Sex differences in improved efficacy of doxorubicin chemotherapy in *Cbr1+/−* mice

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The anthracycline chemotherapeutic agent doxorubicin is converted by the enzyme carbonyl reductase 1 (CBR1) into its cardiotoxic metabolite doxorubicinol. *Cbr1+/−* mice have been shown to be protected from doxorubicin-induced cardiotoxicity, and the inhibition of CBR1 activity may be a useful means of ameliorating the side effects of doxorubicin in patients undergoing chemotherapy. Because reduced conversion to doxorubicinol increases circulating levels of the more effective parent drug doxorubicin, it was hypothesized that therapeutic efficacy against tumors might also be enhanced. *Cbr1+/−* mice were bred to mice transgenic for the polyomavirus middle T antigen (*PyVT*) to create offspring with palpable mammary tumors. Latency to initial tumor formation was similar in *Cbr1+/−* and *Cbr1+/+* animals. Tumor regression was improved in *Cbr1+/−* animals, but only in male mice. Western blotting showed a marked sex difference in protein levels, with a much higher expression of Cbr1 in the female kidney and liver. Thus, the combined effects of a naturally low expression and the heterozygous *Cbr1* null allele seem to have enhanced tumor regression in *Cbr1+/−* males. Future efforts to design a clinical CBR1 inhibitor to protect patients from the cardiac side effects of doxorubicin treatment should evaluate the effect of sex on anticancer efficacy. *Anti-Cancer Drugs* 23:584–589 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Doxorubicin (Adriamycin) is a common anthracycline chemotherapeutic used to treat a wide variety of cancers including breast and esophageal carcinomas, osteosarcomas, Kaposi's sarcomas, and soft tissue sarcomas, and Hodgkin's and non-Hodgkin's lymphomas [1]. The antineoplastic effects of doxorubicin have been linked to topoisomerase II inhibition, DNA intercalation, RNA synthesis inhibition, the creation of free radicals, and iron chelation [1–7]. Doxorubicin is converted by the enzyme carbonyl reductase 1 (human CBR1 or mouse Cbr1) into its metabolite, doxorubicinol [8,9]. CBR1 is a member of the short-chain dehydrogenase/reductase family that reduces a wide range of carbonyl substrates to alcohols [9]. CBR1 is expressed in almost all tissues, with a higher expression in both the liver and the kidney [10], and is the primary reducer of doxorubicin in the liver [11,12].

The parent drug doxorubicin is primarily responsible for chemotherapeutic efficacy. Doxorubicin has been shown to have 5–50-fold higher anticancer effect compared with the doxorubicinol metabolite, depending on the tumor type [4,13]. Doxorubicin efficacy is inversely correlated with Cbr1 levels in hepatocarcinomas *in vitro* [14].

In addition, the metabolite doxorubicinol causes cardiac toxicity [1,13,15]. Attempts to attenuate this potentially life-threatening side effect have focused on many different aspects of doxorubicin and its pharmacology, including coadministration of antioxidants, structural alterations, and various biochemical inhibitors [2,5,16–21]. Although many of these potential solutions have been successful *in vitro*, few treatments have progressed past animal modeling, and there is still a dire clinical need.

Modification of the metabolism of doxorubicin has been shown to prevent doxorubicinol-induced cardiotoxicity. Removing one allele of *Cbr1* in mice, and therefore decreasing conversion to doxorubicinol, markedly prevents cardiac damage. In fact, heterozygous *Cbr1+/−* mice treated with doxorubicin have heart parameters indistinguishable from saline-treated controls [22].

Therefore, the inhibition of CBR1 is a potential method for preventing cardiac damage as well as improving therapeutic efficacy in cancer patients by increasing circulating levels of the parent drug doxorubicin. The MMTV-*PyVT* mouse, transgenic for the polyomavirus middle T antigen, is a mammary tumor model that can be used to assess tumor regression rates [23]. The polyomavirus middle T antigen activates *src* tyrosine kinases and downstream signal transduction pathways [24].

