Elucidation of the Mechanism Enabling Tumor Selective Prodrug Monotherapy

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ABSTRACT

Elucidation of the mechanism enabling tumor selective PMT in vivo with appropriate glucuronyl-spacer-doxorubicin prodrugs, such as HMR 1826, is important for the design of clinical studies, as well as for the development of more selective drugs.

Enzyme histochemistry, immunohistochemistry, and the terminal deoxy transferase technique were applied using human cryopreserved cancer tissues, normal human, monkey, and mouse tissues, and human tumor xenografts to examine mechanisms underlying the selectivity of successful PMT with HMR 1826.

It could unambiguously be shown by enzyme histochemistry that necrotic areas in human cancers are the sites in which lysosomal β-glucuronidase is liberated extracellularly in high local concentrations. The cells responsible for the liberation of the enzyme are mainly acute and chronic inflammatory cells, as shown by IHC. Furthermore, it could be demonstrated that β-glucuronidase liberated in necrotic areas of tumors can activate HMR 1826, resulting in increased doxorubicin deposition in human tumor xenografts or in human lung cancers subjected to extracorporeal perfusion, compared to chemotherapy with doxorubicin. Additionally, the doxorubicin load to normal tissues was significantly reduced compared to chemotherapy with doxorubicin. Surprisingly, the increased doxorubicin deposition in tumors also resulted in strong antitumor effects also in cancers resistant to maximum tolerated doses of systemic doxorubicin. Finally, toxicity studies in mice and monkeys revealed an excellent tolerability of HMR 1826, up to a dose of 3 g/m² (monkeys). These data suggest that HMR 1826 is a promising candidate for clinical development.

INTRODUCTION

Selective delivery of systemically administered chemotherapeutic drugs to solid cancers in vivo remains one of the major challenges facing chemotherapy. Due to the fact that only a small fraction of drug reaches the tumor (1, 2), the vast majority of the applied drug dose is taken up by normal tissues, resulting in unfavorable side effects.

Despite the development of complex drug-targeting systems for the treatment of Kaposi sarcoma, such as liposomally encapsulated daunorubicin (DaunoXome, NexStar) or doxorubicin (Doxil, Sequus Pharmaceuticals), only marginal improvements with respect to tumor selective drug deposition are achieved in the patient (3).

Recently, our group reported on a novel one-step, tumor-selective prodrug activation system, designated PMT1 (4, 5), involving the activation of a nontoxic, hydrophilic glucuronyl-spacer-doxorubicin prodrug, Activation of HMR 1826 in a necrotic human tumor xenograft.

In the present paper, we present data elucidating the mode of action enabling PMT in vivo and report on the quantitative aspects of doxorubicin and HMR 1826 deposition in normal and malignant tissues after PMT or extracorporeal perfusion in comparison to chemotherapy with doxorubicin. Finally, we present a comprehensive set of efficacy data from a broad panel of human tumor xenografts demonstrating the superiority of PMT over standard chemotherapy with doxorubicin.

MATERIALS AND METHODS

EHC. Cryopreserved human tumors or normal tissues from cancer surgery and human tumor xenografts were investigated using an improved EHC method for β-glucuronidase activity. This method is based on the protocol elaborated by Murray et al. (6) but optimized by us for sensitivity and minimal β-glucuronidase diffusion.

Briefly, after cryosectioning and air drying, tissue sections were fixed for 10 min at room temperature with 4% formaldehyde. After extensive washing, naphthol 3-Hydroxy-2-naphthoic acid anilide glucuronic acid and hexazotized neufuchsin was added for 90 min at 45°C. After washing and drying, the insoluble product of the enzymatic substrate reaction was evaluated using a Laborlux S light microscope (Leitz, Wetzlar, Germany).

IHC. Cryopreserved tissue sections were stained with MAbs, followed by addition of alkaline phosphatase labeled goat antimouse immunoglobulin second antibody, and tissue sections were incubated with naphthol AS biphosphate and hexazotized neufuchsin following established procedures (7).

The designation, source, and specificity of the MAbs used is summarized in Table 1.

TdT Reaction. The TdT (13) reaction was performed using cryopreserved tissue sections fixed for 10 min with 4% formaldehyde according to the suppliers protocol (Oncor, Gaithersburg, Maryland).

