Research Article

Cancer Epidemiology, Biomarkers & Prevention

TD-GC-MS Analysis of Volatile Metabolites of Human Lung Cancer and Normal Cells *In vitro*

Wojciech Filipiak[1,3], Andreas Sponring[1,3], Anna Filipiak[1,3], Clemens Ager[1,3], Jochen Schubert[3,4], Wolfram Miekisch[3,4], Anton Amann[1,3], and Jakob Troppmair[1,2]

Abstract

The aim of this study was to confirm the existence of volatile organic compounds (VOC) specifically released or consumed by the lung cancer cell line A549, which could be used in future screens as biomarkers for the early detection of lung cancer. For comparison, primary human bronchial epithelial cells (HBEpC) and human fibroblasts (hFB) were included. VOCs were detected in the headspace of cell cultures or medium controls following adsorption on solid sorbents, thermodesorption, and analysis by gas chromatography mass spectrometry. Using this approach, we identified VOCs that behaved similarly in normal and transformed cells. Thus, concentrations of 2-pentanone and 2,4-dimethyl-1-heptene were found to increase in the headspace of A549, HBEpC, and hFB cell cultures. In addition, the ethers methyl *tert*-butyl ether and ethyl *tert*-butyl ether could be detected at elevated levels in the case of A549 cells and one of the untransformed cell lines. However, especially branched hydrocarbons and alcohols were seen increased more frequently in untransformed than A549 cells. A big variety of predominantly aldehydes and the ester *n*-butyl acetate were found at decreased concentrations in the headspace of all cell lines tested compared with medium controls. Again, more different aldehydes were found to be decreased in hFB and HBEpC cells compared with A549 cells and 2-butenal was metabolized exclusively by both control cell lines. These data suggest that certain groups of VOCs may be preferentially associated with the transformed phenotype. *Cancer Epidemiol Biomarkers Prev; 19(1); 182–95. ©2010 AACR.*

Introduction

Analysis of exhaled breath is a noninvasive method for diagnosis and therapeutic monitoring (1-3). Paradigmatic examples are the ^{13}C-urea breath test for detection of *Helicobacter pylori* (4, 5) and the hydrogen-based breath test for carbohydrate malabsorption (6). Promising investigations included critically ill persons (7, 8), patients suffering from renal and liver diseases (9-13), and cancer patients (14-21). Typical compounds in exhaled breath comprised hydrocarbons, ketones, aldehydes, alcohols, amides, sulfides, and ethers (21).

Because the field of breath analysis is relatively new, and the advances in analytic technology occur so fast, many compounds in exhaled breath have been detected; compounds whose biochemical origin has not yet been studied. In addition, little is known about their relationship to cellular processes such as malignant transformation. Investigations of exhaled breath from cancer patients showed that concentrations of specific compounds may be increased *or* decreased in comparison with healthy age-matched controls. This also applies to compounds in the headspace of cell cultures. Tumors are complex systems with a high degree of heterogeneity. Apart from the transformed cells, nontumorous components may also contribute to volatile organic compounds (VOC) present in the exhaled air of a lung cancer patient. Such potential sources of VOCs, which have not been studied here, are the activated immune system (22-25) and, possibly, microorganisms (26-28). The aim of the present work was to test for the existence of cancer-derived VOCs through the analysis of established cell lines

Authors' Affiliations: [1]Department of Operative Medicine and [2]Daniel-Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Center of Operative Medicine, Innsbruck Medical University, Innsbruck, Austria; [3]Breath Research Institute of the Austrian Academy of Sciences, Dornbirn, Austria; and [4]University of Rostock, Department of Anesthesiology and Intensive Care, Rostock, Germany

Authors' Contributions: The original plan of the cell culture workpackage in the EU-project BAMOD was devised and written by J. Schubert, W. Miekisch, A. Amann, and J. Troppmair. W. Filipiak developed the protocol for TD-GC-MS analyses of volatile compounds in headspace of cell cultures (conditions of sample collection, thermal desorption, gas chromatography temperature program, and mass spectrometry settings). Additionally, W. Filipiak performed the gas chromatographic analysis of all samples, performed the calibrations, and wrote a draft of the manuscript. A. Sponring contributed to cell culture sampling system development, performed the cell culture experiments and wrote the draft of the manuscript. A. Filipiak performed the chromatographic data analysis. C. Ager did the data analysis. J. Schubert and W. Miekisch developed the composition of sorption traps and chose the chromatographic column. A. Amann and J. Troppmair designed the study, supervised the experiments, discussed the results and continuous improvement of measurements using different analytic techniques, and finalized the manuscript. All authors read and approved the manuscript.

Corresponding Author: Anton Amann and Jakob Troppmair, Innsbruck Medical University, Innrain 66, Innsbruck 6020, Austria. Phone: 43-512-50427819; Fax: 43-512-50424624. E-mail: anton.amann@i-med.ac.at and jakob.troppmair@i-med.ac.at

doi: 10.1158/1055-9965.EPI-09-0162

[5] Sponring A, Filipiak W, Mikoviny T, et al. Release of volatile organic compounds (VOCs) from the lung cancer cell line NCI-H1666 *in vitro*. 2009:submitted.

(29-32). In previous experiments, we investigated three lung cancer cell lines, NCI-H2087 (32), CALU-1 (31), and NCI-H1666.[5] In NCI-H2087 cells, the release of the alcohol 2-ethyl-1-hexanol and the branched alkane 2-methylpentane was observed as well as a decline of acetaldehyde, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, and *n*-butyl acetate (32). The cell line CALU-1 showed a significant release of branched hydrocarbons such as 2,3,3-trimethylpentane, 2,3,5-trimethylhexane, and 2,4-dimethylheptane and 4-methyloctane, whereas decreased concentrations were found for acetaldehyde, 3-methylbutanal, *n*-butyl acetate, acetonitrile, acrolein, methacrolein, 2-methylpropanal, 2-butanone, methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and hexanal (31). However, no significantly increased release of VOCs could be shown for NCI-H1666 cells,[5] whereas a decrease in methacrolein, 3-methylbutanal, hexanal, and *n*-butyl acetate was observed. Thus, in our studies, compounds especially belonging to the class of branched hydrocarbons were released from lung cancer cells *in vitro*, whereas, in particular, aldehydes and *n*-butyl acetate decreased in concentration.

In the work presented here, we studied an additional lung cancer cell line, A549, to obtain a better defined spectrum of potential tumor cell–derived VOCs, as well as two nontransformed cell lines to filter out substances potentially restricted to transformed cells.

Materials and Methods

Cell Culture

A549 cells, which carry a mutated *K-Ras* but a wild-type *B-Raf* gene, have been obtained from American Type Culture Collection. They have been isolated originally from a lung carcinoma of a 58-y-old man and showed epithelial morphology and grew adherent (33-36). Human fibroblasts (hFB) derived from the dermis are a generous gift of Prof. Gabriele Werner-Felmayer, Section of Biological Chemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria. A549 and hFB cells were grown in DMEM high-glucose culture medium containing sodium pyruvate (110 mg/L) supplemented with 10% FCS, penicillin (100,000 units/L), streptomycin (100 mg/L), and L-glutamine (293 mg/L).

Human bronchial epithelial cells (HBEpC) are primary cells (PromoCell GmbH) isolated from the mucosa of the main bronchi of a 42-y-old male Caucasian. The cells were cultivated in Airway Epithelial Cell Growth Medium (PromoCell GmbH) supplemented with the Airway Epithelial Cell Growth Medium Supplement Pack (PromoCell GmbH) according to the manufacturer's instructions.

