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In vivo Effects of a Histone Deacetylase Inhibitor, FK228, on Human Acute Promyelocytic Leukemia in NOD/Shi-*scid*/*scid* Mice

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Histone acetylation and deacetylation are closely linked to transcriptional activation and repression, respectively. In acute promyelocytic leukemia (APL), histone deacetylase inhibitors (HDACIs) have a synergistic effect with all-*trans* retinoic acid (ATRA) *in vitro* to induce differentiation. Here we report *in vitro* and *in vivo* effects of a HDACI, FK228 (formerly FR901228 or depsipeptide), on the human APL cell line NB4. FK228 had a strong and irreversible cytotoxicity compared with another HDACI, trichostatin A. *In vivo* administration of ATRA or FK228 alone partly inhibited the growth of established tumors of NB4 subcutaneously transplanted in NOD/Shi-*scid*/*scid* mice, and the combination was synergistically effective. Histopathological examination revealed that the combination induced apoptosis and differentiation as well as histone acetylation. Intravenous injection of NB4 in NOD/Shi-*scid*/*scid* mice followed by combination treatment significantly prevented leukemia death, whereas single administration did not. These findings suggest that FK228 is a promising agent to enhance ATRA-sensitivity in the treatment of APL.

Key words: Acute promyelocytic leukemia — All-*trans* retinoic acid — Differentiation — Histone deacetylase inhibitor — FK228

All-*trans* retinoic acid (ATRA) induces differentiation of acute promyelocytic leukemia (APL) cells *in vitro* and *in vivo*, and has provided the first success in differentiation therapy against malignancies.[1–3] APL is characterized by a chromosomal translocation, t(15;17), which fuses the *PML* gene to the *retinoic acid receptor α* (*RARα*) gene resulting in the expression of PML-RARα chimeric gene product.[4, 5] It has been shown that PML-RARα causes transcriptional repression of ATRA-responsive genes, which is associated with differentiation block in APL.[6, 7] ATRA-therapy targets PML-RARα as well as RARα and relieves the repressed transcription, resulting in therapeutic differentiation.[2, 8, 9]

Histone acetylation/deacetylation is an important mechanism to control transcription.[10, 11] The wild-type RARα forming a heterodimer with retinoid-X receptor (RXR) binds to the retinoic acid response element in target genes. In the absence of ligand, the RARα/RXR heterodimer associates with a transcriptional repressor complex, including N-CoR, mSin3a, and a histone deacetylase (HDAC). The binding of ATRA causes a conformational change in RARα/RXR resulting in the release of the repressor complex and recruitment of a transcriptional activator complex, which has histone acetyltransferase (HAT) activity and is associated with transcriptional activation.[12, 13] A rare variant of APL carrying t(11;17)/PLZF-RARα responds poorly to ATRA therapy in contrast to APL with t(15;17)/PML-RARα.[14, 15] The difference in sensitivity to ATRA was explained as follows. APL-specific fusion proteins, PML-RARα and PLZF-RARα, recruit the N-CoR/HDAC complex even in the presence of a physiological concentration of ATRA. The therapeutic concentration of ATRA dissociates the N-CoR/HDAC complex from PML-RARα, but not from PLZF-RARα, because PLZF-RARα has high affinity with the N-CoR/HDAC complex.

This hypothesis was confirmed by the finding that a HDAC inhibitor (HDACI), trichostatin A (TSA), relieved the suppressed transcription and differentiated ATRA-resistant APL *in vitro* when used in combination with ATRA.[16–18] We also observed that the combination of HDACI and ATRA induced differentiation of ATRA-resistant leukemia cell lines and fresh APL cells with PLZF-RARα.[19, 20] HDACIs are considered to be anti-cancer agents inducing growth arrest, differentiation, or apoptotic cell death of transformed cells.[21–23] However, the *in vivo* efficacy of HDACIs for human leukemia remains unknown. One successful case was reported in which a patient with therapy-resistant t(15;17) APL was given sodium phenylbutyrate, which has HDACI activity, in combination with ATRA.[24] Recently, a new cyclic depsipeptide, FK228 (formerly FR901228),[25] originally

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developed as an anti-cancer agent, was found to have strong HDACI activity,[26] and its clinical application has been started in phase I trials in the USA.[27, 28]

Here, we report that FK228 enhanced the sensitivity of APL cells to ATRA not only *in vitro*, but also *in vivo*. Furthermore, we suggest that *in situ* detection of acetylated histone is useful to evaluate the molecular effects of HDAC-targeted therapy.