Briefly, after blocking of endogenous peroxidase with H2O2 and addition of TdT, digoxigenin-labeled dUTP and unlabeled dATP were added as an enzyme substrate. As a negative control, dideoxy-ATP was added to separate tissue sections. TdT activity was visualized by adding a peroxidase antidigoxigenin antibody followed by incubation with the substrate for peroxidase (3,3′- diaminobenzidine). Thereafter, sections were briefly counterstained with hematoxylin (Mayers Hämaluna, Merck KGaA, Darmstadt, Germany). Sections were evaluated as described below.

In double-staining experiments, the TdT reaction was performed after the EHC studies.

Processing of Data from EHC, IHC, and TdT Reactions. Computer-assisted image analysis was performed using the personal computer-based Quantimet 500 MC image analysis system (Leica, Bensheim, Germany) connected with a three-CCD DONPISHA color vision camera (Sony Corporation, Tokyo, Japan) and a Laborlux S microscope (Leitz, Wetzlar, Germany). Each image was processed using the Q-win color software (Leica, Bensheim, Germany). Each microscope field was quantified using specific color detection of single pixels in an acquired image of 512 × 512 pixels. Correlations between images obtained in double-staining experiments (TdT, brown stain; EHC, red stain) were processed using the Q-win color software package.

Prodrug Supply and Quality. HMR 1826 was synthesized and purified according to published procedures (14). The doxorubicin content was less than 0.1% (w/w).

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2 To whom requests for reprints should be addressed, at Hoechst Marion Roussel Deutschland GmbH, P.O. Box 1140, D-35001 Marburg, Germany.

3 The abbreviations used are: PMT, prodrug monotherapy; EHC, enzyme histochemistry; IHC, immunohistochemistry; TdT, terminal deoxytransferase; AUC, area under the curve; MAb, monoclonal antibody.
Table 1: Specificity of MAbs used for IHC

<table>
<thead>
<tr>
<th>MAb designation</th>
<th>Source (Ref.)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 E 10</td>
<td>8</td>
<td>Acute inflammation marker, detects macrophages producing IL-1, IL-6, and TNF, as well as granulocytes in acute inflammation (MRP 18/14 complex)</td>
</tr>
<tr>
<td>25 F 9</td>
<td>9</td>
<td>Human late stage inflammation, detects mature macrophages, Kupffer cells, and histiocytes</td>
</tr>
<tr>
<td>BW 495/36</td>
<td>10</td>
<td>All epithelial cells (gastrointestinal carcinoma antigen)</td>
</tr>
<tr>
<td>BW 250/183</td>
<td>11</td>
<td>All human granulocytes (NCA 95)</td>
</tr>
<tr>
<td>BW 2156/94</td>
<td>12</td>
<td>Human β-glucuronidase</td>
</tr>
</tbody>
</table>

* NCA 95, nonspecific cross-reacting antigen.

Reverse-Phase High-Performance Liquid Chromatography Analysis of HMR 1826 and Doxorubicin. Normal tissues or human tumor xenografts from nude mice receiving pharmacodynamically relevant doses of doxorubicin or HMR 1826 were processed as described previously (12). Tissues derived from lung cancer surgery and subjected to extracorporeal perfusion were processed and analyzed as described (15).

Analysis of Therapeutic Efficacy. CD-1-nu/nu or National Medical Research Institute nu/nu mice (seven animals/group) bearing s.c. human carcinoma xenografts (tumor diameter, 3 mm) of different origin received three i.v. bolus injections of 250-400 mg/kg of HMR 1826 in 0.5 ml of vehicle (vehicle: 0.4 mg of CaCl2 X 2 H2O in 5% mannitol, pH 7.35) at days 1, 4, and 7 (8), or three doses of 4.5 mg/kg doxorubicin, or three doses of 0.5 ml of vehicle (saline) at the same study days. Tumor growth was monitored every third day by measurement of two perpendicular tumor diameters. Mean relative tumor areas were calculated from tumor diameters measured at individual days divided by tumor diameters measured at start of therapy. For treated groups the Treated/Control (%) at individual days was calculated as follows:

\[ \frac{\text{mean relative tumor volume of treated group}}{\text{mean relative tumor volume of control group}} \times 100 \]

As a parameter for maximum efficacy, the minimal T/C % value is given at the day it was obtained during the experiment.

