



## Original Article

# Rocaglamide breaks TRAIL-resistance in human multiple myeloma and acute T-cell leukemia *in vivo* in a mouse xenograft model



Yin Wu<sup>a</sup>, Marco Giaisi<sup>b</sup>, Rebecca Köhler<sup>b</sup>, Wen-Ming Chen<sup>a, \*\*</sup>, Peter H. Krammer<sup>b</sup>,  
Min Li-Weber<sup>b, \*</sup>

<sup>a</sup> Department of Hematology, Beijing Chao-Yang Hospital, Capital Medical University, 8 Gongren Tiyuchang Nanlu, Chaoyang District, Beijing 100020, China

<sup>b</sup> Tumorimmunology Program (D030), German Cancer Research Center (DKFZ), D-69120, Heidelberg, Germany

## ARTICLE INFO

## Article history:

Received 7 October 2016

Received in revised form

1 December 2016

Accepted 9 December 2016

## Keywords:

TRAIL

Rocaglamide

Apoptosis

c-FLIP

Myeloma

Leukemia

## ABSTRACT

Multiple myeloma (MM) is an incurable malignancy by the presently known therapies. TRAIL is a promising anticancer agent that virtually not shows any toxicity to normal cells. We have recently carried out clinical trials with a human circularly permuted TRAIL, CPT, against MM saw a partial response in approximate 20–30% of patients. In the current study, we investigated the cause of CPT resistance and revealed that the majority of the MM patients express elevated levels of c-FLIP. Knockdown of c-FLIP expression by siRNA alone was sufficient to increase CPT-mediated apoptosis in a CPT-resistant human MM cell line U266. To overcome CPT resistance, we investigated the combination of CPT with Rocaglamides(s) in MM which has been shown to inhibit c-FLIP expression *in vitro*. We show that Rocaglamide(s) overcomes CPT resistance in U266 *in vitro* and significant increases in anti-tumor efficacies of CPT in mice xenografted with U266. Similar results were also obtained in mice xenografted with the CPT-resistant human acute T-cell leukemia cell line Molt-4. Our study suggests that the combination of Rocaglamide(s) with CPT may provide a more efficient treatment against myeloma and leukemia.

© 2016 Elsevier Ireland Ltd. All rights reserved.

## Introduction

Induction of apoptosis is one of the strategies against different types of cancer. So far, radio- and chemotherapy are the main treatments of cancer in addition to surgical resection of the tumor. However, toxicity to normal tissues is the main obstacle of radio- and chemotherapy. The tumor necrosis factor apoptosis inducing ligand (TRAIL) has emerged as one of the promising anticancer agents and shows virtually no toxicity to normal cells [1]. However, the results of the early clinical trials using recombinant TRAIL or TRAIL-R agonists showed little antitumor efficacy [2–4]. The low efficiency of TRAIL therapy has been recognized to be largely due to poor activity of the agonists used in the clinical trials [4,5]. Besides, many cancer cells are partially or completely resistant to TRAIL due to over-expression of the cellular caspase-8 (FLICE)-inhibitory protein (c-FLIP) and/or the inhibitor of apoptosis proteins (IAPs),

particularly the X-linked IAP (XIAP), which blocks activation of caspase-8 and caspase-3, respectively [5]. Therefore, large efforts have been made to develop more efficient TRAIL-R-targeting agents and/or inhibitors of c-FLIP and IAPs [4].

Circularly permuted TRAIL (CPT) is a new generation of recombinant human TRAIL made by Beijing Sunbio Biotech LTD [6]. The primary difference of CPT from the wild-type TRAIL is that the N-terminus of amino acids 121–135 sequence is connected to the C-terminus of the amino acids 135–281 sequence of TRAIL by a flexible linker. This mutated molecule forms stable homotrimers and has potent apoptosis-inducing activity *via* enhanced binding to its death receptors TRAIL-R1/R2 (DR4/DR5) [6]. Comparison with the wild-type TRAIL, CPT shows improved stability and longer half-life. CPT also displays a better antitumor activity without significant toxicity against normal cells [6]. Recently, CPT has been studied in clinical trials in patients with relapsed and/or refractory multiple myeloma (RRMM) [7,8]. Multiple myeloma (MM) is a disease that affects plasma cells and can lead to devastating clinical features such as anemia, lytic bone lesions, hypercalcemia, and renal disease [9]. The clinical trials show that CPT mono-therapy is well tolerated but, unfortunately, only 20–30% of patients showed a partial or better response [7,8]. Currently, CPT is in a phase II clinical trial in

\* Corresponding author. Tumor Immunology Program D030, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Fax: +49 6221 411715.

\*\* Corresponding author. Fax: +86 10 65031910.

E-mail address: [m.li-weber@dkfz-heidelberg.de](mailto:m.li-weber@dkfz-heidelberg.de) (M. Li-Weber).

combination with the anti-angiogenesis drug Thalidomide in RRMM patients. It is thought that CPT and Thalidomide have different antitumor mechanisms and, thus, a combination of the two drugs would give a synergistic antitumor effect. However, the initial trial did not show the expected effect [10]. Therefore, it is necessary to find a rational CPT partner that can enhance TRAIL anticancer therapy in MM.

We have previously shown that a group of natural phytochemicals Cyclopenta[b]benzofuran compounds, collectively named Rocaglamides (Rocs) (also referred to as Flavaglines) (for reviews see Ref. [11] and Ref. [12]), selectively induce apoptosis in malignant cell by activation of the stress-response mitogen-activated protein kinases (MAPK) p38 and inhibit tumor growth by inhibition of the survival Raf-MEK-ERK signaling pathway leading to inactivation of eIF4E, a key factor for controlling the rate-limiting step of translation [13–15]. Due to translation inhibition, Rocs down-regulate expression of anti-apoptotic proteins which have a short half-life such as c-FLIP and Mcl-1 [14,16]. C-FLIP is a well-known inhibitor of the receptor-mediated signaling pathway and, thus, is the key determinant of tumor resistance to TRAIL-induced cell death [17,18]. Many cancers have been shown to over-express c-FLIP proteins including colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, Hodgkin's lymphoma, B cell chronic lymphocytic leukemia, melanoma, ovarian carcinoma, cervical carcinoma, bladder urothelial carcinoma, and prostate carcinoma [19]. We have recently shown that Rocs can sensitize resistant cancer cells towards TRAIL-induced cell death in HTLV-1-associated adult T-cell leukemia/lymphoma (ATL) and in Hodgkin's lymphoma *via* inhibition of c-FLIP expression [14,20]. So far, the expression levels of c-FLIP in MM have not been thoroughly studied. Therefore, we asked whether c-FLIP expression levels interfere with CPT therapy in MM and, if so, whether Rocs can overcome CPT-resistance in MM.

To answer the above questions, we investigated expression levels of c-FLIP proteins in primary MM samples isolated from patients. We show that the majority of MM patients express elevated levels of c-FLIP. We also show that over-expression of c-FLIP correlates with CPT resistance in MM cells. We further show that Rocs synergistically enhance CPT-induced apoptotic cell death in resistant MM cells *in vitro*. To confirm the *in vitro* data, we further carried out animal experiments and xenografted mice with either the CPT-resistant human MM cell line U266 or the CPT-resistant human T-cell leukemia cell line Molt-4, respectively. We show that combination treatment with Rocs and CPT significantly enhances efficacy of cancer treatment *in vivo*.

