Ability of 16 priority PAHs to be photocytotoxic to a cell line from the rainbow trout gill

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Abstract

Sixteen polycyclic aromatic hydrocarbons (PAHs) were screened for their ability to be photocytotoxic to a cell line from the rainbow trout gill, RTgill-W1. PAHs could be divided into one of three groups: incapable of being photocytotoxic, able to be both photocytotoxic and directly cytotoxic, or capable of being only photocytotoxic. Photocytotoxicity was distinct from direct cytotoxicity in that EC50 values were lower with the neutral red assay immediately after the PAH/UV treatment than with alamar Blue or CFDA-AM, indicating a more specific action on lysosomes. As well, in photocytotoxicity but not in direct cytotoxicity, the three assays showed increased impairment 24 h after treatment. Most PAHs were found to be strictly photocytotoxic; however, only six compounds were photocytotoxic at concentrations theoretically achievable in water. When photocytotoxic PAHs were ranked relative to fluoranthene to establish fluoranthene equivalent factors (FEFs), benzo[a]pyrene and benzo[g,h,i]perylene were found to be most potent. However, when the water solubility of each compound was taken into account in order to calculate the potential environmental photocytotoxic potency (PEPP), fluoranthene and pyrene appeared to have the most potential to impact fish through photocytotoxicity. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Photocytotoxicity; Polycyclic aromatic hydrocarbons; Rainbow trout gill cell line

1. Introduction

The toxicity that arises from simultaneous exposure to polycyclic aromatic hydrocarbons (PAHs) and UV radiation has been studied in a number of aquatic organisms, including fish (Bowling et al., 1983; Kagan et al., 1985; Oris and Giesy, 1985, 1986; Kagan et al., 1987). In fish, the dorsal epidermis and the gill epithelium have been identified as target tissues of the photoinduced toxicity caused by anthracene and fluoranthene (Oris and Giesy, 1985; Weinstein et al., 1997), and the disruption of cell membranes has been proposed as the major cause (Oris and Giesy, 1985; McCloskey and Oris, 1993). However, because
determining the potential of other PAHs to be photocytotoxic is done more easily with fish cells in culture, we previously developed a methodology that allows environmentally important PAHs to be tested rapidly and inexpensively, using confluent cell cultures from the rainbow trout gill as a model system (Schirmer et al., 1997).

Upon absorbing UV radiation, PAHs undergo photochemical reactions that involve the formation of singlet oxygen, free radicals and, potentially, photomodified PAH products (Foote, 1976; Girotti, 1983; Huang et al., 1995; Arfsten et al., 1996). The damaging or killing of cells that results from these photochemical reactions is defined as photocytotoxicity (MacRobert et al., 1989; Schirmer et al., 1997). This is in contrast to PAHs eliciting a cytotoxic response rapidly in the absence of UV radiation which is referred to as direct cytotoxicity (Schirmer et al., 1998).

Both photocytotoxicity and cytotoxicity can be monitored efficiently for various cellular endpoints using fluorescent indicator dyes that can be read with a fluorescent plate reader. Indicator dyes that have previously been used with fish cells are alamar Blue (metabolic activity), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; cell membrane integrity) and neutral red (lysosomal membrane integrity) for quantifying the cytotoxicity of PAHs (Schirmer et al., 1998) and alamar Blue and CFDA-AM for measuring photocytotoxicity (Schirmer et al., 1997). Thus, the neutral red assay has yet to be applied in studies on the photocytotoxicity of PAHs.

In this paper, we have used previously developed methodologies to demonstrate the potential of 16 priority PAHs to be photocytotoxic to the rainbow trout gill cell line, RTgill-W1. Our goals were three-fold. Firstly, we wanted to identify PAHs that are toxic in the presence of UV radiation, and secondly, compare how photocytotoxic PAHs affect different cellular endpoints either immediately after UV irradiation or 24 h later. This involved exposing cells to individual PAHs and UV radiation, and applying the three fluorescent indicator dyes, alamar Blue, CFDA-AM and neutral red. Thirdly, we determined the photocytotoxic potencies of PAHs relative to fluoranthene. This required the calculation of $EC_{50}$ values and resulted in the proposal of fluoranthene equivalent factors ($FEFs$) for photocytotoxicity. In addition, a second ranking value was calculated in order to indicate relative to fluoranthene the potential environmental photocytotoxic potency ($PEPP$) of each PAH.

