In vitro cultured lung cancer cells are not suitable for animal-based breath biomarker detection

This content has been downloaded from IOPscience. Please scroll down to see the full text.

2015 J. Breath Res. 9 027103

(http://iopscience.iop.org/1752-7163/9/2/027103)

View the table of contents for this issue, or go to the journal homepage for more

Download details:

IP Address: 141.63.186.166
This content was downloaded on 24/07/2015 at 13:17

Please note that terms and conditions apply.
In vitro cultured lung cancer cell lines are not suitable for animal-based breath biomarker detection

Kristin Schallschmidt¹, Roland Becker¹, Hanna Zwaka², Randolf Menzel², Dorothea Johnen³, Carola Fischer-Tenhagen³, Jana Rolff⁴ and Irene Nehls¹

¹ Federal Institute for Materials Research and Testing (BAM), Berlin, Germany
² Freie Universität Berlin, Institute of Biology: Neurobiology, Berlin, Germany
³ Freie Universität Berlin, Faculty of Veterinary Medicine, Berlin, Germany
⁴ Experimental Pharmacology & Oncology Berlin-Buch GmbH, Berlin, Germany

E-mail: roland.becker@bam.de

Keywords: A549, honey bees, odour discrimination task, sniffer dogs, solid phase microextraction, volatile organic compounds, volatilome

Abstract

In vitro cultured lung cancer cell lines were investigated regarding the possible identification of volatile organic compounds as potential biomarkers. Gas samples from the headspace of pure culture medium and from the cultures of human lung adenocarcinoma cell lines A549 and Lu7466 were exposed to polypropylene fleece in order to absorb odour components. Sniffer dogs were trained with loaded fleeces of both cell lines, and honey bees were trained with fleeces exposed to A549. Afterwards, their ability to distinguish between cell-free culture medium odour and lung cancer cell odour was tested. Neither bees nor dogs were able to discriminate between odours from the cancer cell cultures and the pure culture medium. Solid phase micro extraction followed by gas chromatography with mass selective detection produced profiles of volatiles from the headspace offered to the animals. The profiles from the cell lines were largely similar; distinct differences were based on the decrease of volatile culture medium components due to the cells’ metabolic activity. In summary, cultured lung cancer cell lines do not produce any biomarkers recognizable by animals or gas chromatographic analysis.

1. Introduction

Lung cancer is one of the most frequent forms of cancer in Europe. In 2012 it was the leading type of cancer for males and the third most common type for females with 353,000 deaths overall [1]. The 5-year survival rates for lung cancer patients are generally poor; in the period from 2005 to 2007 the European mean was 13.4% [2]. The survival rate depends on the tumour stage at the time of diagnosis and is highest for the earliest tumour stage [3]. Therefore, various approaches consider the identification of biomarkers for an early-stage diagnosis based on a non-invasive sampling procedure.

Animals’ odour discrimination abilities are widely used in military and civil tasks; first and foremost dogs are used to detect explosives, drugs or trails [4]. Moreover, their olfactory ability enables them to differentiate biological samples including urine, blood, faeces, and tissue from cancer patients and from healthy people by their sniffing ability [5–13]. A further example is the detection of lung cancer using breath samples [14–19]. In principle, the use of breath has the advantage of being a non-invasive sampling procedure.

Fruit flies were used to discriminate between the air over breast cancer cells and non-pathogenic mammary epithelial cells kept in vitro based on in vivo calcium imaging of olfactory receptor neurons on the fly’s antennae [20].

Honey bees were able to detect explosives such as 2,4-dinitrotoluene and trinitrotoluene [21] and were trained to indicate methyl phenyl acetate, methyl nicotinate and methyl p-anisate which were proclaimed as signature compounds of tuberculosis [22]. However, there is no scientific publication proving the applicability of honey bees for tuberculosis detection or other medical diagnosis.