## Materials and methods

### Cell lines and cell culture

The human malignant cell lines used in this study are the T-cell acute lymphoblastic leukemias (T-ALL) Sup-T1, Molt-4 and Jurkat; the lymphoma T-cell cell line SeAx; the MM cell lines U266 and L363. All cells were cultured in RPMI 1640 or DMEM medium (GIBCO laboratories, Grand Island, USA), supplemented with 10% FCS, 100 Units/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO) and 2 mM L-glutamine (GIBCO) at 37 °C and 5%.

### Isolation of primary MM cells

Bone marrow aspirates from patients (detailed information about the patients will be provided upon request) were first subjected to Ficoll-Hypaque (GE Healthcare Bio-Sciences AB) gradient centrifugation to obtain the mononuclear cell fraction. The red cells were lysed by ammonium chloride and followed by wash with phosphate-buffered saline (PBS) several times. Bone marrow plasma cells were enriched by using the directly conjugated monoclonal mouse anti-CD138 microbeads (Miltenyi Biotec, Auburn, CA, USA) and by an immunomagnetic bead selection process as described previously [21]. Purity of cells more than 95% was confirmed by 2-color flow cytometry (CD138+/CD45-and CD38+/CD45-) (Becton Dickinson, San Jose, CA, USA).

### Preparation of human proliferating T cells

Primary T cells from human peripheral blood T cells were prepared to more than 90% CD3 positivity as described previously [13]. For generation of proliferating T cells, freshly isolated T cells were cultured at  $2 \times 10^6$  cells/ml and were activated with 1 µg/ml PHA overnight. Activated T cells were then washed three times and cultured for additional 5 days in the presence of 25 U/ml IL-2.

### CPT and Rocs

CPT, the recombinant human mutant of TRAIL, is made by Beijing Sunbio Biotech LTD (Beijing, China). Roc-A (>98% pure) was purchased from Active Biochem., Hong Kong, China. The other Rocaglamide derivatives isolated from different species of the plant genus *Aglaiia* are kindly provided by Dr. Peter Proksch (Institute of Pharmaceutical Biology and Biotechnology, University of Duesseldorf, D-40225 Duesseldorf, Germany). All compounds are >98% pure assessed by HPLC (more information see Ref. [15]). The synthetic Roc derivative FL3 is kindly provided by Dr. Laurent Désaubry (Laboratoire d'Innovation Thérapeutique (UMR 7200), Faculté de Pharmacie, F-67401 Illkirch cedex, France).

### Determination of apoptosis

Rocs were solved in dimethyl sulfoxide (DMSO; Roth, Karlsruhe, Germany) at a stock concentration of 50 mM. CPT was solved in cell culture medium. Cells were treated with different concentrations of Rocs or CPT for 48 h. Apoptotic cell death was examined by analysis of DNA fragmentation as previously described [13]. Results are presented as % specific DNA fragmentation using the formula: (percentage of experimental apoptosis – percentage of spontaneous apoptosis) / (100 – percentage of spontaneous apoptosis) × 100.

### Knockdown of c-FLIP proteins

U266 cells were transfected with 200 nM of nonsense siRNA (#AM4611, Thermo fisher scientific Germany), c-FLIP<sub>L</sub> siRNA (5'-GAGCUUCUUCGAGACACCUCC-3') and c-FLIP<sub>S</sub> siRNA (5'-CACCCUAUGCCCAUUGUCCU-3') using the Amaxa Nucleofector solution (Nucleofector kit c) by the Amaxa program x-005 (Amaxa Biosystems, Cologne, Germany). After transfection, cells were allowed to rest for 24 h in culture and then treated with different concentrations of CPT for 24 h. After CPT treatment, cells were collected for apoptosis measurement. Knockdown efficiency of c-FLIP proteins was controlled by Western blot analysis 24, 48 and 72 h after transfection.

### Western blot analysis

For each sample,  $1 \times 10^7$  cells were lysed as previously described [13]. Equal amounts of protein were separated on 7–13% SDS-PAGE depending on the molecular sizes of the proteins and blotted onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfon, UK) as previously described. The following antibodies were used: The Caspase-9 (sc-17784) from Santa Cruz, Dallas, Texas, USA; Caspase-3 (9662), XIAP (2042) and TRAIL-R2 from Cell Signaling Technology, Danvers, USA; TRAIL-R1 from Prosci, CA; tubulin and actin from Sigma, Saint Louis, USA; PARP (556362) from BD Biosciences, Heidelberg, Germany. The caspase-8 mAb C15 (mouse IgG2b) and c-FLIP mAb NF6 were generated in our lab as described previously [22,23].

### Cell surface staining TRAIL receptors

For analysis of surface expression levels of TRAIL receptors, cells ( $5 \times 10^5$ ) were washed with PBS and incubated with 1 µg/ml of corresponding antibodies for 30 min at 4 °C, washed with PBS and incubated for 30 min with phycoerythrin-conjugated goat anti-mouse antibody (Dianova, Duabiva, Hamburg, Germany), and analyzed by flow cytometry with a FACScan Cytometer (Becton Dickinson, Heidelberg, Germany). The following anti-TRAIL receptor antibodies were used: HS101 (TRAIL-R1/DR4), HS201 (TRAIL-R2/DR5), HS301 (Dc-R1) and HS402 (Dc-R2) (Enzo livesciences Lausen, Germany).

### In vivo mouse studies

Immunodeficient mice (NOD CB17-Prkdc scid/NcrCrI) were implanted subcutaneously in the dorsal flank region with U266 ( $2.5 \times 10^7$ ) or Molt-4 ( $5 \times 10^6$ ) cells. In the U266 mouse experiment, once small tumors were visible (about two weeks after grafting), mice were randomly separated into four groups (8 mice in each group) and were treated by intraperitoneal injection (i.p.) with vehicle (DMSO) or with CPT (50 mg/kg) in the presence or absence of Roc-A (0.5 mg/kg) 5 times per week for 2 weeks and then further at 1 week on each second day. The tumor size was measured with a micrometer caliper and the tumor volume (V) was calculated by the formula  $V = (\text{width}^2 \times \text{length})/2$ . In the Molt-4 mouse experiment, one week after grafting, tumor-size-matched mice (approximativ 15 – 25 mm<sup>3</sup>) were treated with vehicle or with CPT (50 mg/kg) in the presence or absence of Roc-A (0.5 mg/kg) (each group contains seven mice) five times per week by i.p. for two weeks. The mice were killed and the body and tumor weight were measured. All protocols using and

maintaining animals were approved by the German Animal Protection Authority (Office Regierungspräsidium Karlsruhe) in Karlsruhe.

