Efficacy of Radioprotective Agents in Preventing Small and Large Bowel Radiation Injury

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PURPOSE: A variety of adjuvant treatments and cytoprotective agents have been proposed to lessen the toxicity of radiation therapy. The following study was designed to evaluate the benefit of six agents or combinations using anastomotic bursting strength as a measure of transmural radiation injury. METHODS: The 40-Gy study consisted of the following. Seventy-two male Sprague-Dawley rats were divided into eight equal groups: nonradiated control, radiated untreated control, and six radiated treated groups. The radioprotective treatments included ribose-cysteine (RibCys), WR-2721, glutamine, vitamin E, $MgCl_2$/adenosine triphosphate, and RibCys/glutamine in combination. Radiated animals received 40 Gy to the abdomen. Two weeks after radiation, all animals underwent small bowel and colonic resection with primary anastomosis. Animals were sacrificed one week postoperatively, at which time anastomoses were evaluated and bursting strengths determined. The 70-Gy study consisted of the following. The same protocol was repeated for five groups of nine rats divided into nonradiated, radiated untreated, and three radiated treated groups receiving RibCys (8 mmol/kg), RibCys (20 mmol/kg), and WR-2721. All radiated animals received 70-Gy doses. RESULTS: In the 40-Gy group, there were 10 radiation-related deaths and 6 anastomotic leaks among 70 rats studied. None of the differences between groups were significant. Nonradiated control group small bowel and large bowel anastomotic bursting pressures were significantly elevated compared with all radiated groups. Compared with radiated controls, there were significant improvements in small bowel bursting strength in the RibCys, WR-2721, RibCys-glutamine, and vitamin E groups and significant improvement in colonic bursting strength in $MgCl_2$/adenosine triphosphate, WR-2721, and RibCys groups. In the 70-Gy group, all nine nonradiated control rats survived. All eight untreated radiated control rats died, four of eight WR-2721 animals died ($P = 0.03$), all RibCys (8 mmol/kg) animals died ($P = 0.03$), and three of nine treated with RibCys (20 mmol/kg) survived ($P = 0.08$). CONCLUSIONS: WR-2721 and RibCys gave consistent protection against large and small bowel radiation injury. The lower incidence of treatment-related toxicity and potentially equal or greater radioprotective effects may make RibCys more clinically useful than WR-2721. [Key words: Glutamine, RibCys; WR-2721; Radiation injury; Radioprotectant]

Carroll MP, Zera RT, Roberts JC, Schlafmann SE, Feeney DA, Johnston GR, West MA, Bubrick MP. Efficacy of radioprotective agents in preventing small and large bowel radiation injury. Dis Colon Rectum 1995;38:716-722.

Although radiation therapy remains an important treatment modality for many pelvic tumors, including adenocarcinoma of the rectum, early and late intestinal complications of radiation therapy remain a major clinical problem, which has limited more widespread use of this treatment. Up to 17 percent of patients receiving abdominal or pelvic radiation will develop major complications, and as many as 5 percent of all patients treated will eventually require surgery for radiation injury.[1] In addition, the healing of colorectal anastomoses following radiation therapy has been associated with high rates of anastomotic dehiscence and mortality, both experimentally[2] and clinically.[3-5] To lessen toxicity and risk of radiation injury, a variety of adjuvant treatments and cytoprotective agents have been proposed for clinical use.[6-9]

Sulfhydryl-containing compounds in particular have long been known to possess radioprotective capabilities, although the clinical use of these compounds have previously been limited by toxicity. One approach to overcome this problem has been to construct prodrugs that can function as slow-release forms of the active compound, thereby eliminating the presence of high, toxic doses at any given time. WR-2721, a prodrug form of a free thiol compound WR-1065, has been shown to be an effective intestinal radioprotective agent both experimentally[10] and clinically.[11] Despite these encouraging findings, the use of WR-2721 may be limited by toxicity. We hypothesized that other related prodrugs may have similar