2. Materials and methods

2.1. Cell culture, cytotoxicity tests and assays

The cell line from rainbow trout (*Oncorhynchus mykiss*) gills, RTgill-W1, was developed in this laboratory (Bols et al., 1994). Unlike several other established fish cell lines, RTgill-W1 was found to lack inducible 7-ethoxyresorufin-O-deethylase (EROD) activity, which is a measure of cytochrome P4501A. Cells were cultured in 75 cm$^2$ culture flasks at 22°C in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (FBS), using culture supplies and subcultivation procedures previously described (Bols and Lee, 1994; Schirmer et al., 1994).

Confluent monolayers of RTgill-W1 cells in 48 well tissue culture plates were used to study the photoinduced cytotoxic effects of PAHs. Confluent cultures were achieved and cells dosed with the PAHs as described in Schirmer et al. (1998). Immediately after the dosing, UV irradiation was performed as described below. As well as preparing plates for UV irradiation, control plates were prepared for exposure of cells in the dark. Fluoranthene was included as a positive control in most experiments because of its ability to be photocytotoxic to RTgill-W1 cells (Schirmer et al., 1997).

Upon termination of the UV radiation exposure, cytotoxicity assays were performed immediately and 24 h later. Three cytotoxicity assays were used that are based on the fluorescent indicator dyes, alamar Blue, CFDA-AM and neutral red. A detailed description of the preparation and application of these dyes is given in Schirmer et al. (1998). All cell cultures received L-15 containing 10% FBS in the 24 h between the assays.
2.2. UV radiation exposure

After the cells had been dosed and before the cytotoxicity assays were carried out, UV radiation was applied to the cell monolayers. UV radiation was supplied with one UV-A and one UV-B fluorescent lamp (Southern N.E. Ultraviolet, Branford, CT). Cells were irradiated at room temperature in an atmosphere of air and in the presence of tissue culture plate lids as described previously (Schirmer et al., 1997). Irradiation was measured frequently with an InstaSpec™II photodiode array spectroradiometer calibrated with a 1-kW quartz halogen lamp (Oriel Corporation, Stratford, CT). With one UV-A and one UV-B lamp, and varying distances between the radiation source and the tissue culture plates, the photon fluence rate was adjusted to $10^{-3}$ mols/m$^2$/s ($\pm 10\%$) for UV-A and $1 \mu$mols/m$^2$/s ($\pm 6\%$) for UV-B. These values represent the photon fluence rates at the surface of the medium in the wells. A 500-μl/well aliquot of L-15 had previously been shown to have little discernible effect on these fluence rates (Schirmer et al., 1997). The duration of irradiation was 2 h for all experiments.

The absorption by each PAH of the UV radiation emitted by the UV photoreactor lamps was calculated by multiplying the values of the UV emission- and PAH absorption-spectra at each wavelength and integrating the area under the resulting curve (Krylov et al., 1997). This integrated absorption value, which is designated $J$, was normalized for the largest absorption value, which was obtained for benzo[a]pyrene (Table 1).

2.3. Preparation of PAH solutions and HPLC analysis

PAHs were prepared in dimethyl sulphoxide (DMSO, BDH, Toronto, ON, Canada) and PAH concentrations confirmed by HPLC analysis as described in Schirmer et al. (1998). DMSO has previously been shown to have a slightly sensitizing effect on cells in the presence of a UV radiation treatment (Schirmer et al., 1997). However, for screening purposes, DMSO qualifies as a suitable solvent. In contrast to the previously introduced method of solubilizing fluoranthene in the absence of a carrier, DMSO required smaller volumes and allowed the preparation of a wider dose range for each of the PAHs tested.

2.4. Data analysis

Data were analyzed and fitted to a logistic function as previously described (Schirmer et al., 1998). All statistical tests were performed using SYSTAT™ software (SPSS Inc., 1996).

