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SHORT COMMUNICATION

Inhibition of tumour and non-tumour cell proliferation by pygidial gland secretions of four ground beetle species (Coleoptera: Carabidae)

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Abstract
Inhibition of the proliferation of human tumour cells and porcine non-tumour cells by the pygidial gland secretion released by adults of four ground beetle species was observed in this study. The sulphorhodamine B (SRB) assay was applied to establish the percentages of inhibition of the net growth of four human tumour cell lines and porcine liver primary non-tumour cells. The secretions of all tested ground beetle species were shown to have an antiproliferative effect on the tested cell lines. Special emphasis is put on the secretion of *Abax parallelepipedus*, which showed the highest antitumour potential and weakest inhibition of non-tumour cell proliferation. The antitumour and antiproliferative potential of the pygidial gland secretions of ground beetles is here demonstrated for the first time. It is suggested that certain organic acids are responsible for the action. Further investigation needs to be conducted in order to better understand the mechanisms governing the observed cytotoxic and antitumour activity.

Keywords Pygidial gland secretions · Ground beetles · Antiproliferative effect · Cell lines · Ellipticine

Introduction

The pygidial gland secretions of ground beetles are chiefly composed of organic acids, aldehydes, alcohols, and terpenes (Eisner et al. 1977; Moore 1979; Dazzini-Valcurone and Pavan 1980; Dettner 1987; Giglio et al. 2011). Previous investigators determined the chemical composition of pygidial gland secretions of the ground beetle species *Carabus ullrichii* Germar, 1824; *C. coriaceus* L., 1758; *Abax parallelepipedus* (Piller & Mitterpacher, 1783); and *Laemostenus punctatus* (Dejean, 1828) (Lečić et al. 2014; Vesović et al. 2015, 2017). According to their analyses, *C. ullrichii* produces the following acids: methacrylic (78.7%); angelic (17.7%); tiglic (2.5%); and benzoic, isobutyric, butyric, and 2-methyl butyric (less than 1% each) (Lečić et al. 2014). *Carabus coriaceus* was found to produce methacrylic (63.5–79.2%), tiglic (20.8–36.5%), and benzoic (less than 1%) acids (Vesović et al. 2017). *Abax parallelepipedus* synthesizes the following acids: methacrylic (76.5%); tiglic (22.9%); and isobutyric, crotonic, and senecioic (less than 1% each) (Lečić et al. 2014). Finally, *L. punctatus* releases undecane (40.4%); dodecyl acetate (34.2%); formic acid (19.4%); 9-methyltetracosane (2.6%); dodecan-1-ol (1.2%); and acetic acid, 7-hexyldocosane, palmitic acid, decyl acetate, and undecyl acetate, as well as stearic, oleic, and caproic acids (less than 1% each) (Vesović et al. 2015).

Nenadić et al. (2016a, b, 2017) recorded that pygidial gland secretions of the mentioned ground beetle species possess an antimicrobial potential against human pathogens and pathogens in the environment. This fact underlines the need to test the toxicity of these mixtures in order to establish their possible medical significance (Nishikawa and Kitani 2011). There is evidence indicating antimicrobial properties of mixtures of these compounds in the Adephaga, but their toxicity for human tissues has been insufficiently studied. In addition to cytotoxicity for normal proliferating cells, there is the question of cytotoxicity for human tumour cell lines. Among the chemical components produced by Coleoptera, certain ones such as the monoterpene cantharidin have shown a quite high level of antitumour activity (Suh and Kang 2012). It is detected in

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droplets of haemolymph released from the joints of legs and antennas in a process known as reflex bleeding, which is common in blister beetles (Meloidae) and false blister beetles (Oedemeridae). Cantharidin induces apoptosis of human cancer cells of hepatomas, colorectal carcinomas, oral carcinomas, oesophageal carcinomas, leukemias, bladder carcinomas, and melanomas (Ghaoneim 2014).

Compared to glandular secretions (particularly pygidial gland secretions), the medical potential of polypeptides from the haemolymph of some insects has been relatively well investigated (Wu et al. 2009; Chernysh et al. 2015; Tonk and Vilcinskas 2017).

The main aim of the current paper was to investigate the antiproliferative potential of pygidial gland secretions of four ground beetle species (*C. ullrichii*, *C. coriaceus*, *A. parallelepipedus*, and *L. punctatus*), particularly against human tumour cells.

