Tirapazamine Administered as a Neoadjuvant to Radiotherapy Reduces Metastatic Dissemination

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Abstract

Purpose: The level of hypoxia in primary tumors has been linked both clinically and experimentally to the incidence of metastases. This study was designed to address the effect of selectively targeting hypoxic cells in primary tumors on subsequent presentation of metastasis.

Experimental Design: The murine KHT model was used as a reproducible temporal and spatial onset of metastases is revealed following treatment of primary ($\sim$400 mm^3) s.c. tumors with a 25 Gy radiation dose. The bioreductive drugs tirapazamine and RB6145 were administered in multiple doses before radiotherapy.

Results: Fractionated treatment with both tirapazamine and RB6145 significantly reduced the hypoxic fraction of the primary tumor, as assessed by pimonidazole binding, and had no effect on the overall growth rate of the primary tumor. Excision assays showed an increased level of cell kill in tirapazamine-treated versus RB6145-treated tumors consistent with tirapazamine targeting hypoxic cells at a broader range of oxygen tensions than RB6145. Tirapazamine treatment significantly reduced the presentation of metastases following radiotherapy (P = 0.003 versus saline controls) whereas RB6145 had no effect. Local control rates increased from 20% to 32% and 50% when radiation was combined with RB6145 and tirapazamine, respectively.

Conclusions: These data provide direct evidence that selective targeting of hypoxic cells in primary tumors is a viable approach in the control of metastatic disease. The enhanced efficacy of tirapazamine versus RB6145 suggests that the radioresistant cells at intermediate oxygen tensions, conducive to targeting with tirapazamine but not with the more stringent bioreductive RB6145, predominate in terms of linking primary tumor hypoxia and metastases.

Metastatic disease is responsible for the majority of cancer-related deaths and has been linked both clinically and experimentally to the extent and severity of hypoxia within the primary tumor (1–3). The causal basis for this link was originally thought to be the refractive nature of hypoxic cells to common anticancer treatment modalities (4–10). A modification of this initial hypothesis had to be made in light of the observations of Hockel et al. (7) who found that hypoxia was of equal prognostic significance for overall and disease-free survival for patients treated with either radiotherapy with curative intent or with surgery. Of particular relevance in the latter treatment group was the finding that eight of thirteen recurrences from hypoxic primary tumors had distant metastases compared with only two of seven recurrences in the non-hypoxic primary tumor group. The apparent association between hypoxia and aggressive disease phenotype observed clinically is supported by various experimental studies (1, 11, 12). In this work, we describe experiments designed to eliminate the burden of hypoxic cells in primary tumors using bioreductive drugs and determine the effect of this treatment on the subsequent presentation of metastatic disease. The hypoxia selectivity of bioreductive agents is achieved because low oxygen tensions facilitate enzymatic bioactivation required to yield the cytotoxic products. In the presence of oxygen, futile cycling occurs whereby the cytotoxin is back-oxidized to the parental drug. In the present study, two bioreductive agents have been used, RB6145 and tirapazamine (SR4223). These two drugs were selected on the basis of their differing oxygen concentration dependence for toxicity. RB6145 is a brominated less emetic prodrug form of RSU1069 (13, 14). It is a 2-nitroimidazole with a potentially alkylating aziridine group as its active species and requires oxygen levels below 0.1% to elicit a cytotoxic response (14, 15). In contrast, the aromatic di-*N*-oxide tirapazamine becomes increasingly cytotoxic with decreasing levels of oxygen (15). The reduced stringency of tirapazamine in terms of hypoxic dependence enables this drug to target cells that would be considered to be at intermediate oxygen concentrations (0.1-1.5%) in a radiobiology context. The C3H syngeneic KHT murine sarcoma was selected for these studies because spontaneous lung metastases can be revealed following controlling radiotherapy of primary s.c. KHT tumors.

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These metastases that occur in the lungs appear in a reproducible temporal and spatial manner. In addition, previous studies have established the presence of hypoxic tumor cells within s.c. KHT tumors and that metastatic dissemination to the lung can occur in a manner related to the oxygenation status of the primary tumor (16, 17).

