of gp100/pmel17 was also observed by RT-PCR assay (Figure S3b). Thus, these data demonstrate that a limited dose of depsipeptide ($2\,mg\,kg^{-1}$) sufficiently sensitized B16/F10 cells *in vivo* for adoptive immunotherapy. Regarding the perforin modulation, we could not determine whether perforin induction occurred *in vivo* by FACS analysis.

We further investigated whether s.c. tumor growth of B16/F10 cells could be inhibited by the adoptive transfer of Pmel-1 CTLs in combination with depsipeptide (Figure 7c). After waiting about a week for tumor growth, $2\,mg\,kg^{-1}$ of depsipeptide was administered via the intra-peritoneal route, followed by the adoptive transfer of Pmel-1 CTLs. In contrast to either CTL transfer alone or depsipeptide pretreatment alone, this combinatorial treatment strikingly suppressed B16/F10 tumor growth. Moreover, we examined whether the above depsipeptide pretreatment and a similar CTL transfer could suppress metastatic tumor growth (Figure 8). Luc-B16/F10 cells were intravenously injected into the tail vain of C57BL/6 mice, and pulmonary metastasis of luc-B16/F10 cells was monitored through luciferase-based luminescent imaging. After waiting about a week for tumor growth, animals were treated with $2\,mg\,kg^{-1}$ of depsipeptide for 3 days, followed by the adoptive transfer of CTLs, which were generated by immunization using irradiated IL-12/IL-18-transduced B16/F10 cells. This combinatorial treatment significantly suppressed tumor-derived photons in pulmonary metastases 21 days following tumor injection. Similar suppression of pulmonary metastases was obtained by adoptive transfer of Pmel-1-derived CTLs (data not shown). Thus, these results demonstrate that sensitization using a limited dose of depsipeptide increases the efficacy of adoptive immunotherapy for established tumors.

DISCUSSION

Cellular unresponsiveness of solid tumor through aberrant transcriptional regulation represents a critical barrier that limits the therapeutic potential of adoptively transferred autologous CTLs in patients with cancer. Herein, we have demonstrated that tumor sensitization with depsipeptide is effective for adoptive immunotherapy against murine B16/F10 melanoma. The remarkable features presented in this study include the following: (1) depsipeptide upregulated gp100/pmel17 melanoma antigen; (2) a limited dose of depsipeptide was able to sufficiently sensitize B16/F10 cells for Fas-mediated apoptosis; (3) depsipeptide increased the perforin-expressing CTLs in post-transcriptional levels; and (4) adoptive cell transfer in combination with depsipeptide led to effective tumor growth suppression.

Emerging evidence suggests that there are a variety of factors that limit tumor regression in the host–tumor interaction. For example, cancer progression often takes place despite the presence of circulating cancer-specific CTLs. Even with patients in whom large numbers of highly activated tumor-specific CTLs have been infused, clinical improvement has been difficult to achieve (Dudley *et al.*, 2001; Rosenberg, 2004). For example, recent evidence concerning host factors suggests that regulatory elements of the immune responses, including $CD4^+CD25^+$ regulatory T cells (Tregs), inhibit the ability of CTLs to produce effective antitumor responses (Antony and Restifo, 2005; Dannull *et al.*, 2005). In regard to tumor-escape factors, many aggressive tumors do not express the tumor antigen or MHC (HLA) antigen (Ferrone and Marincola, 1995; Cabrera *et al.*, 2003). Moreover, many cancer types lack sufficient apoptotic cell death pathways through the aberrant transcription (Johnstone *et al.*, 2002; Maecker *et al.*, 2002). It is theoretically essential to overcome these tumor-escape factors for efficient cancer immunotherapy, and perhaps to manipulate the tumor, as well as the host, before adoptive immune cell transfer.

HDACs are considered among the most promising targets in drug development for cancer, and some HDACi, including depsipeptide (FK228), are currently being tested in phase I and II clinical trials (Minucci and Pelicci, 2006). HDACi is capable of inducing varying degrees of growth arrest,

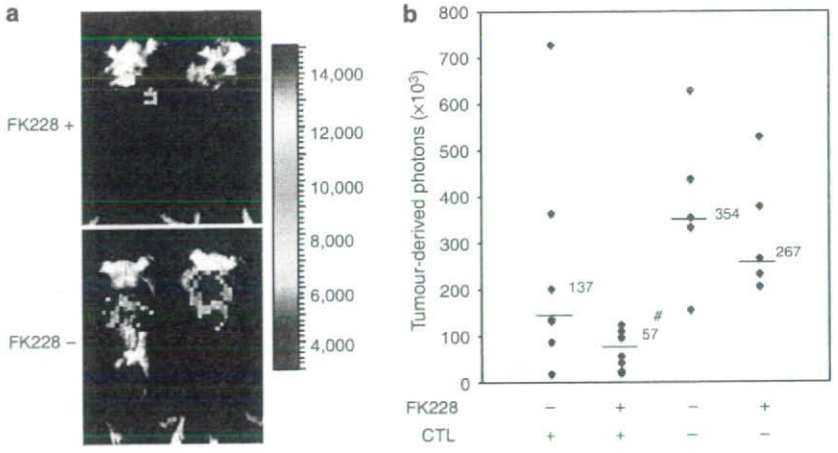

Figure 8. Depsipeptide pretreatment and B16-specific CTLs transfer suppress metastatic tumor growth of B16/F10 cells. (**a**) Representative luciferase images of luc-B16/F10 metastatic lung tumor at day 15 after combinatorial therapy of depsipeptide ($2\,mg\,kg^{-1}$) and immune cell adoptive transfer from immunized mice using irradiated IL-12/IL-18-transduced B16/F10 cells. (**b**) Photon counting of luc-B16/F10-pulmonary metastasis at day 21 ($n=6$–7). $^{\#}P<0.01$ (Kruskal–Wallis test). One of three independent experiments with similar results is shown.

differentiation, or apoptosis of cancer cells (Johnstone *et al.*, 2002; Minucci and Pelicci, 2006). In addition, normal fibroblasts and melanocytes are almost always considerably more resistant than tumor cells to depsipeptide (Kobayashi *et al.*, 2006; Minucci and Pelicci, 2006), suggesting this effect may be specific to malignant cells. We demonstrated the potential upregulation of MHC class I molecules by depsipeptide treatment. As the MHC class I molecule is released from the endoplasmic reticulum only after the peptide has bound and is allowed to reach the cell surface (Williams *et al.*, 2002), this upregulation could provide substantial benefits for the immunological recognition. However, there are abnormalities in the expression and/or function of various components of the MHC class I antigen-processing pathway in human malignant cells (Seliger *et al.*, 2000; Chang and Ferrone, 2007), and it remains to be elucidated whether HDACi affects various components of the protein-processing machinery.

Although the clinical use of depsipeptide when administered alone showed partial and complete responses in patients with hematological malignancy (Piekarz *et al.* 2001, 2004; Byrd *et al.*, 2005), only a partially objective response was observed in solid cancer patients (Sandor *et al.*, 2002; Stadler *et al.*, 2006), suggesting that depsipeptide alone appears far from beneficial in the treatment of cancer patients. However, moving the focus onto the modulation of tumor factors and targeting HDACs could provide great benefits, particularly for selective immunotherapy against cancer. Intriguingly, HDACi could activate components of death receptor pathways, including FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Nakata *et al.*, 2004; Singh *et al.*, 2005; Earel *et al.*, 2006). In fact, B16/F10 cells are less immunogenic and highly resistant to a variety of apoptotic stimuli if they are not manipulated (Avent *et al.*, 1979; Tsai *et al.*, 1997; Kalechman *et al.*, 1998). Nonetheless, exposure of B16 cells to a limited dose of depsipeptide induced cell surface expression of Fas and MHC class I, and the enforced Fas-engagement synergistically increased caspase-3/7 activity of B16/F10 cells in the presence of depsipeptide (Figure 3). Furthermore, these changes successfully provided CTLs with an enhanced ability to recognize and destroy target tumor cells (Figures 4 and 5).

Indeed, it has been demonstrated that HDACi synergizes with exogenously added TRAIL to induce apoptosis of various human solid tumor cell lines *in vitro* (Inoue *et al.*, 2004; Nakata *et al.*, 2004; Singh *et al.*, 2005; Lundqvist *et al.*, 2006). While the TRAIL system seems to be relatively major in the effect of HDACi on the human death receptor pathway, it has been suggested that the effect of HDACi on the death receptor pathway may not be universal (Minucci and Pelicci, 2006). We could not obtain a synergistic effect of depsipeptide with FasL in Fas-negative human MM-LH cells, and mouse Pmel-1 T cells did not express TRAIL on the cell surface (unpublished data). The Fas-FasL system is well known as the major pathway of CTL-mediated tumor destruction in murine models (Kagi *et al.*, 1994; Caldwell *et al.*, 2003; Lee *et al.*, 2006), and therefore some differences between species should be considered for the major death receptor pathway. However, current animal studies have demonstrated that the augmented tumoricidal effects of tumor-specific CTLs induced as a consequence of depsipeptide sensitization resulted in melanoma cells that produced CTL-mediated cytotoxicity *in vivo*. A limited administration of depsipeptide also sufficiently modulated the acetylation level of histone H3 at the tumor site (Figure 7b). These findings may provide the rationale for protocols that pretreat cancer patients with depsipeptide to potentiate adoptive immune therapy.

Emerging experimental data indicate that lymphodepletion using cyclophosphamide before adoptive transfer of tumor-specific T lymphocytes plays a key role in enhancing treatment efficacy by eliminating regulatory T cells and competing elements of the immune system (Ghiringhelli *et al.*, 2004; Lutsiak *et al.*, 2005). Furthermore, pretreatment with cyclophosphamide contributes to the elimination of immunosuppressive cells such as $CD4^+CD25^+$ Treg in patients with cancer and the depletion of endogenous cells that compete for the activation of cytokines (known as the "cytokine sink") to maximize the exposure of homeostatic cytokines to the transferred CTLs (Gattinoni *et al.*, 2006). In addition to this pretreatment, depsipeptide may safely be added to reduce barrier tumor factors limiting the therapeutic potential of adoptively transferred CTLs. We further demonstrated that depsipeptide increased the level of perforin in activated T cells to varying degrees: the number of perforin-expressing CTLs increased in mice, whereas an accumulation of perforin was observed in humans. Perforin release by T cells in conjunction with granzymes induces an apoptotic cascade in target cells (Kagi *et al.*, 1994). In fact, Palmer *et al.* (2004) showed that there was a B16 cell-dependent release of perforin after adoptive cell transfer of pmel-1 T cells, in which caspase-3 activation was also shown at the tumor site as a consequence of the downstream activation of perforin. Therefore, the residual depsipeptide in the plasma of pretreated hosts could be expected to release large amounts of toxic granules to the target tumor at the sites and, in combination with cyclophosphamide, the use of depsipeptide may be considered for host and tumor modulation before adoptive tumor-specific CTL transfer.

The therapeutic options for patients with metastatic disease remain limited, and the majority of these patients will develop a local or systemic recurrence. A variety of human cancers such as melanoma and breast, colon, and prostate cancers aberrantly express the chemokine receptor CXCR4 (Balkwill, 2004; Kakinuma and Hwang, 2006; Zlotnik, 2006), and its activation through prosurvival pathways such as Akt has been implicated as a mechanism by which cancer cells evade host immunity (Murakami *et al.*, 2003) and increase their metastatic properties (Kakinuma and Hwang, 2006). Recently, Lee *et al.* (2006) reported that sensitization of B16 cells with a CXCR4 antagonistic peptide increases the efficacy of immunotherapy for pulmonary metastases, suggesting that the inhibition of tumor factor is an effective strategy for melanoma immunotherapy. Herein, we also provide compelling *in vitro* and *in vivo* data suggesting that sensitization of less immunogenic B16 cells with depsipeptide facilitates the efficacy of immunotherapy

for established pulmonary metastases. Further investigations based on the findings of this study, especially those incorporating *in vivo* techniques, should improve the design of optimized clinical protocols.

MATERIALS AND METHODS

Animals, cells, and reagents

Male C57BL/6J mice (8–12 weeks old) were purchased from Charles River Japan Inc. (Tsukuba, Japan). Pmel-1 TCR pmel-1 +/Thy1.1 + transgenic mice (Overwijk *et al.*, 2003) were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments in this study were approved by the animal ethics review board of Jichi Medical University and performed in accordance with the Jichi Medical University Guide for Laboratory Animals, following the principles of laboratory animal care formulated by the National Society for Medical Research.