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Expression of the *PyVT* oncogene triggers four stages in the development of mammary tumors: hyperplasia, adenoma/mammary intraepithelial neoplasia, early carcinoma, and late carcinoma. These four stages can be compared with classifications ranging from benign to invasive in human breast cancer [25].

We hypothesized that tumors in MMTV-*PyVT* mice that were heterozygous for the *Cbr1* null allele would have improved tumor regression due to the reduced conversion of doxorubicin to doxorubicinol. Here, we report that this improved efficacy appears to be dependent on the animals' sex.

Methods

Animals

Cbr1+/− mice [22] were obtained from R. Reeves at Johns Hopkins University School of Medicine (Baltimore, Maryland, USA). Heterozygotes were used because *Cbr1*−/− homozygotes are embryonic lethal. *Cbr1*+/− mice were maintained on two separate genetic backgrounds by mating them to C57B16/J (Jackson Labs, Bar Harbor, Maine, USA) or 129SVE mice (Taconic Labs, Hudson, New York, USA). Mice with the *PyVT* oncogene were obtained from the Mouse Models of Human Cancers Consortium (MMHCC, Bethesda, Maryland, USA) and maintained on an FVB genetic background (Jackson Labs). Thus, the genetic background of offspring was either F1(C57Bl6/JxFVB) or F1(129SVExFVB). Mice from both of these F1 backgrounds were combined for this analysis in order to achieve sufficient sample size. Mice were genotyped for the null *Cbr1* allele as in Olson *et al.* [22] and for the *PyVT* allele according to the MMHCC website. The number of animals in each experimental group is shown in Table 1. The protocol was prospectively approved by the Institutional Animal Care and Use Committee.

Offspring with the *PyVT* oncogene were assessed for tumor formation by palpation twice weekly. Once tumors were noted, tumor volume was estimated daily by caliper measurement using the equation: tumor volume = $(L \times W^2)/2$ [26]. Once the total tumor volume reached at least 200 mm^3, 2 mg/kg doxorubicin was injected intraperitoneally daily for 9 days [26]. Tumor volume was then monitored daily during treatment and post-treatment. The initial protocol included 5 days of post-treatment monitoring. Because preliminary results showed a post-treatment period effect, we extended the monitoring to 15 days post-treatment or when the total tumor burden reached approximately 1000 mm^3, in which case the animal was euthanized to prevent distress.

Table 1 Sample sizes applicable to Figs 1–3

	Males	Females
Cbr1+/+	17	22
Cbr1+/−	18	22

Western blotting

One wild-type and *Cbr1*+/− animal of each sex and of C57Bl6/J and 129SVE backgrounds was killed by cervical dislocation to allow for immediate removal of organs. Protein was extracted with standard methods using nonidet P-40 buffer and a protease inhibitor cocktail (Sigma, St Louis, Missouri, USA). SDS polyacrylamide gel electrophoresis was performed with 10 μg protein per well as calculated from a Bradford assay and the gels were blotted onto nitrocellulose membranes (BioRad, Hercules, California, USA) using standard protocols [27]. Rabbit IgG anti-HCbr monoclonal primary antibody (donated by Dr Akira Hara) was used at a 1 : 10 000 dilution in Tris-buffered saline with 0.1% Tween. The secondary antibody was horseradish peroxidase-linked antirabbit immunoglobulin from donkey (GE Healthcare, Chalfont St Giles, UK). Blots were visualized using the Amersham ECL Plus western blotting detection system (GE Healthcare). Membranes were then reprobed with a control anti-α-tubulin mouse primary antibody (Calbiochem, San Diego, California, USA) and horseradish peroxidase-linked antimouse immunoglobulin from sheep (GE Healthcare) as a secondary antibody.

The developed film was scanned into a digital format and analyzed by optical densitometry using the Labworks (Ultraviolet Products, Upland, California, USA) program. Measurements were made using the total density minus background as determined by a standard optical curve in optical density units and normalized by tubulin control values. The Cbr1/tubulin ratios were then compared between sexes, setting the female value as 100%.

