The Thalidomide Analogue, CC-4047, Induces Apoptosis Signaling and Growth Arrest in Childhood Acute Lymphoblastic Leukemia Cells In vitro and In vivo

Shabnam Shalapour,1 Andrea Zelmer,4 Madlen Pfau,1 Eva Moderegger,1 Cristiane Costa-Blechschmidt,2 Frank K.H. van Landeghem,2 Tillmann Taube,1 Iduna Fichtner,3 Christoph Bührer,4 Günter Henze,1 Karl Seeger,1 and Sven Wellmann4

Abstract

Purpose: Thalidomide and its analogues have shown promise in the treatment of multiple myeloma but their therapeutic potential has not been evaluated in models of acute lymphoblastic leukemia (ALL).

Experimental Design: We assessed the effects of the thalidomide analogue, CC-4047, on the growth and apoptosis signaling of human B cell precursor (BCP) ALL cell lines and freshly obtained childhood BCP-ALL cells grown with or without stromal cells. In addition, we studied the effects of CC-4047 on the progression and dissemination of xenotransplanted human BCP-ALL cells in nonobese diabetic/severe combined immunodeficiency mice.

Results: CC-4047 reduced the proliferation of human BCP-ALL cell lines in vitro. In contrast with the antileukemic effect of cytarabin, this was more pronounced when cell lines or freshly obtained childhood BCP-ALL cells were cocultured with stromal cells. CC-4047 induced the cleavage of caspase-3, caspase-9, and poly(ADP-ribose) polymerase in stroma-cocultured BCP-ALL cells. The inhibition of tumor growth, caspase-3 cleavage, and reduced microvessel density was observed in nonobese diabetic/severe combined immunodeficiency mice inoculated s.c. with childhood BCP-ALL cells upon CC-4047 treatment. After i.v. BCP-ALL xenotransplantation, CC-4047 reduced splenic dissemination.

Conclusions: The thalidomide analogue, CC-4047, displays profound cytostatic effects on stroma-supported human ALL cells both in vitro and in vivo.

Acute lymphoblastic leukemia (ALL) is the most common leukemia in childhood. The sustained improvement of ALL therapy regimens has led to high survival rates, however, 20% of all children remain incurable despite contemporary improved risk assessment, chemotherapy, hematopoietic stem cell transplantation, and supportive care (1). The dismal outcomes are associated with early relapse from the bone marrow (BM; refs. 2, 3), and novel treatment approaches that target mechanisms which allow ALL cells to grow and survive in the BM are needed.
Materials and Methods

Substances
The thalidomide analogue, CC-4047 (Celgene, Summit, NJ), was dissolved in DMSO (Sigma-Aldrich, Taufkirchen, Germany) for cell culture experiments and stored at −20°C until use. CC-4047 was diluted in culture medium (0.1-100 μmol/L) with <0.1% DMSO immediately before use. Cytarabine (ARA-C; 2-chloro-2'-deoxyadenosine, 1-t-arabinofuranosylcytosine) was purchased from Sigma-Aldrich. For in vivo use, CC-4047 was suspended in 0.5% carboxymethylcellulose according to the instructions of the supplier.

B cell precursor-ALL–derived cell lines, patient cells, and stromal cell lines
The human B cell precursor (BCP)-ALL cell line Z-181 [t(9;22); CD10+, CD19+] was a gift from Dr. Z. Estrov (Anderson Cancer Center, Houston, TX; ref. 15). The four BCP-ALL cell lines, REH [t(12;21); CD10+, CD19+, CD34−, Nalm-6 [t(6;12); q33.2;p13.2]; CD10+, CD19+, CD34−], RS4;11 [t(4;11); CD10−, CD19+, CD34+], and MHH-CALL-2 [CD10+, CD19+, CD34−], as well as the acute monocytic leukemia cell line, MV4-11 [t(4;11);q21;q23]; CD10−, CD19−, CD34+], were purchased from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany; http://www.dsmz.de/). Two stromal cell lines were used, the mouse stromal cell line MS-5 (DSMZ) and the human stromal cell line L87/4, kindly provided by Dr. K. Thalmeier (Institute of Pathology, University of Munich, Munich, Germany; ref. 16). Patient BCP-ALL cells (CD10+, CD19+, CD34+, BCR-ABL negative, and TEL-AML1 negative) were kindly provided by Dr. K. Thalmeier. Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany; http://www.dsmz.de/).

