

# Apoptosis of t(14;18)-positive lymphoma cells by a Bcl-2 interacting small molecule

David R. Abbott · Robert T. Abbott ·  
Stephen D. Jenson · G. Chris Fillmore ·  
Kojo S. J. Elenitoba-Johnson · Megan S. Lim

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**Abstract** Overexpression of Bcl-2 protein occurs via both t(14;18)-dependent and independent mechanisms and contributes to the survival and chemoresistance of non-Hodgkin lymphomas. HA14-1 is a nonpeptidic organic small molecule, which has been shown to inhibit the interaction of Bcl-2 with Bax, thereby interfering with the antiapoptotic function of Bcl-2. In this study, we sought to determine the *in vitro* efficacy of HA14-1 as a therapeutic agent for non-Hodgkin lymphomas expressing Bcl-2. Assessment of cell viability demonstrated that HA14-1 induced a dose- ( $IC_{50}=10\ \mu\text{M}$ ) and time-dependent growth inhibition of a cell line (SudHL-4) derived from a t(14;18)-positive, Bcl-2-positive, non-Hodgkin lymphoma. HA14-1 effectively induced apoptosis via a caspase 3-mediated pathway but did not affect either the p38 MAPK or p44/42 MAPK pathways. Western blot analyses of Bcl-2 family proteins and other cell cycle-associated proteins were performed to determine the molecular sequelae of HA14-1-induced apoptosis. The results show down-regulation of Mcl-1 but up-regulation of p27<sup>kip1</sup>, Bad, Bcl-xL, and Bcl-2 proteins, without change in Bax levels during HA14-1-mediated apoptosis. Our findings further elucidate the cellular mechanisms accompanying Bcl-2 inhibition and demonstrate the potential of Bcl-2 inhibitors as therapeutic agents for the treatment of non-Hodgkin lymphomas.

**Keywords** Follicular lymphoma · Bcl-2 · HA14-1 · Antiapoptosis · Small molecule inhibitor

D. R. Abbott · R. T. Abbott · S. D. Jenson · G. C. Fillmore ·  
K. S. J. Elenitoba-Johnson · M. S. Lim (✉)  
Department of Pathology,  
University of Michigan Medical School,  
M5242 Medical Science 1, 1301 Catherine,  
Ann Arbor, MI 48109-0602, USA  
e-mail: meganlim@umich.edu

## Introduction

The B-cell lymphoma-2 gene product (Bcl-2), a key player in the regulation of cellular apoptosis, is overexpressed in nearly 60% of all non-Hodgkin lymphomas (NHL) [1, 2]. Bcl-2, the prototypic antiapoptotic member of the Bcl-2 protein family, is overexpressed as a result of chromosomal translocation (14;18) [3–5] and/or as a consequence of transcriptional up-regulation in NHLs. The Bcl-2 oncoprotein exerts its effects by inhibiting apoptosis in cells that are normally bound for death [3, 6, 7]. Studies have shown that Bcl-2 can inhibit cell death from a broad array of apoptotic stimuli, including radiation [8, 9], chemotherapy drugs [10–12], and growth factor withdrawal [8, 13]. These characteristics are directly linked to resistance of many forms of conventional cancer therapies.

Efforts to target Bcl-2 overexpression have focused on antisense therapies [14, 15]. Other strategies are being investigated to disrupt the overexpression of Bcl-2. One such approach utilizes the properties of small molecules to antagonize the function of the target protein. HA14-1 [ethyl 2-amino-6-bromo-4(1-cyano-2ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate] is a nonpeptidic organic small molecule that binds to the BH3 binding pocket of Bcl-2, effectively competing with Bak BH3 for interaction with Bcl-2 [16]. The interference of HA14-1 with the surface pocket of Bcl-2 has been shown to successfully impede the function of Bcl-2 and reduce cell viability and induce apoptosis of human acute myeloid leukemia (HL-60) cells [16] and lymphoma cells [17] that overexpress Bcl-2. Its mechanism of action is diverse and has been shown to initiate autophagy [18], reconstitute impaired mitochondrial pathway of apoptosis in renal cell carcinoma cell lines [19], and sensitize tumor cells to immune-mediated destruction [20]. Other reports have shown that HA14-1 has synergis-

tic effects with MEK/MAPK in overcoming the aggressive apoptosis-resistant phenotype of acute myeloid leukemia cells [21]. It has also been observed that sequential exposure of myeloma cells to proteasome inhibitors, followed by treatment of HA14–1, results in a substantial increase in apoptotic effects [22] via the translocation of Bax from the cytosol to the mitochondria, thus enhancing cytochrome release to the cytosol [23]. It has also been shown that HA14–1 interacts synergistically with cyclin-dependent kinase inhibitors to induce mitochondrial injury and apoptosis in myeloma cells through a free radical-dependent and Jun NH<sub>2</sub>-terminal kinase-dependent mechanism [24].