Materials and Methods

Cell line maintenance. The KHT murine sarcoma used in this study was originally provided by Dr. P. Twentyman (Medical Research Council, Cambridge, United Kingdom). Viable tumor pieces of ~2 mm^2 in size were implanted s.c. onto the back of female C3H/He mice, ages 9 to 12 weeks, to initiate primary *in vivo* stocks. Viably frozen pieces of these stocks were disaggregated and passaged twice *in vitro* using RPMI 1640 (GibcoBRL, Paisley, United Kingdom) supplemented with 10% FCS (LabTech International, Ringmer, United Kingdom) and 2 mmol/L glutamine (Invitrogen Life Technologies, Paisley, United Kingdom). This yielded sufficient cells for each of the documented experiments. In each case, the piece used to generate the cells was derived from the same KHT tumor.

Bioreductive agents. Both tirapazamine and RB6145 were synthesized in-house according to published protocols (18, 19). The nuclear magnetic resonance data (^{1}H-NMR, ^{13}C-NMR) for tirapazamine and RB6145 were identical to those in the literature. The purity of the compounds was determined using microanalytic techniques. The concordance between the observed and expected microanalytic data suggests that the compounds were pharmacologically pure [tirapazamine ($C_7H_6N_4O_2$)—expected: C, 47.19; H, 3.39; N, 31.45%; found: C, 46.97; H, 3.26; N, 31.61%; RB6145 ($C_7H_{11}BrN_4O_2 \cdot HBr \cdot H_2O$)—expected: C, 22.24; H, 3.73; N, 14.28%; found: C, 22.31; H, 3.81; N, 14.43%].

Tumor initiation and treatment regimens. Tumors were initiated on the back of female C3H/He mice by the s.c. injection of 5×10^5 KHT cells in a 0.1 mL volume and mice were monitored daily. Once palpable, tumors were measured using callipers and volume was estimated by multiplying the length, breadth, and height measurements. For the metastasis experiments, 12 mice were implanted per treatment group. The experiment was repeated thrice for saline and tirapazamine treatments and twice for RB6145. Bioreductive drugs were administered in a fractionated protocol via i.p. injection of a 0.1 mL volume per 10 g body weight. RB6145 was used at 75 mg kg^{-1} and tirapazamine at 13 mg kg^{-1}. Both drugs were administered every 12 hours for a total of eight doses. Control mice received 0.9% saline. In the experiments combining radiation and bioreductive drug treatment, radiotherapy was administered the day after the final drug dose. Radiotherapy was delivered as a single dose of 25 Gy under ambient conditions to non-anesthetized tumor-bearing mice restrained in lead-shielded containers. The experimental end point was a moribund appearance and labored breathing in 50% of the control saline-treated mice. Drug only growth delay experiments were initiated using six mice per group and were terminated when a s.c. tumor volume of 1,000 mm3 was achieved. Excision assays were done following single drug exposures at the maximum tolerated dose (50 mg kg^{-1} for tirapazamine and 250 mg kg^{-1} for RB6145) using established methods (20). Briefly, tumors were excised 18 hours after treatment, minced, agitated at 37°C in 0.2% trypsin and 0.05% DNase in PBS, and filtered through 35 μm mesh to produce a single cell suspension. This was diluted and plated in 0.3% agar/medium overlaid onto a 0.5% agar/medium base layer. All procedures were carried out in accordance with the Scientific Procedures Act 1986 by approved protocols (Home Office Project license number 40-1770) following Institutional guidelines.

Evaluation of tumor hypoxia and vascularity. Pimonidazole (Chemicon International, Inc., CA) was administered at a dose of 60 mg kg^{-1} (i.p.) 16 hours after the completion of fractionated drug or saline treatment. Tumors were excised 2 hours later (n = 3 per treatment group). Sections of 5 μm were taken from formalin-fixed paraffin-embedded samples at the maximal circumference of the excised tumor. Hypoxyprobe-1 monoclonal antibody (Chemicon International) and the mouse Envision kit (DAKO Ltd., Ely, United Kingdom) were used according to the guidelines of the manufacturer to disclose pimonidazole adduct formation. Sections were counterstained using Gill's haematoxylin. A semiquantitative analysis of pimonidazole staining was undertaken. Sections representing the whole tumor area were scanned (6.3× objective) and the extent and intensity of pimonidazole staining scored in each individual field of view. The extent of binding was scored 1 to 4, representing 0% to 20%, 20% to 40%, 40% to 60%, and 60% to 80% stained area, respectively. Intensity was scored as 1 to 3, with 3 representing intense staining. The two values were multiplied to give a score for each field of view. Generally, five fields of view covered the whole tumor section. The average for all fields of view was determined and taken as the final score for each individual tumor section. At least three sections were analyzed per tumor. Scores were obtained first by S.J.L. and then the assessment was repeated by K.J.W. to ascertain the reproducibility of the grading system used. The effect of bioreductive drug treatment on vascularity was evaluated histopathologically by R.J.F. in serial sections stained with H&E.

