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Activity of TER286 against human tumor colony-forming units

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Glutathione S-transferase (GST) isozymes have been shown to be elevated in many human cancer types as compared to normal tissues. TER286, one in a class of glutathione-based GST-activated cytotoxins, was tested in a soft agar cloning assay to determine its *in vitro* activity against primary human tumor colony-forming units. Breast and lung specimens from patients who had received prior therapy and those who were previously untreated were exposed to TER286 at concentrations of 1, 10 and 50 μM using both 1 h and continuous exposures. Overall *in vitro* responses (50% or less survival compared to untreated controls) were observed in 0% (0/14), 14% (2/14) and 29% (4/14), respectively, in specimens exposed to TER286 for 1 h, and in 5% (2/41), 10% (4/41) and 61% (25/41), respectively, in specimens exposed to TER286 continuously. TER286 has cytotoxic activity against both breast and lung cancer colony-forming units, and demonstrates a concentration–response effect. At 50 μM, there is a significant difference between 1 h and continuous exposures in head-to-head comparisons. These data suggest that TER286 can be activated in human tumor colony-forming units and should be pursued as a treatment candidate for patients whose tumors are resistant to drug treatment based on up-regulation of GST.

Key words: Cancer, cytotoxicity, glutathione S-transferase, soft agar cloning, TER286.

Introduction

Glutathione S-transferases (GSTs) are a group of cytosolic enzymes which catalyze the conjugation of electrophilic xenobiotics with the scavenging peptide glutathione (GSH) via the free sulfhydryl group of GSH. The resulting conjugates are normally metabolized to mercapturates and excreted, generally leading to a detoxification. The human cytosolic GSTs belong to a supergene family comprised of at least four multigene classes: α, μ, π and θ. These isoenzymes are found in normal and malignant cells, but an increase in GST activity has been noted in cancerous areas of tissue compared with matched normal tissue.[1] Overexpression of GSTs in tumor cells is a common occurrence[2,3] and is generally believed to be part of the multifactorial process of tumor resistance to many chemotherapeutic drugs.[4]

Recently, alkylating agents which are catalytically activated by GSTs have been reported.[5] An example of such a compound is TER286, a GSH derivative designed to release a phosphorodiamidate moiety containing two bis(chlorethyl)amines when metabolized by GSTs.[6] The phosphorodiamidate moiety is the presumed alkylating agent.[7] Identification and targeting of cancers with increased GST levels may be useful as an indicator for the selection of treatment. Chemotherapeutics, such as TER286, being activated by GSTs, would be useful in clinical situations where the conventional drugs are normally detoxified by GSTs.[8]

We have undertaken cytotoxicity studies of TER286 utilizing the human tumor cloning system. This system, initiated by Hamburger and Salmon,[9,10] was originally utilized to select the most appropriate anticancer agent for an individual patient's tumor. From several encouraging *in vitro/in vivo* correlations, it became obvious that the cloning system might be utilized to screen for antitumor activity of new compounds.[11–15] In the present study, breast and lung specimens were taken directly from patients who had received prior therapy as well as those who were previously untreated. These human tumors were exposed to TER286 *in vitro* and the respone (less than 50% survival compared to untreated controls) was assessed by quantitating the formation of human tumor colony-forming units. The aim of the study was to determine if TER286 has cytotoxic activity against breast and lung cancer colony-forming units.

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Materials and methods

After obtaining informed consent in accordance with federal and institutional guidelines, malignant effusions, ascites and bone marrow aspirates containing tumor cells, as well as solid tumor specimens were collected from patients undergoing procedures done as part of their diagnostic work-up or as part of treatment for their disease. No samples were obtained solely for research purposes. Solid tumors or lymph nodes were minced into 2–5 mm fragments in the operating room and immediately placed in McCoy's Medium 5A plus 10% heat inactivated newborn calf serum and 1% penicillin/streptomycin. Within 4 h, these solid tumors were mechanically disassociated with scissors, forced through no. 100 stainless steel mesh, through 25 gauge needles and then washed with McCoy's medium as previously described.[10–15] Ascitic, pleural, pericardial fluids and bone marrows were obtained by standard techniques. The fluid or marrow was placed in sterile containers containing 10 units of preservative-free heparin per ml of malignant fluid or marrow. After centrifugation at 150 *g* for 10 min, the cells were harvested and washed with McCoy's plus 10% heat inactivated fetal calf serum. The viability of cell suspensions was determined on a hemocytometer with Trypan blue.