MATERIALS AND METHODS

Cell lines and HDACIs The APL cell line carrying PML-RARα, NB4, was obtained from Dr. M. Lanotte (Hôspital Saint-Louis, Paris, France). NB4 was cultured in RPMI 1640 medium supplemented with L-glutamine (2 m*M*), antibiotics and 10% fetal calf serum (FCS) at 37°C in a humidified CO_2 incubator, maintained at $0.2-1\times10^6$ cells/ml, and seeded at 0.2×10^6 cells/ml in 24-well plates for *in vitro* experiments. TSA was purchased from Wako Pure Chemical Industries Ltd. (Osaka). TSA was dissolved in ethanol at a concentration of 500 mg/ml and was stored at −20°C. FK228 was a generous gift from Fujisawa Pharmaceutical Co. (Osaka). FK228 was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and was stored at −20°C. Cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differentiation was assessed by morphology and nitroblue tetrazolium (NBT) reduction assay.[29]

Wash-out experiment NB4 was treated with a serially diluted TSA or FK228 for various periods. After the treatment, NB4 cells were washed three times with RPMI 1640 medium and cultured again. At a total of 75 h of culture, MTT assay and immunocytological assay for acetylated histone were performed.

Immunological detection of acetylated histone Anti-acetylated histone H4 (Ac-H4) antibody (Upstate Biotechnology Inc., Lake Placid, NY) was diluted at 1:4000 and incubated with specimens for 45 min. The binding antibody was detected by the labeling streptoavidin-biotin (LSAB) method.

***In vivo* treatment** NOD/Shi-*scid*/*scid* (NOD-*scid*) mice were bred and maintained under specific pathogen-free conditions. Six- to eight-week-old male mice were pretreated with 3 Gy of total body irradiation one day before inoculation. Fifty million NB4 cells per mouse were injected into the right side of the back. Treatment was started on day 14 after the injection. ATRA (Sigma, St Louis, MO) was dissolved in salad oil at the concentration of 1 mg/ml and 0.01 mg/g was administered periorally every day for 35 days. FK228 was solved in DMSO at the concentration of 1 mg/ml, and 0.5 μg/g (body weight) of FK228 was injected intraperitoneally 3 times a week. Tumor size was monitored every day. Tumor weight (TW) was evaluated by means of the following formula: TW (mg) = (long diameter) × (short diameter)^2 × 1/2.[28] In the next experiment, 10×10^6 cells were intravenously injected via a tail vein to observe survival of mice. ATRA and FK228 were administered similarly for 14 days from the day after the injection.

This experiment was performed according to the guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine, and was approved by the institutional ethics committee for laboratory animals used in experimental research. Statistical analysis of TW and survival period was performed with the Mann-Whitney *U* test and Kaplan-Meier method using StatView software (Abacus Concepts Inc., Berkeley, CA).

Pathological examination Specimens were fixed with a 10% formalin solution and embedded in paraffin wax. Paraffin sections were stained with hematoxylin-eosin (H-E). α-Naphthol ASD chloroacetate esterase (NASD) was detected by enzyme histochemistry. Anti-myelo-

