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ORIGINAL ARTICLE

MRx102, a triptolide derivative, has potent antileukemic activity *in vitro* and in a murine model of AML

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Triptolide, isolated from the herb *Tripterygium wilfordii*, has been shown to potently induce apoptosis in various malignant cells by inhibiting RNA synthesis and nuclear factor-κB activity. Previously, we showed that triptolide promotes apoptosis in acute myeloid leukemia (AML) cells via the mitochondria-mediated pathway, in part, by decreasing levels of the anti-apoptotic proteins XIAP and Mcl-1. MRx102 is a triptolide derivative, currently in preclinical development. Here we show that MRx102 potently promoted apoptosis in AML cell lines, with EC_{50} values of 14.5 ± 0.6 nM and 37.0 ± 0.9 nM at 48 h for OCI-AML3 and MV4–11 cells, respectively. MRx102, at low nanomolar concentrations, also induced apoptosis in bulk, $CD34^+$ progenitor, and more importantly, $CD34^+CD38^-$ stem/progenitor cells from AML patients, even when they were protected by coculture with bone marrow derived mesenchymal stromal cells. MRx102 decreased XIAP and Mcl-1 protein levels and inhibited RNA synthesis in OCI-AML3 cells. *In vivo*, MRx102 greatly decreased leukemia burden and increased survival time in non-obese diabetic/severe combined immunodeficiency mice harboring Ba/F3-ITD cells. Collectively, we demonstrated that MRx102 has potent antileukemic activity both *in vitro* and *in vivo*, has the potential to eliminate AML stem/progenitor cells and overcome microenvironmental protection of leukemic cells, and warrants clinical investigation.

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Keywords: MRx102; triptolide; XIAP; Mcl-1; AML and AML stem cells; microenvironment

Introduction

Triptolide, isolated from a Chinese herb *Tripterygium wilfordii*, has been used in Chinese medicine for centuries to treat inflammatory and autoimmune diseases. In recent years, triptolide has attracted attention for its ability to inhibit growth and promote death of tumor cells *in vitro* and *in vivo*,[1–5] and to enhance the anti-tumor effects of various chemotherapeutic agents.[3,6–10] Triptolide exerts potent anti-tumor activity through multiple mechanisms. Its ability to inhibit nuclear factor-κB[11,12] and HSP70,[13] decrease levels of the anti-apoptotic proteins XIAP, Mcl-1 and Bcr-Abl,[3,4,14] induce expression of p53 and death receptor DR5,[9] and block the SDF-1/CXCR4 axis[15] are just a few examples. Most, if not all, of these functions are probably attributable to the ability of triptolide to target RNA polymerase, thereby decreasing the levels of short-lived anti-apoptotic proteins,[16–18] and its ability to inhibit tumor cellular proteasome activity, as recently reported.[19]

Antitumor activity of triptolide was first identified in leukemia.[20] We previously showed that triptolide decreased mRNA and protein levels of XIAP and Mcl-1 in myeloid leukemia cells, decreased the level of Bcr-Abl in chronic myeloid leukemia (CML) cells, induced apoptosis in both acute myeloid leukemia (AML) and CML cells, sensitized AML cells to TNF-related apoptosis-inducing ligand-induced apoptosis via decrease of XIAP- and p53-mediated increase of DR5, and importantly, induced apoptosis independent of cellular responses to imatinib in blast crisis CML cells, including quiescent $CD34^+$ primitive progenitor cells.[3,4,9] To optimize the activity of triptolide for clinical applications, new triptolide derivatives with improved pharmacokinetics and bioavailability have been designed and synthesized. Among them, PG490-88Na (F60008)[21,22] has entered clinical development and has been shown to have potent antitumor activity. Importantly, complete remissions were reported in a phase 1 study in patients with refractory or relapsing acute leukemias.[23]

MRx102 is a potent triptolide derivative currently under development by MyeloRx (Vallejo, CA, USA). To determine the therapeutic potential of MRx102 in leukemia, we examined the effects of MRx102 on AML cells, including primary human AML cells and AML stem/progenitor cells, and non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice injected with Ba/F3-ITD cells. Here, we report that MRx102 decreased XIAP and Mcl-1 protein levels, inhibited RNA synthesis in OCI-AML3 cells, and had potent activity not only against AML cell lines and bulk AML blasts, but also against AML progenitor/stem cells, even when they were protected by coculture with bone marrow (BM)-derived mesenchymal stromal cells (MSCs) *in vitro* and in a murine model of AML.

