CI-994 (N-acetyl-dinaline) in combination with conventional anti-cancer agents is effective against acute myeloid leukemia *in vitro* and *in vivo*

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Abstract. N-acetyl-dinaline (CI-994) is an investigational anti-cancer drug which inhibits histone deacetylases. We evaluated the interaction between CI-994 and conventional chemotherapeutics used in acute myeloid leukemia (AML) in a rat model for AML and Brown Norway rat acute myelocytic leukemia (BNML). *In vitro*, CI-994 in combination with cytarabine (ara-C), daunorubicin and mitoxantrone, resulted in moderate synergism. *In vivo*, higher dosages of CI-994 induced complete remissions. CI-994/ara-C was very active against BNML. The combinations of CI-994/daunorubicin and CI-994/mitoxantrone were also active against BNML. This study demonstrates favorable *in vitro* and *in vivo* interactions between CI-994 and conventional anti-cancer agents used for the treatment of AML.

Introduction

N-acetyl-dinaline (CI-994) is a novel, oral analog of the parent compound dinaline (1). This substituted benzamide derivative has shown activity in a broad spectrum of mouse, rat and human tumor models, including HCT-8 xenografts, murine mammary 25, colon 26 and a high anti-leukemic activity in the Brown Norway rat acute myelocytic leukemia model (BNML) (2,3). Moreover, CI-991 had only minor toxicity to normal pluripotent hematopoietic stem cells (4).

In a phase I clinical trial of patients with solid tumors, CI-994 was orally administered daily for 8 weeks. Thrombocytopenia was dose limiting at 8 mg/m^2. Other toxicities included fatigue and gastrointestinal effects such as nausea, vomiting, diarrhea, constipation and mucositis. One partial response was observed in a patient with heavily pre-treated adenocarcinoma of the lung (5). CI-994 is in phase II clinical trials for a number of neoplastic diseases (6,7).

With the exception of the BNML model, in which CI-994 is cytotoxic, tumor models sensitive to CI-994 respond in a cytostatic manner. Although several changes in cellular metabolism induced by CI-994 have been characterized (e.g. effects on cell cycle progression and the phosphorylation and stability of a low molecular weight protein), the primary molecular mechanism of its anti-tumor activity has only recently been described. Kraker *et al* demonstrated that CI-994 is a histone deacetylase (HDAC) inhibitor, which induces hyperacetylation of H3 in a time-and dose-dependent manner (8).

Histone modification is emerging as a central theme in the regulation of gene expression in a variety of cancers (9). Aberrant chromatin remodeling, through deacetylation of histones, results in tightly coiled DNA, restricting the access of transcription factors. HDAC inhibitors are a new class of cancer chemotherapeutics in clinical development that promote acetylation of histones leading to the uncoiling of chromatin and thereby enabling gene transcription. An important question in the clinical development of HDAC inhibitors is how to combine these agents with conventional chemotherapeutics already approved for the treatment of cancer.

Although the treatment of acute leukemia has improved significantly over the past few decades, the prognosis of acute myeloid leukemia (AML) remains relatively poor. Despite successful remission induction and consolidation treatment, ~30-50% of the patients that achieve CR ultimately relapse (10,11). Resistance to chemotherapy remains a major obstacle in the treatment of AML. New treatment modalities are therefore needed. HDAC inhibitors might be particulary effective in AML since this disease is characterized by chromosomal translocations that result in abberant fusion proteins, which act as dominant negative inhibitors and impair normal hematopoietic differentiation by the recruitment of nuclear corepressor complexes, including histone deacetylase. These so-called type II mutations act in consort with type I

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mutations (e.g. activating point mutations in receptor tyrosine kinases) resulting in proliferative and survival advantages of hematopoietic cells and impaired differentiation (12,13). The present study investigates the *in vitro* and *in vivo* interaction between the HDAC inhibitor CI-994 and conventional anti-cancer agents frequently used in the treatment of acute myeloid leukemia (AML): cytarabine (ara-C), daunorubicin and mitoxantrone.

Materials and methods

Materials. RPMI-1640 was purchased from Flow Laboratories and fetal calf serum (FCS) from Gibco. Ara-C was obtained from Upjohn and dissolved in DMSO for the *in vitro* experiments. CI-994 was kindly provided by Parke-Davis Pharmaceuticals (now Pfizer Inc, USA).