Cell cultures
Z-181 was cultured in α-MEM (Biochrom). All other cells including the stromal cells were cultured in RPMI 1640 (Biochrom) at 37°C in 5% CO2 in a humidified incubator. Media were supplemented with 10% FCS (Biochrom) and 1% penicillin/streptomycin (Biochrom), referred to as complete medium. Patient BCP-ALL cells were cultured after centrifugation on a Ficoll gradient prior to experiments for 24 hours in basal Iscoves medium (Biochrom) supplemented with 10% FCS and 1% donor horse serum (Biochrom). For monoculture assays, 3 × 10^5 leukemia cells per well were seeded into a six-well plate (2 mL/well) in complete medium together with DMSO, CC-4047, or cytarabine. For coculture assays, 1 × 10^5 stromal cells per well were seeded into a six-well plate in complete medium (2 mL/well). After 24 hours, supernatants with unattached cells were removed and replaced with fresh medium containing different serum concentrations (1-10%) as indicated and 3 × 10^5 leukemia cells, as well as DMSO, CC-4047, or cytarabine.

Cell proliferation assays
The proliferation rate of leukemic cells in monoculture and coculture assays was examined by the use of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium] tetrazolium assay (Cell Titer96 Aqueous, Promega, Mannheim, Germany). Supernatants of monoculture and coculture assays containing leukemic cells were carefully stirred and pipetted in quadruplicate (100 μL/well) in 96-well plates. MTS (20 μL) was added to each well and after 2 hours of incubation at 37°C in a humidified, 5% CO2 atmosphere, the plates were read in a microplate autoreader (Multiskan Ascent, Thermo Electron Corporation, Dreieich, Germany) at 450 nm wavelength, with 630 nm as reference. The results are expressed as cell number per microliter of medium and were calculated from values obtained with standard cell dilutions. In order to validate these results, cell count and size were determined at the end of monoculture and coculture assays using the multichannel electronic cell counter and analyzer system CASY (Schärfe Systems, Reutlingen, Germany).

Flow cytometry
Apoptotic and necrotic cells were quantitated in monoculture and coculture assays by use of the phosphatidyl serine detection kit (IQProducts, Groningen, Netherlands). Briefly, cells were sequentially incubated at 4°C for 20 minutes with FITC-conjugated Annexin V, and for 5 minutes with propidium iodide, as suggested by the manufacturer. Stained cells (10^4 per sample) were analyzed on a FACScan flow cytometer with standard CellQuest software (BD Biosciences, Palo Alto, CA).

Western blots
At the end of monoculture and coculture assays, all cells were harvested and incubated with CD19 micromagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to separate leukemic cells by magnetic-activated cell separation. Then, the separated leukemic cells were washed in ice-cold PBS and lysed using Chaps Cell Extract buffer according to the manufacturer’s instructions (Cell Signaling, Beverly, MA). Cell lysates were centrifuged at 20,000 × g for 15 minutes at 4°C. Protein concentrations in the supernatants were determined by the reducing agent–compatible/detergent-compatible protein assay (Bio-Rad, Hercules, CA). Equal protein amounts (30 μg) were separated by 12% to 15% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Buckinghamshire, United Kingdom). After blocking with 5% nonfat milk in PBS, the membranes were probed with rabbit antibodies against human cleaved caspase-3 (Asp175), cleaved caspase-9 (Asp315), poly(ADP-ribose) polymerase (Asp214), and Bcl-2, all diluted 1:1,000 and all from Cell Signaling. Blots were subsequently incubated with horseradish peroxidase–linked secondary goat anti-rabbit antibody (Cell Signaling). The signals were detected by chemiluminescence phototope–horseradish peroxidase kit (Amersham) according to the manufacturer’s instructions. As an internal control, blots were stripped and reprobed with mouse anti-β-actin antibody (Sigma-Aldrich) followed by horseradish peroxidase–linked secondary sheep anti-mouse antibody (Cell Signaling).