Materials and methods

Cells, cell culture and treatment of cells

Human AML cell lines OCI-AML3 was kindly provided by Dr M Minden (Ontario Cancer Institute, Toronto, ON, Canada) and MV4-11 was purchased from the American Type Culture Collection (Manassas, VA, USA). Ba/F3-ITD cells, a murine pro-B lymphocyte line stably transfected with expression vector encoding human FLT3 with an internal tandem duplication (ITD) mutation, were generated by Dr D Small's laboratory (Johns Hopkins University School of Medicine, Baltimore, MD, USA). Ba/F3-ITD-GFP/Luc cells, which stably express *Renilla* luciferase and green fluorescent protein (GFP), were generated by infecting Ba/F3-ITD cells with a lentivirus-based construct as previously described.[24] All cell lines were cultured in RPMI

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1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Fresh BM or peripheral blood samples from AML patients with high blast counts, and BM samples from normal subjects were acquired after written informed consent had been obtained according to the Declaration of Helsinki, and the study protocol was approved by the MD Anderson Institutional Review Board. Mononuclear cells were purified by Ficoll-Hypaque (Sigma, St Louis, MO, USA) density-gradient centrifugation and cultured in the same medium as for the cell lines. The clinical characteristics of these samples are summarized in Table 1.

OCI-AML3 and MV4–11 cells (0.4×10^6 per ml), Ba/F3-ITD-GFP/Luc cells (0.1×10^6 per ml), or mononuclear cells from AML and normal BM samples (0.5×10^6 per ml) were treated with various concentrations of MRx102 for up to 72 h. An appropriate amount of dimethyl sulfoxide was used as the control. For treatment of AML cells cocultured with MSCs, early-passage MSCs isolated from human BM, as previously described,[25] were pre-plated at 5×10^3 per cm^2 for 24–96 h; AML cells were then added and treated.

Cell viability assay

Viable cell counts were determined by flow cytometry using CountBright absolute counting beads (Invitrogen, Carlsbad, CA, USA) on annexin V-/7-amino-actinomycin D-negative cell events. Apoptosis was assessed by flow cytometry of phosphatidyl serine[26] externalization with annexin-V-Cy5 (BD Biosciences, San Diego, CA, USA) using a FACSArray Bioanalyzer (BD Biosciences). Membrane integrity was simultaneously assessed by 7-amino-actinomycin D exclusion in the annexin V-stained cells. For AML patient samples, apoptosis was determined in bulk, as well as in primitive and stem/progenitor cell compartments, after cells were stained with anti-CD34 and anti-CD38 antibodies. For AML cells cocultured with MSCs, apoptosis induction in various cell compartments was analyzed separately for detached cells (floaters) and attached (adherent) cells. Floaters were obtained by combining all cells in suspension and cells collected after washing the wells twice with phosphate-buffered saline. Adherent cells were obtained by trypsinization. Leukemic cells were distinguished from MSCs by gating on $CD45^+$ populations. Floaters or adherent cells were stained with CD45-APC-H7, CD34-PE, CD38-PE-Cy7 and annexin-V-Cy5 (BD Biosciences). Apoptosis was determined by flow cytometry of annexin-V-Cy5 positivity in bulk ($CD45^+$), $CD45^+CD34^+$, $CD45^+CD34^+CD38^+$ and $CD45^+CD34^+CD38^-$ cells. To eliminate the variation from spontaneous apoptosis in AML patient samples and normal controls, the apoptosis measured in these samples was expressed as specific apoptosis:

$$\frac{\%\text{ of apoptosis in treated cells} - \%\text{ of apoptosis in untreated cells}}{\%\text{ of apoptosis in untreated cells}} \times 100\%$$

Western blot analysis

Mcl-1 and XIAP protein levels were determined by western blot analysis, as described previously.[27,28] XIAP antibody was purchased from BD-Transduction Laboratories (BD Biosciences) and Mcl-1 antibody from Cell Signaling Technology (Danvers, MA, USA). Signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantitated using Odyssey software version 3.0 (LI-COR Biosciences). β-Actin was used as a loading control.

Measurement of RNA synthesis

RNA synthesis was measured by quantitating incorporation of [^{3}H]uridine into the perchloric acid-insoluble materials. Briefly, MRx102-treated OCI-AML3 cells were labeled with [^{3}H]uridine (10 μCi/ml) for 1 h, washed twice with 10 ml of ice-cold phosphate-buffered saline, and then lysed while vortexing with 0.5 ml of H_2O and 0.5 ml of 0.8 N perchloric acid. The pellet was collected by centrifugation, washed once with 1 ml of 0.4 N perchloric acid, and then dissolved in 1 ml of H_2O with 50 μl of 10 N potassium hydroxide overnight. The supernatant was then transferred to scintillation vials and radioactivity was quantitated.

