Comparison of Three Approaches to Doxorubicin Therapy: Free Doxorubicin, Liposomal Doxorubicin, and β-Glucuronidase-Activated Prodrug (HMR 1826)

RICHARD WOESSNER1, ZILI AN2, XIONG MEI2, ROBERT M. HOFFMAN2, ROBERT DIX1 and ALAN BITONTI3

1Aventis Pharmaceuticals, Route 202-206, Bridgewater, NJ 08807;
2Anticancer Inc, 7917 Ostrow Street, San Diego, CA 92111;
3Current address: Syntex Pharmaceuticals, 9 Fourth Avenue, Waltham, MA 02451, U.S.A.

Abstract. Background: Three approaches to doxorubicin therapy are directly compared: free doxorubicin, liposomal doxorubicin and β-glucuronidase-activated prodrug (HMR 1826). Materials and Methods: The optimal dose of HMR 1826 was determined to be 200 mg/kg once a week and subsequent studies were carried out comparing HMR 1826 at 200 mg/kg 1x/wk, liposomal doxorubicin (Doxil) at 9 mg/kg 1x/wk and free doxorubicin at 7 mg/kg 1x/wk in seven different human tumor xenograft models. Results: All three forms of doxorubicin inhibited tumor growth with similar efficacy in each of the tumor models with the exception of MDA-MB-231 tumor xenografts, which were resistant to free doxorubicin but sensitive to Doxil and HMR 1826. Overall less weight loss was observed with HMR 1826 treatment. Conclusions: The efficacy of HMR 1826 is equal to or better than that of doxorubicin and Doxil at a safe dose and schedule, indicating that the β-glucuronidase activated prodrug approach is safe and effective.

Doxorubicin, an antitumor antibiotic that binds DNA and inhibits topoisomerase activity and nucleic acid synthesis, is widely used to treat various human cancers. Cardiac toxicity and myelosuppression are two major side effects limiting its clinical use (1). Approaches that would facilitate a more tumor-selective chemotheraphy with reduced systemic toxicity have been the subject of intensive research (2). Two such approaches are encapsulation in polyethylene glycol-coated (PEGylated) liposomes (3, 4) and the use of prodrugs (5, 6).

PEGylated liposomal doxorubicin (Doxil), characterized by a long circulating half-life and slow plasma clearance (3, 4), has recently shown promising activity in AIDS-related Kaposi’s sarcoma (7). It is hypothesized that because of their small size and persistence in the circulation, the PEGylated Doxil liposomes are able to penetrate the altered and often compromised tumor vasculature resulting in localization of doxorubicin in the tumor tissue (3, 4, 8). The immune system responds to conventional liposomes by opsonization and subsequent macrophage uptake after injection (4, 9). PEGylation allows the liposomes to evade detection (a "stealth" effect). Tumor drug concentrations 10 times greater than after the same dose of free doxorubicin have been reported (4, 10), as well as up to a 25 fold increase in doxorubicin at the tumor site, vs. normal tissue (11).

One prodrug approach to doxorubicin involves the attachment of a glucuronide to doxorubicin via a nitrobenzene spacer (6). This compound, N-[4-β-glucuronyl-3-nitrobenzyloxy carbonyl]doxorubicin (HMR 1826), is inactive and non-cytotoxic until cleaved to release free doxorubicin when in contact with extracellular β-glucuronidase, which is found in necrotic areas of tumors (5, 6, 12). Due to its limited permeation into intact cells, HMR 1826 can not be activated by the lysosomal β-glucuronidase present inside the cells. Tumors contain an elevated level of β-glucuronidase (13, 14, 15), which is most likely liberated by invading monocytes and granulocytes in the necrotic areas of tumors, with only marginal contribution from disintegrating tumor cells (5). Studies with human tumor xenografts (5) and ex vivo human lung tumor specimens (2) have demonstrated that the mechanism of prodrug activation utilized by HMR 1826 leads to increased levels of free doxorubicin in tumor tissue and decreased levels in normal tissues compared to that of free doxorubicin. In tumor bearing nude mice, accumulation of doxorubicin in normal tissue was 6 to 15 fold greater than in tumor tissue after administration of free doxorubicin, but was up to 15 fold greater in tumor than in normal tissue after administration of HMR 1826 (5). In human lung tissue perfused with free doxorubicin or HMR 1826 ex vivo, doxorubicin accumulated preferentially in normal tissue by a ratio of 7:1 after administration of free doxorubicin, but the distribution was about equal (1:1) after administration of HMR 1826 (2, 5).

