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Biomedicine & Pharmacotherapy 154 (2022) 113546

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

NA1—115—7, from *Zygogynum pancheri,* is a new selective MCL-1 inhibitor inducing the apoptosis of hematological cancer cells but non-toxic to normal blood cells or cardiomyocytes

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ARTICLE INFO

Keywords:
MCL-1 inhibitor
Drimane sesquiterpenoids
B-cell lymphoproliferation
BH3-mimetics
BAK
BAX

ABSTRACT

The overexpression of antiapoptotic members (BCL-2, BCL-xL, MCL-1, etc.) of the BCL-2 family contributes to tumor development and resistance to chemotherapy or radiotherapy. Synthetic inhibitors targeting these proteins have been developed, and some hematological malignancies are now widely treated with a BCL-2 inhibitor (venetoclax). However, acquired resistance to venetoclax or chemotherapy drugs due to an upregulation of MCL-1 has been observed, rendering MCL-1 an attractive new target for treatment. Six MCL-1 inhibitors (S64315, AZD-5991, AMG-176, AMG-397, ABBV-467 and PRT1419) have been evaluated in clinical trials since 2016, but some were affected by safety issues and none are currently used clinically. There is, therefore, still a need for alternative molecules. We previously described two drimane derivatives as the first covalent BH3 mimetics targeting MCL-1. Here, we described the characterization and biological efficacy of one of these compounds (NA1—115—7), isolated from *Zygogynum pancheri,* a plant belonging to the *Winteraceae* family. NA1—115—7 specifically induced the apoptosis of MCL-1-dependent tumor cells, with two hours of treatment sufficient to trigger cell death. The treatment of lymphoma cells with NA1—115—7 stabilized MCL-1, disrupted its interactions with BAK, and rapidly induced apoptosis through a BAK- and BAX-mediated process. Importantly, a similar treatment with NA1—115—7 was not toxic to erythrocytes, peripheral blood mononuclear cells, platelets, or cardiomyocytes. These results highlight the potential of natural products for use as specific BH3 mimetics non-toxic to normal cells, and they suggest that NA1—115—7 may be a promising tool for use in cancer treatment.

1. Introduction

Apoptosis plays a fundamental role in the normal development of multicellular organisms and is crucial for tissue homeostasis. Its dysregulation can lead to various diseases, such as neurodegenerative diseases and cancer. Apoptosis is induced by two major pathways, one involving extrinsic signaling via death receptors and the other involving intrinsic signaling via mitochondria. The main regulators of the mitochondrial pathway are proteins from the B-cell lymphoma 2 (BCL-2) family, all of which containing at least one BCL-2 homology (BH) domain. Some of the proteins of this family are pro-apoptotic, whereas others are antiapoptotic. The proapoptotic proteins of these family form two subclasses: BH3-only proteins (BIM, BID, Puma, NOXA, etc.) which play a regulatory role and effectors (BAK, BAX, etc.). Under physiological conditions, the antiapoptotic proteins of this family (MCL-1, BCL-2, BCL-xL, BCL-w, BFL-1/A1, etc.) inhibit apoptosis by interacting with the

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https://doi.org/10.1016/j.biopha.2022.113546
Received 16 June 2022; Received in revised form 6 August 2022; Accepted 11 August 2022
Available online 18 August 2022

BH3 domain of the proapoptotic proteins via a hydrophobic groove on their surface. In conditions of cell stress, BH3-only proteins are upregulated, and apoptosis is induced either directly, through activation of the proapoptotic effectors BAK or BAX, or indirectly, through disruption of the complexes between antiapoptotic proteins and effectors, which are, thus, released. Following their activation, BAK and BAX oligomerize to form pores, leading to mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic factors (cytochrome *c*, SMAC/DIABLO, etc.) into the cytosol, where they trigger caspase-mediated apoptotic signaling pathways [1–3].

In tumor cells, the balance between pro- and antiapoptotic proteins is often disrupted by the downregulation of proapoptotic proteins or the upregulation of antiapoptotic proteins, rendering these cells resistant to conventional treatments that induce apoptosis. In recent years, many laboratories have worked on the development of new tools targeting antiapoptotic proteins, particularly BCL-2. Most of the tools developed to date are small molecules known as "BH3 mimetics", one of which, ABT199/venetoclax has been approved by the FDA and shown to be effective in several preclinical models of hematological malignancies [4]. This molecule is now used for various indications [5–8]. However, during the development of BCL-2 inhibitors, acquired resistance to treatment due to the upregulation of MCL-1 was observed in a number of studies [9–13]. Conversely, a loss of MCL-1 expression has been shown to sensitize cells to BCL-2 inhibitors [14]. MCL-1 has also been implicated in resistance to chemotherapy drugs [15–17].

MCL-1 is an unusual member of the BCL-2 family with specific and essential functions during embryogenesis. MCL-1 knockout is embryolethal during the peri-implantation period. MCL-1 is also required for the survival of various cell lineages, including neutrophils, lymphocytes, neurons, and cardiomyocytes [18–22]. Furthermore, unlike other members of the family, MCL-1 has a long N-terminus that is relatively unstructured and contains two PEST (proline/glutamic acid/serine/threonine-containing) domains involved in the regulation of its degradation and responsible for its short half-life (1–2 h) [23,24]. MCL-1 gene amplification is one of the most common events in cancers [25], and overexpression of the MCL-1 protein has been reported in several types of solid tumor (lung, pancreas, prostate, ovaries, etc.) and in hematological malignancies. MCL-1 has been implicated, in particular, in the survival of multiple myeloma (MM), acute myeloid leukemia (AML), B-lineage acute lymphoblastic leukemia (ALL), and B-cell lymphoma [26–29] cells. A loss of the MCL-1 gene in the B-lymphoid progenitors of *Eμ-Myc* transgenic mice has also been shown to delay MYC-dependent lymphomagenesis [30], and the sustained growth of Burkitt lymphoma (BL) cells is dependent on MCL-1 [31]. MCL-1 is, thus, an interesting potential target for new therapies.

New BH3 mimetics targeting this protein have recently been developed by various pharmaceutical companies, and their efficacy, alone or in combination with venetoclax or other drugs, has been demonstrated in preclinical studies [32–36]. All these molecules target one or more of the four pockets within the hydrophobic groove of MCL1 known to interact with the BH3 domain of pro-apoptotic proteins. Six of these MCL-1 inhibitors (S64315/MIK665, AZD-5991, AMG-176, AMG-397, ABBV-467 and PRT1419) have been evaluated in phase I clinical trials for the treatment of MM, AML and various types of lymphoma. Some of these trials were suspended for safety reasons, due to cardiac toxicity (AMG-176, NCT02675452 and NCT03797261), or for potential safety reasons (AZD-5991, NCT03218683) and the trials of AMG-397 and ABBV-467 were stopped early for strategic reasons (NCT03465540 and NCT04178902). Trials of PRT1419, as single agent (NCT04543305) and S64315/MIK665, in combination (NCT04702425 and NCT03672695) are still underway. Interestingly, these inhibitors bind to MCL-1 with a subnanomolar affinity, potentially accounting for the cardiotoxicity observed in some patients [32,35,37,38]. In any case, the safety issues encountered in trials of the MCL-1 inhibitors described to date suggest that there is still a need for safer alternative molecules.