Human melanoma cell lines RPM-MC, MM-LH, MM-BP, and MM-RU were kindly provided by Dr H. Randolph Byers (Boston University Medical School) and maintained in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Byers *et al.*, 1991). Murine B16/F10 melanoma cells (Fidler, 1973) and EL-4 thymoma cells (Ralph, 1973) were grown in DMEM (GIBCO, Gaithersburg, MD) with 10% FCS and supplements (Sato *et al.*, 2006). Luciferase-expressing B16/F10 (luc-B16/F10) cells were generated previously (Sato *et al.*, 2006) and maintained in DMEM with 10% FCS and supplements, including puromycin (10 $\mu g\,ml^{-1}$; Sigma-Aldrich, St Louis, MO). Normal human epidermal melanocytes were purchased from Kurabo Biomedicals (Osaka, Japan) and maintained in Medium 154S with supplement (Kobayashi *et al.*, 2006). The cultures were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

Anti-acetyl-histone H3 (Lys 9), anti-acetyl-histone H3 (Lys 18), anti-histone H3, and anti-phospho-Rb (Ser 780) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse $p21^{Waf1/Cip1}$ (BD Pharmingen, San Diego, CA), anti-human perforin (clone δG9; BD Pharmingen), and anti-actin (sc-1616, Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies were used for western blotting. Flow cytometric analysis involved the use of phycoerythrin (PE)-conjugated anti-mouse H-$2D^b$ mAb, PE-conjugated anti-mouse Fas (CD95) mAb, PE-conjugated anti-mouse FasL (CD178) mAb, and isotype-matched IgG controls, all of which were purchased from BD Pharmingen. FITC-conjugated anti-mouse perforin mAb (clone: eBioOMAK-D) was purchased from eBioscience (San Diego, CA). Anti-mouse FasL mAb (clone MFL3; BD Pharmingen) was used for the FasL neutralization.

The expression plasmids for mouse IL-12 and IL-18, pCAGGS-IL-12 and pcDNA-mproIL-18-mICE respectively, have been described previously (Ajiki *et al.*, 2003). Depsipeptide (FK228) was obtained from Gloucester Pharmaceuticals (Cambridge, MA).

Reverse transcription-PCR

Total RNA was extracted from cells using Isogen (Nippon Gene, Toyama, Japan). Two micrograms of total RNA was used for first-strand synthesis using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was then performed using ExTaq polymerase (Takara, Ohtsu, Japan). The following primers were used for gp100/pmel-17 and perforin expression analysis: human gp100/pmel-17 sense, 5′-CCTCCTTCTCTATTGCCTTG-3′; human gp100/pmel-17 anti-sense, 5′-TGTAGGAGAGGTGAGCTTCA-3′; mouse gp100 sense, 5′-GGCCAACAACACCATCATCA-3′; mouse gp100 anti-sense, 5′-GGGCAAAGATGAGAGGATGA-3′; mouse perforin sense, 5′-ACAATAACAATCCCCGGTGG-3′; mouse perforin anti-sense, 5′-TGGGATTAAAGGCGTGTGCT-3′; glyceraldehyde-3-phosphate dehydrogenase sense, 5′-GTATCGTGGAAGGACTCATG-3′; and glyceraldehyde-3-phosphate dehydrogenase anti-sense, 5′-AGT GGGTGTCGCGCTGTTGAAG-3′. PCR conditions for each set of primers included an initial treatment at 95°C for 2 minutes, followed by 30 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 57°C for 30 seconds, and then extension at 72°C for 2 minutes. PCR products were analyzed using a 1% agarose gel.

Transfection and ELISA

B16/F10 cells (1×10^6) were transfected with pCAGGS-IL-12 (5 μg) and pcDNA-mproIL-18-mICE (5 μg) using Lipofectamine 2000 (Invitrogen). Immunization experiments involved irradiating IL-12/IL-18-transfected B16 cells with 80 Gy 36 hours after transfection. Irradiated cells ($1–2 \times 10^5$) were then injected twice into the subcutaneous space of C57BL/6 mice during the remaining weeks.

To analyze IFN-γ production, splenocytes ($1–2 \times 10^5$) that were isolated from immunized mice were co-cultured for 24 hours with 1×10^5 irradiated target cells, and the IFN-γ concentration of supernatants was then measured using a mouse IFN-γ immunoassay kit. All samples were assayed in triplicate.

Apoptosis and cytotoxic assay

To detect caspase-3/7 activity, B16/F10 cells (2×10^4 per well of a 96-well plate) were plated, and the Caspase-Glo 3/7 Assay system (Promega, Madison, WI) was used for analysis in accordance with the manufacturer's instructions 16 hours after the addition of depsipeptide. The background luminescence associated with the cell culture and assay reagent (blank reaction) was subtracted from experimental values. Means of triplicates were used to represent caspase-3/7 activity for the given cells. Each experiment was performed three times with similar results.

For enhancement of Fas-mediated apoptosis, B16/F10 cells were exposed to 10 $ng\,ml^{-1}$ recombinant human FLAG-tagged FasL (Apotech, San Diego, CA) in combination with 1 $mg\,ml^{-1}$ anti-FLAG M2 mAb (Sigma, St Louis, MO) for 16 hours with 5 nM depsipeptide at 37°C in the presence of 0.5% FCS, as described previously (Murakami *et al.*, 2003). After exposure of B16/F10 cells to apoptosis-enhancing conditions for 16 hours, attached (and detached) cells were collected from tissue culture plates for annexin-V staining according to the manufacturer's instructions (MEBCYTO Apoptosis Kit; MBL, Nagoya, Japan). Analysis of caspase-3/7 activity involved the assessment of 2×10^4 cells using the Caspase-Glo 3/7 Assay system (Promega).

Western blotting and flow cytometry

For western blotting, cells were lysed by sonication in radio-immuno-protein assay (RIPA) buffer (Sato *et al.*, 2006) and then centrifuged for 10 minutes at 4°C. Each cell extract (10 μg of protein) was assayed using appropriate antibodies and protein G-conjugated horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, UK).

For the flow cytometric analysis, cells (1×10^6) were washed with phosphate buffered saline (PBS) and incubated with mAb for

30 minutes at 4°C. Following washing with 0.1% FCS-PBS, cells were analyzed using FACS Calibur (Becton Dickinson, Mountain View, CA) and FlowJo analysis software (Tree Star, San Carlos, CA).

Subcutaneous and intravenous tumor inoculation

Cells in an exponential growth phase were harvested by trypsinization and washed twice in PBS before injection. For the s.c. injections, cells (1×10^6) were injected into the abdominal subcutaneous space of C57BL/6 mice. Tumor growth at the skin was monitored by measurement of the two maximum perpendicular tumor diameters. For the intravenous injections to the lungs, luc-B16/F10 cells (5×10^4 in 0.2 ml PBS) were injected into the tail vein of C57BL/6 mice. Each experiment was performed 2–4 times with similar results.

In vivo bioluminescence imaging

In vivo tumor progression was examined using the noninvasive bioimaging system IVIS (Xenogen, Alameda, CA). Tumor-implanted mice were anesthetized with a mixture of ketamine and xylazine, and D-luciferin (potassium salt; Biosynth, Postfach, Switzerland) was injected into the peritoneal cavity at 2 mg per animal, which was followed immediately by the measurement of luciferase activity. The imaging system consisted of a cooled, back-thinned charge-coupled device camera to capture both a visible light photograph of the animal taken with light-emitting diodes and a luminescent image. After acquiring photographic images of each mouse, luminescent images were acquired with a 1–15 minutes exposure time (Ohsawa *et al.*, 2006; Sato *et al.*, 2006). Images were obtained with a 25 cm field of view, a binning (resolution) factor of 8, 1/f stop, and an open filter. The resulting gray scale photographic and pseudo-color luminescent images were automatically superimposed by software to facilitate the identification of any optical signal and the location on the mouse. Optical images were displayed and analyzed using Igor (WaveMetrics, Lake Oswego, OR) and IVIS Living Image (Xenogen) software packages. The signal from tumors was quantified as photons flux in units of photons per second per cm^2 per sr.

Immunohistochemistry

Removed specimens were fixed with 10% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) deparaffinized in xylene were passed through graded alcohols before being treated with 1% H_2O_2 (v/v) in H_2O for 20 minutes at room temperature. After washing the sections three times with PBS, sections were blocked for 20 minutes with 10% FCS diluted in PBS. All incubations were performed at room temperature in a moist chamber. The slides were incubated overnight at 4°C with anti-acetyl-histone H3 (Lys 18) antibody (no. 9675, Cell Signaling Technology) diluted 1:100 in blocking solution. The sections were washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), and staining was visualized using a streptavidin-peroxidase conjugate (Vector Laboratories). A diaminobenzidine substrate kit (Vector Laboratories) was used for color (brown) visualization, and sections were counterstained with hematoxylin.

Statistical analysis

P-values based on two-sided Student's *t*-test, Mann–Whitney test, or Kruskal–Wallis test were obtained using the Instat software package (GraphPad, San Diego, CA). Differences between groups were considered significant if $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank Ms Yasuko Sakuma, Ms Yumi Ohde, and Ms Masayo Kumagai for their skillful technical assistance. This study was supported by a grant to T.M. from the Kowa Life Science Foundation (2004) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (Project No. 17591181; 2005–2007) and by a grant from the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT (2003–2007).

SUPPLEMENTARY MATERIAL

Figure S1. Expression of HLA class I (A, B, C) and Fas (CD95/Apo-1) in human melanoma cell lines following exposure to depsipeptide.

Figure S2. Increase of perforin in PHA-stimulated human T cells by exposure to depsipeptide (4 nM).

Figure S3. Effect of depsipeptide on the subcutaneous tumor of B16/F10 cells.

REFERENCES

Ajiki T, Murakami T, Kobayashi Y, Hakamata Y, Wang J, Inoue S *et al.* (2003) Long-lasting gene expression by particle-mediated intramuscular transfection modified with bupivacaine: combinatorial gene therapy with IL-12 and IL-18 cDNA against rat sarcoma at a distant site. *Cancer Gene Ther* 10:318–29

Antony PA, Restifo NP (2005) CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother* 28:120–8

Avent J, Vervaert C, Seigler HF (1979) Non-specific and specific active immunotherapy in a B16 murine melanoma system. *J Surg Oncol* 12: 87–96

Balkwill F (2004) Cancer and the chemokine network. *Nat Rev Cancer* 4: 540–50

Byers HR, Etoh T, Doherty JR, Sober AJ, Mihm MC Jr (1991) Cell migration and actin organization in cultured human primary, recurrent cutaneous and metastatic melanoma. Time-lapse and image analysis. *Am J Pathol* 139:423–35

Byrd JC, Marcucci G, Parthun MR, Xiao JJ, Klisovic RB, Moran M *et al.* (2005) A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. *Blood* 105:959–67

Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F (2003) Analysis of HLA expression in human tumor tissues. *Cancer Immunol Immunother* 52:1–9

Caldwell SA, Ryan MH, McDuffie E, Abrams SI (2003) The Fas/Fas ligand pathway is important for optimal tumor regression in a mouse model of CTL adoptive immunotherapy of experimental CMS4 lung metastases. *J Immunol* 171:2402–12

Chang CC, Ferrone S (2007) Immune selective pressure and HLA class I antigen defects in malignant lesions. *Cancer Immunol Immunother* 56: 227–36

Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D *et al.* (2005) Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115: 3623–33

Dudley ME, Wunderlich J, Nishimura MI, Yu D, Yang JC, Topalian SL *et al.* (2001) Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J Immunother* 24:363–73

Earel JK Jr, VanOosten RL, Griffith TS (2006) Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells. *Cancer Res* 66: 499–507

Ferrone S, Marincola FM (1995) Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today* 16:487–94

Fidler IJ (1973) Selection of successive tumour lines for metastasis. *Nat New Biol* 242:148–9

Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP (2006) Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol* 6: 383–93

Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C *et al.* (2004) $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34:336–44

Herman JG, Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349:2042–54

Hoshikawa Y, Kwon HJ, Yoshida M, Horinouchi S, Beppu T (1994) Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. *Exp Cell Res* 214:189–97

Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ, Cohen GM (2004) Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* 11(Suppl 2):S193–206

Johnstone RW, Licht JD (2003) Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell* 4:13–8

Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108:153–64

Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S *et al.* (1994) Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265:528–30

Kakinuma T, Hwang ST (2006) Chemokines, chemokine receptors, and cancer metastasis. *J Leukoc Biol* 79:639–51

Kalechman Y, Strassmann G, Albeck M, Sredni B (1998) Up-regulation by ammonium trichloro(dioxoethylene-0,0') tellurate (AS101) of Fas/Apo-1 expression on B16 melanoma cells: implications for the antitumor effects of AS101. *J Immunol* 161:3536–42

Klisovic DD, Katz SE, Effron D, Klisovic MI, Wickham J, Parthun MR *et al.* (2003) Depsipeptide (FR901228) inhibits proliferation and induces apoptosis in primary and metastatic human uveal melanoma cell lines. *Invest Ophthalmol Vis Sci* 44:2390–8