Statistics

Data were entered into Microsoft Excel (Redmond, Washington, USA) spreadsheets. Data management, including double key entry comparisons, was carried out using SAS 9.2 (Cary, North Carolina, USA) and all analyses and plotting were performed in R 2.13.1 (*http://www.r-project.org/*).

Tumor latency was defined as age at first tumor detection. Latency was analyzed both graphically (boxplots and Kaplan–Meier plots) and numerically (Cox proportional hazards regression).

Tumor response to doxorubicin treatment over time was visualized with locally weighted scatterplot smoothing (LOESS) [28] using approximate 95% confidence bounds. LOESS is a nonparametric scatter plot smoother used to observe general trends in data that are not easily characterized by simple functions. Both normal residual and bootstrap confidence bounds for the LOESS curves were generated. As the two sets of confidence bounds appeared to be similar, those generated using the normality assumption are presented. Percent of the total tumor volume on the first day of treatment (day 0) was used to correct for the between-animal differences seen in initial tumor volume. Because the sample size at later time points is small, due partly to an extension of the

protocol and partly to tumor burdens requiring euthanasia to prevent distress (see the Animals section), the LOESS curves should be interpreted cautiously at these later time points.

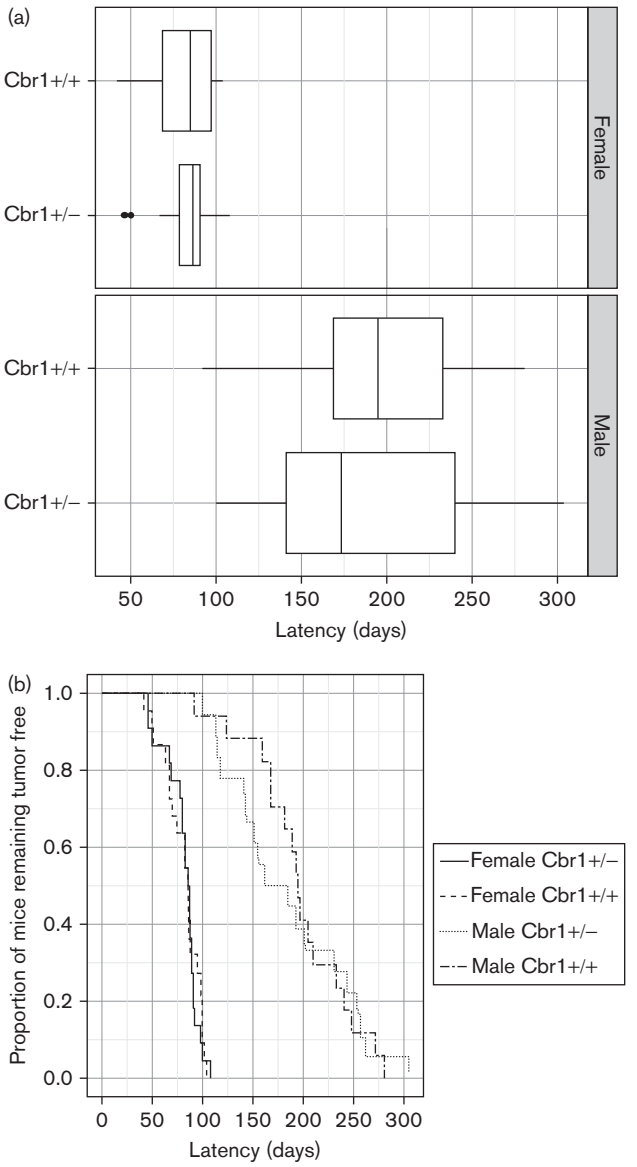
Results

Cbr1+/– mice were bred to *PyVT* mice to generate tumor-susceptible offspring with either a wild-type or a heterozygous Cbr1 expression. Tumor latency (age at first tumor detection) was much lower in females than in males, with a median of 85.5 days [95% confidence interval (82, 89)] in females and 192.0 days [95% confidence interval (168, 231)] in males, as expected due to the high levels of circulating estrogen in females [29]. Cox regression confirmed this highly significant effect of sex on tumor latency ($P < 0.001$; Fig. 1). There was no statistically significant interaction effect between sex and genotype ($P = 0.94$), and the *Cbr1* genotype alone did not significantly affect tumor latency ($P = 0.69$; Fig. 1).