Scoring of metastases. For the analysis of metastatic burden, lungs were excised and fixed in Bouin's solution (2.5% in 0.9% saline) to disclose tumor. The lungs were awarded a grade of 0 to 5 depending on the severity of metastasis. Grade 0 indicated that there were no visible metastases; grade 1 indicated a single lesion of less than 1 mm3; grades 2 to 5 were given for lung metastastic burdens that approximated to 10% to 20%, 20% to 40%, 40% to 80%, and >80% of the lung surface, respectively. Metastatic burden was assessed first by S.J.L. and then a random series of 50 specimens was reanalyzed by K.J.W. using the same scoring variables to assess intraindividual variability.

Statistical analyses. Spearman's rank correlations were used to evaluate the reproducibility of the scoring methods used to evaluate both the extent of tumor hypoxia and metastatic burden. Concordance was tested using Bland-Altman plots. Highly significant correlations were revealed between the pimonidazole binding (r = 0.94, P = 0.0002) and metastatic burden (r = 0.88, $P < 0.0001$) scores obtained by the two independent observers (S.J.L. and K.J.W.). Excellent concordance was also shown with Bland-Altman plot equations of $y = -0.0014\ x - 0.5443$ (R^2 = 0.00006) and $y = 0.0134\ x - 0.3194$ (R^2 = 0.0028) for the pimonidazole and metastatic burden scores, respectively. The significance of the effect of bioreductive drug treatment on the metastatic burden was evaluated using Mann Whitney U tests unless stated otherwise. Data cited within the text are mean values ± SE.

Results

Fractionated bioreductive drug treatment alone has no effect on primary tumor growth and vascularity but reduces the hypoxic fraction. Mice bearing s.c. KHT tumors were treated with eight drug fractions at 12-hour intervals and tumor volume was monitored daily. The average volume of the tumors at the start of treatment was 100 ± 8 mm^3 (saline), 100 ± 5 mm^3 (tirapazamine), and 94 ± 8 mm^3 (RB6145). The growth profile of tumors treated with either tirapazamine or RB6145 was similar to that observed in control tumors treated with saline (Fig. 1). A small effect on tumor growth was apparent for tirapazamine-treated tumors but this did not achieve statistical significance in terms of tumor volume relative to saline-treated controls at the point of excision of the latter group (778 ± 49 versus 966 ± 49 mm^3, $P > 0.05$). To evaluate whether drug treatment effectively reduced overall hypoxia within the s.c. tumor, the hypoxic cell marker pimonidazole was given 16 hours after the final drug dose. Tumors were excised 2 hours later and pimonidazole adduct formation evaluated by immunohistochemistry. In this