For the *in vivo* experiments, CI-994 was dissolved in 0.5% (w/v) carboxy-methyl cellulose, solubilized in purified water (Millipore Reagent Q system; Millipore, Bedford, USA) and protected against light. The drug solution was stored at -20˚C. Ara-C was prepared freshly at the start of each experiment (Pharmacia and Upjohn BV, Woerden, The Netherlands) by dissolving ara-C powder in 0.9% NaCl solution (Baxter BV, Utrecht, The Netherlands). Mitoxantrone (AHP Pharma BV, Hoofddorp, The Netherlands) was dissolved in 0.9% NaCl solution and stored at 15-25˚C. Daunorubicin (Rhône-Poulenc Rorer BV, Amstelveen, The Netherlands) was dissolved in water and diluted with 0.9% NaCl solution. The drug solution was stored at -20˚C.

Cell lines. Rat leukemia BCLO is the *in vitro* model isolated from Brown Norway myeloid leukemia (BNML). BCLO cells were cultured in RPMI-1640 as previously described (14), supplemented with 10% fetal calf serum, 3.2 mM glutamine and 250 ng/ml gentamycin (Gibco). Cells were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 and regularly screened for mycoplasma contamination by using a rapid detection system with a ^{3}H-labeled DNA probe (Gen-Probe) and were found to be negative. BCLO was generously provided by Dr A. Hagenbeek (Utrecht, The Netherlands).

In vitro growth inhibition. The *in vitro* growth inhibition induced by CI-994, ara-C, daunorubicin and mitoxantrone as single agents was determined after 72 h by cell counting. For the combination experiments, CI-994 was co-incubated with ara-C, daunorubicin and mitoxantrone at a fixed ratio for 72 h (CI-994:ara-C, 20:1; CI-994:mitoxantrone, 2000:1 and CI-994: daunorubicin, 250:1). The effect of the combinations was evaluated by median drug effect analysis (Chou & Talalay; Calcusyn, Biosoft, UK) and combination index (CI) values were calculated to determine drug interactions. Synergy was defined as CI<0.9; additivity as 0.9<CI<1.1 and antagonism as CI>1.1 (15).

In vivo studies - maximum tolerated dose. Initial toxicity studies were performed with groups of 3 male Brown Norway rats each (Harlan, Horst, The Netherlands). The maximum tolerated dose (MTD) was determined using schedules based on regimens currently used in the clinic. Drugs were administered in a maximum volume of 1 ml/kg body weight. CI-994 was administered intraperitoneally (i.p.) at 12 mg/kg once a day for 5 days, followed by a 2 day rest, after which CI-994 was administered again for 5 days [(q1d x 5) 2]. Ara-C was given subcutaneously (s.c.) at 160 mg/kg twice a day for 3 days (q0.5d x 6). Daunorubicin and mitoxantrone were administered i.p. at a dose of 2 and 1 mg/kg, respectively, once every 4 days for 3 times (q4d x 3). A 25% increase or decrease in dose was given when a drug was either ineffective or too toxic. Toxicity of the treatment was assessed on the basis of body weight (%) and survival rate after treatment. Body weight was determined on the first day of treatment (day 0) and set at 100%. Rats were weighed at least five times a week (starting on the first day of the treatment). Signs of sickness and mortality were recorded and moribund animals were sacrificed and examined macroscopically for side effects. Two weeks after the final drug injection, the MTD was defined as a maximum weight loss (MWL) of 15%.

In order to determine the MTD for the combination schedules, 66% of the MTD of each single agent was used with steps of 33% for an increase/decrease in dosage of the combination. The dosage of the conventional anti-cancer agents (ara-C, daunorubicin and mitoxantrone) was kept as high as possible.