Supported by the 1993 ASCRS/ETHICON Surgical Research Fellowship Award and the Minneapolis Medical Research Foundation.
Read at the meeting of The American Society of Colon and Rectal Surgeons, Orlando, Florida, May 8 to 13, 1994.
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efficacy, without the dose-limiting toxicity of WR-2721. Ribose-cysteine (RibCys) is a prodrug of L-cysteine, which stimulates glutathione biosynthesis. Previous studies have shown that RibCys protects swine against radiation-related deaths and anastomotic leaks following high doses of pelvic irradiation.[12]

The following study was designed to evaluate the cytoprotective benefit of one agent already in early clinical use (WR-2721) and four other agents. These agents were chosen from the many known radioprotective agents, based on promising earlier work and applicability to clinical use. Anastomotic healing measured by gross healing and bursting strength have not been widely used as parameters of radioprotection, although these measures may be highly relevant to clinical surgery. The study was designed to measure the effects of these radioprotective agents on survival and anastomotic and histologic healing parameters in animals that have undergone moderate and high-dose abdominal radiation.

MATERIALS AND METHODS

The protocol was approved by the Animal Care and Use Committee of the Minneapolis Medical Research Foundation, and the U.S. Public Health Service guide for the care and use of laboratory animals was followed.

40-Gy Study

Seventy-two male Sprague-Dawley rats (Harlan Sprague-Dawley Co., Madison, WI) weighing approximately 250 g were randomly divided into eight equal groups. The first group served as a nonradiated untreated control group. The second group served as a radiated untreated control group, and the other six were radiated treated groups. All radiated groups received 40 Gy whole abdomen radiation equivalent to 2 Gy daily fractions according to the Ellis[13] nominal standard dose equation. Animals in the treated groups received radioprotectant pretreatments before radiation. The six treatments were as follows: 1) RibCys, 8 mmol/kg solubilized in 5 ml of 0.9 percent sodium chloride solution and injected intraperitoneally (IP) fifteen minutes before radiation; 2) WR-2721, 250 mg/kg Amifostine™ (U.S. Bioscience, West Conshohocken, PA) solubilized in 5 ml of 0.9 percent sodium chloride solution and injected IP fifteen minutes before radiation; 3) glutamine, 3 percent L-glutamine (Sigma Chemical Co., St. Louis, MO) oral solution *ad libitum* for one week before and one week following radiation; 4) vitamin E, 20 mg/kg DL-α-tocopherol (Sigma Chemical Co.) IP daily for six days before radiation; 5) magnesium chloride/adenosine triphosphate, 100 mmol/kg $MgCl_2$ and 100 mmol/kg adenosine 5′-triphosphate disodium salt (Sigma Chemical Co.) injected intravenously immediately before radiation as described by Chaudry[14]; 6) RibCys/glutamine, in combination as prepared individually for Groups 1 and 3. above. Both control groups and treatment Groups 3, 4, and 5 received sham IP injections of 5 ml of 0.9% sodium chloride solution before radiation. All rats were fed a commercial rat diet *ad libitum* throughout the study, except for the day before radiation treatment, surgery, or sacrifice.

An eight-unit, partitioned, wooden box was designed for this protocol to hold seven rats in a calibrated ionization chamber (Victoreen R. Meter, Model 500, and Thimble Chamber, Model 550-A, Victoreen Inc., Cleveland, OH) during each radiation treatment (Fig. 1). The ionization chamber was surrounded by tissue-equivalent bolus material (Super Stuff Bolus Material, Model 489, Radiation Products Design, Inc., Albertville, MN) in a cellophane bag to simulate the size of the rats in the adjacent compartments and to assure that the dose measured was a minimum dose to whole abdomen depth. A single rat from each of the six treatment groups and one from the radiated control group were randomly selected. All rats received a ketamine hydrochloride (10 mg/100 g of