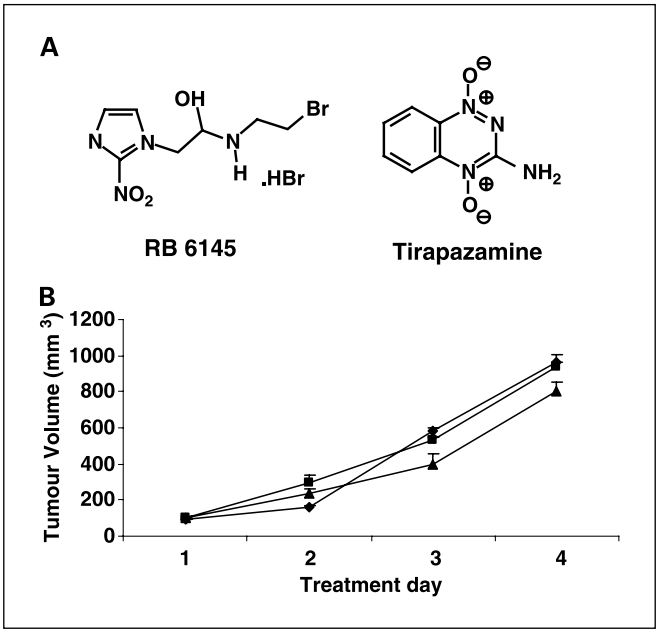

Fig. 1. *A,* chemical structures of the bioreductive drugs RB6145 and tirapazamine. *B,* fractionated bioreductive drug treatment has no effect on the growth of s.c. KHT tumors. Drug treatment was initiated on treatment day 1. Tirapazamine (▲), RB6145 (■), or saline (◆) was administered every 12 hours over a 4-day period (eight doses in total). Tirapazamine and RB6145 were administered at 13 and 75 mg kg^{-1}, respectively. *Points,* average values (n = 6 per group); *bars,* SE.

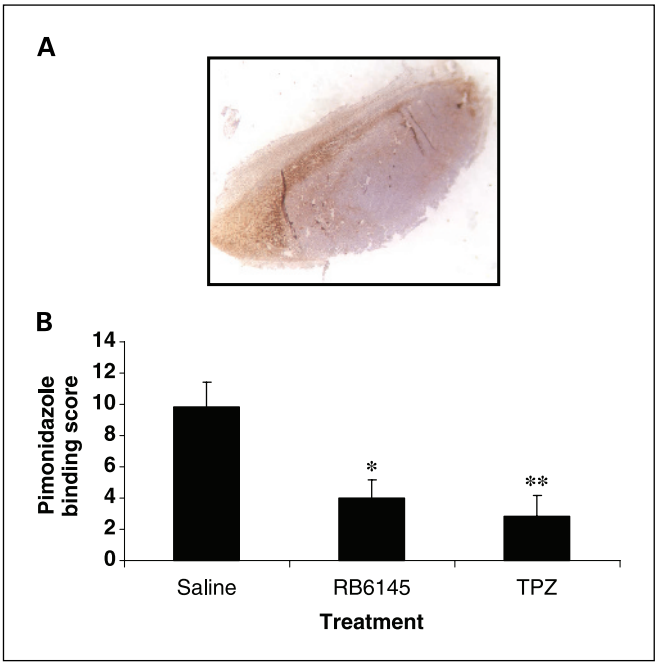

Fig. 2. Fractionated bioreductive drug treatment significantly reduces the hypoxic fraction of s.c. KHT tumors. Pimonidazole (60 mg kg^{-1}) was administered 16 hours after the completion of saline, RB6145 (75 mg kg^{-1}), or tirapazamine (*TPZ*, 13 mg kg^{-1}) treatment (eight doses at 12-hour intervals). Tumors were excised 2 hours later and pimonidazole adduct formation was evaluated by immunohistochemistry. *A,* representative example of pimonidazole binding that was scored 5 using the semiquantitative scoring system detailed in Materials and Methods. *B,* pimonidazole scores following fractionated saline, RB6145, or tirapazamine treatment (±SD, n = 3 per group). *, P = 0.02; **, P = 0.001, versus saline controls (paired t test).

series (n = 3 per treatment group), the size at excision was 533 ± 150 mm^3 (saline), 400 ± 54 mm^3 (tirapazamine), and 396 ± 170 mm^3 (RB6145). Saline-treated control s.c. KHT tumors were found to show intense pimonidazole staining covering 60% to 80% of the tumor section area. The pimonidazole binding score (see Materials and Methods for scoring details) was 9.85 ± 0.9 for these tumors This was significantly reduced by pretreatment with either tirapazamine or RB6145 (Fig. 2; binding scores of 2.8 ± 0.8 and 4 ± 0.7, respectively, $P < 0.05$, paired t test). There was no significant correlation between tumor size and pimonidazole binding score (r = 0.1, P = 0.8, n = 9). Assessment of vascularity in serial sections of these tumors revealed no differences associated with the individual treatments. Capillary density was increased around the margins of focal necrosis and ranged from 5/mm^2 to 15/mm^2 within each of the treatment groups.