Animal experiments
All animal experiments were done in female nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (5-8 weeks of age) lacking T, B, and natural killer cells and macrophage function (The Jackson Laboratory, Bar Harbor; refs. 17, 18). The mice were maintained under sterile and standardized environmental conditions (20 ± 1°C room temperature, 50 ± 10% relative humidity, 12-hour light/dark rhythm) and received autoclaved food and bedding (sniff Specialdiäten, Soest, Germany) and acidified (pH 4.0) drinking water ad libitum. Mice were tested for leukemia and only animals with IgG levels <100 ng/mL were used. All experiments were done according to the German Animal Protection Law with permission from the responsible local authorities. In compliance with such regimens, mice were euthanized when tumor volumes exceeded 10% of the body weight.

Xenograft models of therapy-resistant ALL
To determine the antitumor activity of CC-4047, 5- to 8-week-old female NOD/SCID mice were inoculated in the s.c. challenge model with 1 × 10^7 leukemia blasts from a 1-year-old boy with first manifestation of MLL/AF4-positive BCP-ALL (s.c. model ALL-SCID 19) in Matrigel (Sigma-Aldrich; ref. 19). In the i.v. challenge model, 1 × 10^7 leukemia blasts were inoculated from a 5-year-old girl with second relapse of BCR/ABL-positive BCP-ALL (i.v. model ALL-SCID 7; ref. 19). In both models, the total injection volume of the cell suspension was 0.2 mL/mouse. Once the tumors reached a size of 200 mm^3 in the s.c. model, and 3 days after inoculation in the i.v. model,
animals were randomized (n = 8 per group). Each group was treated i.p. daily with either the drug vehicle (0.5% carboxymethylcellulose) or CC-4047 at 50 mg/kg. Prior to our tumor challenge experiments, we did an in vivo tolerability study with CC-4047 in which 5- to 8-week-old female NOD/SCID mice were given daily i.p. injections of either the vehicle (0.5% carboxymethylcellulose) or CC-4047 at 25 to 100 mg/kg for 2 weeks. Three animals were included in each dose group. Necropsy was done by veterinary pathologists and depicted no signs of toxicity. In the tumor challenge model (thrice a week), serial caliper measurements of perpendicular diameters were used to calculate tumor volume using the formula: (shortest diameter)² × (longest diameter) × 0.5. The animals were observed daily, and when mice of the control group were moribund, all animals were sacrificed by CO₂ asphyxiation. Harvested tumors were snap-frozen in liquid nitrogen and spleens were removed and weighed.

**Tumor histology**

*Microvessel staining and counting.* After removal, the tumors were immediately snap-frozen in liquid nitrogen and cut to 5-μm-thick cryostat sections. Sections were fixed in cold acetone for 10 minutes and washed with PBS. Nonspecific antibody binding was inhibited with 10% normal rabbit serum in PBS for 30 minutes. The incubation with the monoclonal antibody rat anti-mouse CD31 (clone 390, 1:50 dilution; Serotec, Oxford, United Kingdom) was done for 90 minutes at room temperature. As secondary antibody F(ab')2 rabbit anti-rat IgG was used and applied at a dilution of 1:50 for 30 minutes. The signal was detected using the avidin-biotin complex method according to the manufacturer (Dako, Hamburg, Germany). 3,3′-Diaminobenzidine hydrochloride was used as chromogen. Finally, the sections were counterstained with hematoxylin. In control sections, the primary antibody was omitted.

