Volatile organic compounds cytotoxicity and expression of HSP72, HSP90 and GRP78 stress proteins in cultured human cells

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Abstract

The aim of this study was to determine whether overexpression of stress proteins (SPs) could be a sensitive biomarker for cell injury due to exposure to low doses of volatile organic compounds (VOCs) such as benzene, ethylbenzene, toluene, xylene, and chlorinated derivatives (ClB). Sublethal and cytotoxic threshold concentrations of the VOCs were determined by studying the growth rate of normal (fibroblasts) or tumor-derived human cell lines (A549, HepG2) exposed for 4 days to VOCs. Changes in SP expression as a function of concentrations were investigated by Western blotting.

VOC toxicity was found to be correlated with their degree of chlorination and their hydrophobicity. Cytotoxic threshold concentrations (no-observed effect concentration, NOEC) were found to be similar for the three cell lines. It was observed that using a mixture of VOCs, each of them at concentration below the NOEC, resulted in an actual toxicity to the cells. This finding reveals a synergistic effect and should be taken into account when assessing threshold risk and exposure limit values in the worker’s environment when several pollutants may be present. HSP72 and HSP90 expression levels were not affected whereas GRP78 expression was increased by all the VOCs. Taking into account the specific molecular function of GRP78, it suggests that VOC exposure results in misfolded or underglycosylated protein accumulation in the endoplasmic reticulum. GRP78 overexpression was closely related to the magnitude of growth inhibition due to increasing concentrations of each VOC. The overexpression was found to be significant for concentrations 5 to 30 times higher than NOEC, indicating that, under our experimental conditions, GRP78 expression cannot be considered as a sensitive biomarker of exposure to environmental VOCs.

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1. Introduction

The monocyclic aromatic hydrocarbons: benzene, toluene, ethylbenzene, xylene (collectively BTEX), and chlorobenzenes (ClB) are potential harmful substances commonly found in occupational or nonoccupational environments. BTEX are present in fuel, paint, rubber products and adhesives. Benzene and ethylbenzene are present in commercial gasoline at concentrations of 3–5% and up to 15%, respectively. ClB are current intermediates in dye and pesticide industrial synthesis.

Recent studies highlight the fact that exposure levels to these volatile organic compounds (VOCs) are more important indoors than outdoors. It was calculated that the major source of exposure (45%) of the US population to benzene arises from cigarette smoke [1,2] which accumulates inside homes, offices and vehicles. Other emission sources of these VOCs are stored household products (white spirit, paint, glue...). Tichenor and Guo [3] reported that common household activities (e.g., painting, staining, varnishing) can produce air concentrations of VOCs as high as 1000 mg/m³. p-Dichlorobenzene (p-diClB) is present in toilet disinfectants and room deodorizers and the 2–5 dichlorophenol (metabolite of p-diClB) can be detected in urine of the general population [4,5].

Benzene is a well-known carcinogen that has been causally linked to leukaemia [6]. Although toluene and xylene are not currently classified as carcinogens, Lundberg and Milatou-Smith [7] reported an increase of cancer
incidence in paint industry workers with long-term exposure to these organic solvents. Furthermore, increased risk for esophageal, rectal and colon cancers was suspected to be related to occupational exposure to toluene and xylene [8]. Besides, a decrease of semen quality was observed in workers after occupational exposure to respective concentrations of ethylbenzene, toluene, xylene and benzene of 230, 200, 50 and 40 mg/m³ [9].

Various adverse effects of CIB have been reported in industrial workplaces [10]. Liver appears to be the primary site of injury by p-diClB as subsequent cases of jaundice and cirrhosis have been reported [11]. Painful irritations of the eyes and nose and breathing difficulties among workers were observed for p-diClB airborne concentrations of 80–160 ppm [12].

The potential toxicity of these compounds is increased by the fact that they are strongly lipophilic with a capacity to concentrate in fat deposits throughout long-term exposures [13–15] and to accumulate in the lipid bilayer of the cellular membranes [16]. Hence, there is a pressing need to find “stress indicators” to rigorously evaluate the impact of these xenobiotics on biological processes.