As a parameter for the duration of growth inhibition a growth delay 200% and 400% value is given representing the tumor volume doubling or quadrupling time of the drug treated group minus the tumor volume doubling time of the control group and is calculated using the following formula for a growth delay of 200%:

\[ T - C = \text{doubling time of tumor volume}_{\text{treated group}} - \text{doubling time of tumor volume}_{\text{control group}} \]

and the following formula for a growth delay of 400%:

\[ T - C = \text{quadrupling time of tumor volume}_{\text{treated group}} - \text{quadrupling time of tumor volume}_{\text{control group}} \]

Calculations. Time courses of HMR 1826 concentrations resulting from i.v. injection in mice, rats, and monkeys were adjusted according to a biexponential function, corresponding to an open two-compartment model with characteristics as follows:

\[ C(t) = A \exp(-\alpha t) + B \exp(-\beta t) \]

\[ \alpha = \ln(2)/t_{1/2}\alpha \]

\[ \beta = \ln(2)/t_{1/2}\beta \]

The model equation was fitted to the data by means of nonlinear regression (personal computer HoeReP software) with weighed least squares and by putting inverse quadratic weights on individual plasma concentrations.

Safety Studies. MF1 mice, as recommended by the Cancer Research Campaign/European Organization of Research and Treatment of Cancer (16), were used in this acute toxicity experiment. The mice were of specific pathogen-free quality and supplied by Harlan Winkelmann (Borchen, Germany). The body weights ranged from 20.6 to 32.3 g at the start of study. Twenty animals (10 males and 10 females) were assigned to each group and treated with doses of 0, 300, 625, 1250, or 1875 mg/kg by i.v. injection into the tail veins to determine the LD50 value. Mice in the higher dose groups received either two or three application within an interval of 2 h. The animals were observed after treatment and daily thereafter for 28 days. LD10 values were transformed from mg/kg into mg/m2 according to the European Organization of Research and Treatment of Cancer Guideline (16).

Cynomolgus monkeys (Macaca fascicularis) were supplied by Shamrock (Small Dole, West Sussex, United Kingdom). The body weight of the animals, which were approximately 26–37 months old, ranged from 1.8 to 2.9 kg at the start of the study. Four animals (two males and two females) were placed for each dose group. The doses given were 0, 60, 120, or 250 mg/kg by single i.v. injection. Thereafter, the monkeys were observed for 13 weeks to identify possible late-occurring adverse reactions. The applied doses were transformed from mg/kg into mg/m2 as described (17).

RESULTS

Histochemical Studies Using Human Tumor Xenografts and Human Cancers from Surgery Confirm Similar Modes of Action. A collection of cryopreserved human tumor tissues and human tumor xenografts was studied using three cytochemical methods (EHC, IHC, and TdT) to clarify the mode of action underlying PMT. Results of these investigations are summarized in Table 2.

From the EHC data presented in Table 2, it is clear that β-glucuronidase activity in cryopreserved lung cancer surgery specimens is concentrated preferentially within areas of necrosis, because the depostition of the red stain from EHC was found in the same areas as the brownish stain from the TdT reaction. Some red spots representing functionally active β-glucuronidase were, however, also found in living tumor cell areas. Furthermore, the cells or cell debris within necrotic areas mainly were seen to bear markers present on human monocytes and/or granulocytes and only occasionally markers for human epithelial cells, as shown by IHC using MAb selective for the appropriate cell surface antigens (Table 2 and Fig. 1). From these results, it can be concluded that monocytes/granulocytes are most likely the cells that liberate β-glucuronidase in areas of necrosis within human surgical lung cancer specimen and that the contribution to liberated β-glucuronidase activity resulting from disintegrating cancer cells is only marginal.

In contrast to the findings with human lung cancer specimen, investigations using human stomach, ovarian, pancreatic, and breast carcinomas showed that the degree of necrosis is less pronounced or is absent (ovarian carcinomas) and that β-glucuronidase is more heterogeneously distributed as individual spots, corresponding with monocytes/granulocytes, all over the tumor sections (data not shown).

From these observations, it can be concluded that the host inflammatory component is the major source of β-glucuronidase present in human cancer tissue biopsies.