Excision assays revealed a greater proportion of cells were killed following tirapazamine treatment compared with that following RB6145 when drugs were administered at their maximum tolerated doses (50 and 250 mg kg^{-1}, respectively). Values of the surviving fraction of cells derived from tumors excised 24 hours after single drug doses were 0.43 for tirapazamine and 0.73 for RB6145 (n = 4 per group).[3] This is consistent with the ability of tirapazamine to kill hypoxic cells at a broader range of oxygen tensions than RB6145.

Treatment of KHT subcutaneous tumors with tirapazamine before radiation reduces subsequent metastatic burden. Fractionated tirapazamine or RB6145 treatment and radiotherapy were given according to the schedule depicted in Fig. 3A. Drug treatment was initiated when tumors achieved a volume of 60 to 80 mm^3. Controls received saline. Tumor sizes at radiation treatment were 334 ± 17 mm^3 (saline), 362 ± 18 mm^3 (tirapazamine), and 287 ± 12 mm^3 (RB6145). The experiment was terminated when 50% of the control animals were moribund. At this point, the average size of the irradiated primary tumors was 225 mm^3 ± 57 (SE n = 35) for saline controls, and 7 of 35 showed no signs of regrowth. The average sizes of both the tirapazamine-treated and RB6145-treated primary tumors were significantly reduced compared with saline-treated controls (tirapazamine, 51 ± 13 mm^3, n = 35; RB6145, 81 ± 19 mm^3, n = 22; P = 0.004 and 0.05 versus saline, respectively). There was no significant difference in the residual primary tumor burden between tirapazamine-treated and RB6145-treated groups ($P > 0.1$). No primary tumor regrowth was evident in 18 of 35 tirapazamine-treated and 7 of 22 RB6145-treated tumors. Residual primary tumor volume bore no significant correlation with tumor size at the time of radiation although a weak inverse relationship was observed in the saline-treated group (saline, $r = -0.28$, P = 0.1; tirapazamine, r = 0.12, P = 0.5; RB6145, $r = -0.04$, P = 0.9).

Lung metastatic burden was graded 0 to 5 depending on the severity of the visible metastases. The images in Fig. 3B are representative examples of excised lungs presenting with each metastatic grade. Tirapazamine significantly reduced the grade of metastases presented (P = 0.003 versus saline; Figs. 4 and 5). When data were combined from three independent experiments (two for RB6145; Fig. 5), 60% of tirapazamine-treated mice presented with grade 2 or lower metastases as opposed to

[3] S. Cole and H.S. Edwards, unpublished observations.

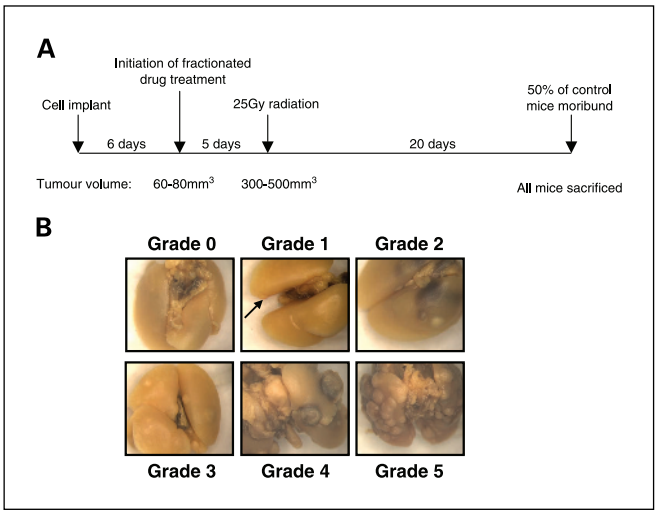

Fig. 3. *A,* schematic representation of the treatment schedule used. Fractionated drug treatment was initiated ~6 days post-implantation at a size of 60 to 80 mm^3. Tirapazamine (13 mg kg^{-1}), RB6145 (75 mg kg^{-1}), or saline was administered every 12 hours for 4 days (eight doses in total). Radiotherapy (25 Gy) was given the day after the completion of the fractionated drug treatment to restrain primary s.c. tumor growth. Mice were monitored for evidence of the onset of lung metastases (labored breathing, weight loss). When 50% of saline-treated control mice had a moribund appearance (~20 days post-radiotherapy), the experiment was terminated. Lungs were excised and fixed in Bouin's solution before grading according to the scoring system detailed in Materials and Methods. *B,* representative images of metastasis-bearing lungs of each grade.