Kobayashi Y, Ohtsuki M, Murakami T, Kobayashi T, Sutheesophon K, Kitayama H *et al.* (2006) Histone deacetylase inhibitor FK228 suppresses the Ras-MAP kinase signaling pathway by upregulating Rap1 and induces apoptosis in malignant melanoma. *Oncogene* 25:512–24

Lee CH, Kakinuma T, Wang J, Zhang H, Palmer DC, Restifo NP *et al.* (2006) Sensitization of B16 tumor cells with a CXCR4 antagonist increases the efficacy of immunotherapy for established lung metastases. *Mol Cancer Ther* 5:2592–9

Lundqvist A, Abrams SI, Schrump DS, Alvarez G, Suffredini D, Berg M *et al.* (2006) Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res* 66:7317–25

Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H (2005) Inhibition of CD4(+)25(+) T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* 105:2862–8

Maecker HL, Yun Z, Maecker HT, Giaccia AJ (2002) Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. *Cancer Cell* 2:139–48

Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK (2001) Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer* 1:194–202

Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* 6:38–51

Murakami T, Cardones AR, Finkelstein SE, Restifo NP, Klaunberg BA, Nestle FO *et al.* (2003) Immune evasion by murine melanoma mediated through CC chemokine receptor-10. *J Exp Med* 198:1337–47

Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T (2004) Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. *Oncogene* 23:6261–71

Ohsawa I, Murakami T, Uemoto S, Kobayashi E (2006) *In vivo* luminescent imaging of cyclosporin A-mediated cancer progression in rats. *Transplantation* 81:1558–67

Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA *et al.* (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 198:569–80

Palmer DC, Balasubramaniam S, Hanada K, Wrzesinski C, Yu Z, Farid S *et al.* (2004) Vaccine-stimulated, adoptively transferred CD8+ T cells traffic indiscriminately and ubiquitously while mediating specific tumor destruction. *J Immunol* 173:7209–16

Piekarz RL, Robey R, Sandor V, Bakke S, Wilson WH, Dahmoush L *et al.* (2001) Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. *Blood* 98:2865–8

Piekarz RL, Robey RW, Zhan Z, Kayastha G, Sayah A, Abdeldaim AH *et al.* (2004) T-cell lymphoma as a model for the use of histone deacetylase inhibitors in cancer therapy: impact of depsipeptide on molecular markers, therapeutic targets, and mechanisms of resistance. *Blood* 103:4636–43

Ralph P (1973) Retention of lymphocyte characteristics by myelomas and theta+-lymphomas: sensitivity to cortisol and phytohemagglutinin. *J Immunol* 110:1470–5

Rosenberg SA (2004) Shedding light on immunotherapy for cancer. *N Engl J Med* 350:1461–3

Rosenberg SA, Dudley ME (2004) Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. *Proc Natl Acad Sci USA* 101(Suppl 2):14639–45

Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 10:909–15

Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J *et al.* (2002) Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin Cancer Res* 8:718–28

Sato A, Ohtsuki M, Hata M, Kobayashi E, Murakami T (2006) Antitumor activity of IFN-lambda in murine tumor models. *J Immunol* 176: 7686–94

Seliger B, Maeurer MJ, Ferrone S (2000) Antigen-processing machinery breakdown and tumor growth. *Immunol Today* 21:455–64

Singh TR, Shankar S, Srivastava RK (2005) HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in breast carcinoma. *Oncogene* 24:4609–23

Stadler WM, Margolin K, Ferber S, McCulloch W, Thompson JA (2006) A phase II study of depsipeptide in refractory metastatic renal cell cancer. *Clin Genitourin Cancer* 5:57–60

Tsai V, Southwood S, Sidney J, Sakaguchi K, Kawakami Y, Appella E *et al.* (1997) Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary *in vitro* immunization with peptide-pulsed dendritic cells. *J Immunol* 158: 1796–802

Ueda H, Manda T, Matsumoto S, Mukumoto S, Nishigaki F, Kawamura I *et al.* (1994a) FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum No. 968. III. Antitumor activities on experimental tumors in mice. *J Antibiot (Tokyo)* 47: 315–23

Ueda H, Nakajima H, Hori Y, Goto T, Okuhara M (1994b) Action of FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum no. 968, on Ha-ras transformed NIH3T3 cells. *Biosci Biotechnol Biochem* 58:1579–83

Williams A, Peh CA, Elliott T (2002) The cell biology of MHC class I antigen presentation. *Tissue Antigens* 59:3–17

Zlotnik A (2006) Chemokines and cancer. *Int J Cancer* 119:2026–9

Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil

Takako Eguchi Nakajima[1,2], Masahiro Yasunaga[2], Yasuhiko Kano[3], Fumiaki Koizumi[4], Ken Kato[1], Tetsuya Hamaguchi[1], Yasuhide Yamada[1], Kuniaki Shirao[1], Yasuhiro Shimada[1] and Yasuhiro Matsumura[2*]

[1]*Gastrointestinal Oncology Division, National Cancer Center Hospital, Tokyo, Japan*
[2]*Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan*
[3]*Hematology Oncology, Tochigi Cancer Center, Tochigi, Japan*
[4]*Shien Lab Medical Oncology Division, National Cancer Center Hospital, Tokyo, Japan*

Int. J. Cancer: **122,** 2148–2153 (2008)

Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil

Takako Eguchi Nakajima[1,2], Masahiro Yasunaga[2], Yasuhiko Kano[3], Fumiaki Koizumi[4], Ken Kato[1], Tetsuya Hamaguchi[1], Yasuhide Yamada[1], Kuniaki Shirao[1], Yasuhiro Shimada[1] and Yasuhiro Matsumura[2]*

[1]*Gastrointestinal Oncology Division, National Cancer Center Hospital, Tokyo, Japan*
[2]*Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan*
[3]*Hematology Oncology, Tochigi Cancer Center, Tochigi, Japan*
[4]*Shien Lab Medical Oncology Division, National Cancer Center Hospital, Tokyo, Japan*

The authors reported in a previous study that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)-releasing nano-system, exhibited high antitumor activity against human colorectal cancer xenografts. This study was conducted to investigate the advantages of NK012 over irinotecan hydrochloride (CPT-11) administered in combination with 5-fluorouracil (5FU). The cytotoxic effects of NK012 or SN-38 (an active metabolite of CPT-11) administered in combination with 5FU was evaluated *in vitro* in the human colorectal cancer cell line HT-29 by the combination index method. The effects of the same drug combinations was also evaluated *in vivo* using mice bearing HT-29 and HCT-116 cells. All the drugs were administered i.v. 3 times a week; NK012 (10 mg/kg) or CPT11 (50 mg/kg) was given 24 hr before 5FU (50 mg/kg). Cell cycle analysis in the HT-29 tumors administered NK012 or CPT-11 *in vivo* was performed by flow cytometry. NK012 exerted more synergistic activity with 5FU compared to SN-38. The therapeutic effect of NK012/5FU was significantly superior to that of CPT-11/5FU against HT-29 tumors ($p = 0.0004$), whereas no significant difference in the antitumor effect against HCT-116 tumors was observed between the 2-drug combinations ($p = 0.2230$). Cell-cycle analysis showed that both NK012 and CPT-11 tend to cause accumulation of cells in the S phase, although this effect was more pronounced and maintained for a more prolonged period with NK012 than with CPT-11. Optimal therapeutic synergy was observed between NK012 and 5FU, therefore, this regimen is considered to hold promise of clinical benefit, especially for patients with colorectal cancer.

Key words: NK012; SN-38; 5-fluorouracil; drug delivery system; colorectal cancer

The 5-year survival rates of colorectal cancer (CRC) have improved remarkably over the last 10 years, accounted for in large part by the extensively investigated agents after 5-fluorouracil (5FU). Irinoetcan hydrochloride (CPT-11), a water-soluble, semi-synthetic derivative of camptothecin, is one such agent that has been shown to be highly effective, and currently represents a key-drug in first- and second-line treatment regimens for CRC. CPT-11 monotherapy, however, has not been shown to yield superior efficacy, including in terms of the median survival time, to bolus 5FU/leucovorin (LV) alone.[1] In 2 Phase III trials, the addition of CPT-11 to bolus or infusional 5FU/LV regimens clearly yielded greater efficacy than administration of 5FU/LV alone, with a doubling of the tumor response rate and prolongation of the median survival time by 2–3 months.[1,2]

CPT-11 is converted to 7-ethyl-10-hydroxy-camptothecin (SN-38), a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases in the liver and the tumor. SN-38 has been demonstrated to exhibit up to a 1,000-fold more potent cytotoxic activity than CPT-11 against various cancer cells *in vitro*.[3] The metabolic conversion rate is, however, very low, with only <10% of the original volume of CPT-11 being metabolized to SN-38[4,5]; conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of carboxylesterases.[6] Direct use of SN-38 itself for clinical cancer treatment must be shown to be identical in terms of both efficacy and toxicity.

Some drugs incorporated in drug delivery systems (DDS), such as Abraxane and Doxil, are already in clinical use.[7,8] The clinical benefits of DDS are based on their EPR effect.[9] The EPR effect is based on the pathophysiological characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Several types of DDS can be used for incorporation of a drug. A liposome-based formulation of SN-38 (LE-SN38) has been developed, and a clinical trial to assess its efficacy is now under way.[10,11]

Recently, we demonstrated that NK012, novel SN-38-incorporating polymeric micelles, exerted superior antitumor activity and less toxicity than CPT-11.[12] NK012 is characterized by a smaller size of the particles than LE-SN38; the mean particle diameter of NK012 is 20 nm. NK012 can release SN-38 under neutral conditions even in the absence of a hydrolytic enzyme, because the bond between SN-38 and the block copolymer is a phenol ester bond, which is stable under acidic conditions and labile under mild alkaline conditions. The release rate of SN-38 from NK012 under physiological conditions is quite high; more than 70% of SN-38 is released within 48 hr. We speculated that the use of NK012, in place of CPT-11, in combination with 5FU may yield superior results in the treatment of CRC. In the present study, we evaluated the antitumor activity of NK012 administered in combination with 5FU as compared to that of CPT-11 administered in combination with 5FU against CRC in an experimental model.

Material and methods

Cells and animals

The human colorectal cancer cell lines used, namely, HT-29 and HCT-116, were purchased from the American Type Culture Collection (Rockville, MD). The HT-29 cells and HCT-116 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin, streptomycin, and amphotericin B (100 units/mL, 100 μg/mL, and 25 μg/mL, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

BALB/c *nu/nu* mice were purchased from SLC Japan (Shizuoka, Japan). Six-week-old mice were subcutaneously (s.c.)

***Correspondence to:** Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. Fax: +81-4-7134-6866. E-mail: yhmatsum@east.ncc.go.jp
Received 2 September 2007; Accepted after revision 20 November 2007
DOI 10.1002/ijc.23381
Published online 14 January 2008 in Wiley InterScience (www.interscience.wiley.com).

inoculated with 1×10^6 cells of HT-29 or HCT-116 cell line in the flank region. The length (a) and width (b) of the tumor masses were measured twice a week, and the tumor volume (TV) was calculated as follows: $TV = (a \times b^2)/2$. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Drugs

The SN-38-incorporating polymeric micelles, NK012, and SN-38 were prepared by Nippon Kayaku (Tokyo, Japan).[12] CPT-11 was purchased from Yakult Honsha (Tokyo, Japan). 5FU was purchased from Kyowa Hakko (Tokyo, Japan).

Cell growth inhibition assay

HT-29 cells were seeded in 96-well plates at a density of 2,000 cells/well in a final volume of 90 μL. Twenty-four hours after seeding, a graded concentration of NK012 or SN-38 was added concurrently with 5FU to the culture medium of the HT-29 cells in a final volume of 100 μL for drug interaction studies. The culture was maintained in the CO_2 incubator for an additional 72 hr. Then, cell growth inhibition was measured by the tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). WST-1 labeling solution (10 μL) was added to each well and the plates were incubated at 37°C for 3 hr. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader. Cell viability was measured and compared to that of the control cells. Each experiment was carried out in triplicate and was repeated at least 3 times. Data were averaged and normalized against the nontreated controls to generate dose-response curves.

Drug interaction analysis

The nature of interaction between NK012 or SN-38 and 5FU against HT-29 cells was evaluated by median-effect plot analyses and the combination index (CI) method of Chou and Talalay.[13] Data analysis was performed using the Calcusyn software (Biosoft, NY, USA). NK012 or SN-38 was combined with 5FU at a fixed ratio that spanned the individual IC_{50} values of each drug. The IC_{50} values were determined on the basis of the dose-response curves using the WST assay. For any given drug combination, the CI is known to represent the degree of synergy, additivity or antagonism. It is expressed in terms of fraction-affected (Fa) values, which represents the percentage of cells killed or inhibited by the drug. Isobologram equations and Fa/CI plots were constructed by computer analysis of the data generated from the median effect analysis. Each experiment was performed in triplicate with 6 gradations and was repeated at least 3 times. The resultant dose-response curves were averaged, to create a single composite dose-response curve for each combination.

