Selective in Vivo Tumor Localization of Uroporphyrin Isomer I in Mouse Mammary Carcinoma: Superiority over Other Porphyrins in a Comparative Study

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ABSTRACT

Twenty-eight porphyrins were evaluated for tumor localization as delineated by fluorescence using a transplantable KHJJ mammary carcinoma in BALB/c mice as the tumor model. Five of the 28 porphyrins were found to localize and of these, one, i.e., uroporphyrin I (UROP I), showed a higher tumor:skin ratio than any of the others; moreover, as no measurable UROP I was present in the gut, the tumoralintestinal porphyrin ratio under the conditions of assay was infinity. Because hematoporphyrin derivative (HPD), a complex mixture of porphyrins has been studied extensively as a tumor localizer, we compared HPD with UROP I at differing doses (2–40 mg/kg) and at different times (3–96 h) following i.v. administration. Dose response curves showed tissue levels of porphyrin to plateau out at doses above 20 mg/kg. Peak tumor HPD and UROP I levels attained 6–18 h after i.v. administration (40 mg porphyrin/kg) were comparable, but tumor retention of HPD over the ensuing 96 h was higher. The ratio of UROP I in tumor compared to skin was significantly greater throughout the period of observation. At all times, no UROP I was detectable in gastrointestinal mucosa. At differing doses (10–40 mg/kg), the tumor:skin ratio for HPD ranged from 1.47–1.85, and for UROP I from 6.06–12.33. As a function of time (6–72 h), the tumor:skin ratios respectively were 1.85–2.38, and 11.9 to infinity. At all times, the mucosal:muco ratio at different doses for HPD approached 1 and for UROP I was infinity. We conclude that the greater specificity of tumor uptake by UROP I and its lack of retention by gut mucosa warrants further study to determine its potential clinical application as a diagnostic marker, particularly for early mucosal cancer, and in photoradiation therapy.

INTRODUCTION

HPD4 has been studied extensively as a tumor localizer, a property exploited both for photoradiation therapy (1–5) and for the diagnosis of pre- and early malignancy (6–13). Diagnosis is dependent on the property of porphyrins to fluoresce red when excited by blue light; thus, endoscopic photoexcitation of premalignant lesions retaining HPD has led to their detection in the bladder (6, 9) and the bronchus (7,12, 13). The potential for using porphyrins to diagnose mucosal malignancy before it is visible offers a new frontier in cancer detection at a curative phase of the disease; however, high volume cancer surveillance for bronchogenic, gastrointestinal, and bladder carcinoma will be limited because HPD, while preferentially retained by tumor, is still present in significant amounts in the skin and mucosa. Skin porphyrin retention results in clinical photocutaneous sensitivity 4–6 weeks after HPD administration requiring patients to meticulously avoid sunlight (1). The presence of HPD in normal mucosa reduces the sensitivity and specificity of porphyrin fluorescence in mucosal malignancy even with the use of an image intensifier equipped with appropriate light filters to minimize the effects of non-porphyrin autofluorescence (12).

Broad application of HPD in clinical oncology has been hampered by a lack of understanding of the structural properties of the tumor localizing active components (14). HPD, like its crude parent compound, HP, is a complex mixture of vinyl porphyrins, HP monoacetate, HP diacetate, protoporphyrin, and several additional derivatives of deuteroporphyrin (15–17). Moan et al. (18) by high performance liquid chromatography demonstrated 3 major and at least 17 additional minor components. In 1955, Schwartz et al. (19) proposed that the selective fluorescence of malignant tissue following injection of HPD might be due to an “impurity” in the solution rather than HP itself. Recent studies from Dougherty et al. (20) suggest the active component to be bis-1-[8-(1-hydroxyethyl)]deuteroporphyrin-3-yl)ethyl ether, known more commonly as dihematoporphyrin ether. This provisional data will set the stage for reevaluation of this compound in clinical oncology.

A drawback to contemporary studies has been the quantitation of tumor and tissue HPD content where methods used have been described as tedious, where complete porphyrin extraction is difficult, and accuracy of measurements is hindered by quenching of fluorescence by tissue components (21). In this manuscript, we have adapted a rapid, simple and reproducible assay for tissue porphyrin content without the above disadvantages (22). We also report a study of the tumor localizing behavior of 28 different porphyrins, mostly single pure porphyrins, and have identified UROP I to exhibit the most selective tumor specific uptake. Under the conditions of this study, at different doses and over a 96 h time period, uptake of UROP I by mammary carcinoma tissue was comparable to HPD but with significantly less skin and gastrointestinal mucosal retention. These properties could permit more sensitivity with regard to tumor detection without prolonged photocutaneous side effects.