All the compounds tested to date are synthetic molecules obtained by fragment-based methods and structure-based design. However, natural products and their derivatives often form the basis for drug discovery and development processes. Indeed, highly original bioactive molecules can be identified by screening plant extracts or marine organisms, and molecules discovered in this way account for a large proportion of the agents currently used to treat cancer. Natural products are of particular interest because their physicochemical characteristics and structural complexity have evolved to interact with biological systems. Various natural compounds targeting MCL-1 have been described by different teams, including ours. However, these compounds, whether isolated from cottonseed (gossypol and derivatives), various plants (coumarins and derivatives), streptomycetes of marine origin (maritoclax) or Malaysian plants (meiogynin A1 and derivatives), were all found to be pan- or dual BCL-2 family inhibitors or yielded conflicting results concerning specificity (review in [39]). We also recently demonstrated that various drimanes are potent inhibitors of MCL-1 and BCL-xL and that their activity against the target protein can be modified by subtle changes to their structure [40]. We describe here the characterization and biological efficacy of one of these new MCL-1 inhibitors, isolated from *Zygogynum pancheri,* a plant of the *Winteraceae* family endemic to the New Caledonian rainforest. We previously showed, in fluorescence polarization and NMR studies, that this compound (compound 5, now named NA1—115—7) is selective, with a high affinity for the BH3-binding pocket of MCL-1. We also showed, in MTT assays, that it is cytotoxic to MCL-1-dependent cells. Remarkably, we also demonstrated that it inhibits MCL-1 through a covalent interaction between its two aldehyde functional groups and a lysine residue of the MCL-1 protein [40]. We show here that treatment with NA1—115—7 stabilizes MCL-1 and specifically induces the apoptosis of MCL-1-dependent cell lines by activating BAK and BAX, leading to the release of cytochrome *c* and SMAC/DIABLO from mitochondria. Treatment with NA1—115—7 for two hours was sufficient to trigger the apoptosis of tumor cells. We observed no toxicity to erythrocytes, peripheral blood mononuclear cells, platelets, or cardiomyocytes. An absence of cardiotoxicity was also confirmed for NA1—115—7 by hERG testing.

2. Materials and methods

2.1. Reagents

NA1—115—7 was obtained from the trunk bark and leaves of *Z. pancheri* subsp. *pancheri* collected from the rainforest of Nodela (Southern Province of New Caledonia). It was extracted, purified and characterized as previously described [41]. ABT-737 was kindly provided by Abbott Laboratories (Chicago, IL, USA). S63845 and ABT-199 were purchased from MedChem Express. Doxorubicin was provided by the Gustave Roussy Hospital pharmacy.

Rabbit anti-BAX pAb (N-20), mouse anti-LDH mAb (H-10), rabbit anti-MCL-1 pAb (S-19), mouse anti-BCL-2 mAb (C2), mouse anti-BCL-xL-HRP mAb (7B2.5) and goat anti-BAK pAb (G-23) were purchased from Santa Cruz Biotechnology Inc. Mouse anti-PARP mAb (Ab-2) was purchased from Calbiochem. Mouse β-actin mAb (AC-74) was purchased from Sigma Aldrich. Rabbit anti-SMAC/DIABLO pAb was purchased from Imgenex. Rabbit anti-VDAC was purchased from Cell Signaling. Mouse anti-cytochrome *c* mAb (7H8–2C12) was purchased from BD.

Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG secondary antibodies were purchased from GE Healthcare.

2.2. Cell lines

The BL2 and Remb1 cell lines were kindly provided by the International Agency for Research on Cancer (IARC, Lyon). The NCI-H929 and RS4;11 cell lines were purchased from DSMZ (Braunschweig, Germany) and the H9C2 cell line was obtained from ATCC. The BL2, Remb1, and RS4;11 cell lines were cultured in RPMI 1640 medium (Invitrogen)

supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum. The NCI-H929 and H9C2 cell lines were cultured in RPMI 1640 advanced medium (Invitrogen) and DMEM (Invitrogen), respectively, both supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% heat-inactivated fetal calf serum. All cells were incubated at 37 °C in a humidified incubator under an atmosphere containing 5% CO_2.

2.3. MTT assay

Cells (7 $\times 10^4$, in triplicate) were incubated for 24 h at 37 °C with various concentrations (0/0.312/0.625/1.25/2.5/5/10/20 μM) of NA1—115—7 in 100 μL complete RPMI medium. Cell proliferation assays were performed as previously described [42]. LC_{50} (50% lethal concentration) values were calculated by plotting dose-response curves with GraphPad Prism software. Experiments were performed at least three times.

2.4. Quantification of apoptosis

Cells (3 $\times 10^5$) were treated for the times indicated, in the corresponding complete medium. Apoptosis was then assessed by labeling cells with annexin V-FITC and PI, as previously described [42]. For PBMCs and platelets, cells were labeled with anti-CD3-FITC (BD Pharmingen) or anti-CD41-FITC (BD Pharmingen) antibodies, respectively, to identify the population isolated, and apoptosis was assessed by annexin V-APC staining. Experiments were performed at least three times.

2.5. Preparation of mitochondrial and cytosolic fractions

Cells (2 $\times 10^6$) were resuspended in 100 μL ice-cold cell lysis and mitochondria intact (CLAMI) buffer (250 mM sucrose, 70 mM KCl, 200 μg/mL digitonin, supplemented with protease inhibitor cocktail) and cell fractionation was performed as previously described [43]. Total protein concentration was determined in a Bradford assay (Biorad) and western blotting was then performed.

2.6. MCL-1 immunoprecipitation

After treatment with DMSO or NA1—115—7 (4 μM), MCL-1 was immunoprecipitated by overnight incubation at 4 °C with beads coupled to either an anti–MCL-1 mouse mAb (B-6) (Santa Cruz Biotechnology Inc.) or a control mouse IgG (sc-2343) (Santa Cruz Biotechnology Inc). The immunoprecipitate obtained was resuspended in Laemmli buffer and boiled. The immunoprecipitates obtained with anti-MCL-1 (IP) or an isotype control Ab (IgG) were analyzed by western blotting, together with 15% of the cell lysates (input).

2.7. Western-blot analysis

Whole-cell extracts were prepared and immunoblotted as previously described [44]. The blots were visualized with an ImageQuant LAS4000 imager (GE healthcare) or on X-ray film (Amersham). Quantity One software version 4.6 (Bio-Rad) was used for densitometric analysis of the blots.

2.8. Activation of BAX and BAK

After 4 h of incubation with or without NA1—115—7 (4 μM), cells were washed with ice-cold PBS and fixed by incubation for 5 min at room temperature (RT) in 0.25% paraformaldehyde (PFA) in PBS. The cells were washed with PBS and incubated for 30 min at RT with a conformational anti-BAX mAb (clone 6A7; Santa Cruz Biotechnology) or an anti-BAK mAb (clone Ab-1; Merck Millipore) diluted in 200 μg/mL digitonin in PBS (Sigma-Aldrich). Cells were washed with PBS, incubated for 30 min at RT with a goat anti-mouse Alexa 488-conjugated secondary Ab (Beckman Coulter) in PBS, and washed twice with PBS before flow cytometry analysis (BD Accuri C6).