For all experiments, cells were cultivated under standard conditions at 37°C in a humidified atmosphere with 92.5% air/7.5% CO_2. For VOC measurements, 25, 75, or 100 million A549 cells and 50 million HBEpC or hFB cells, respectively, were inoculated in 100 mL phenol red–free DMEM high-glucose medium (supplements: 5% FCS, 100,000 units/L penicillin, 100 mg/L streptomycin, 293 mg/L L-glutamine, and 110 mg/L sodium pyruvate) or standard tissue culture medium (HBEpC cells). The concentration of FCS in DMEM during the experiment was lowered to 5% to reduce the high background of VOCs in the analyzed headspace. Culture vessels were then flushed with clean, synthetic air from a gas cylinder (defined gas mixture, Linde) containing 5% CO_2 to reduce background contamination. The rinsing was done for 10 min at a flow of 100 mL/min. Subsequently the fermenters were sealed for 21 h. At the end of the incubation time, 200 mL of air from the headspace was sampled and analyzed by gas chromatography mass spectrometry (GC-MS).

Sampling

Glass tubes (Gerstel) filled with the following sorbents were used as traps for sample collection with simultaneous preconcentration: 25 mg Tenax TA (60/80 mesh), 35 mg Carboxen 569 (20/45 mesh), and 250 mg Carboxen 1000 (80/100 mesh; each from Supelco). Sorbents were separated by glass wool. To decrease the relative humidity, the gaseous samples were diluted 1:6 (5 mL/min sample:25 mL/min air) with dry, additionally purified air taken from a gas cylinder (Linde). This procedure prevents the excessive adsorption of water on carbon molecular sieves and thereby avoids problems during sample preconcentration, cryofocusing during desorption, and finally chromatographic separation. The volume of collected sample originating from the fermenter was 200 mL with a total flow through sorption trap of 30 mL/min.

Thermal Desorption

The sampled analytes were released from sorbents by thermal desorption in a TDS3 unit equipped with a TDSA2 auto sampler (both from Gerstel). The flow rate of carrier gas through the sorption trap during desorption was 90 mL/min. The initial temperature of 30°C was increased to 300°C with a heating rate of 100°C/min (held for 10 min). Liquid nitrogen was used for cryofocusing the desorbed analytes at −90°C. For subsequent sample injection into the capillary column, the CIS-4 injector, which contained a glass liner filled with Carbotrap B (Gerstel), was heated at a rate of 12°C/s up to 320°C (than hold 2 min in splitless mode).

GC-MS Analyses

The TD-GC-MS analysis (thermal desorption coupled with gass chromatography mass spectrometry) were done on a 6890N gas chromatograph equipped with a mass selective detector 5973N (both from Agilent Technologies) with sample injection by means of thermal desorption (described in the previous sections). The PoraBond Q capillary column 25 m × 0.32 mm × 5 μm (Varian) was used. The oven temperature program was as follows: initial 50°C held for 5 min, then ramped 5°C/min up to 140°C, held for 5 min, again ramped 5°C/min to 280°C, and held for 4 min. The constant flow rate of helium carrier gas was 2 mL/min. The MS analyses were done in a full scan mode (TIC mode), with a scan range of 20 to 200 amu. Ionization of the separated compounds was done by electron impact ionization

Table 1. Quantification of VOCs released or taken up (consumed or degraded) by A549 cancer cells

Group	Class	Compound	CAS	R^2	LOD [ppb_v]
INCREASED	Hydrocarbons	2-Methyl-1-pentene	763-29-1	0.996	0.133
		n-Octane	111-65-9	0.999	0.827
		2,4-Dimethyl-1-heptene	19549-87-2	0.998	0.199
	Alcohols	Ethanol	64-17-5	0.921	2.723
	Ethers	Methyl *tert*-butyl ether	1634-04-4	0.999	0.508
		Ethyl *tert*-butyl ether	637-92-3	0.999	0.372
	Ketones	Acetone	67-64-1	0.999	0.382
		2-Pentanone	107-87-9	0.997	0.164
DECREASED	Esters	*n*-Butyl acetate	123-86-4	0.999	0.140
	Aldehydes	Methacrolein	78-85-3	0.999	0.806
		2-methylpropanal	78-84-2	0.996	0.180
		2-Ethylacrolein	922-63-4	0.993	0.391
		2-Methyl-2-butenal	1115-11-3	0.985	0.745
		3-methylbutanal	590-86-3	0.994	0.406
	Aromatic amines	Pyrrole	109-97-7	0.979	0.716

NOTE: CAS numbers (Chemical Abstracts Service), correlation coefficients (R^2), and LODs expressed in concentration unit [ppb_v] are presented. Average concentrations (ppb_v) are given with SDs. The ratio of the average concentrations of the target analyte compared with medium control and the *p* values of Kruskal-Wallis tests have been calculated for each cell density.

at 70 eV. The chromatographic data was acquired using the Agilent Chemstation Software (GC-MS Data Analysis from Agilent). The mass spectrum library NIST 2005 was applied for the identification of detected compounds.

Reagents and Standards

2,4-Dimethyl-1-heptene, 2,3,5-trimethylhexane and 2,3,3-trimethylpentane were purchased from Chem-SampCo, and 2-pentanone was from Acros Organics.

Table 1. Quantification of VOCs released or taken up (consumed or degraded) by A549 cancer cells (Cont'd)

Mean medium (ppb_v)	SD medium (ppb_v)	Mean cells (ppb_v)	SD cells (ppb_v)	*p*	Ratio cell/medium
1.741	1.357	4.247	2.130	0.086	3.13
		5.295	2.500	0.043	3.90
		6.996	1.873	0.014	5.16
1.659	0.636	2.566	0.734	0.142	4.03
		2.567	0.563	0.149	4.03
		2.902	0.522	0.027	4.56
3.089	1.777	6.001	3.107	0.142	3.38
		6.999	2.527	0.021	3.94
		9.927	3.282	0.014	5.59
63.23	50.51	186.5	75.21	0.050	3.69
		229.4	112.7	0.083	4.54
		211.0	120.7	0.040	3.34
0.949	0.240	2.306	0.771	0.014	9.60
		1.949	0.497	0.021	8.11
		2.050	0.383	0.014	8.54
4.726	0.767	8.574	2.399	0.014	11.18
		7.657	2.973	0.149	9.98
		9.258	2.251	0.014	12.07
193.4	44.80	303.9	81.42	0.050	6.78
		296.7	87.47	0.083	6.62
		357.8	74.30	0.014	7.99
0.551	0.139	1.809	0.485	0,014	13.01
		2.071	0.722	0.021	14.89
		2.333	0.732	0.014	16.77
52.67	9.555	31.34	6.906	0.027	3.28
		8.096	2.838	0.021	0.85
		5.303	1.828	0.014	0.55
7.931	5.713	0.832	0.469	0.014	0.15
		<LOD	—	0.018	—
		<LOD	—	0.013	—
59.79	9.019	0.197	0.270	0.013	0.02
		0.000	—	0.014	—
		2.501	5.591	0.011	0.28
0.802	0.332	0.000	—	0.007	—
		0.000	—	0.014	—
		0.000	—	0.007	—
1.833	1.534	0.000	—	0.007	—
		0.000	—	0.014	—
		0.000	—	0.007	—
191.8	24.33	2.249	1.477	0.014	0.09
		2.215	0.510	0.021	0.09
		2.589	0.829	0.014	0.11
1.009	0.620	<LOD	—	0.007	—
		<LOD	—	0.014	—
		<LOD	—	0.007	—

All other compounds were purchased from Sigma Aldrich.