In this study, we have examined the effect of HA14–1 on the viability of cell lines derived from mature B-cell NHLs, the mechanism of growth inhibition, and its effect on key cell cycle-associated proteins. Our results suggest that inhibitors of Bcl-2 may have utility in the treatment of patients with NHLs.

## Materials and methods

### Cell culture and drug treatment

The SudHL-4 [t(14;18)+;Bcl-2+] and SudHL-5 [t(14;18)-;Bcl-2-] cell lines were obtained from ATCC (Manassas, VA, USA) and grown in RPMI 1640 (ISC Bioexpress, Kaysville, UT, USA) supplemented with 4.5 g/L glucose, 9.5 g/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 1.6 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 10% heat-inactivated fetal bovine serum, and 100 U/ml of penicillin–streptomycin mixture (Gibco Life Technologies, Grand Island, NY, USA). Each cell line was incubated with varying concentration of HA14–1 (Maybridge Chemical Company, Trevillet, UK, and Calbiochem, La Jolla, CA, USA) dissolved in dimethyl sulfoxide (DMSO). The initial concentration of each cell line was  $1 \times 10^5$  cells per milliliter. The cells were harvested at various time points for viability assays, proliferation assay, apoptosis analysis, and lysate preparation. Separate control cells were sustained under identical conditions as the treated cells and were exposed to equal volumes of DMSO diluent.

### Cell viability and proliferation

Cell viability was assessed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay] (MTT) assay [25]. The desired standard of cell viability at the initiation of each experiment was 90% for each cell line. Briefly, 0.1 ml cells ( $0.5 \times 10^5$  cells) per well were seeded in a 96-well plate. MTT (10 mg/ml, 10  $\mu$ l/well; Sigma, St. Louis, MO, USA) was added to each well and incubated at 37°C for 2 h. Cells were then lysed by adding 0.1 ml per well of lysis

solution (20% sodium dodecyl sulfate, 50% *N,N*-dimethyl formamide, pH 4.7). Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. All MTT assays were performed in triplicate.

### Caspase-3 activity assay

Caspase-3 activity (CaspACE Assay System, Colormetric, Promega, Madison, WI, USA) measurements were conducted on cells grown under the previously stated conditions at a concentration of  $10^5$  cells per milliliter. HA14–1 was added to SudHL-4 cells to create a positive (induced apoptosis) control. Z-VAD-FMK inhibitor was used at a final concentration of 50  $\mu$ M. A negative control was prepared using untreated cells. Following a 23-h incubation period, the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) and resuspended in cell lysis buffer at a concentration of  $10^8$  cells per milliliter. The cells were lysed by freezing and thawing and then incubated on ice for 15 min. The cell lysates were centrifuged at  $15,000 \times g$  for 20 min at 4°C and the supernatant fraction was collected for use as cell extract. Replicate wells were prepared according to the CaspACE assay system protocol and then incubated for 4 h at 37°C. The absorbance was measured at 405 nm using an ELISA reader.

### Cell lysate preparation

Cells were collected and washed twice with cold PBS and then resuspended in cell lysis buffer at a concentration of  $10^7$  cells per milliliter. The cells were lysed by sonication. Protein concentrations of cell extracts were measured using the Bradford protein assay (BioRad Protein Assay, Hercules, CA, USA).

### Western blot analysis

Forty micrograms of protein from the prepared lysates was resolved in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel using a BioRad minigel system. Separated proteins were then transferred onto a nitrocellulose film using a semidry transfer apparatus (BioRad). The antibody dilutions and sources used for immunoblot analysis are shown in Table 1. Protein bands were visualized using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Densitometric analysis

Densitometric analyses of Western blots were performed using ImageMaster Total Lab v1.11 (Amersham Pharmacia Biotech, Uppsala, Sweden). p38 MAPK was used as loading control for normalization.