Ba/F3-ITD-GFP/Luc murine model

Ba/F3-ITD-GFP/Luc cells (0.3×10^6) were injected into the tail vein of 7-week-old female NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME, USA). Starting on day 3, mice (11/group) were treated with vehicle control (phosphate-buffered saline/dimethyl sulfoxide), 1.5 mg of MRx102/kg/day, or 3.0 mg of MRx102/kg/day for 5 weeks intraperitoneally. The percentage of circulating Ba/F3-ITD-GFP/Luc cells was determined by flow cytometry of GFP in blood samples. Tumor infiltration was monitored by non-invasive bioluminescence imaging using the IVIS-200 *in vivo* imaging system (Xenogen, Hopkinton, MA, USA) after mice were injected with the luciferase substrate coelenterazine (Biotium, Hayward, CA, USA). The extent of leukemic infiltration of various organs was assessed by hematoxylin and eosin or anti-GFP staining of selected mice from each group. Mouse survival time was recorded.

Statistical analysis

Experiments for cell lines were conducted three times, and results are expressed as mean ± s.d. For AML patient samples, results are expressed as mean ± s.e. IC_{50}, the dose at which MRx102 inhibits cell growth by 50%, and EC_{50}, the dose at which MRx102 induces apoptosis in 50% of cells were calculated using CalcuSyn software (Biosoft, Ferguson, MO, USA). Statistical differences between groups or cell populations at each dose level were determined using paired Student's *t*-test, in which significant differences were indicated (*) when $P<0.05$. Data of mouse survival time were analyzed using log-rank test.

Results

MRx102 potently induces apoptosis in AML cell lines

To determine the efficacy of MRx102 *in vitro* in AML cells, we first treated AML cell lines with MRx102. We found that MRx102 had a potent antileukemic effect. MRx102, at low nanomolar concentrations, induced pronounced apoptosis, decreased viability and inhibited growth of OCI-AML3 and MV4–11 cells (Figure 1). At 24 h, $EC_{50} = 108.0 \pm 1.0$ nM and $IC_{50} = 92.4 \pm 10.5$ nM for MV4–11 cells, and $EC_{50} = 101.3 \pm 3.5$ nM and $IC_{50} = 76.1 \pm 6.5$ nM for OCI-AML3 cells; at 48 h, $EC_{50} = 37.0 \pm 0.9$ nM and $IC_{50} = 16.9 \pm 1.1$ nM for MV4–11 cells, and $EC_{50} = 14.5 \pm 0.6$ nM and $IC_{50} = 6.9 \pm 0.3$ nM for OCI-AML3 cells, respectively.

MRx102 decreases XIAP and Mcl-1 protein levels and inhibits RNA synthesis in OCI-AML3 cells

We showed previously that triptolide promotes apoptosis in leukemic cells, in part, by decreasing expression of the short-lived anti-apoptotic proteins XIAP and Mcl-1.[3,4] Subsequently,