Calibration

For the quantification of compounds detected in the headspace of cells and of the medium, an external standard calibration was done. The preparation of gaseous standards was done by evaporating liquid substances in glass bulbs. Each bulb (Supelco) was cleaned with methanol (Sigma-Aldrich), dried at 85°C for at least 20 h, purged with clean nitrogen for minimally 20 min, and subsequently evacuated using a vacuum pump (Vacuubrand) for 30 min. Liquid standards (1-3 μL, according to the desired concentration) were

injected through a septum by using a GC syringe. After the evaporation of standards, the glass bulb was filled with nitrogen of purity 6.0 (i.e., 99.9999%, Linde) to equalize the pressure to ambient pressure. Then, the appropriate volume (μL) of vapor mixture was transferred by a gas tight syringe (Hamilton) into Tedlar bags (SKC 232 Series), which were previously filled with 1.5 liters of nitrogen (99,9999%, additionally purified by means of carbon molecular sieves Carboxen 1000).

Statistical Analyses

Putative statistical significance was calculated by the Kruskal-Wallis test, which is a test to compare samples from two or more groups of independent observations (37). It is a one-way ANOVA and does not assume a normal population, unlike the analogous one-way ANOVA. The Kruskal-Wallis test is a nonparametric version of the classic one-way ANOVA, and an extension of the Wilcoxon rank-sum test to more than two groups (37). Additionally, results are presented as mean values with SDs.

Results

Our study with CALU-1 cells had shown that only longer incubation times (18 hours) allowed for the reproducible detection of significant differences in VOC concentrations (31). Here, we consistently kept the incubation time at 21 hours and observed that after this time, the average viability was 97.7 ± 1.1% for 25 million, 95.3 ± 2.9% for 75 million, and 96.4 ± 6.2% for 100 million A549 cells. Thus, cell culture conditions did not cause substantial cell death, which ensured that the release of potential VOCs was mostly due to living cells. Under the same conditions, the average viability was 95.4 ± 2.29% for hFB and 77.9 ± 9.90 for HBEpC cells, which proved more fragile under the experimental setting used. The considerable cell death observed in the case of the HBEpC cells may additionally contribute to differences in VOC profiles in ways, which will be addressed in future studies.

Identification and Quantification of VOCs Released by Cells *In vitro*

Among all compounds detected, 132 compounds were identified not only by spectral library match using the NIST 2005 library but also by determination of their retention time based on calibration mixtures of the respective pure standards. The peaks, for which proper identification was not possible (too low library match and no confirmation by retention time), are not discussed. Generally, the applied TD-GC-MS method is characterized by good linearity (even for the lowest concentrations detected) with correlation coefficients R^2 being predominantly higher than 0.99 in calibration measurements. The limits of detection (LOD) for almost all compounds of interest were at the ppt_v level, being lowest for hydrocarbons such as 2,4-dimethylhexane (0.044 ppb_v) or 3-methylheptane (0.048 ppb_v). The applied method was also very sensitive for polar analytes such as propyl acetate (0.078 ppb_v), methyl acetate (0.106 ppb_v), or 2-methylbutanal (0.134 ppb_v). The compounds with LOD at the single ppb_v level were alcohols, such as 2-ethyl-1-hexanol (9.885 ppb_v), ethanol (2.723 ppb_v), or acetaldehyde (1.517 ppb_v). The use of Tedlar bags for the preparation of standard mixtures for TD-GC-MS calibration could be the reason of relatively high LODs (single ppb_v level), especially for alcohols that are partly absorbed in Tedlar material. It should also be noted that the selected ion-monitoring mode, which improves the sensitivity of MS analyses, was not applied. Instead, full scan mode (Total Ion Chromatogram, TIC mode) was chosen to be adequate for the correct identification of a wide range of VOCs detected in the samples. Thus, the measured low LOD with simultaneous low errors (expressed by correlation coefficients) testify very good precision and sensitivity of the applied TD-GC-MS method.

TD-GC-MS Analyses of VOCs in the Headspace of A549 Cells

Our experiments with A549 cells included the analyses of medium controls ($n = 4$) and 25 ($n = 5$), 75 ($n = 4$), or 100 million cells ($n = 5$). Eight compounds were found to be increased and seven compounds to be decreased in the headspace of A549 cells compared with medium control (Table 1). The concentrations of methyl *tert*-butyl ether and 2-pentanone were found to be increased in all A549 cancer cell samples (Fig. 1). Besides that, the unsaturated branched hydrocarbons 2-methyl-1-pentene and 2,4-dimethyl-1-heptene were found to be significantly elevated in the headspace of 75 and 100 million A549 cells. No significant differences to medium controls were found for these two compounds in experiments with 25 million cells although concentrations were increased. Furthermore, isobutene and octane showed significantly increased concentrations in experiments with 100 million cells ($P = 0.01$ and 0.03, respectively) but not with lower cell amounts. In general, relatively low concentrations of VOCs released by cells and high background levels originating from medium controls resulted in big SDs. Therefore, a considerable amount of cells is required to detect statistically significant differences in the level of VOCs released by A549 cancer cells and other tested cell lines. Moreover, ethyl *tert*-butyl ether, acetone, and ethanol were present at significantly higher concentrations in the headspace of 25 million and 100 million cancer cells compared with medium controls. No statistically significant difference in the amounts of these three VOCs compared with medium controls was found in experiments with 75 million A549 cells (Fig. 1).

Among the *decreased* compounds, the aldehydes 2-ethylacrolein and 2-methyl-2-butenal were found exclusively in the headspace of medium controls and not in the headspace of cell samples. 2-Methylpropanal was not detected in measurements with 75 million cells

(n = 4) but occasionally in measurements of 25 million (2 of 5) and 100 million cells (1 of 5; see Table 1). Similarly, pyrrole was found in several but not all measurements. For a few other significantly decreased compounds, the levels detected were below their LOD (pyrrole and methacrolein). Only n-butyl acetate and 3-methylbutanal were present in all samples at concentrations above their LODs and at significantly lower concentrations in the headspace of cancer cells (Fig. 1). n-Butyl acetate showed p values below 0.05 in all experiments and, in the case of 100 million cells, a p value of 0.01. More detailed information can be found in Table 1.

TD-GC-MS Analyses of VOCs in the Headspace of HBEpC and hFB Cells

In the case of the HBEpC, four independent measurements were done for medium controls (n = 4) and three for 50 million HBEpC cells (n = 3). The concentrations of 10 compounds were *increased* and of 8 compounds *decreased* in the headspace of HBEpC cells compared with medium control (Table 2). As already observed in A549 cells, acetone, ethyl *tert*-butyl ether, 2-pentanone, and 2,4-dimethyl-1-heptene were released by HBEpC cells in significant amounts (Fig. 2). The remaining six VOCs with increased concentrations in the headspace of HBEpC included the three branched hydrocarbons 2,3,3-trimethylpentane, 4-methylheptane, and 3-methylheptane. The concentration of 2,3,3-trimethylpentane was below the LOD in the headspace of medium controls and the other two were not detected in the controls at all. In addition, the alcohol 2-methyl-2-propanol and the esters methyl acetate and n-propyl acetate (not detected in the medium control headspace) had increased concentrations in HFB cells.