Most organisms can adjust their spectrum of active genes to deal with environmental adversity. Because stress-related gene overexpression appears as a pleiotropic response to a range of environmental injuries, there is a growing interest in considering stress proteins (SPs) as biomarkers to provide a quantitative indication that organisms have been exposed to, or affected by, environmental pollutants [17–20]. SPs encompass a large ubiquitous group of proteins whose common function is to stabilize aggregation-prone folding intermediates and facilitate the acquisition of the functional native state [21]. In cells experiencing, or recovering from, protein perturbing stresses, they act by assisting protein refolding and preventing aggregation of damaged proteins [22]. The synthesis of many SPs, also referred as molecular chaperones, is enhanced in stressed cells. We focused our attention on two cytosolic heat shock proteins (HSP72, HSP90) and a glucose regulated stress protein (GRP78) resident in the lumen of the endoplasmic reticulum (ER). HSP72 is known to be highly induced by a wide variety of agents or conditions including heat, anoxia, heavy metals and oxidizing agents [21,22]. HSP90 is now understood to be involved in the regulation of signal transduction pathways from a variety of hormones and cell cycle regulators [23,24]. GRP78 is implicated in the regulation of secretion of N-glycosylated proteins synthesized within the endoplasmic reticulum [25].

Our previous work [26] indicated that GRP78 was the most overexpressed after exposure to benzene and some of its chlorinated derivatives. In the present work, we intend to determine whether GRP78, HSP72 or HSP90 stress proteins could be used as sensitive indicators of VOCs’ adverse effects.

HSP expression was quantified in cells exposed for 4 days to pollutant concentrations ranging from no-observed effect (NOEC) on cellular proliferation up to sublethal doses.

Experiments were carried out on two human tumor-derived cells in culture: A549 pneumocytes, as lung alveolar epithelial cells are the first target of volatile pollutants, and HepG2 hepatocytes, as liver plays a key role in metabolism and detoxification of chemicals. Besides, primary cultures of human gingival fibroblasts were used as nontumoral reference cells.

2. Materials and methods

2.1. Chemicals and reagents

Benzene, toluene, xylene, ethylbenzene and chlorobenzenes were obtained from Aldrich Chemical Company. 1,2,3-CIB, 1,3,5-CIB and 1,2,3,4-CIB were dissolved in dimethyl-ctetone to make 100 mM stock solutions. Monoclonal mouse antibodies raised against HSP72 (SPA810), HSP72/73 (SPA822), HSP90 (SPA830) or GRP78 (SPA827) were from StressGen Biotechnologies. Peroxidase goat anti-mouse IgG antibody (A2554) was purchased from Sigma (France). Proteases inhibitors (aprotinin, leupeptin, pepstatin) were from Boehringer-Mannheim (France). Fetal calf serum (FCS) and cell culture reagents were purchased from BIOMEDIA (France). SuperSignal West Pico chemiluminescent substrate for detection of peroxidase was from PIERCE (USA).

2.2. Cell culture

Both A549 (human lung adenocarcinoma) and HepG2 (human liver hepatoma) cells were obtained from the American Type Culture Collection. Normal fibroblast cultures have been established from gingival explants in our laboratory. Cells were grown at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with nonessential amino acids, 4.5 g/l glucose, 10% fetal calf serum and 20 μg/ml gentamycin.

2.3. Experimental cultures for investigating the toxicity of the pollutants and to determine the threshold concentrations (NOEC)

Because we found that aromatic hydrocarbons interacted with polystyrene walls of culture flasks [26], cells were cultured in glass flasks (volume: 130 ml, culture surface: 70 cm²) containing 10 ml of culture medium. After seeding (3 × 10⁵ cells/flask), cells were allowed to attach and the pH of the medium allowed to equilibrate for 16 h. Then, pollutant was introduced and flasks were rapidly tightly closed. Cell cultures were left in the permanent presence of the pollutant for 4 days, at 37 °C. Cell proliferation was estimated by counting cells in a suspension obtained by trypsin–EDTA treatment, using a Coulter Counter (Coul-
tronics, France). In all cases, three cultures were treated in parallel for each pollutant concentration and three control cultures (not submitted to pollutant) were processed similarly for comparison. Percents of growth inhibition were calculated as follows: \( \frac{100}{C_0} \left( \frac{\text{cell number in experimental cultures}}{\text{mean of cell number in controls}} - 1 \right) \times 100 \).