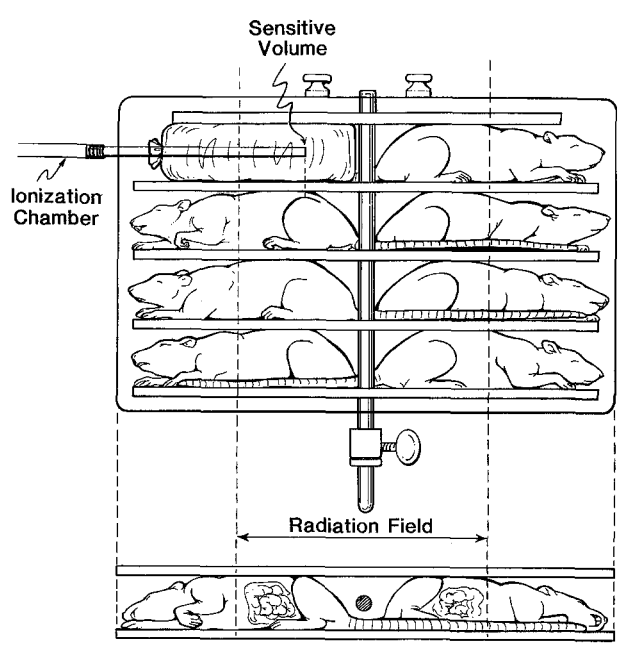

Figure 1. Diagram of animals positioned for abdominal radiation in eight-unit partitioned box.

body weight) and xylazine (0.5 mg/100 g of body weight) mixture *via* IP injections to maintain anesthesia during radiation. Rats were placed in a right decubitus position for one-half of the required treatment, then they were transferred to a left decubitus for completion of the dose.

Radiation doses were calculated using the NSD as follows: NSD = dose (total in rads) × (days on treatment)$^{-0.11}$ × (number of fractions)$^{-0.24}$. An isocentric cobalt-60 teletherapy machine was used to deliver the 40-Gy NSD-equivalent whole abdomen dose *via* equally weighted, parallel-opposed lateral fields. The sensitive volume of the ionization chamber (surrounded by tissue-equivalent phantom material) was positioned at the level of the adjacent rat's mid abdomen. The total dose administered to each of the two opposed portals was approximately 7.5 Gy.

Two weeks following radiation treatment, all controls and treated animals underwent simultaneous wedge resections of the small and large bowel with primary anastomoses. Before surgery, rat diet was withheld for one day. Preoperatively, all rats received 20 mg of cefotaxime IP, and anesthesia was maintained with a ketamine and xylazine mixture injected IP. The bowel resections were performed through a midline epigastric incision. The site of the resection was approximately 10 cm proximal to the cecum for the small bowel and 2 cm distal for the large bowel. A primary anastomosis was performed in a single layer with multiple, simple 6–0 silk sutures. The abdominal wound was closed as a single layer with a running 4–0 Vicryl™ (Ethicon, Somerville, NJ) suture. Postoperatively, water with acetaminophen (40 mg/200 ml of water) was provided *ad libitum* for analgesia. In addition, the rat diet was restricted for one day.

All animals underwent a second operation under ketamine and xylazine anesthesia after one week, at which time anastomotic segments were resected and examined. Before resection, the peritoneal cavity was examined for adhesions, intraperitoneal abscess, or other evidence of anastomotic leak. After the anastomotic segments were resected, animals were killed with an intracardiac injection of potassium chloride.

To determine bursting pressure, an arterial line tubing connected to a pressure recorder and containing methylene blue was secured in the proximal end of the anastomotic segment, and the distal end was clamped. The segment was placed in a water bath, and increasing pressure of methylene blue solution was run into the bowel lumen. Bursting strength was defined as a pressure at which methylene blue appeared in the water bath. The site of anastomotic segment failure was noted, and the anastomotic segment was divided longitudinally for gross examination and histologic sectioning. Histologic evaluation was done by light microscopy, and radiation injury was graded from 0 to 3 based on mucosal ulceration, epithelial atypia, submucosal edema, submucosal fibrosis, and vascular change.

70-Gy Study

The above protocol was repeated for five groups of nine rats, divided into a nonradiated untreated control group, a radiated untreated group, and three treated groups receiving RibCys (8 mmol/kg), RibCys (20 mmol/kg), and WR-2721 (250 g/kg). All radiated animals received 70-Gy equivalent by the NSD equation.

Statistical Analysis

Tests of significance included analysis of variance for continuous data and Kruskal-Wallis nonparametric test for noncontinuous data. Variance is expressed as standard deviation throughout the text.