Once the total tumor volume reached at least 200 mm^3, mice were treated with 2 mg/kg doxorubicin by an intraperitoneal injection for 9 consecutive days. Tumor regression was measured daily during the treatment period and for varying time frames following the treatment period (see the Methods section). The trend in total tumor volume as a percent of tumor volume on the initial day of treatment (day 0) was plotted using LOESS with 95% confidence bounds. Although later time points are a less reliable indicator of treatment effect due to a small sample size (see the Methods section), the lack of an overlap of the confidence bounds is consistent with improved regression in male *Cbr1*+/– mice compared with male *Cbr1*+/+ mice (Fig. 2). Tumor regression in females did not appear to be influenced by the *Cbr1* genotype. As an alternative way to view these data, we plotted the male versus female tumor response (Fig. 3). The *Cbr1* + /– genotype appears to have a synergistic effect with sex for the enhanced tumor regression in males compared with females.

To examine this sex difference, we analyzed the expression of Cbr1 by western blot in the liver and kidney (Fig. 4 and Supplementary Table 1). In wild-type mice, males expressed only 11% of the female protein level in the liver; even more markedly, males expressed only 0.3% of the female level in the kidney. This sex difference appeared similar in heterozygous *Cbr1*+/– mice, although it could not be quantified because the expression in males was too close to the background optical density levels.

Fig. 1

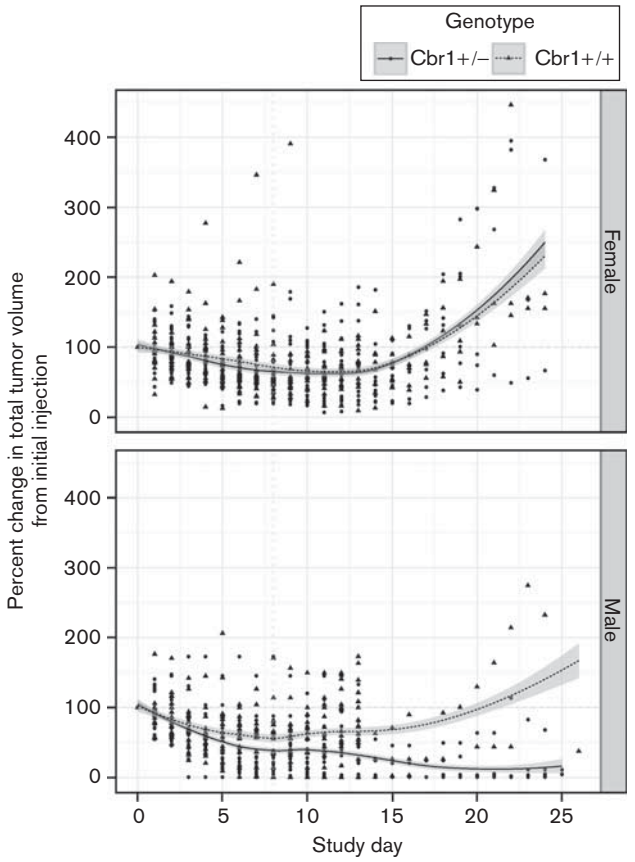


Cbr1+/– mice have unchanged latency to form mammary tumors. Mice were palpated twice a week to detect tumor formation. (a) Boxplot depicting latency in days from birth to first tumor detection separated by sex and genotype. The vertical black line is the median, the box represents the interquartile range (the range of the middle 50% of observations), and the points outside of the whiskers are possible outliers. (b) Kaplan–Meier curves of latency in days from birth to first tumor detection separated by sex and genotype. Cox regression indicates that, after correcting for a significant sex difference ($P<0.0001$), genotype is not a significant predictor ($P=0.69$) of time to tumor development. CBR1, carbonyl reductase 1.