The highest concentration of each pollutant that leads to a nonsignificant decrease (as determined by Post-Hoc Scheffe test) of the growth rate, as compared to controls, was considered as the threshold concentration for toxicity (cell growth-NOEC).

### 2.4. Quantitative determinations of the pollutants

Each pollutant was set on cotton wool put into the neck of culture flasks and its actual concentration in the culture medium (10 ml) and in the upper atmosphere (120 ml) was determined by headspace Solid Micro Extraction (SPME) [27,28] in combination with Gas Chromatography Flame Ionization Detection (GC-FID) as previously reported [26].

### 2.5. Analysis of HSP expression on Western blot

Just after exposure to pollutant, cell layers were rinsed with ice-cold phosphate buffered saline (PBS), scraped with a rubber policeman and collected into a lysis buffer (PBS containing 0.5% NP-40 and protease inhibitors: 10 mM EDTA, 1 mM PMSF, 1 µg/ml of aprotinin, leupeptin and pepstatin, pH 7.4), and stored at −80 °C; 100 µl of lysis buffer was used for about 7×10^5 cells.

Prior to use, cell lysates were thawed, sonicated for 10 s and protein concentrations were determined using the Lowry method [29]. Equal amounts of protein (20 µg) from each homogenate were separated by SDS-PAGE according to Laemli’s method [30]. Separated proteins were electroblotted onto 0.45-µm nitrocellulose membranes. In order to saturate the unspecific sites, the membranes were incubated for 1 h at 37 °C into a Tris-buffered saline (TBS: 10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.3% Tween20 (TBST). The membranes were then incubated for 1 h at room temperature under continuous shaking in the presence of mouse monoclonal antibodies: GRP78, HSP90, HSP72, HST2/73 diluted to 1:15000, 1:10000, 1:40000, 1:30000, respectively in TBST and then allowed to stand overnight at 4 °C. After washing into (1) TBS for 5 min, (2) TBS containing 0.1% Nonidet-P40 for 15 min, (3) TBS for 5 min (twice), the blots were incubated at room temperature, in darkness, for 2 h under continuous shaking in the presence of a goat anti-mouse IgG peroxidase-conjugated antibody diluted 1:10000 in TBS containing 3% dried skimmed milk. After washing, membranes were incubated with the chemiluminescent substrate for 2 min at room temperature and then exposed to Bio Max light-1 film (KODAK). HSP levels were determined by computer-assisted densitometric analysis of the resulting autoradiographs.

### 2.6. Statistical evaluation

The data are presented as the mean ± standard deviation. Dose–effect relationships were analyzed by Anova and Post-Hoc Scheffe tests. Difference between treatments was considered significant at * \( P < 0.01 \).

### 3. Results

#### 3.1. Partitioning of volatile hydrocarbons between air and culture medium

Preliminary experiments showed that adding pollutants directly to the culture medium resulted in highly toxic transient concentrations that decreased over the first 30 min as these volatile agents evaporated into the atmosphere of the flask (not shown). To avoid this problem, pollutants

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Concentrations after introducing 1 µl of each hydrocarbon in the flasks</th>
<th>Partitioning (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air (ppm)</td>
<td>Medium (µM)</td>
</tr>
<tr>
<td>Benzene</td>
<td>1550</td>
<td>390</td>
</tr>
<tr>
<td>Mono-C1B</td>
<td>1450</td>
<td>310</td>
</tr>
<tr>
<td>1,2-C1B</td>
<td>1500</td>
<td>190</td>
</tr>
<tr>
<td>1,3-C1B</td>
<td>1500</td>
<td>185</td>
</tr>
<tr>
<td>1,2,4-C1B</td>
<td>1450</td>
<td>125</td>
</tr>
<tr>
<td>1,2,3-C1B, 100 mM in dimethyl-cetone</td>
<td>5</td>
<td>8.1</td>
</tr>
<tr>
<td>1,3,5-C1B, 100 mM in dimethyl-cetone</td>
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<td>2.3</td>
</tr>
<tr>
<td>1,2,3,4-C1B, 100 mM in dimethyl-cetone</td>
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<td>4.4</td>
</tr>
<tr>
<td>Toluene</td>
<td>1400</td>
<td>291</td>
</tr>
<tr>
<td>Xylene</td>
<td>1300</td>
<td>216</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>1310</td>
<td>211</td>
</tr>
</tbody>
</table>

One microliter of each volatile pollutant was deposited onto cotton that was quickly put into the neck of the culture flasks. Then the flasks were immediately and hermetically closed with bakelite screw caps containing a rubber septum with a face in Teflon. Assays were performed 24 h later. Values are means of three separate assays.
were deposited onto cotton wool that was quickly introduced into the neck of culture flasks, which were then immediately tightly closed.

