Advances in Brief

Synergistic Interaction between Tirapazamine and Cyclophosphamide in Human Breast Cancer Xenografts

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Abstract

This study examined the efficacy of combining cyclophosphamide and the hypoxic cytotoxin, tirapazamine, in the treatment of human breast cancer xenografts grown in nude mice. A single dose of tirapazamine was followed 2 h later by a single dose of cyclophosphamide. As determined from tumor regrowth delay, the effectiveness of combined therapy was greater than the additive effects of each treatment given alone. Possible mechanisms of this synergistic interaction include enhancement of DNA damage, inhibition of repair of DNA damage, or induction of apoptosis. Apart from some loss in body weight, the only other toxicity of interest in mice treated with tirapazamine was necrosis of the skin on the distal tail, which appeared to be vascular in origin.

Introduction

Tirapazamine (also known as SR 4233) is a benzoazaine di-N-oxide that has been shown to be selectively toxic to hypoxic cells (1). Severe hypoxia is not required, and the level of hypoxia found in many human tumors (<10–20 mm Hg) is sufficient for toxicity (2). Tirapazamine has been shown to interact with radiation (both fractionated external beam radiation and radioimmunotherapy) to enhance tumor cell killing (3–5). Multiple doses of tirapazamine are thought to be necessary to allow reestablishment of the hypoxic fraction between doses, thus allowing more than the initial fraction of hypoxic cells to be killed by tirapazamine. However, recent studies in murine tumors using tirapazamine combined with cisplatin or alkylating agents, using a single dose of each agent, have resulted in significantly enhanced toxicity over either agent administered alone (6, 7). Because only a single dose was given, this enhanced toxicity cannot be explained by the same mechanism as suggested for fractionated radiotherapy. This paper presents the results of combining tirapazamine with cyclophosphamide in human breast cancer xenografts grown in nude mice.

Materials and Methods

Cell Line. The cell line used for this preliminary study was MDA-MB468, a human breast adenocarcinoma that is known to grow in nude mice. It was obtained from the American Type Culture Collection and was maintained in Dulbecco’s modified Eagle’s medium with 10% iron supplemented bovine serum. It was originally established in 1977 from a pleural effusion in a patient who had received 5-fluorouracil, doxorubicin, and CTX® until 3 months prior to obtaining the tumor specimen (8). The cell line is estrogen receptor negative, who had received 5-fluorouracil, doxorubicin, and CTX® until 3 months prior to obtaining the tumor specimen (8). The cell line is estrogen receptor negative,

Clonogenic Assay. Tumors were excised under sterile conditions, weighed, minced in a solution of PBS, and centrifuged gently. The slurry was then resuspended in an enzyme cocktail (0.02% DNase, 0.025% Pronase, and 0.025% collagenase in PBS) and incubated with mixing for 1 h at 37°C. The mixture was then passed through a stainless steel mesh (80 holes/cm2), centrifuged, and resuspended in fresh complete medium. Cells were plated to yield 25 to 100 colonies per plate, with replicates for each treatment group, so that 9–18 plates were evaluated. Cell counts were performed with a hemocytometer, and only trypan blue-excluding cells were counted. A feeder layer of lethally irradiated cells (1 × 106 cells/60-mm dish) was plated before adding the treated cells. After 21 d incubation, the colonies were fixed and stained with crystal violet, and colonies with more than 50 cells were scored. Corrections were made for multiplicity when necessary. Surviving fractions were calculated relative to untreated controls.

Results

Tumor Growth Delay. Tumors in mice treated with PBS or tirapazamine alone continued to grow at the same rate. Tumors treated with CTX or CTX plus tirapazamine decreased in size to 45% and 23% of their original mean volumes, respectively, by 3 weeks after treat-
TIRAPAZAMINE AND CTX IN BREAST CANCER XENOGRAFTS

Fig. 1. Response of MDA-MB468 tumors to SR 4233 treatment combined with cyclophosphamide. Tumor volume divided by the volume on the day treatment started (V/V₀) is plotted as a function of time after treatment. Bars, SEM.