In human tumor xenografts, a clear-cut correlation between the presence of high local concentrations of β-glucuronidase in necrotic areas and the brownish staining for the degrading DNA, as visualized by the TdT reaction product, was observed. Necrotic cells or cell debris in necrotic areas were only marginally stained by the MAb BW 495. Due to the lack of reliable IHC markers for mouse monocytes/granulocytes, the conclusion that the cells contributing most to β-glucuronidase activity in necrotic areas may be mouse monocytes/granulocytes is only indirect. This assumption is furthermore supported by the finding that MAb 2156/94, which is selective for human β-glucuronidase, does not stain the β-glucuronidase within necrotic areas to a significant extent, but only detects the enzyme within living human tumor cells.
**β-Glucuronidase Is Preferentially Localized Intracellularly in Normal Tissues of Mice, Monkeys, and Humans.** In addition to the panel of human cancers and human tumor xenografts, a broad panel of normal tissues derived from mice, monkeys, and humans were investigated by EHC to determine the localization of β-glucuronidase. β-Glucuronidase activity was found intracellularly in the majority of tissues investigated. Very weak extracellular staining was detected in lymph nodes (humans) and the tubuli of the kidney (humans and monkeys). No staining at all was observed in heart, brain, muscle, and pituitary gland (data not shown).

Similarly, β-glucuronidase was found to be intracellular in most tissues of mice except lung and kidney, where a weak extracellular reaction was observed. These results extend earlier histochemical findings in rats (18) and support the opinion that β-glucuronidase is an enzyme with preferential intracellular localization in mice, monkeys, and humans.

**PMT in Human Tumor Xenografts Generates Superior Therapeutic Effects Using a Broad Panel of Tumors.** Preliminary data presented by our group suggested that PMT with HMR 1826 results in superior therapeutic effects in a human colon carcinoma xenograft model (LoVo) in nude mice compared to chemotherapy with doxorubicin (4). Using a panel of 20 different human tumor xenografts derived from human lung, colon, breast, ovary, and stomach cancers, we could confirm and extend these findings. Tumors resistant to treatment with the MTD of doxorubicin could be significantly influenced in their growth or brought into complete remission (Table 3). These data confirm the superiority of PMT compared to standard chemotherapy with doxorubicin.

**Superior Efficacy of PMT Compared to Chemotherapy with Doxorubicin Is Due to Increased Doxorubicin Deposition in Tumor Tissues.** Tissues from mice treated with HMR 1826 or with doxorubicin were analyzed using C-18-reverse-phase high-performance liquid chromatography to determine the content of HMR 1826 and doxorubicin at different time points. After a first i.v. injection of 400 mg/kg of HMR 1826 (Fig. 2A), the concentration of doxorubicin in the tumor increased during the first 8 h to approximately 5–8 μg of doxorubicin/g of tumor. Following the second injection at day 4, doxorubicin content increased to approximately 6–16 μg/g of tumor tissue. Similarly, the tissue receiving the highest doxorubicin concentration after administration of HMR 1826 is clearly the tumor.

Furthermore, the distribution of HMR 1826 was determined in the same set of organs, including the tumor, and found to decrease within a few hours in all tissues (Fig. 2B). The organs containing the highest concentrations of HMR 1826 were kidney, lung, and liver.

For comparison, doxorubicin was injected as a single dose i.v. and its deposition was determined at the same time points as done for HMR 1826 (Fig. 2C). Doxorubicin deposition in tumor was minimal compared to the doxorubicin deposition achieved after HMR 1826 injection (10-fold lower). In contrast, the deposition of doxorubicin in normal tissues, especially heart, was significantly higher after doxorubicin injection compared to therapy with HMR 1826.

Taken together, these data yield a clear-cut rationale for the superior pharmacodynamic effects achieved in the human tumor xenograft...
studies (see above) and for the good tolerability of HMR 1826 even at very high doses (see below).

According to the IHC investigations presented above, the therapeutic effects, as well as the tumor-selective drug deposition after PMT, are most likely mediated by β-glucuronidase liberated from host (mouse) monocytes/granulocytes. To evaluate whether human inflammatory cells infiltrating human lung cancers are able to cleave HMR 1826 as efficaciously as mouse monocytes/granulocytes can, doxorubicin deposition and HMR 1826 concentrations were determined immediately after PMT in an extracorporal human lung cancer perfusion system (for details, see Ref. 15). A summary of the doxorubicin and HMR 1826 concentrations in surgical normal human lung specimens separated from human lung cancers, as well as from the respective human lung cancers, is given in Table 4.