Morphometrical analysis was done according to Weidner et al. (20). The sections were independently examined by two observers blinded with respect to the treatment of mice, sections were subsequently screened for the tumor areas with the highest vascularization. In these areas, the number of blood vessels were determined in at least five standardized microscopic fields of 625 μm², defined by an ocular grid at a final magnification of ×400. Values represent the number of blood vessels per square millimeter.

**Fluorescence immunohistochemistry of cleaved caspase-3 and CD10.** After blocking of unspecific binding, sections were incubated with monoclonal primary antibodies, mouse anti-human CD10 biotin-labeled (dilution 1:50, incubation for 90 minutes at room temperature, clone 138; Leinco Technologies, St. Louis, MO) or rabbit anti-human cleaved caspase-3 (1:100, overnight at 4°C; Cell Signaling), respectively. Sections were then washed with PBS and further incubated with an avidin-FITC conjugate (1:200, 90 minutes at room temperature; Sigma-Aldrich) and a donkey anti-rabbit IgG conjugated to Alexa 594 (1:100, for 60 minutes at room temperature; Molecular Probes, Invitrogen, USA).

**Immunoblotting** was done to detect cleavage of the proapoptotic molecules caspase-3, caspase-9, and poly(ADP-ribose) polymerase. Actin served as a control for equal protein loading. All data shown are representative of three independent experiments.

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**Fig. 1.** CC-4047 inhibits the proliferation of BCP-ALL cell lines in a dose-dependent manner. Cells were grown in complete medium (containing 10% FCS) with increasing concentrations of CC-4047. After 72 hours, proliferation was measured by the use of the MTS tetrazolium assay. Points, mean values plotted from triplicate determinations, representative of three independent experiments; bars, SE.

**Fig. 2.** CC-4047 induces growth arrest and activates proapoptotic molecules in monocultures and cocultures of MHH-CALL-2 cells. MHH-CALL-2 cells were grown alone or in coculture with murine MS-5 stromal cells in the presence of different FCS concentrations as indicated, and were treated with or without CC-4047 and ARA-C for 72 hours. Proliferation was measured by the use of the MTS tetrazolium assay and cell numbers per microliter were calculated from appropriate well dilution series. Columns, mean of at least three independent experiments; bars, SE (*, P < 0.05; **, P < 0.01). Immunoblotting was done to detect cleavage of the proapoptotic molecules caspase-3, caspase-9, and poly(ADP-ribose) polymerase. Actin served as a control for equal protein loading. All data shown are representative of three independent experiments.
Karlsruhe, Germany), respectively. Finally, sections were covered with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) prior to examination under fluorescence microscope (Axioplan 200; Zeiss, Jena, Germany).

**Statistical analysis**

The significance of the differences between groups was determined by Student’s *t* test. The Kruskal-Wallis one-way ANOVA on ranks was used for statistical analysis in case of microvessel counting. *P* < 0.05 was assumed to denote statistical significance.

**Results**

**CC-4047 inhibits growth of ALL cell lines when cultured in monoculture.** The initial characterization of CC-4047 for activity in BCP-ALL included proliferation assays using different BCP-ALL cell lines. In these assays, we observed that CC-4047 directly inhibits the proliferation of leukemic cells in a dose-dependent manner when cultured in complete medium (Fig. 1). The growth arrest of CC-4047 was less distinct when leukemic cells were cultured in serum-starved medium (for MHH-CALL-2 cells; Fig. 2). The most sensitive of all tested cell lines were MHH-CALL-2 cells (50% inhibition), followed by Nalm-6, RS4;11, REH, and Z-181, whereas proliferation of the acute monocytic leukemia cell line, MV4-11, was only slightly affected by treatment with CC-4047 (Fig. 1).