MATERIALS AND METHODS

Pilot Study

Animals and Tumor Systems. The tumor model used was a transplantable KHJJ mammary carcinoma (23). BALB/c mice weighing approximately 25 g were recipients of small pieces of died tumor transplanted s.c. using a 20-gauge needle. The animals were tested for porphyrin uptake by tumor 7–14 days posttransplantation when the diameter of the s.c. tumor nodule varied between 0.25 and 1 cm. At this size, the small tumor was homogeneously white, and spontaneous necrosis was minimal or absent.

Porphyrins. The following 28 porphyrins were studied: uroporphyrin I; uroporphyrin I and III octamethyl ester; heptacarboxylic porphyrin I; pentacarboxylic porphyrin I; coproporphyrin I, II*, and III; deuteroporphyrin IX; deuteroporphyrin 2,4-disulfonic acid; deuteroporphyrin 2,4-bisglycol; deuteroporphyrin 2,4-bisacetal; deuteroporphyrin 2-acetyl; diacetyl deuteroporphyrin*; deuteroporphyrin dimethyl ester; di-
acetyl deuteroporphyrin dimethyl ester*; deuteroporphyrin 2-acetyl dimethyl ester*; proto- and zinc protoporphyrin; mesoporphyrin IX; mesotetraethylporphine; meso-tetra (4-pyridyl) porphine; TCPP; TPPS; porphine; phthalocyanine; hematoporphyrin derivative; and a derivative thereof, Photofrin II. The latter two products were purchased from Oncology Research & Development, Inc., Cheektowaga, NY, and those porphyrins annotated by asterisks were synthesized by Dr. Kevin Smith, Professor of Chemistry, University of California, Davis, who kindly supplied these for the study. Other porphyrin compounds were purchased from Porphyrin Products, Logan, UT.

Four mg of each of the crystalline porphyrin compounds were dissolved in 100 μl of dimethyl sulfoxide and diluted to a final concentration of 4 mg/ml with 0.5% sodium bicarbonate in Dulbecco's phosphate-buffered physiological saline according to the method of Berenbaum et al. (15). The pH of the final solution was adjusted to 7.5 and injections were made a minimum of 3 h following preparation of the porphyrin solution. The commercially purchased HPD and Photofrin II (2.5 mg/ml) were used as such but with the addition of dimethyl sulfoxide to a final concentration equivalent to porphyrin, 100 μl/4 mg.

Porphyrin- Screening Experimental Protocol. Because free porphyrins have the property of orange-red fluorescence in response to photoexcitation by long wavelength UV and in view of the potential to exploit this characteristic as a diagnostic marker of early malignancy, tumor fluorescence was used as a screening measure of porphyrin uptake. We anticipated minimal quenching of the fluorescent signal by the homogeneously white tumor. Porphyrins were injected i.v. at a dose of 40 mg/kg body weight. A minimum of four animals bearing tumor were used for testing each porphyrin. Initial efficacy of porphyrin localization was assessed 18–20 h postinjection in the following way. The tumors were dissected free from the s.c. tissue, excised in toto, divided in two with a scalpel so that surface and cross-section red porphyrin fluorescence could be assessed with the naked eye following photoexcitation with a black-ray long wavelength UV lamp (Model B-100A; Ultraviolet Products Inc., San Gabriel, CA). Porphyrin fluorescence, graded 1 to 4+, was similarly assessed in the skin, dissected free of hair, and the entire gastrointestinal tract from the gastroesophageal junction to the rectum was excised. The gastrointestinal mucosal fluorescence of the stomach, small intestine, and colon was separately screened before and after an intraluminal cleaning lavage.