Purified TER286 (see Figure 1) provided by Terrapin Technologies, Inc. (South San Francisco, CA) was diluted to create concentrated stocks. One-half milliliter (0.5 ml) aliquots of each stock solution were labeled and stored at −70°C; aliquots were thawed for each new tumor sample tested. The final concentrations tested were 1, 10 and 50 μM at both 1 h and continuous exposures.

For the 1 h exposure studies, the cells were incubated for 1 h with TER286 in McCoy's medium, then washed to simulate the disappearance of the drug from the body. Cells were suspended in 0.3% agar in enriched CMRL 1066 supplemented with 15% heat-inactivated horse serum, penicillin (100 units/ml), streptomycin (2 mg/ml), glutamine (2 mM), insulin (3 units/ml), asparagine (0.6 mg/ml) and HEPES buffer (2 mM). Cells were plated in 35 mm Petri dishes in a top layer of agar over an underlayer of 0.3% agar to prevent growth of fibroblasts. Three plates were prepared for each data point. For the continuous exposure, cells were combined with TER286 in the CMRL medium described above, then plated as for the 1 h exposure. It should be noted that the 2 week exposure only means that the compound was not washed from the culture during the 2 week incubation period (additional drug was not added to the culture). It is not known how long TER286 is stable in this test system, but it is estimated to have a half-life of less than 5 h. The plates were placed in a 37°C incubator and removed on day 14 for counting of the number of colonies in each plate. The number of colonies (defined as more than 50 cells) formed in the three compound-treated plates were compared to the number of colonies formed in the three untreated control plates and the percent colonies surviving at each concentration was calculated.

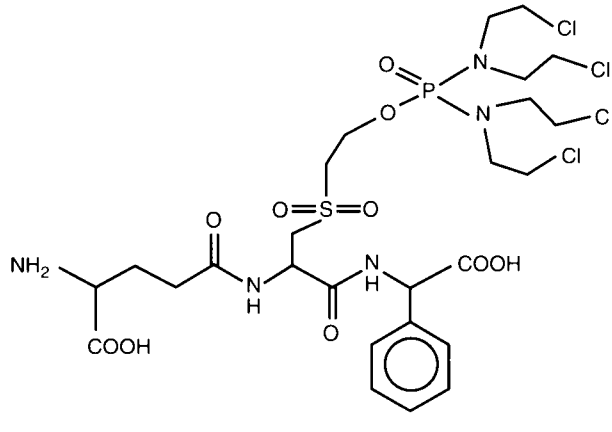

Figure 1. Chemical structure for TER286.

To assure the presence of an excellent single-cell suspension on the day of plating, positive controls were used. For each tumor tissue sample tested, three positive control plates were set up to contain the cell poison orthosodium vanadate at 200 μg/ml. This positive control agent (orthosodium vanadate) should destroy all clonogenic cells. If there was no effect of positive control on colony formation, then the single cell suspension on day 0 was poor (since orthosodium vanadate does not affect clumps) and the tumor sample test was considered non-evaluable.

A test is defined as an experiment, performed on a unique tumor tissue sample, that contains untreated control, positive control and three specified compound concentration levels. An evaluable test is one having an average of 20 or more colonies present on day 14 in the untreated control plates and less than 30% survival in the positive control (orthosodium vanadate) plates when compared to untreated control plates.

Results

Breast and lung specimens were obtained from patients who had received prior therapy as well as

from those who were previously untreated. A total of 80 specimens were tested and 51% were evaluable. The group of 41 evaluable human tumors tested with TER286 consisted of breast tumors with prior therapy ($n = 10$), breast tumors with no prior therapy ($n = 10$), non-small cell lung cancers with prior therapy ($n = 10$), non-small cell lung cancers with no prior therapy ($n = 10$) and one small cell lung tumor with no prior therapy. Fourteen specimens in this group were tested at 1 h exposure. All 41 specimens were exposed to TER286 at concentrations of 1, 10 and 50 μM using continuous exposures. Results with the different histologic types are shown on Table 1. Overall *in vitro* responses (50% or lower survival compared to untreated controls) were observed in 0% (0/14), 14% (2/14) and 29% (4/14) of specimens exposed to TER286 for 1 h, respectively, and in 5% (2/41), 10% (4/41) and 61% (25/41) of specimens exposed to TER286 continuously. Tumor specimens respond to TER286 with cytotoxic activity seen for both breast and lung tumors in a clear concentration–response effect.