Table 1 Clinical characteristics of samples from AML patients

Sample #	*Source*	*Blast %*	*New/ relapsed*	*Clinical treatments and responses*	*Cytogenetics*	*FLT3 status*	*CD34+38 ±*	*Co-culture*
#01	PB	85	Refractory	Did not respond to SAR103168. Had 2 allogeneic stem-cell transplants.	46,XY,t (1;18)(p13;q21)[20]	ITD	Y	—
#02	PB	78	New		47,XY,+11[14];46,XY[6]	wt	Y	Y
#03	BM	42	(Restricted access requested by the patient)			Y	Y	
#04	BM	67	Relapsed	Evolved from MDS. Responded to clofarabine, followed by low-dose Ara-C, then relapsed. Responded to Vidaza then developed leukocytosis with circulating blast; no response to fludarabine+Ara-C.	45,XX,−7,−12,+mar[1];46,XX[10]	D835	—	—
#05	PB	92	Refractory	Resistant to idarubicin+Ara-C+sorafenib. Some response to AC220 and then underwent allogeneic transplant. Relapsed 70 days post-transplant.	46,XY[20]	D835	—	—
#06	BM	91	Refractory	Refractory secondary AML transformed from MDS on aranesp+azacitidine. Failed amonofide+Ara-C.	47,XY,+8[20]	ITD	Y	Y
#07	PB	69	Relapsed	Achieved remission with idarubicin, Ara-C and vorinosttat, and later relapsed. Failed to respond to decitabine, JAK2 inhibitor, INCB and BIDFA.	45,XX,der(16)t(16;17)(q11.2;q11.2), −17[4];46,XX[4]	wt	Y	Y
#08	PB	99	Refractory	History of Ph+AML. Achieved remission on idarubicin+Ara-C+Gleevec, then relapsed. Received bone-marrow transplant and later relapsed. Was on thiarabine.	46,XY[20]	wt	Y	Y
#09	PB	64	Refractory	No response to 3+7, decitabine, VP-16+mitoxantrone.	Complex cytogenetics	wt	Y	Y
#10	PB	100	Refractory	Secondary AML. Failed idarubicin+Ara−C, bendamustine and AS703026. Was on AP24534.	Complex cytogenetics	D835	Y	Y
#11	PB	76	Refractory	Failed 7+3 (idarubicin/Ara−C); achieved remission on 299 regimen, followed by HDAC. Later relapsed and failed mitoxantrone+etoposide, decitabine, FLAG+idarubicin.	Complex cytogenetics	ITD	Y	—
#12	PB	95	Refractory	Failed 7+3+3 (Ara-C/daunorubicin/etoposide).	46–47,XX,−7,+1–2mar[cp5];46,XX[20]	ITD	—	—

Abbreviations: AML, acute myeloid leukemia; BIDFA, 2x/day fludarabine+ Ara-c; BM, bone marrow; FLAG, fludarabine + high dose Ara-C + G-CSF; HDAC, histone deacetylase; INCB, ruxolitinib; ITD, internal tandem duplication; MDS, myelodysplastic syndrome; PB, peripheral blood; Ph, Philadelphia chromosome; wt, wild type; Y, apoptosis analysis was performed.

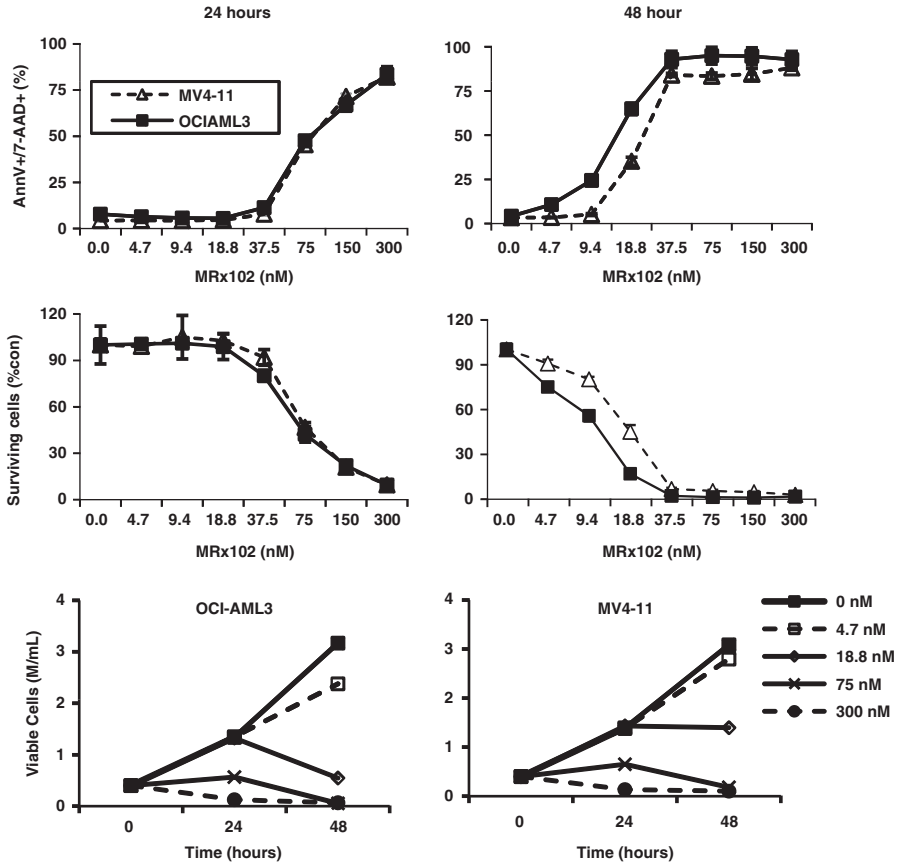

Figure 1 MRx102 induces apoptosis, decreases viability and inhibits growth in AML cell lines. OCI-AML3 and MV4–11 cells were treated with MRx102, and cell viability and apoptosis were determined at 24 and 48 h. M/ml, million cells/ml.