Like in A549 cells, methacrolein, 2-methylpropanal, 3-methylbutanal, and n-butyl acetate showed diminished concentrations in the headspace of HBEpC cells. Methacrolein was not detected at all in the headspace of cell cultures, and n-butyl acetate was reduced to 19.1% of medium control concentration. The aldehyde 2-methylpropanal was found only in one of three cell samples, whereas 3-methylbutanal, which was detected in all samples measured, was reduced to 1.1% of medium control concentration (Fig. 2). Moreover, acetaldehyde and

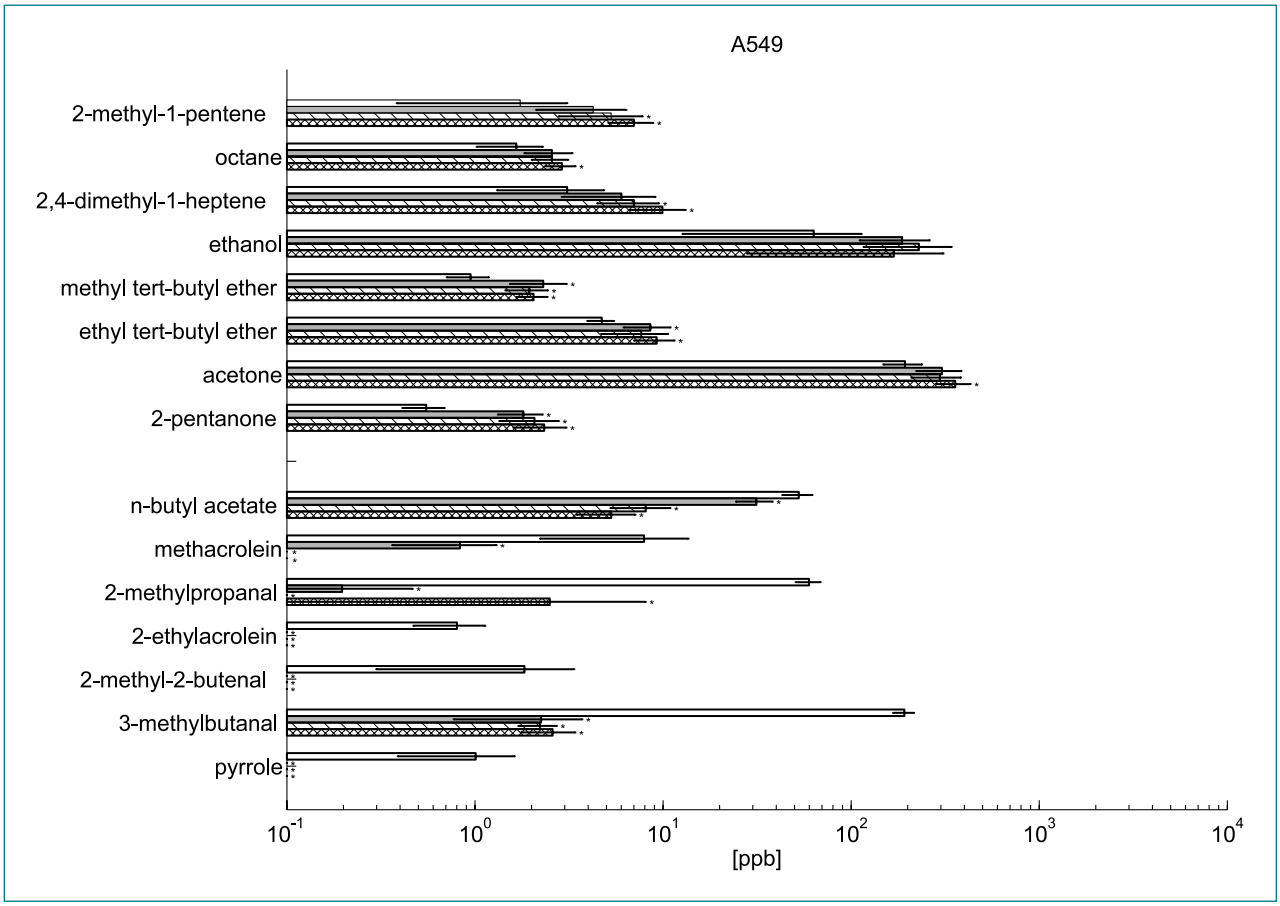

Figure 1. VOCs present at higher or lower concentrations in the headspace of A549 cells than in the medium control. Shown are average concentrations (ppb_v) in logarithmic scaling with SD for 25 million cells (n = 5; *dashed columns*), 75 million cells (n = 4; *squared columns*), and 100 million cells (n = 5; *crossdashed columns*) compared with medium (n = 4; *empty columns*). *, significant differences.

Table 2. Quantification of VOCs released or taken up (consumed or degraded) by nontransformed cells—hFB and HBEpC

Cell type	Group	Class	Compound	CAS	R^2
hFBs	Increased	Hydrocarbons	Benzene	71-43-2	0.999
			2,3,3-Trimethylpentane	560-21-4	0.998
			2,3,4-Trimethylpentane	565-75-3	0.995
			2,4-Dimethylhexane	589-43-5	0.999
			4-Methylheptane	589-53-7	0.993
			3-Methylheptane	589-81-1	0.998
			n-Octane	111-65-9	0.998
			2,4-Dimethyl-1-heptene	19549-87-2	0.998
			2,3,5-Trimethylhexane	1069-53-0	0.997
		Alcohols	2-Methyl-1-propanol	78-83-1	0.995
			3-Methyl-1-butanol	123-51-3	0.996
			2-Ethyl-1-hexanol	104-76-7	0.918
		Ethers	Methyl tert-butyl ether	1634-04-4	0.999
		Ketones	2-Pentanone	107-87-9	0.997
			2-Hexanone	591-78-6	0.997
	Decreased	Esters	*n*-Butyl acetate	123-86-4	0.999
		Aldehydes	Acetaldehyde	75-07-0	0.993
			(E)-2-Butenal	123-73-9	0.997
			2-Methylpropanal	78-84-2	0.996
			2-Methylbutanal	96-17-3	0.996
			3-Methylbutanal	590-86-3	0.994
			Benzaldehyde	100-52-7	0.990
		Ketones	3-Penten-2-one	3102-33-8	0.995
HBEpC	Increased	Hydrocarbons	3-Methylheptane	589-81-1	0.999
			2,3,3-Trimethylpentane	560-21-4	0.998
			4-Methylheptane	589-53-7	0.993
			2,4-Dimethyl-1-heptene	19549-87-2	0.998
		Alcohols	2-Methyl-2-propanol	75-65-0	0.997
		Esters	Methyl acetate	79-20-9	0.999
			n-Propyl acetate	109-60-4	0.998
		Ethers	Ethyl tert-butyl ether	637-92-3	0.998
		Ketones	Acetone	67-64-1	0.999
			2-Pentanone	107-87-9	0.997
	Decreased	Esters	*n*-Butyl acetate	123-86-4	0.999
		Aldehydes	Acetaldehyde	75-07-0	0.993
			Methacrolein	78-85-3	0.998
			(E)-2-Butenal	123-73-9	0.999
			2-Methylpropanal	78-84-2	0.995
			3-Methylbutanal	590-86-3	0.994
			Hexanal	66-25-1	0.981
			Octanal	124-13-0	0.914

NOTE: CAS numbers (Chemical Abstracts Service), correlation coefficients (R^2), and LODs expressed in concentration unit [ppb_v] are presented. Average concentrations (ppb_v) are given with SDs. The ratio of the average concentrations of the target analyte compared with medium control and the *P* values of Kruskal-Wallis tests have been calculated for each cell density.