In vivo analysis of the effects of NK012 combined with 5FU as compared to those of CPT-11 combined with 5FU

When the mean tumor volumes reached ~93 mm^3, the mice were randomly divided into test groups consisting of 5 mice per group (Day 0). The drugs were administered i.v. *via* the tail vein of the mice. In the groups administered NK012 or 5FU as single agents, the drug was administered on Days 0, 7 and 14. In the combined treatment groups, NK012 or CPT-11 was administered 24 hr before 5FU on Days 0, 7 and 14, according to the previously reported combination schedule for CPT-11 and 5FU.[14] Complete response (CR) was defined as tumor not detectable by palpation at 90 days after the start of treatment, at which time-point the mice were sacrificed. Tumor volume and body weight were measured twice a week. As a general rule, animals in which the tumor volume exceeded 2,000 mm^3 were also sacrificed.

Experiment 1. Evaluation of the effects of NK012 combined with 5FU and determination of the maximum tolerated dose (MTD) of NK012/5FU. By comparing the data between NK012 administered as a single agent and NK012/5FU, we evaluated the effects of the combined regimen against the s.c HT-29 tumors. A preliminary experiment showed that combined administration of NK012 15 mg/kg + 5FU 50 mg/kg every 6 days caused drug-related lethality (data not shown). To determine the MTD, therefore, we set the dosing schedule of the combined regimen at 5 or 10 mg/kg of NK012 + 50 mg/kg of 5FU three times a week.

Experiment 2. Comparison of the antitumor effect of NK012/5FU and CPT-11/5FU. Based on a comparison of the data between NK012/5FU and CPT-11/5FU against the s.c. HT-29 and HCT-116 tumors, we investigated the feasibility of the clinical application of NK012/5FU for the treatment of CRC. CPT-11/5FU was administered three times a week at the respective MTDs of the 2 drugs as previously reported, that is, CPT11 at 50 mg/kg and 5FU at 50 mg/kg, respectively.[14] NK012/5FU was administered once three times a week at the respective MTDs of the 2 drugs determined from Experiment 1.

Cell cycle analysis

Samples from the HT-29 tumors that had grown to 80–100 mm^3 were removed from the mice at 6, 24, 48, 72 and 96 hr after the administration of NK012 alone at 10 mg/kg or CPT-11 alone at 50 mg/kg. The samples were excised, minced in PBS and fixed in 70% ethanol at −20°C for 48 hr. They were then digested with 0.04% pepsin (Sigma chemical Co., St Lous, MO) in 0.1 N HCL for 60 min at 37°C in a shaking bath to prepare single-nuclei suspensions. The nuclei were then centrifuged, washed twice with PBS and stained with 40 μg/mL of propidium iodide (Molecular Probes, OR) in the presence of 100 μg/mL RNase in 1 mL PBS for 30 min at 37°C. The stained nuclei were analyzed with B-D FACSCalibur (BD Biosciences, San Jose, CA), and the cell cycle distribution was analyzed using the Modfit program (Verity Software House Topsham, ME).

Statistical analyses

Data were expressed as mean ± SD. Data were analysed with Student's t test when the groups showed equal variances (F test), or Welch's test when they showed unequal variances (F test). $p < 0.05$ was regarded as statistically significant. All statistical tests were 2-sided.

Results

Antiproliferative effects of NK012 or SN-38 administered in combination with 5FU

Figure 1*a* shows the dose-response curves for NK012 alone, 5FU alone and a combination of the two. The IC_{50} levels of NK012 and 5FU against the HT-29 cells were 39 nM and 1 μM, respectively, and the IC_{50} level of SN-38 was 14 nM (data not shown). Based on these data, the molar ratio of NK012 or SN-38:5FU of 1:1,000 was used for the drug combination studies.

Figures 1*b* and 1*c* show the median-effect and the combination index plots. Combination indices (CIs) of <1.0 are indicative of synergistic interactions between 2 agents; additive interactions are indicated by CIs of 1.0, and antagonism by CIs of >1.0. Figure 1*c* shows the combination index for NK012 and 5FU, when 2 drugs are supposed to be mutually exclusive. Marked synergism was observed between Fa 0.2 and 0.6. Theoretically, the CI method is the most reliable around an Fa of 0.5, suggesting synergistic effects of the combination of NK012 and 5FU. This synergistic effect was more evident than that of SN-38/5FU (Fig. 1*d*).

In vivo *effect of combined NK012 and 5FU*

Experiment 1. Dose optimization and effect of combined NK012 and 5FU against HT-29 tumors. Comparison of the relative tumor volumes on Day 40 revealed significant differences between

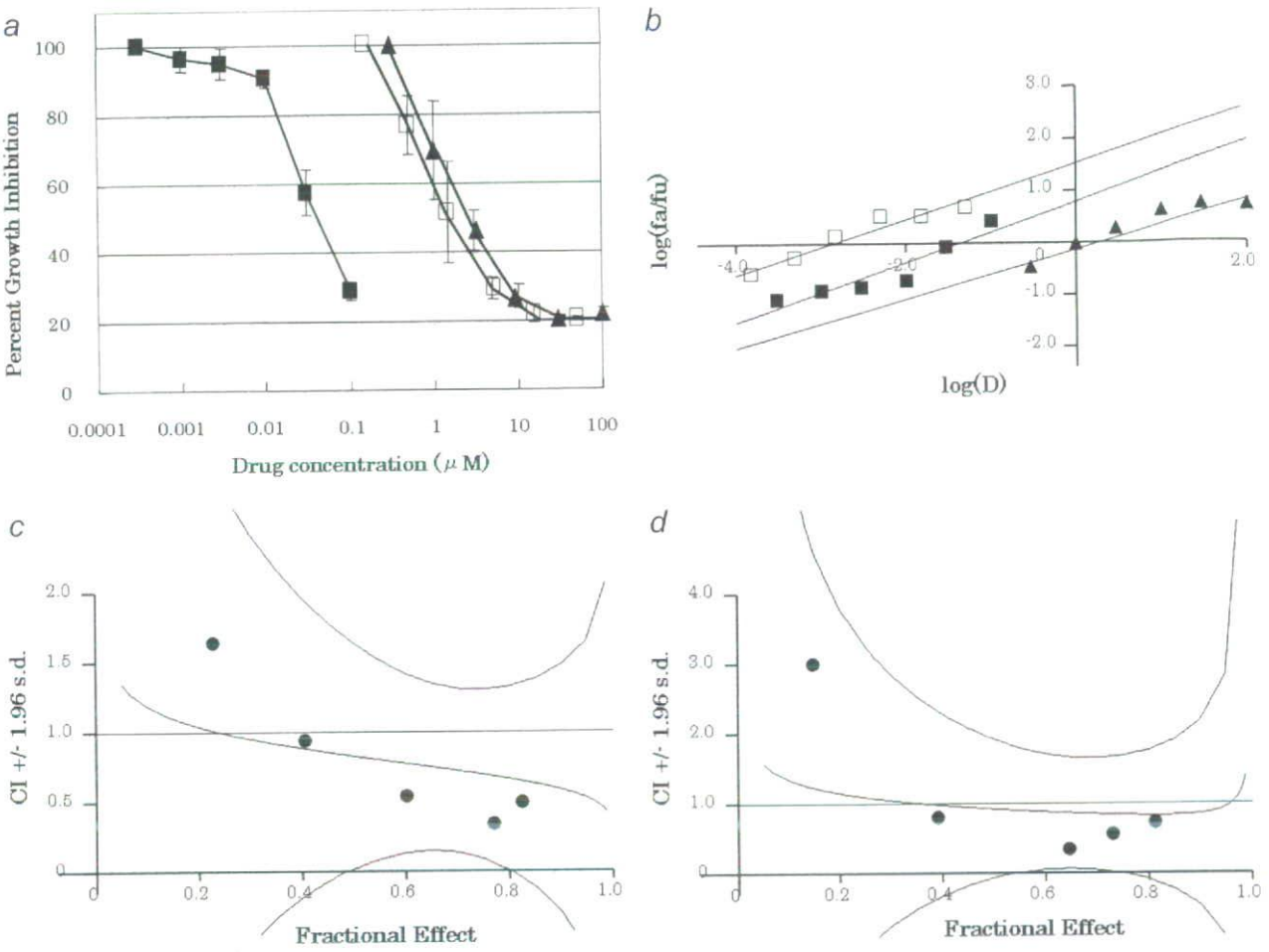

FIGURE 1 – Interaction of NK012 and 5FU *in vitro*. (*a*) Dose-response curves for NK012 alone (■), 5FU alone (▲) and their combination (□) against HT-29 cells. HT-29 cells were seeded at 2,000 cells/well. Twenty-four hours after seeding, a graded concentration of NK012 or 5FU was added to the culture medium of the HT-29 cells. Cell growth inhibition was measured by WST assay after 72 hr of treatment. Cell viability was measured and compared with that of the control cells. Each experiment was carried out independently and repeated at least 3 times. Points, mean of tripricates; bars, SD. (*b*) Median effect plot for the interaction of NK012 and 5FU. (*c*, *d*) Combination index for the interaction as a function of the level of effect (fraction effect = 0.5 is the IC_{50}). The straight line across the CI value of 1.0 indicates additive effect and CIs above and below indicate antagonism and synergism, respectively. The molar ratio of NK012/5FU (*c*) or SN-38/5FU (*d*) at 1:1,000 was tested by CI analysis. Black circles represent the CIs of the actual data points, solid lines represent the computer-derived CIs at effect levels ranging from 10 to 100% inhibition of cell growth, and the dotted lines represent the 95% confidence intervals.

those in the mice administered NK012 alone and those administered NK012/5FU at 5 mg/kg of NK012 ($p = 0.018$) (Fig. 2*a*). Although there was no statistically significant difference in the relative tumor volume measured on Day 54 between the mice administered NK012 alone and NK012/5FU at 10 mg/kg of NK012 ($p = 0.3050$), a trend of superior antitumor effect was demonstrated in the group treated with NK012/5FU at 10 mg/kg of NK012 (Fig. 2*a*). The CR rates were 20, 40 and 60% for 5 mg/kg NK012 + 50 mg/kg 5FU, 10 mg/kg NK012 alone and 10 mg/kg NK012 + 50 mg/kg 5FU, respectively. The schedule of 10 mg/kg NK012 + 50 mg/kg 5FU resulted in no remarkable toxicity in terms of body weight changes, and these doses were determined as representing the MTDs (Fig. 2*b*).

Experiment 2. Comparison of the antitumor effect of combined NK012/5FU and CPT-11/5FU against HT-29 and HCT-116 tumors. The therapeutic effect of NK012/5FU on Day 60 was significantly superior to that of CPT-11/5FU against the HT-29 tumors ($p = 0.0004$) (Fig. 3*a*). A more potent antitumor effect, namely, a 100% CR rate, was obtained in the NK012/5FU group as compared to the 0% CR rate in the CPT-11/5FU group. Although no statistically significant difference in the relative tumor volume on Day 61 was demonstrated between the NK012/5FU and CPT-11/5FU in the case of the HCT-116 tumors ($p = 0.2230$), a trend of superior antitumor effect against these tumors was observed in the NK012/5FU treatment group (Fig. 3*b*). The CR rates for the case of the HCT-116 tumors were 0% in both NK012/5FU and CPT-11/5FU groups.

Specificity of cell cycle perturbation

We studied the differences in the effects between NK012 10 mg/kg and CPT-11 50 mg/kg on the cell cycle (Fig. 4*a*). The data indicated that both NK012 and CPT-11 tended to cause accumulation of cells in the S phase, although the effect of NK012 was stronger and maintained for a more prolonged period than that of CPT-11; the maximal percentage of S-phase cells in the total cell population in the tumors was 34% at 24 hr after the administration of CPT-11, whereas it was 39% at 48 hr after the administration of NK012 (Figs. 4*b*, and 4*c*).