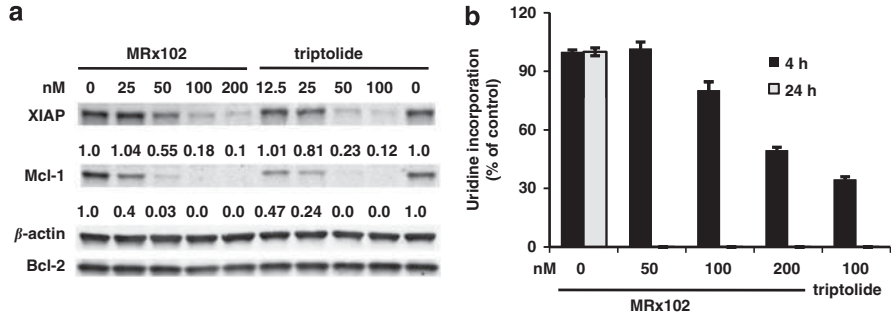

Figure 2 MRx102 decreases XIAP and Mcl-1 protein levels and inhibits RNA synthesis. OCI-AML3 cells were treated with MRx102 and triptolide. (**a**) XIAP and Mcl-1 protein levels were determined at 24 h by western blot analysis, and (**b**) RNA synthesis was assessed at 4 and 24 h by quantitating uridine incorporation.

triptolide was reported to suppress RNA polymerase II-mediated RNA synthesis.[16–18] To determine whether the triptolide derivative MRx102 exerts its activity through similar mechanisms, we treated OCI-AML3 cells with MRx102 and examined XIAP and Mcl-1 protein levels at 24 h, by western blot analysis, and RNA synthesis at 4 and 24 h, by quantitating uridine incorporation into these cells. We found that, like triptolide, MRx102 greatly decreased protein levels of XIAP and Mcl-1 at 24 h, but had minimal effect on the long-lived Bcl-2 protein at 24 h (Figure 2a). At 4 h, RNA synthesis was inhibited by approximately 50% by 200 nM MRx102, whereas at 24 h, RNA synthesis was completed inhibited even by 50 nM MRx102 (Figure 2b).

MRx102 induces apoptosis in leukemic cells, including leukemic stem/progenitor cells, from patients with AML

We next determined the efficacy of MRx102 *in vitro* in primary samples from patients with AML. Mononuclear cells from AML patient samples ($n=12$; Table 1) and normal BM samples ($n=3$) were treated with MRx102, and apoptosis was determined at 24 h. We found that MRx102, at low nanomolar concentrations, effectively induced apoptosis in all the AML samples treated, regardless of the cytogenetics or clinical responses to various therapies of the patients at the time of sampling (Figure 3a, $ED_{50}=132.1\pm7.5$ nM; Table 1). MRx102 also promoted apoptosis in normal BM cells, but with

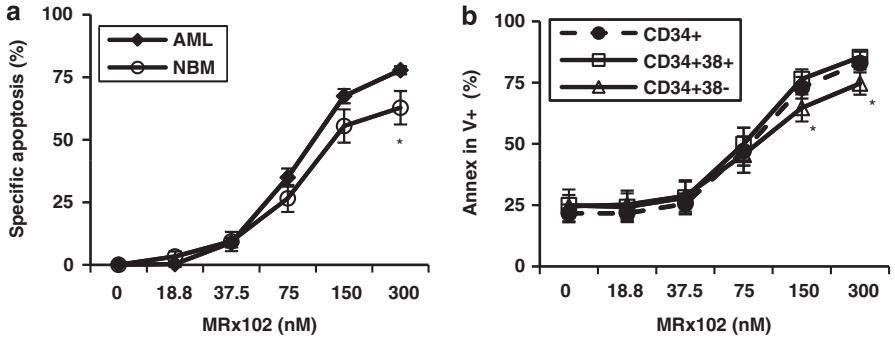

Figure 3 MRx102 induces apoptosis in leukemic cells, including leukemic stem/progenitor cells from patients with AML. BM or peripheral blood samples obtained from patients with AML and BM samples from normal subjects ($n=3$) were treated with MRx102 for 24 h and apoptosis was determined in (**a**) bulk AML ($n=12$) and normal BM cells and (**b**) $CD34^-$, $CD34^-CD38^-$ and $CD34^-CD38^-$ AML ($n=9$) cells. *$P<0.05$.