In preclinical studies, doxil (6, 17) and HMR 1826 (5, 18, 19) both exhibit a superior safety profile when compared to free doxorubicin, particularly in the area of cardiotoxicity.
Figure 1. Effect of HMR 1826 on the growth of MDA-MB-231 tumor xenografts in nude mice. Tumor implantation and drug treatment as described in Materials and Methods. Error bars represent standard error (n=6 per group). Doses are 50 mg/kg (a), 100 mg/kg (b), 200 mg/kg (c) and 400 mg/kg (d). Dosing schedules are: filled circles - untreated control; open circles - 1x/wk on days 25, 32, 39, 46; filled triangles, 1/2x/wk on days 25, 39, 53; open triangles, 1x/3wk on days 25, 46; filled squares, qd x 3 on days 25-29, 53-57. Hind limb paralysis was observed as follows: 2/6 at 200 mg/kg qd x 3, 6/6 at 400 mg/kg 1x/1wk, 6/6 at 400 mg/kg 1x/2wk, 2/6 at 400 mg/kg 1x/3wk.
Doxil has a reduced tendency to accumulate in myocardium (four times lower in heart than in tumor tissue in tumor bearing nude mice), possibly because liposomes do not easily cross the continuous capillaries of myocardial tissue (3). In humans with breast cancer or non-small cell lung cancer, peak doxorubicin concentration in tumors was 4 to 16 fold greater after treatment with Doxil than after the same dose of free doxorubicin (20). In cardiotoxicity studies using the isolated perfused heart model, with i.v. injection every other day for 11 days, both HMR 1826 and Doxil were largely devoid of cardiotoxicity. HMR 1826 appeared about 100-fold less cardiotoxic than doxorubicin (19).

Doxil (3, 11, 21-27) and HMR 1826 (5, 6, 18) exhibit superiority to free doxorubicin in several human tumor xenograft / nude mouse models. However, there are no studies reported that directly compare the efficacy of these two strategies. In the study reported here, we compare the antitumor efficacy of different doses and schedules of HMR 1826 against human tumor xenografts to select an optimized dose and schedule and then compare the antitumor efficacy of HMR 1826 with that of Doxil and free doxorubicin in subcutaneous nude mouse models of seven tumor lines.

Materials and Methods

Materials. HMR 1826, synthesized at Hoechst Marion Roussel, was dissolved in 5% mannitol and diluted into the required concentrations prior to use. Liposomal doxorubicin, manufactured by Alza (Palo Alto CA) was purchased from Besse Medical Supply (Cincinnati, OH). Doxorubicin was purchased from Sigma Chemical Company (St. Louis, MO) for the MDA-MB-231, PC-3 and HCT-15 studies, or preformulated from Besse Medical Supply for the renal and pancreatic xenograft studies.

Animals and Tumor implantation. MDA-MB-231, PC-3 and HCT-15 tumors: Tumors were grown in athymic nude mice (Harlan). MDA-MB-231 and HCT-15 tumors were grown in female mice, PC-3 in male mice. 1 - 2 mm³ tumor pieces were implanted subcutaneously using a trocar needle. Mice were 5 - 6 weeks of age at the time of implant.

Pancreatic and renal tumors: Two pancreatic carcinoma cell lines, Panc-1 and BxPC-3 and two renal carcinoma cell lines, SN12C and ACHN, were used in the study. The cell lines were cultured in vitro according to the protocol provided by ATCC. Suspended cells were injected into the subcutaneous space of nude mice (athymic CD-1 nude, Charles River Laboratories, Wilmington, MA) with each mouse receiving 5 × 10⁶ cells. Stock tumors were harvested in log phase. Viable tissues were cut into small fragments of 1 mm³ each prior to subsequent implantation into mice 4 - 5 weeks of age. Animals were anesthetized with isoflurane prior to surgery. The left flank was disinfected with iodine and alcohol and a small incision was made on the skin. 8-10 fragments of the stock tumor tissue were inserted into the subcutaneous sac. The incision was closed with a 6-0 silk surgical suture.

Study design. Animals without tumor takes as well as those with exceedingly small or large tumors were excluded from the study. The study animals were selected to establish groups of similar mean tumor size as determined by caliper measurement. Groups for each of the treatment conditions were randomly chosen, with 6 animals per group for the MDA-MB-231, PC-3 and HCT-15 tumors, or 10 animals per group for the pancreatic and renal tumors. HMR 1826, Doxil and free doxorubicin were tested side-by-side against control. The dosing schedules chosen for Doxil (9 mg/kg, once a week) and free doxorubicin (7 mg/kg, once a week) have been reported to be optimal for these compounds in nude mouse xenograft models (11, 28). Drug administration by i.v. tail vein injection was begun when the tumors reached 100-150 mm³ in volume. Treatment schedules for the HMR 1826 optimization studies were as described in the results section. Experiments were terminated at various time points, based on the length of the observation period and the rate of tumor growth.