From the data presented in Table 4, it is clear that perfusion with HMR 1826 leads to a preferential deposition of doxorubicin within lung cancer tissues compared to normal human lung (18 μg of doxorubicin/g of cancer tissue versus 12 μg of doxorubicin/g of lung tissue). Perfusion with doxorubicin, however, resulted in a preferential accumulation of doxorubicin in lung tissues (23 μg of doxorubicin/g of lung tissue versus 3 μg of doxorubicin/g of cancer tissue). The actual concentrations of HMR 1826 or doxorubicin used during perfusion were selected because similar concentrations were reached in plasma of mice and monkeys during efficacy or toxicology studies (see below).

From these data, it can be concluded that the human inflammatory cells within human lung cancer tissues are able to activate HMR 1826 to an extent similar to mouse monocytes/granulocytes in the xenograft model system.

HMR 1826 Is Well Tolerated in Mice and Monkeys. The acute toxicity study in MF1 mice revealed LD_{10} values of 307.3 mg/kg in males and 623.9 mg/kg in females. These LD_{10} values are equivalent to 904.8 mg/m^2 and 1761.8 mg/m^2. Signs of neurotoxicity were observed at doses above the LD_{10} in a progressive manner, starting with difficulties in coordination of the hind limb movements in the third week after treatment, which increased later on to complete hind limb paralysis. For comparison, the LD_{10} values for doxorubicin in these mice are in the range of 15–20 mg/kg if doxorubicin is administered according to an identical study protocol. Again, similar signs of progressive neurological disorder were noticed in the animals treated with 10–15 mg/kg doxorubicin in the third week of this particular study. These observations are in accord with previously reported findings after single dose exposure to anthracyclines (19). These progressive neurological disorders are related to the selective damage of ganglia of the peripheral nervous system and are only observed in rodents. This objective was addressed to another safety experiment using cynomolgus monkeys. These animals were treated at a dosage of up to 250 mg/kg, which is equivalent to approximately 3000 mg/m^2 by single i.v. injection. No mortality was found, and the
clinical observation did not reveal any signs of overt toxicity. The ability to coordinate movements was not altered at any time within the observation period. The profile of marker enzymes and substrates for proper liver, heart, and kidney function were not influenced. However, the leukocyte counts were severely reduced in the animals of the high-dose group (250 mg/kg). The reduction was transient, and physiological values were reached again at the end of the third week after administration. The macroscopic and histopathological examinations did not indicate adverse side effects related to HMR 1826. The good tolerability of HMR 1826 in monkeys is clearly superior to the tolerability of doxorubicin in this animal model. According to published results from cynomolgus monkeys, the single-dose lethality value of doxorubicin is 40 mg/kg (one-third of 120 mg/kg) in monkeys should be the pharmacodynamically most relevant factor influencing therapeutic efficacy, 40 mg/kg (one-third of 120 mg/kg) in monkeys should generate therapeutic effects in humans at doses that do not induce detectable side effects. This is based on the studies with monkeys, which are considered to be relevant for the evaluation of HMR 1826 because the distribution of β-glucuronidase is most similar to humans.

DISCUSSION

The preferential delivery of drugs to solid cancers remains an unresolved problem in chemotherapy today. Various drug-targeting systems based on an assortment of complicated mechanisms, such as antibody enzyme conjugates (21) or gene therapeutic vector constructs combined with prodrugs (22), hydrophilic polymeric drug conjugates (23), or stealth liposomes containing cytotoxic drugs (24), were developed to achieve tumorselective delivery of drugs. Nevertheless, none of these drug-targeting systems has proved thus far to be clinically satisfactory (3). Therefore, we thought that the development of a less complicated drug-targeting system should be pursued.

In this report, we describe in more detail the mode of action underlying a novel drug-targeting system (PMT) that our group recently described (4). In contrast to the above mentioned drug-targeting systems, the PMT system is a low molecular weight, one-component system, consisting of an appropriately formulated glucuronyl-spacer-doxorubicin prodrug (HMR 1826; Ref. 4).

