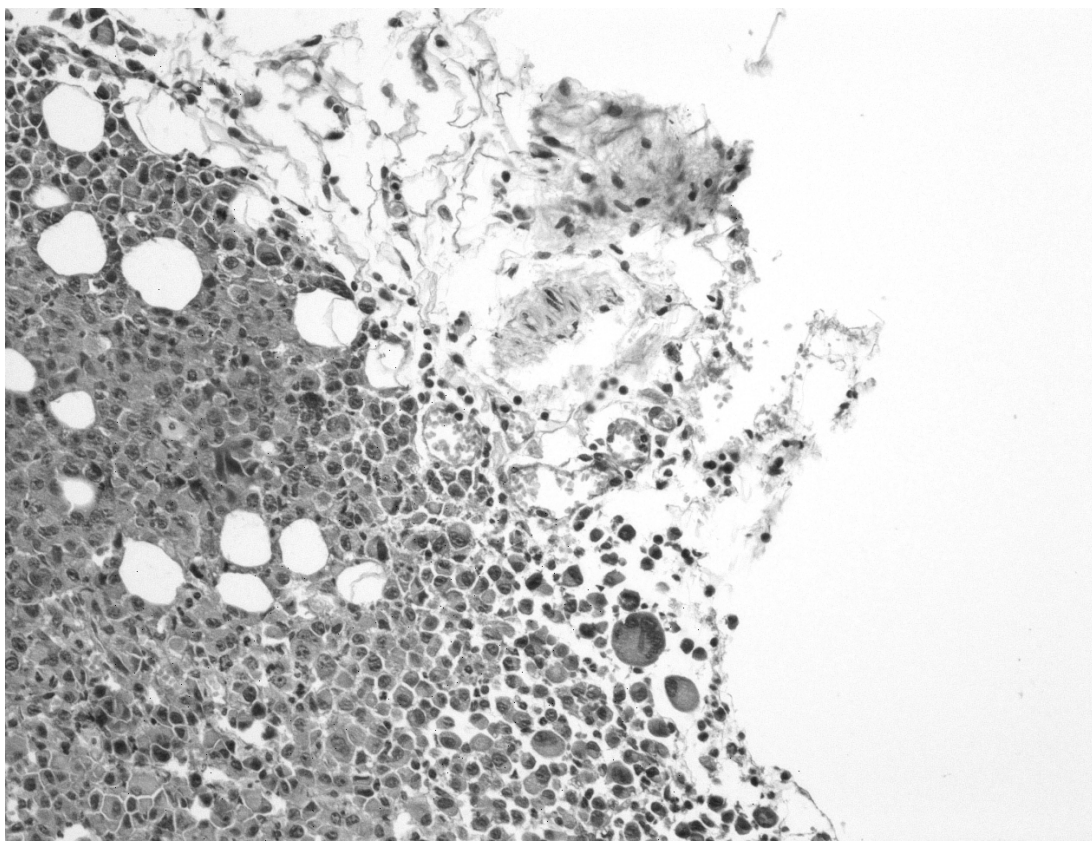


Fig. 10. Blood vessels at a tumor margin. Photo taken under 20 x lens. Control group.

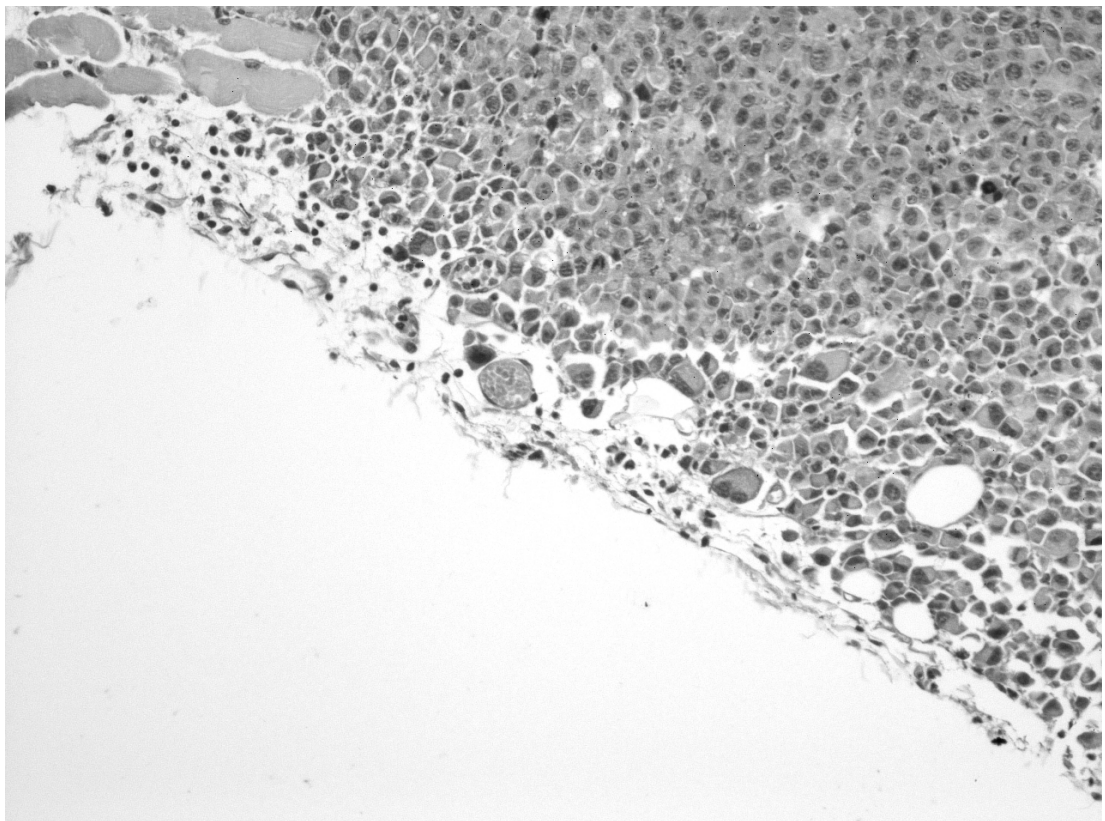


Fig. 11. Blood vessels at a tumor margin. Photo taken under 20 x lens. Experimental group.

odorifera, S(+) undecan -2-ol has been found in cacao butter. *Ruta graveolens* is also a natural, rich source of undec-2-ol acetate (Lawrence and Reynolds 1998).

Ethylene acetal of undecan-2(4)-one and propylene acetal of undecan- 2 (4)-one are newly prepared compounds and they have not been found in nature. They have characteristic pleasant odors and therefore can be used as potential starting materials for the creation of perfumes or flavors for food industries.

In this paper we have described new interesting properties of the propylene acetal of 2-undecanone: negative regulation of tumor angiogenesis and tumor VEGF production.

Previously, we have reported strong immunostimulatory effect of this compound on antibody production in mice (Gibka et al. 2008a).

We feel, that propylene acetal of 2-undecanone might became in the future (after additional toxicological studies) a beneficial food and cosmetics additive for patients with malignant tumors, immunocompromised by chemotherapy.

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