Fig. 2. Examples of mild (A) and severe (B) degrees of tail toxicity 2 weeks after tirapazamine administration.

Table 1 VDT and tumor growth delay relative to PBS-treated controls

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>VDT ± SEM (days)</th>
<th>Tumor growth delay (days)</th>
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<tbody>
<tr>
<td>Control (PBS)</td>
<td>21.6 ± 3.6</td>
<td>0</td>
</tr>
<tr>
<td>Tirapazamine alone</td>
<td>20.2 ± 2.5</td>
<td>-1.4</td>
</tr>
<tr>
<td>Cyclophosphamide alone</td>
<td>52.1 ± 3.3</td>
<td>30.5</td>
</tr>
<tr>
<td>Tirapazamine plus cyclophosphamide</td>
<td>62.8 ± 4.0</td>
<td>41.2</td>
</tr>
</tbody>
</table>

ment, at which time the tumors started to regrow (Fig. 1). Mean VDT ± SEM are given in Table 1. The differences in the mean VDT among the four treatment groups was greater than would be expected by chance (P < 0.001). After performing all pairwise comparisons, the only pair that was not significantly different (P > 0.05) was the PBS versus tirapazamine comparison. Tumor growth delays relative to PBS-treated controls, calculated from the VDTs, are also given in Table 1. From these data, the extra growth delay achieved by combined treatment over that predicted from the additive effect of each treatment given alone is 12.1 days. The final slope of the growth curve for combined treatment is steeper than that for the other three groups, suggesting that there may have been selection of a more aggressive cell population by therapy. However these slope differences were not statistically significant.

Normal Tissue Toxicity. At this high single dose of tirapazamine (68% of the 50% lethal dose), there was weight loss to 92 and 88% of initial body weight for tirapazamine alone and combined therapy, respectively, but no lethality. Maximum weight loss after CTX administration was to 95% of initial body weight. Maximum weight loss occurred 7 days after treatment, and all mice resumed a normal growth rate after this.

An unexpected toxicity that occurred in this study and has not been reported previously was damage to the skin of the tail. This occurred only in the mice treated with tirapazamine. Beginning approximately 1 week after treatment, punctate reddish lesions appeared on the distal tail. Many of these went on to form small ulcers associated with mild swelling and, in a few mice, frank necrosis of the distal tail occurred with loss of this portion of the tail (Fig. 2). At no time did the mice appear systemically ill as a result of these lesions. In most of the mice, the lesions resolved by 8 weeks after treatment. Histological analysis at the end of the experiment was dominated by a chronic inflammatory response which prevented determination of the underlying lesion.

In a subsequent study, we will perform histological analysis as soon as the lesions appear.

The tail lesions that occurred in the tirapazamine-treated mice appeared to be a vascular event, presumably at the arteriolar level. It is not known what oxygen concentrations are present in mouse tails. No lesions were seen in the ears or toes. A subsequent study using tirapazamine from a completely different source had the same effect on the tails. We have used lower doses of tirapazamine (up to 0.12 mmol/kg/dose) given in 10 daily doses and have not seen this toxicity at these dose levels. A subsequent dose-response analysis suggested that the lesions occur only within a narrow dose range because a dose of 62.7 mg/kg (9% higher than in this study) produced a lower incidence and severity of lesions. Other similar bioreductive agents (pyrazine mono-N-oxides) have been reported to have vasoactive properties, but visible lesions were not reported (9). If this effect is found in animals other than nude mice, it may be important to examine the possible role of vascular effects in tumor and normal tissue toxicity.

Clonogenic Assay. The number of clonogenic cells relative to untreated controls 24 h after treatment for tirapazamine and tirapazamine plus CTX were 0.66 and 0.061, respectively. We do not have data for CTX alone. The estimates of surviving fractions from the regressions on the data in Fig. 1 were 0.20, 1.1, and 0.046 for CTX, tirapazamine, and combined treatment, respectively. These results are comparable to those found by clonogenic assay.
Discussion

This study demonstrates the enhanced efficacy of single doses of tirapazamine combined with CTX in human breast cancer xenografts grown in nude mice compared with either agent given alone. Because tirapazamine is bioreductively activated, it is likely that it was cytotoxic primarily to hypoxic cells in these tumors (pO₂ < 20 mm Hg; Ref. 2). Oxygen electrode studies in human tumor xenografts and in breast cancers in humans have shown that a significant fraction of tumor cells are within this O₂ tension range (10, 11). We do not have data on O₂ tension for the small tumors used in this study.