## Results

### The majority of MM patient samples over-express c-FLIP proteins

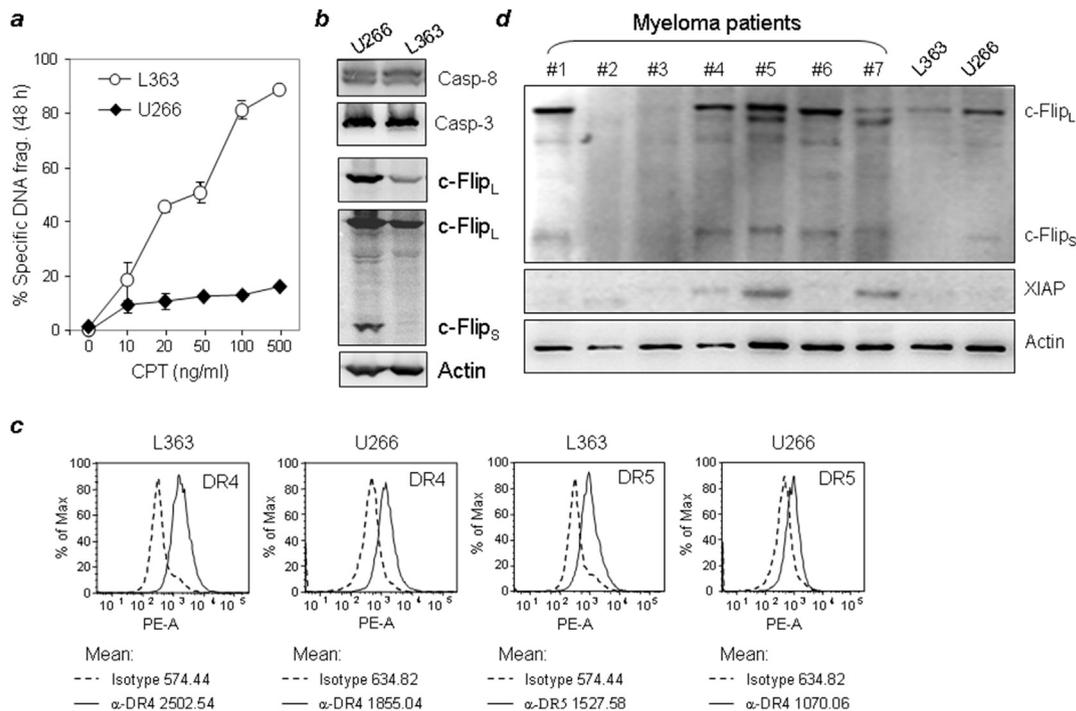
As only 20–30% of MM patients show partial response to CPT, we decided to investigate the reasons that cause CPT resistance. We first selected two MM cell lines, a CPT-sensitive cell line L363 and a CPT-resistant cell line U266, as model systems (Fig. 1a). Western blot analysis showed that both cell lines express similar levels of Caspase-8 and Caspase-3 (Fig. 1b). Also, both cell lines express TRAIL receptors DR4 and DR5 (TRAIL-R1/R2) at the cell surface with a slightly higher level in L363 (Fig. 1c). No detectable expression of decoy receptor 1 (DcR1) and DcR2 were seen in both cell lines by Western blot analysis (data not shown) and by cell surface expression analysis (Supplementary Fig. S1). C-FLIP is the key inhibitor of the receptor-mediated signaling pathway [5,17,18]. Notably, U266 expresses significantly higher levels of c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> compared to L363 (Fig. 1b). We then compared the c-FLIP expression levels of primary tumor samples from seven MM patients with L363 and U266. The analysis showed that 5 out of 7 MM samples (approximately 71% of the patients) express c-FLIP proteins at the levels similar to or even higher than U266 (Fig. 1d). XIAP is another inhibitor of the receptor-mediated signaling pathway [5]. Three patient samples show elevated XIAP expressions (about 43% of the patients) (Fig. 1d). The ratios of c-FLIP over-expressing patients consist with the CPT-response ratios in the clinical trials (20–30% of patients). Thus, high levels of c-FLIP expression are likely the cause of CPT resistance in MM patients.

### Knockdown of c-FLIP increases CPT sensitivity in U266 cells

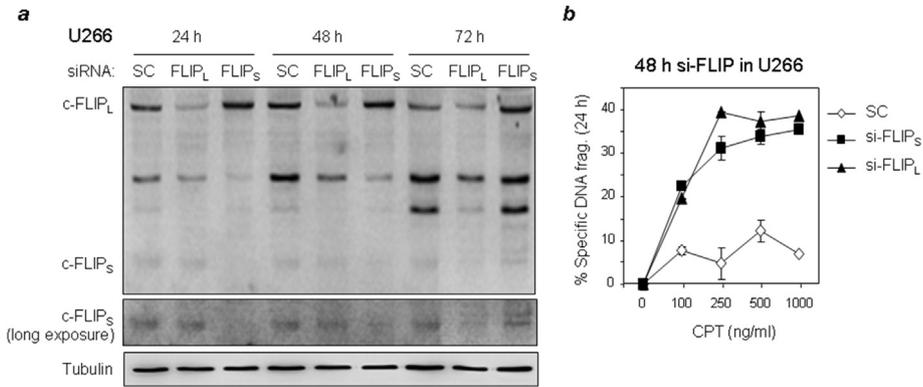
To investigate the role of c-FLIP in regulation of CPT sensitivity and resistance in MM cells, we performed a siRNA approach to knockdown c-FLIP expression in the CPT-resistant U266 cells. The experiment showed that specific knockdown of the expression of either c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> in U266 cells resulted in increase in CPT-mediated apoptotic cell death (Fig. 2). Despite the fact that U266 cells have less surface expression of DR4 and DR5 compared to L363 (Fig. 1c), down-regulation of c-FLIP protein expression is sufficient to enhance sensitivity of U266 towards CPT-induced killing. The experiment suggests that suppression of TRAIL expression is a promising strategy to improve the TRAIL-based therapy in MM.