Data Collection and Analysis. During the course of the study, tumor size was assessed once or twice a week, depending on growth rate, by measuring the perpendicular minor (a) and major (b) dimensions using sliding calipers. Tumor volume was calculated by the formula (a² × b) × 1/2. Body weight was determined with an electronic balance at the time of tumor measurement. Tumor weight was measured at autopsy. Student’s 3-sided t-test was used to analyze tumor and animal body weights.

Results

Dose and Schedule Optimization. Studies to optimize the dose and schedule of HMR 1826 were carried out using mice bearing MDA-MB-231 xenografts (Figure 1). No significant antitumor effect was observed at 70 days with any of the
Figure 3. Effect of HMR 1826 (200 mg/kg), Doxil (9 mg/kg) and doxorubicin (7 mg/kg) on the growth of MDA-MB-231 (a), PC-3 (b) and HCT-15 (c) xenografts in nude mice. Tumor implantation and drug treatment as described in Materials and Methods. n=6 per group. Data presented are the tumor volumes measured at day 35 (MDA-MB-231) or day 61 (PC-3 and HCT-15) after tumor implantation.
schedules at 50 mg/kg (Figure 1a). A dose dependent effect was observed with all of the schedules beginning at 100 mg/kg (Figure 1b) and continuing through 200 mg/kg (Figure 1c) and 400 mg/kg (Figure 1d). Examination of the treated animals revealed the presence of hind limb paralysis at 400 mg/kg with all of the schedules and at 200 mg/kg with the qd x 5 schedule (indicated in the legends to Figure 1). Results similar to those obtained with the MDA-MB-231 xenografts were seen when mice bearing PC-3 xenografts were treated with HMR 1826 at the same doses and schedules (data not shown), with hind limb paralysis seen with all schedules at 400 mg/kg. A study with HCT-15 xenografts, comparing 1x/wk at 200 mg/kg with several more frequent dosing schedules (Figure 2) showed that the 1x/wk schedule was as efficacious as more frequent dosing schedules, except for the qd x 5 schedule, which had slightly higher efficacy. Since the purpose of these experiments was to find the maximal effective and safe dose of HMR 1826 for further studies, the 400 mg/kg dose and the 200 mg/kg dose at qd x 5 were eliminated from further consideration, due to the appearance of hind limb paralysis.

The data from the above studies indicated that the 200 mg/kg 1x/wk dose and schedule combined the properties of maximal total dose, greatest efficacy and consistent lack of side effects. Since 1x/wk administration at 7 mg/kg (doxorubicin) or 7 - 9 mg/kg (doxil) have been reported to be optimal, safe schedules for treatment with these compounds (11, 28), selection of the 1x/wk schedule for HMR 1826 also allowed comparison of the three drugs at the same schedule.

**Effects of HMR 1826, Doxil and Doxorubicin on MDA-MB-231, PC-3 and HCT-15 tumors.** Comparison of the effect of free doxorubicin, Doxil and HMR 1826 on the growth of MDA-MB-231, PC-3 and HCT-15 tumor xenografts is shown in Figure 3. For MDA-MB-231 (Figure 3a), all schedules of Doxil and HMR 1826 exhibited similar, significant efficacy. The efficacy of free doxorubicin was indistinguishable from the untreated control. For PC-3 (Figure 3b), doxil at 1x/wk, doxil at 2x/wk and HMR 1826 at 1x/2wk were the most efficacious, with doxorubicin at 1x/wk or 1x/2wk and HMR 1826 at 1x/wk being slightly less effective. The apparent decrease in efficacy of the 1x/wk HMR 1826 treatment is likely due to the fact that two of the tumors appeared to escape the effect of the drug in this particular study (data not shown). For HCT-15 xenografts (Figure 3c), doxorubicin and HMR 1826 at 1x/wk showed similar efficacy.
Effect of HMR 1826, Doxil and Doxorubicin on Pancreatic and Renal Tumors. The efficacy of HMR 1826 at 200 mg/kg was statistically significant vs. the untreated controls in all four of the tumor lines (Table I and Figure 4). In all four of the models, there was some difference in average tumor size among the HMR 1826, Doxil and doxorubicin treatments, but none of the differences were statistically significant vs. HMR 1826 (p > 0.05). The in vitro IC50s for the cell lines, measured by MTT assay, are 0.060 µg/ml (Panc-1), 0.26 µg/ml (BxPC-3), 1.5 µg/ml (SN12C) and 0.010 µg/ml (ACHN) (data not shown).

At the time when the body weight loss in each group reached the lowest point during the experiment, HMR 1826 had caused less significant body weight loss than Doxil at the chosen doses (Table I and Figure 5). The differences were statistically significant in all cases. In the two renal models, body weights in the HMR 1826 treated groups were also significantly better than those in the free doxorubicin treated group. Statistically-significant differences in body weight between the HMR 1826 treated groups and the controls were not observed in any of the four studies.