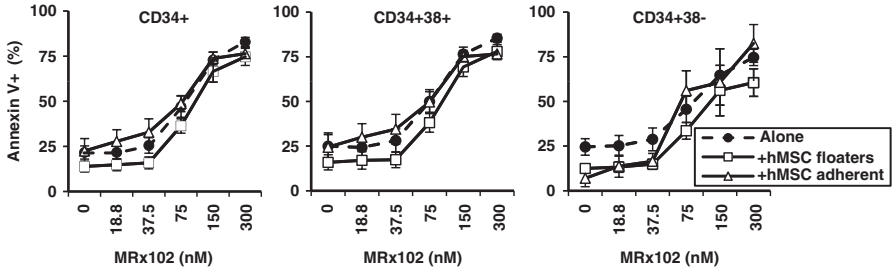

Figure 4 MRx102 induces apoptosis in leukemic cells, including leukemic stem/progenitor cells, from patients with AML protected by the BM microenvironment. BM or peripheral blood samples obtained from patients with AML ($n=7$) were treated with MRx102 for 24 h alone or cocultured with BM-derived MSCs. Apoptosis of floaters and adherent cells was determined in $CD34^-$, $CD34^-CD38^-$ and $CD34^-CD38^-$ cells.

less efficacy (Figure 3a, $ED_{50}=209.9\pm56.8$ nM; $P=0.003$ at 300 nM MRx102).

To examine the ability of MRx102 to eliminate AML stem/progenitor cells, which, in general, are more resistant than bulk AML blasts to chemotherapy, we treated cells from AML patients with MRx102 and determined the viabilities of primitive $CD34^+$ and $CD34^+CD38^+$ AML cells, and $CD34^+CD38^-$ AML progenitor/stem cells at 24 h. Among 12 samples treated, 9 had sufficient cells for apoptosis analysis in all cell compartments (Table 1). We found that MRx102 potently promoted apoptosis in $CD34^+$, $CD34^+CD38^+$ and $CD34^+CD38^-$ AML cells, although $CD34^+CD38^-$ cells were less sensitive than $CD34^+$ cells at 150 nM ($P=0.033$) and 300 nM ($P=0.015$) (Figure 3b).

MRx102 effectively induces apoptosis in AML cells even when protected by the BM microenvironment

Further, to examine the efficacy of MRx102 in eradicating AML cells protected by the BM microenvironment, we cocultured AML cells obtained from patients with BM-derived MSCs, treated them with MRx102, and determined apoptosis in cells detached (floaters) from and attached (adherent) to MSCs in $CD34^+$, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells. We found that MRx102 efficiently promoted apoptosis in AML cells even when they were cocultured with MSCs, in all cell compartments for both floaters and adherent cells (Figure 4). Note that for adherent $CD34^+CD38^-$ cells under coculturing conditions, apoptosis was determined only in three patient samples because of low numbers of cells; seven samples were analyzed for all the other conditions.

MRx102 significantly prolongs the life of NOD/SCID mice injected with Ba/F3-ITD-GFP/Luc cells

To test the antileukemic activity of MRx102 *in vivo* in a Ba/F3-ITD-GFP/Luc NOD/SCID murine model, we first treated Ba/F3-ITD-GFP/Luc cells *in vitro* with MRx102 and found that MRx102 induced apoptosis and suppressed growth in these cells (Figure 5a). We then injected Ba/F3-ITD-GFP/Luc cells intravenously into NOD/SCID mice and started treatment on day 3 after the injection with 1.5 or 3.0 mg of MRx102/kg/day for 5 weeks. Antileukemic activity of MRx102 was monitored by bioluminescence imaging of mice, by recording mouse survival time, and by hematoxylin and eosin and GFP staining for organ infiltration by leukemic cells.

No significant differences were observed in luciferase intensity between control and MRx102-treated groups at day 7 and day 14. At day 33, lower luciferase activity was detected in both MRx102 treatment groups compared with the untreated control group, especially in the group treated with 3.0 mg of MRx102/kg/day (Figure 5b), suggesting that treatment with MRx102 decreased the leukemic burden in Ba/F3-ITD NOD/SCID mice. We found that MRx102, administered at 1.5 mg/kg/day had no significant effect on the survival of Ba/F3-ITD NOD/SCID mice ($P=0.095$, median survival time 35 days versus 36 days for control, Figure 5c), suggesting that at this dose, although MRx102 decreased the leukemic burden, it was not effective in prolonging survival of these mice. Mice treated with MRx102 at 3.0 mg/kg/day had significantly longer survival time (median survival time 43 days) than control mice ($P=0.010$) or mice treated with MRx102 at 1.5 mg/kg/day ($P=0.027$), indicating that MRx102 has antileukemic activity *in vivo*.