2.9. Platelet, erythrocyte, and PBMC isolation

Platelets, erythrocytes and PBMCs were obtained by the Ficoll density gradient centrifugation of blood diluted in sterile PBS (1:1). Ficoll was added to the bottom of a tube and gently overlaid with the diluted blood (1:2.5). After centrifugation at RT for 20 min at 900 x *g* with the brake off, cells at the interface (PBMCs), in the pellet (erythrocytes), and in the supernatant (platelets) were collected. PBMCs were washed in PBS and centrifuged twice, for 10 min each, at 300 x *g*. Finally, the pellet was resuspended in RPMI 1640 supplemented with 10% FBS and incubated at 37 °C in a humidified incubator, under an atmosphere containing 5% CO_2, for up to 48 h. Erythrocytes were washed in PBS and then cultured at 37 °C, in RPMI 1640 supplemented with 10% FBS, under an atmosphere containing 5% CO_2. The supernatant fraction was mixed (1:1) with HEP buffer (140 mM NaCl, 2.7 mM KCL, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) and centrifuged at 100 x *g* for 15 min at RT, with the brake off, to obtain a pellet of cells other than platelets. The supernatant was then centrifuged at 800 x *g* for 15 min at RT, with the brake off, and the pellet obtained was washed in PBS. Platelets were gently resuspended in RPMI 1640 supplemented with 10% FBS and cultured at 37 °C, in a humidified incubator, under an atmosphere containing 5% CO_2, for up to two days.

2.10. Erythrocyte lysis

Erythrocytes (13 $\times 10^6$) were resuspended in PBS and treated with various concentrations of NA1—115—7 (2, 4, 8, and 16 μM) or S63845 (0.5 μM). Cells resuspended in water were used as a positive control. After 24 h of treatment, cells were either examined under an inverted microscope (Zeiss Axiovert S100), with image capture, or transferred to hemolysis tubes and centrifuged at 100 x *g*. Hemolysis rates were evaluated on the basis of the color of the supernatant.

2.11. hERG inhibition assay

We assessed hERG channel binding in the Predictor™ hERG Fluorescence Polarization Assay (PV5665 Thermo Fisher Scientific), as previously described [45]. IC_{50} values were determined graphically, by plotting the percent inhibition against the logarithm of eight NA1—115—7 concentrations with GraphPad Prism software (version 6.01, GraphPad Software, La Jolla, CA, USA).

2.12. Statistical analysis

The data plotted on graphs are expressed as means and standard deviations (SD). Statistical analyses were performed with two nonparametric tests in GraphPad Prism software: the Mann-Whitney test for paired comparisons and the Kruskal-Wallis test for multiple comparisons. Values of $p < 0.05$ were considered significant, for all comparisons.

3. Results

3.1. Evaluation of NA1—115—7 cytotoxicity in various B-lymphoid cell lines

We first performed a BH3-profiling assay on various blood cancer cell lines to determine the specificity of NA1—115—7. We selected four such cell lines that are primed and dependent on BCL-2, BCL-xL, or MCL-1 (Table 1). We then assessed the levels of these three major

Table 1
NA1—115—7 is only cytotoxic for MCL-1-dependent cell lines.

Cell lines	Dependency*	Lethal concentration 50% (LC50) (μM)			
		NA1—115—7	S63845	ABT-737	ABT-199
BL2	MCL-1 / BCL-xL	3.1	0.16	> 20	> 5
Remb1	BCL-xL	12	> 2	0.89	4.6
RS4;11	BCL-2	9.4	> 2	0.8	0.2
H929	MCL-1	5.6	0.008	> 20	> 5

Cell viability was determined by MTT after 24 h of treatment with NA1—115—7 and ABT-737 at 20, 10, 5, 2.5, 1.25, 0.625, and 0.312 μM. ABT-199 was tested at doses between 5 and 0.312 μM. S63845 was tested at doses between 2 and 0.03 μM
*Cell dependency for antiapoptotic molecules was determined by BH3-profiling.

antiapoptotic proteins by western blotting (Fig. 1A). Consistent with the results of BH3 profiling, H929 cells were found to have high levels of MCL-1, BL2 cells had high levels of both MCL-1 and BCL-xL (but also a smaller amount of BCL-2), Remb1 cells had high levels of BCL-xL (but also smaller amounts of MCL-1 and BCL-2) and RS4;11 cells had similar high levels of BCL-2 and BCL-xL and a very low level of MCL-1. These cells were then treated for 24 h with various concentrations of NA1—115—7, and a cell proliferation assay (MTT) was performed (Fig. 1B). For confirmation of the BH3-profiling results, cells were also treated with characterized BH3 mimetics: ABT-737 (inhibitor of both BCL-xL and BCL-2, Fig. 1B), S63845 (MCL-1 inhibitor, Supplemental Fig. 1A) and ABT-199 (BCL-2 inhibitor, Supplemental Fig. 1B). MTT assays were then performed.

BL2 cells, which are dependent on MCL-1 and BCL-xL, were killed by NA1—115—7 (LC_{50} of 3.1 μM) and S63845 (LC_{50} of 0.16 μM), but not by ABT-737 or ABT-199. Remb1 cells, which are dependent on BCL-xL, were susceptible to ABT-737 (LC_{50} of 0.89 μM) but not to ABT-199, S63845 or NA1—115—7. RS4;11 cells, which are dependent on BCL-2, were susceptible to ABT-737 (LC_{50} of 0.8 μM) and ABT-199 (LC_{50} of 0.2 μM) and, in accordance with previous results [33] showing its partial dependence on MCL-1, these cells were also mildly susceptible to S63845 and NA1—115—7. Finally, the H929 MCL-1-dependent cells were killed by NA1—115—7 (LC_{50} of 5.6 μM) and S63845 (LC_{50} of 0.008 μM), but not by ABT-737 or ABT-199. All the results of the MTT