The partitioning of the pollutants between air and the culture medium was assessed by introducing a standard amount (1 μl) of each pollutant in the flasks and measuring the concentrations in the aqueous phase (culture medium) and the upper atmosphere (air) after 30 min, 1 h, 2 h, 24 h, 2, 3 and 4 days. As previously reported [23], pollutants quickly volatilized and an equilibrium between the two phases, at 37 °C, was reached within 1 h (not shown). Then concentrations were found constant, confirming that culture flasks were leak-proof. Table 1 shows the concentrations of the pollutants at equilibrium between the two phases. Except for 1,2,3- and 1,2,3,4-ClB, the concentration in the aqueous phase of chlorinated derivatives of benzene was lower than that of benzene. With respect to the total amount of pollutant introduced in the flasks, the quantity of chlorinated derivatives, mono-ClB, di-ClB and 1,2,4-tri-ClB found in the aqueous phase decreased from 31% to 17%. Ethylbenzene and xylene were also observed to be less soluble than benzene. Surprisingly, the solubility of 1,2,3,4-ClB and 1,2,3-ClB in the culture medium was high, representing 43% and 80%, respectively, of the total amount introduced in the atmosphere. The presence of dimethyl-cetone used to dissolve the crystals of these compounds may explain the high solubility in the aqueous phase.

On the basis of their percent of partitioning of each pollutant, an appropriate amount was introduced in the neck of the flasks to obtain the desired concentration in the culture medium, i.e., at the cell contact.

3.2. Evaluation of the cytotoxic threshold concentrations (cell growth-NOEC) of VOCs on A549 cells

Fig. 1 shows the percent of growth inhibition of A549 cells, resulting from 4 days exposure to increasing concentrations of pollutants. It appears that the decrease of the proliferation rate is dose-dependent for all hydrocarbons studied. These concentrations did not lead to cell death, as verified by trypan blue exclusion (not shown).

Among BTEX, benzene was found to be the less cytotoxic as a significant decrease of proliferation rate was induced for doses higher than 1 mM and the NOEC was 500 μM. Toluene toxicity appeared at about five times lower doses (NOEC = 100 μM). Xylene and ethylbenzene showed a NOEC of 50 μM and induced almost complete arrest of cell growth at 1 mM.

The NOEC of monochlorobenzene (mono-ClB) was found to be similar to that of xylene and ethylbenzene. Conversely, the toxicity of others chlorobenzenes was higher.

Table 2

<table>
<thead>
<tr>
<th>VOCs</th>
<th>1,2,3,4-ClB; 1,2,4-ClB</th>
<th>1,2,3-ClB; 1,2-ClB</th>
<th>1,3-CIB; 1,3,5-CIB</th>
<th>Mono-ClB, xylene, ethylbenzene</th>
<th>Toluene</th>
<th>Benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell growth-NOEC</td>
<td>1 μM</td>
<td>5 μM</td>
<td>10 μM</td>
<td>50 μM</td>
<td>200 μM</td>
<td>500 μM</td>
</tr>
<tr>
<td>GRP78-NOEC</td>
<td>30 μM</td>
<td>50 μM</td>
<td>100 μM</td>
<td>500 μM</td>
<td>500 μM</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

Cell growth-NOEC corresponds to highest pollutant concentration in the culture medium leading to a nonsignificant decrease (as determined by Post-Hoc Scheffe test) of the cell proliferation after 4 days of culture. GRP78-NOEC corresponds to the VOC threshold concentration leading to a significant overexpression of the GRP78 stress protein.
than that of BTEX and increased as a function of the number of Cl, as shown in Fig. 1 and Table 2. Indeed, cell growth-NOEC decreased from benzene to mono-ClB, 1,3-ClB, 1,2,3-ClB and 1,2,3,4-ClB. Surprisingly, the 1,2-ClB showed a toxicity similar to that of 1,2,3-ClB (NOEC ≈ 5 μM) and higher than that of 1,3-ClB (NOEC ≈ 10 μM), suggesting that Cl in position 2 was more toxic than in position 3. In the same way, Cl in position 4 was more toxic than in position 3 since 1,2,4-ClB and 1,2,3,4-ClB NOEC were lower than that of 1,2,3-ClB.