3. Results

The response of RTgill-W1 cells to PAH/UV treatments allowed the 16 priority PAHs to be divided into three basic groups. Under the conditions of the assays, PAHs were either incapable of being photocytotoxic, able to be both photocytotoxic and directly cytotoxic, or capable of being only photocytotoxic.

<table>
<thead>
<tr>
<th>PAH</th>
<th>$J_{\text{norm}}$</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>0.023</td>
<td>0.80$^c$</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>0.242</td>
<td>d.n.a.$^e$</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>0.047</td>
<td>0.58$^a$</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.045</td>
<td>0.31$^a$</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.049</td>
<td>0.80$^a$</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.334</td>
<td>0.60$^a$</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>0.476</td>
<td>0.60$^a$</td>
</tr>
<tr>
<td>Pyrene</td>
<td>0.579</td>
<td>0.27$^c$</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>0.390</td>
<td>0.80$^a$</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0.191</td>
<td>0.67$^c$</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>0.717</td>
<td>d.n.a.$^e$</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>0.687</td>
<td>d.n.a.$^e$</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>1.000</td>
<td>0.40$^a$</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>0.654</td>
<td>0.98$^d$</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>0.788</td>
<td>0.60$^a$</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>0.874</td>
<td>d.n.a.$^e$</td>
</tr>
</tbody>
</table>

$^a$ $J_{\text{norm}}$ = Absorption of UV radiation by the PAH, normalized for benzo[a]pyrene.

$^b$ $\Phi$ = quantum yield of triplet-state formation; values were obtained from available literature: ‘Krylov et al., 1997; d.Birks, 1970, pp. 251–253; d.n.a. = data not available.
Fig. 1. Impairment of RTgill-W1 cells after being exposed to increasing concentrations of naphthalene in the presence or absence of UV radiation. Confluent cultures were exposed to naphthalene in L-15/ex and either kept in the dark (○) or simultaneously UV irradiated for 2 h (□). Immediately afterwards and 24 h later, effects on cells were assayed with a mixture of alamar Blue as a measure of the cellular redox potential (top panels) and CFDA-AM as an indirect measure of cell membrane integrity (middle panels), as well as with neutral red as an indicator of lysosomal membrane integrity (bottom panels). The results were expressed as a percentage of the readings in control cultures that received the appropriate dark or UV treatment but no naphthalene. One representative experiment is shown. Each data point is the mean of four culture wells with the vertical lines indicating the standard deviation. Similar relative dose-response curves were obtained for fluorene.

3.1. Non-photocytotoxic PAHs

Three PAHs showed no photocytotoxicity. For naphthalene and fluorene, this was judged from the similarities of dose-response curves that were obtained after exposure for 2 h in the dark or after 2 h of UV irradiation (Fig. 1). For chrysene, toxicity was not observed for either treatment, even when chrysene was tested at concentrations that exceeded its water solubility limit by approximately 25 times (Fig. 2).

3.2. Photocytotoxic and cytotoxic PAHs

Acenaphthylene, acenaphthene and phenanthrene, which were previously found to be cytotoxic (Schirmer et al., 1998), also showed some photocytotoxicity. This was judged from dose-response curves that were shifted towards lower PAH concentrations in the presence of UV radiation (Fig. 3). Unlike cytotoxicity, photocytotoxic-

Fig. 2. Impairment of RTgill-W1 cells immediately and 24 h after being exposed to increasing concentrations of chrysene in the presence (□) or absence (○) of UV radiation. Exposure of cell cultures to chrysene and subsequent cytotoxicity assays were performed as described in Fig. 1. One representative experiment is shown. Each data point is the mean of four culture wells with the vertical lines indicating the standard deviation. The arrows indicate the concentration at which chrysene is maximally soluble in water (13 nM).
Fig. 3. Impairment of RTgill-W1 cells immediately and 24 h after being exposed to increasing concentrations of acenaphthylene in the presence (□) or absence (●) of UV radiation. Exposure of cell cultures to acenaphthylene and subsequent cytotoxicity assays were performed as described in Fig. 1. One representative experiment is shown. Each data point is the mean of four culture wells with the vertical lines indicating the standard deviation. The arrows indicate the concentration at which acenaphthylene is maximally soluble in water (26 μM). Similar relative dose-response curves were obtained foracenaphthene and phenanthrene.