Discussion

Our primary endpoint was to clarify the advantages of NK012 over CPT-11 administered in combination with 5FU. We demonstrated that combined NK012 and 5FU chemotherapy exerts more

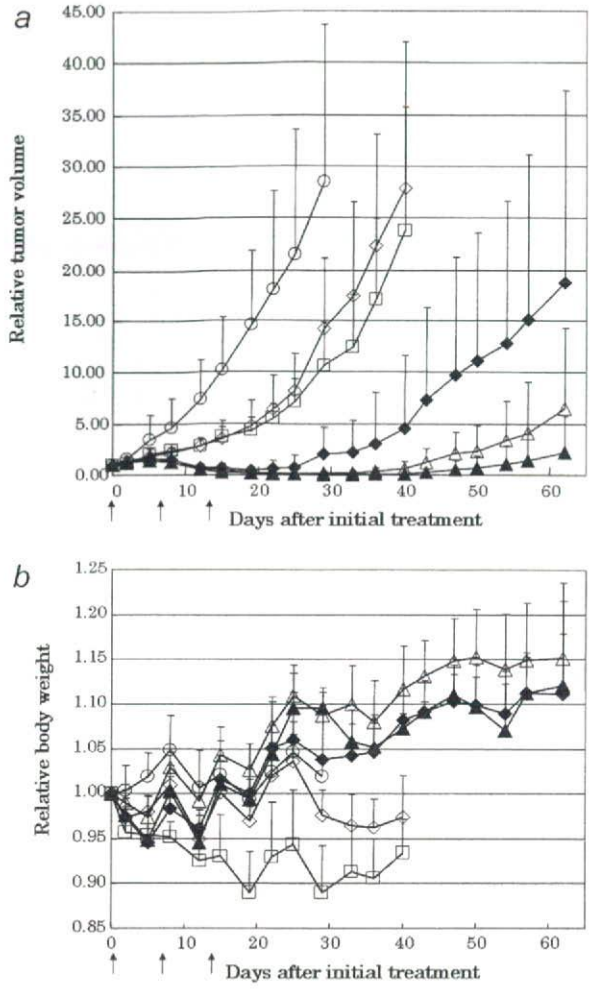

FIGURE 2 – Effect of NK012 alone or NK012 in combination with 5FU against HT-29 tumor-bearing mice. Points, mean; bars, SD. (*a*) Antitumor effect of each regimen on Days 0, 7 and 14. (○) control, (□) 5FU 50 mg/kg alone, (◇) NK012 5 mg/kg alone, (◆) NK012 5 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg alone, (▲) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. (*b*) Changes in the relative body weight. Data were derived from the same mice as those used in the present study.

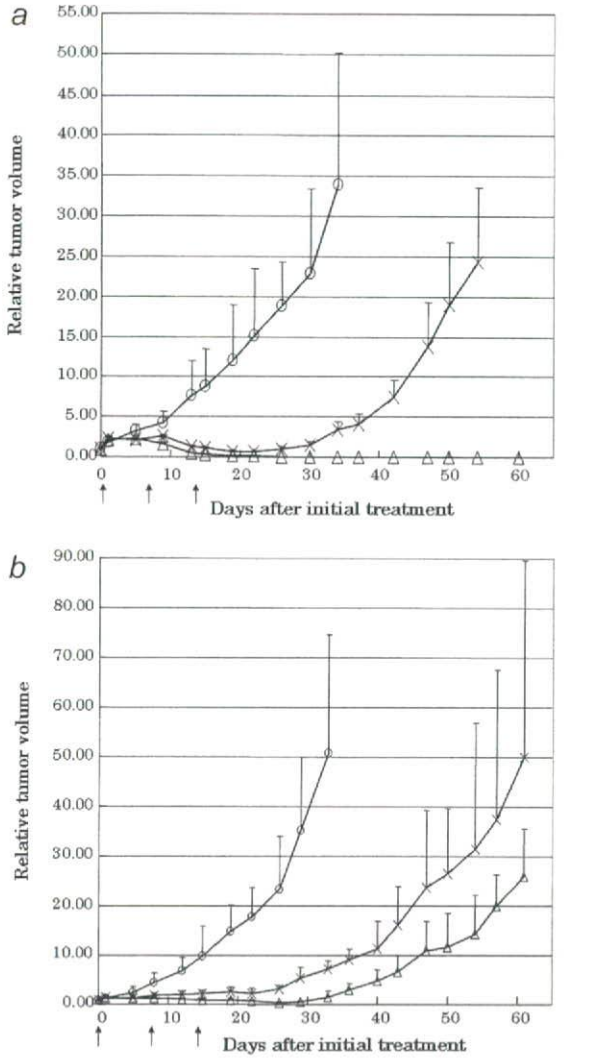

FIGURE 3 – Effect of NK012/5FU as compared with that of CPT11/5FU against HT-29 (*a*) or HCT-116 (*b*) tumor-bearing mice. Antitumor effect of each schedule on Days 0, 7 and 14. (○) control, (×) CPT-11 50 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. Points, mean; bars, SD.

synergistic activity *in vitro* and significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU. The combination of NK012 and 5FU is considered to hold promise of clinical benefit for patients with CRC.

CPT-11, a topoisomerase-I inhibitor, and 5FU, a thymidilate synthase inhibitor, have been demonstrated to be effective agents for the treatment of CRC. A combination of these 2 drugs has also been demonstrated to be clearly more effective than either CPT-11 or 5FU/LV administered alone *in vivo* and in clinical settings.[1,2,14] Administration of 5FU by infusion with CPT-11 was shown to be associated with reduced toxicity and an apparent improvement in survival as compared to that of administration of the drug by bolus injection with CPT-11.[1,2] This synergistic enhancement may result from the mechanism of action of the 2 drugs; CPT-11 has been reported to cause accumulation of cells in the S phase, and 5FU infusion is known to cause DNA damage specifically in cells of the S phase.[14] On the basis of this background, our results suggesting the more pronounced and more prolonged accumulation of the tumor cells in the S phase caused by NK012 as compared with that by CPT-11 may explain the more effective synergy of the former administered with 5FU infusion. This may be attributable to accumulation of NK012 due to the enhanced permeability and retention (EPR) effect.[9] It is also speculated that NK012 allows sustained release of free SN-38, which may move more freely in the tumor interstitium.[15] Otherwise NK012 itself could internalize into cells to localize in several cytoplasmic organelles as reported by Savic *et al.*[16] These characteristics of NK012 may be responsible for its more potent antitumor activity observed in this study, because CPT-11 has been reported to show time-dependent growth-inhibitory activity against the tumor cells.[17]

The major dose-limiting toxicities of CPT-11 are diarrhea and neutropenia. SN-38, the active metabolite of CPT-11, may cause CPT-11-related diarrhea as a result of mitotic -inhibitory activity.[18] Because it undergoes significant biliary excretion, SN-38 may have a potentially long residence time in the gastrointestinal tract that may be associated with prolonged diarrhea.[19,20] In our previous report, we evaluated the tissue distribution of SN-38 after administration of an equimolar amount of NK012 (20 mg/kg) and CPT-11 (30 mg/kg), and found no difference in the level of SN-38 accumulation in the small intestine.[12] A significant antitumor effect of NK012 with a lower incidence of diarrhea was also dem-

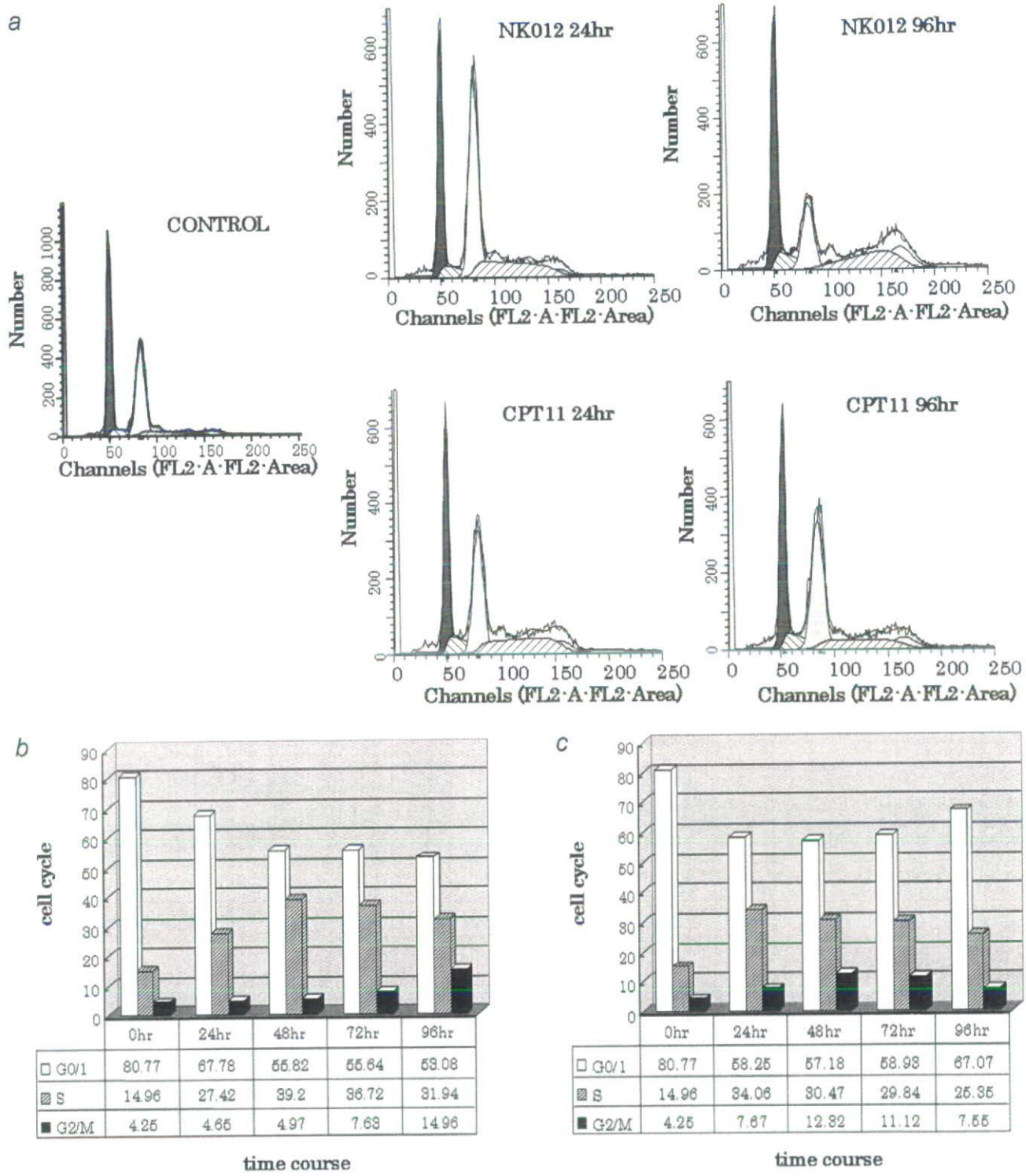

FIGURE 4 – Cell cycle analysis of HT-29 tumor cells collected 24, 48, 72 and 96 hr after administration of NK012 at 10 mg/kg alone or CPT-11 at 50 mg/kg alone using the Modfit program (Verity Software House Topsham, ME). (*a*) Cell cycle analysis of HT-29 tumor cells 24 and 96 hr after administration of NK012 at 10 mg/kg or CPT-11 at 50 mg/kg, respectively. (*b*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with NK012 at 10 mg/kg. (*c*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with CPT-11 at 50 mg/kg.

onstrated as compared to that observed with CPT-11 in a rat mammary tumor model.[21] Combined administration of CPT-11 with 5FU/LV infusion appears to be associated with acceptable toxicity in patients with CRC. In addition, no significant difference in the frequency of Grade 3/4 diarrhea was noted between patients treated with FOLFIRI (CPT-11 regimen with bolus and infusional 5FU/LV) and those treated with FOLFOX6 (oxaliplatin regimen with bolus and infusional 5FU/LV).[22,23] Our *in vivo* data actually revealed no severe body weight loss in the NK012/5FU group. Consequently, we expect that the NK012/5FU regimen, especially

with infusional 5FU, may be an attractive arm for a Phase III trial in CRC, with CPT-11/5FU as the control arm. We have already initiated a Phase I trial of NK012 in patients with advanced solid tumors based on the data suggesting higher efficacy and lower toxicity of this preparation than CPT-11 *in vivo*.[12]

In conclusion, we demonstrated that combined NK012 and 5FU chemotherapy exerts significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU, indicating the necessity of clinical evaluation of this combined regimen.