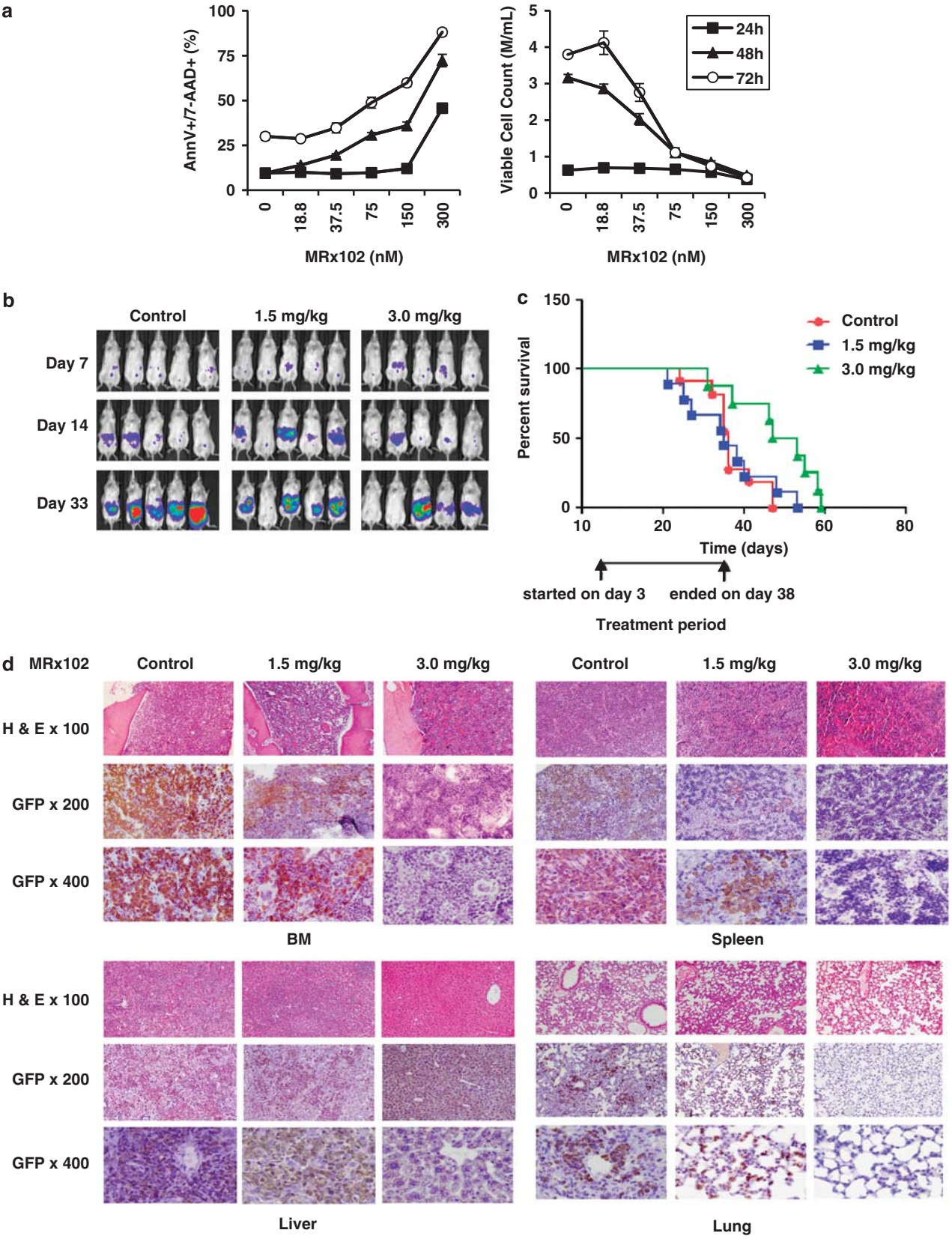

Figure 5 MRx102 shows antileukemic activity *in vivo* in a Ba/F3-ITD NOD/SCID mouse model. (**a**) MRx102 promotes apoptosis and suppresses growth of Ba/F3-ITD cells *in vitro*. In NOD/SCID mice harboring Ba/F3-ITD cells, MRx102 treatment (**b**) decreased luciferase activity, (**c**) increased survival time of mice and (**d**) lowered the leukemic burden in various organs.