Based on the tumor types, a 40% response (2/5) was observed in non-small cell lung tumors, 25% response (2/8) in breast and 0% (0/1) of small cell lung tumors with 1 h exposure. At a continuous exposure, a 50% response (10/20) was noted in breast tumors, 70% response (14/20) in non-small cell lung and 100% response (1/1) of small-cell lung tumors.

McNemar's test has been used to compare the rates of sensitivity of the tumors to TER286. There were no significant differences among concentrations used as a 1 h exposure, although the sample size ($n = 14$) may be too small to yield statistically significant results. As continuous exposure, sensitivity rates for concentrations 1 and 10 μM were not different ($p = 0.48$), but the sensitivity rate at 50 μM was significantly greater than at 10 μM ($p = 0.00001$). Increased sensitivity to a continuous exposure is seen in specimens tested head-to-head 1 h versus continuous ($p = 0.034$ by McNemar test). This trend was observed in non-paired specimens as well. There is no significant difference in response rates between specimens from patients who had received prior therapy and those who did not.

Discussion

The GSTs are a family of detoxification enzymes that offer protection to cells against exogenous and endogenous factors. While this is advantageous in normal cells, this capability provides tumor cells with resistance to some cancer drugs such as chlorambucil, doxorubicin and melphalan.[16] Comparison of GST activity between normal cells and tumor cells showed a higher level in tumor cells and an even greater elevation in drug-resistant tumor cells.[1,2,4,16] Recently, Nishimura *et al.*[17] examined the relationship between expression of GSTs determined by immunohistochemistry and clinical response to platinum-based chemotherapy in head and neck cancer patients. The report described a strong inverse correlation between expression of GSTs and the effectiveness of platinium-based therapy. The level of GSTs and the classes of GSTs within malignant tumors varies, providing an explanation for varibility of individual response to treatment. Determination of GST levels and substrate specificity for a particular patient could provide a guide for selection of drug treatment.[8]

Treatment of tumor cells with GST-activated alkylating agents such as TER286 may be actually more effective in the drug-resistant tumors. This new chemotherapeutic strategy could offer an additional

Table 1. Tumor-specific *in vitro* response[a] to TER286 in a human tumor cloning system

Tumor type	1 h exposure (μM)			Continuous exposure (μM)		
	1.0	10.0	50.0	1.0	10.0	50.0
Breast (with prior therapy)	0/3	0/3	1/3	0/10	1/10	3/10
Breast (with no prior therapy)	0/5	1/5	1/5	0/10	0/10	7/10
Lung, non-small cell (with prior therapy)	0/1	0/1	1/1	1/10	1/10	8/10
Lung, non-small cell (with no prior therapy)	0/4	1/4	1/4	1/10	2/10	6/10
Lung, small cell	0/1	0/1	0/1	0/1	0/1	1/1
	0/14 (0%)	2/14 (14%)	4/14 (29%)	2/41 (5%)	4/41 (10%)	25/41 (61%)

[a]Inhibition: 50% or less survival.

benefit to patients by targeting tumor cells. A side effect of many cytotoxic drugs is damage to the granulocyte/macrophage progenitor cells in bone marrow, leaving patients immunocompromised. GST targeting drugs would likely do little damage to critical normal cells, such as the GM progenitor cells, which express little GST.[8]

Further work is needed to establish optimal exposure to TER286, as the continuous exposure conditions here do not take into account the spontaneous decay of the compound at physiological pH.

Conclusion

TER286 is a glutathione-based GST-activated cytotoxin that demonstrated *in vitro* activity against primary human tumor specimens. Our study showed that it is an interesting compound because it can potentially be activated in a patient's tumor which is resistant based on up-regulation of GST. The study suggests that development of TER286 should be pursued in clinical trials.

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