3.3. Strictly photocytotoxic PAHs

The majority of the PAHs was found to be only photocytotoxic (Tables 2 and 3). These PAHs could be divided into two groups.

The first group consisted of PAHs that were photocytotoxic only at relatively high concentrations (Fig. 4). As estimated from dose-response data, benzo[k]fluoranthene and indeno[1,2,3-cd]pyrene elicited no toxicity at concentrations at which these compounds are maximally soluble in water. For benzo[b]fluoranthene and dibenzo[a,h]anthracene, cell viability was decreased by less than 10% at their respective water solubility limits in the assays that were performed 24 h after UV irradiation. EC$_{50}$ values, which were lowest in the assays 24 h after UV exposure, ranged from approximately 14–23 times above water solubility for benzo[k]fluoranthene, 50–72 times for benzo[k]fluoranthene, and 57–89 and 144–163 times above water solubility for dibenzo[a,h]anthracene and indeno[1,2,3-cd]pyrene, respectively. At these high concentrations, PAHs are likely present in the culture medium in microcrystalline form, which potentially affects their UV absorption and uptake characteristics (Lakowicz et al., 1980; Weinberger and Cline Love, 1984). Therefore, the toxicity that was observed for benzo[k]fluoranthene, benzo[b]fluoranthene, dibenzo[a,h]anthracene and indeno[1,2,3-cd]pyrene likely was governed by factors more complex than those present for the second group of photocytotoxic PAHs.

This second group consisted of PAHs that were photocytotoxic at concentrations below the concentrations which these compounds could theoretically achieve in water (Fig. 5; Table 2). In the presence of UV radiation, fluoranthene, pyrene, anthracene, benzo[a]pyrene, benzo[a]anthracene and benzo[g,h,i]perylene caused the cellular activities of all three assays to be impaired immediately after UV irradiation (Fig. 3). When EC$_{50}$ values for either of the three cytotoxicity assays were compared by analysis of variance, followed by Tukey’s post-hoc test (α = 0.05), the rank order for photocytotoxicity was similar to that for cytotoxicity with acenaphthylene ≥ acenaphthene > phenanthrene.
Table 2

<table>
<thead>
<tr>
<th>PAH</th>
<th>EC_{50} values (nM) 2 h after UV irradiation</th>
<th>EC_{50} values (nM) 24 h after UV irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alamar Blue</td>
<td>CFDA-AM</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>56 ± 23 n = 5</td>
<td>66 ± 24 n = 5</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>253 ± 35 n = 3</td>
<td>201 ± 34 n = 3</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>69 ± 21 n = 4</td>
<td>87 ± 35 n = 4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>201 ± 72 n = 5</td>
<td>238 ± 110 n = 4</td>
</tr>
<tr>
<td>Pyrene</td>
<td>184 ± 51 n = 2</td>
<td>204 ± 6 n = 2</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>255 ± 55 n = 9</td>
<td>225 ± 44 n = 13</td>
</tr>
</tbody>
</table>

*a The EC_{50} value obtained for neutral red was significantly lower than the EC_{50} value for CFDA-AM 2 h after UV irradiation (Tukey’s test; α = 0.025).

*b The EC_{50} value obtained for neutral red was significantly lower than the EC_{50} values for CFDA-AM and alamar blue 2 h after UV irradiation (Tukey’s test; α = 0.025).

*c The EC_{50} values obtained 24 h after UV irradiation were significantly lower than the EC_{50} values for the same cellular endpoint 2 h after UV irradiation (Student’s t-test; α = 0.05).

exposure to any one of the six PAHs (Table 2). For an individual PAH, the EC_{50} values obtained in the alamar Blue and the CFDA-AM assays were very similar. In the assays that were performed 24 h later, all three fluorescent indicator dyes gave similar EC_{50} values (Table 2). Furthermore, when EC_{50} values for the immediate and 24 h assays were compared for each of the three dyes, it was found that in all cases of alamar Blue and CFDA-AM the EC_{50} values decreased significantly over the 24 h period, whereas the neutral red EC_{50} values did not, with one exception: benzo[a]pyrene. Finally, slightly U- or L-shaped dose-response curves were obtained in the neutral red assay with fluoranthene, pyrene, benzo[g,h,i]perylene (Figs. 5 and 6), and in a few cases for benzo[a]pyrene (data not shown).