Tissue Porphyrin Quantitation. The distribution of HPD and a commercially available derivative thereof, Photofrin II, was compared to UROP I in tumor, skin, and gut 18–20 h post i.v. injection of a dose, 40 mg/kg. Porphyrin content was quantitated by a modification of the method of Granick et al. (22). Tissues were freeze-dried in the dark for 4 h using Virtis research equipment, Gardiner, NY. The freeze-dried tumor was ground into a fine powder and a known weight was subjected to porphyrin extraction by homogenization with 1 N aqueous perchloric acid and methanol (1:1, v/v) in the dark using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was then centrifuged (4000 rpm) using a Sorvall bench top centrifuge for 10 min. The tissue precipitate was reextracted until no fluorescence was observed under UV, and the combined supernatant was filtered through Whatman no. 1 filter paper and its volume recorded. The supernatant porphyrin content was quantitated fluorometrically using Farrand spectrophuorimeter Model MK1. The concentration was determined using the porphyrin under study as the standard under optimal conditions for photoexcitation and fluorescence emission. Under conditions of assay, a standard curve confirmed that the fluorescence bore a linear relationship to the test porphyrin under study at the concentrations of the extracted test porphyrins. Each determination, which was performed in duplicate, was reproducible with little variance.

Dose and Time Response Studies

Experimental Protocol. This protocol was similar to that of the pilot study with the following exceptions. (a) As dimethyl sulfoxide did not influence the tumor localization of UROP I or HPD (24), this was not added to the injection solution. HPD commercially obtained was diluted with Dulbecco's phosphate-buffered physiological saline, pH 7.5. (b) To ensure accurate dose studies, the i.v. injection of the study porphyrin was deemed i.v. if the solution went in easily and there was no minimal extravasation of porphyrin into the tail vein as judged by UV fluorescence of the tail postinjection. If there were any doubt, that animal was not used for the study. The mice did not appear to suffer short- or long-term systemic side effects from the volume injected, which usually was 0.25 ml and never more than 0.4 ml. (c) The dose and time response studies were performed approximately 9 months following the pilot studies, the tumor line being kept viable by serial passage in mice. The native tumor used to reinoculate the study animals was small (±0.25 (SD) cm diameter) rather than 0.25–1 cm as previously. (d) To ensure minimal volumetric differences in these studies, the animals were sacrificed when the "split pea" tumor nodule was about 0.25 cm diameter. Whether due to biological variation of the tumor over 9 months of passage or because in larger tumors (±1 cm) porphyrin concentration in 30% of tumors is more intense peripherally (see "Discussion"), tumor porphyrin concentrations both in HPD- and UROP I-treated mice were 1.5–2 times greater than in the pilot study.

Dose and Time Schedules. In two sequential studies, the tumor, skin, and gastrointestinal mucosal porphyrin contents were studied as a function of dose and time. In the former study, batches of 6 animals bearing s.c. tumor nodules received HPD or UROP I i.v. at doses ranging from 2–40 mg/kg. Six h after administration, the animals were sacrificed and the tumor, skin, and GI porphyrin contents were determined as above. In the time response studies, after an i.v. dose of either HPD or UROP I, 40 mg/kg, batches of 6 animals each were sacrificed at 3, 6, 12, 18, 24, 48, 72, and 96 h after the initial injection. The porphyrin contents of tumor, skin, and gastrointestinal mucosa were quantitated fluorometrically. In all these studies, the diameter of the tumor nodules by palpation and subsequent measurement following excision very closely approximated 0.25 cm. This was chosen to keep the tumor volume as a minimal variable and to avoid spontaneous in vivo tumor necrosis.

RESULTS

In the pilot study, obvious tumor fluorescence was noted in only 5 of the 28 porphyrins tested, namely, HPD, Photofrin II, TCPP, TPPS, and UROP I. The tumor fluorescence in about 30% of animals studied was more intense in the periphery of the tumor than in the center. Skin and intestinal fluorescence was marked (3+) in animals treated with HPD, Photofrin II, TCPP, and TPPS 18–20 h after injection; in contrast, following UROP I, there was no naked eye fluorescence in the skin or in any portion of the gut. Because previous studies have indicated the superiority of HPD to TCPP and TPPS with regard to tumor localization, we confined further comparative studies to UROP I.

The tumor, skin, and gut porphyrins were quantitated in those animals 18–20 h after receiving i.v. UROP I, HPD, and Photofrin II at a dose of 40 mg/kg. Results are set out in Table 1. Note the significant and comparable retention by the tumor of HPD, Photofrin II, and UROP I. The absence of detectable porphyrins in the gut (stomach, small intestine, and colon) in animals treated with UROP I indicates a tumor: gut porphyrin ratio which for practical purposes is infinity. This is in contrast to the 1:1 ratio observed using HPD and Photofrin II. In the skin, despite the absence of naked eye fluorescence at 18–20 h, minor amounts of porphyrins were detectable by quantitative analysis. The tumorskin porphyrin content ratio with UROP I of about 18 was more than 7 times that of the 2.5:1 ratio observed with HPD and Photofrin II. This is consistent with a rapid clearance of the UROP I from the skin. We observed that the intense porphyrin fluorescence of ears, tail, and urine of the treated mice decreased over the first 2–3 h following injection and virtually had disappeared beyond 6 h.