RESULTS

Animals Treated with 40-Gy Equivalent

Radiation-Related Complications and Deaths. Two of the original 72 animals died before radiation. Of the remaining 70, there were 10 radiation-related deaths in the eight groups. All ten developed progressively bloody diarrhea and dehydration before death, clinically consistent with acute radiation enteritis. A total of six anastomotic leaks were identified, but none of the differences between groups was significant. Collectively, there were no significant differences in radiation-related complications (deaths or leaks) between groups (Fig. 2).

Weights. There was a significant decrease in weight among all radiated animals two weeks after receiving radiation compared with the nonradiated control group. In addition, there was a significant further decrease in weight among glutamine-treated animals compared with the RibCys, WR-2721, and $MgCl_2$/adenosine triphosphate groups (Fig. 3).

Anastomotic Bursting Strength. All of the small bowel bursting strength studies indicated a significant decrease in mean bursting strength among the radiated groups compared with the nonradiated group. Mean bursting strength for the nonradiated controls was 297.1 ± 29.1 mmHg and ranged from 122.6 ±

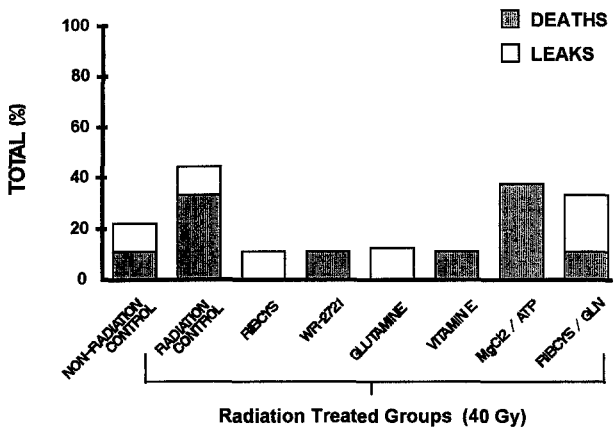

Figure 2. Postradiation complications—deaths and leaks at 40 Gy. RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

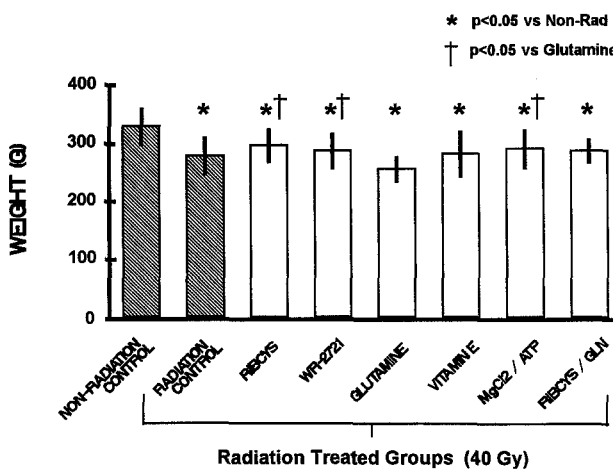

Figure 3. Preoperative rat weight (g). Non-Rad = nonradiated; RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

64.3 mmHg for radiated untreated controls to 214.3 ± 49.7 mmHg for rats treated with vitamin E. Significant differences were found among bursting strength in the RibCys, WR-2721, RibCys/glutamine, and vitamin E groups compared with radiated controls. Differences in bursting strength between animals treated with glutamine and $MgCl_2$/adenosine triphosphate compared with radiated controls were not significant (Fig. 4).

The mean large bowel anastomotic bursting pressure for the control group was 318.8 ± 53.2 mmHg. Mean bursting pressures were significantly decreased for all radiated groups compared with nonradiated controls, and pressures ranged from 179.3 ± 93.2 mmHg for radiated untreated control rats to 299.5 ± 68.8 mmHg for $MgCl_2$/adenosine triphosphate-treated rats. Mean bursting pressures for animals treated with $MgCl_2$/adenosine triphosphate, WR-2721, and RibCys