Evaluation of therapeutic efficacy. The BNML rat model is characteristic of human AML in its progression and hematological pathology. It has been used extensively for the appraisal of therapeutic index, optimization of dose schedules and combinations of anthracyclines, ara-C and several other therapeutic drugs (16). BNML was established by injecting $1x10^7$ BCLO cells intravenously into the tail vein. Subsequently, BNML was maintained by isolation of BNML cells from the spleen of a Brown Norway rat with leukemia, as previously described (17). Recipient rats were inoculated with a total of $1x10^7$ BNML cells by an intravenous injection in the tail vein. Death was expected around day 25 after injection. Anti-leukemic effect studies were performed with the MTDs from the dose finding studies and various passages of BNML (4, 7 and 10). The randomized control and treatment groups consisted of 5 animals (except the control group at passage 4, which consisted of 8 animals). All drugs were also tested as single agents at the same dose that was used in the combination schedules. Weight, behavior, general condition and survival were monitored. Rats were taken off the experiment one day before expected death, animals were sacrificed and one day was added to the survival time. Survival time of the animal was used to calculate a median life span of the group (starting with the first day of treatment). The increase in life span (ILS) was determined using the following calculation: % ILS = T/C x 100 (T and C are the median days of death for the treated and control groups, respectively). Efficacy criteria were defined as follows: Inactive = ILS <125%, active = ILS >125% and very active = ILS >200%.

Evaluation of blood cell counts. The anti-leukemic effect was also evaluated by measuring blood cell counts. Fifteen male Brown Norway rats were injected with BNML passage 10. On day 7 after the injection, animals were divided into 3 groups: control group (n=5), CI-994 (5 mg/kg) treatment group (n=5)

Table I. *In vitro* growth inhibition by single drugs in BCLO cells.

Drug		IC_{50} value
Ara-C	nM	113.2±58.5
Daunorubicin	nM	7.5±1.4
Mitoxantrone	nM	1.2±0.5
Cl-994	μM	2.5±0.9

Values are means ± SD of 4 experiments.

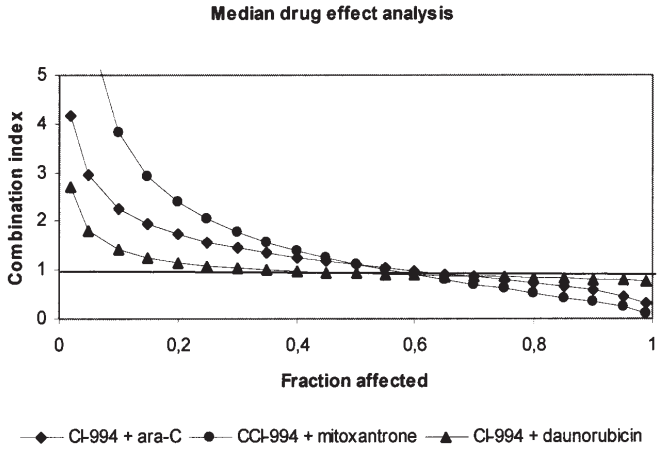

Figure 1. Median drug effect analysis. CI-994 was combined with ara-C, mitoxantrone and daunorubicin in the rat leukemia cell line BCLO at a fixed ration. Median combination indices: CI-994/ara-C = 0.83 (moderate synergism), CI-994/mitoxantrone = 0.68 (moderate synergism) and CI-994/daunorubicin = 0.86 (nearly additive).

and the CI-994 + ara-C (5 + 75 mg/kg) group (n=5). Blood samples were taken twice a week by a tail cut and 250 μl was collected in a microcontainer with EDTA (Becton Dickinson, Meylan Cedex, France). White blood cells (WBC), lymphocytes, red blood cells and platelets were measured by analyzed 50 μl of full blood on a Sysmex K-4500 automated hematology analyzer (TOA Medical Electronics Co., Ltd, Kobe, Japan). All measurements were expressed relative to the cell number on day -10 which was set at 100%.

All protocols were approved by the Ethics Committee for animal experiments of the VU University Medical Center in Amsterdam.

Results

In vitro growth inhibition and combination studies. The *in vitro* growth inhibition of BCLO cells by ara-C, daunorubicin, mitoxantrone and CI-994 as single agents was determined (Table I). All drugs were cytotoxic as single agents, ara-C, daunorubicin and mitoxantrone at nanomolar and CI-994 at micromolar levels. In the combination study, median drug effect analysis showed moderately synergistic interactions between CI-994/ara-C (mean CI = 0.83), CI-994/daunorubicin (mean CI = 0.86) and CI-994/mitoxantrone (mean CI = 0.68) (Fig. 1).