2-butenal (below LOD in the headspace of cells) showed decreased concentrations. Exclusively decreased in HBEpC cells were the aldehydes hexanal and octanal.

In the case of the second control cell line hFB, the concentrations of 15 compounds were *increased* and of 8 were *decreased* (Table 2). Altogether, three independent experiments with 50 million cells and four independent measurements with medium control were done. Like in A549 cancer cells, the concentrations of methyl *tert*-butyl ether, 2-pentanone, and 2,4-dimethyl-1-heptene

Table 2. Quantification of VOCs released or taken up (consumed or degraded) by nontransformed cells—hFB and HBEpC (Cont'd)

LOD [ppb_v]	Mean medium (ppb_v)	SD medium (ppb_v)	Mean cells (ppb_v)	SD cells (ppb_v)	*p*	Ratio cell/medium
0.201	5.291	1.264	6.914	0.689	0.025	130.68
0.127	1.389	0.616	4.688	0.155	0.025	337,46
0.137	0.147	0.251	1.106	0.140	0.022	752.78
0.044	0	—	0.396	0.216	0.010	—
0.202	0.831	0.387	2.866	0.057	0.025	344.68
0.048	2.463	0.528	4.059	0.706	0.025	164.80
0.064	7.996	2.248	12.28	1.731	0.025	153.59
0.199	6.105	2.413	16.22	2.723	0.025	265.75
0.267	0	—	7.213	4.925	0.010	—
1.283	0.803	1.124	6.242	3.401	0.022	776.91
1.141	0	—	11.38	1.225	0.010	—
4.199	488.7	161.5	1,082.5	98.009	0.025	221.49
0.293	0.346	0.489	1.692	0.933	0.047	488.57
0.164	1.059	0.681	9.450	10.218	0.025	892.39
0.152	0.596	0.375	2.322	0.995	0.025	389.70
0.134	55.51	28.83	11.22	2.904	0.025	20.20
1.517	883.1	462.2	167.7	52.968	0.025	18.99
0.254	4.289	0.844	0	—	0.022	0
0.180	98.41	40.43	0.644	0.287	0.025	0.65
0.134	214.8	117.4	1.620	0.119	0.025	0.75
0.406	158.9	60.19	3.800	0.624	0.025	2.39
0,320	36.26	11.39	2.798	2.170	0.025	7.71
0.160	1.611	0.704	0	—	0.022	0
0.048	0	—	1.400	0.910	0.006	—
0.127	<LOD	—	1.988	0.943	0.011	—
0.202	0	—	0.604	0.562	0.006	—
0.169	1.077	1.239	4.636	2.004	0.039	430.55
0.525	0.740	0.815	9.624	6.539	0.018	1,300.67
0.106	0.883	0.355	5.169	1.611	0.020	585.21
0.078	0	—	0.555	0.448	0.006	—
0.369	0	—	1.754	1.644	0.006	—
0.382	13.88	7.901	39.53	19.18	0.039	284.89
0.164	<LOD	—	1.337	0.765	0.020	—
0.134	13.90	8.014	2.651	4.591	0.038	19.07
1.517	741.8	198.7	365.7	250.7	0.020	49.30
0.798	4.281	1.699	0	—	0.018	0.00
0.254	3.430	0.771	<LOD	—	0.020	3.19
0.182	18.71	5.538	0.216	0.374	0.020	1.15
0.406	54.08	18.25	0.604	1.046	0.020	1.12
0.936	1,093.2	305.6	192.0	211.7	0.020	17.56
1.020	6.457	1.856	1.219	2.112	0.038	18.88

were found to be significantly increased for hFB cells (Table 2; Fig. 3).

Among the remaining 12 VOCs with increased concentrations in hFB cells, 6 were branched saturated hydrocarbons including 2,4-dimethylhexane, which was not detected in the medium control, 2,3,4-trimethylpentane, 2,3,3-trimethylpentane, 4-methylheptane, 3-methylheptane, and 2,3,5-trimethylhexane (not detected in the medium control headspace). Increased concentrations were also found for octane and several alcohols, such as 2-methyl-1-propanol (below LOD in medium control), 3-methyl-1-butanol (not detected in medium control), and 2-ethyl-1-hexanol (221.5% of medium control concentration). Two unique analytes released by hFB cells were 2-hexanone and benzene.

As in A549 cells, *n*-butyl acetate, 2-methylpropanal, and 3-methylbutanal were significantly decreased in the headspace of hFB cells. Other aldehydes with decreased concentrations were acetaldehyde, 2-methylbutanal, 2-butenal (not detected in the headspace of cells), and

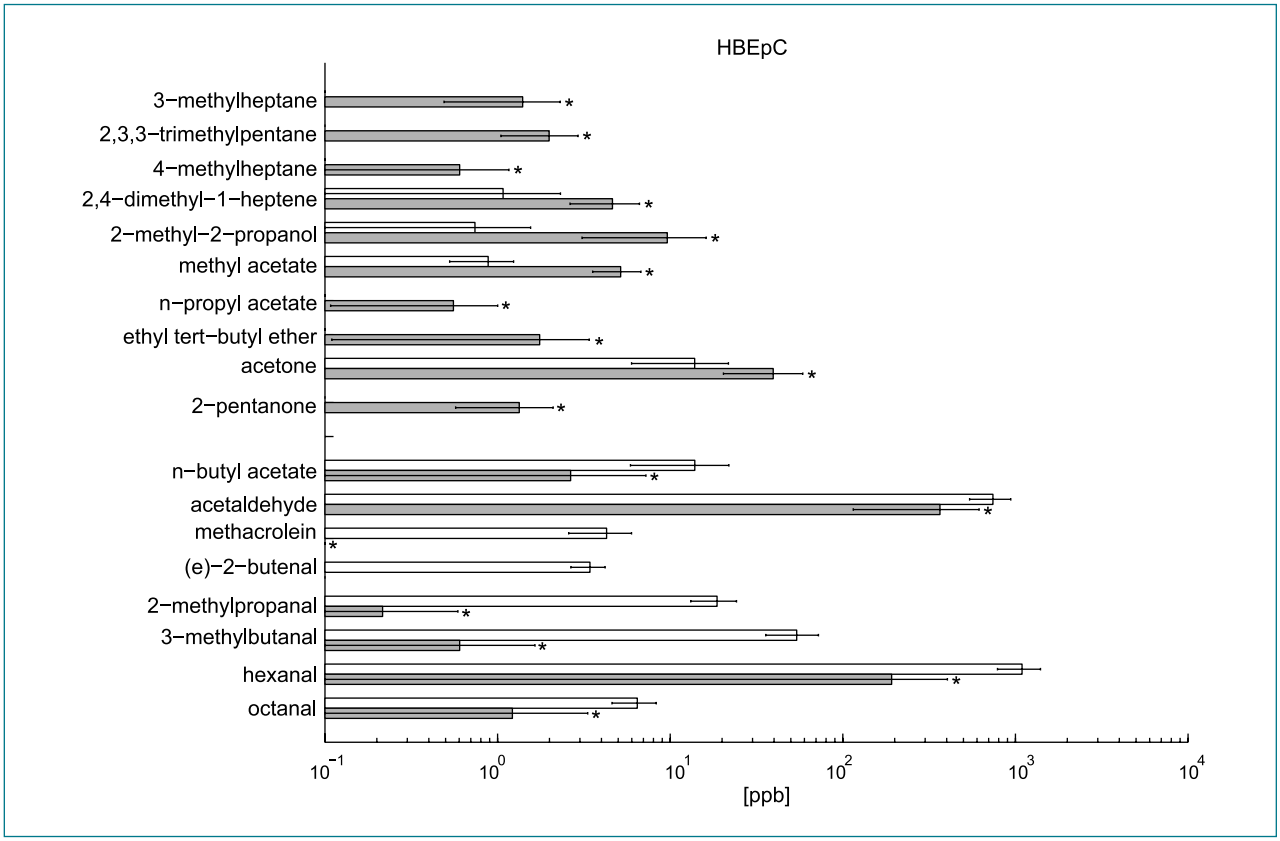

Figure 2. VOCs present at higher or lower concentrations in the headspace of the HBEpC cell line than in medium controls. Presented are average concentrations (ppb_v) in logarithmic scaling with SD for 50 million cells (n = 3; *gray columns*) compared with medium (n = 4; *empty columns*). *, significant differences.