**Table 1** List of antibodies used for the study

Antibody	Source	Dilution	Company
Bcl-2	Mouse mono	(1:1,000)	Santa Cruz Biotechnology
Bcl-xl	Rabbit poly	(1:1,000)	Upstate Biotechnology, Billerica, MA, USA
p38	Mouse mono	(1:1,000)	Cell Signaling Technology, Danvers, MA, USA
Bax	Rabbit poly	(1:1,000)	Upstate Biotechnology
Mcl-1	Mouse mono	(1:1,000)	Santa Cruz Biotechnology
p27	Mouse mono	(1:1,000)	Santa Cruz Biotechnology
Bad	Mouse mono	(1:1,000)	Cell Signaling Technology
Caspase 3	Mouse mono	(1:1,000)	Cell Signaling Technology
Phospho p44/42	Mouse mono	(1:1,000)	Cell Signaling Technology
p44/42	Mouse mono	(1:1,000)	Cell Signaling Technology
Phospho H2A	Mouse mono	(1:1,000)	Upstate Biotechnology
Parp	Mouse mono	(1:1,000)	Upstate Biotechnology
Phospho p38	Mouse mono	(1:1,000)	Cell Signaling Technology
Anti-Mouse IgG	Donkey	(1:1,000)	Cell Signaling Technology
Anti-Rabbit IgG	Goat	(1:1,000)	Cell Signaling Technology

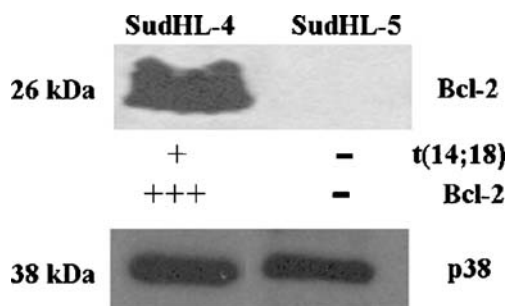
**Statistical analysis**

Statistical calculations were performed for MTT assay to determine standard error of mean representing three separate experiments done in triplicate. Standard error of mean was calculated using Excel software (Microsoft, Redmond, WA, USA).

**Results**

**HA14-1 decreases cell viability**

To examine the effects of HA14-1 on the viability of NHLs, we initially evaluated two cell lines with varying Bcl-2 expression: SudHL-4 (Bcl-2 positive) and SudHL-5 (Bcl-2 negative) (Fig. 1). Preliminary experiments using MTT assay showed that SudHL-4 cells demonstrated a

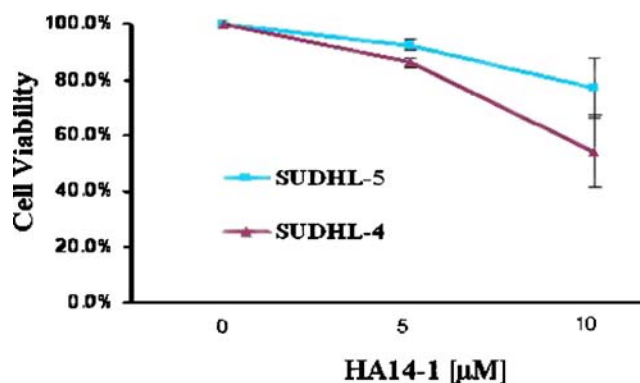


**Fig. 1** Basal Bcl-2 expression levels of two NHL cell lines visualized by Western blot analysis as described in the “Materials and methods” section. The level of Bcl-2 expression is high in SudHL-4 cells and negligible in SudHL-5 cells with equivalent protein loading determined by BCA protein concentration assay. p38 was used as loading control

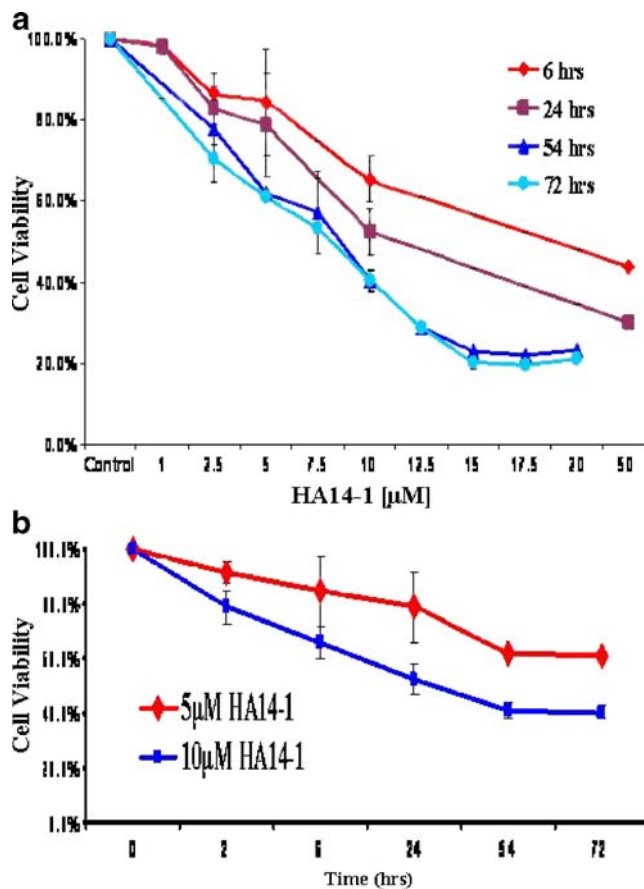
significant decrease in cell viability in response to HA14-1, while the SudHL-5 were less sensitive. After a 24-h treatment with 10 μM HA14-1, SudHL-5 cells demonstrated cell viability of 80%, where as SudHL-4 cells showed a significant reduction in viability at approximately 50% compared to control (Fig. 2). For subsequent experiments, we focused our studies on SudHL-4 cells.

