Effect of $^{67}\text{Cu}$-2IT-BAT-Lym-1 Therapy on $BCL-2$ Gene and Protein Expression in a Lymphoma Mouse Model$^1$

Linda A. Kroger,$^2$ Sally J. DeNardo,
Gerald L. DeNardo, Cheng Yi Xiong,
Michelle D. Winthrop, and Paul H. Gumerlock
Molecular Cancer Institute, Sacramento, California 95816

Abstract
Radioimmunotherapy using monoclonal antibodies against tumor-associated antigens has been particularly promising in the treatment of radiosensitive malignancies such as lymphoma. $^{67}\text{Cu}$ has excellent physical and biochemical properties for radioimmunotherapy. $^{67}\text{Cu}$-2IT-BAT-Lym-1 has been used in preclinical and clinical trials, where an exceptionally long residence time of $^{67}\text{Cu}$ on tumor was observed. $BCL-2$, a proto-oncogene that promotes cell survival by blocking apoptotic cell death, is overexpressed in most B-cell lymphomas including Raji human Burkitt’s lymphoma cells. In this study, therapeutic efficacy and $BCL-2$ gene and protein expression levels were examined in Raji xenografts in mice after $^{67}\text{Cu}$-2IT-BAT-Lym-1 radioimmunotherapy. $^{67}\text{Cu}$-2IT-BAT-Lym-1 therapy induced a response rate (complete and partial responses) of $\sim 50\%$. $BCL-2$ gene expression was decreased 3 h after radioimmunotherapy, followed by a decrease in Bcl-2 protein by 24 h. Decreases in $BCL-2$ gene and protein expression preceding observations of $^{67}\text{Cu}$-2IT-BAT-Lym-1 therapeutic effect suggest that down-regulation of $BCL-2$ leaves cells more likely to be killed by low dose-rate radiation from radioimmunotherapy.

Introduction
Despite advances in conventional therapy for NHL,$^3$ 40–70% of patients with intermediate and high grade NHL fail to achieve long-term disease-free survival, and no curative treatment has been established for patients with low grade NHL (1, 2). RIT using MoAbs against tumor-associated antigens to deliver cytotoxic radionuclides to tumors has been particularly promising in the treatment of radiosensitive malignancies such as lymphoma (3–8).

$^{67}\text{Cu}$ has excellent physical and biochemical properties for RIT. Its half-life (62 h) is well matched to the uptake and residence time of antibodies in the tumor (9). $^{67}\text{Cu}$ has $\beta$ emissions (mean energy, 141 keV; $e_{\text{max}} = 577$ keV) for therapy, and $\gamma$ emissions (185 keV, 47%; 93 keV, 17%) that are excellent for imaging but contribute minimal radiation exposure to medical personnel. An advantage of $^{67}\text{Cu}$ is its enhancement of the therapeutic index compared with other radionuclides. $^{67}\text{Cu}$-radiolabeled MoAbs have been shown in preclinical studies to deliver higher doses to tumor and higher tumor to nontumor dose ratios when compared with their iodinated counterparts (10, 11). These results have been substantiated by data from clinical trials of $^{67}\text{Cu}$-2IT-BAT-Lym-1, in which an exceptionally long residence time of $^{67}\text{Cu}$ in tumors was observed (12, 13). The macrocyclic chelating agent, 1,4,7,11-tetraazacyclotetradecane-$N,N',N'',N'''$-tetracetic acid, specifically designed to stably bind copper (14), is the chelating moiety in the immun conjugate 2IT-BAT-Lym-1. Under well-characterized conditions, 2IT-BAT-Lym-1 binds $^{67}\text{Cu}$ rapidly and selectively, providing $^{67}\text{Cu}$-2IT-BAT-Lym-1 with high specific activity and complete retention of structural and functional integrity (15). $^{67}\text{Cu}$-2IT-BAT-Lym-1 radiolabeling has been optimized to the extent that product yield and quality are comparable to those for iodinated MoAbs.

$BCL-2$ is the acronym for the $B$-cell lymphoma/leukemia-$2$ gene, which was first discovered in association with $B$-cell malignancies in which chromosomal translocations were found to activate the gene in the majority of follicular NHLs (16). $BCL-2$ is a proto-oncogene that regulates cell survival by blocking a common pathway leading to apoptotic cell death (17). It is overexpressed, and thus oncogenic, in most $B$-cell lymphomas, including Raji human Burkitt’s lymphoma cells (18). Cells blocked at the $G_2$-$M$ interface appear to be much more radiosensitive to radiation (19–22). Macklis et al. (23) studied radiation-induced cell cycle changes and demonstrated that Raji cells are efficiently blocked at the $G_2$-$M$ interface despite overexpression of $BCL-2$.