Dimethyl-cetone (DMC) was used to dissolve 1,2,3-ClB, 1,3,5-ClB and 1,2,3,4-ClB crystals to make 100 mM stock solutions. By introducing DMC as a blank in some controls flasks, it was verified that the low concentrations of this

Table 3
Evidence for additive effects of VOCs

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Percent of growth inhibition after 4 days exposure to pollutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzene (500 μM)</td>
</tr>
<tr>
<td></td>
<td>−3 ± 5%</td>
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<tr>
<td>2</td>
<td>Benzene (500 μM)</td>
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<td></td>
<td>−2 ± 6%</td>
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</tbody>
</table>

A549 cells were exposed for 4 days, either to each of the pollutants at concentration equal to NOEC, or to mixtures (A, B) of the four pollutants at the same concentration as above. (A/4) and (B/4) mean that concentration of each pollutant was four times lower than NOEC. Values represent the mean of growth inhibition (as percent ± standard deviation), as compared to controls normalized to 100%.

* Post-Hoc Scheffe test: *P < 0.01.

Table 4
Comparative sensitivity of 3 human cell lines (pneumocytes A549, hepatocytes HepG2, fibroblasts) toward VOCs

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Benze (2 μM)</th>
<th>5 μM</th>
<th>10 μM</th>
<th>20 μM</th>
<th>50 μM</th>
<th>100 μM</th>
<th>200 μM</th>
<th>500 μM</th>
<th>1 mM</th>
<th>2 mM</th>
<th>4 mM</th>
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<tr>
<td></td>
<td>A549</td>
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<tr>
<td>Toluene</td>
<td>0 ± 5</td>
<td>−3 ± 3</td>
<td>−9 ± 7</td>
<td>−22 ± 5*</td>
<td>−45 ± 6*</td>
<td>−70 ± 5*</td>
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<td></td>
<td>−1 ± 6</td>
<td>−17 ± 7*</td>
<td>−39 ± 2*</td>
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<td></td>
<td>−4 ± 3</td>
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<td>−31 ± 6*</td>
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<td>−38 ± 7*</td>
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<td>−37 ± 5*</td>
<td>−89 ± 2*</td>
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<tr>
<td>1,2,4-CIB</td>
<td>−15 ± 2*</td>
<td>−20 ± 6*</td>
<td>−36 ± 5*</td>
<td>−40 ± 6*</td>
<td>−75 ± 5*</td>
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<tr>
<td></td>
<td>−5 ± 4</td>
<td>−10 ± 3*</td>
<td>−29 ± 5*</td>
<td>−64 ± 3*</td>
<td>−95 ± 7*</td>
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<tr>
<td></td>
<td>−6 ± 3</td>
<td>−15 ± 5*</td>
<td>−20 ± 4*</td>
<td>−59 ± 2*</td>
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</tbody>
</table>

Values represent mean of growth inhibition (as percent ± standard deviation) as compared to controls (normalized to 100%), induced by 4 days exposure to each pollutant.

* Post-Hoc Scheffe test: *P < 0.01.
DMC present in the culture medium did not involve additional cytotoxicity (not shown).

3.3. Evaluation of the effects of a VOC mixture on A549 cells growth

Benzene, toluene, xylene and mono-ClB were together added (mixture A) in culture flasks at concentrations corresponding to cell growth-NOEC of each of these pollutants. Results (Table 3) show that mixture A led to a significant growth inhibition approximately equal to the sum of the slight inhibitions induced by each pollutant. Similarly, a mixture of benzene, mono-Clb, 1,2-Clb and 1,2,4-Clb (mixture B) significantly inhibited cell growth, whereas each separate pollutant did not. Cytotoxic effects disappeared when cells were exposed to a mixture of the same pollutants at concentrations reduced to a quarter (A/4, B/4).