the saline and RB6145 groups at 34% and 36%, respectively. Fourteen percent of all tirapazamine-treated mice had no visible lung metastases and the proportion of mice presenting with grade 5 metastastic disease was less than 10% compared with 49% and 45% for saline-treated and RB6145-treated animals, respectively. There was a weak positive correlation between residual primary tumor size and metastatic grade for

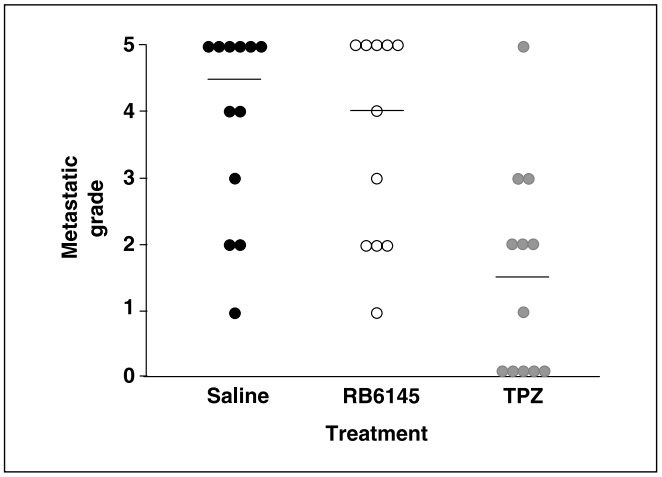

Fig. 4. Tirapazamine treatment before radiotherapy significantly reduces subsequent presentation of metastases. Mice bearing s.c. KHT tumors received eight doses of saline, tirapazamine (13 mg kg^{-1}), or RB6145 (75 mg kg^{-1}) administered at 12 -hour intervals. This treatment was followed a day later by 25 Gy of localized radiotherapy. Upon a moribund appearance in 50% of the control animals that received saline, the experiment was ended and lungs were excised, fixed, and scored for metastatic burden. Data from a single experiment; each symbol represents the metastatic grade obtained for the lungs of an individual mouse. *Bar,* median metastatic grade for each treatment group.