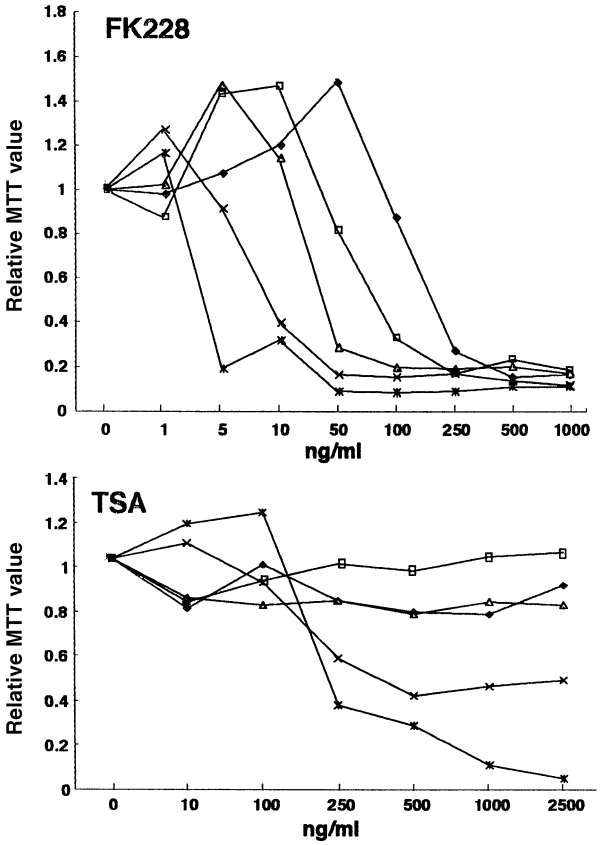

Fig. 1. Cytotoxic effects of TSA and FK228 on NB4 cells. A_{540} values of MTT assay relative to untreated sample are shown. NB4 cells were exposed to various doses (horizontal scale) of each HDACI for 3 (◆), 6 (□), 9 (△), 15 (×) and 30 (∗) h.

peroxidase (MPO) (DAKO Co., Carpinteria, CA) antibody and anti-Ac-H4 antibody were used in the immunohistological examination. Apoptosis was detected by using the ApopTag plus kit (Intergen, Gaithersburg, MD) according to the manufacturer's instructions. Tumor cells suspended in phosphate-buffered saline were studied for NBT-reducing activity.[20)]

RESULTS

***In vitro* effects of HDACI on NB4 cells** To study the relationship between exposure to HDACI and cell viability, wash-out experiments were performed on NB4 cells. TSA or FK228 was added to the culture at various concentrations for 1, 3, 6, 9, 15 or 30 h. After a total of 75 h culture, MTT assay was performed. A concentration of more than 300 ng/ml TSA and an exposure time of more than 15 h were required for cytotoxicity, and the effect depended on exposure time. On the other hand, the cytotoxicity by FK228 was concentration-dependent but exhibited only a slight dependency on exposure time (Fig. 1). The concentration required to induce half-maximum cytotoxicity (IC_{50}) after 3 days of culture was 150 ng/ml for TSA and 2.5 ng/ml for FK228. The *in vitro* differentiation experiment was performed at lower concentrations than IC_{50}.

We examined the effect of HDCIs on NB4 cells. Treatment with either FK228 or TSA alone failed to induce differentiation. In combination with ATRA, however, the HDACIs increased the NBT-positivity in a dose-dependent manner (Fig. 2).

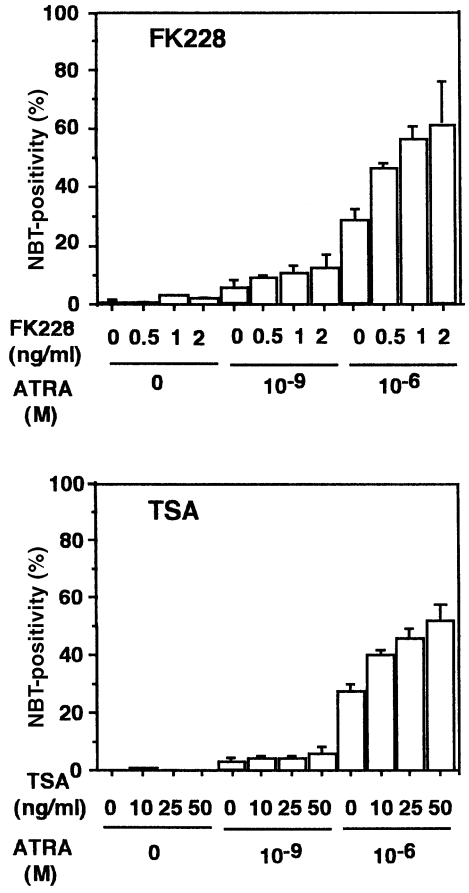

Fig. 2. Synergistic effect of HDACI on differentiation of NB4 cells. NBT reduction activity was assayed as a differentiation marker *in vitro*. FK228 or TSA synergistically induced NBT-positivity with ATRA in NB4 cells in a dose-dependent manner.