The dose response data (Figs. 1 and 2; Table 2) indicate peak values of tumor and skin porphyrin were achieved at about 20 mg/kg. At all doses, the tumor levels of HPD were similar to that observed with UROP I whereas the skin levels of the former were significantly higher (P < 0.001) (25). The tu-
Table 1 Tumor, skin, and gastrointestinal porphyrin content 20 h after i.v. administration of UROP I, HPD, and Photofrin II

<table>
<thead>
<tr>
<th>Tissue porphyrin contents (µg/g dry wt)</th>
<th>Porphyrin (40 mg/kg)</th>
<th>Tumor</th>
<th>Skin</th>
<th>Gastrointestinal tract</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>UROP I</td>
<td>16.93 ± 3.58* (15)</td>
<td>0.833 ± 0.188 (15)</td>
<td>None detected</td>
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<tr>
<td></td>
<td>HPD</td>
<td>25.0 ± 2.55 (5)</td>
<td>10.0 ± 2.45 (5)</td>
<td>None detected</td>
</tr>
<tr>
<td></td>
<td>Photofrin II</td>
<td>20.6 ± 3.36 (5)</td>
<td>7.0 ± 0.79 (5)</td>
<td>None detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.5 ± 1.87 (5)</td>
<td>28.5 ± 2.74 (5)</td>
<td>21.0 ± 1.87 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.0 ± 2.54 (5)</td>
<td>26.0 ± 2.35 (5)</td>
<td>20.0 ± 2.41 (5)</td>
</tr>
<tr>
<td>* Mean ± SD 18-20 h after porphyrin administration.</td>
<td></td>
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<td></td>
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<td>* Numbers in parentheses, number of animals studied.</td>
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DISCUSSION

Of the 28 porphyrins tested individually for tumor localization in a malignant squamous mammary carcinoma mouse model, tumor uptake of 5 was noted. In two, TCPP and TPPS, there are no carboxyl groups linked to the porphyrin ring. HPD and Photofrin II are lipophilic dicarboxylic porphyrin complexes (14-17) and the hydrophilic octacarboxyl UROP I used was a pure single porphyrin. These observations suggest that tumor uptake of porphyrins is not dependent on lipophilicity or the number of carboxyl side chains. More than 30% of the neoplasms exhibited fluorescence which was more intense in the periphery of the tumor. This is not a novel observation and has been reported with HP diacetate by Henderson et al. (17) in their mice with squamous carcinoma, and by Cortese et al. (7) who noted “maximum fluorescence along the leading edge of the tumor” in human patients with bronchial carcinoma receiving HPD. Thus UROP I appears to behave similarly in this regard to other porphyrins studied. In the pilot study, the tumors:skin ratio for HPD and photofrin II was 2.5 and 2.3,
Animals were sacrificed at 6 h. Points, mean ± SD (bars) of six individual studies. Administration of HPD at a dose of 40 mg/kg. Points, mean ± SD (bars) of six individual studies.

Mammary carcinoma were sacrificed at varying time intervals following i.v. administration of HPD at a dose of 40 mg/kg. These values are similar to the 72-h ratio of that observed for HPD (Table 1). There was no visible fluorescence or measurable porphyrin in the gastrointestinal tract with UROP I was approximately 18:1, more than 7 times that observed for HPD (Table 1).

The dose and time response studies extend and confirm the dose-response study at 18 h following i.v. administration of the test porphyrin of 40 mg/kg was about 1.5–2 times greater than in the pilot study performed 9 months previously. Whether this represents a biological change in tumor behavior over this period or the smaller volume of tumors resected (0.25 cm compared to 0.25–1 cm in the pilot study) is not known. The latter is a likely explanation because the smaller tumors were more homogeneously fluorescent.