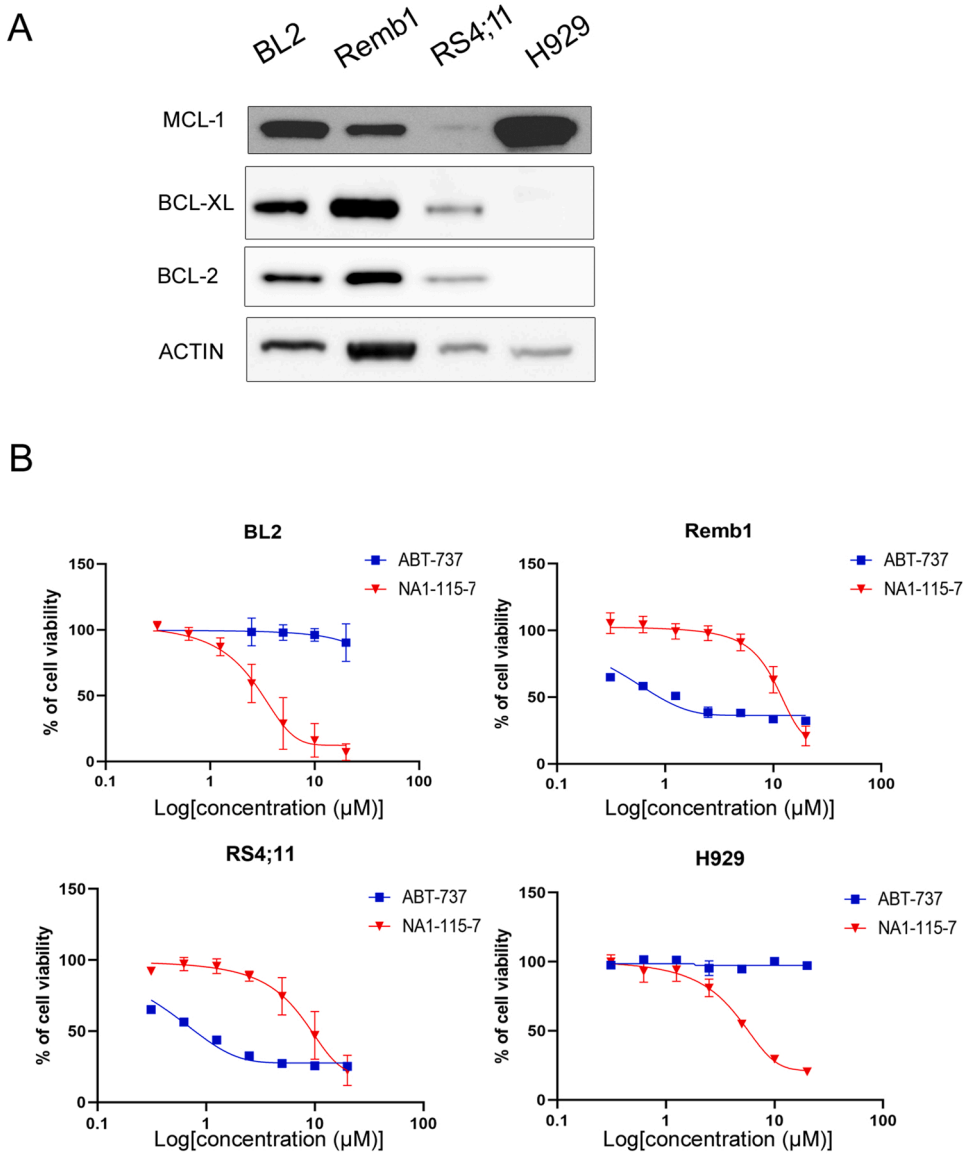


Fig. 1. *NA1—115—7 is cytotoxic only to MCL-1-dependent cell lines.* A – Total extracts of BL2, Remb1, RS4;11 and H929 cells were analyzed by western blotting with an anti-MCL-1 pAb, an anti-Bcl-xL-HRP mAb, an anti BCL-2 mAb and an anti-actin mAb. B – Susceptibility to NA1—115—7 and ABT-737 was assessed with the MTT assay. The values shown (mean ± S.D.) were obtained from at least three independent experiments.

assays (Table 1) are consistent with the dependence profiles of the cells determined by BH3 profiling and strongly suggest that NA1—115—7 is an MCL-1 inhibitor.

3.2. NA1—115—7 induces apoptosis via the mitochondrial pathway in MCL-1-dependent cells

We then investigated the mechanism of NA1—115—7-induced cell death. Cells were first incubated for 24 h with or without 4 μM NA1—115—7. They were then labeled with annexin V-FITC and propidium iodide (PI), and apoptosis was assessed by flow cytometry. Cells

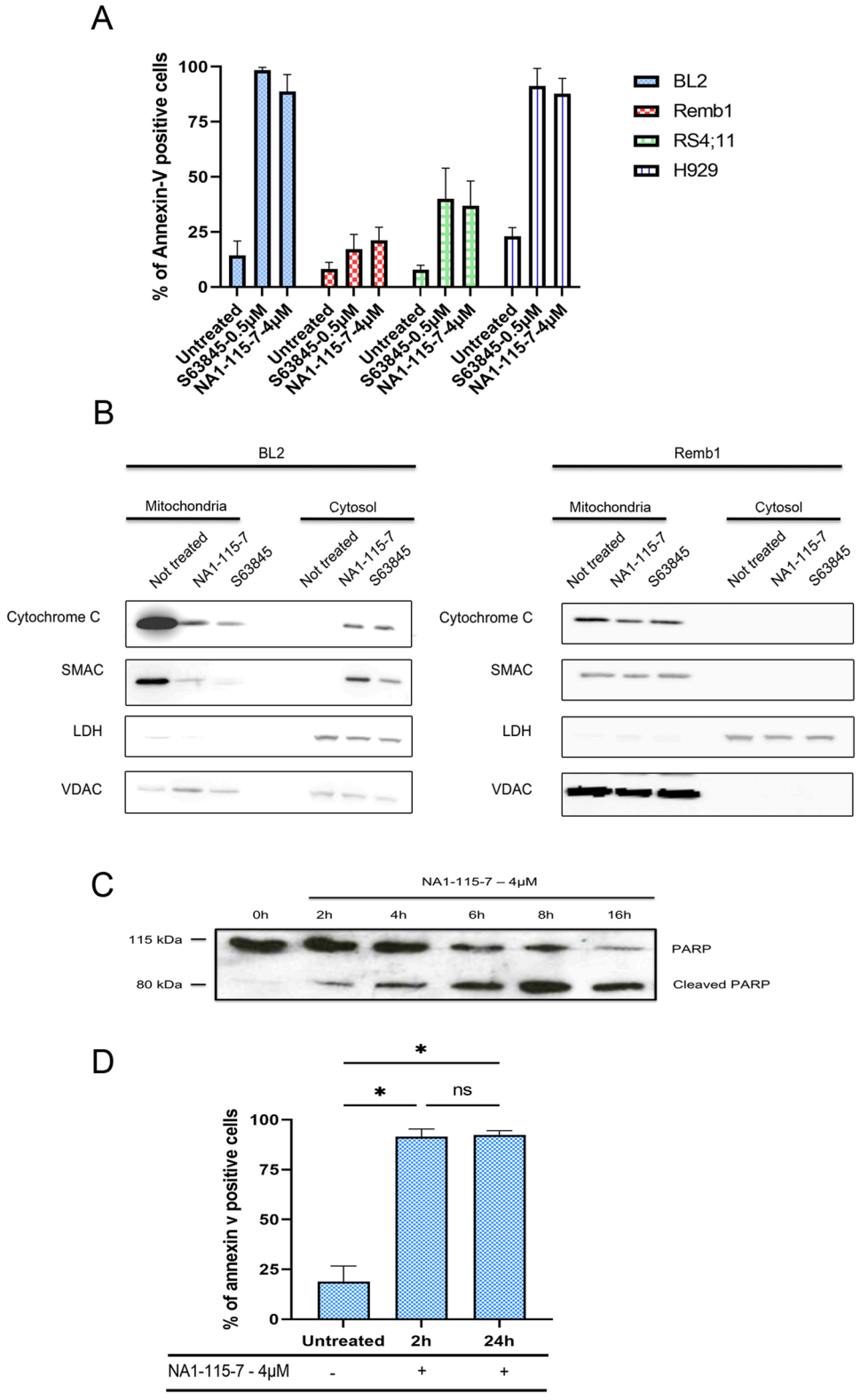


Fig. 2. *NA1—115—7 specifically induces apoptosis in MCL-1-dependent cell lines by activating the mitochondrial pathway*. A – Cells were incubated for 24 h with and without NA1—115—7 (4 μM) or S63845 (0.5 μM). The cells were labeled with annexin V-FITC and PI and apoptosis was assessed. The values (mean ± S.D.) were obtained from at least five independent experiments. B – Cells were incubated for 7 h with or without NA1—115—7 (4 μM) or S63845 (0.5 μM). Cytosolic and mitochondrial fractions were then analyzed by western blotting with an anti-cytochrome *c* mAb and an anti-SMAC/DIABLO pAb. LDH and VDAC were used as cytosolic and mitochondrial markers, respectively. C – BL2 cells were incubated for various time periods with or without NA1—115—7 (4 μM) and PARP cleavage assessed by western blotting. D – BL2 cells were incubated for 2 or 24 h in the presence or absence of NA1—115—7 (4 μM). The cells that were treated for 2 h were then washed and incubated for an additional 22 h with complete medium without NA1—115—7. The cells were labeled with annexin V-FITC and PI and apoptosis was assessed by flow cytometry. The values shown (mean ± S.D.) were obtained from at least three independent experiments. Kruskal-Wallis tests were performed for statistical analysis (*: $p < 0.05$).

were also treated with 0.5 μM S63845, which has been reported to be a BH3 mimetic specific for MCL-1 [33], as a positive control. NA1—115—7 strongly induced apoptosis in the BL2 and H929 cell lines (88.7 ± 8% and 87.8 ± 6.5%, respectively), whereas RS4;11 was less susceptible, with only 36.9 ± 10% of cells undergoing apoptosis in response to treatment. Remb1 was much less susceptible, with only 21 ± 6% of cells undergoing apoptosis (Fig. 2A). Very similar results were obtained with S63845, confirming the specificity of NA1—115—7 for MCL-1.