Some researchers in the field worked with lung cancer cell lines cultured in vitro as a model for the search for volatile organic compounds (VOCs) which might be promising biomarkers for diagnosing lung cancer in the early stages. Filipiak et al and Sponring...
et al investigated the headspace (HS) of different lung cancer cell lines with thermal desorption-gas chromatography mass spectrometry (GC-MS) after pre-concentration on multibed sorption tubes. They observed significant changes between the adenocarcinoma cell line A549 compared to the cell-free culture medium as well as significant differences between lung cancer cells and non-pathogenic cells [23–26]. Pyo et al used HS solid phase microextraction (HS-SPME) with GC-MS to examine the influence of induced cell death on the VOC profile of A549 [27], whereas Barash et al first absorbed the VOC on Tenax TA and, subsequently, compared the thermally desorbed compounds with HS-SPME-GC-MS and nanoparticle sensors in order to discriminate between different types of lung cancer cell lines and non-pathogenic cells [28]. Wang et al also applied HS-SPME-GC-MS to characterize VOC profiles of lung cancer cell lines [29]. Proton transfer reaction MS [30] and single ion flow tube MS [31, 32] were used for VOC analysis in the HS of lung cancer cell lines, as well. Other approaches for biomarker identification did not use cell cultures kept in vitro but in vivo cancer models [33] and resected tumour tissues [34–36], which were analysed with respect to differences in VOC profiles of cancerous and non-cancerous samples.

Despite these efforts, none of the proposed biomarker sets achieved clinical relevance. Amongst other reasons, this could be due to a lack of biological evidence of the declared substances.

In the following, the attempt to use cultured cell lines as a model for the generation of volatile cancer markers is described. Animal-based detection principles employing sniffer dogs and honey bees as well as the characterization of VOC profiles using chemical trace analysis were investigated.

2. Materials and methods

2.1. Cell cultivation

The commercially available cell lines A549 and Lu7466 (established at Experimental Pharmacology & Oncology Berlin-Buch GmbH, Berlin, Germany) were used for the in vitro studies. Both cell lines showed adenocarcinoma histology. A549 with a cell count of $5 \times 10^6$ was grown in Ham’s F-12 medium with L-glutamine and 10% foetal bovine serum added. Lu7466 cells with a cell count of $3 \times 10^6$ were cultivated in Dulbecco’s modified eagle medium (DMEM)/Ham’s F-12 media with L-glutamine and 20% foetal bovine serum added. Cells were grown under standard conditions at 37 °C in a humidified (90–98% relative humidity) and CO₂-enriched (5 vol% CO₂, 95 vol% ambient air) atmosphere in T150 culture flasks (TPP GmbH, Trasadingen, Switzerland). The penultimate sampling of the last passage was conducted with exactly 25 ml of the culture medium. Instead of the normal filter caps, special septum caps (figure 1) in vent position were used, after disinfection with ethanol. This cap allows the introduction of a syringe needle through the slotted septum and sampling of air from the culture flask without dilution by ambient air, which would stream into the flask if the usual filter cap was used. Culture flasks containing only 25 ml of the culture medium without cells were prepared in parallel. One hour prior to sampling, the flasks were closed by turning the caps so that they were gas-tight.

2.2. Sampling procedure for dog training and testing

HS samples were derived from A549 and Lu7466 cell cultures and the related culture medium.

The sampling tubes were made of glass (length 150 mm, inner diameter 20 mm) with GL25 sockets on both sides (Gassner Glastechnik GmbH, Munich, Germany). The tubes contained two polypropylene fleeces (70 × 43 mm), one hydrophilic (asota® olefin hydrophil) and the other hydrophobic (asota® olefin hydrophob) modified (Asota GmbH, Linz, Austria). The tubes were closed either with red polybutylene-terephthalate screw caps with polytetrafluoroethylene (PTFE)/ silicone gaskets or black polyphenylsiloxane screw caps with PTFE/ silicone gaskets (Bohlender GmbH, Grünsfeld, Germany).