In vivo studies - dose finding experiments. MTDs for each of the single drugs and for the combinations, are listed in Table II. We did not observe any toxicity signs after administration of CI-994. After administration of several doses of ara-C (180, 200 and 250 mg/kg) toxicity signs were observed macroscopically, namely red secretion of eyes and nose, thick lips, thick salivary and lachrymal glands of the throat. A red-colored liver was seen at the 180 mg/kg dose and a (slight) yellow discoloration of the liver was observed at the 200 and 250 mg/kg dose.

Administration of 2.5 and 3.0 mg/kg daunorubicin (q4d x 3) resulted in weight loss of >15% and these schedules were therefore considered too toxic. At 2.5 mg/kg, one rat was found dead and two rats had to be sacrificed on day 22. These animals had a very small spleen and a colorless liver. At 3 mg/kg, all rats had to be sacrificed due to excessive weight loss, but no visible toxicity signs were found after obduction.

Administration of 1.6 and 2.0 mg/kg mitoxantrone (q4d x 3) resulted in moderate MWL on day 18 of 4.1 and 5.1%, respectively, though long-term toxicity effects were seen. One rat in the 1.6 mg/kg group died on day 23 with diarrhea and a very small spleen was observed at obduction. Another rat in this group died on day 64 and also had diarrhea with bleeding in the abdominal cavity. This animal had a very small spleen and liver. In the 2.0 mg/kg group, one rat died on day 50, with diarrhea, pink colored small intestine, coecum and colon and a very small spleen and liver.

Based on these data, initial combinations were performed on approximately two thirds of the MTD of each drug. Toxicity in these normal rats was a mixture to that observed for the single agents. Therefore these doses were used for initial anti-leukemic experiments.

Therapeutic efficacy and toxicity signs. Anti-leukemic effect studies were performed with different passages of BNML (Table III). Treatment with CI-994 alone was very effective and the highest dose induced complete remissions. At obduction, the animals in the 5 mg/kg treatment group had a light-red liver. In the 10 mg/kg treatment group, one animal became paralyzed and was sacrificed on day 110. Treatment with ara-C alone (100 mg/kg) was also effective against BNML and induced complete remissions. Lower doses gave a suboptimal effect. Single agent treatment with daunorubicin was inactive against BNML. Toxicity signs were seen at obduction, animals had diarrhea and a yellow colored liver. Mitoxantrone alone was active against BNML, but long-term toxic side effects were observed in the abdomen. At a lower dose mitoxantrone was not active.

The combination of high-dose CI-994/ara-C was also effective in all animals. No toxicity signs were observed. The combination schedule of high-dose CI-994/daunorubicin was active against BNML and induced complete remissions. In this treatment group, one animal died on day 8, a very small spleen and a yellow colored liver were observed at obduction. The median survival time of the group treated with high-dose CI-994/mitoxantrone was 123 days, although this regimen was too toxic. At obduction a yellow colored liver and a tough peritoneum were observed and the guts stuck together.

Since the activity of high-dose CI-994 alone against BNML was considerable, this masked the effect of

Table II. Dose finding studies of CI-994, ara-C, daunorubicin and mitoxantrone as single agents and in combinations in the BNML rat model.

Drug	Dose (mg/kg)	Scheme	Weight loss (%)	Days	
Cl-994	12	(q1dx5:) x 2	7.4	4	
	15	(q1dx5:) x 2	13.0	15	MTD
Ara-C	160	q0.5d x 6	4.7	4	MTD
	180	q0.5d x 6	12.4	8	
	200	q0.5d x 6	12.2	8	
	250	q0.5d x 6	20.4	8	
Daunorubicin	2.0	q4d x 3	2.8	9	MTD
	2.5	q4d x 3	19.0	21	
	3.0	q4d x 3	18.0	15	
Mitoxantrone	1.0	q4d x 3	0.6	1	
	1.6	q4d x 3	4.1	18	MTD
	2.0	q4d x 3	5.1	18	
Cl-994 + ara-C	10+100	[a]	12.7	4	MTD
Cl-994 + daunorubicin	10+1.3	[a]	11.6	11	MTD
Cl-994 + mitoxantrone	10+1.0	[a]	12.7	4	MTD

[a]See single drug. MTD, maximum tolerated dose.