The EHC studies performed to determine the localization of functionally active β-glucuronidase clearly show that active enzyme is present extracellularly in large amounts in necrotic areas of cancers taken from human lung cancer surgery (Table 2) and from nude mouse xenografts. In contrast, in normal tissues from mice, monkeys, and humans, β-glucuronidase activity is found mainly intracellularly (Table 1), it could be shown that the cells present in necrosis with β-glucuronidase activity are mainly monocytes/granulocytes, epithelial cells (tumor cells) or human monocytes/granulocytes but not (or only to a low extent) tumor cells (Fig. 1). Furthermore, the enzyme activity that is detectable by EHC in necrosis of xenografts did not stain with MAb selective for human β-glucuronidase and is therefore not of human origin. Therefore, the preferential

<table>
<thead>
<tr>
<th>Tumor of origin</th>
<th>Histology</th>
<th>Dose (mg/kg)</th>
<th>Schedule (day)</th>
<th>Minimum T/C [% (day)]</th>
<th>200% (T-C)</th>
<th>400% (T-C)</th>
<th>200% (T-C)</th>
<th>400% (T-C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma of lung</td>
<td>SCLC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5</td>
<td>1, 4, 8</td>
<td>60 (8)</td>
<td>1</td>
<td>2</td>
<td>400</td>
<td>1, 4, 8</td>
</tr>
<tr>
<td>B 98</td>
<td>SCLC</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>61 (25)</td>
<td>2</td>
<td>3</td>
<td>340</td>
<td>1, 4, 7</td>
</tr>
<tr>
<td>B 527</td>
<td>SCLC</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>92 (11)</td>
<td>0</td>
<td>1</td>
<td>400</td>
<td>1, 4, 8</td>
</tr>
<tr>
<td>GOT 1</td>
<td>SCLC</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>57 (15)</td>
<td>3</td>
<td>11</td>
<td>400</td>
<td>1, 4, 7</td>
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<tr>
<td>OAT 75</td>
<td>SCLC</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>83 (28)</td>
<td>1</td>
<td>6</td>
<td>300</td>
<td>0, 3, 7</td>
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<tr>
<td>LXFA 289</td>
<td>Adeno</td>
<td>3.0</td>
<td>0, 3, 7</td>
<td>63 (14)</td>
<td>7</td>
<td>4</td>
<td>300</td>
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<td>Adeno</td>
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<td>0, 3, 7</td>
<td>76 (41)</td>
<td>2</td>
<td>0</td>
<td>300</td>
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<tr>
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<td>Adeno</td>
<td>3.0</td>
<td>0, 3, 7</td>
<td>43 (28)</td>
<td>11</td>
<td>20</td>
<td>350</td>
<td>0, 3, 7</td>
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<tr>
<td>LXFA 211</td>
<td>Epidermoid</td>
<td>3.0</td>
<td>1, 4, 8</td>
<td>42 (25)</td>
<td>0</td>
<td>1</td>
<td>400</td>
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<tr>
<td>LXFA 397</td>
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<td>1, 4, 8</td>
<td>74 (28)</td>
<td>2</td>
<td>6</td>
<td>300</td>
<td>1, 4, 11</td>
</tr>
<tr>
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<td>Adeno</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>76 (28)</td>
<td>2</td>
<td>2</td>
<td>350</td>
<td>1, 4, 7</td>
</tr>
<tr>
<td>LoVo</td>
<td>Adeno</td>
<td>3.0</td>
<td>0, 3, 7</td>
<td>100 (0)</td>
<td>1</td>
<td>1</td>
<td>350</td>
<td>0, 3, 7</td>
</tr>
<tr>
<td>CXT 1103/7</td>
<td>Adeno</td>
<td>3.0</td>
<td>1, 4, 7</td>
<td>63 (16)</td>
<td>1</td>
<td>1</td>
<td>400</td>
<td>1, 4, 8</td>
</tr>
<tr>
<td>Carcinoma of breast</td>
<td>Adeno</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>38 (18)</td>
<td>1</td>
<td>9</td>
<td>375</td>
<td>1, 4, 7</td>
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<tr>
<td>MX-1</td>
<td>Adeno</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>70 (36)</td>
<td>5</td>
<td>13</td>
<td>250</td>
<td>1, 4, 7</td>
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<tr>
<td>O 5</td>
<td>Adeno</td>
<td>4.5</td>
<td>1, 4, 7</td>
<td>60 (46)</td>
<td>6</td>
<td>15</td>
<td>250</td>
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<td>OVXF 899</td>
<td>Adeno</td>
<td>3.0</td>
<td>0, 3, 7</td>
<td>24 (28)</td>
<td>13</td>
<td>25</td>
<td>400</td>
<td>0, 3, 7</td>
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<tr>
<td>OVXF 1023</td>
<td>Adeno</td>
<td>3.0</td>
<td>0, 3, 7</td>
<td>59 (24)</td>
<td>11</td>
<td>16</td>
<td>350</td>
<td>0, 3, 7</td>
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<tr>
<td>Carcinoma of stomach</td>
<td>Adeno</td>
<td>10.0</td>
<td>1</td>
<td>87 (13)</td>
<td>NR</td>
<td>NR</td>
<td>250</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> SCLC; small cell lung carcinoma; adeno, adenocarcinoma; NR, not reached.