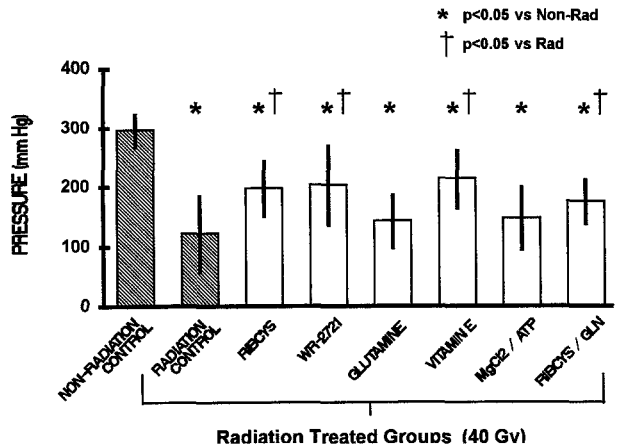

Figure 4. Small bowel burst strength at 40 Gy. Non-Rad = nonradiated; RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

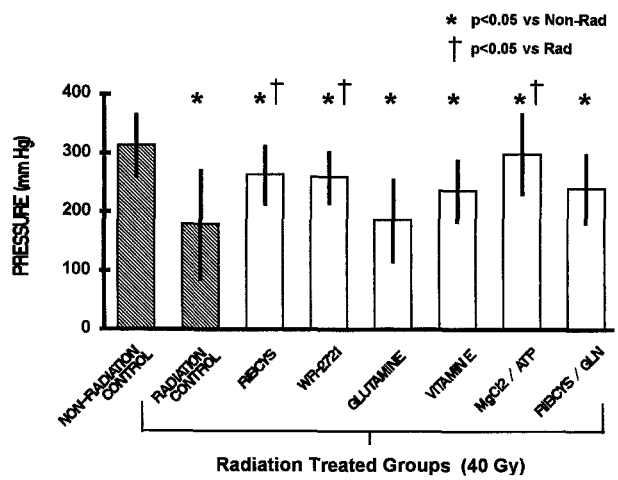

Figure 5. Large bowel burst strength at 40 Gy. Non-Rad = nonradiated; RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

were significantly increased compared with radiated untreated controls. There was no significant benefit in mean bursting pressure demonstrated among animals treated with glutamine, vitamin E, and RibCys/glutamine (Fig. 5).

Histologic Evaluation. There were no significant differences in histologic grading of injury for either the small bowel (Fig. 6) or large bowel (Fig. 7) among any groups.

Animals Treated with 70-Gy Equivalent

Rats treated with 70-Gy equivalent dose could only be evaluated for mortality. The nine nonradiated control rats all survived, and all eight untreated radiated control rats died of acute radiation enterocolitis. Four of the eight animals treated with WR-2721 survived,

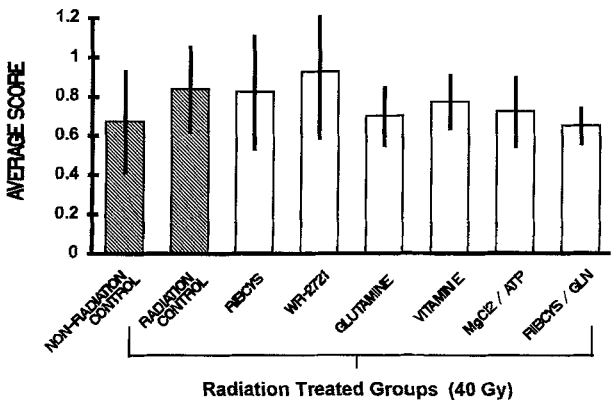

Figure 6. Small bowel histologic evaluation. RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

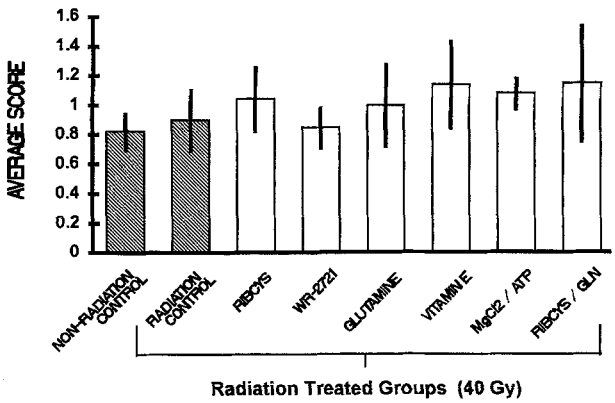

Figure 7. Large bowel histologic evaluation. RIBCYS = ribose-cysteine; GLN = glutamine; ATP = adenosine triphosphate.