For air sampling, 100 ml gas-tight glass syringes were equipped with 19-gauge needles (length 115 mm; SGE Europe Ltd, Milton Keynes, United Kingdom). The culture flasks were pierced by the cannula through the septum and 100 ml of air were drawn from the HS in the culture flask into the syringe. The sampling tubes were opened at both sides, the cannula was positioned in the fleeces, and the gas was purged into the sampling tubes. This procedure (figure 2) was repeated twice before closing the sampling tube again. Tubes with air from cell cultures were sealed with red caps (positive), whereas tubes with air from flasks with culture medium only were closed with black caps (negative). In total 300 ml of air were sampled per flask and tube. The loaded tubes were stored at 6 °C until used (up to three months).
2.3. Sampling procedure for honey bee conditioning

Air samples for honey bee conditioning were taken only from the A549 cell culture and the corresponding culture medium. Fleece pieces of about 1 cm² were put into disposable 20 ml plastic syringes equipped with Luer locks (B. Braun Melsungen AG, Melsungen, Germany, see figure 3) and 115 mm needles (see section 2.2). Each culture flask was pierced by the cannula through the slotted septum and 15 ml of air from the HS in the culture flask were drawn into the syringe. Afterwards, the syringe was closed with syringe caps (B. Braun Melsungen AG). Each culture flask was used to fill twenty syringes, amounting to a total sampled volume of 300 ml. In total, 180 syringes with air from six culture flasks with cells and 180 syringes with air from six flasks with pure culture medium were prepared. The filled syringes were stored for about one week at 4 °C until used.

2.4. Sampling procedure for instrumental analysis

The instrumental analysis was carried out for both cell lines and the corresponding cell culture media. Baked-out (420 °C, 24 h) 20 ml SPME vials (Gerstel GmbH & Co. KG, Mülheim a. d. Ruhr, Germany) were flushed with synthetic air (Air Liquide, Berlin, Germany) for 30 s and sealed with crimp caps with Silicone/PTFE septum (1.5 mm, 55° Shore A; Gerstel). A 25 ml gas-tight syringe (Restek GmbH, Bad Homburg, Germany) was equipped with a 27-gauge needle (length 20 mm; Th. Geyer GmbH & Co. KG, Renningen, Germany) and 15 ml of air were evacuated from the closed vials. Then, 15 ml of air from the cell culture HS were drawn into the syringe and discharged into the evacuated vials (figure 3). The procedure was executed three times for each cell culture and culture medium (n = 3), each time using a new culture flask. The filled vials were analysed within 12 h.

2.5. Sniffer dog training and testing

Two privately owned dogs were trained successfully in this study. Dog 1 was a 3 year-old spayed female Labrador retriever and Dog 2 was a 3 year-old castrated male cross-breed. Both dogs had basic obedience training and were experienced in scent discrimination [37, 38].

Training took place in a separate room at the Faculty of Veterinary Medicine, FU Berlin. For training and testing, cell culture and culture medium samples were loaded in specially designed cones (BAM, division ‘Test Devices and Equipment’, patent DE 10 2013 109 901.7, see figure 4). The training was clicker-enhanced and food-rewarded. It was configured in three consecutive phases according to a training plan developed by veterinarians. In the first phase the dogs had to learn to indicate the positive (lung cancer cell culture) sample. Indication was either by sitting (Dog 1) or standing still with nose pointing (Dog 2). The second phase was designed to train the discrimination between one positive and one negative (culture medium) sample. In the third phase the design was changed to one positive sample and three negative samples in parallel. Training was carried out with both lung cancer cell lines. Both dogs participated in the training sessions regularly two to three times a week. Data recording started with the first training phase. The training lasted for eight months.

Figure 2. Preparation of glass tubes for sniffer dogs: Sampling of air from a culture flask (a), addition of sampled air to the fleece tubes (b), fleece tubes with positive (air from lung cancer cell culture, red) and negative (air from pure culture medium without cells, black) samples for the sniffer dog tests (c).

Figure 3. Left: Sampling equipment for bee conditioning: Culture flask with special septum cap, one-way 20 ml syringe with two pieces of fleece, syringe cap, and needle. Right: Sampling procedure for instrumental analysis: equipment consisting of 25 ml gas-tight syringe, 27-gauge needle, SPME vial with silicone/PTFE crimp cap, and culture flask with special septum cap (a), sampling of 15 ml of air from culture flask (b), discharge of sampled air into evacuated SPME vial (c).
At the end of the training both dogs were able to discriminate the training samples with an average sensitivity and specificity of 80%.