3.4. Photocytotoxicity rankings relative to fluoranthene

For the PAHs that were identified as photocytotoxic to RTgill-W1 cells, relative photocytotoxic potencies were calculated in two ways. Fluoranthene was chosen as a reference compound because it is an ubiquitous environmental contaminant (Ankley et al., 1994) and because it was phototoxic in this and a previous study with fish cells at concentrations well below its water solubility limit (Schirmer et al., 1997). Calculations were based on the mean of the mean EC_{50} values for the three assays at 24 h after UV irradiation. These values were used because they were similar for all three indicator dyes and comparable to the EC_{50} values obtained for the neutral red assay 2 h after UV irradiation, which was the most sensitive indicator of immediate photocytotoxicity (Table 2). A FEF ranked a PAH according to its relative toxicity at concentrations equimolar to fluoranthene and was calculated by dividing the EC_{50} for fluoranthene by the EC_{50} for the PAH. In addition to FEFs, another ranking value was calculated in order to indicate relative to fluoranthene the PEPP of a PAH. A PEPP incorporated the photocytotoxicity of a PAH together with its highest possible concentration in aquatic environments and was calculated by dividing the water solubility to EC_{50} ratio for a PAH by the water solubility to EC_{50} ratio for fluoranthene. As the UV exposures and toxicity assays were carried out in this report, benzo[a]pyrene was the most photocytotoxic PAH (Table 3, FEF) but fluoranthene was the PAH most likely to occur in the environment at concentrations at which photocytotoxicity could occur (Table 3, PEPP).
Table 3

EC50 values for the photocytotoxicity of PAHs and their potencies relative to fluoranthene

<table>
<thead>
<tr>
<th>PAH</th>
<th>Molecular Structure</th>
<th>W.S. (nM) (1)</th>
<th>EC50 (nM) (2)</th>
<th>W.S./EC50</th>
<th>PEPP (3)</th>
<th>FEF (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td></td>
<td>240 x 10^3</td>
<td>none</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td></td>
<td>26 x 10^3</td>
<td>24 x 10^3 (5)</td>
<td>1.1</td>
<td>0.078</td>
<td>0.003 800</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td></td>
<td>23 x 10^3</td>
<td>26 x 10^3 (5)</td>
<td>0.880</td>
<td>0.063</td>
<td>0.003 600</td>
</tr>
<tr>
<td>Fluorene</td>
<td></td>
<td>12 x 10^3</td>
<td>none</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td></td>
<td>7000</td>
<td>54 x 10^3 (5)</td>
<td>0.130</td>
<td>0.009</td>
<td>0.001 700</td>
</tr>
<tr>
<td>Anthracene</td>
<td></td>
<td>409</td>
<td>49</td>
<td>8.3</td>
<td>0.593</td>
<td>1.898</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td></td>
<td>1 285</td>
<td>93</td>
<td>14</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Pyrene</td>
<td></td>
<td>667</td>
<td>55</td>
<td>12</td>
<td>0.857</td>
<td>1.691</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td></td>
<td>48</td>
<td>28</td>
<td>1.7</td>
<td>0.121</td>
<td>3.321</td>
</tr>
<tr>
<td>Chrysene</td>
<td></td>
<td>13</td>
<td>none</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td></td>
<td>6.0</td>
<td>111</td>
<td>0.054</td>
<td>0.004</td>
<td>0.838</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td></td>
<td>3.0</td>
<td>184</td>
<td>0.016</td>
<td>0.001</td>
<td>0.505</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td></td>
<td>16</td>
<td>11</td>
<td>1.5</td>
<td>0.107</td>
<td>8.454</td>
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<tr>
<td>Dibenzo[a,h]anthracene</td>
<td></td>
<td>1.8</td>
<td>132</td>
<td>0.014</td>
<td>0.001</td>
<td>0.704</td>
</tr>
<tr>
<td>Benzo[ghi]perylene</td>
<td></td>
<td>20</td>
<td>12</td>
<td>1.7</td>
<td>0.121</td>
<td>7.750</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td></td>
<td>0.7</td>
<td>176</td>
<td>0.004</td>
<td>0.000 280</td>
<td>0.528</td>
</tr>
</tbody>
</table>