Discussion

The ability to target cytotoxic agents to tumor tissue can enhance the effectiveness of cytotoxic agents by increasing the amount of drug taken up by the tumor (leading to an enhanced cytotoxic effect), while decreasing the amount of drug absorbed by normal tissues (leading to a decrease in toxicity to those tissues). HMR 1826 and Doxil both demonstrate these properties (3-5, 8, 10, 16-20). This report compares the antitumor efficacy of doxorubicin, Doxil and HMR 1826 in seven different xenograft models: HCT-15 (colon), MDA-MB-231 (breast), PC-3 (prostate), SN12C and ACHN (renal) and BXPC3 and Panc-1 (pancreas). The doses for the three drugs were selected to deliver the maximum amount of drug and achieve the best efficacy, while maintaining an acceptable toxicity profile. The results demonstrate that HMR 1826 at 200 mg/kg has antitumor efficacy equivalent to Doxil and free doxorubicin in the models and doses tested. The relative efficacy of the three drugs varied depending on the tumor line, but the differences were not statistically significant. The one exception was the MDA-MB-231 tumor (Figure 3a), where Doxil and HMR 1826 were much more effective than free doxorubicin.

The fact that HMR 1826 achieves efficacy equivalent to that of Doxil with less drug-induced weight loss, even though the equivalent dose of doxorubicin delivered with 200 mg/kg HMR 1826 is 120 mg/kg (well above the toxic dose) supports the utility of the β-glucuronidase-activated prodrug approach. Earlier studies (5) reported that HMR 1826 was superior to free doxorubicin in some xenograft models of lung, colon,
breast, ovary and stomach cancer. These studies clearly established the efficacy of the β-glucuronidase-activated prodrug approach for HMR 1826, but they compared doxorubicin at submaximal doses (3.5 and 4.0 mg/kg) with HMR 1826 at a dose above that found in this study to induce hindlimb paralysis (300-400 mg/kg).

The variability of relative efficacy of the three compounds in different tumor xenograft models is not surprising, since Doxil and HMR 1826 rely on different tumor properties for uptake or activation. The enhanced pharmacological profile of Doxil is believed to be due to enhanced t1/2 and enhanced tumor uptake. The t1/2 of Doxil in humans is on the order of days (10, 29, 30). The preferential accumulation of Doxil in tumors has been explained by the fact that tumor vasculature is often leaky and/or badly formed and the liposomal particles, which circulate for a prolonged time, can extravasate into the tumor (4, 8). The antitumor activity of HMR 1826 relies on the presence of extracellular β-glucuronidase in the tumor. Since the extracellular β-glucuronidase appears to come primarily from granulocytes and macrophages and is most frequently associated with necrotic regions (5), the amount of macrophage infiltration and the extent of necrosis will have an impact on the prodrug cleavage and efficacy of HMR 1826. Similarly, the efficacy of Doxil could be affected by the condition of the tumor vasculature, which can vary among tumor lines. In this regard, the improved efficacy of HMR 1826 and Doxil compared to free doxorubicin in MDA-MB-231 tumors (Figure 3a) is notable, since in our hands, MDA-MB-231 subcutaneous xenografts exhibit a particularly high degree of necrosis and a high level of the vascular permeablizing factor VEGF/VPF (unpublished observations). The observations that HMR 1826 is also effective in a rat adjuvant arthritis model (31) and in a mouse DTH model (32) (unpublished results) also support the role of the local host inflammatory component as the mediator for HMR 1826 activation. A more specific evaluation of the contribution of these factors will require correlation of vascular leakage and extracellular β-glucuronidase levels with antitumor efficacy in specific tumors.

A potential issue for administration of HMR 1826 to patients is the high dose required to achieve efficacy. The 200 mg/kg dose in mice has the potential to release up to 120 mg/kg of free doxorubicin - an acutely toxic concentration. The high dose required is due to the fact the only a small percentage of the total dose of the prodrug is cleaved within the tumor. The high dose HMR 1826 required could present a problem for patients with an inflammatory condition that included release of β-glucuronidase. However, HMR 1826 could provide an advantage in patients with a high inflammatory or necrotic component in their tumors. An approach that has been investigated to further increase the utility of HMR 1826 involves an ADEPT strategy. Smaller tumor metastases will most likely not contain sufficient necrosis and inflammation to contain enough extracellular β-glucuronidase to activate the HMR 1826 prodrug. The utility of an ADEPT strategy for targeting HMR 1826 has been demonstrated, using a carcinoembryonic antigen (CEA) - β-
glucuronidase fusion protein to target the enzyme to tumor tissue (14, 33-35).

References


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