For the test procedure, 20 cones for presenting sampling tubes were numbered. Numbers 1–10 held samples with air from the cell culture (positive samples, five from A549 and Lu7466 each) and number 11–20 contained samples with air from the culture medium only (five from F-12 medium and DMEM/F-12 medium each). The negative samples were used repeatedly during the test procedure. As during the last training phase, the dogs had to identify one positive sample out of four.

The test procedure was conducted as a double-blind test. The experimenter, initially not knowing the sample positioning plan, filled the cones with sample tubes while documenting the corresponding tube for each cone. Then the experimenter set up the first trial according to the sample positioning plan which had been created by another person who was not present in the testing area. The position of the positive sample was randomized for every trial according to the plan. The dog trainer and investigator were not observing the scene at this point. Afterwards, the experimenter turned around to face the wall of the room and covered her ears with her hands. The trainer commanded Dog 1 to search for the positive probe. After the trainer had acknowledged the dog’s indication (figure 5), the dog was rewarded without any feedback as to whether the indication was correct or not, and the indicated position was documented. Trainer and investigator turned then around and the experimenter changed the positions of the cones already in use according to the test plan for the second half of the first trial. Afterwards, Dog 2 had to indicate a cone unseen by the experimenter but watched by trainer and investigator. The procedure was repeated nine times (in total 10 trials) with a break of half an hour after the fifth trial.

After the last trial, the dogs’ indications and the test plan were compared. Sensitivity was calculated as the number of correctly indicated positive samples divided by the total number of positive samples (10), while specificity was calculated as the number of negative samples which were never falsely indicated as positive divided by the total number of negative samples (10).

2.6. Honey bee conditioning

Honey bees (∥'es melliferax) from one colony held in an indoor flight room at the Institute of Neurobiology, FU Berlin were used for differential conditioning of the proboscis extension response (PER) following the procedure described by Bitterman et al [39] and by Matsumoto et al [40]. Therefore, 20 worker bees were caught the day prior to the experimental day at the hive entrance. The insects were immobilized on ice and harnessed in plastic tubes (figure 5).

They were able to move antennae and mouthparts freely. Each bee was fed with aqueous sucrose solution (30%, weight/weight) to saturation. The insects were kept in a dark and moist room at about 25°C overnight. Thirty minutes before odour conditioning started, they were placed near the experimental location for acclimatization. Odour conditioning consisted of ten training trials followed by two test trials.

The training trials were set up as follows: first, the conditioned, reinforced stimulus (CS+, cell culture air) was presented for 5 s and then paired with the unconditioned stimulus (US, 30% aqueous sucrose solution) for 3 s. The US was presented to the antennae and then
to the reflexively extended proboscis. The US onset was 3 s after CS+ onset, thus CS+ and US overlapped for 2 s. In the second trial, the contrasting odour of the cell-free culture medium samples was presented as the unreinforced stimulus (CS−). This time no US was presented to the bees. A new syringe was used for each animal and every trial. Every honey bee had an interval of 10 min between two trials. Trials with CS+ and CS− were repeated in pseudorandom order with a total of 10 training trials (table 1). The test trials followed 1 h later.

In the first test trial, 10 honey bees were presented with CS+ while the other 10 bees were presented with CS−. This set-up was changed in the second test trial. During test trials no US was presented.

During all training trials the occurrence of the PER was recorded at three points in time: placement of the honey bee in front of an exhaust, presentation of CS, and presentation of US in CS+ trials. During the test trials PER was recorded for placement of bees and CS presentation.

All honey bees that died during the procedure or showed no PER for US presentation in more than one training trial with CS+ were classified as non-responders and were excluded from further consideration. Animals showing PER at the moment of placement for more than one trial were excluded as context responders. The remaining honey bees (US responders) were considered as the basic population. Subsequently, the responders showing PER at the moment of CS presentation were counted for each trial. This number was set in relation to the basic population. An analysis of variance (ANOVA) was then conducted for trials 1–12.