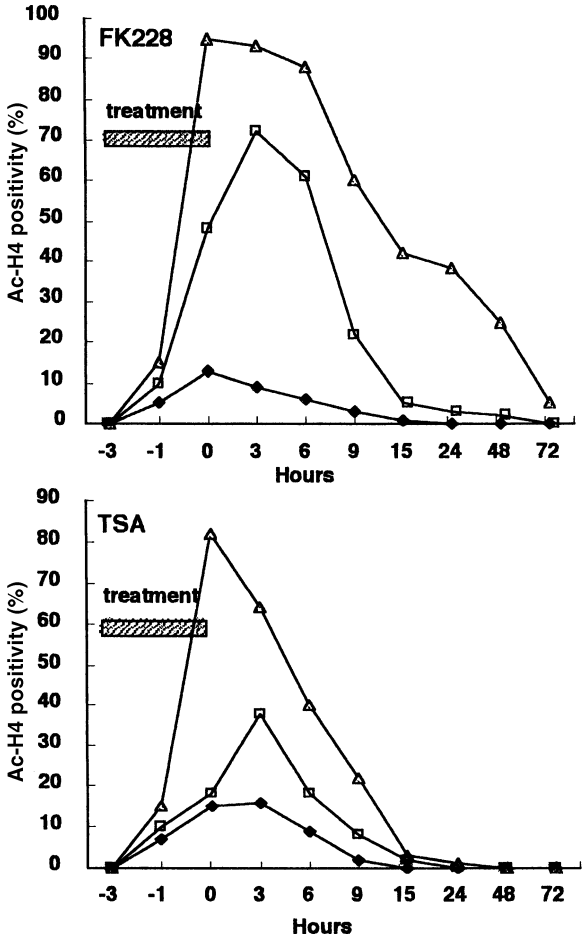

Fig. 3. Histone acetylation during the treatment and after wash-out of FK228 and TSA. Histone acetylation was immunologically evaluated using anti-Ac-H4 antibody. NB4 cells were treated with FK228 or TSA for 3 h, washed with medium, then cultured for the indicated period. FK228: △ 100 ng/ml, □ 10 ng/ml, ◆ 1 ng/ml. TSA: △ 10 μg/ml, □ 1 μg/ml, ◆ 0.1 μg/ml.

Histone acetylation on HDACI treatment We then studied whether *in vitro* treatment with HDACI effectively caused histone acetylation. In acid/urea/Triton gel electrophoresis,[26] highly acetylated histone was detected on FK228 treatment for 24 h (data not shown). Using Ac-H4 antibody, the acetylation was detected *in situ* (Fig. 3). After 1 h of treatment, cells positive for acetylated histone appeared and gradually increased in number. At 3 h, almost all NB4 cells were stained with the antibody. After 3-h treatment followed by a wash-out, the Ac-H4-positive cells gradually decreased in number. On the other hand, the acetylation by TSA rapidly diminished (within 15 h). These findings suggest that the histone acetylation induced by FK228 is more sustained than that of TSA.

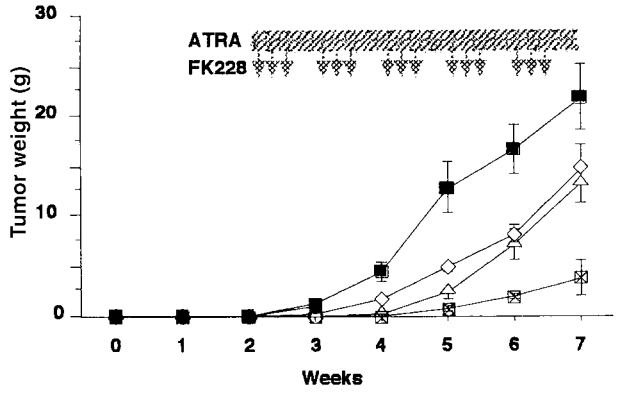

Fig. 4. Tumor growth of NB4 in NOD-*scid* mice. Twenty mice were divided into 4 groups (non-treated (N=5, ■), FK228 (N=5, ◇), ATRA (N=5, △) and ATRA plus FK228 (N=5, ⊠)). Treatment was started on day 14 when subcutaneous tumors became palpable and continued for 5 weeks.