benzaldehyde. The only compound that was significantly decreased (consumed or degraded) exclusively by hFBs was the ketone (E)-3-penten-2-one (not detected in the headspace of cells; Table 2; Fig. 3).

Discussion

VOCs belonging to various classes of chemical compounds have been linked previously to lung cancer by different authors (14, 17-21). For most of these compounds, the cellular and biochemical origin has not been determined and some of them might be of exogenous origin. For the production or consumption of the compounds found, different types of cells could be responsible including nontumorous cells, i.e., normal surrounding tissue, immune cells, or even infectious agents. In the study presented here, we attempted not only to provide further insight into VOCs specifically released by cancer cells (31, 32),[5] but also to look for the presence of VOCs, which may help to discriminate normal from transformed cells. This knowledge will be essential to introduce VOCs into routine screening procedures. A common feature of the cell lines we have studied thus far (31, 32),[5] with the exemption of NCI-H1666, is the fact that similar hydrocarbons are released at significant level. In contrast, several aldehydes and *n*-butyl acetate were consumed by these cell lines. In addition, no hydrocarbon was taken up (consumed or degraded) and no aldehydes or *n*-butyl acetate were ever released by these cells.

Merely a few compounds found with the tested lung cancer cell lines A549, NCI-H2087, and CALU-1 are unique and not found in the control cells tested here. Particularly interesting among them is 2-methylpentane (released by NCI-H2087), which has been detected at higher concentrations in the breath of patients suffering from non–small cell lung cancer (20). 4-Methyloctane, exclusively found in CALU-1 cells, has been reported by Phillips et al. (14-16, 38) and has been used to differentiate between lung cancer patients and healthy volunteers. Similarly, in our work with lung cancer patients (21), we have obtained evidence that some branched hydrocarbons are important VOCs, but also alcohols and ketones are found to be increased in concentration in the breath of cancer patients. Nevertheless, it should be noted that no hydrocarbon was significantly *released* by at least two of the different cancer cell lines studied here. This may be due to the fact that every tumor cell line

is only a limited representation of the human primary tumor it has been derived from, and only the comparison on many more such lines and the inclusion of primary material will allow to pinpoint VOCs, which can serve as biomarkers.

Interestingly, more compounds with significant differences in their concentration to medium controls were released by healthy than tumor cells. This can be seen for the release of the branched hydrocarbons 2,3,4-trimethylpentane, 2,4-dimethylhexane, 4-methylheptane, and 3-methylheptane, where no differences to the medium control were found in the cancer cell line studies (Table 3). Furthermore, the differences in the concentration of four hydrocarbons, 2,3,3-trimethylpentane, *n*-octane, 2,3,5-trimethylhexane, and 2,4-dimethyl-1-heptene, between cancer cells and medium controls were higher for nontransformed than A549 cells.

Higher activity of nontransformed cells in production of VOCs could also be suspected because of the more abundant release of alcohols. In particular, 2-methyl-1-propanol, 2-methyl-2-propanol, and 3-methyl-1-butanol were found to be significantly released only by healthy cell lines and not by any of cancer cell lines tested in this or earlier studies (Table 3). These data suggest that the metabolic pathways, which have lead to the generation of these compounds, have been more active in normal than in transformed cells and point to a possible tumor suppressive function. Interestingly, 2-ethyl-1-hexanol, which was released at nearly the same level by hFB and NCI-H2087 cells, was also released from cells of the dermis as described previously (39). The only alcohol produced exclusively by cancer cells was ethanol (Table 1; Fig. 1). Restricted to primary bronchial cells was the release of the esters methyl acetate and *n*-propyl acetate. No esters at all were found to be significantly released by any cancer cell line investigated in this and previous studies (31, 32).[5] However, this difference between carcinogenic and noncarcinogenic cells, suggested by our studies, needs to be confirmed by additional investigations.

The only two ethers significantly released by cancer cells are methyl *tert*-butyl ether and ethyl *tert*-butyl ether (released by A549 cells). Interestingly, the highest concentration of methyl *tert*-butyl ether was observed for 25 million A549 cells, perhaps reflecting better growth conditions at lower cell numbers. However, because the concentration of methyl *tert*-butyl ether released is very similar among cells, the concentration profile of