The goal of this study was to examine modulation of $BCL-2$ in the response of Raji xenografts to $^{67}\text{Cu}$-2IT-BAT-Lym-1 therapy. In these studies, nude mice bearing Raji tumors were treated with 335, 400, and 500 $\mu$Ci of $^{67}\text{Cu}$-2IT-BAT-Lym-1 and were followed for efficacy and toxicity (10). Selected mice that received 335 $\mu$Ci of $^{67}\text{Cu}$-2IT-BAT-Lym-1 were sacrificed, and their tumors were analyzed for $BCL-2$ gene expression and Bcl-2 protein levels.

Materials and Methods
Cell Lines. The Raji human lymphoma cell line was obtained from American Type Culture Collection (Rockville, MD). The human renal carcinoma cell line, G2101, was isolated from a rib metastasis of a renal cell carcinoma and has been shown to be positive for $BCL-2$ expression (24). Cells were

---

$^1$Presented at the “Seventh Conference on Radioimmuno detection and Radioimmunotherapy of Cancer,” October 15–17, 1998, Princeton, NJ. This research was supported by grants from the National Cancer Institute (PHS CA 47829) and the Department of Energy (DE FG03-84ER60233).

$^2$To whom requests for reprints should be addressed, at Molecular Cancer Institute, 1508 Alhambra Boulevard, Room 3100, Sacramento, CA 95816.

$^3$The abbreviations used are: NHL, non-Hodgkin’s lymphoma; RIT, radioimmunotherapy; MoAb, monoclonal antibody; 2IT, 2-iminothiolane; BAT, 6-L-p(bromocetamido)benzyl]-1,4,7,11-tetraazacyclotetradecane-$N,N',N'',N'''$-tetracetic acid; RT-PCR, reverse transcription-PCR.
maintained in RPMI 1640 (Raji) or DMEM (G2101) growth media (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum. Cells used in these studies were harvested in their logarithmic growth phase.

**Pharmaceutical.** Lym-1 (Techniclone, Inc., Tustin, CA) is an IgG2a mouse MoAb with high affinity against a discontinuous epitope on the β chain of the HLA-DR antigen located on the surface membrane of malignant B lymphocytes (25, 26). Lym-1 can activate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity against Raji cells in vitro but has little effectiveness in vivo (27). The immunoconjugate 2IT-BAT-Lym-1 was prepared by conjugating BAT to Lym-1 via 2IT (Sigma Chemical Co., St. Louis, MO). Radiolabeling of 2IT-BAT-Lym-1 with 67Cu was performed by previously described methods (10). 67Cu-2IT-BAT-Lym-1 was examined by cellulose acetate electrophoresis, molecular sieving high-performance liquid chromatography, and radioimmunoassay. All preparations passed quality control tests.

**Animal Studies.** Female athymic BALB/c/c-rnu/rnu mice (Harlan Sprague Dawley, Frederick, MD) weighing 20–25 g were maintained according to University of California Animal Care guidelines on a normal diet ad libitum and under pathogen-free conditions. Prior to tumor implantation, mice received a single treatment with ~'7Cu-2IT-BAT-Lym-1 or until tumors were harvested two times per week for 84 days after tumor implantation. Subgroups of mice treated with 335 pCi of ~'7Cu-2IT-BAT-Lym-1 had tumors harvested at 3 and 24 h after therapy. Untreated tumors were used as controls for gene expression. Untreated tumours were used as controls for gene expression. Untreated tumours were used as controls for gene expression.

**Results**

**Tumor Response to 67Cu-2IT-BAT-Lym-1.** Tumor volume in untreated mice increased rapidly, whereas tumor volume in treated mice characteristically decreased after 5 days. The overall response rate (complete and partial response and cure) for tumors in all three treated groups of mice was 47%, and 33% of the tumors were cured (Fig. 1). In mice that received 335 μCi of 67Cu-2IT-BAT-Lym-1, 5 of 18 (28%) tumors disappeared and did not regrow during the 84-day study (Fig. 1, Cure), 1 of 18 (6%) tumors disappeared but later regrew (complete response; Fig. 1, CR) and 3 of 18 (17%) tumors decreased by at least 50% (partial response; Fig. 1, PR). In mice that received 400 μCi of 67Cu-2IT-BAT-Lym-1, 5 of 12 (42%) tumors were cured. In mice that received 500 μCi of 67Cu-2IT-BAT-Lym-1, two of six (33%) tumors were cured, and one of six (17%) tumors decreased by at least 50%. Toxicity was modest at all three dose levels of 67Cu-2IT-BAT-Lym-1 (335, 400, or 500 μCi) with 11, 20, and 9% mouse mortality occurring within 30 days of therapy, respectively.

**Modulation of BCL-2 Expression Following RIT.** To study the effect of RIT on BCL-2 expression, 4–5 Raji xenografts were harvested at each time point (3 and 24 h) after 67Cu-2IT-BAT-Lym-1 therapy and were evaluated for changes in gene expression. Untreated tumors were used as controls for gene expression. BCL-2 expression was significantly decreased...
BCL-2 in Raji Xenografts after $^{67}$Cu-2IT-BAT-Lym-1

Fig. 1 Tumor responses to $^{67}$Cu-2IT-BAT-Lym-1 (335, 400, or 500 μCi) or no treatment. The response rates were similar at all dose levels. Overall response rate for all treated groups and cure rate were 47 and 33%, respectively.