Establishment of a murine model of human APL, NB4-NOD-*scid* NB4 cells were inoculated subcutaneously into mice, because the anti-tumor effects could be quantitatively and pathologically assessed. When 50×10^6 cells or more were injected into the back right side of mice, subcutaneous tumors appeared after 2 weeks with 100% incidence. The mice died from 8 to 16 weeks after inoculation. Histopathologic examination of the tumors revealed intermediate-sized blasts and active mitosis in about 10% of tumor cells. The tumor was weakly positive for MPO (data not shown), comparable to an immature myeloid derived from NB4. However, organs such as the lymphnodes, liver, spleen and bone marrow were not involved.

***In vivo* treatment of established subcutaneous tumor of NB4 with FK228** Previous *in vivo* studies indicated that intraperitoneal administration of 0.5 μg/g FK228 3 times

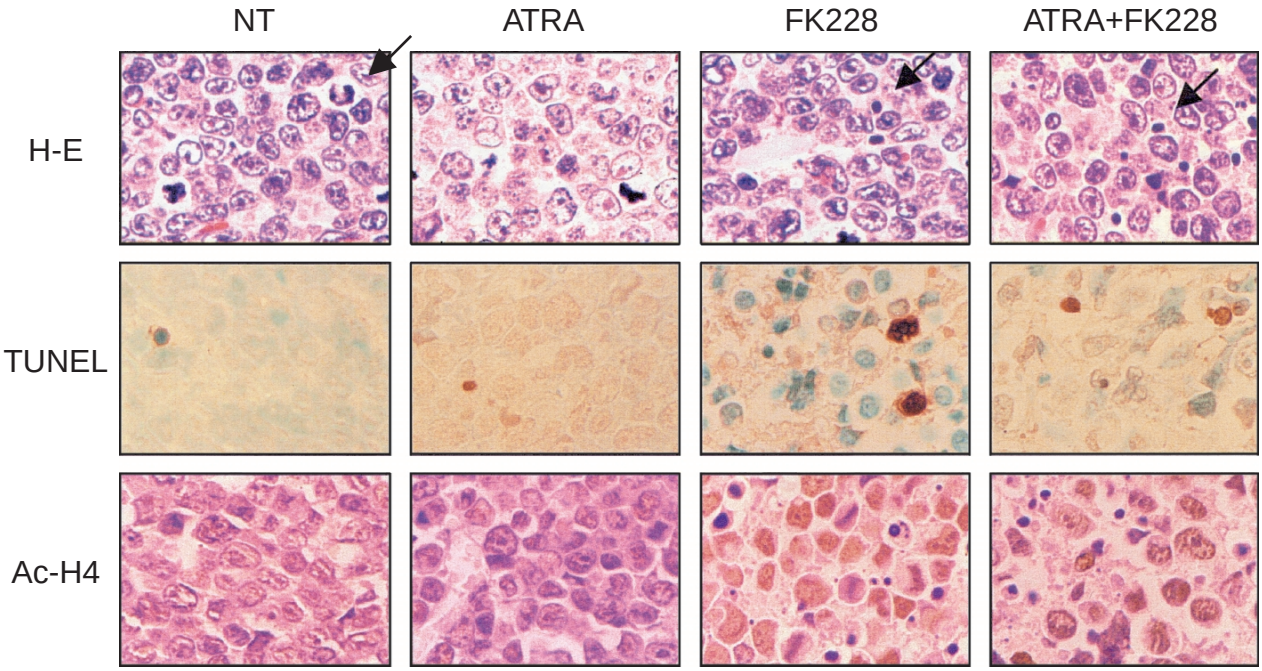

Fig. 5. Pathological examination of NB4 tumors in NOD-*scid* mice. After treatment with ATRA, FK228, or ATRA+FK228, the mice were sacrificed for pathological examination. Growth and apoptosis could be evaluated by morphological observation of mitosis (indicated by an arrow in non-treated (NT) mice) and condensed nuclei (indicated by arrows in FK228-treated mice), respectively. TUNEL-staining confirmed apoptotic cell death. The pathological findings are summarized in Table I.