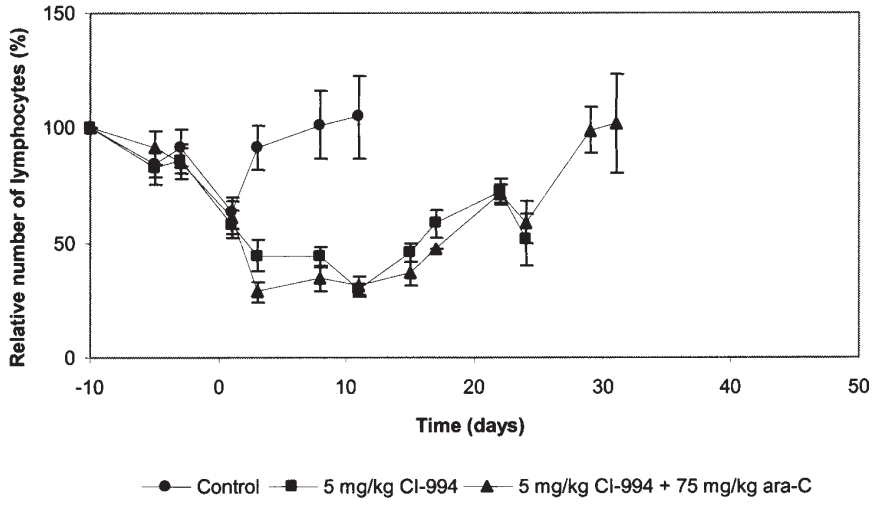

Figure 2. Relative number of lymphocytes in full blood of Brown Norway rats after inoculation with BNML (day -10) and treatment (start day 0). The cell number at day -10 ($14.7x10^6$ cells/ml) was set at 100% and a minimum of 4 rats were measured in each group. The number of lymphocytes decreased significantly in both treatment groups during treatment group (5 mg/kg CI-994 group, p=0.02; 5 mg/kg CI-994 + 75 mg/kg ara-C, p=0.02).

combination chemotherapy. Subsequent combination studies were therefore performed with a lower dose of CI-994 (5 mg/kg), while for the other drugs lower doses were used because toxicity in leukemia bearing rats was more than in normal rats. The combination of CI-994/ara-C was very active (ILS = 231 and 257%) against BNML. The median survival time of the animals treated with this combination was 37 days. The combinations of CI-994/daunorubicin and CI-994/mitoxantrone were classified as active against BNML, with a median survival time of 29 days in the two groups. However, the ILS of these combinations was similar to the ILS afforded by CI-994 as a single agent at this dose.

Blood cell counts. The cellular effects of treatment with CI-994 (5 mg/kg) alone and CI-994/ara-C (5 + 75 mg/kg) were determined by measuring blood cell counts in full blood of Brown Norway rats after inoculation with BNML (day -10) followed by treatment (day 0). These counts reflect the change in leukemic cells in the blood and the effect of the leukemia on other blood cell components. Blood cell counts on day -10

Table III. Anti-leukemic effect of Cl-994 alone and in combination with standard anti-cancer agents in the BNML rat model.

Treatment	Dose (mg/kg)	Median survival (days)	ILS (%)[a]	Dead/alive (at day 127)
BNML passage 4				
Control		26	100	7/8
Cl-994	5	43	165	5/5
Cl-994	10	126	>485	1/5
Cl-994	15	126	>485	0/5
Ara-C	100	126	>485	2/5
Daunorubicin	1.3	31	119	4/5
Daunorubicin	2	29	111	4/5
Mitoxantrone	1	60	231	5/5
Mitoxantrone	1.5	37	142	5/5
Cl-994 + ara-C	5+100	126	>485	0/5
Cl-994 + daunorubicin	10+1.3	126	>485	1/5
Cl-994 + mitoxantrone	10+1.0	123	473	3/5
BNML passage 7				
Control		16	100	5/5
Cl-994	5	29	181	5/5
Ara-C	75	23	144	5/5
Daunorubicin	1.3	22	138	5/5
Mitoxantrone	1.0	16	100	5/5
Cl-994 + ara-C	5+75	37	231	4/5
Cl-994 + daunorubicin	5+1.3	29	181	5/5
Cl-994 + mitoxantrone	5+1.0	29	181	5/5
BNML passage 10				
Control		14	100	4/5
Cl-994	5	28	200	5/5
Ara-C	75	23	144	5/5
Cl-994 + ara-C	5+75	36	257	4/5

[a]ILS <125%, inactive; ILS >125%, active; ILS >200%, very active.

were: White blood cells 19.1x10^9/l, lymphocytes 14.7x10^9/l and red blood cells 8.6x10^{12}/l.