Fig. 2 Gene expression of BCL-2 by untreated tumors and tumors obtained from mice 3 and 24 h after 335 μCi of $^{67}$Cu-2IT-BAT-Lym-1. mRNA expression was obtained by scanning the photographic negative of the ethidium bromide-stained agarose gels and normalizing to N-RAS and G2101 cell expression levels. Decreased expression of BCL-2 is seen at 3 h with diminished expression persisting at 24 h. Bars, SD.

3 h after RIT ($P = 0.006$) and remained decreased at 24 h ($P = 0.005$) when compared with the BCL-2 expression of the control tumors (Fig. 2).

Bcl-2 Protein Expression. Four Raji xenografts harvested at 3 and 24 h after $^{67}$Cu-2IT-BAT-Lym-1 therapy were evaluated for Bcl-2 protein expression by Western blotting and compared to Bcl-2 protein levels in untreated tumors. Western blot analysis revealed that Bcl-2 protein expression in xenografts treated with $^{67}$Cu-2IT-BAT-Lym-1 was unchanged at 3 h but decreased to 64% of the control level at 24 h (Fig. 3).

Discussion

BCL-2 is a proto-oncogene that regulates cell survival by blocking a common pathway leading to apoptotic cell death (17). BCL-2 is overexpressed in most B-cell lymphomas, including the Raji cells used for these studies (18). Additionally, Burkitt's lymphoma cells have p53 mutations that cluster in two regions (codons 209–216 and 234–243). Raji Burkitt's cells harbor two abnormal p53 alleles, one at codon 213 and one at codon 234 (32). p53 protein promotes apoptosis in DNA-damaged cells, such as those that might be induced by radiation.

To assess therapeutic efficacy, mice bearing Raji tumors were treated with $^{67}$Cu-2IT-BAT-Lym-1. At doses translatable to patients, $^{67}$Cu-2IT-BAT-Lym-1 provided a therapeutic, and frequently curative (33%), dose of radiation to tumored mice with modest toxicity (10). Lym-1 antibody by itself has no therapeutic effect on lymphoma in patients (27). A major low dose-rate radiation effect, such as that induced by $^{67}$Cu-2IT-BAT-Lym-1 RIT, is abrupt intermitotic cell death, referred to as apoptosis, through mediation of cell cycle processes and the activation of endogenous endonucleases (19, 33–35). Although some have observed this effect in response to antibody alone (36), the combination of antibody binding and low dose-rate radiation appear to be synergistic in inducing this effect (19, 37). In studies on human B-lymphoma cells, low dose-rate radiation has been associated with arrest of cells in the G$_2$-M phase of the cell cycle and ultimately cell death by necrosis or apoptosis (19, 23, 38).

To examine a potential factor contributing to RIT efficacy, BCL-2 gene and protein expression was determined in Raji...
tumors after 335 μCi of 67Cu-2IT-BAT-Lym-1, a dose associated with substantial efficacy and minimal toxicity. BCL-2 gene expression was decreased at 3 h with a subsequent decrease in Bcl-2 protein by 24 h. This orderly decrease in BCL-2 gene and protein expression after 67Cu-2IT-BAT-Lym-1 RIT suggests that the events were related. These gene and protein expression events occurred within 24 h of administration of the 67Cu-2IT-BAT-Lym-1 and preceded evidence of tumor regression. Additional evidence that the decrease in BCL-2 proto-oncogene represented a significant mechanism for the therapeutic effect of RIT on the tumor is provided by the description of an analogous decrease in BCL-2 expression associated with response to RIT in a breast cancer model (30).

The oncogene BCL-2 acts to decrease the probability of apoptosis because the protein encoded by the BCL-2 gene blocks programmed cell death. Despite inherent overexpression in-tmunotherapy. This mechanism may play a significant role in more likely to be killed by low dose-rate radiation from radioimmunotherapy. The coordination preceding observations of 67Cu-2IT-BAT-Lym-1 pharmacokinetics, radiation dosimetry, toxicity and tumor regression in patients with lymphoma. J. Nucl. Med., in press, 1999.


Acknowledgments

We would like to acknowledge David Kukis and Laird Miers for assistance in generating data.

References


3014s BCL-2 in Raji Xenografts after 67Cu-2IT-BAT-Lym-1


Effect of \(^{67}\text{Cu-2IT-BAT-Lym-1}\) Therapy on \(\text{BCL-2}\) Gene and Protein Expression in a Lymphoma Mouse Model

Linda A. Kroger, Sally J. DeNardo, Gerald L. DeNardo, et al.

_Clin Cancer Res_ 1999;5:3010s-3014s.

**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/10/3010s

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.