Table I. Summary of Pathological Findings

NB4 in NOD-*scid*	Treatment			
	NT (%)	ATRA (%)	FK228 (%)	ATRA+FK228 (%)
Mitotic index	10.0	2.5	4.0	5.0
Apoptotic index	1	1	5	8
Differentiation NBT	0	26	0	38

Mitotic and apoptotic indexes mean the ratio of cells in mitosis and apoptosis, respectively. NBT-positivity was measured in suspended tumor cells.
NT, non-treated.

per week was tolerable to mice.[30, 31] The plasma concentration was over 1 μg/ml at maximum but rapidly decreased to less than 10 ng/ml within 1 h (unpublished data from Fujisawa Pharmaceutical Co.). Since this condition was enough to acetylate histone, the same dose and schedule were used for *in vivo* treatment. The dose of ATRA was according to the previous report.[41]

NB4 was inoculated into 20 NOD-*scid* mice. The mice inoculated with NB4 were divided into four groups; non-treatment (NT) (N=5), FK228 alone (N=5), and ATRA alone (N=5), ATRA plus FK228 (N=5). Treatment was done from day 14 to day 49. Tumor growth inhibition was significant from day 35 (P=0.05, by Mann-Whitney U test), and continued throughout the observation period. On day 35, tumor weight was 12.7±2.5, 4.8±0.3, 2.5±0.7 and 0.9±0.2 g in the four groups, respectively (Fig. 4). However, all the treated mice gradually developed subcutaneous tumors after 5 weeks. On day 49, tumor progression was significantly suppressed only in the combination treatment group (P=0.05). None of the treated mice displayed obvious signs of toxicity. This experiment indicates that the combination of FK228 and ATRA suppressed the progression of the tumor even when treatment was started after tumor formation.

On day 50, all mice were sacrificed for pathological examination (Fig. 5, Table I). H-E staining revealed that mitosis was less frequent in the tumors from the three treatment groups than in those from the non-treatment group. Apoptotic features such as condensed and fragmented nuclei were observed in the FK228 group and the ATRA plus FK228 group, and were confirmed by TUNEL staining. The NBT-reduction assay using suspension cells from the tumors showed that the percentage of NBT-positive cells was 0%, 0%, 26% and 38% in the non-treatment, FK228, ATRA, and ATRA plus FK228 groups, respectively. These data suggest that both differentiation and apoptosis are needed for the *in vivo* suppression of NB4 cells.

To observe the molecular effects of FK228, Ac-H4 was immunologically studied *in situ*. Treatment with FK228 induced hyperacetylation of histone H4 in the tumors (Fig. 5) and other organs such as liver, kidney and spleen (data not shown). However, apoptotic features were detected only in the tumors.

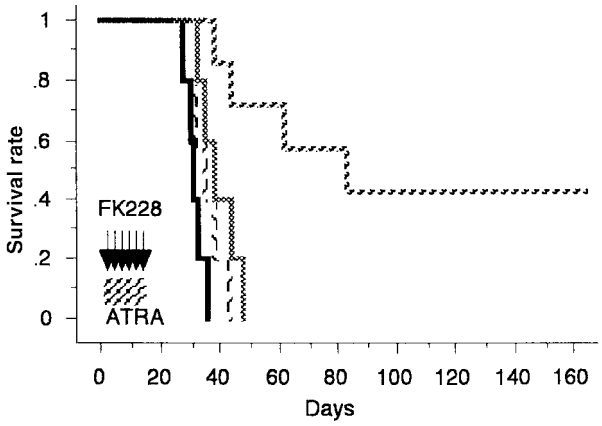

Fig. 6. Twenty mice were treated with FK228 (N=5, ——), ATRA (N=5, ----), or ATRA plus FK228 (N=7, ⋯⋯) or non-treated (N=5, ——). Treatment was started on the day after the injection and performed for 2 weeks. While the treatment with ATRA alone or FK228 alone had no significant effect on survival, the combination prevented tumor death in 3 of the 7 mice treated.

***In vivo* treatment of intravenously injected NB4 with FK228** In the above model, the effect of FK228 on survival could not be obtained, because the tumor size did not necessarily correlate with survival time. Thus, we generated another model by intravenous injection of NB4 cells. Treatment was started on the day after the injection and performed for 2 weeks; ATRA was administered daily and FK228 three times a week. The mice of the NT group (N=5) died of systemic leukemia involvement in lymph-nodes, the surface of the brain and the post-orbital space on day 28 to 35. While treatment with ATRA alone (N=5) or FK228 alone (N=5) had no significant effect on survival, the combination prevented tumor death in 3 mice of the ATRA plus FK228 group (N=7). Thus, the prognosis was significantly improved by the combination treatment (P=0.0001 by the Kaplan-Meier method, Fig. 6).