and this differences was significant compared with the radiated controls ($P = 0.03$). All animals treated with RibCys (8 mmol/kg) died, but three of the nine treated with 20 mmol/kg RibCys survived ($P = 0.08$) *vs.* radiated control (Fig. 8).

DISCUSSION

The effect of increasing radiation dose on the probability of both tumor control and radiation complications is a well-established principle of radiation therapy. The close relationship between tumor control and complications illustrates the narrow therapeutic index of radiation therapy (Fig. 9). As a consequence of this relationship, a small increase in radiation dose above conventional therapeutic levels generally yields an unacceptable increase in radiation-related complications. This has limited widespread use of adjuvant radiation therapy for rectosigmoid cancer and precludes the use of higher, more tumoricidal levels of radiation for advanced or recurrent tumors. A

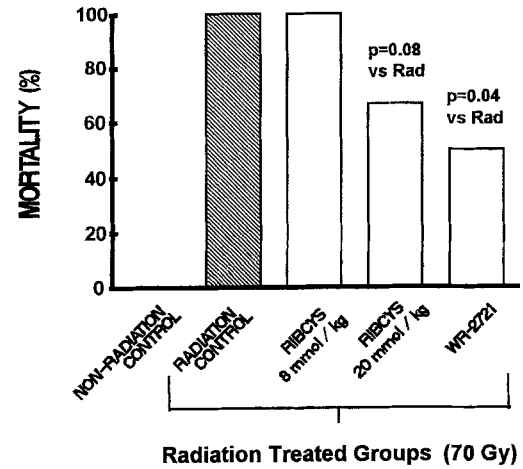

Figure 8. Postradiation mortality at 70 Gy. Rad = radiated; RIBCYS = ribose-cysteine.

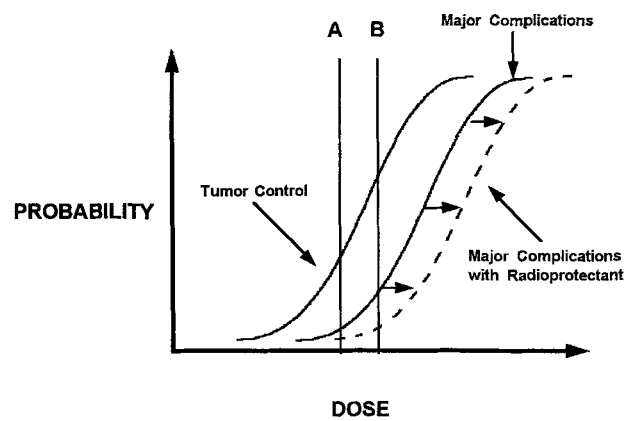

Figure 9. Effect of radioprotectants on the probability of major complications.

multitude of different methods have been tried to minimize postradiation complications, including radiation dose fractionation, radiation planning, and patient positioning,[1] small bowel suspension procedures,[15] and use of radioprotectant compounds.[6–12] The goal of these techniques is to shift the complication curve to the right, thereby increasing the radiation dose that can be given without promoting complications and widening the therapeutic index of radiation therapy for tumor control.

WR-2721 and RibCys were the most consistently effective agents in this study. WR-2721 (*S*-2-(3-aminopropyl-amino) ethylphosporothioate), a sulfhydryl-containing compound was developed during the United States Army Antiradiation Drug Development Program at Walter Reed Hospital. In 1969, Yuhas and Storer[16] demonstrated the capacity of this compound to provide differential selective radioprotection of normal tissue, without limiting tumoricidal activity.