### 2.7. Instrumental volatilome analysis

Instrumental analysis took place at BAM. SPME was performed using a carboxen-polydimethylsiloxane-coated 75 µm fibre conditioned according to the manufacturer’s instructions (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The bake-out period was 15 min at 300 °C. The vials were preheated for 5 min at 37 °C. Then, the extraction was carried out for 60 min at 37 °C. Afterwards the fibre was desorbed in the injector for 5 min, the first 3 min with closed split valve.

Analyses were performed with a 7890B gas chromatograph coupled with a 5977 A mass selective detector, and equipped with a VF 624 ms column (60 m × 0.32 mm × 1.8 µm, Agilent Technologies, Waldbronn, Germany). The autosampler was a MPS 2 (Gerstel). The oven temperature program was: initially 40 °C, held for 5 min, ramped with 4 °C min−1 to 140 °C, then held for 5 min, then ramped with 20 °C min−1 to 260 °C, and held for another 4 min. Helium 5.0 (Air Liquide) was chosen as the carrier gas and a constant flow rate of 1.4 ml min−1 was used. The split-splitless injector was set to a temperature of 280°C. Electron impact (70 eV) spectra were recorded in the scan mode within the range m/z 35–300. The transfer line was heated to 280 °C, while the ion source had a temperature of 230°C. The NIST MS Search 2.0a software was used for peak identification by comparison of mass spectra. Signal areas were determined using the extracted ion mode.

### 3. Results and discussion

#### 3.1. Sniffer dogs training and testing

During the double-blind test, dog 1 indicated two positive samples correctly, while negative samples were falsely indicated in eight trials. Four out of 10 negative samples were never indicated by dog 1. Dog 2 indicated one positive sample correctly, while negative samples were falsely indicated in nine trials. Five out of 10 negative samples were never indicated by dog 2 (table 2). During the test trials it was observed that the dogs became increasingly tired, recovering only a little after the break.

The double-blind test trials with odour samples from A549 and Lu7466 versus the culture medium revealed an indication of random responses, as shown by the p-values of the binomial distribution (table 2). Hence, the dogs were not able to discriminate between air from in vitro cultured lung cancer cells and cell-free culture medium. The dogs’ indication was notably better in the training phase with an average sensitivity and specificity of 80%. This might be influenced by the fact that training is necessarily carried out without blinding the trainer and the dog reacts to the trainer’s response, unnoticed by the trainer [18]. It is most evident from the results of training and testing in this study.

#### Table 1. Pseudorandom ordering of CS+ and CS− presentation during honey bee conditioning (conditioned stimulus (CS): air of A549 cell culture (+) or culture medium (−), unconditioned stimulus (US): sucrose solution).

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>CS presentation</th>
<th>US presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>−</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>+/−</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>+/−</td>
<td>No</td>
</tr>
</tbody>
</table>

#### Table 2. Test results of the cell culture discrimination by dogs (one out of four set up, 10 trials).

<table>
<thead>
<tr>
<th>Dog</th>
<th>Negatives never falsely indicated as positive</th>
<th>Specificity</th>
<th>Correctly indicated positive samples s</th>
<th>Sensitivity</th>
<th>p-value of binomial distribution for s indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>40%</td>
<td>2</td>
<td>20%</td>
<td>0.28</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>50%</td>
<td>1</td>
<td>10%</td>
<td>0.19</td>
</tr>
</tbody>
</table>
that blinded test trials are essential for the generation of unbiased results. Furthermore, training was conducted with a limited number of samples, whereas samples used for testing were completely new to the dogs. It is possible that the dogs learned to identify the individual odour samples from A549 and Lu7466 during training instead of detecting a common odour of two different lung cancer cell lines kept in vitro. A number of authors reported on sniffer dogs’ ability to discriminate between samples of urine, blood and breath from cancer patients and from healthy controls, in particular in the case of lung cancer [14–19]. In contrast, we observed that dogs were not able to distinguish between the odours of two different lung cancer cell lines kept in vitro from their culture medium. Therefore, we assume that they would not perform better in discriminating between pathogenic and healthy cells. Consequently, the in vitro lung cancer cell model is not appropriate for the breath biomarker search based on sniffer dogs’ discrimination ability. There is evidence that the dogs’ olfactory ability to detect samples derived from cancer patients is not based on the alternated tumour cells themselves, but requires further characteristics of tumour metabolism. It should be noted that the cell lines were grown under standard conditions. In tumour tissue, however, the cancer cells have to deal with a hypoxic atmosphere [41]. The oxygen content strongly influences the metabolism and is therefore assumed to affect the VOC profile.