**Stromal cells support leukemic cell growth but do not prevent CC-4047-induced growth arrest and apoptosis.** To ascertain whether the stroma might exert a protective effect on leukemic cells as it was seen when treated with cytarabine (Figs. 2 and 3), CC-4047 was added to cocultures of different ALL cell lines with stromal cells. Coculture of MHH-CALL-2, REH, and Z-181 with stromals did not alter the antiproliferative effect of CC-4047 (Figs. 2 and 3). Moreover, the CC-4047-induced inhibition of proliferation was more severe when ALL cell lines grew supported by stromal cells. To exclude the possibility that the antiproliferative effect of CC-4047 results from damaging stromal cells, we investigated the potential effect of CC-4047 on the proliferation of both stromal cell lines, MS-5 and L87/4. Treatment with CC-4047 in different doses did not alter the
The beneficial effect of stromal cells on leukemic cell proliferation seems to depend mainly on a direct cell-cell contact between leukemic and stromal cells, which could not be compensated for by the addition of conditioned stromal cell medium to leukemic cells, or by the separation of cocultures with filters (Fig. 4).

Having shown that CC-4047 inhibits leukemic cell proliferation, we next evaluated its effect on the level of protein expression of proapoptotic and antiapoptotic markers. As shown in Fig. 2, treatment of leukemic cells in coculture with CC-4047 induced the cleavage of caspase-3, caspase-9, and poly(ADP-ribose) polymerase similar to cytarabine-treated cells. Again, whereas stromal cells protected leukemic cells from cytarabine-induced cytotoxicity, cleavage of the caspases was more pronounced, when ALL cell lines grew supported by stromal cells (Fig. 2). In addition, the expression of the antiapoptotic marker, Bcl-2, was down-regulated in monocultures and cocultures after treatment with CC-4047 in Z-181 and MHH-CALL-2 cells (Fig. 3B).

**CC-4047 inhibits proliferation and induces apoptosis in ALL patient cells.** Freshly isolated leukemic cells from the BM of five pediatric patients with BCP-ALL were cocultured with human stromal cells (L87/4) in serum-starved medium and treated for 72 hours with 10 μM of CC-4047. As shown in Fig. 5, treatment with CC-4047 induced a pronounced increase in apoptosis in all ALL samples. There was also reduced proliferation in four of five ALL samples.

**CC-4047 leads to tumor regression and reduces dissemination in ALL in vivo models.** To examine the influence of CC-4047 on therapy-resistant ALL in vivo, we injected s.c. leukemic blasts into NOD/SCID mice (s.c. model ALL-SCID 19) and started treatment with 50 mg/kg/d of CC-4047 as described (21) once the tumor was palpable (average size, 200 mm³). Our previous in vivo toxicity studies depicted no signs of toxicity up to 100 mg/kg (see Materials and Methods). All animals were sacrificed at day 20 when the tumors of the control animals had reached a critical size. Animals treated with CC-4047 showed significant inhibition of tumor growth (Fig. 6A).

Next, we asked whether CC-4047 was also able to inhibit ALL when grown in the i.v. challenge model (i.v. model Fig. 6).

**Fig. 5.** Treatment of diagnostic patient BCP-ALL cells in coculture with CC-4047 induces growth arrest and apoptosis. Fresh BCP-ALL cells of five different pediatric patients were collected at diagnosis and cocultured with human stromal cells (L87/4). Patient nos. 1, 2, and 5 had a first relapse, patient no. 3 had a second relapse after SCT, and patient no. 4 had primary disease. After treatment with or without CC-4047 in serum-starved medium (1% FCS), proliferation was quantitated by the use of the MTS tetrazolium assay and cell numbers per microliter were calculated from appropriate cell dilution series. Apoptosis was determined by use of the phosphatidylserine detection kit. Columns, means of triplicate cultures; bars, SE (*, P < 0.05; **, P < 0.01).