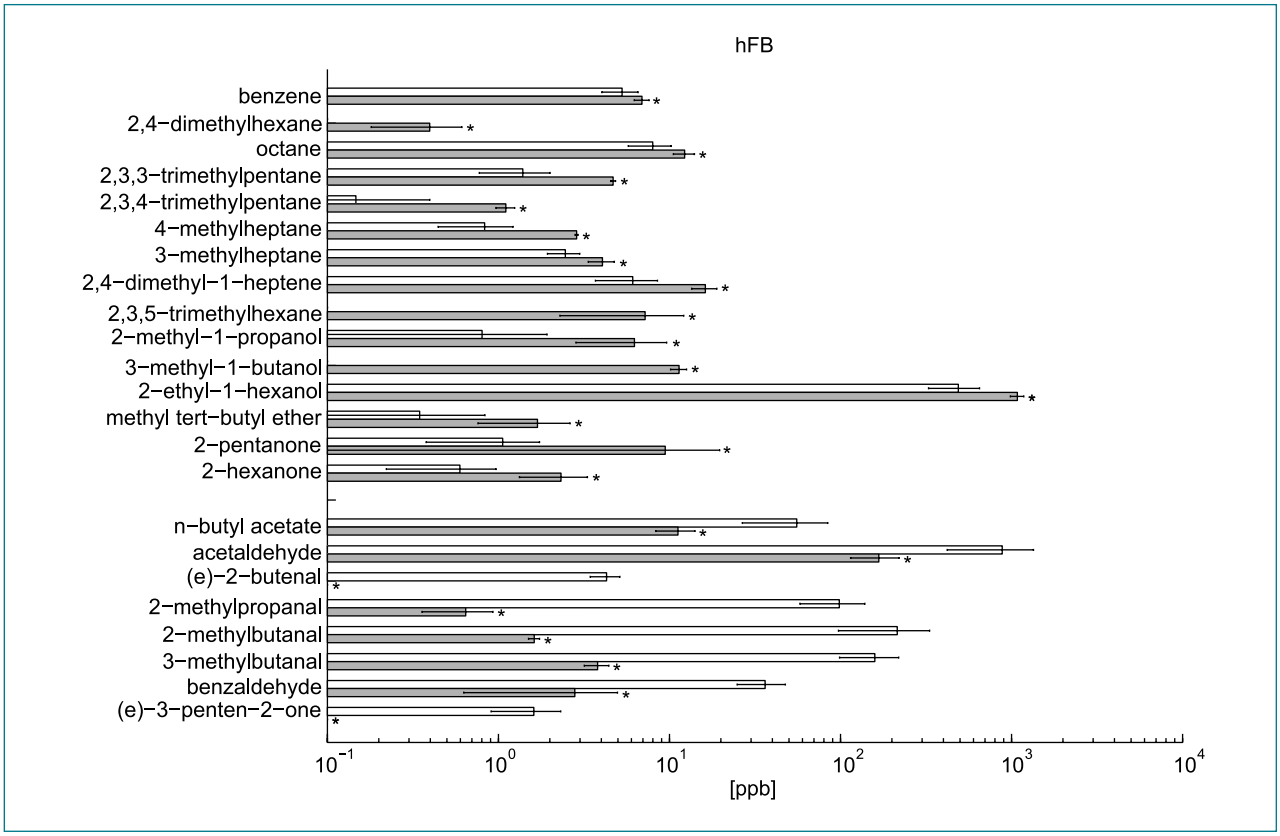

Figure 3. VOCs present at higher or lower concentrations in the headspace of the hFB cell line than in medium controls. Presented are average concentrations (ppb_v) in logarithmic scaling with SD for 50 million cells ($n = 3$; *gray columns*) compared with medium ($n = 4$; *empty columns*). *, significant differences.

Table 3. Overview over VOCs released or taken for HBEpC, hFB, A549, NCI-H2087 (32), CALU-1 (31), and NCI-H1666 cells

Group	Class	Normal cells	
		Bronchia	Dermis
		HBEpC	hFB
Increased	Hydrocarbons		
			2,4-Dimethylhexane
			2,3,4-Trimethylpentane
		2,3,3-Trimethylpentane	2,3,3-Trimethylpentane
		4-Methylheptane	4-Methylheptane
			Octane
		3-Methylheptane	3-Methylheptane
			2,3,5-Trimethylhexane
		2,4-Dimethyl-1-heptene	2,4-Dimethyl-1-heptene
	Ketones	Acetone	
		2-Pentanone	2-Pentanone
			2-Hexanone
	Alcohols		
		2-Methyl-2-propanol	
			2-Methyl-1-propanol
			3-Methyl-1-Butanol
			2-Ethyl-1-hexanol
	Esters	Methyl acetate	
		n-Propyl acetate	
	Ethers		Methyl tert-butyl ether
		Ethyl tert-butyl ether	
	Aromatics		Benzene
Decreased	Aldehydes	Acetaldehyde	Acetaldehyde
		Methacrolein	
		2-Methylpropanal	2-Methylpropanal
		2-Butenal	2-Butenal
		3-Methylbutanal	3-Methylbutanal
			2-Methylbutanal
		Hexanal	
			Benzaldehyde
		Octanal	
	Esters	*n*-Butyl acetate	*n*-Butyl acetate
	Ketones		
			(E)-3-Penten-2-one
	Ethers		
	Furans		
	N containing		

NOTE: For NCI-H1666: Sponring A, Filipiak W, Mikoviny T, et al. Release of VOCs from the lung cancer cell line NCI-H1666 *in vitro*. 2009:submitted.

Table 3. Overview over VOCs released or taken for HBEpC, hFB, A549, NCI-H2087 (32), CALU-1 (31), and NCI-H1666 cells (Cont'd)

Lung cancer cell lines			
A549	**NCI-H2087**	**NCI-H1666**	**CALU-1**
	2-Methylpentane		
2-Methyl-1-pentene			
			2,3,3-Trimethylpentane
Octane			
			2,3,5-Trimethylhexane
			2,4-Dimethylheptane
2,4-Dimethyl-1-heptene			
			4-Methyloctane
Acetone			
2-Pentanone			
Ethanol			
	2-Ethyl-1-hexanol		
Methyl tert-butyl ether			
Ethyl tert-butyl ether			
	Acetaldehyde		Acetaldehyde
			Acrolein
Methacrolein		Methacrolein	Methacrolein
2-Methylpropanal	2-Methylpropanal		2-Methylpropanal
Butanal			
2-Ethylacrolein			2-Ethylacrolein
3-Methylbutanal	3-Methylbutanal	3-Methylbutanal	3-Methylbutanal
	2-Methylbutanal		
2-Methyl-2-butenal			2-Methyl-2-butenal
		Hexanal	Hexanal
			Benzaldehyde
n-Butyl acetate	*n*-Butyl acetate	*n*-Butyl acetate	*n*-Butyl acetate
			2-Butanone
			Methyl tert butyl ether
			Ethyl tert butyl ether
			Tetrahydrofuran
Pyrrole			
			Acetonitrile

this analyte is most likely within the range of random error and the sensitivity of the applied methods is insufficient to detect any increase. Both ethers were also significantly released by one of the nontransformed cell types, methyl *tert*-butyl ether by hFB and ethyl *tert*-butyl ether by HBEpC. In contrast, these ethers were found to be significantly decreased (degraded) by CALU-1 cancer cells.

For ketones, a representative analyte was acetone. In the experiments discussed here, acetone was released by nontransformed (HBEpC) and cancer (A549) cell lines. Among other ketones, 2-pentanone was secreted by all three currently tested cell lines (hFB, HBEpC, and A549), whereas 2-hexanone was only released by hFB cells. It should be noted that as for some of previously mentioned metabolites, hFB cells released the highest amounts of 2-pentanone. On the other hand, two other ketones were taken up (consumed or degraded), namely 3-penten-2-one by hFBs and 2-butanone, by previously investigated CALU-1 cancer cells.

A significant observation in this study is the strong decrease in the concentration of numerous aldehydes and *n*-butyl acetate in the headspace of A549 cell cultures and control cells (Tables 1 and 2; Figs. 1-3). Among the decreased VOCs, the ester *n*-butyl acetate and the aldehyde 3-methylbutanal were found to be lowered in all tested cell lines. Previous work on NCI-H2087 (32), NCI-H1666,[5] and CALU-1 (31) lung cancer cell lines showed that both 3-methylbutanal and *n*-butyl acetate were decreased in all cells investigated *in vitro*. Typically, but not always, the aldehydes acetaldehyde, 2-methylpropanal, and methacrolein were also found to be decreased (Table 3). This decrease could be observed either in the lung cancer cell lines A549, NCI-H2087, or CALU-1, or in one of the tested control cell lines (Table 3; Figs. 1-3). 2-Ethylacrolein and 2-ethyl-2-butenal were only degraded by lung cancer cell lines. A HBEpC-specific feature not found in any of the other investigated cell lines was the degradation of *n*-octanal, whereas only hFB cells showed a decrease in 3-penten-2-one. It should be noticed that besides the ester *n*-butyl acetate (and 3-penten-2-one for hFB), nontransformed cells only degrade aldehydes, whereas cancer cells could also degrade nitrogen-containing compounds (pyrrole by A549 and acetonitrile by CALU-1), ketone, and ethers (acetone, methyl *tert*-butyl ether, and ethyl *tert*-butyl ether, respectively, all degraded by the CALU-1 line).

Overall, the reasons for differences in VOC release or consumption among the investigated cell lines are currently unknown, but may result from phenotypic or genotypic differences. Clarification of this issue will require an understanding of the underlying molecular mechanisms for VOC production, which is currently lacking for the mentioned compounds.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Member of the Tyrolean regional government Dr. Erwin Koler and the Director of the University Clinic of Innsbruck (TILAK) Mag. Andreas Steiner for their generous support.