3.4. Comparative sensitivity of three human cell lines

VOC toxicity toward A549, HepG2 cells and human normal fibroblasts was compared. As shown in Table 4, tumor-derived cells (A549, HepG2) and normal human fibroblasts were found to display similar growth inhibitions, after 4-day exposure to the pollutants.

3.5. Dose-related expression of SPs

Fig. 2 illustrates the levels of expression (as compared to controls) of HSP72, GRP78 and HSP90 in A549 cells exposed for 4 days to benzene as a function of its concentration in the culture medium. No significant change of expression level of HSP72 and HSP90 was observed for benzene concentrations inhibiting cell growth up to 80%. In the same way, no change of expression of these two SPs was observed after treatment with the others pollutants, i.e., toluene, xylene, ethylbenzene and chlorinated derivatives (not shown).

Conversely, a dose-dependent increase of GRP78 expression was induced by benzene (Fig. 2) and by all the others pollutants (Fig. 3). A direct relationship between the extent of growth inhibition and the levels of GRP78 overexpression induced by pollutants was observed. As determined by Post-Hoc Scheffe test, overexpression was only significant at doses leading to growth inhibitions over 40% (Fig. 4). Similar results were found with HepG2 cell line and normal fibroblasts (not shown). So, GRP78-NOEC appeared to be 4 to 30 times higher than cell growth-NOEC (Table 2).
4. Discussion

The aim of this study was to estimate the threshold concentrations for toxicity of some volatile organic chemicals (VOCs) and to analyze their ability to induce SPs in cultured human cells after a 4 days exposure. Changes in the rate of HSP synthesis was expected to be useful markers to assess the degree of stress exerted on mammalian cells.

To accurately estimate the actual pollutant concentrations, exposure protocols were carried out as previously described [26]. It is important to underscore that tightly closed glass culture flasks were used to avoid both the interactions of the pollutants with the flask walls and the loss of VOCs throughout the caps. Moreover, because of their volatility, VOCs were not introduced directly in the culture medium, but deposited into the neck of the culture flasks, i.e., in the aerial part of the culture flasks. This avoids the transient high concentration leading to a wrong estimation of the toxicity of the pollutants, as observed in preliminary experiments (unpublished data).

For precise evaluation of actual VOC concentrations obtained in the culture medium around the cells, their partitioning between the aqueous culture medium and the overlying air was determined. Except for 1,2,3- and 1,2,3,4-CIB, our results showed that hydrophobicity of BTEX and chlorobenzenes was increased as a function of the degree of chlorination.

Cytotoxicity studies showed that substituted derivatives of benzene were much more harmful than benzene, their toxicity increasing as a function of the number of methyl (for toluene and xylene) or chlorine (for chlorobenzenes). Potency of chlorinated derivatives to impair cell growth was found to be 10 times (mono-CIB) to more than 100 times (1,2-CIB) higher than that of benzene, as growth-NOEC were, respectively, 10 and 100 times lower. Similar results were reported by Ait-Aissa et al. [20] who showed that cytotoxicity was related to the degree of chlorination of the molecules of chlorophenol. Referring to the works of Connel et al. [31] and Poulin and Krishnan [32], it appears that cytotoxicity of VOCs is related to their lipophilicity. Indeed, these authors reported that lipophilicity increases as follows: benzene < toluene < mono-CIB < xylene, which well correlates with hydrophobicity and the relative toxicity of these chemicals.

Literature indicates that toxicity of benzene depends on its metabolism in the liver. Therefore, benzene toxicity was evaluated in human HepG2 hepatoma cells since these cells retained many of the properties of primary cells, including the ability to metabolize a wide variety of toxicants [33,34]. No significant difference of toxicity of benzene compounds was observed when comparing HepG2 and A549 cells.

Besides, sensitivity to VOCs of the tumoral cells (A549, HepG2) and of the normal cell line of gingival fibroblasts was found to be almost similar, which suggests that toxicity of the monocyclic aromatic hydrocarbons studied could be in part due to by their solvent properties. Indeed, the primary target of lipophilic solvents is the cell membrane. It has been shown that such hydrocarbons accumulate in the lipid bilayer [16] according to their octanol/water partition coefficient, which is used as an index of the solubility of organic chemicals in lipids [35,36]. In agreement with these observations, we see that toxicity of the studied VOCs increases in direct relationship to their partition coefficient.