Material and methods

Collection and handling of ground beetle specimens

The secretion samples were taken from adult individuals of both genders of four ground beetle species, viz., *C. ullrichii* (12 specimens), *C. coriaceus* (eight specimens), *A. parallelepipedus* (46 specimens) [collected by hand on 5 June 2015 in the villages of Beli Potok and Pinosava, near Belgrade, Central Serbia (air temperature 28 °C, atmospheric humidity 20%) by the authors], and *L. punctatus* (30 specimens) [collected by hand on 22 March 2015 in the Ogorelička Pećina Cave, village of Sićevo, near Niš, Southeast Serbia (air temperature 12–14 °C, atmospheric humidity 60–81%) by the authors]. Live beetle specimens were stored under controlled conditions in portable plastic coolers. Pygidial gland secretions were extracted by stimulating the beetles' abdomens and milking into glass vials containing a 5% aqueous solution of DMSO [dimethyl sulfoxide, $(CH_3)_2SO$), Sigma-Aldrich, Munich, Germany] (Fig. 1). The amount of secretions discharged from the four ground beetle species was measured on a Sartorius Model 2405 analytical balance for weighing quantities up to a maximum of 30 g with a resolution of 0.001 mg (Sartorius Group, Göttingen, Germany). Upon completion of analyses, the samples were subjected to further testing for inhibition of cell proliferation.

Biological material

The pygidial gland secretion of each tested species was dissolved in 3000 μl of a 5% DMSO-water solution (5 ml of DMSO in 95 ml of distilled water). The highest tested concentrations of secretion samples were: 4.159 mg/ml (*C. ullrichii*), 0.501 mg/ml (*C. coriaceus*), 0.061 mg/ml (*A. parallelepipedus*), and 0.022 mg/ml (*L. punctatus*). All samples were stored in a refrigerator at −20 °C.

Commercial anticancer drug

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) (Sigma Chemical Co., St. Louis, MO, USA) was applied as a positive control up to a maximum concentration of 25 μg/ml. It was used because of its powerful antitumour activity, having been previously observed to induce remission of tumour growth (Paoletti et al. 1980).

Tested cell lines

Four human tumour cell lines were used in this study, viz., MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) from DSMZ (Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), as well as one non-tumour primary culture of porcine liver cells (PLP2) (Fig. 1). Liver tissues were obtained and prepared from a harvested porcine liver purchased from a local slaughterhouse (Bragança, Portugal). We selected these cell lines because they are widely used, well-established, homogeneous, and stable.

Inhibition of cell proliferation in human tumour cell lines

Secretion samples were prepared as stock solutions in 5% DMSO and kept at −20 °C. Appropriate serial dilutions were prepared in DMSO/H_2O solvent [a maximum concentration of DMSO (0.25%) was used in order to confirm the absence of toxicity]. Human tumour cells were routinely maintained as adherent cell cultures in RPMI-1640 medium (10% heat-inactivated FBS and 2 mM glutamine at 37 °C) in an incubator with humidified air containing 5% CO_2. Each cell line was plated at an appropriate density (1.0×10^4 cells/well) in 96-well plates and allowed to attach for 24 h. Afterwards, cultivated cells were treated for 48 h with various concentrations of each secretion sample. Thereafter, cold 10% (*w*/*v*) trichloroacetic acid (TCA, 100 μl) was used to fix the adherent cells, which were incubated for 60 min at 4 °C. The plates were then rinsed with deionized water and dried. Afterwards, a solution of sulphorhodamine B (SRB) (0.1% in 1% acetic acid, 100 μl) was added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, while bound SRB was solubilized with 10 mM Tris buffer (200 μl). Absorbance was measured at 540 nm using an ELX800 Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) (Calhelha et

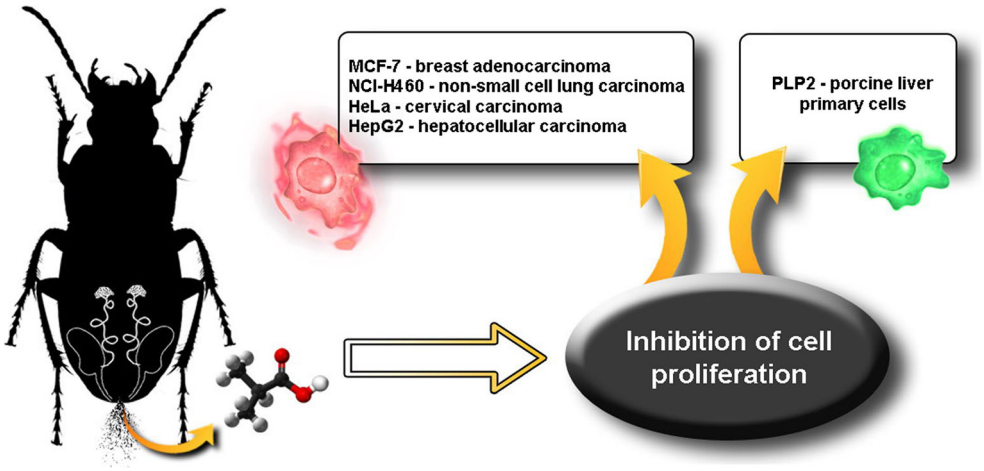

Fig. 1 Diagram of the performed experiment

al. 2012). The results were expressed as percentages of inhibition of cell proliferation.