Discussion

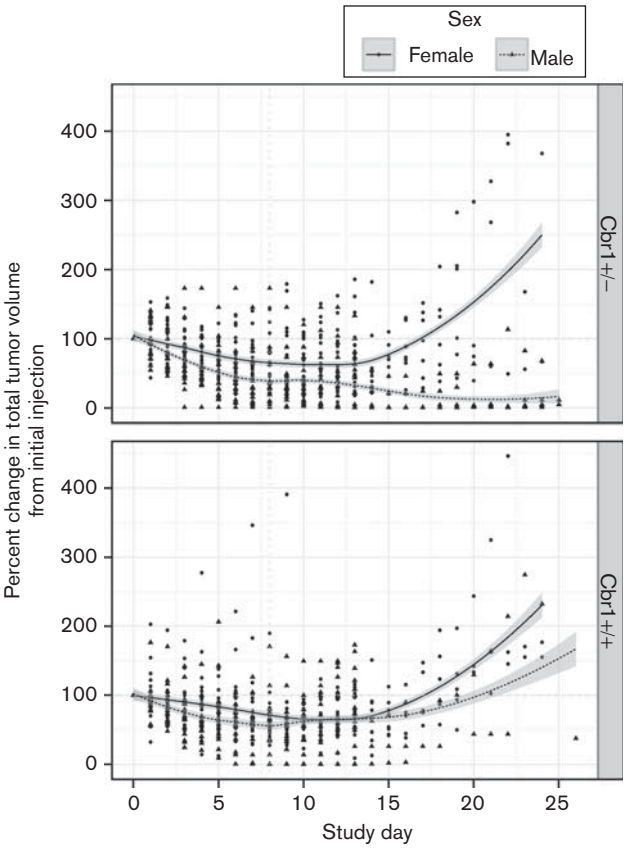
Previous work [22,30,31] has suggested the development of a pharmacological inhibitor of the doxorubicin-reducing enzyme, CBR1, in order to prevent the cardiotoxicity associated with the metabolism of doxorubicin to doxorubicinol. We aimed to investigate whether such an inhibition of metabolism (as modeled in a *Cbr1*+/– mouse) would affect drug efficacy in tumor regression. Our results show that *PyVT*-induced mammary tumors have an enhanced response to doxorubicin treatment in male *Cbr1*+/– mice. Males may be particularly sensitive to the loss of a single *Cbr1* allele because of their naturally occurring low levels of expression of Cbr1. In contrast, the higher basal levels of Cbr1 in female mice seem to

Fig. 2



Genotype comparison of locally weighted scatter plot smoothing (LOESS) of total tumor volume trend over time with approximate 95% confidence bounds (shaded). Triangles indicate *Cbr1+/+* observations; circles are *Cbr1+/−* observations. On days 0–8, mice were treated with 2 mg/kg doxorubicin and tumor volume decreased; the lack of an overlap of the confidence bounds is consistent with enhanced tumor regression in *Cbr1+/−* male mice. Because of different protocols, mice were monitored post-treatment for varying time intervals on days 9–25 (see the Methods section) to evaluate tumor regrowth. Although LOESS trend lines should be compared cautiously due to small sample sizes at later time points (particularly ≥ day 20 in females and ≥ day 14 in males), note that male *Cbr1+/−* mice are the only group to show a lack of return of the tumor volume to the baseline level. CBR1, carbonyl reductase 1.

Fig. 3



Sex comparison of locally weighted scatter plot smoothing (LOESS) of total tumor volume trend over time with approximate 95% confidence bounds (shaded). Triangles indicate male observations; circles are female observations. Particularly in the post-treatment period days 10–14, with a reliable number of observations (see Fig. 2 legend), the enhanced antitumor effect in males is only seen in *Cbr1+/−* animals. CBR1, carbonyl reductase 1.