We then measured the release of the apoptogenic factors cytochrome *c* and SMAC/DIABLO from the mitochondria into the cytosol, to elucidate the apoptotic pathway involved. We treated BL2 and Remb1 cells with NA1—115—7 (4 μM) or S63845 (0.5 μM) for 7 h and then prepared mitochondrial and cytosolic fractions, which were analyzed by western blotting. Fractionation quality was checked by probing blots with antibodies recognizing proteins known to localize to the mitochondria (VDAC) or cytosol (LDH). As expected, cytochrome *c* and SMAC/DIABLO were found exclusively in the mitochondrial fraction of untreated cells. After the treatment of BL2 cells with NA1—115—7, both apoptogenic factors were found principally in the cytosol, whereas they remained in the mitochondria in Remb1 cells (Fig. 2B). Similar results were obtained after S63845 treatment.

Another hallmark of apoptosis is the cleavage of poly (ADP-ribose) polymerase (PARP) – a DNA repair enzyme – by caspases. We, thus,

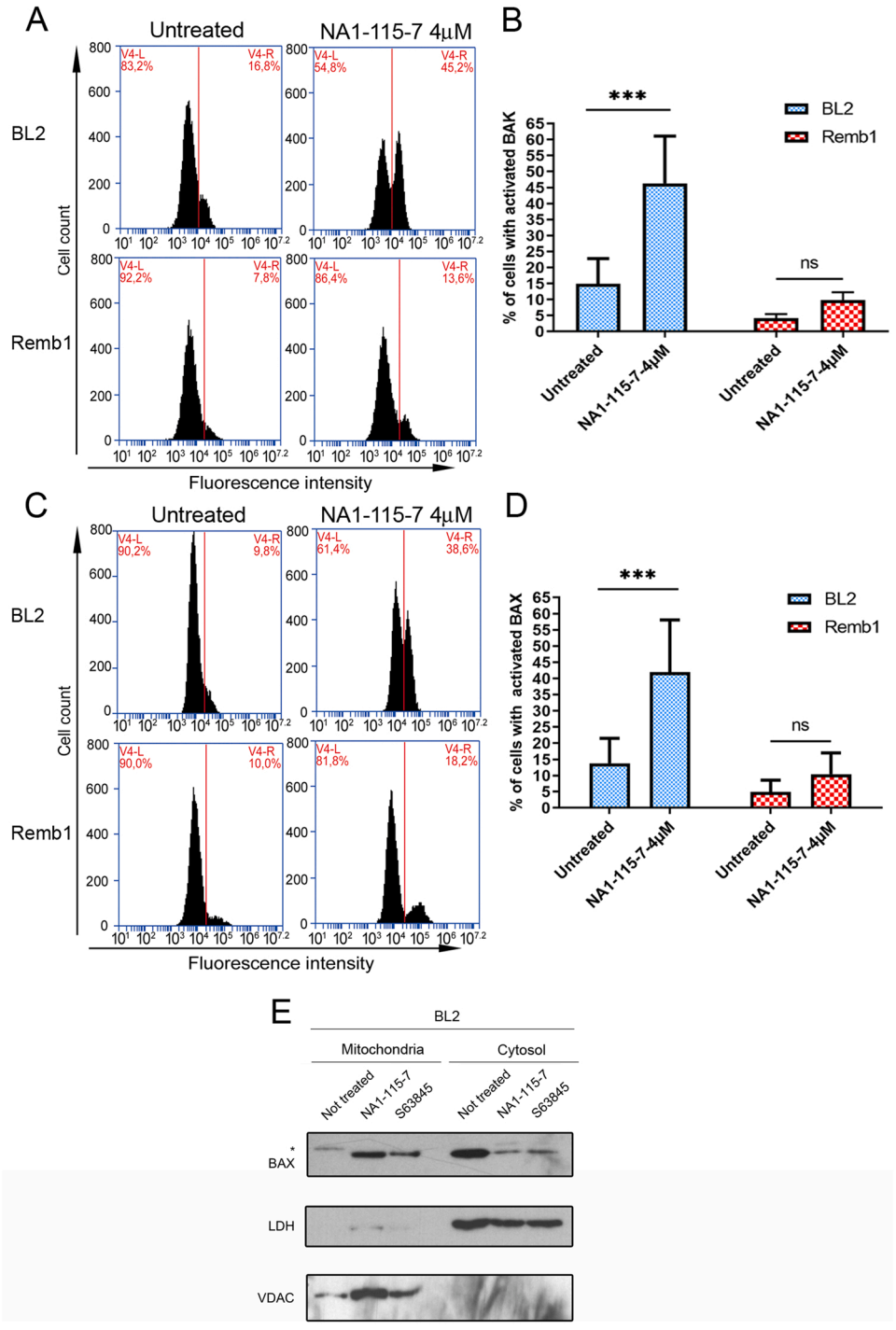


Fig. 3. *The apoptosis induced by NA1—115—7 treatment is dependent principally on the activation of BAX and BAK.* BL2 and Remb1 cells were incubated for 4 h with or without NA1—115—7. Cells were then fixed, permeabilized, labeled with a conformational BAK mAb (ab-1) or with the 6A7 conformational BAX mAb, and analyzed by flow cytometry. A – Representative plots for BAK activation. B – The values shown (mean ± S.D.) were obtained from at least five independent experiments for BAK activation. Mann-Whitney tests were performed for statistical analysis (***: $p < 0.01$). C – Representative plots for BAX activation. D – The values shown (mean ± S.D.) were obtained from at least five independent experiments for BAX activation. Mann-Whitney tests were used for statistical analysis (***: $p < 0.01$). E – BL2 cells incubated for 7 h with or without NA1—115—7 (4 μM) or S63845 (0.5 μM). Cytosolic and mitochondrial fractions were prepared and analyzed by western blotting with an anti-BAX mAb. LDH and VDAC were used as cytosolic and mitochondrial markers, respectively. *The upper bands correspond to prior staining with an anti-SMAC/DIABLO antibody.

performed western blots to assess the cleavage of PARP after the treatment of BL2 cells for various periods of time. The 85 kDa PARP cleavage product was detectable as early as 2 h after treatment, and its level gradually increased, reaching a peak at 16 h of treatment (Fig. 2C). Based on these findings, we compared apoptosis levels in BL2 cells treated with NA1—115—7 (4 μM) for 24 h, or for 2 h followed by 22 h of incubation in complete medium devoid of this compound. Both treatments resulted in 92 ± 0.5% annexin V/PI-positive cells (Fig. 2D). These findings indicate that short periods of exposure to NA1—115—7 are sufficient to trigger the mitochondrial apoptotic pathway in a specific manner, in MCL-1-dependent cell lines. They are consistent with our previous MALDI data demonstrating a covalent interaction (Paal-Knorr reaction) of NA1—115—7 with a lysine residue in the MCL-1 BH3 groove [40].