DISCUSSION

In this study, we have examined the feasibility of using FK228 in *in vivo* therapy for APL.

First, we observed that the differentiation of NB4 by ATRA was enhanced by FK228 or by TSA. Second, from the viewpoint of clinical application, we focused on the pharmacological difference between TSA and FK228. It was demonstrated that the cytotoxic effect depends on exposure time and dose, and is accompanied by the acetylation of histones. TSA[32] and suberoylanilide hydroxamic acid (SAHA)[33] are reportedly reversible inhibitors, and FK228 and trapoxins[34] are irreversible inhibitors. Other HDACIs reported (i.e., depudecin,[35] oxamflatin[36] and MS-275[37]) have not been characterized. Moreover, TSA and SAHA were demonstrated to bind with and to inhibit HDAC1,[38] but it remains unclear how other HDACIs inhibit HDACs. The mode of inhibition is likely to have a significant influence on the biological effects.

Another important consideration is the molecular pathway between HDAC inhibition and the biological effects. Indeed, inhibition of HDAC is the primary effect of HDACIs as many investigators have reported. However, it remains unknown whether HDACIs cause all of their biological effects through histone acetylation. It is not known whether histones are the sole substrate of HDAC or not. HDAC itself is a component of the complex mediating transcriptional repression and chromatin remodeling, and such machinery is conserved from yeast to mammals.[39] One possibility is that the treatment with HDACI increases the acetylation of proteins other than histones. Some transcriptional factors such as p53[40] are acetylated in association with biological function. It is still under investigation whether the treatment with HDACI augments the acetylation of transcriptional factors. Alternatively, a particular chromatin structure may be associated with the transcriptional preference. Thus, the targeting of the acetylation process possibly causes direct and indirect alterations of the transcriptional profile. Furthermore, since there are reportedly several different HDACs, it is important to distinguish the HDAC specificity of HDACIs for the development of selective transcription therapy.

In this study, we established *in vivo* APL models using NOD-*scid* mice. In the subcutaneous model, NB4 cells did not reconstitute leukemia in peripheral blood or bone marrow. However, this system was useful for directly measuring *in vivo* anti-tumor effects. The pharmacokinetics of FK228 is very important to its application *in vivo*.[30, 31] In murine plasma, FK228 had a short half-life ($t_{1/2}$), 0.05 and 0.47 h in the α and β phases, respectively. In rat, the plasma concentration was 20 ng/ml 3 h after 10 μg/g was intravenously injected and the $t_{1/2\beta}$ was 1.45 h. According to the wash-out experiments *in vitro*, the anti-tumor effect of FK228 was both dose- and time-dependent. Since the *in vitro* treatment with 10 ng/ml FK228 for 9 h did not decrease the viability of NB4 cells, we had speculated that the administration at 5 μg/g *in vivo* would be non-toxic to NB4 cells. Actually at this dose *in vivo*, FK228 had little effect on tumors.

To date, two methods have been developed to generate murine APL models. One is implantation of an APL cell line,[41] as used in this study, and the other is generating transgenic mice of the *PML-RAR*α chimeric gene.[42] The former model responded poorly to ATRA, although ATRA seems to be more effective in the transgenic model. In the present study, treatment with ATRA alone partly suppressed the tumor development. Pathological examination revealed that ATRA therapy induced immunohistochemical changes associated with differentiation, but not the morphological changes observed *in vitro*. On combination treatment with FK228 and ATRA, marked apoptosis and suppression of mitosis were observed. Although terminal differentiation was not detected, myeloid differentiation markers including NBT-reduction activity were induced. In this model, however, apoptosis and suppression of mitosis seem to contribute more than differentiation to the anti-tumor effect.

The *in vivo* model revealed that the HDACI therapy caused histone acetylation in various organs as well as the tumors. The degree of histone acetylation was similar in equivalent cells of the liver but varied in the kidney and spleen (data not shown). Many factors (i.e. pharmacokinetics, cell-cycle, and expression of HDAC and HAT) possibly make a difference. Notably, apoptotic damage was not observed in tissues other than the tumors. The sensitivity to HDACI may differ between tumors and normal tissue, or the combination may specifically target tumors. Thus, the use of FK228 is promising to enhance ATRA-sensitivity and presumably overcome ATRA-resistance in the *in vivo* treatment of APL.

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