The virtual absence of UROP I in the gastrointestinal tract and the more rapid disappearance of this porphyrin from tumor and skin when compared to HPD might reflect in part the rapid clearance of uroporphyrin by the kidneys rather than the liver with no or minimal enterohepatic circulation as reported by Koskelo and Kekki (26) in their multicompartment analysis of 14C-labeled uroporphyrin kinetics in human beings. This also is consistent with the findings in human porphyric disorders where uroporphyrin is found almost exclusively in the urine whereas porphyrins with a lesser number of carboxyl groups (26) are more efficiently cleared by the liver and excreted in the urine and feces, or in the case of protoporphyrin, exclusively in the feces. The enteral absorption of porphyrin excreted in the bile is highly efficient, more than 95% being absorbed in the proximal 15 cm of the small intestine (27). The magnitude of the enterohepatic circulation as it relates to the plasma porphyrin pool (26, 27) and its major influence on circulating plasma porphyrin levels in the systemic circulation (28, 29) may well explain why HPD, which appears in the gut lumen, is retained by the body for a far longer period than is uroporphyrin.

Tumor uptake of UROP I is greater than that observed with uroporphyrin III (24) which probably relates to the greater affinity of the latter for circulating plasma proteins, in particular albumin (30, 31). Porphyrin-binding serum proteins (30, 31), especially albumin, may play a significant role in governing tissue uptake of porphyrins (24). This is consistent with in vitro observations that in cell culture, serum proteins in the media modulate tumor cell uptake of mesoporphyrin as well as HPD components (32–34).

Can UROP I be used in photoradiation therapy? Using the rat liver as a tumor model (35, 36), we have observed photo-

<table>
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<tr>
<th>Time after injection (h)</th>
<th>Porphyrin</th>
<th>Tumor</th>
<th>Skin</th>
<th>GI mucosa</th>
<th>Tumor:skin</th>
<th>Tumor:mu cosa</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>HPD</td>
<td>29.7 ± 2.3*</td>
<td>18.4 ± 2.9</td>
<td>29.17 ± 3.1</td>
<td>1.61</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>UROP I</td>
<td>30.0 ± 6.1</td>
<td>2.53 ± 0.6</td>
<td>0</td>
<td>11.9</td>
<td>Infinity</td>
</tr>
<tr>
<td>18</td>
<td>HPD</td>
<td>36.91 ± 1.2</td>
<td>35.75 ± 3.7</td>
<td>34.62 ± 2.8</td>
<td>1.03</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>UROP I</td>
<td>33.84 ± 4.6</td>
<td>2.56 ± 1.7</td>
<td>0</td>
<td>13.2</td>
<td>Infinity</td>
</tr>
<tr>
<td>72</td>
<td>HPD</td>
<td>31.6 ± 2.4</td>
<td>13.3 ± 1.8</td>
<td>28.7 ± 2.4</td>
<td>2.38</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>UROP I</td>
<td>14.9 ± 2.2</td>
<td>0</td>
<td>0</td>
<td>Infinity</td>
<td>Infinity</td>
</tr>
</tbody>
</table>

* After i.v. administration of either HPD or UROP I (40 mg/kg) animals were sacrificed at 6, 18, and 72 h.
* Mean of six individual experiments ± SD.
dynamic necrosis of UROP I-sensitized liver in animals receiving 200 J of light energy at an intensity of 50 mW/cm². Control animals receiving an identical dose and intensity of light exhibited no necrosis, confirming the effect to be nonthermal. Using a tuneable dye laser (Spectra Physics, Mountain View, CA) with DCM (Exciton Chemical Co., Inc., Dayton, OH) as the lasing medium, we determined the optimal photosensitizing wavelength to be 612–615 nm. Using this wavelength, Hartman et al. (37) reported regression of UROP I-treated human melanoma implanted in athymic mouse skin.

The observations of tumor localization of UROP I with a high therapeutic ratio and rapid clearance from the body and the photodynamic activity of this porphyrin have obvious clinical implications which require further studies in other malignant neoplasms, in other experimental animals, and ultimately in humans. The lack of UROP I uptake by gut mucosa with a potential tumor to mucosal ratio of infinity renders this porphyrin as a potential diagnostic marker of early colonic carcinoma, not visible on routine colonoscopy. Because colonic malignancy ranks second in mortality in both men and women in the United States (38), the data of pending studies are particularly relevant in regard to cancer surveillance in the gastrointestinal tract. It is unlikely that uroporphyrin will result in organ toxicity. In porphyrnia cutanea tarda, the most common human porphyria, the only apparent tissue side effect of chronic elevations of circulating plasma uroporphyrin is that of photocutaneous sensitivity (39). Further investigations are required to extend the observations in this report to other tumor models in different animals and in humans to test the diagnostic and therapeutic utility of uroporphyrin and light in malignant disorders (40).

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REFERENCES

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