3.3. BAK and BAX are activated in MCL-1-dependent cells treated with NA1—115—7

One of the key steps in the mitochondrial apoptosis pathway is the formation of pores in the mitochondrial outer membrane by the pro-apoptotic proteins BAK and BAX, leading to the release of apoptogenic factors. When activated, both these proteins undergo changes in conformation that expose the N-terminal epitopes. The labeling of cells with conformation-dependent antibodies can, therefore, be used to determine whether or not BAK and BAX are activated. Flow cytometry analysis with such antibodies showed that BAK was activated in BL2 cells after 4 h of treatment with NA1—115—7, whereas it was not activated in Remb1 cells (activation in 46 ± 15% and 10 ± 2% of cells, respectively, Fig. 3A, 3B). Similarly, BAX was activated in BL2 cells, but not in Remb1 cells (in 41 ± 16% and 10 ± 6% of cells, respectively, Fig. 3C, 3D). Thus, following treatment with NA1—115—7, BAK and BAX are activated only in MCL-1-dependent cells.

Under physiological conditions, BAX is translocated from the cytosol to the mitochondria. However, it is continually translocated back to the cytosol by BCL-xL, which remains the principal compartment in which it is found. By contrast, once activated, BAX remains in the mitochondria [46]. A western-blot analysis of subcellular fractions of BL2 cells showed that BAX was present only in the cytosolic fraction in untreated cells, whereas the amount of BAX in the cytosol of cells treated with NA1—115—7 (4 μM) or S63845 (0.5 μM) for 7 h was much lower and accompanied by an increase in the amount of BAX present in the mitochondrial fraction (Fig. 3E). These results confirm the activation of BAX following the treatment of MCL-1-dependent cells with NA1—115—7.

3.4. Disruption of the MCL-1/BAK complex and MCL-1 stabilization during NA1—115—7 treatment

Anti- and proapoptotic members of the BCL-2 family are engaged in dynamic interactions that regulate cell fate and can be disrupted by BH3 mimetics. We, therefore, further investigated the apoptotic pathway induced by NA1—115—7, by analyzing its effect on MCL-1/BAK complexes. Total protein extracts from BL2 cells with and without four hours of NA1—115—7 (4 μM) treatment were subjected to immunoprecipitation with an anti-MCL-1 Ab and the co-immunoprecipitation of BAK was assessed by western blotting. BAK co-immunoprecipitated with MCL-1 in untreated cells, whereas this interaction was 50% weaker in NA1—115—7-treated cells (Fig. 4A).

Previous studies have shown that treatment with various MCL-1-specific BH3 mimetics extend the half-life of the protein [32,33]. We therefore assessed MCL-1 level after the treatment of BL2 cells with NA1—115—7 (4 μM) and cycloheximide (CHX, a protein synthesis inhibitor that blocks translational elongation), or CHX alone (Fig. 4B). As expected, the amount of MCL-1 decreased rapidly (half-life of 30–60 min) in cells treated with CHX alone, but clearly remained stable for much longer (half-life of between 2 and 4 h) in cells treated with a

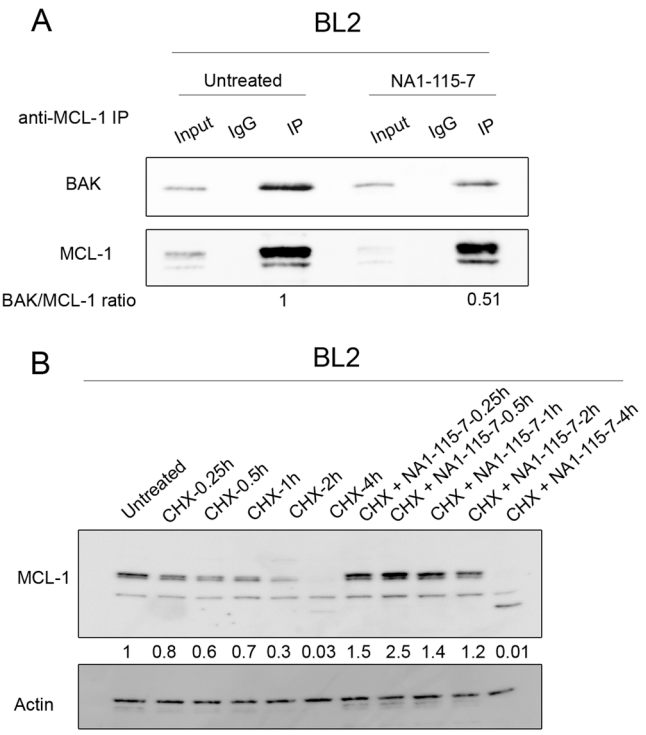


Fig. 4. *NA1—115—7 disrupts the MCL-1/BAK complex and stabilizes MCL-1 at early stages*. A – BL2 cells were incubated for 4 h with or without NA1—115—7. Whole-cell lysates were then prepared and subjected to immunoprecipitation with an anti-MCL-1 mAb (B-6) or a control mouse IgG. The immunoprecipitated proteins were then analyzed by western blotting with an anti-BAK pAb or an anti-MCL-1 mAb. "Input" corresponds to 15% of the total lysate. IP: immunoprecipitates. B – Western-blot characterization of MCL-1 protein half-life in BL2 cells treated with CHX (500 μM), with or without NA1—115—7 (4 μM), for various times. Quantity One software, version 4.6 (Bio-Rad) was used for the densitometric analysis of the blots. Fold-change values are shown under the MCL-1 blot and were calculated relative to the untreated control and normalized against β-actin levels.

combination of NA1—115—7 and CHX.

Overall, these results show that NA1—115—7 specifically targets and stabilizes MCL-1, disrupts its binding to BAK, and triggers an apoptotic signaling pathway involving BAK and BAX activation.

3.5. NA1—115—7 is nontoxic to normal blood cells and is not cardiotoxic

If new specific MCL-1 inhibitors are to be used in clinical practice, they will need to succeed in clinical trials. It will, therefore, be necessary to demonstrate an absence of major toxicity to normal cells. We first tested the effect of various concentrations of NA1—115—7 on human erythrocytes isolated from healthy donors (Fig. 5). After 24 h of treatment, the erythrocytes were centrifuged and hemolysis was assessed by visual examination (Fig. 5A and Supplemental Fig. 1). NA1—115—7, at concentrations of 2 μM and 4 μM, the optimal concentrations for inducing tumor cell apoptosis, had little (at 4 μM) or no (at 2 μM) hemolytic activity. The treatment of erythrocytes with 0.5 μM S63845 induced an effect similar to that obtained with 4 μM NA1—115—7. These results were confirmed by an analysis of erythrocytes by microscopy after 24 h of treatment with 4 μM NA1—115—7 or 0.5 μM S63845 (Fig. 5B), and are consistent with those of Fratoni et al. showing that other drimane sesquiterpenes have almost no hemolytic activity [47].