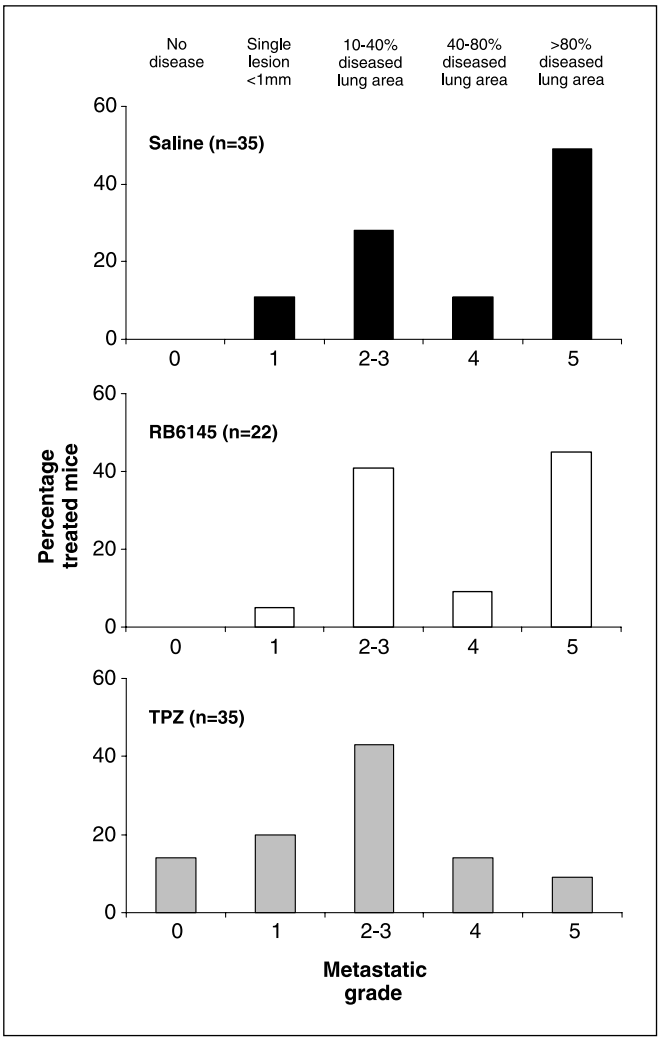

Fig. 5. Combined data for three individual experiments (two for RB6145) showing the effect of bioreductive drug treatment on the frequency distribution of metastatic grade. KHT tumor–bearing mice received tirapazamine (13 mg kg^{-1}; *shaded columns*), RB6145 (75 mg kg^{-1}; *open columns*), or saline (*closed columns*) as eight fractionated doses followed by radiotherapy (25 Gy) 1 day later. Lung metastases were evaluated ~20 days later when 50% of saline-treated mice were moribund in appearance. In each experiment, 12 mice were assigned to each treatment group. Loss of condition that was not attributable to metastases was evident in one saline-treated, one tirapazamine-treated, and two RB6145-treated mice and these were removed from the experiment. The distribution of the metastatic grades obtained is given as a percentage of the mice treated within each group. Descriptions of the metastatic presentation in each grade are given above the histograms.

the saline-treated group ($r = 0.28$) but this did not achieve statistical significance ($P > 0.1$). No correlations were observed for the tirapazamine-treated ($r = -0.17$, $P > 0.3$) and RB6145-treated groups ($r = -0.16$, $P > 0.5$).

Discussion

In this study, we have shown that the bioreductive drug tirapazamine can be used as a successful neoadjuvant to radiotherapy in the control of metastatic disease. Although both tirapazamine and RB6145 reduced the primary tumor burden to the same extent when used in conjunction with radiotherapy, only tirapazamine was efficacious in reducing metastatic spread. This suggests that the more effective local

control of the primary tumor is not the mechanism by which tirapazamine exerts its antimetastatic effect. The difference in efficacy of tirapazamine versus RB6145 as neoadjuvants to radiotherapy may therefore lie in the precise hypoxic population that the two drugs target within the primary tumor mass. Considering the properties of tirapazamine activity (15), cells at intermediate levels of oxygen, which are resistant to the more stringent bioreductive drug RB6145/RSU1069 but still at sufficiently low oxygen concentrations to be resistant to radiation, may be predominantly responsible for the connection between primary tumor hypoxia and metastatic progression. The finding that tirapazamine treatment reduced to a greater extent than RB6145/RSU1069 the tumor fraction able to bind pimonidazole, further supports the contention that tirapazamine targets cells at a broader range of oxygen tensions *in vivo*. Pimonidazole forms adducts at oxygen concentrations of $\sim$1.5% (product information, Chemicon International) and below, giving an oxygen-dependent binding profile that mirrors that of tirapazamine cytotoxicity. The presence of a subpopulation of hypoxic KHT tumor cells that could be targeted only by tirapazamine was evinced from the excision assays showing a greater level of cell kill with tirapazamine compared with that observed following RB6145 treatment.

The suggestion that primary tumor cells at intermediate oxygen concentrations are causatively linked with metastases is supported by a previous study (1) in which mice bearing KHT tumors were subjected to repeated rounds of 5% to 7% O_2 breathing (12×10 minutes per day for 7 days). This treatment resulted in tumor pO_2 values that fluctuated within the intermediate range conducive to tirapazamine but not RB6145/RSU1069 targeting. An increased incidence of lung micrometastases was observed compared with air breathing or the induction of prolonged chronic hypoxia ($pO_2 < 1$ mm Hg induced by 2 hours of 5% to 7% O_2 breathing per day for 7 days). Mechanistically, recent observations have suggested that hypoxic exposure can increase the metastatic potential of KHT cells through an acquired resistance to stress-induced cell death (21). Although exhaustive studies were not undertaken, as we did not observe any gross modification in vascularity following bioreductive drug treatment, our data are more supportive of a mechanism by which tirapazamine directly targets a tumor population with an inherently enhanced metastatic potential rather than having modified the potential exit points for metastatic dissemination.

Overall, we have provided evidence that selective targeting of hypoxic cells pre-radiotherapy can have profound effects on metastatic progression. By using bioreductive drugs with diverse oxygen dependencies, we have been able to show subtle differences in the nature of the hypoxic fraction, which contribute to metastatic dissemination. These observations may prove useful in the future preclinical and clinical development of bioreductive agents for use in the control of metastatic disease.

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