### Rocs synergize with CPT to enhance apoptosis induction in U266 cells

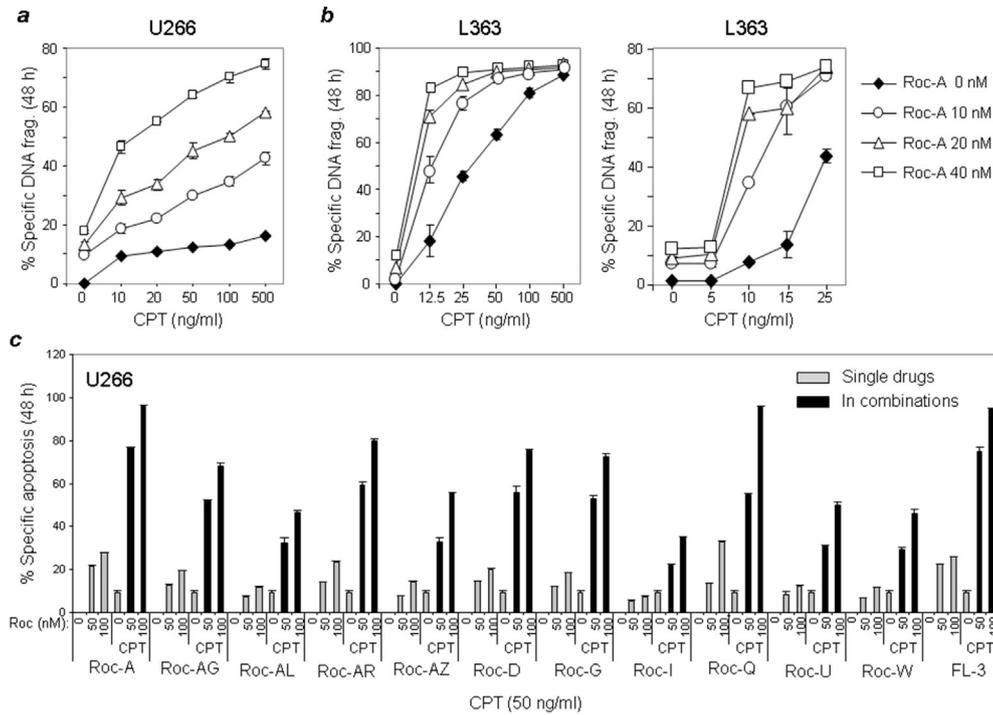
We have previously shown that Rocs are potent suppressors of c-FLIP expression in HTLV-1-associated ATL and Hodgkin's lymphoma cells [14,20]. Therefore, we asked whether Rocs could also overcome CPT-resistance in MM cells. To answer this question, we first tested the effect of Roc-A, the first Roc compound identified in 1982 [11,12], on U266 and L363. Cells were treated with an increasing concentrations of CPT in the absence or presence of different doses of Roc-A. U266 cells were highly resistant to CPT with only 10% cell death when treated with CPT alone even with a very high concentration (500 ng/ml), the concentration that killed almost 90% of L363 cells (Figs. 1a and 3a). Roc-A at the concentrations 10–40 nM induced also little cell death (10–20%). However, in combination of both drugs, Roc-A was shown to synergistically



**Fig. 1.** Most of primary tumor cells from MM patients show elevated levels of c-FLIP. a. The human MM cell lines U266 and L363 show different sensitivities to CPT treatment. Cells were treated with different concentrations of CPT as indicated for 48 h. Apoptotic cell death was determined by DNA fragmentation. Means  $\pm$  SD are shown. Results are representative of three independent experiments each performed in duplicate assays. b. Comparison of protein expression levels of caspase-8, -3, and c-FLIP in U266 and L363. Total cell lysates of U266 and L363 were subjected to Western blot analysis for expression levels of the proteins involved in receptor-mediated apoptosis. Actin was used for the loading control. Results are one of three independent experiments. c. Comparison of cell surface expression levels of the TRAIL receptors DR4 and DR5 in U266 and L363. Results are from one of two independent similar experiments. d. Analysis of c-FLIP and XIAP expression levels of MM patients samples. MM cells were isolated from seven patients and subjected to Western blot analysis for the expression levels of c-FLIP and XIAP. Actin was used as protein loading control.



**Fig. 2. Inhibition of c-FLIP expression is sufficient to enhance CPT-induced apoptosis.** a. Knockdown of c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> in U266. U266 cells were transfected with either siRNA specific for c-FLIP proteins or scrambled siRNA as described in Materials and Methods. Knockdown efficiency was controlled by Western blot analysis 24, 48 and 72 h after transfection. b. Knockdown of c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> enhances CPT-induced cell death. Cells 48 h after transfection with siRNA were subjected to CPT treatment for 24 h. Apoptotic cell death was determined by DNA fragmentation. Means ± SD are shown. The result is representative of two independent knockdown experiments.



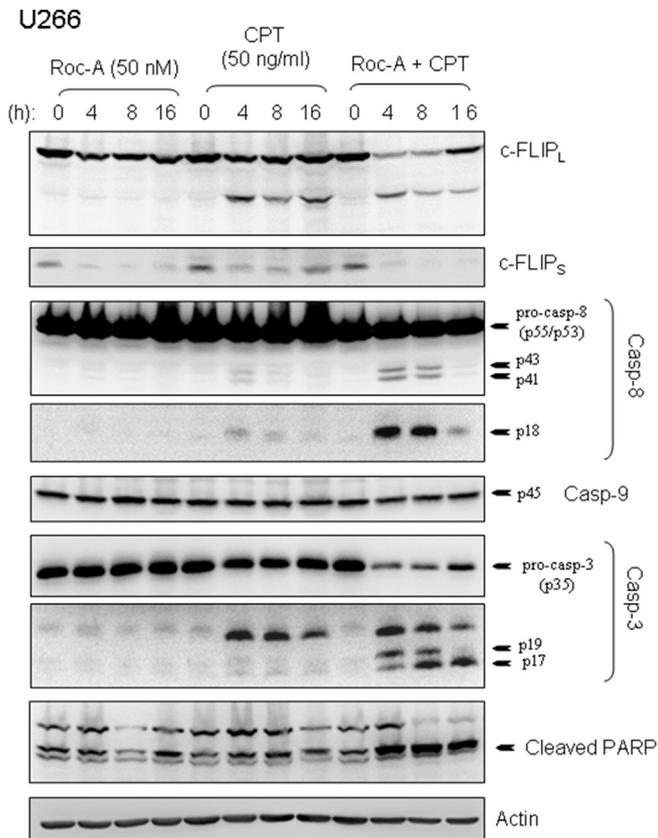
**Fig. 3. Rocs enhance CPT-induced apoptosis in U266 and L363 cells.** a. Roc-A overcomes CPT resistance in U266 cells. U266 cells were treated with different concentrations of CPT in the absence or presence of indicated concentrations of Roc-A. Apoptotic cell death was determined by DNA fragmentation after 48 h treatment. Means ± SD are shown. Results are representative of three independent experiments each performed in duplicate assays. b. Roc-A enhances CPT-induced apoptosis in L363 cells. L363 cells were treated with different concentrations of in the absence or presence of indicated concentrations of Roc-A as in A. Means ± SD are shown. Results are representative of two independent experiments each performed in duplicate assays. c. Overcome CPT resistance in U266 cells by different Rocs. U266 cells were treated with different concentrations of CPT in the absence or presence of indicated concentrations of different Roc derivatives for 48 h. Means ± SD are shown. Results are representative of two independent experiments each performed in duplicate assays.

enhance CPT-mediated cell death in a dose-dependent manner leading to almost 80% death in U266 cells (Fig. 3a). Synergistic effects were also observed in L363 cells treated by combinations of CPT and Roc-A (Fig. 3b).

To investigate whether other Rocs could also enhance the efficacy of CPT, we tested additional 11 Roc derivatives. All Rocs tested showed synergies with CPT to kill U266 cells (Fig. 3c). These experiments demonstrate that Rocs can enhance anticancer efficacy of CPT.