We then assessed the effect of NA1—115—7 on platelets, which play a key role in cases of blood vessel damage. Platelets isolated from healthy donors were incubated for 24 h with NA1—115—7 (4 μM),

A

Treatment (24h)	Untreated	H2O	S63845	NA1-115-7			
			0.5μM	2μM	4μM	8μM	16μM
Cell lysis	-	+++	+/-	-	+/-	++	+++

B

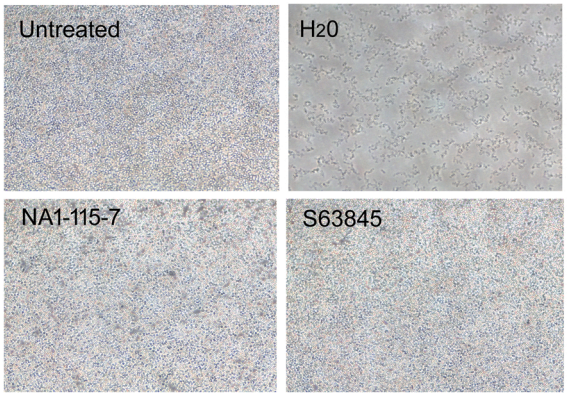


Fig. 5. *NA1—115—7 shows very weak hemolytic activity at effective doses.* A – Erythrocytes were treated for 24 h with various doses of NA1—115—7 (2, 4, 8, and 16 μM), S63845 (0.5 μM), or water as a positive control. Erythrocyte lysis was estimated by eye (see the images presented in Supplemental Fig. 1). B – Erythrocytes were treated for 24 h with NA1—115—7 (4 μM), S63845 (0.5 μM), or water as a positive control and examined under an inverted light microscope (100x).

S63845 (0.5 μM), or ABT-737 (10 μM) as a positive control, and apoptosis measured by FACS analysis after annexin V/PI labeling of the cells. ABT-737 induced > 90% apoptosis in platelets, whereas NA1—115—7 and S63845 had only a very small effect (22 ± 16% and 21 ± 17% of cells apoptotic, respectively, Fig. 6A).

MCL-1 is known to play an essential role in the survival of various types of cell, including lymphocytes and cardiomyocytes [20,48]. We therefore evaluated the toxicity of our compound (and S63845) to human PBMCs obtained from healthy donors. After 24 h of treatment, apoptosis was evaluated by labeling the cells with annexin V and PI. A weak induction of apoptosis occurred in PBMCs treated with NA1—115—7 (22 ± 4% apoptotic cells), whereas a higher proportion of the PBMCs treated with S63845 became apoptotic (58 ± 13%) (Fig. 6B). Finally, we also assessed the cardiotoxicity of NA1—115—7 by two methods. We first determined the LC_{50} of NA1—115—7 and S63845 in the H9C2 cell line derived from rat heart myoblasts, which is classically used in assessments of the cardiotoxicity of drugs. Doxorubicin, which is known to be cardiotoxic, was used as a positive control. The LC_{50} was > 20 μM for NA1—115—7 and > 1 μM for S63845. These two concentrations are much higher than the corresponding effective doses for these compounds (Fig. 6C). We confirmed these results in the hERG (human *ether-a-go-go*-related gene) inhibition assay. hERG is a potassium channel that plays a key role in the repolarization of cardiac action potential. Decreases in its activity are associated with a strong increase in the risk of potentially fatal cardiac arrhythmias. It is, therefore, essential to determine whether a new drug binds to an inhibits hERG, as a means of assessing its potential cardiotoxicity. We assessed the binding of NA1—115—7 to hERG in the Predictor™ hERG polarization assay. The low affinity of NA1—115—7 made it impossible to determine an IC_{50} value for hERG channel inhibition (Fig. 6D). At a concentration of 10 μM NA1—115—7, only 46.2% of the channels were inhibited.

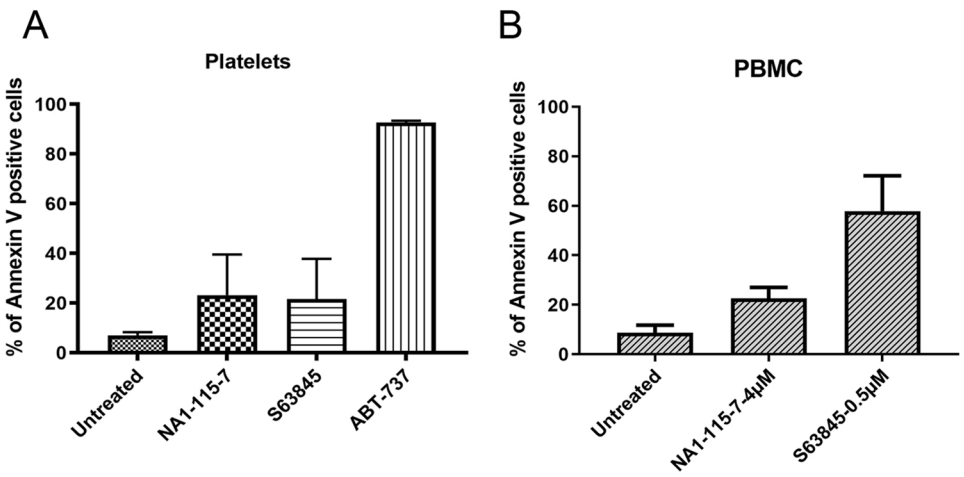
Overall, these results show that NA1—115—7 is nontoxic for normal blood cells and that it is not cardiotoxic at doses inducing tumor cell apoptosis.

4. Discussion

MCL-1 is a crucial regulator of cell survival in both normal and neoplastic cells and is often responsible for resistance to anticancer therapy. Here, we investigated the therapeutic potential of a natural product, NA1—115—7, for use as a novel specific inhibitor of MCL-1. We showed that NA1—115—7 disrupted the BAK/MCL-1 interaction, leading to the activation of BAK and BAX and the induction of apoptosis in MCL-1-dependent cells. Moreover, treatment for as little as two hours was sufficient to induce the apoptosis of MCL-1-dependent cells, consistent with our previous NMR and MALDI data showing that NA1—115—7 forms a covalent bond with a lysine residue in the MCL-1 BH3-groove via Paal-Knorr condensation [40]. We also found that BL2 cells, which were shown to be dependent on both MCL-1 and BCL-xL in BH3 profiling experiments, were very efficiently killed by NA1—115—7, but not by ABT-737, which targets BCL-xL (in addition to BCL-2). These results are consistent with previous findings that the sustained survival and growth of BL cell lines is highly dependent on MCL-1, even though some of these cell lines were also partly dependent on BCL-xL [31,49].

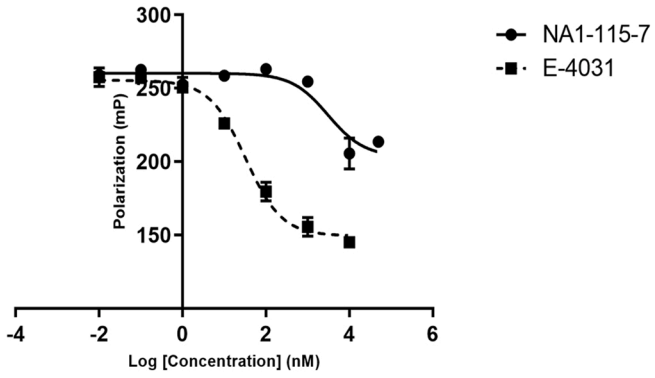
MCL-1 knockout in mice has harmful hepatic, hematological, and cardiac effects, including the rapid development of heart failure [50]. Cardiotoxicity problems were also encountered during the phase I clinical trials initiated for six different BH3-mimetic drugs targeting MCL-1 in various hematological malignancies. Several trials were stopped and trials are currently continuing for only two of these compounds. The tolerability of MCL-1 inhibitors is, therefore, an important concern in their clinical development. Our results show that NA1—115—7 was not toxic to erythrocytes, peripheral blood mononuclear cells, platelets, or cardiomyocytes. In light of all these results, NA1—115—7 appears to be a promising lead of true therapeutic interest, particularly for MCL-1-dependent B-cell lymphoproliferative disorders.