Assuming that bioreduction was responsible for the drug interaction described here, a mechanistic explanation of this phenomenon must take into account the known chemical and biological properties of reduced tirapazamine. Cell death following exposure to tirapazamine has been attributed to DNA dsb caused by an oxidizing one-electron reduction species produced under hypoxia (12–14). It has been suggested that the ability of the reduced drug to oxidize pyrimidine residues could cause DNA damage in cells (15). These mechanistic ideas are consistent with observations of DNA single strand breaks and dsb in hypoxic cells exposed to tirapazamine. In addition, the finding of a correlation between the rate of drug metabolism by cellular reductases and dsb repair capability (14) supports the hypothesis that chromosome breaks may account for lethality to hypoxic cells. Evidence has been presented that these dsb are more refractory to repair than those caused by ionizing radiation, suggesting structural differences resistant to enzymatic repair activity (16).

Previous analysis has dealt with a synergistic interaction reported for tirapazamine and radiation (3, 4). In a theoretical study of this phenomenon, it was assumed that radiation killed mainly normoxic cells and that the drug killed hypoxic cells (17). The increased effectiveness of the combined treatment was attributed to the reestablishment of hypoxia after each cycle of therapy, with subsequent elimination of the new hypoxic fraction of cells by tirapazamine. Thus, the drug killed more than the initial hypoxic fraction of cells. However, this mechanism cannot explain the synergistic cytotoxicity of a single dose of tirapazamine and chemotherapy reported here.

The results of this study demonstrate that CTX, like cisplatin and some alkylating chemotherapy agents (6, 7), can synergize with tirapazamine cytotoxicity in vivo. Because the damage to cellular macromolecules associated with exposure to these drug molecules is different from that caused by tirapazamine, the basis of this synergism is not clear. However, some hypotheses can be advanced. The importance of delaying the administration of cisplatin after tirapazamine exposure (7) suggests that the efficiency of DNA adduct or cross-link repair can be compromised by the damage caused by the bioreductive drug. This hypothesis is reasonable if it is assumed that the number of drug-induced chromosomal breaks increases with time and eventually inhibits repair processes requiring genetic recombination, such as cross-link repair. Another possibility is that the DNA damage produced by tirapazamine may somehow make the DNA more accessible to subsequent attack. It is also conceivable that the activation of G₁ or G₂ checkpoints in a subpopulation of tumor cells damaged by tirapazamine and then exposed to another cytotoxic agent may initiate some form of programmed cell death (apoptosis). Evidence of this cytotoxic mechanism has been reported using rodent cell lines arrested for prolonged times in G₂/M following treatment with cisplatin and other agents (18). Tirapazamine has been observed to cause a significant enhancement over untreated controls of a G₂/M arrest in hypoxic human LS174T colon adenocarcinoma spheroids, although a direct role in cell death has not been established (4). It is worth mentioning that tirapazamine aerobic toxicity, perhaps arising from redox cycling, may contribute to the drug interaction.

Drug-resistant cell lines may be useful for investigating the mechanism of this interaction. For example, it was reported that exposure of a cisplatin-resistant human breast cancer cell line to tirapazamine under hypoxic conditions eliminated resistance to cisplatin given later under aerobic conditions (19). It has been suggested that increased levels of cellular sulfhydryl compounds such as glutathione or reduced drug uptake could account for cisplatin resistance in these cells (20). Because tirapazamine has not been observed to deplete glutathione levels (13), it probably alters cisplatin resistance in these cells by another mechanism. Thus, drug-resistant cell lines can be used to guide further study of the ability of tirapazamine to chemosensitize experimental tumors. Ultimately, this drug could provide a means to overcome some forms of clinically derived drug resistance.

References

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