**Fig. 6.** CC-4047 blocks in vivo growth of established tumors in the ALL-SCID 19 model and reduces leukemic dissemination in the ALL-SCID 7 model. A, NOD/SCID mice were inoculated s.c. with 1 × 10⁷ patient BCP-ALL cells. When tumor volume reached 200 mm³, groups of eight mice were treated daily with either CC-4047 (●, 50 mg/kg) or vehicle (○) via i.p. injection. Mice were observed daily for signs of toxicity. Points, mean of tumor volume (n = 8) after start of treatment day + 1; bars, SD. Tumor volumes are significantly different for CC-4047 versus control (P < 0.001, day 20). B, NOD/SCID mice were inoculated i.v. with 1 × 10⁷ patient BCP-ALL cells. Three days after tumor inoculation, groups of eight mice were treated daily with either CC-4047 (50 mg/kg) or vehicle via i.p. injection. Mice were observed daily for signs of toxicity and when the first mice became moribund, all mice were sacrificed.
ALL-SCID 7). This model represents the characteristics of leukemogenesis much better than the s.c. model. Treatment was started 3 days after i.v. inoculation with 50 mg/kg/d of CC-4047 and was continued until day 27. At day 28, mice were sacrificed when the first animals of the control group became moribund, whereas the treated animals depicted no signs of disease or toxicity. Dissemination of leukemic cells into the spleen was significantly reduced in the treated animals, both when absolute spleen weight and when spleen weight as a percentage of the total body weight was calculated (Fig. 6B).

**Reduced MVD and activated caspase-3 in CC-4047-treated tumors.** Because CC-4047 has been shown to be a potent antiangiogenic drug, we were interested in studying the antiangiogenic activity in addition to the proapoptotic activity in vivo. CC-4047-treated tumors of the ALL-SCID 19 model showed significantly lower MVD than did tumors of the control group (Fig. 7A). Cleaved caspase-3 was observed with moderate to strong intensity in all tumors of CC-4047-treated mice, whereas vehicle-treated tumors displayed no cleaved caspase-3 (Fig. 7B).

**Discussion**

This study identified the thalidomide analogue, CC-4047, as a potent drug targeting ALL cell growth and survival. CC-4047 overcomes stromal support of leukemic cells and exerts its antitumor activity in at least three different ways. The combination of these mechanisms may explain the dramatic tumor regression observed in vivo.

First, a variable direct antitumor effect of CC-4047 was shown in monoculture experiments of various BCP-ALL cell lines. Rates of growth inhibition ranged between 10% and 50%, which is less than those of lymphoma cells (HSSultan) and MM cells (MM.1S and MM.1R; refs. 22, 23). Apoptosis of BCP-ALL cells induced by CC-4047 was associated with activation of caspase-3 and caspase-9, whereas CC-4047-mediated apoptosis of MM cells involves the activation of caspase-8 but not caspase-9 [24], suggesting that the mechanism of cell death may be tumor cell type–dependent.

Second, when investigating the growth of BCP-ALL cell lines or childhood BCP-ALL cells supported by stromal cells, CC-4047...
inhibited leukemic cell proliferation up to 60%. Thus, the antiproliferative effect of CC-4047 was not attenuated by stromal cells, in contrast to the attenuation of cytarabine cytotoxicity after stroma coculture (6).

Third, antiangiogenesis, as a hallmark of thalidomide action (25), was also observed with CC-4047, as CC-4047 reduced MVD in xenotransplanted leukemic tumors. Although MVD in the untreated ALL tumors was low (±9 microvessels/×400 field), as compared with the published results of Hs Sultan tumors (±29; ref. 21), there is unequivocal evidence that increased MVD occurs in the BM of patients with various hematologic malignancies including childhood ALL (12–14). Expression of the key angiogenetic growth factor, vascular endothelial growth factor, and its receptor have been shown in childhood ALL samples (26–28).

In conclusion, we have shown that CC-4047 is a powerful antileukemic agent in preclinical in vitro and in vivo models. CC-4047 stopped the growth of relapsed childhood ALL cells transplanted into NOD/SCID mice without evident side effects. This was also observed for disseminated ALL in which established cytotoxic drugs such as cytarabine, daunorubicin, and asparaginase, have failed to reduce tumor burden (29). As CC-4047 is well tolerated by adult patients with MM (30), its use in relapsed childhood ALL refractory to conventional chemotherapy is a promising approach.

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