The mechanism of BAX/BAK activation remains a key issue in apoptosis signaling. We expected to observe BAK activation after NA1—115—7 treatment, as BAK interacts directly with MCL-1 [51,52]. By contrast, the observed activation and redistribution of BAX was more surprising, even though Smith et al., recently showed that the MCL-1 inhibitor S63845 mostly induces BAX-dependent apoptosis in diffuse large B-cell lymphomas (DLBCL) [53]. Interestingly, these authors showed that BAX activation could be mediated by the displacement of BAK, BIM, and NOXA from MCL-1 rather than a direct interaction between MCL-1 and BAX [53]. Similarly, in our model, MCL-1 interacted with NOXA and BAK, but not with BAX (data not shown). Germain *et al.* also demonstrated that BAX activation did not require a direct interaction between MCL-1 and BAX, and that MCL-1 inhibited BAX downstream from its translocation to mitochondria [54]. Anti-apoptotic BCL-2 family members may sequester BAX and BAK monomers to prevent their homo-oligomerization, but also BH3 activators to prevent BAX and BAK activation. Through its covalent interaction with MCL-1, NA1—115—7 may liberate BH3 activators, such as BIM and NOXA. Indeed, the displacement of BIM and/or NOXA may be functionally important for NA1—115—7-induced apoptosis, as the BIM and/or NOXA released might initiate apoptosis by binding directly to BAX and BAK, causing their activation. Alternatively, BAK may play a direct role in BAX activation. For example, Smith et al. showed that S63845 treatment decreased the level of BAX activation in BAK-deleted cells, suggesting a role for BAK in BAX activation [53]. Another informative study revealed that an association with the mitochondrial outer membrane via helix 9 of BAX and BAK is required and sufficient for the oligomerization and activation of these proteins in the absence of BH3-only and anti-apoptotic BCL-2 proteins [55]. It is, therefore, possible that NA1—115—7-mediated disruption of the BAK/MCL-1 interaction led to the activation of BAK, which was then able to oligomerize with inactive BAX in the mitochondrial outer membrane, activating the resulting hetero-oligomers.



C

Cell lines	Lethal Concentration (LC50) (µM)		
	NA1-115-7	S63845	Doxorubicin
H9C2	> 20	> 1	0.69



Compound	IC_{50} (nM)	% inhibition
E-4031	33.9	
NA	ND	46.2 at 10 µM

Fig. 6. *NA1—115—7 is not cytotoxic to platelets, PBMCs, or cardiac cells*. A – Platelets were treated for 24 h with NA1—115—7 (4 µM), S63845 (0.5 µM), or ABT-737 (10 µM). The cells were labeled with annexin V-FITC and PI and apoptosis was assessed by flow cytometry. The values shown (mean ± S.D.) were obtained from two independent experiments. B – PBMCs were treated for 24 h with NA1—115—7 (4 µM) or S63845 (0.5 µM) and apoptosis was assessed as in (A). The values shown (mean ± S.D.) were obtained from at least four independent experiments. C – H9C2 cells were treated with various doses of NA1—115—7, S63845, or doxorubicin (as a positive control). Cell viability was determined in MTT assays and the LC_{50} was calculated. D – The cardiotoxicity of NA1—115—7 was determined in the hERG inhibition assay. The values shown (mean ± S.D.) were obtained from two independent experiments. IC_{50} values were determined graphically with GraphPad Prism software. E-4031 is an inhibitor of the hERG channel.

The binding of NA1—115—7 to MCL-1 leads to the stabilization of MCL-1 and then its degradation, two outcomes already reported for the MCL-1 inhibitors AMG-176 [32] and S63845 [33]. In previous ligand-protein NMR experiments [40], we showed that NA1—115—7 is selective for MCL-1, binding in the groove formed by its BH1, BH2, and BH3 regions (more precisely helices α2, α3, α4, and α5). The signal of a lysine (K234), close to the BH3 domain, and, thus, close to the hydrophobic groove of MCL-1, is strongly affected by the presence of NA1—115—7. MALDI experiments also showed that our compound formed a stable and irreversible complex via a Paal-Knorr reaction. We assume that this reaction involves the lysine 234 residue, although this remains to be confirmed. Interestingly, a previous report showed that aryl boronic acid carbonyl warheads formed a reversible covalent bond with the lysine 234 residue of MCL-1 [56]. However, to our knowledge, the ability of these compounds to induce the apoptosis of MCL-1-dependent cells has never been evaluated. NA1—115—7 may therefore be considered the first covalent MCL-1 inhibitor described to date with an ability to function as a "suicide" molecule: once attached to the target, it is degraded with it. This may explain the micromolar doses required to induce apoptosis with NA1—115—7, whereas nanomolar doses are sufficient for other molecules, such as S63845. This may limit the use of NA1—115—7 for certain cancers, as tumors overexpressing MCL-1 would require high doses of NA1—115—7, which might prove cytotoxic. By contrast, diseases such as multiple myeloma, which is predominantly MCL-1-dependent but without amplification of the 1q arm (in about 60% of *de novo* cases), suggesting that it may not overexpress MCL-1, may be very good candidate diseases for treatment with this molecule [57].

5. Conclusion and future outcomes

Our data reveal that, on entering cells, NA1—115—7 rapidly forms a covalent bond with a lysine residue in the MCL-1 BH3-groove, leading to the release of BAK and, probably, of the NOXA BH3-only protein. BAK and BAX are then activated and form pores in the mitochondrial outer membrane via which apoptogenic factors, such as SMAC and cytochrome *c* are released, thereby triggering apoptosis. At doses toxic to tumor cells, NA1—115—7 does not induce the apoptosis of normal cells.

The lipophilic structure of NA1—115—7 and its sensitivity to acidic conditions might hinder its clinical development. It will, therefore, be crucial to find ways of increasing its solubility and stability so that experiments can be performed in animal models. We have developed NA1—115—7-loaded nano-emulsions (NE-) for this purpose and have shown, that such formulations increase the bioavailability of the compound and its ability to penetrate cells (unpublished data), which should facilitate its evaluation *in vivo*.

CRediT authorship contribution statement

Florian Daressy, Aude Robert, and Joëlle Wiels: **Conceptualization**. Florian Daressy, Loëtitia Favre, Sophie Corvaisier, Cécile Apel, Marc Litaudon, Vincent Dumontet, Sandy Desrat, Aude Robert: **Acquisition of data**. Florian Daressy, Line Séguy, Loëtitia Favre, Sophie Corvaisier, Anne-Claire Groo, Aurélie Malzert-Fréon, Fanny Roussi, Aude Robert, and Joëlle Wiels: **Analysis and interpretation of data**. Florian Daressy, Anne-Claire Groo, Aurélie Malzert-Fréon, Fanny Roussi, Aude Robert and Joëlle Wiels: **Writing, review, and/or revision of the manuscript.** Aude Robert and Joëlle Wiels: **Study supervision.** Aurélie Malzert-Fréon, Fanny Roussi, and Joëlle Wiels: **Funding acquisition**.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Yann Lécluse (Imaging and Cytometry Platform, Institut Gustave Roussy) for expert technical assistance for flow cytometry analyses. We are also very grateful to the Northern and Southern Provinces of New Caledonia, which facilitated our field investigation.

Funding sources

This work was supported by *Université Paris-Saclay* (PhD fellowship to F.D.) through the "IDI 2017" project (grant 165236); *Institut National du Cancer* (INCa) (2016–1-PL-BIO) managed by *Cancéropole Ile-de-France*; an *Investissement d'Avenir* grant managed by *Agence Nationale de la Recherche* (CEBA, ANR-10-LABX-25–01); and by the *Ligue Contre le Cancer* (Calvados committee, NEMIBLY Project).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113546.

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