Hematoxylin and eosin and GFP staining of various tissues showed that BM, spleens, livers and lungs of control mice were infiltrated with leukemic cells, whereas the amounts of leukemic cells in these organs were lower in mice treated with MRx102 at 1.5 mg/kg/day and lowest in mice treated with MRx102 at 3.0 mg/kg/day (Figure 5d). However, we observed no decrease, but rather an increase in the number of circulating Ba/F3-ITD cells at day 35 in mice treated with MRx102 at 3.0 mg/kg/day, compared with mice treated with MRx102 at 1.5 mg/kg/day and untreated control mice, as determined by flow cytometry of GFP in blood ($n=3$, results not shown), probably because in the group treated with MRx102 at 1.5 mg/kg/day and in untreated control mice, only animals with lower numbers of circulating leukemic cells survived until day 35. No apparent treatment-related toxicities such as weight loss or diarrhea were observed. After prolonged treatment with MRx102 at 3.0 mg/kg/day, mice showed signs of physical weakness that improved on stopping the treatment.

Discussion

Triptolide has been shown to be a potent antitumor agent, and various triptolide derivatives have been developed or are being developed to optimize the activity and/or pharmacokinetic properties of triptolide for clinical applications. In this study, we showed that the triptolide derivative MRx102 is effective against AML cells *in vitro* and also *in vivo* in a murine model of AML. MRx102 promoted apoptosis not only in bulk AML cells, but also in AML stem/progenitor cells even when they were protected by the BM microenvironment. In addition, MRx102 greatly decreased the leukemic burden and increased the survival time of NOD/SCID mice injected with Ba/F3-ITD cells.

MRx102 is believed to exert its biological activity after being converted to triptolide. Our findings that MRx102 decreases XIAP and Mcl-1 levels and inhibits RNA synthesis in AML cells, as triptolide does, support the notion that MRx102 acts through mechanisms similar to those of triptolide.

Despite a high response rate to therapy, most AML patients relapse, largely because AML stem/progenitor cells are not targeted effectively by available therapeutic regimens and are responsible for drug resistance and relapse. Importantly, we found that MRx102 induced apoptosis in all compartments of AML cells, with similar efficacies in bulk, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells. This result is not surprising given that as in the primitive $CD34^+$ AML cells, nuclear factor-κB is constitutively activated in AML stem cells[29] and that triptolide is known to inhibit nuclear factor-κB. We previously showed that triptolide is effective in killing not only proliferating, but also quiescent CML progenitor cells, independent of cellular response to imatinib.[4] The ability of MRx102 to eliminate AML stem/progenitor cells will likely enable improvement of the current treatment regimens for AML. Furthermore, increasing evidence demonstrates the critical role of the BM microenvironment in supporting the survival of leukemic cells; protecting AML cells, especially AML stem cells, from therapeutic agents and maintaining the self-renewal of AML stem cells. Here, we showed that MRx102 was effective against AML stem/progenitor cells even when leukemic cells were cocultured with BM-derived MSCs, suggesting the potential of MRx102 to overcome microenvironmental protection.

We found that MRx102 also killed normal BM cells, but with lower potency. A careful MRx102 dosing schedule is needed to minimize toxicity and maximize the antileukemic effect. Previous toxicology tests of MRx102 in rats with a 7-day sub-acute injection up to the top dose of 1.5 mg/kg and an acute single dose up to the top dose of 3.0 mg/kg showed no deaths and no adverse signs, and the histopathology report showed no findings related to administration of MRx102.[30] The no-observable adverse-effects level is $\geqslant$1.5 mg/kg/day for MRx102, whereas it is $\leqslant$0.1 mg/kg/day for other related compounds.[30] After prolonged treatment of Ba/F3-ITD NOD/SCID mice with MRx102 at 3.0 mg/kg/day, we did observe signs of physical weakness; these improved on stopping the treatment, suggesting that treatment-related toxicity can be alleviated with proper scheduling. The fact that MRx102 markedly lowered the leukemic burden and prolonged the life of Ba/F3-ITD NOD/SCID mice suggests that MRx102 has *in vivo* antileukemic activity, supported by our previous data in a nude mouse subcutaneous xenograft model.[30]

Collectively, our results showed that MRx102, a triptolide derivative, has potent antileukemic activity *in vitro* and in a murine model of AML. MRx102 is a potential therapeutic agent in AML and warrants further investigation.

Conflict of interest

JMF is an employee of MyeloRx and all the other authors have no conflict of interest.

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