This principle of radioprotection was recently applied in a clinical trial of WR-2721 for inoperable, unresectable, or recurrent rectal carcinoma. Results of this study showed that WR-2721 is an effective agent, which decreases both acute and chronic postradiation complications without adversely influencing tumoricidal activity.[11] The use of WR-2721 as a radioprotectant may be limited by the side-effect profile noted in Phase I clinical trials.[17] The adverse effects include nausea, vomiting, hypotension, and malaise, which have limited WR-2721 to a maximum dose of 340 mg/m.^{2} A radioprotectant agent with a lower toxicity profile would be much more desirable.

RibCys [(2RS)-D-Ribo (1′, 2′, 3′, 4′-tetrahydroxybutyl) thiazondine-4 (R) (carboxylic acid)], also a sulfhydryl-containing compound, has been shown to be a viable prodrug of L-cysteine.[18] Patt *et al.*[19] previously demonstrated the radioprotectant properties of L-cysteine, but its clinical use was limited by unacceptable toxicity at radioprotective doses. The synthesis of RibCys was accomplished by chemical condensation of L-cysteine and D-ribose so that the sulfhydryl-containing amino acid is released by a nonenzymatic ring opening and solvolysis mechanism. L-Cysteine is one of the three amino acid building blocks of glutathione and may be the limiting precursor for glutathione biosynthesis *in vivo*.[20] RibCys has been demonstrated to significantly enhance glutathione biosynthesis *in vivo*.[18] RibCys or related amino acid derivatives may ultimately become more useful than WR-2721 because they may prove to have less toxicity than WR-2721. If toxicity can be controlled, it is conceivable that RibCys or related amino acid derivatives could allow radiation doses to be pushed to higher levels without toxicity or injury.

Other radioprotectant agents used in this study included the antioxidant vitamin E, $MgCl_2$/adenosine triphosphate, and glutamine. Although vitamin E and $MgCl_2$/adenosine triphosphate have shown promise as cytoprotective agents, they may have their greatest benefit when used in combination with amino acid derivatives or other agents. The synergism of such compounds has been shown to materially reduce the toxicity seen with higher doses of the individual drugs.[18]

The relative ineffectiveness of glutamine was surprising and conflicts with other studies,[9] but this finding may be consistent with its role as a nutrient that promotes intestinal proliferation. Increased blood flow, oxygenation, metabolic activity, or proliferation induced by the glutamine pretreatment may have sensitized the intestinal mucosal to worsening radiation effect. The comparative success of intraperitoneal WR-2721 and RibCys in this model also suggests that radioprotectants may need to be given systemically because this route provides radioprotection that is not limited to the mucosal enterocytes. Glutamine may prove to be more beneficial after radiation or as a form of posttreatment nutritional support.

Use of anastomotic bursting strength in the moderate dose (40 Gy) phase of this study serves as a useful screening model to measure clinically significant intestinal radiation injury, and we prefer this test to histologic grading, which was not useful for us. The parameters traditionally used to measure the radioprotective benefits of individual drugs have included either survival[12] or histologic measurements such as crypt numbers and mucosal height.[21, 22] Although anastomotic healing, as measured by bursting strength, has not previously been widely used to assess radioprotection, it is probably more relevant to clinical surgery. Bursting strength measures the effects of healing on the entire intestinal wall as opposed to histologic parameters, which measures only mucosal enterocyte stability and proliferation.

The consistent benefits seen with the use of WR-2721 and RibCys at moderate doses of radiation (40 Gy) led us to study the effects of these agents at a decidedly lethal dose (70 Gy), and results with WR-2721 are striking. Results with RibCys were encouraging and suggest that a dose-response relationship may exist for this drug. We were unable to evaluate this further with the rat model because the maximum RibCys dose had to be limited to 20 mmol/kg, a dose that requires the maximum tolerable volume for intraperitoneal injection in the rat. Use of doses many times this amount may be feasible in clinical practice. Studies with RibCys in swine using doses as small as 1 mmol/kg[12] showed some efficacy, and this animal may be the suitable model for further study of intravenous effects of RibCys at increasing doses. If the incremental increase in benefit with RibCys persists without associated toxicity, then RibCys or related amino acid derivatives may prove to be even more useful than WR-2721, and this can significantly expand the therapeutic role of radiation therapy.

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