To investigate the mechanism by which Roc-A sensitizes CPT-mediated cell death, we carried out a kinetic analysis of the

status of proteins involved in receptor- and mitochondria-mediated cell death (Fig. 4). U266 cells were treated with Roc-A and CPT alone or in combination for 4, 8, and 16 h. The cell lysates were subjected to Western blot analysis. As shown in Fig. 4, Roc-A (50 nM) alone was not able to activate pro-caspases and to induce PARP cleavage up to 16 h of treatment. Although treatment with CPT (50 ng/ml) alone slightly activated caspase-8, the amount of activated caspase-8 (p18) was too little to further activate pro-caspase-3 to generate the active p17 caspase-3. CPT alone was also not sufficient to induce PARP cleavage. In contrast, the combination of Roc-A and CPT resulted in strong activation of caspase-8 which in turn activated



**Fig. 4. Kinetic analysis of the effects of combinations of Roc-A with CPT by Western blot.** U266 cells were treated with either Roc-A (50 nM) or CPT (50 ng/ml) alone or in combination of both for 2–16 h. The cell lysates were subjected to Western blot with antibodies against c-FLIP, caspase-8, 9, 3 and PARP. Actin was used as control for equal loading of proteins. Results shown are from one of two independent Western blots.

caspase-3 to generate the p19 and p17 cleavage products at 4–8 h and to completely activate caspase-3 (p17) at 16 h treatment. Consequently, PARP was completely cleaved at 8–16 h by the combination treatment (Fig. 4). No cleavage of caspase-9 was seen with the combination treatment indicating that the enhanced cell death was mainly through the receptor-mediated apoptosis pathway. The analysis also showed that strong inhibition of protein expression of c-FLIP<sub>L</sub>, in particular c-FLIP<sub>S</sub>, was associated with the enhanced caspase-8 activation (Fig. 4). The data demonstrate that suppression of c-FLIP expression led to sensitization of U266 to CPT-mediated cell death.

#### *In vivo* evaluation of the effect of CPT and Roc-A on MM

To explore the anti-tumor effect of combination therapy *in vivo*, we carried out an animal study.  $2.5 \times 10^7$  U266 cells each were implanted subcutaneously into immunodeficient mice. Two weeks after grafting, as small tumors were visible, mice were randomly separated into four groups (8 mice in each group) and were treated by intraperitoneal injection (i.p.) with vehicle (DMSO) or CPT (50 mg/kg) in the absence (DMSO) or presence of Roc-A (0.5 mg/kg) 5 times per week for 2 weeks and then further treated for 1 week on each second day. Tumor size was measured on days as indicated in Fig. 5a. The experiment showed that in the control group (DMSO), all 8 mice developed tumors. In the CPT treated group, 6 out of 8 mice showed delay in tumor growth for 26 days but developed tumors after stop of the treatment at the growth rate

similar to the control group. In the Roc-A group, 4 mice (50%) were shown absence of tumor growth. In contrast, treatment with CPT in combination with Roc-A resulted in inhibition of tumor growth in 6 tumor bearing mice (75%) (Fig. 5). This experiment demonstrates that combination of Roc-A with CPT significantly enhances efficacy of the therapy.

#### *In vivo* evaluation of the effect of CPT and Roc-A on leukemia

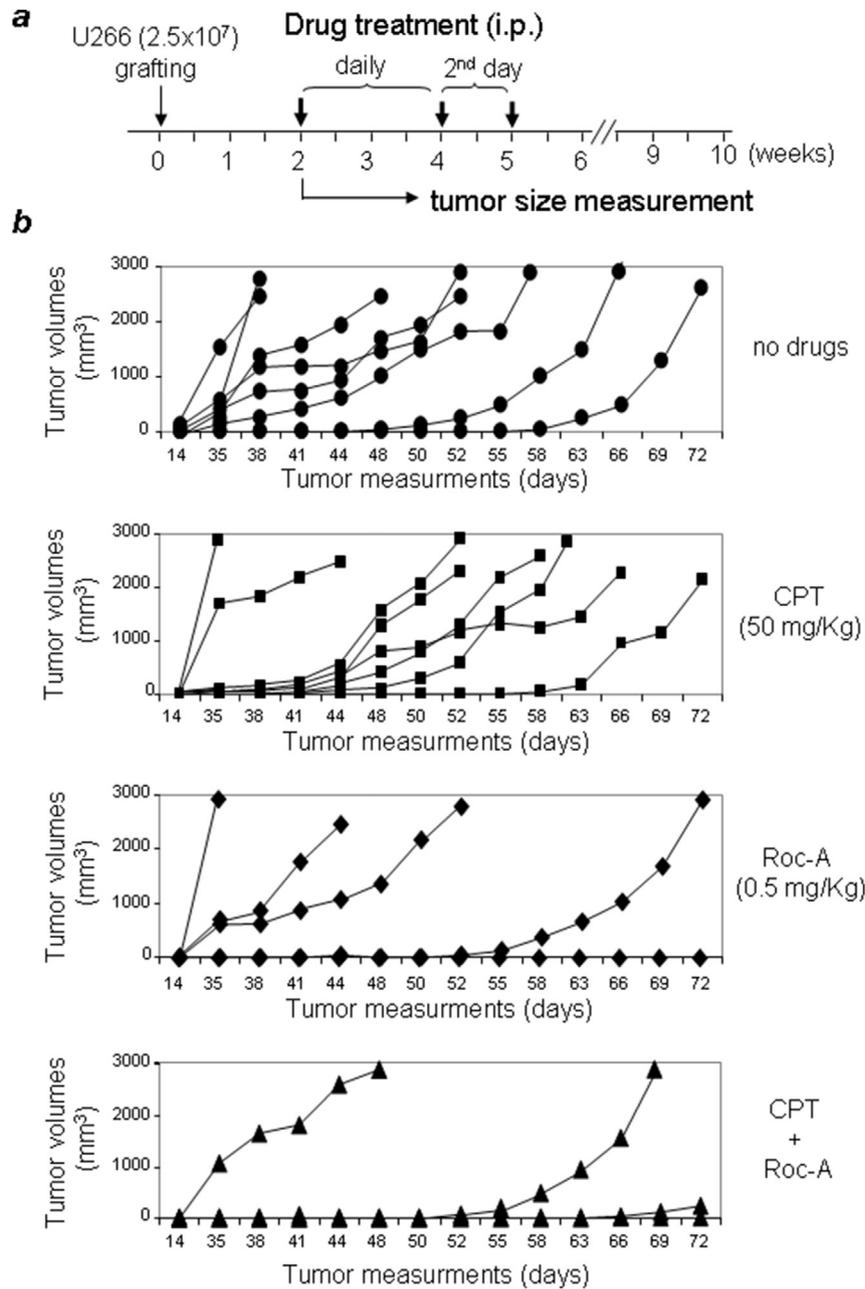
We further asked whether Roc-A could also improve TRAIL therapy in other types of hematopoietic malignancies. To investigate this question, we tested the effects of Roc-A and CPT on the human cutaneous T-cell lymphoma cell line SeAx and the human acute T-cell leukemia cell lines Jurkat, Sup-T1 and Molt-4. Treatment with CPT alone showed only 15–30% cell death in SeAx and Jurkat cells. Sup-T1 and Molt-4 cells were completely resistant to CPT treatment (Fig. 6a). Similar to MM cells, synergistic increases in apoptotic cell death were obtained by treatment with CPT in the presence of Roc-A in all four cell lines (Fig. 6a). Western blot analysis showed that down-regulation of c-FLIP expression correlated with increase in caspase-8 and 3 activities and enhanced cleavage of PARP (Fig. 6b). Thus, Roc-A can also enhance CPT-induced cell death in leukemia and lymphoma cells.