Inhibition of cell proliferation in a porcine liver primary cell culture

Liver tissues were rinsed in Hank's balanced salt solution (100 U/ml penicillin, 100 μg/ml streptomycin) and divided into 1 × 1 mm^3 explants. Some of these explants were placed in 25-cm^2 tissue flasks in DMEM medium [supplemented with 10% fetal bovine serum (FBS), 2 mM non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin]. Incubation was performed at 37 °C in a humidified atmosphere (5% CO_2), after which the medium was changed every 48 h. Finally, a cell culture, which was designated as PLP2, was monitored directly every 2–3 days using a phase contrast microscope. Prior to reaching confluence, cells were subcultured and plated in 96-well plates at a density of 1.0 × 10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. We performed the SRB assay according to the procedure previously described by Calhelha et al. (2012). The results were expressed as percentages of inhibition of net cell growth.

Statistical analysis

The results are shown as mean values ± standard deviation (SD) using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test with $\alpha = 0.05$, coupled with Welch's statistic. This analysis was performed using SPSS Statistics, ver. 22.0 (IBM Corp., Armonk, NY, USA). All samples were analysed in triplicate.

Results and discussion

We previously obtained the following amounts (mean values) of pygidial gland secretions from the four analysed ground beetle species: 1.039 ± 0.001 mg per specimen of *C. ullrichii* (12 specimens), 0.187 ± 0.001 mg per specimen of *C. coriaceus* (eight specimens), 0.004 ± 0.001 mg per specimen of *A. parallelepipedus* (46 specimens), and 0.002 ± 0.001 mg per specimen of *L. punctatus* (30 specimens) (Nenadić et al. 2016a, b). All results of inhibition of the tested cell cultures were achieved at maximum concentrations of the applied pygidial gland secretions.

The pygidial gland secretion of *L. punctatus* showed the strongest inhibition of porcine liver primary cell proliferation (14.72 ± 0.86%) (Table 1), followed by the secretions of *C. coriaceus*, *C. ullrichii*, and *A. parallelepipedus*, the last two of which being without significant differences (1.61 ± 0.21% and 1.26 ± 0.27%, respectively). The secretion of *L. punctatus* also showed the strongest antiproliferative effect for the MCF7 (42.77 ± 3.56%) and HeLa (16.09 ± 1.23%) cell lines, with no significant difference in relation to that of *A. parallelepipedus* (17.06 ± 1.25%). However, the secretion of *A. parallelepipedus* was the most effective cell inhibitor for the NCI-H460 (34.01 ± 2.17%) and HepG2 (36.49 ± 3.89%) cell lines.

The ellipticine positive control proved to be a much more effective inhibitor of proliferation in all cases, since at very low concentrations it inhibited 50% of net cell growth (at higher concentrations it reached full inhibition; data not shown), whereas all values of inhibition obtained using the tested samples were invariably lower than 50%.

With respect to the antitumour potential of various kinds of secretions, the coleopteran family Carabidae has not yet been tested. However, for other beetle families (e.g., Tenebrionidae), there are studies pointing to biological activities, including an antitumour potential, of some defensive secretions (Crespo et al. 2011; Xiao et al. 2017). Xiao et al. (2017) refer to a species well-known in Chinese alternative medicine, viz., *Blaps rynchopetera* Fairmaire, 1886, whose defensive secretion [containing not only two dominant compounds (2-methyl-2,5-cyclohexadiene-1,4-dione and 2-ethyl-2,5-cyclohexadiene-1,4-dione), but also benzoquinones] was

Table 1 Inhibition of net cell proliferation in % (lower than 50%) after application of highest concentrations of pygidial gland secretions from four ground beetle species

	C. ullrichii	*C. coriaceus*	*A. parallelepipedus*	*L. punctatus*
NCI-H460	4.24 ± 0.52 c	4.37 ± 0.62 c	34.01 ± 2.17 a	17.02 ± 0.96 b
HeLa	7.81 ± 1.29 b	0.14 ± 0.01 c	17.06 ± 1.25 a	16.09 ± 1.23 a
MCF7	20.14 ± 1.87 c	36.56 ± 4.11 b	31.53 ± 2.85 b	42.77 ± 3.56 a
HepG2	6.06 ± 0.87 c	13.51 ± 1.56 b	36.49 ± 3.89 a	13.40 ± 1.41 b
PLP2	1.61 ± 0.21 c	11.16 ± 0.98 b	1.26 ± 0.27 c	14.72 ± 0.86 a