<sup>b</sup> Response after 8 days.

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DISCUSSION

The preferential delivery of drugs to solid cancers remains an unresolved problem in chemotherapy today. Various drug-targeting systems based on an assortment of complicated mechanisms, such as antibody enzyme conjugates (21) or gene therapeutic vector constructs combined with prodrugs (22), hydrophilic polymeric drug conjugates (23), or stealth liposomes containing cytotoxic drugs (24), were developed to achieve tumorselective delivery of drugs. Nevertheless, none of these drug-targeting systems has proved thus far to be clinically satisfactory (3). Therefore, we thought that the development of a less complicated drug-targeting system should be pursued.

In this report, we describe in more detail the mode of action underlying a novel drug-targeting system (PMT) that our group recently described (4). In contrast to the above mentioned drug-targeting systems, the PMT system is a low molecular weight, one-component system, consisting of an appropriately formulated glucuronyl-spacer-doxorubicin prodrug (HMR 1826; Ref. 4).

The EHC studies performed to determine the localization of functionally active β-glucuronidase clearly show that active enzyme is present extracellularly in large amounts in necrotic areas of cancers taken from human lung cancer surgery (Table 2) and from nude mouse xenografts. In contrast, in normal tissues from mice, monkeys, and humans, β-glucuronidase activity is found mainly intracellularly and in lower concentrations. Using IHC with MAb selective for monocytes/granulocytes, epithelial cells (tumor cells) or human β-glucuronidase (Table 1), it could be shown that the cells present in necrosis with β-glucuronidase activity are mainly monocytes/granulocytes but not (or only to a low extent) tumor cells (Fig. 1). Furthermore, the enzyme activity that is detectable by EHC in necrosis of xenografts did not stain with MAb selective for human β-glucuronidase and is therefore not of human origin. Therefore, the preferential...
B-GLUCURONIDASE-MEDIATED PRODRUG ACTIVATION

Fig. 2. A, tissue distribution of doxorubicin (ng/g of tissue) after repetitive i.v. injection of 400 mg/kg HMR 1826 in NMRI nu/nu mice at days 1, 4, and 8. B, tissue distribution of HMR 1826 (µg/g of tissue) after repetitive i.v. injection of 400 mg/kg HMR 1826 in NMRI nu/nu mice at days 1, 4, and 8. C, tissue distribution of doxorubicin (µg/g of tissue) after a single i.v. injection of 4.5 mg/kg doxorubicin in NMRI nu/nu mice.

The liberated β-glucuronidase activity in areas of necrosis seems to be responsible for the activation of HMR 1826, resulting in a preferential accumulation of doxorubicin within carcinomas as shown by extracorporeal perfusion studies performed with fresh human lung cancer surgical materials (Table 4). Addition of an excess of saccharolactone, a β-glucuronidase inhibitor, into the perfusion fluid during extracorporeal perfusion results in a complete blockade of HMR 1826 activation, showing that β-glucuronidase, rather than other factors like pH, is necessary for activation of HMR 1826 (15).

Taken together, PMT results in preferential activation of HMR 1826 in cancer tissues compared to normal tissues, despite the well-known heterogeneity in intracellular levels of β-glucuronidase in normal tissues (25).

PMT is a refinement of the early studies of Fishman et al. (26, 27), who found increased β-glucuronidase levels in a high percentage of tumors, such as carcinomas of the breast, stomach, and colon, compared to adjacent normal tissues. Due to the lack of reliable EHC techniques for β-glucuronidase and the absence of MAbs selective for various cell components, Fishman et al. (26, 27) could not determine at that time whether the enzyme originates from tumor cells or from host components, such as monocytes/granulocytes, nor whether β-glucuronidase was present in necrosis or only intracellularly in living tumor cells.