(1) W.S. = Water solubility; Source: Mackay et al., 1992
(2) EC50 values were calculated as the mean of the mean EC50 values obtained for each fluorescent indicator dye 24 hr after UV irradiation.
(3) PEPP = potential environmental photocytotoxic potency ([W.S./EC50(Par)];[W.S./EC50(Fluoranthene)]).
(4) FEF = Fluoranthene equivalent factor (EC50(Par)/EC50(Fluoranthene)).
(5) n.a. = not applicable
(6) Toxicity is partly due to cytotoxicity.
4. Discussion

Most of the priority PAHs were photocytotoxic, and the ability to be photocytotoxic appeared to depend on the proper combination of photochemical and solubility properties. Photocytotoxicity appeared to correlate with the PAHs' photochemical properties that others have identified as favoring the formation of the triplet excited state and singlet oxygen (Foote, 1976; MacRobert et al., 1989). One photochemical property is the absorption of UV radiation which varies among PAHs and can be quantified by the overlap of the absorption spectrum of each compound and the spectrum of the UV radiation source (Huang et al., 1997a; Krylov et al., 1997). Secondly, PAHs differ in their ability to undergo the conversion from the singlet excited state to the longer-lived triplet excited state. This photochemical property is commonly expressed as the triplet state quantum yield ($\Phi$; MacRobert et al., 1989), and values for $\Phi$ can be obtained from the literature (Birks, 1970; Krylov et al., 1997). Naphthalene and fluorene showed the least overlap of their respective absorption spectra with that of the UV-radiation source and were not photocytotoxic. Benzo[a]pyrene showed the greatest overlap and was most photocytotoxic as judged from the $EC_{50}$ values. Acenaphthene and phenanthrene...
showed an overlap similar to that of fluorene but their triplet state quantum yields are much higher than that of fluorene. Chrysene showed a different behavior. Although intermediate between phenanthrene and anthracene in absorption and triplet state quantum yield, it is not photocytotoxic. Therefore, an additional factor to be considered is the ability of PAHs to accumulate at critical cellular sites, which we have argued previously are cell membranes (Schirmer et al., 1997, 1998). Thus, additional properties influencing the ability of a PAH to be photocytotoxic are water solubility and lipophilicity (Schirmer et al., 1998). Consequently, it is likely that chrysene did not accumulate in cells sufficiently to elicit a photocytotoxic response.

Although the occurrence of additional mechanisms working at different levels and having different degrees of specificity is possible, the general mechanism behind the photocytotoxicity of the PAHs appears to be the impairment of membranes generally. This is because the three assays of cellular viability indirectly measure the maintenance of membrane integrity and gave broadly similar results. In this way the photocytotoxicity of PAHs is similar to the direct cytotoxicity of PAHs (Schirmer et al., 1998). Yet, one important observation shows that the origins of the impairment are different between the two modes of toxicity. Most of the PAHs that are photocytotoxic are not directly cytotoxic. However, in the presence of UV radiation, these photocytotoxic PAHs have properties that would be expected to lead to the formation of reactive oxygen species (ROS). ROS can damage membranes, and therefore, are the likely cause of membrane impairment in photocytotoxicity (Valenzo, 1987).