In each row different letters mean significant differences ($p < 0.05$) between secretion samples. Applied concentrations: *C. ullrichii* – 4.159 mg/μl, *C. coriaceus* – 0.501 mg/μl, *A. parallelepipedus* – 0.061 mg/μl, *L. punctatus* – 0.022 mg/μl. NCI-H460 - non-small cell lung carcinoma, HeLa - cervical carcinoma, MCF7 - breast adenocarcinoma, HepG2 - hepatocellular carcinoma, PLP2 - porcine liver primary cells. GI_{50} values for ellipticine (concentrations that inhibited 50% of the net cell growth, expressed in μg/ml): NCI-H460: 1.03 ± 0.09, HeLa: 0.91 ± 0.11, MCF7: 1.21 ± 0.02, HepG2: 1.10 ± 0.09, PLP2: 2.29 ± 0.18 (therefore, direct comparisons with values within the columns should not be made)

shown to possess a high cytotoxic potential against human tumour cell lines such as those of gastric cancer (AGS), colorectal adenocarcinoma (Caco-2), liver cancer (HepG2), glioma (U251), and hepatoma (Bel 7402). This species is traditionally used in China for the treatment of fever, gastritis, boils, and tumours (Xiao et al. 2017). Benzoquinones are also detected in the defensive secretions of other species within the family Tenebrionidae, such as *Ulomoides dermestoides* (Fairmaire, 1893), and are specifically responsible for inhibition of proliferation of the A549 human lung carcinoma epithelial cell line (Crespo et al. 2011). It will be interesting to investigate the antitumour properties of pygidial gland secretions produced by representatives of the tribe Brachinini of ground beetles in the future, since the mixtures also contain benzoquinones (Eisner et al. 1977). In other beetle families, ones such as Staphylinidae, (*Paederus fuscipes*) Curtis, 1826 contains bioactive compounds in the haemolymph (paederin) that possess cytotoxic properties and block DNA and protein synthesis at minimum concentrations (Samani et al. 2014).

Regarding the composition of a mixture of pygidial gland secretion obtained from *A. parallelepipedus*, several carboxylic acids were previously recorded, with special emphasis on isobutyric acid (0.4%, the highest percentage of the compound in comparison with the secretions of other tested ground beetle species) (Lečić et al. 2014). At the present time, there is no precise proof of the anticancer effect of isobutyric acid, but there is evidence indicating that sodium butyrate (SB), a short-chain fatty acid salt, is a histone deacetylase inhibitor (HDACi) that induces growth arrest and apoptotic death in various human cancer cells (Kazemi-sefat et al. 2015). Entin-Meer et al. (2005) showed that treatment of glioma cell lines with two butyric acid derivatives induced hyperacetylation, increased p21 expression, inhibited proliferation, and enhanced apoptosis. A significant level of cytotoxicity in non-tumour fibroblast cells was previously proven to be caused by methacrylic acid (Kurata et al. 2012), which is the most prevalent compound in mixtures of all of the pygidial gland secretions tested (constituting even 78.7% in the secretion of *C. ullrichii*), and cytotoxicity was also demonstrated for isobutyric acid (Kurata et al. 2012), which specifically occurs in the secretion of both *C. ullrichii* and *A. parallelepipedus* (Lečić et al. 2014). Interestingly, we found that the highest percentage of inhibition of non-tumour cell proliferation was achieved after application of the secretion of *L. punctatus*, while the secretion of *A. parallelepipedus* was the weakest inhibitor of non-tumour cell proliferation, but potentially has a strong anticancer effect, since a very low concentration of it was used in comparison with the other tested mixtures. Similarly, Sakurazawa and Ohkusa (2005) pointed out that low concentrations of short-chain fatty acids such as isobutyric, butyric, and isovaleric acids obtained from intestinal bacteria can trigger a cascade of apoptotic events in a human colonic epithelial cell line, probably by increasing the expression of bax via activation of JNK/AP1. In addition, it has been previously noted that short-chain fatty acids possibly have a significant role in antimicrobial activity of the pygidial gland secretions of *C. ullrichii* and *A. parallelepipedus* (Nenadić et al. 2016a).

It can be postulated that the components of secretions have an additive effect (as in *A. parallelepipedus*) (Nenadić et al. 2016a), that the effect of the single compound with the highest percentage in a mixture (e.g., methacrylic acid in *Carabus* spp.) is prevalent, or that an antagonistic effect of components could be responsible for antiproliferative action on tumour cells. A second question that needs to be considered is the ecological role of these effects in the life cycles of ground beetles.

Conclusions

In summary, it can be stated that this study indicates for the first time that the pygidial gland secretions of ground beetles

cause some inhibition of the proliferation of human tumour cells and primary porcine liver non-tumour cells.

Opening new pathways and possibilities, our study suggests ideas for further research aimed at obtaining a better understanding of mechanisms governing inhibition of the proliferation of tumour and normal human and murine cells (including screening of the activity of each separate compound of secretions against tumours). The results of such research perhaps could have medical significance in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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