Our results also indicate that toxicity of the components of a mixture of VOCs was additive. This observation disagree with the proposal of Cassee et al. [37] who concluded that, “as a rule, exposure to mixtures of chemicals at (low) nontoxic doses of the individual constituents is of no health concern”. In so far as ambient air to which workers are exposed is often a mixture of pollutants, our results support the idea that additivity of pollutant effects should be considered when setting up occupational exposure limits.

Our studies showed that HSP72 and HSP90 levels in A549, HepG2 cells and normal fibroblasts were not significantly modified by exposure to benzene or substituted derivatives.

Some organochlorinated compounds (e.g., 1-chloro-2,4 dinitrobenzene) have been reported to activate the promoter of HSP70 at sublethal doses [20]. Studying bromobenzene, Salimen et al. [38] reported an induction of HSP70/I in HepG2 cells after 24-h exposure to a high concentration (1.5 mM), which also produced 40–70% cell mortality, unlike what happens under our experimental conditions.

Numerous observations indicate that gene activation of HSP72, and to a lesser extent HSP90, is related to oxidative stress leading to increased levels of abnormal proteins in the cytoplasm [39,40].

Studies to explain the mechanism of toxicity of benzene [41] and halogenated benzene [42] argue that the oxidative attack by cytochrome P450 results in the formation of electrophilic intermediates able to react with sulfurhydryl groups, leading to depletion of reduced glutathione. More recently, it has been shown [34] that exposure of HepG2 cells to a panel of polycyclic aromatic hydrocarbons induce the synthesis of cytochrome P450 and aldo-keto reductase, which led to the formation of reactive oxygen species (ROS). On the basis of these observations, it is likely that exposure to BTEX or CIB generates an oxidative stress, at least in HepG2 cells. Nevertheless, we did not find any change of HSP72 expression in these cells. We may suggest that, under our conditions of exposure to VOCs, ROS production was too low to significantly induce HSP72 expression and/or that the steady-state of antioxidant molecules was sufficient to prevent protein denaturation. Another explanation could be that some ROS only do regulate HSP expression. Indeed, Polla and Cossarizza [43] concluded that H2O2 and •OH, but not O2·− or NO·, lead to sufficient alterations in protein structure and/or function to trigger SPs induction.

In our work, GRP78 overexpression appeared for VOC concentrations which already inhibit cell growth by 40%
although not involving cell mortality. These concentrations are too high to be reasonably encountered in ambient atmosphere and/or occupational environment. It follows that GRP78 overexpression cannot be a convenient biomarker of an effect of these VOCs.

Besides, all VOCs studied so far, i.e., benzene, methyl-, ethyl- and chlorinated benzene were found to induce quantitatively similar overexpressions of GRP78. This suggests that GRP78 induction is more related to the benzene molecule itself (or to its metabolites) than to the methyl, ethyl or chlorine groups. GRP78 is a resident protein of the endoplasmic reticulum (ER) that transiently binds to nascent polypeptides translocated in the ER. However, GRP78 keeps stably associated to misfolded or glycosylated-defective proteins, a process which leads to their retention in the pre-Golgi compartments and, therefore, hinders their secretion [25,44]. This supports the idea that benzenic compounds, by promoting protein misfolding in the ER, have adverse effects on protein secretion.

5. Conclusion

By investigating the effects of various VOCs on cell growth, the high toxicity of halogenated benzene derivatives, as compared to benzene, was demonstrated. Besides, the effects of low (nontoxic) concentrations were found to be additive, able to exert a resulting toxic effect, a fact which should be taken into account when setting up current occupational exposure limits.

From our work, we may conclude that HSP70 and HSP90 are not convenient biomarkers of adverse exposure to benzene and that GRP78 induction is not a very sensitive indicator of VOC effects. Nevertheless, our work points out that all benzene compounds (either substituted or not) induce a similar overexpression of GRP78. This suggests that the benzene ring is, by itself, responsible for this induction. Moreover, induction of GRP78 supports the idea that exposure to benzene promotes a misfolding of nascent proteins in the ER, leading to a defect of protein secretion.

Acknowledgements

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