3.2. Honey bee conditioning

The honey bee conditioning was executed as differential learning with air from the A549 cell culture (reinforced stimulus) and the corresponding culture medium (unreinforced stimulus, see section 2.3). Of 20 honey bees, five were classified as non-responders and one as a context responder, so that 14 individuals were considered for the evaluation. Figure 6 displays the ratio of honey bees showing a PER during presentation of CS. Taking into account the last two training trials, it can be seen that 86% of honey bees showed PER for cell-free culture medium air, whereas only 71% reacted to A549 cells. In the consecutive test trials the number of honey bees showing PER was the same for both kinds of samples. Therefore, none of the bees learned the difference between the two odours. The repeated measurement ANOVA revealed no significant discrimination between cell culture and culture medium by honey bees (p = 0.062).

Fruit flies were proven to code in vitro cultured breast cancer cells and healthy cells differently at the level of first order neural integration, the antennal lobe [20]. Such experiments have yet to be performed with honey bees. At the behavioural level, honey bees demonstrated a high degree of generalization of air samples from the lung cancer cell and the culture medium samples. If the same coding strategy applies to honey bees as to fruit flies, we might conclude that the two test odours were not distinguished because common components were more salient than differing components, thus leading to generalization between both test odours.

Since the honey bees showed no discrimination ability for the cell line A549 and the corresponding medium and the sniffer dog experiments demonstrated no differentiation for both cell lines, it was expected that training the honey bees with the Lu7466 cell line would not produce different results, so this training was omitted.

3.3. Instrumental odour analysis

The VOC profiles from the culture flasks were investigated by automated HS-SPME-GC-MS (section 2.7). Figure 7(a) displays a section of a typical chromatogram of the HS samples taken from A549 cultures and the corresponding cell-free medium.

Of 25 assigned peaks, 19 were identified by library spectra and comparison of retention times with authentic compounds, four identified by library spectra and two narrowed down to one chemical class with a presumed sum formula (table 3). The signal intensity of each identified hydrocarbon was lower in the presence of cells or remained unchanged in the A549 cell culture compared to cell-free culture medium. Aldehyde concentrations were also lower in cell samples, whereas the amounts of alcohols, esters, acetic acid, dichloromethane (DCM) and ketones were higher in the A549 culture.

Thirteen volatiles were identified in the HS of the Lu7466 cell line cultures and the corresponding culture medium. Very few obvious differences were found in the VOC profiles of Lu7466 cells compared to the culture medium (figure 7(b)). Significant changes in signal intensity were seen only for DCM, 1-propanol, and acrylic acid methyl ester, all increasing. No decrease of peak intensities due to cells compared to culture medium was observed; most signals did not change at all.

The sampling procedure of the air from the culture flasks for analysis by HS-SPME-GC-MS was designed...
to detect the very compounds actually offered in the sniffer dogs’ test. The method used was sensitive enough to detect a variety of compounds typically associated with the breath volatilome and to reveal differences in VOC profiles between both cell lines and culture medium.

A comparison of our results for A549 with the literature (table 3) confirms some stated trends, such as the decrease of aldehydes or the increase of ethanol and acetic acid. Barash et al [28] also observed a decrease of decanal for cancer cells compared to the cell-free medium. But then, if apoptosis was induced in A549 cancer cells as described in [27], the nonanal concentration increased. We observed no significant increase of acetone, which was mentioned by Filipiak et al [25], as there was a large variance for the acetone signal in the A549 samples.