References

1. Saltz LB, Douillard JY, Pirotta N, Alakl M, Gruia G, Awad L, Elfring GL, Locker PK, Miller LL. Irinotecan plus fluorouracil/leucovorin for metastatic colorectal cancer: a new survival standard. Oncologist 2001;6:81–91.
2. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000;355:1041–7.
3. Takimoto CH, Arbuck SG. Topoisomerase I targeting agents: the camptothecins. In: Chabner BA, Lango DL, eds. Cancer chemotherapy and biotherapy: principal and practice, 3rd ed. Philadelphia, PA: Lippincott Williams and Wilkins, 2001. 579–646.
4. Slatter JG, Schaaf LJ, Sams JP, Feenstra KL, Johnson MG, Bombardt PA, Cathcart KS, Verburg MT, Pearson LK, Compton LD, Miller LL, Baker DS, et al. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients. Drug Metab Dispos 2000;28:423–33.
5. Rothenberg ML, Kuhn JG, Burris HA, III, Nelson J, Eckardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI, Rock MK, Von Hoff DD. Phase I and pharmacokinetic trial of weekly CPT-11. J Clin Oncol 1993;11:2194–204.
6. Guichard S, Terret C, Hennebelle I, Lochon I, Chevreau P, Fretigny E, Selves J, Chatelut E, Bugat R, Canal P. CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. Br J Cancer 1999;80:364–70.
7. Gradishar WJ, Tjulandin S, Davidson N, Shaw H, Desai N, Bhar P, Hawkins M, O'Shaughnessy J. Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. J Clin Oncol 2005;23:7794–803.
8. Muggia FM. Liposomal encapsulated anthracyclines: new therapeutic horizons. Curr Oncol Rep 2001;3:156–62.
9. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 1986;46:6387–92.
10. Zhang JA, Xuan T, Parmar M, Ma L, Ugwu S, Ali S, Ahmad I. Development and characterization of a novel liposome-based formulation of SN-38. Int J Pharm 2004;270:93–107.
11. Kraut EH, Fishman MN, LoRusso PM, Gorden MS, Rubin EH, Haas A, Fetterly GJ, Cullinan P, Dul JL, Steinberg JL. Final result of a phase I study of liposome encapsulated SN-38 (LE-SN38): safety, pharmacogenomics, pharmacokinetics, and tumor response [abstract 2017]. Proc Am Soc Clin Oncol 2005;23:139S.
12. Koizumi F, Kitagawa M, Negishi T, Onda T, Matsumoto S, Hamaguchi T, Matsumura Y. Novel SN-38-incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor-secreting bulky tumors. Cancer Res 2006;66:10048–56.
13. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.
14. Azrak RG, Cao S, Slocum HK, Toth K, Durrani FA, Yin MB, Pendyala L, Zhang W, McLeod HL, Rustum YM. Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts. Clin Cancer Res 2004;10:1121–9.
15. Jain RK. Barriers to drug delivery in solid tumors. Sci Am 1994; 271:58–65.
16. Savic R, Luo L, Eisenberg A, Maysinger D. Micellar nanocontainers distribute to defined cytoplasmic organelles. Science 2003;300:615–18.
17. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res 1991;51:4187–91.
18. Slater R, Radstone D, Matthews L, McDaid J, Majeed A. Hepatic resection for colorectal liver metastasis after downstaging with irinotecan improves survival. Proc Am Soc Clin Oncol 2003;22(abstract 1287).
19. Araki E, Ishikawa M, Iigo M, Koide T, Itabashi M, Hoshi A. Relationship between development of diarrhea and the concentration of SN-38, an active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11. Jpn J Cancer Res 1993;84:697–702.
20. Atsumi R, Suzuki W, Hakusui H. Identification of the metabolites of irinotecan, a new derivative of camptothecin, in rat bile and its biliary excretion. Xenobiotica 1991;21:1159–69.
21. Onda T, Nakamura I, Seno C, Matsumoto S, Kitagawa M, Okamoto K, Nishikawa K, Suzuki M. Superior antitumor activity of NK012, 7-ethyl-10-hydroxycamptoyhecin-incorporating micellar nanoparticle, to irrinotecan. Proc Am Assoc Cancer Res 2006;47:720s(abstract 3062).
22. Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 2004;22:229–37.
23. Colucci G, Gebbia V, Paoletti G, Giuliani F, Caruso M, Gebbia N, Carteni G, Agostara B, Pezzella G, Manzione L, Borsellino N, Misino A, et al. Phase III randomized trial of FOLFIRI versus FOLFOX4 in the treatment of advanced colorectal cancer: a multicenter study of the Gruppo Oncologico Dell'Italia Meridionale. J Clin Oncol 2005; 23:4866–75.

Oncology Research, Vol. 17, pp. 137–149

0965-0407/08 $90.00 + .00
E-ISSN 1555-3906
www.cognizantcommunication.com

Long-Term Results of Dose-Intensive Chemotherapy With G-CSF Support (TCC-NHL-91) for Advanced Intermediate-Grade Non-Hodgkin's Lymphoma: A Review of 59 Consecutive Cases Treated at a Single Institute

Miyuki Akutsu,* Saburo Tsunoda,* Tohru Izumi,* Masaru Tanaka,* Susumu Katano,† Koichi Inoue,* Seiji Igarashi,‡ Kaoru Hirabayashi,‡ Yusuke Furukawa,§ Ken Ohmine,*§ Kazuya Sato,*§ Hiroyuki Kobayashi,*§ Keiya Ozawa,§ Keita Kirito,*¶ Takahiro Nagashima,*¶ Satoshi Teramukai,# Masanori Fukushima,# and Yasuhiko Kano*

*Division of Hematology, Tochigi Cancer Center, Tochigi, 320-0834, Japan
†Division of Radiation Oncology, Tochigi Cancer Center, Tochigi, 320-0834, Japan
‡Division of Pathology, Tochigi Cancer Center, Tochigi, 320-0834, Japan
§Division of Hematology, Jichi Medical University, Tochigi 329-0498, Japan
¶Division of Hematology, Yamanashi University, Yamanashi 409-3898, Japan
#Division of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, 606-8507, Japan

(Submitted September 25, 2007; revision received January 9, 2008; accepted January 15, 2008)

We evaluated the long-term outcome of very dose-intensive chemotherapy (TCC-NHL-91) for advanced intermediate-grade lymphoma, in which an eight-cycle regimen with 11 drugs was given with granulocyte colony-stimulating factor (G-CSF) support (total 18 weeks). Fifty-nine patients were treated during February 1, 1991 and March 31, 2001 (median age: 48 years). Forty-three patients (73%) were in a high-intermediate risk or high-risk group (HI/H) according to the age-adjusted International Prognostic Index (aa-IPI). Forty-six patients received 7 or 8 cycles of therapy. Ten of 15 patients over age 60 stopped before 7 cycles. Forty-three patients with an initial bulky mass or a residual mass received involved-field radiation. Overall, 56 patients (95%) achieved complete remission (CR). Grade 4 hematotoxicity was observed in all patients. With a median follow-up of 128 months, the 10-year overall survival (OS) and progression-free survival (PFS) rates were 76% and 61%, respectively. Neither aa-IPI risk factors nor the index itself was associated with response, OS, or PFS. One patient died of sepsis during the therapy and one died of secondary leukemia. This retrospective study suggests that the TCC-NHL-91 regimen achieves high CR, OS, and PFS in patients with advanced intermediate-grade lymphoma up to 60 years old and may be a valuable asset in the management of this disease. Further evaluation and prospective studies of the TCC-NHL-91 are warranted.

Key words: TCC-NHL-91; G-CSF; Aggressive lymphoma; CNS lymphoma

INTRODUCTION

The prognosis of non-Hodgkin's lymphoma (NHL) remains unsatisfactory in spite of significant advances in treatment. The 5-year survival rate of patients with advanced aggressive NHL is about 30–40% (1,2). Many patients die of relapsed or refractory diseases. Although second- or third-generation regimens were initially reported to improve survival (3–6), a large-scale randomized study showed that these regimens had no superiority to the standard CHOP regimen (cyclophosphamide, doxorubicin, vincristine, prednisone) (2).

Recently, a chimeric monoclonal antibody, rituximab, which targets the CD20 antigen on B cells, has been introduced for clinical use. The outcome for patients with diffuse large B-cell lymphoma (DLBCL) is significantly improved (by 10–20%) by the addition of rituximab to CHOP or other regimens (7–9). However, the prognosis is still unsatisfactory for especially high-risk patients. Patients with DLBCL classified into high-intermediate and high IPI groups (HI/H) showed a long-term chance of cure in the range of 50% (9), and the survival rate in patients with mantle cell lymphoma and T-cell lymphoma was much lower than that in advanced DLBCL. No consensus was obtained during the 1990s about using high-dose therapy as a first-line therapy for poor-risk intermediate-grade lymphoma (10). It is obvious that CHOP-based therapy is far from ideal and there

Address correspondence to Yasuhiko Kano, Divison of Hematology, Tochigi Cancer Center, Yonan 4-9-13, Utsunomiya, Tochigi, 320-0834, Japan. Tel: 011-81-28-658-5151; Fax: 011-81-28-658-5488; E-mail: ykano@tcc.pref.tochigi.jp

is clearly a need for better combination chemotherapy to improve the survival.

Refractory and relapsed lymphoma may be viewed as the clinical consequence of drug resistance and rapid cell regrowth. The Norton-Simon model predicts that the total effect of therapy is related to the cell kill for each dose, the length of time drugs are administered, and the rate of tumor growth between each treatment, and that the most efficient way to treat heterogeneous cancer cells is to eradicate the numerically dominant, faster growing cells first, followed by the more slow-growing, resistant cells (11). The theoretical reason for the advantage afforded by dose-intensive treatment is that regrowth of resistant cells between cycles of chemotherapy is reduced by shortening the available time between doses and increasing the dose of drugs (11,12).

At the beginning of the 1990s, colony-stimulating factor (G-CSF) was introduced for clinical use, and it has made it possible to increase the dose intensity compared with dosages that can be delivered without G-CSF support. If so, more dose-intensive regimens than second- or third-generation regimens might improve the survival of patients with intermediate-grade NHL with poor prognosis features. Accordingly, a dose-intensive regimen (TCC-NHL-91) against advanced intermediate-grade lymphoma was started in 1991 in our center.

Here we report the long-term results of the TCC-NHL-91 regimen, which was employed before the incorporation of rituximab against advanced intermediate-grade lymphoma. Our results suggest that the dose-intensive sequential regimen may improve the survival of patients with advanced intermediate-grade NHL.

PATIENTS AND METHODS

Patient Eligibility

A total of 64 patients, treated with TCC-NHL-91 from February 1, 1991 to March 31, 2001 in our cancer center, were analyzed retrospectively. Patients' characteristics were as follows: age, 16–69 years; stage II bulky (≥7 cm), III , or IV disease as defined by the Ann Arbor staging criteria; no prior therapy; normal cardiac, renal, pulmonary, and hepatic function, unless abnormal because of disease involvement; no active double cancer. The study was conducted in accordance with the Helsinki declaration. The trial was approved by our institution's ethics committee, and informed consent for treatment was obtained from all patients.

Pretreatment Evaluations

Pretreatment evaluations included a thorough history, physical examination, blood cell count and differential blood chemistry tests, lumbar puncture, bone marrow aspiration, electrocardiography, chest X-ray, computed tomography of the chest and abdomen, abdominal echo, and gallium scans. Computed tomography of the head, gastrointestinal X-ray examination, endoscopy, and magnetic resonance imaging were performed as required clinically.

Treatment Strategy

The dose-intensive TCC-NHL-91 regimen was designed by reference to the CHOP and LNH84 (13) regimens, which at that time were considered as standard regimens for intermediate-grade lymphoma. Cyclophosphamide, doxorubicin (topoisomerase-II inhibitors), vincristine, and prednisone were "core" agents for the treatment of intermediate-grade lymphoma, and thus the total dose and dose intensity of these agents were not decreased in the regimen. The dose-intensive schedule was accomplished by using G-CSF to permit 2-week cycling of the drugs at their optimal dose levels rather than the conventional 3-week cycling. The consolidation phase referred to the application of the concept that multiple drugs are needed to perturb cancers maximally that are composed of cells of heterogeneous drug sensitivity. Drugs commonly used in salvage therapy, such as methotrexate (MTX), ifosfamide, cytarabine, and carboplatin, were included in this phase.

In experimental studies, prednisone, cyclophosphamide, doxorubicin, etoposide, bleomycin, mitoxantrone, and ifosfamide have shown additive effects with most antilymphoma agents [(14,15), unpublished data]. These agents can be combined simultaneously. MTX produced marked antagonistic effects with other antilymphoma agents in simultaneous exposure, while MTX followed by a variety of agents including vincristine and cytarabine produced synergistic effects [(16–18), unpublished data]. A schedule of MTX followed by vincristine was employed in this study. As MTX is highly time dependent (19), 24-h continuous infusion with late low-dose leucovorin rescue was used. Simultaneous exposure to cytarabine and mitoxantrone or carboplatin has shown synergistic effects and these agents were combined (15,17).

In advanced aggressive lymphoma, CNS relapse is not rare and the prognosis of secondary CNS lymphoma is extremely poor (20–26). To prevent CNS relapse, inthrathecal MTX, moderate-dose MTX, ifosfamide, and high-dose cytarabine, which cross the blood–brain barrier, were employed in the regimen.