In addition to a general mechanism of damage, a more specific action on lysosomes appears to contribute to photocytotoxicity. This is suggested by the neutral red assay, which measures the uptake and retention of the dye in lysosomes (Babich and Borenfreund, 1992). With acenaphthylene, acenaphthene and phenanthrene, only the neutral red assay detected photocytotoxicity immediately after the PAH/UV treatments. When all three assays of cellular viability were performed immediately after concurrent PAH and UV treatments, the EC50s were lower with the neutral red assay than with the other two assays. This means that at some PAH concentrations significant lysosomal damage was occurring while mitochondrial activity and plasma membrane integrity was being maintained as normal. The greater sensitivity of lysosomes was likely due to the specific accumulation of PAHs in this organelle. Preferential PAH accumulation in lysosomes has been found to occur in animal cells generally (Allison and Malucci, 1964; Kocan et al., 1983) and would cause lysosomal damage to occur at lower nominal concentrations.

The specific action on the lysosome was not apparent 24 h after termination of the PAH/UV treatment. At this time the neutral red assay gave very similar results to the CFDA-AM and alamarBlue assays. This means that after the PAH/UV treatments had ended, impairment of mitochondrial activity and plasma membrane integrity continued more rapidly than damage to lysosomes. Three different scenarios, which likely act together, can be advanced to explain this. One possibility is that early damage to lysosomes caused the release of lysosomal enzymes which during the next 24 h caused further damage to mitochondria and plasma membranes more so than impairment to lysosomes. This is reminiscent of a proposal by Allison et al. (1966). Secondly, UV irradiation might have led to the formation

Fig. 6. Impairment of RTgill-W1 cells immediately (○) and 24 h (△) after being simultaneously exposed for 2 h to UV radiation and increasing concentrations of pyrene (panel A) and benzo[g,h,i]perylene (panel B), and as measured with the neutral red assay. Exposures of cell cultures to pyrene and benzo[g,h,i]perylene, followed by the neutral red assay were performed as described in Fig. 1. One representative experiment is shown for each compound. Each data point is the mean of four culture wells with the vertical lines indicating the standard deviation.
within the cells of photomodified PAH products that had more effect on electron transport (Huang et al., 1997b) and membrane integrity than on lysosomes. A third scenario is that lysosomes can slow down the formation of ROS better than other cellular sites. Once initiated, the generation of ROS proceeds in a cascade fashion unless disrupted by protective cellular actions, such as vitamin E (Halliwell and Gutteridge, 1985). For example, in mammalian cells, chain peroxidation has been found to continue for at least 2 h after simultaneous exposure to UV radiation and a photosensitizer, merocyanine 540 (Geiger et al., 1997). ROS formation might overwhelm any local protective actions in mitochondria and plasma membranes and cause damage to continue at these sites after termination of the PAH/UV treatments, while in the lysosome damage due to ROS would be slowed down or halted. In support of this idea, rat liver lysosomal membranes have been shown to have much higher vitamin E (\( \alpha \)-tocopherol) levels than mitochondrial membranes and microsomes (Rupar et al., 1992).

Several PAHs among the photocytotoxic PAHs appeared to differ in the manner or extent to which they acted at the lysosome. This was seen by comparing the results from neutral red assays that had been done immediately after the termination of the concurrent exposure to UV/PAH and 24 h later. Fluoranthenone and B[a]P most clearly showed contrasting behavior. With fluoranthenone, the neutral red assay showed little change between the two time periods, although alamar Blue and CFDA-AM assays indicated more damage at 24 h than at 2 h. By contrast, with B[a]P, the neutral red assay indicated more damage at 24 h than at 2 h, as did the other two assays. In molluscs, the isomeric PAHs phenanthrene and anthracene have been observed to act differently in their interaction with lysosomes (Nott and Moore, 1987). Perhaps, specific features of the lysosomal membrane could account for some PAHs having a unique action on the lysosome in addition to a more general one.