Grant Support

European Commission (project BAMOD, project No LSHC-CT-2005-019031) and the Austrian Ministry for Science and Research (BMWF, Vienna, Austria, grants GZ 651.019/1-VI/2/2006, GZ 651.019/3-VI/2/2006).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 2/20/09; revised 10/13/09; accepted 11/3/09; published online 1/7/10.

References

1. Amann A, Smith D, editors. Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring. Singapore: World Scientific; 2005.
2. Amann A, Spanel P, Smith D. Breath analysis: the approach towards clinical applications. Mini Rev Med Chem 2007;7:115–29.
3. Risby T. Current status of clinical breath analysis. In: Amann A, Smith D, editors. Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring. Singapore: World Scientific; 2005, p. 251–65.
4. Chen CP, Chen CY, Lu CL, et al. Infrared spectrometry based 13C-octanoic acid breath test in measuring human solid gastric emptying. J Gastroenterol Hepatol 2003;18:41–6.
5. Liao CC, Lee CL, Chiang TC, et al. The 13C-urea breath test to detect Helicobacter pylori infection: a validated simple methodology with 50 mg 13C-urea. Aliment Pharmacol Ther 2002; 16:787–92.
6. Eisenmann A, Amann A, Said M, Datta B, Ledochowski M. Implementation and interpretation of hydrogen breath test. J Breath Res 2 (2008) 046002 (9pp).
7. Schubert J, Miekisch W, Nöldge-Schomburg G. VOC breath markers in critically ill patients: potentials and limitations. In: Amann A, Smith D, editors. Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring. Singapore: World Scientific; 2005, p. 267–92.
8. Miekisch W, Schubert JK, Noeldge-Schomburg GF. Diagnostic potential of breath analysis-focus on volatile organic compounds. Clin Chim Acta 2004;347:25–39.
9. Davies S, Spanel P, Smith D. A new 'online' method to measure increased exhaled isoprene in end-stage renal failure. Nephrol Dial Transplant 2001;16:836–9.
10. Davies S, Spanel P, Smith D. Quantitative analysis of ammonia on the breath of patients in end-stage renal failure. Kidney Int 1997; 52:223–8.
11. Davies SJ, Spanel P, Smith D. Quantitative analysis of metabolites on the breath of patients in renal failure. J Am Soc Nephrol 1996;7: A0352–A.
12. Perri F, Marras RM, Ricciardi R, Quitadamo M, Andriulli A. 13C-breath tests in hepatology (cytosolic liver function). Eur Rev Med Pharmacol Sci 2004;8:47–9.
13. Candelli M, Armuzzi A, Nista EC, Fini L, Gasbarrini G, Gasbarrini A. 13C-methacetin breath test for monitoring hepatic function in

cirrhotic patients before and after liver transplantation. Aliment Pharmacol Ther 2004;19:243.
14. Phillips M, Altorki N, Austin JH, et al. Prediction of lung cancer using volatile biomarkers in breath. Cancer Biomark 2007;3:95–109.
15. Phillips M, Cataneo RN, Cummin AR, et al. Detection of lung cancer with volatile markers in the breath. Chest 2003;123:2115–23.
16. Phillips M, Cataneo RN, Ditkoff BA, et al. Volatile markers of breast cancer in the breath. Breast J 2003;9:184–91.
17. Wehinger A, Schmid A, Mechtcheriakov S, et al. Lung cancer detection by proton transfer reaction mass spectrometric analysis of human breath gas. Int J Mass Spec 2007;265:49–59.
18. Machado RF, Laskowski D, Deffenderfer O, et al. Detection of lung cancer by sensor array analyses of exhaled breath. Am J Respir Crit Care Med 2005;171:1286–91.
19. Di Natale C, Macagnano A, Martinelli E, et al. Lung cancer identification by the analysis of breath by means of an array of non-selective gas sensors. Biosens Bioelectron 2003;18:1209–18.
20. Poli D, Carbognani P, Corradi M, et al. Exhaled volatile organic compounds in patients with non-small cell lung cancer: cross sectional and nested short-term follow-up study. Respir Res 2005;6:71.
21. Ligor M, Ligor T, Bajtarevic A, et al. Determination of volatile organic compounds appearing in exhaled breath of lung cancer patients by solid phase microextraction and gas chromatography mass spectrometry. Clin Chem Lab Med 2009;47:550–60.
22. Brandacher G, Cakar F, Winkler C, et al. Non-invasive monitoring of kidney allograft rejection through IDO metabolism evaluation. Kidney Int 2007;71:60–7.
23. Bonatti H, Brandacher G, Boesmueller C, et al. Hemolytic uremic syndrome following Campath-1H induction. Transpl Int 2007;20:386–9.
24. Brandacher G, Winkler C, Schroecksnadel K, Margreiter R, Fuchs D. Antitumoral activity of interferon-γ involved in impaired immune function in cancer patients. Curr Drug Metab 2006;7:599–612.
25. Josting A, Rudolph C, Mapara M, et al. Cologne high-dose sequential chemotherapy in relapsed and refractory Hodgkin lymphoma: results of a large multicenter study of the German Hodgkin Lymphoma Study Group (GHSG). Ann Oncol 2005;16:116–23.
26. Bunge M, Araghipour N, Mikoviny T, et al. On-line monitoring of microbial volatile metabolites by proton transfer reaction-mass spectrometry. Appl Environ Microbiol 2008;74:2179–86.
27. Syhre M, Scotter JM, Chambers ST. Investigation into the production of 2-Pentylfuran by Aspergillus fumigatus and other respiratory pathogens *in vitro* and human breath samples. Med Mycol 2008; 46:209–15.
28. Syhre M, Chambers ST. The scent of Mycobacterium tuberculosis. Tuberculosis (Edinb) 2008;88:317–23.
29. Smith D, Wang TS, Sule-Suso J, Spanel P, El Haj A. Quantification of acetaldehyde released by lung cancer cells *in vitro* using selected ion flow tube mass spectrometry. Rapid Commun Mass Spectrom 2003; 17:845–50.
30. Chen X, Xu F, Wang Y, et al. A study of the volatile organic compounds exhaled by lung cancer cells *in vitro* for breath diagnosis. Cancer 2007;110:835–44.
31. Filipiak W, Sponring A, Mikoviny T, et al. Release of volatile organic compounds (VOCs) from the lung cancer cell line CALU-1 *in vitro*. Cancer Cell Int 2008;8:17.
32. Sponring A, Filipiak W, Mikoviny T, et al. Release of volatile organic compounds from the lung cancer cell line NCI-H2087 *in vitro*. Anticancer Res 2009;29:419–26.
33. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
34. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 1976;17:62–70.
35. Giard DJ, Aaronson SA, Todaro GJ, et al. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973;51:1417–23.
36. Hay RJ, Caputo CJ, Macy ML. ATCC Quality Control Methods for Cell Lines. 2nd Edition. Published by ATTC; 1992.
37. Kleinbaum D, Kupper L, Muller A, Nizam K. Applied Regression Analysis and Other Multivariable Methods. Pacific Grove (CA): Brooks/Cole Publishing Company; 1998.
38. Phillips M, Altorki N, Austin JH, et al. Detection of lung cancer using weighted digital analysis of breath biomarkers. Clin Chim Acta 2008; 393:76–84.
39. Acevedo CA, Sanchez EY, Reyes JG, Young ME. Volatile organic compounds produced by human skin cells. Biol Res 2007;40: 347–55.