Although CPT and Roc-A alone are nontoxic to normal cells [6,13], the effect of combination of the two drugs on normal cells have not been examined. To investigate the tumor selectivity of the combination therapy, we examined the effect of CPT and Roc-A on proliferating normal blood T cells isolated from healthy donors. Consistent with previous data, no substantial cell death was seen by the combination of CPT and Roc-A in normal proliferating T cells (Supplementary Fig. S2). Thus, Roc-A does not enhance CPT toxicity in normal blood T cells.

To evaluate the effect of Roc-A and CPT on leukemia cells *in vivo*,  $5 \times 10^6$  of Molt-4 cells were grafted subcutaneously into mice. One week after xenografting, tumor-size-matched mice (approximately  $15 - 25 \text{ mm}^3$ ) were treated with vehicle (DMSO) or with CPT (50 mg/kg) in the presence or absence of Roc-A (0.5 mg/kg) (each group contains 7 mice) five times per week by i.p. injection as described in Fig. 7a (upper panel). After two weeks of treatment, the body weight and tumor weight were measured. The experiment showed that CPT alone had almost no inhibitory effect on tumor growth ( $P = 0.529$ ) (Fig. 7a and b). Treatment with Roc-A alone showed no significant reduction in tumor size but a substantial reduction in tumor weight ( $P = 0.040$ ) (Fig. 7a and b). In contrast, the combination of both drugs significantly reduced tumor size as well as tumor weight ( $P = 0.005$ ) (Fig. 7a and b). No body weight loss was seen in mice after treatment (Fig. 7c). The experiment demonstrates that Roc-A can also improve the efficacy of TRAIL-based anticancer therapy in leukemia.

#### Discussion

So far, MM is a malignancy incurable by the presently known therapies. Despite of the development of several classes of new drugs, the median survival following treatment has remained at about 5 years for the past decade [24,25]. Thus, it is urgent to find new strategies for MM treatment. TRAIL is a promising anticancer agent which kills various cancer cells with no toxicity to normal tissues. So far, many efforts have been made to improve TRAIL-R-targeting agents. Although CPT has a much higher anti-cancer activity than the original TRAIL *in vitro* in cell culture and also *in vivo* in animal tumor experiments [6], in clinical trials many MM patients remain resistant to CPT therapy [7,8,10]. Our current study aimed at exploring a rational approach to overcome CPT-resistance in MM.

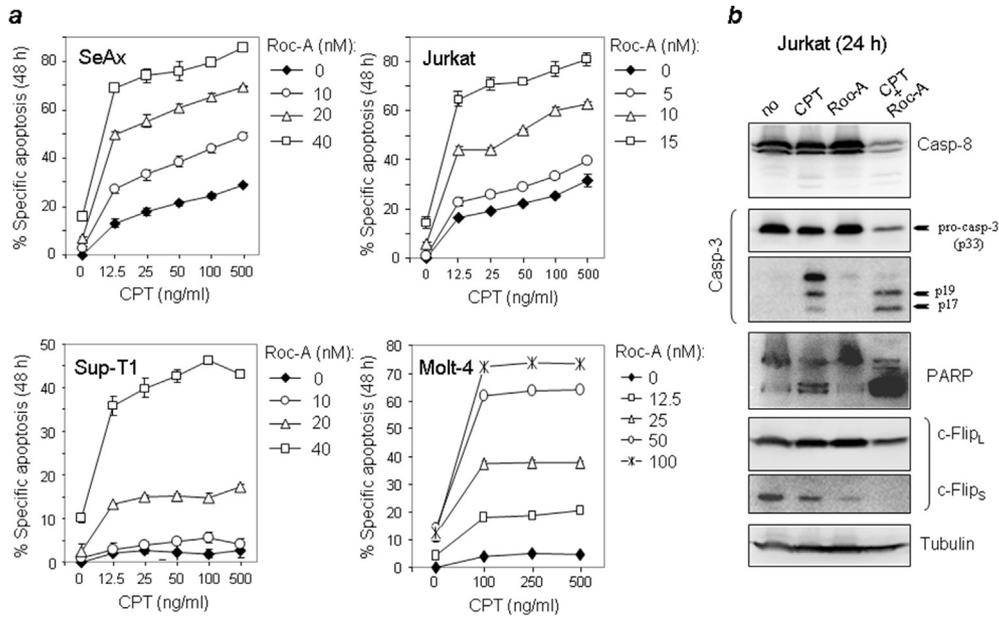


**Fig. 5.** *In vivo* study of combination treatment with Roc-A and CPT in mice grafted with U266 cells. a. U266 cells ( $2.5 \times 10^7$ ) each were implanted subcutaneously into immunodeficient mice. Two weeks after grafting, as small tumors were visible, mice were randomly separated into four groups (8 mice in each group) and were treated by intraperitoneal injection (i.p.) with vehicle (DMSO) or CPT (50 mg/kg) in the presence or absence of Roc-A (0.5 mg/kg) 5 times per week for 2 weeks and then further treated for 1 week on each second day. b. Tumor size was measured on days as indicated.

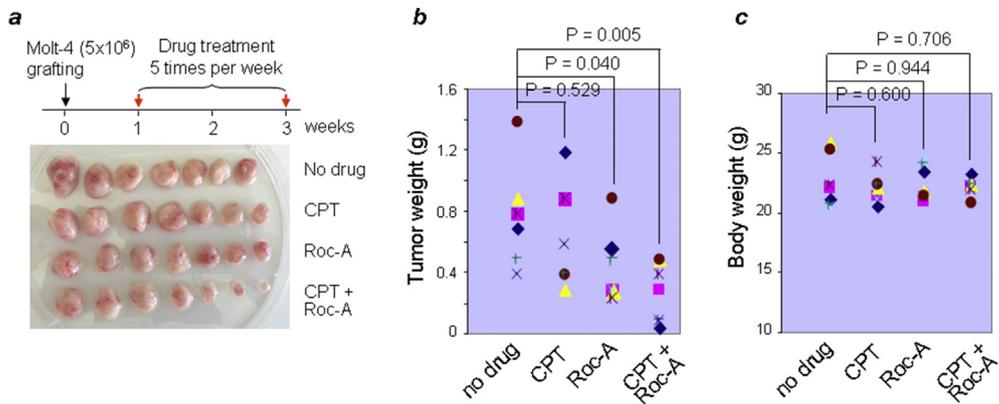
We and others have previously shown that c-FLIP expression levels play an essential role in regulation of sensitivity and resistance of cancer cells towards TRAIL-mediated apoptotic cell death [14,17,18,20]. Previously, an analysis of c-FLIP expression levels in five different MM cell lines including U266 showed that all cells express comparable amounts of c-FLIP<sub>L</sub> and low to undetectable levels of c-FLIP<sub>S</sub>. Based on this observation, the authors concluded that the levels of FLIP<sub>L</sub> were not predictive of the sensitivity of MM to TRAIL [35]. Therefore, we checked c-FLIP expression levels of primary MM cells and found that the majority of MM patient samples express elevated c-FLIP proteins (Fig. 1d). Knockdown of c-FLIP expression in the CPT-resistant U266 cells by c-FLIP specific siRNAs enhances CPT-mediated apoptosis (Fig. 2). This data

indicates that c-FLIP also plays an important role in TRAIL resistance in MM cells.