There was considerable variation in blood cell counts between different days. During treatment, the number of white blood cells decreased significantly in both treatment groups, compared to the control group (5 mg/kg CI-994 group, p=0.02; 5 mg/kg CI-994 + 75 mg/kg ara-C, p=0.02). The decrease was most pronounced in the CI-994/ara-C treatment group. The number of white blood cells increased in all groups at the end of lifetime, including the control group. During treatment, the number of lymphocytes decreased significantly (5 mg/kg CI-994 group, p=0.02; 5 mg/kg CI-994 + 75 mg/kg ara-C, p=0.02), though after the end of treatment numbers returned to the level before treatment (Fig. 2). The number of red blood cells did not change significantly.

Discussion

CI-994 is an investigational anti-cancer drug with a broad spectrum of activity in murine and human tumor xenografts. It has been demonstrated that CI-994 possesses histone deacetylation activity, although other mechanisms may also contribute to its action (8). HDAC inhibitors act by promoting acetylation of histones, leading in turn to the uncoiling of chromatin and activation of a variety of genes involved in the regulation of cell survival, proliferation, differentiation and apoptosis (18). The present study demonstrates favorable *in vitro* and *in vivo* interactions between CI-994 and several conventional anti-cancer agents that are routinely used for the treatment of AML.

Our *in vitro* results demonstrated that CI-994 potentiated the cytoxicity of ara-C, daunorubicin and mitoxantrone in the BCLO rat leukemia cell line. *In vivo*, the combination of a low-dose of ara-C and CI-994 was very active against BNML rat leukemia, without signs of toxicity. At high dosages, CI-994 alone was also able to induce complete remissions in the BNML rat leukemia model. When CI-994 was combined with mitoxantrone and daunorubicin, an increased lifespan was observed compared to the control group, especially in the case of mitoxantrone where long term toxicity was observed.

There is relatively little known about the interaction between HDAC inhibitors and conventional chemotherapeutics. Previous *in vitro* findings have demonstrated that loosening-up the chromatin structure by histone acetylation increased the efficacy of several DNA damaging agents, including doxorubicin, in human cancer cell lines (19). In addition, HDAC inhibitors have been shown to be synergistic with other conventional chemotherapeutics such as etoposide, gemcitabine, vincristine and cisplatin *in vitro* (20-22). The mechanism of this synergistic interaction is not understood. The anti-cancer activity of HDAC inhibitors does not solely result from their ability to regulate histone acetylation, non-histone substrates are deacetylated as well (23). It is noteworthy that the mechanisms by which HDAC inhibitors promote growth arrest, differentiation and/or apoptosis appear to be highly variable. Besides alterations in gene expression, HDAC inhibitors have been shown to induce perturbations in cell cycle regulatory proteins (e.g. p21CIP1), down-regulation of survival signaling pathways (e.g. Raf/MAP-kinase/ERK) and the disruption of cellular redox state (e.g. reactive oxygen species, ROS). Recently, TRAIL has been identified as a mediator of the anti-cancer effect of HDAC inhibitors in AML (24,25).

Clinically, CI-994 has been combined with several conventional chemotherapeutics in a phase I setting, e.g. capecitabine (26). A phase I trial of oral CI-994 in combination with gemcitabine established the maximum tolerated dose for this combination (4 mg/m^2, po x 7) and reported 2 minor responses and 12 patients with stable disease (27). A recent phase II study in pancreatic cancer showed no advantage of gemcitabine plus CI-994 over gemcitabine alone in terms of overall survival, response rate or time to progression (28). In advanced solid tumors, CI-994 in combination with carboplatin and paclitaxel could safely be co-administered and an association between histone H3 acetylation levels and disease response was suggested. Five patients achieved a partial response (3 non-small cell lung cancer, 1 colorectal and 1 unknown primary) and 2 patients achieved a complete response (esophageal and bladder cancer) (29). These recent studies demonstrate that the use of CI-994 in combination with conventional agents is clinically feasible. Our *in vitro* and *in vivo* results demonstrated that a low-dose of CI-994 increased the activity of conventional anti-leukemic drugs at therapeutic doses, with acceptable side effects in AML. We therefore conclude that CI-994 may be a suitable drug for use in future trials with combination schedules in AML.

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