Other differences in VOC profiles, such as the obvious decrease of specific alkanes in the presence of the cells, have not been published before. According to Kalluri et al [41] the application of more realistic hypoxic cell culturing conditions, would most likely lead to increased oxidative stress in the cells and, hence, to an increase in alkane and methylated alkane formation.

Because in this study the adenocarcinoma cell line Lu7466 was investigated for the first time with regard to the VOC profile, it was not possible to compare the results with data from the literature. The used DMEM/F12 medium showed a lower number of peaks compared to the A549’s medium, including no occurrence of aldehydes. Even if the Lu7466 cell line was able to decrease the aldehyde content of the media similar to A549, this could not be revealed by this setup.

The startling presence of DCM in the HS of both cell lines as a result of the metabolism is unlikely. Empty culture flasks that had never been in contact with culture medium or cells (data not shown) also displayed detectable amounts of DCM. It appears to have been used during production of the flasks and then released in presence of cell cultures.

In addition to the HS analysis of the culture flasks we also conducted the analysis of the sampling tubes (data

![Figure 7. Profiles of volatile compounds in the HS of A549 (a) and Lu7466 (b) cell cultures and the respective cell-free culture medium (SPME-GC-MS analysis). For a discussion of individual compounds see table 3.](image-url)
The sampling tubes were identical to those presented to sniffer dogs with the exception that screw caps with aperture and silicone septa were used to allow SPME and instrumental analysis similar to the one performed on the gas in vials. The fleece caused additional signals such as 2-methylpentane and tetrahydrofuran. Since there was a relevant background of acetic acid, the increasing trend due to the cells that was observed for the pure gas samples was no longer in evidence. Some trends observed for the gas samples in vials were shown by the sampling tubes, too, for example the increase of 1-propanol for both cell lines. Other VOC including the esters, almost all aldehydes, DCM, and some alkanes which were observed in the separated gas phase were not detected in the sampling tubes. It is likely that these compounds were absorbed by the fleeces and therefore not accessible to the dogs and honey bees.

### 4. Conclusions and outlook

It was shown that a blinded test setup is absolutely necessary to obtain unbiased results from sniffer dogs. The sampling of cell culture air was successful for the analytical characterization of the respective VOC profiles. The presented approach does not facilitate the identification of breath biomarkers for lung cancer detection by sniffer dogs. Honeybees may generalize between the test odours, although their neural coding strategy may allow for discrimination. This proposal needs to be tested, and different training procedures need to be adopted.

Further investigations must show that the very sniffer dogs used for this experimental setup are able to discriminate between the breath odours of lung cancer patients and healthy people. Some trends observed for the gas samples in vials were shown by the sampling tubes, too, for example the increase of 1-propanol for both cell lines. Other VOC including the esters, almost all aldehydes, DCM, and some alkanes which were observed in the separated gas phase were not detected in the sampling tubes. It is likely that these compounds were absorbed by the fleeces and therefore not accessible to the dogs and honey bees.

### Acknowledgments

The animal experiments were conducted in accordance with the Guiding Principles for Research Involving Animals and Human Beings as adopted by the
American Physiological Society, and in compliance with German regulations.

The authors kindly thank TPP GmbH, Trasadingen, Switzerland, for developing septum caps to meet our requirements and the provision of prototypes free of charge for application tests.

References

[22] Suckling D M and Sagar R I L 2011 Honeybees apis mellifera can detect the scent of mycobacterium tuberculosis Tuberculosis (Edinb), 91 327–8
[35] Filippiak W et al 2014 Comparative analyses of volatile organic compounds (VOCs) from patients, tumors and transformed cell lines for the validation of lung cancer-derived breath markers J. Breath. Res. 8 027111
[38] Fischer-Tenhagen C, Tenhagen B A and Heuwieser W 2013 Short communication: ability of dogs to detect cows in estrus from sniffing saliva samples J. Dairy Sci. 96 1081–4