Recently, we have shown in *in vitro* cell culture systems that the anti-cancer phytochemical Rocs can break TRAIL-resistance in HTV-1 ATL and Hodgkin's lymphoma cells through inhibition of c-FLIP expression [14,20]. Rocs are inhibitors of translation initiation [14,20,26]. Since c-FLIP proteins have a very short half-life [27,28], *de novo* inhibition of protein synthesis would consequently lead to down-regulation of c-FLIP expression and thereby enhance TRAIL-mediated cell death [14,20]. In this study, we extend our studies to MM and T-ALL. Consistent with our previous studies, the experiments show that Rocs synergize with CPT to enhance apoptotic cell death in MM and T-ALL cells (Figs. 3 and 6). These data suggest that



**Fig. 6. Roc-A enhances CPT-induced apoptosis in different human leukemic T cells.** a. Roc-A enhances CPT-induced cell death in human T-ALL cell lines SeAx, Jurkat, Sup-T1 and Molt-4. Cells were treated with different concentrations of CPT in the absence or presence of indicated concentrations of Roc-A. Apoptotic cell death was determined by DNA fragmentation after 48 h treatment. Means  $\pm$  SD are shown. Results are representative of two independent experiments each performed in duplicate assays. b. Western blot analysis of the effect of combination treatment with Roc-A and CPT. Jurkat cells were treated with either Roc-A (50 nM) or CPT (50 ng/ml) alone or in combination of both for 24 h. The cell lysates were subjected to Western blot analysis with antibodies against c-FLIP, caspase-8, 3 and PARP. Tubulin was used as control for equal loading of proteins. Results are from one of two independent Western blots.



**Fig. 7. In vivo study of combination treatment with Roc-A and CPT in mice grafted with Jurkat cells.** a. Molt-4 cells ( $5 \times 10^6$ ) were grafted subcutaneously per mouse. One week after xenografting, tumor-size-matched mice (approximativ 15 – 25 mm<sup>3</sup>) were treated with vehicle or with CPT (50 mg/kg) in the presence or absence of Roc-A (0.5 mg/kg) (each group contains seven mice) five times per week by i.p. injection as described in the upper panel. After two weeks of treatment, mice were sacrificed. Photographs of the tumors are shown in the lower panel. b. The tumor weights. P values were 0.529, 0.040 and 0.005 for CPT, Roc-A and Roc-A plus CPT, respectively. c. After two weeks of treatment, the body weight was measured. P values show no significance.

combinations of Rocs and CPT may be a rational strategy for cancer treatment.

So far, the effects of the combination of TRAIL and Rocs on cancer cells have not been tested *in vivo*. Therefore, we carried out animal experiments and xenografted mice with the human MM U266 and the human T-ALL Molt-4 cells, respectively. U266 and Molt-4 cells were resistant to CPT-mediated killing as shown in the apoptosis assays (Figs. 1a and 6a). Consistent with the *in vitro* data, the *in vivo* mouse experiments showed that treatment with CPT alone was not able to kill the tumor cells. The U266 tumor mouse experiment showed that CPT treatment for three weeks significantly delayed tumor growth. However, when treatment was stopped, tumors grew up with a similar rate as in the non-treated control group (Fig. 5b). This result may reflect the situation in

CPT clinical trials. Treatment with CPT alone in the T-ALL Molt-4 mouse tumor model also showed no statistical significance in reduction of tumor weight ( $p = 0.529$ ) (Fig. 7b). Natural Rocs have been shown to inhibit growth of several tumor cell lines *in vivo* in animal models including the mouse leukemia p388 [29], the mouse RAM lymphoma RMA [30], the human oral epidermis cancer KB [31], and the human breast cancer BC1 and MDA-MB-231 [32,33]. In our mouse experiments, treatment with Roc-A alone for two weeks in the T-ALL Molt-4 model showed a reduction in tumor weight ( $p = 0.040$ ) (Fig. 7b). Also, treatment with Roc-A alone for three weeks resulted in tumor free mice (50%) grafted with U266 cells at the end of the experiment (10 weeks) (Fig. 5b). Significantly, treatment with a combination of CPT and Roc-A further reduced tumor weights ( $p = 0.005$ ) in the T-ALL Molt-4 mouse model and in

the MM U266 mouse model 75% of mice were tumor free (Figs. 5b and 7b). Importantly, there were no influence in body weight on mice in the combination treatment in the T-ALL Molt-4 mouse model ( $p = 0.706$ ) (Fig. 7c). There was also no loss of body weight in the MM U266 mouse model controlled on the day the mice were sacrificed (data not shown). Roc-A has no obvious toxicity up to a dose of 10 mg/kg body weight [32]. Acute toxicity of Roc-A was reported with an LD<sub>50</sub> of >300 mg/kg [34]. The mouse experiments demonstrate that Rocs can be used to potentiate the anti-cancer activity of CPT and to increase the efficacy of cancer treatment.

In summary, we demonstrate that Rocs can break TRAIL resistance in MM and T-ALL *in vivo*. Our study raises the possibility to develop Rocs as a TRAIL adjuvant for the treatment of hematopoietic malignancies.

### Acknowledgements

This work was supported in part by the Beijing Health System High Level Health Technical Personnel Training Plan 2013-2-007. We thank Proksch P for providing different Roc derivatives and Désaubry L for providing FL3.

### Conflicts of interest

The authors have no conflicts of interest to disclose.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.canlet.2016.12.010>.