On the basis of our observations that β-glucuronidase is mainly an intracellular enzyme that gets preferentially liberated during the pathophysiological process of necrosis within tumors (4), a comprehensive glucuronide prodrug synthesis program was initiated in which many different anthracyclin- or alkylating agent-based prodrugs with self-immolative spacers were synthesized and evaluated (14, 28, 29). Out of this program, HMR 1826 (12) was selected due to its unique stability at 37°C in human plasma, its fast enzymatic activation by β-glucuronidase (12), its appropriate hydrophilicity inhibiting significant nonspecific uptake into living cells, and its excellent efficacy in an animal model for human cancers (5).

I.v. injection of HMR 1826 in nude mice bearing a large panel of human tumor xenografts generated strong antitumor effects in all human tumor xenografts tested, most of which being resistant to treatment with doxorubicin. Sometimes, durable complete regressions were observed (Table 3). The superior efficacy of PMT with HMR 1826 was due to the increased doxorubicin deposition and retention of doxorubicin in the tumor connected with reduced drug concentrations in normal tissues (Fig. 2). Repetitive injection further increased the doxorubicin dose in the tumor but did not lead to an increased

| Table 4 | Quantitative determination of doxorubicin and HMR 1826 content in eight human lung cancers and eight normal human lung tissues extracorporally perfused with 400 µg/ml of HMR 1826 or 5 µg/ml of doxorubicin |
| Tissue type | Median HMR 1826 content (µg/g tissue) after perfusion with PMT | Median doxorubicin content (µg/g tissue) after perfusion |
| Normal human lung | 145 | 12 |
| Human lung cancer | 46 | 18 |

| Table 5 | Pharmacokinetic parameters of HMR 1826 in MF 1 mice and monkeys |
| Pharmacokinetic parameter | Monkeys (120 mg/kg) | Mice (300 mg/kg) |
| Weight (g) | 2053 | 22.9 |
| Dose (mg) | 247 | 6.87 |
| Cmax (µg/ml) | 641 | 1486 |
| A (µg/ml) | 489 | 1765 |
| B (µg/ml) | 181 | 410 |
| t1/2 α (min) | 6.6 | 1.5 |
| t1/2 β (min) | 85.8 | 9.0 |
| AUC (µg · min/ml) | 25977 | 9251 |
| Cl (ml/min) | 9.70 | 0.65 |
| Vd (liters) | 1.03 | 0.007 |
doxorubicin retention in normal tissues. This drug-targeting effect results not only in, as already mentioned, very strong antitumor effects but also in good tolerability, as shown by the minimal to moderate body weight loss of tumor-bearing nude mice treated with HMR 1826 at doses up to 1200 mg/kg (body weight loss <15% in most studies; data not shown).

Acute toxicity studies in M1F mice with HMR 1826 revealed a LD10 value 20–40-fold higher (mass basis) than that of doxorubicin. Additionally, signs of neurotoxicity were observed for the HMR 1826 treated mice at LD10 doses. This disorder resembles signs of toxicity observed with high single dose treatment with anthracyclines. In human cancer treatment, however, the neurotoxicity of anthracyclines seen in rodents is not a clinically relevant factor (19).

The lack of neurotoxicity in humans is supported by the tolerability studies in macaque monkeys with HMR 1826, showing that a single dose of 250 mg/kg (3000 mg/m²) is well tolerated. On the basis of the published single dose lethality value for doxorubicin in monkeys (20), tolerability was improved by a factor >60 (mass basis). However, repetitive doses of 250 mg/kg of HMR 1826 induced a severe reduction of peripheral leukocyte counts, supporting the assumption that immunosuppression might be the dose-limiting toxicity in humans if very high treatment doses are administered (data not shown).

Extrapolation of toxicology and toxicokinetic data (Table 5) from macaque monkeys and mice, as well as efficacy data from human tumor xenografts to human patients results in a significant therapeutic window, enabling antitumor therapy with minimal side effects for cancer patients with necrotic solid tumors.

For nonnecrotic tumors, HMR 1826 may not be used as a single-agent therapy (PMT) but may instead be used as a component in conjunction with a recently described humanized antibody-β-glucuronidase fusion protein (12), or as an adjuvant to novel antiangiogenic (30) or antineovascularization approaches (31).

Finally, it should be pointed out that HMR 1826 is also therapeutically effective in the adjuvant arthritis model of the rat (32), in a DTH-model in mice (33) and an allogeneic heart transplantation model in rats (34),4 supporting our finding that the local host inflammatory component is the mediator for HMR 1826 activation, due to liberation of β-glucuronidase.

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