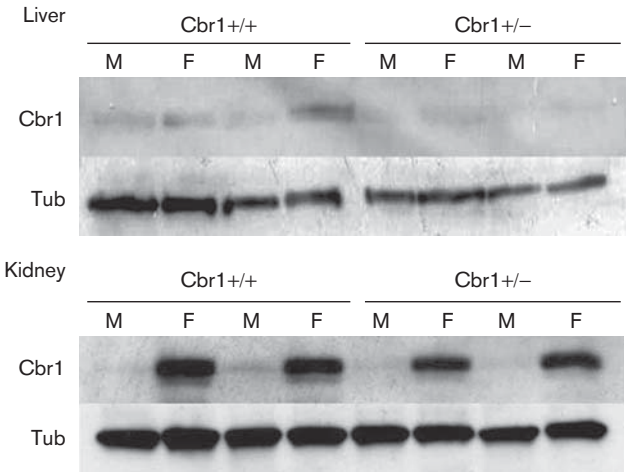
prevent the heterozygous state from conferring an enhanced doxorubicin response.

To our knowledge, a sex-specific difference in Cbr1 expression has not been previously reported. However, clinical studies have shown that doxorubicin-induced cardiotoxicity is more common in females than in males, which would be consistent with an elevated level of CBR1 metabolism and increased doxorubicinol formation [2,7,32]. The liver and the kidney are particularly important in the clearance and metabolism of doxorubicin and doxorubicinol [33], and Cbr1 has been shown to be the primary reducer of doxorubicin in the liver [11]. We predict that the markedly low expression of Cbr1 in males compared with females would impact plasma levels of doxorubicin.

It is also possible that the enhanced antitumor effect in males may be related to the increased doxorubicin-induced lipid peroxidation in males compared with females seen over 25 years ago [34], as Cbr1 protects cells from lipid peroxidation [14]. Differences in doxorubicin efficacy on the basis of sex have been shown in a mouse model of T-cell lymphoma [35], and it would be interesting to see whether this sexual dimorphism is intensified in a *Cbr1+/−* state as in our model. In addition, the observation of sex differences in doxorubicin treatment of both lymphoma and mammary tumors suggests that the phenomenon may be generalizable across tumor types.

Therefore, it is possible that the combined effect of the low basal expression of *Cbr1* in males and the heterozygous null *Cbr1* allele resulted in enhanced chemotherapeutic efficacy. This supports the continued attempts to find a pharmacological inhibitor of Cbr1 to accompany doxorubicin treatment. Multiple groups have identified potential inhibitors of Cbr1, including hydroxyl-PP [14,36], the

Fig. 4



Males express substantially less Cbr1 protein in the liver and kidney. Tubulin serves as a loading control for the western blot. The reduced expression in *Cbr1+/–* heterozygotes is expected; the difference between males and females has not been previously published, to our knowledge. Optical density quantitation can be found in Supplementary Table 1. CBR1, carbonyl reductase 1; F, female; M, male; Tub, tubulin.

flavonoid 7-monohydroxythylrutoside [37], a zearalenone analog [38], indazole-dione derivatives [39], and (–)-epigallocatechin gallate [31], several of which have been shown to increase doxorubicin cytotoxic efficacy *in vitro*.

Our data, showing the lack of *Cbr1* effect on tumor latency, also support the goal of creating a CBR1 inhibitor. Because several studies [40–43] have associated reduced CBR1 expression to increased cancer progression (unrelated to doxorubicin treatment), it will be important to balance this phenomenon by modulating doxorubicin metabolism. We have demonstrated that removing one allele of *Cbr1* does not alter the basal tumor formation rate in *PyVT* mice.

CBR1 and the highly conserved paralog CBR3 have been recent subjects of pharmacogenetic analyses [44–51], with several single-nucleotide polymorphisms being reported as having an effect on doxorubicin metabolism. Work aiming to tailor cancer treatment on the basis of polymorphisms in these genes or by administering pharmacologic inhibitors of the enzymes should include a robust evaluation of the effect of sex on doxorubicin efficacy.

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Conflicts of interest

There are no conflicts of interest.

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