### References

- [1] R.W. Johnstone, A.J. Frew, M.J. Smyth, The TRAIL apoptotic pathway in cancer onset, progression and therapy, *Nat. Rev. Cancer* 8 (2008) 782–798.
- [2] R.S. Herbst, S.G. Eckhardt, R. Kurzrock, S. Ebbinghaus, P.J. O'Dwyer, M.S. Gordon, Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic agonist, in patients with advanced cancer, *J. Clin. Oncol.* 28 (2010) 2839–2846.
- [3] J.C. Soria, E. Smit, D. Khayat, B. Besse, X. Yang, C.P. Hsu, et al., Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer, *J. Clin. Oncol.* 28 (2010) 1527–1533.
- [4] D. de Miguel, J. Lemke, A. Anel, H. Walczak, L. Martinez-Lostao, Onto better TRAILs for cancer treatment, *Cell Death Differ.* 23 (2016) 733–747.
- [5] D. Mahalingam, E. Szegezdi, M. Keane, S. de Jong, A. Samali, TRAIL receptor signalling and modulation: are we on the right TRAIL? *Cancer Treat. Rev.* 35 (2009) 280–288.
- [6] F. Fang, A.P. Wang, S.F. Yang, Antitumor activity of a novel recombinant mutant human tumor necrosis factor-related apoptosis-inducing ligand, *Acta Pharmacol. Sin.* 26 (2005) 1373–1381.
- [7] W.M. Chen, L.G. Qiu, J. Hou, X. Zhang, X. Ke, Z. Wang, et al., Phase Ib study of recombinant circularly permuted TRAIL (CPT) in relapsed or refractory multiple myeloma patients, *Blood (ASH Annual Meeting Abstracts)* 120 (2012) 1857.
- [8] W.M. Chen, L.G. Qiu, J. Hou, Y. Zhao, L. Pan, S. Yang, et al., Recombinant circularly permuted TRAIL (CPT) for the treatment of relapsed or refractory multiple myeloma: an open-label, multicenter phase II clinical trial, *Blood (ASH Annual Meeting Abstracts)* 120 (2012) 78.
- [9] W.M. Kuehl, P.L. Bergsagel, Molecular pathogenesis of multiple myeloma and its premalignant precursor, *J. Clin. Invest.* 122 (2012) 3456–3463.
- [10] C. Geng, J. Hou, Y. Zhao, X. Ke, Z. Wang, L. Qiu, et al., A multicenter, open-label phase II study of recombinant CPT (Circularly Permuted TRAIL) plus thalidomide in patients with relapsed and refractory multiple myeloma, *Am. J. Hematol.* 89 (2014) 1037–1042.
- [11] S.S. Ebada, N. Lajkiewicz, J.A. Porco Jr., M. Li-Weber, P. Proksch, Chemistry and biology of rocaglamides (= flavaglines) and related derivatives from aglaia species (meliaceae), *Prog. Chem. Org. Nat. Prod.* 94 (2011) 1–58.
- [12] M. Li-Weber, Molecular mechanisms and anti-cancer aspects of the medicinal phytochemicals rocaglamides (=flavaglines), *Int. J. Cancer.* 137(201) 1791–1799.
- [13] J.Y. Zhu, I.N. Lavrik, U. Mahlknecht, M. Gaiasi, P. Proksch, P.H. Krammer, et al., The traditional Chinese herbal compound rocaglamide preferentially induces apoptosis in leukemia cells by modulation of mitogen-activated protein kinase activities, *Int. J. Cancer* 121 (2007) 1839–1846.
- [14] M. Bleumink, R. Köhler, M. Gaiasi, P. Proksch, P.H. Krammer, M. Li-Weber, Rocaglamide breaks TRAIL resistance in HTLV-1-associated adult T-cell leukemia/lymphoma by translational suppression of c-FLIP expression, *Cell Death Differ.* 18 (2011) 362–370.
- [15] G. Polier, J. Neumann, F. Thuaud, N. Ribeiro, C. Gelhaus, H. Schmidt, et al., The natural anticancer compounds rocaglamides inhibit the Raf-MEK-ERK pathway by targeting prohibitin 1 and 2, *Chem. Biol.* 19 (2012) 1093–1104.
- [16] G. Polier, M. Gaiasi, R. Köhler, W.W.M. üller, C. Lutz, E.C. Buss, et al., Targeting CDK9 by wogonin and related natural flavones potentiates the anti-cancer efficacy of the Bcl-2 family inhibitor ABT-263, *Int. J. Cancer* 136 (2015) 688–698.
- [17] S. Mathas, A. Lietz, I. Anagnostopoulos, F. Hummel, B. Wiesner, M. Janz, et al., c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis, *J. Exp. Med.* 199 (2004) 1041–1052.
- [18] X. Zhang, T.G. Jin, H. Yang, W.C. DeWolf, R. Khosravi-Far, A.F. Olumi, Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer, *Cancer Res.* 64 (2004) 7086–7091.
- [19] J.K. Yang, FLIP as an anti-cancer therapeutic target, *Yonsei Med. J.* 49 (2008) 19–27.
- [20] M. Gaiasi, R. Köhler, S. Fulda, P.H. Krammer, M. Li-Weber, Rocaglamide and a XIAP inhibitor cooperatively sensitize TRAIL-mediated apoptosis in Hodgkin's lymphomas, *Int. J. Cancer* 131 (2012) 1003–1008.
- [21] F. Zhan, J. Hardin, B. Kordsmeier, K. Bumm, M. Zheng, E. Tian, et al., Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells, *Blood* 99 (2002) 1745–1757.
- [22] C. Scaffidi, J.P. Medema, P.H. Krammer, M.E. Peter, FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b, *J. Biol. Chem.* 272 (1997) 26953–26958.
- [23] C. Scaffidi, I. Schmitz, P.H. Krammer, M.E. Peter, The role of c-FLIP in modulation of CD95-induced apoptosis, *J. Biol. Chem.* 274 (1999) 1541–1548.
- [24] A. Mahindra, J. Laubach, N. Raje, N. Munshi, P.G. Richardson, K. Anderson, Latest advances and current challenges in the treatment of multiple myeloma, *Nat. Rev. Clin. Oncol.* 9 (2012) 135–143.
- [25] L. Naymagon, M. Abdul-Hay, Novel agents in the treatment of multiple myeloma: a review about the future, *J. Hematol. Oncol.* 9 (2016) 52.
- [26] M.E. Bordeleau, F. Robert, B. Gerard, L. Lindqvist, S.M. Chen, H.G. Wendel, et al., Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model, *J. Clin. Invest.* 118 (2008) 2651–2660.
- [27] L. Chang, H. Kamata, G. Solinas, J.L. Luo, S. Maeda, K. Venuprasad, et al., The E3 ubiquitin ligase itch couples JNK activation to TNF $\alpha$ -induced cell death by inducing c-FLIP(L) turnover, *Cell* 124 (2006) 601–613.
- [28] M. Gao, M. Karin, Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli, *Mol. Cell* 19 (2005) 581–593.
- [29] M.L. King, C.-C. Chiang, H.-C. Ling, E. Fujita, M. Ochiai, A.T. McPhail, X-ray crystal structure of rocaglamide, a novel antileukemic 1H-cyclopenta[b] benzofuran from *Aglaia elliptifolia*, *J. Chem. Soc. Chem. Commun.* (1982) 1150–1151.
- [30] J.Y. Zhu, M. Gaiasi, R. Köhler, M.M. Müller, A. Mühleisen, P. Proksch, et al., Rocaglamide sensitizes leukemic T cells to activation-induced cell death by differential regulation of CD95L and c-FLIP expression, *Cell Death Differ.* 16 (2009) 1289–1299.
- [31] B.Y. Hwang, B.-N. Su, H. Chai, Q. Mi, L.B. Kardono, J.J. Afriastini, et al., Silvestrol and episilvestrol, potential anticancer rocaglate derivatives from *Aglaia silvestris*, *J. Org. Chem.* 69 (2004) 3350–3358.
- [32] S.K. Lee, B. Cui, R.R. Mehta, A.D. Kinghorn, J.M. Pezzuto, Cytostatic mechanism and antitumor potential of novel 1H-cyclopenta[b]benzofuran lignans isolated from *Aglaia elliptica*, *Chem. Biol. Interact.* 115 (1998) 215–218.
- [33] R. Cencic, M. Carrier, G. Galicia-Vázquez, M.E. Bordeleau, R. Sukarieh, A. Bourdeau, et al., Antitumor activity and mechanism of action of the cyclopenta[b]benzofuran, silvestrol, *PLoS One* 4 (2009) e5223.
- [34] K. Umezawa, T. Yamamoto, T. Koyano, Japanese Patent 9067375, (1997).
- [35] M. Gómez-Benito, M.J. Martínez-Lorenzo, A. Anel, I. Marzo, J. Naval, Membrane expression of DR4, DR5 and caspase-8 levels, but not Mcl-1, determine sensitivity of human myeloma cells to Apo2L/TRAIL, *Exp Cell Res.* 313 (2007) 2378–2388.