Chemotherapy Regimen

The doses and schedule of the TCC-NHL-91 regimen are shown in Table 1. The induction phase regimen consisted of five cycles, each of a 2-week duration. Doxorubicin and mitoxantrone were administered by continuous

Table 1. TCC-NHL-91 Protocol (Minimum Seven Cycles)

Drug	Route	Dose	Treatment Day	Cycle (Interval) 1 (2W)	2 (2W)	3 (2W)	4 (2W)	5 (2W)	6 (3W)	7 (2W)	8 (3W)	
Cyclophosphamide	IV	1200 mg/m^2	1	•	•	•	•	•				
Ifosfamide	IV	1000 mg/m^2	1, 2, 3, 4							•		
Doxorubicin	CIV (24 h)	75 mg/m^2	1	•		•		•		•		
Mitoxantrone	CIV (24 h)	8 mg/m^2	1, 2				•					
	CIV (24 h)	8 mg/m^2	2, 3								•	X¶
Etoposide	IV	200 mg/m^2	1, 2, 3		•							R
Vincristine	IV	1.4 mg/m^2	1	•	•	•	•	•		•		T
	IV	1.4 mg/m^2	2, 9					•				
Bleomycin	IV	10 mg/m^2 500 mg/body	1	•	•	•	•	•		•		
Methylprednisolone*	IV	×2	1, 2, 3	•	•	•	•	•	•	•	•	
Methotrexate†	CIV (24 h)	500 mg/m^2 15 mg/body	1, 8							•		
Leucovorin‡	IV	×q6×6	3–4, 10–11						▽			
Cytarabine	IV	1 g/m^2 ×2	1, 2								•	
Carboplatin	IV	150 mg/m^2	1, 2, 3								•	
MTX§	IT	15 mg/body	1	•	•	•	•	•				
G-CSF	SC	1–2 mg/kg	variable	▽	▽	▽	▽	▽		▽	▽	

Until September 30, 1993, the fifth cycle of therapy was deleted for patients who entered in CR after two cycles of therapy.
*After June 1, 2000, dexamethasone 40 mg/body ×2 was delivered, because methylpredinosolone was not permitted for use in lymphoma by the Ministry of Health, Labour, and Welfare.
†When MTX level was higher than 1 μM at 48 h after the beginning of MTX administration, a higher dose and more frequent rescues were given.
‡Leucovorin started 36 h after the beginning of MTX administration.
§3–5 times in cycles 1–5.
¶For primary bulky mass and residual mass.

infusion for 24 h to prevent cardiotoxicity. The consolidation phase regimen, involving agents used for salvage therapies, consisted of the next three cycles: the sixth and eighth cycles of a 3-week duration, and the seventh cycle of a 2-week duration. During all cycles, patients received G-CSF (filgrastim, lenograstim, or nartograstim) at 1–2 μg/kg subcutaneously starting 2 or 3 days after the end of administration of cytotoxic agents until granulocyte recovery (>2000/μl). Chemotherapy subsequent to the first cycle was deferred until the absolute neutrophil count was greater than 2000/μl and the platelet count was greater than 10,000/μl. To prevent CNS relapse, 15 mg MTX and hydrocortisone at 50 mg/body were administered intrathecally in the first to fifth cycles of therapy as much as possible.

Involved-Field Radiation

Patients with primary bulky mass and/or patients with a residual mass after treatment received involved-field radiation (20–50 Gy). Radiotherapy was started within 2 months of the end of chemotherapy.

Cranial Radiation for CNS Prophylaxis. After September 1, 1997, patients with high LDH and/or massive bone marrow involvement received CNS prophylactic radiation (24 Gy in 12 fractions).

Supportive Care. All patients had an indwelling central venous catheter. Cotrimoxazole therapy (two tablets daily throughout the course of treatment except during the sixth cycle of therapy) was given to prevent pneumocystis carinii infection, and broad-spectrum antibiotics were given for a persistent undiagnosed fever ≥37.5°C.

Response Criteria

Response to treatment was assessed after every two cycles of therapy. The definition of complete remission (CR) used in the literature has been variable (27). We defined CR as the complete disappearance of all measurable and evaluable disease. Patients with a residual mass with no progression at 3 months after the end of therapy were also regarded as being in CR. Partial remission (PR) was defined as the diminution of >50% of all the initial masses.

Statistical Analysis

Overall survival (OS) was defined as the time from the beginning of treatment to any cause of death or the last follow-up. Progression-free survival (PFS) was defined the time from the beginning of treatment to lymphoma progression or death as a result of any cause or the last follow-up (28). The survival proportions were estimated with the Kaplan-Meier method. The log rank test was performed to test associations between patient characteristics and OS or PFS. Two-sided $p < 0.05$ was regarded as statistically significant. All statistical analyses were done by using SAS version 8.2 (SAS Institute, Cary, NC, USA).

RESULTS

Patients' Characteristics and Response to Therapy

Among the 64 patients treated with TCC-NHL-91, 5 patients were deleted from this study because they were diagnosed with lymphoblastic lymphoma ($n = 1$), follicular mix lymphoma ($n = 3$), or Hodgkin's lymphoma ($n = 1$) by an external pathological review. All these excluded patients are now alive in the first CR. The study was analyzed based on the clinical data of 59 patients on February 28, 2007. The main characteristics of the 59 patients and their response to therapy are listed in Table 2. There were 27 male patients (46%), with a median age of 48 (range 17–69 years), including 15 patients (27%) older than 60. Fifty-five patients (93%) had disease of stages III–IV. Forty-two patients (71%) had a bulky mass. Thirty-seven patients (63%) had two or more extranodal sites. Thirty-six patients (61%) had elevated serum lactate dehydrogenase (LDH). We retrospectively analyzed patients using the age-adjusted International Prognostic Index (aa-IPI) criteria (30). Sixteen patients (27%) had L- or LI-risk factors and 43 (73%) had HI- and H-risk factors. Fifty-six of the 59 patients entered CR (95%), 1 patient PR (2%), and 2 patients stable disease (3%). There was no significant association between any of the validation IPI risk factors, phenotype, or histological type, and response.

Number of Treatment Cycles and Dose Delivery

Forty-six patients (78%) received either seven (17 patients) or eight (29 patients) cycles of therapy (Table 3). Dose reduction was not performed in 55 patients. Dose reduction was performed in three patients over 60 and one patient with hepatic damage during the course of therapy. Thirteen patients (22%), including 10 patients over 60, stopped TCC-NHL-91 treatment before the seventh cycle of therapy for the following reasons: nine patients due to toxicity (one hemorrhagic cystitis; two interstitial pneumonitis; one ischemic heart disease; five intolerance), two due to refusal of further intensive treatment, and two due to non-treatment-related complications (idiopathic thrombocytopenic purpura and hypothyroidism). These patients were treated with other regimens after that. In the sixth cycle of therapy, six patients received only one cycle of therapy because of MTX toxicity. Intrathecal MTX was not sufficiently delivered with intensive chemotherapy (median, 3 times).

Involved-Field Radiation and Cranial Radiation

After chemotherapy, 43 of the 59 patients (64 sites) received involved-field radiation against the initial bulky mass and/or a residual mass without significant toxicity. The radiation dose ranged between 20 and 50 Gy (median dose of 30.6 Gy), varying with initial tumor size, extent, adjacent organs, response to chemotherapy, and the patient's physical condition. Fourteen patients received cranial radiation for CNS prophylaxis (24 Gy).

Toxicity

Table 3 details the treatment-related toxicity produced by this regimen. Grade 4 neutropenia occurred in 100% of patients and persisted for 1–8 days in the first to seventh cycles of therapy and for 1–15 days in the eighth cycle of therapy. Thrombocytopenia was less severe, but grade 4 toxicity was observed in 39% and 93% of patients in the sixth and eighth cycles of treatment, respectively. Infection associated with neutropenia was the other major complication. Of 403 total cycles of chemotherapy delivered, febrile neutropenic episodes (>38°C) occurred in 72 cycles (18%). Because continuous MTX infusion for 24 h and minimal leucovorin rescue were used, MTX toxicity was not ignorable. More than 50% of patients showed transient liver dysfunctions. Six patients received one but not two treatments of MTX because of severe stomatitis (four patients) or delayed MTX clearance (serum MTX concentration $>1 \times 10^{-6}$ M at 48 h) (two patients).

One patient developed hemorrhagic cystitis and two developed drug-induced interstitial pneumonitis, but all three patients recovered. Two patients died during chemotherapy. One patient died of pseudomonas sepsis during the therapy, and the other patient, who dropped out from the regimen after the first cycle, died of sepsis after CHOP therapy. One patient died of secondary leukemia after high-dose therapy with autologous peripheral blood stem cell transplantation in the third CR (11 years after the onset of lymphoma).

Relapse

Among the 56 CR patients, 17 patients relapsed. Four of them showed isolated CNS relapse, nine local relapse,

Table 2. Patients Characteristics and Outcome

Characteristic	No. of Pts (%)	CR (%)	% PFS at 5 Years	% PFS at 10 Years	*p*-Value	% OS at 5 Years	% OS at 10 Years	*p*-Value
All cases	59	56 (95)	68	61		78	76	
Median age (years, range 17–69)	48							
≤60	44 (75)	42 (95)	70	62	0.781	84	81	0.156
>60	15 (25)	14 (93)	60	60		60	60	
Gender								
Male	27 (46)	25 (93)	62	57	0.715	66	62	0.024
Female	32 (54)	31 (97)	72	65		88	88	
B symptoms								
(−)	25 (42)	23 (92)	72	62	0.683	76	76	0.632
(+)	34 (58)	33 (97)	64	60		79	76	
Stage								
II (bulky)	4 (7)	4 (100)	100	100	0.123	100	100	0.206
III–IV	55 (93)	52 (95)	65	58		76	74	
Bulky disease								
(−)	17 (29)	16 (94)	47	41	0.044	65	59	0.200
(+)	42 (71)	40 (95)	76	70		83	83	
ECOG PS								
0–1	29 (49)	28 (97)	69	57	0.565	79	76	0.804
2–4	30 (51)	28 (93)	66	66		76	76	
LDH								
Normal	23 (39)	22 (96)	65	56	0.604	78	78	0.466
High	36 (61)	34 (94)	69	65		78	74	
Extranodal sites								
0–1	22 (37)	21 (95)	82	77	0.128	86	86	0.244
2–	37 (63)	35 (95)	59	50		73	69	
Phenotype (T/B)								
T	9 (15)	9 (100)	44	44	0.165	78	67	0.735
B	50 (85)	47 (94)	72	64		78	78	
Histological type (WF)								
F. large cell	4 (7)	4 (100)	100	75	0.672	100	100	0.514
D. small cleaved cell	3 (5)	3 (100)	33	33		67	67	
D. mixed	11 (18)	11 (100)	55	55		64	55	
D. large cell	37 (66)	34 (92)	70	62		78	78	
T unclassified	2 (3)	2 (100)						
B unclassified	2 (3)	2 (100)						
aa-IPI								
L/LI	1/15 (27)	1/15 (100)	69	55	0.691	81	81	0.345
HI/H	20/23 (73)	19/21 (93)	67	64		77	74	

PFS, progression free survival; OS, overall survival; aa-IPI, age-adjusted International Prognostic Index. Thee *p*-values derived from log rank test.

and four generalized relapse. Two patients had suffered relapse from the radiation sites (total dose 20 and 30 Gy, respectively). By August 1997, 8 of the 32 patients had relapsed. Among them, four patients (50%) showed CNS relapse. Two patients with CNS relapse received seven cycles and the other two patients received eight cycles of therapy. Two patients received two treatments of intrathecal MTX and the other two patients received four treatments. These results suggested that the regimen was insufficient for CNS relapse. Then CNS prophylactic radiation (24 Gy) was added for 14 patients with high LDH and/or bone marrow involvement among the 27 patients. After that, no patient showed CNS relapse.

Survival

The median duration of follow-up has been 128 months (range 71–174 months). Sixteen of the 59 eligible patients have died (27%). Thirty-six patients are now in first CR, six patients in second CR, and one patient in stable disease. There was no significant association between any of the IPI risk factors and OS or PFS. Fig-

Table 3. Treatment Cycles and Toxicities

	Cycle							
	1 (n = 59)	2 (n = 57)	3 (n = 57)	4 (n = 56)	5* (n = 45)	6 (n = 50)	7 (n = 46)	8 (n = 29)
Interval (days)	12–20	12–24	12–27	12–33	13–29	10–33	14–51	
Median	16	15	16	18	17	23	19	
Neutropenia (<500/μl) (%)	86	79	54	98	90	29	74	100
Duration (days)	1–8	1–7	1–7	1–12	1–11	1–7	1–8	1–15
Median (days)	3	4	3	5	5	3	4	7
Thrombocytopenia (<20,000/μl) (%)	7	5	5	21	20	39	13	93
Anemia (Hb <8 g/dl) (%)	14	9	26	49	45	55	39	90
Liver dysfunction (GOT, GPT > 2.5 N) (%)	19	10	11	11	8	51	11	14
Fever >38°C (%)	14	12	5	20	14	32	15	45

The n value in parentheses represents number of patients.
*Five patients deleted the fifth cycle of therapy.

ure 1 shows the Kaplan-Meier estimate of the survival distribution of all patients. The OS and PFS at 5 years were 77.8% (95% CI 67.2–88.5%) and 67.6% (95% CI 55.6–79.6%), and those at 10 years were 75.9% (95% CI 64.9–86.9%) and 61.2% (95% CI 48.3–74.1%), respectively (Fig. 1).