In the neutral red assay, an additional difference was observed between the photocytotoxic PAHs and might be accounted for by the differential formation of excimers. At high concentrations of some PAHs, increasing photocytotoxicity was not observed with increasing concentration of PAH, resulting in a dose-response curve with a U- or L-shape. This was observed for pyrene, benzo[g,h,i]perylene, fluoranthenone and, less frequently and to a smaller extent, for benzo[a]pyrene. Previously, U-shaped dose-response curves were observed for the phototoxicity of pyrene to the fathead minnow, and excimer formation was thought to be the cause (Kagan et al., 1987). At high concentrations several PAHs form excimers, which are complexes of an electronically excited and a ground state molecule (Birks, 1970; Turro, 1978). Excimers have reduced phototoxic potential because they have less capacity to facilitate photochemical reactions (Kagan et al., 1987). For instance, excimers simultaneously involve two PAH molecules and the energy of the excimer singlet excited state is lower than that of the monomeric PAH molecule in the same state (Turro, 1978). As well, both the quantum yield and the lifetime of excimer triplet excited states are diminished (Birks, 1970). The occurrence of U-shaped dose-response curves only in the neutral red assay, which preferentially measures lysosomal impairment, might be due to PAH concentrations being higher, and in turn, excimer formation being greater in the lysosome. The failure of other photocytotoxic PAHs to show U-shaped dose-response curves in the neutral red assay has several different explanations. For anthracene, this result would be expected because with this PAH others were unable to detect excimer formation in the presence of oxygen (Birks, 1970; Weinberger and Cline Love, 1984). As well, both anthracene and benzo[a]anthracene are among the PAHs with the fastest rates of photooxidation (Mackay et al., 1992; Huang et al., 1997b). Therefore, their concentrations in lysosomes were likely too low to lead to excimers. For the PAHs that showed photocytotoxicity only at concentrations above their water solubility, a change in their dose-response curves at high concentrations was difficult to observe because the curves were incomplete and/or showed rather shallow negative slopes. However, due to the high concentrations necessary to elicit any toxic response with these compounds, the formation of molecule aggregates cannot be ruled out.
Of the 16 priority PAHs, 13 were phototoxic, but only two appear to have the potential to impact on fish in the environment through photocytotoxicity. The most restrictive factor is availability in the water column, which is influenced by water solubility. Water solubility reduces the consideration from thirteen to eight PAHs: acenaphthylene, acenaphthene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[a]pyrene and benzo[g,h,i]perylene. These are the PAHs that showed significant photocytotoxicity at concentrations at or below their water solubility. Among these, the EC$_{50}$ of the PAHs varied greatly with respect to their water solubility limit, and only fluoranthene and pyrene had EC$_{50}$ much lower than their water solubility. The other six had EC$_{50}$ values that were too close to their maximum water solubility to likely have an environmental impact, except at exceptional point sources. For example, benzo[a]pyrene was most photocytotoxic (FEF = 8.454) but its EC$_{50}$ value was close to its maximum solubility in water. This was in contrast to fluoranthene whose EC$_{50}$ value was approximately 14-fold below its water solubility limit, making the PEPP of fluoranthene ten-fold higher than that of benzo[a]pyrene (PEPP = 0.107). An additional factor that limits availability in aquatic environments is stability. For example, anthracene has a half-life of only a few hours to days in UV-irradiated environments compared to weeks for fluoranthene (Mackay et al., 1992). Thus, although anthracene (FEF = 1.898) was found to have approximately twice the photocytotoxic potency of fluoranthene in our study (FEF = 1.000), it appears less likely to play a role as a photosensitizer in the environment.

An additional factor to consider in assessing the potential of PAHs to be photocytotoxic to fish is their ability to persist in the main target tissue, the gill. This has never been measured directly but two interrelated factors that might influence persistence are metabolism and accumulation of the PAH in fish. Metabolism would reduce the likelihood of persistence. On the other hand, accumulation of PAHs in fat depots would provide an endogenous PAH source that could supply the gill through a very small fraction of the PAH partitioning from fat into blood. Generally, PAHs are metabolized rapidly and fail to accumulate substantially in fish, but this generalization is based mostly on research with just B[a]P (Varanasi et al., 1989). How other PAHs would behave is unclear, but because mixed function oxygenases (MFOs) are critical to PAH metabolism, the inability to induce these enzymes likely would make metabolism slower and accumulation more likely. Fluoranthene and pyrene were non-inducers or extremely poor inducers of MFOs in rainbow trout in vivo (Gerhart and Carlson, 1978) and in vitro (Bols, unpublished data), and thus, could persist in fish. Therefore, in the environment, fluoranthene and pyrene appear to be the PAHs with the most potential to impact on fish through photocytotoxicity.

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