Figures 2 and 3 show the OS and PFS by aa-IPI subgroups, respectively. The OS at 10 years was 81.3% (95% CI 62.1–100%) and 73.8% (95% CI 60.5–87.2%), for the patients in the L/LI- and HI/H-risk groups, respectively (Fig. 2). The PFS at 10 years was 54.7% (95% CI 29.5–79.9%) and 63.7% (95% CI 48.9–78.6%) for the patients in the L/LI- and HI/H-risk groups, respectively (Fig. 3). The detailed characteristics of all 59 patient and their outcomes are shown in Table 2. There was no significant difference in OS or PFS between/among the subgroups of patients, except regarding sex (OS at 10 years: male/female 62%/88%, $p = 0.024$).

Among 37 patients with diffuse large-cell lymphoma, 32 patients were B-cell type (DLBCL), and 5 patients T-cell type. At present, 23 patients with DLBCL and 4 patients with T-cell type are alive. We analyzed the data

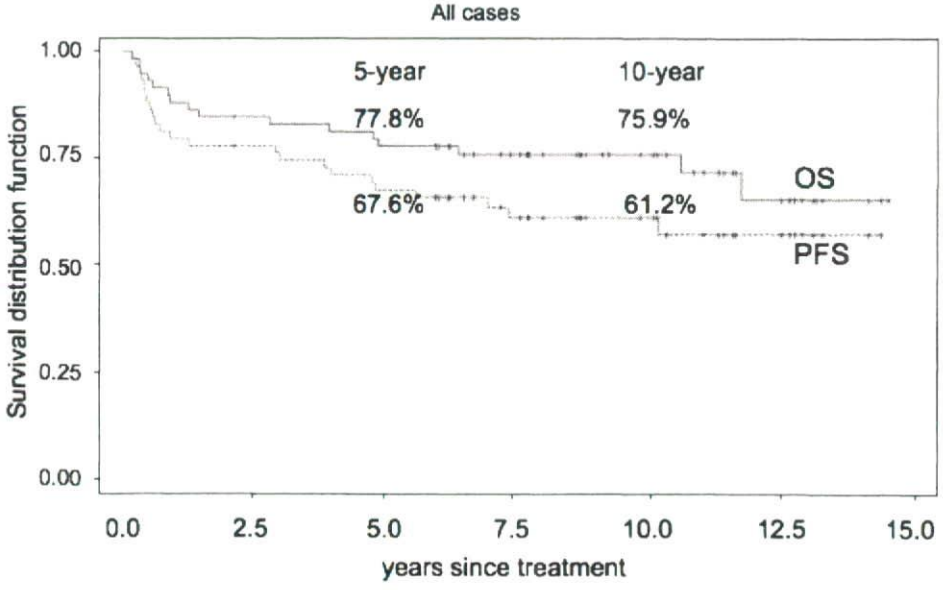

Figure 1. Kaplan-Meier estimate of OS and PFS of the 59 patients treated with TCC-NHL-91. OS at 5 years and 10 years was 77.8% and 75.9%, respectively. PFS at 5 years and 10 years was 67.6% and 61.2%, respectively.

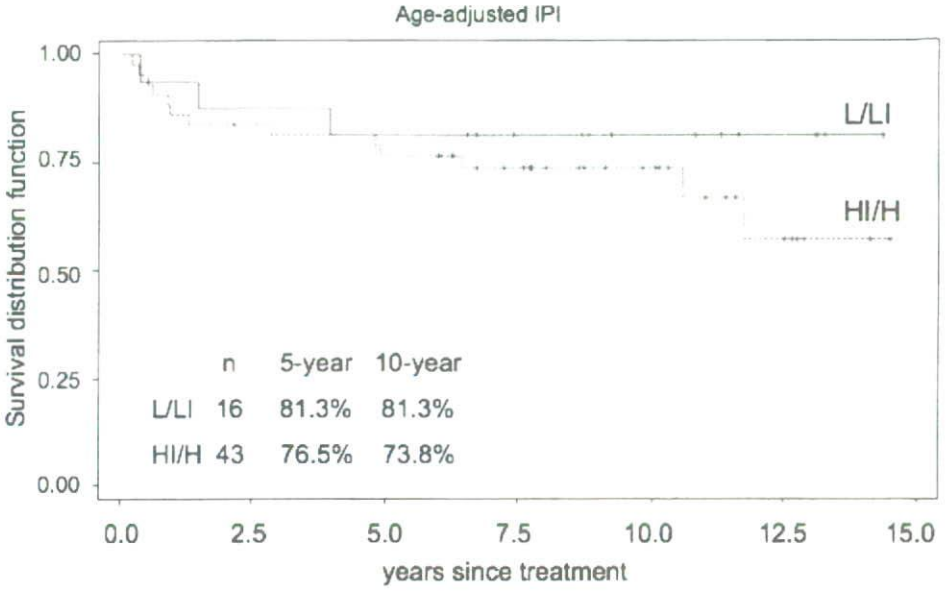

Figure 2. Kaplan-Meier estimate of OS by aa-IPI grouping. OS at 5 and 10 years was 81.3% and 81.3%, respectively, for the L/LI groups. OS at 5 and 10 years was 76.5% and 73.8%, respectively, for the HI/H groups.

for 32 patients with DLBCL. The OS and PFS at 10 years for patients with DLBCL were 78.1% (95% CI 63.8–92.5%) and 61.9% (95% CI 43.4–80.5%), respectively. Figures 4 and 5 show the OS and PFS in patients with DLBCL by aa-IPI subgroups, respectively. The OS at 10 years was 75.0% (95% CI 32.6–100%) and 78.6% (95% CI 63.4–93.8%) for the patients in the L/LI- and HI/H-risk groups, respectively. The PFS at 10 years was 37.5% (95% CI 0–93.6%) and 65.5% (95% CI 46.5–84.5%) for the patients in the L/LI- and HI/H-risk groups, respectively. There was no significant difference in OS or PFS between/among the subgroups of patients,

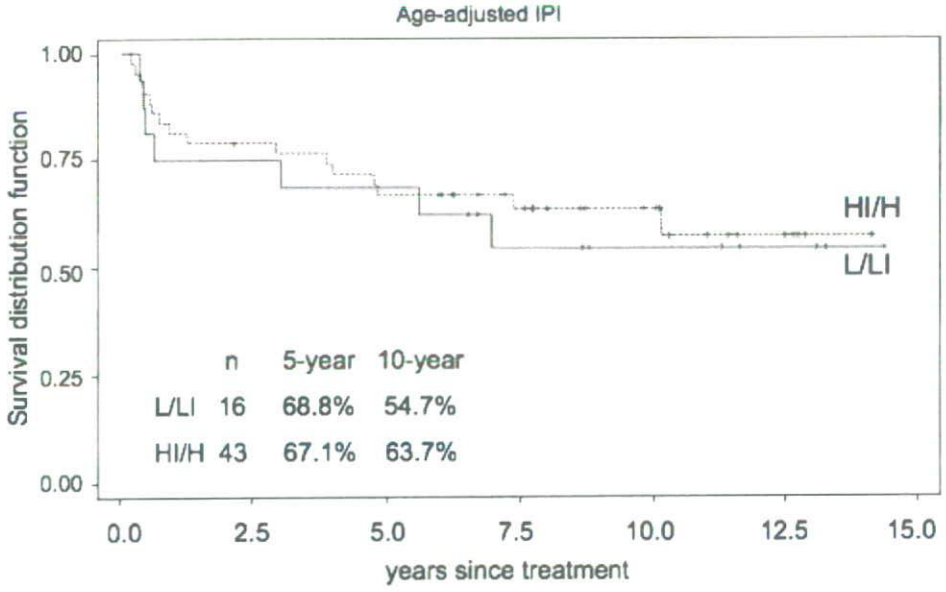

Figure 3. Kaplan-Meier estimate of PFS by aa-IPI grouping. PFS at 5 and 10 years were 68.8% and 54.8%, respectively, for the L/LI group. PFS at 5 and 10 years was 67.1% and 63.7%, respectively, for the HI/H group.

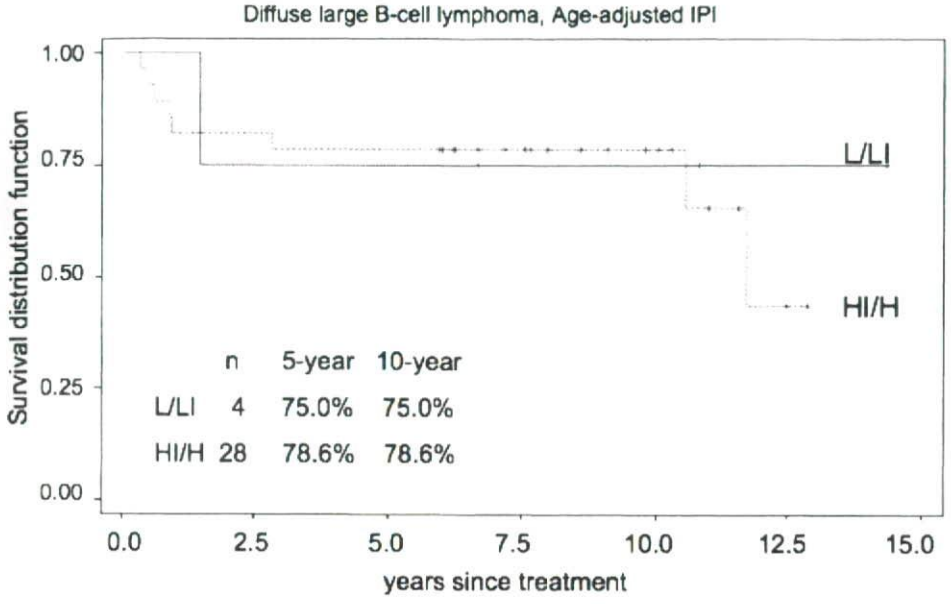

Figure 4. Kaplan-Meier estimate of OS by aa-IPI grouping of DLBCL. OS at 5 and 10 years was 75.0% and 75.0%, respectively, for the L/LI groups. OS at 5 and 10 years was 78.6% and 78.6%, respectively, for the HI/H groups.

but the number of patients for each subgroup was too small for reliable analysis.

DISCUSSION

This is a retrospective study conducted in a single institution involving 59 patients with advanced intermediate-grade lymphoma who underwent intensive chemotherapy with G-CSF support (TCC-NHL-91). We achieved a CR rate of 95% and a projected 10-year OS of 76% and projected 10-year PFS of 61%. The median age of our patient population was approximately 10 years lower than that in most cooperative group lymphoma studies,

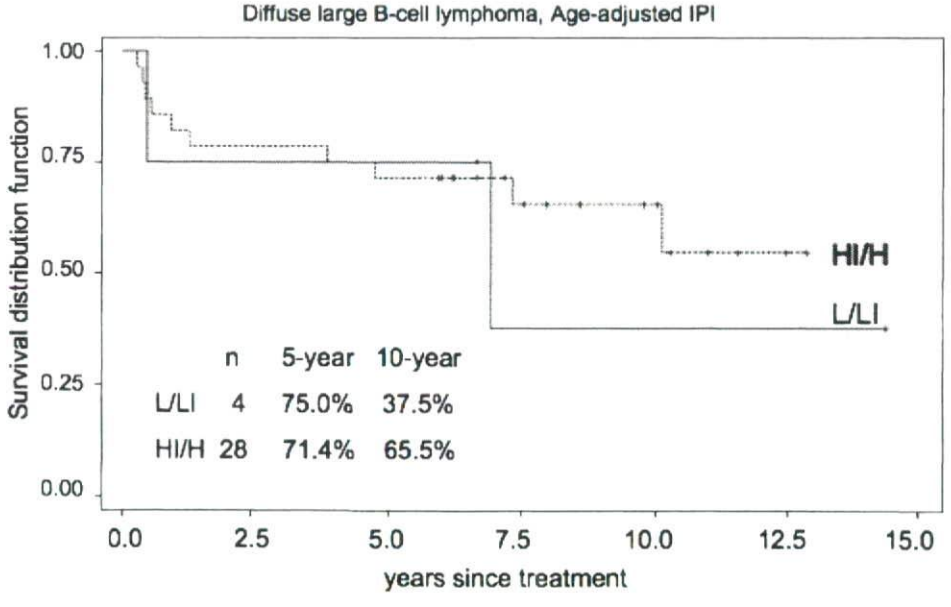

Figure 5. Kaplan-Meier estimate of PFS by aa-IPI grouping of DLBCL. PFS at 5 and 10 years was 75.0% and 37.5%, respectively, for the L/LI groups. PFS at 5 and 10 years was 71.4% and 65.5%, respectively, for the HI/H groups.