SudHL-4 cells were treated with increasing concentrations of HA14-1 for variable time points. HA14-1 decreased the viability of SudHL-4 cells in a dose-dependent manner (Fig. 3a), with an IC<sub>50</sub> of approximately 10 μM at 24 h. A notable reduction in cell viability (10% of control) occurred at 2.5 μM at 6 h. Cell viability decreased with increasing doses of HA14-1 of up to 15 μM, after which the response leveled off to 20–40% of the control.

We also measured the viability of the cells treated with 5 and 10 μM of HA14-1 over a period of 72 h. HA14-1



**Fig. 2** Cell viability measured by MTT assay of two NHL cell lines treated with HA14-1. Each cell line was exposed to 5 and 10 μM of the drug and analyzed after 24 h. The data represent the mean ± standard error of mean of triplicate measurements. Similar data was observed in two additional independent experiments



**Fig. 3** **a** Cell viability of SudHL-4 cells treated with various concentrations of HA14-1, measured at variable time points by MTT assay. Cells were exposed to increasing concentrations of HA14-1 (1–50 µM) and viability evaluated at 6, 24, 54, and 72 h. The data represent the mean ± standard error of mean of triplicate measurements. **b** Cell viability of SudHL-4 cells treated with HA14-1 measured at various time points by MTT assay. Cells were exposed to 5 and 10 µM HA14-1 and viability evaluated at various time points. The data represent the mean ± standard error of mean of triplicate measurements

resulted in a time-dependent decrease on the viability SudHL-4 cells. At both concentrations, a decrease in cell viability was seen as early as 6 h. The 10 µM dose consistently had a twofold effect on the cells over the 5-µM dose. Cells treated with 5 µM HA14-1 showed maximal growth inhibition at 54 h with viability approximately 60% of control, while cells treated with 10 µM HA14-1 showed maximal growth inhibition at 54 h with viability approximately 40% of control (Fig. 3b).

#### HA14-1 induces caspase-3-mediated apoptosis

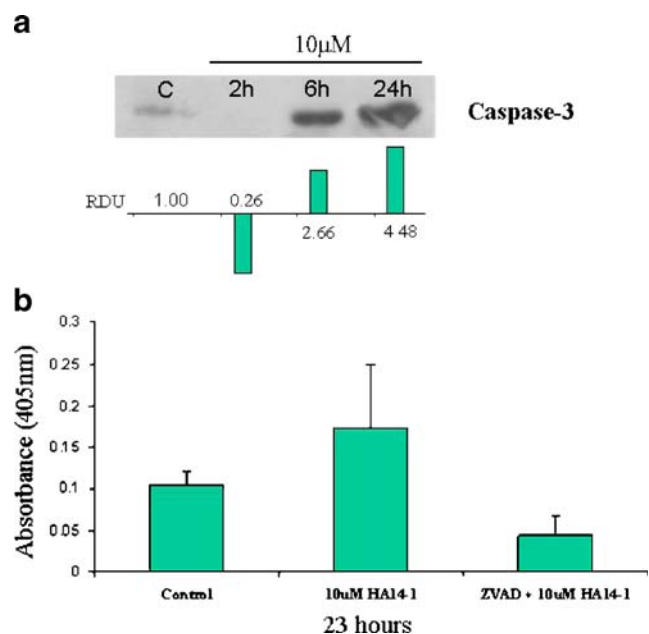
To determine the mechanism of HA14-1-mediated decrease in cell viability, we performed Western blot analysis of drug-treated SudHL-4 cells and evaluated the expression of caspase-3. We observed a significant increase in full-length

caspase-3 protein levels at increasing concentrations and duration of drug exposure.

Densitometric analysis of the Western blot showed a 2.6-fold increase in caspase-3 levels after 6 h, and a 4.5-fold increase after 24 h, in cells treated with 10 µM HA14-1 (Fig. 4a). Caspase-3 activity was also measured using the CaspACE Assay System (Fig. 4b). We observed a 1.6-fold increase in caspase-3 activity after 23 h compared to control, which was abrogated by the pan-caspase inhibitor Z-VAD-FMK.

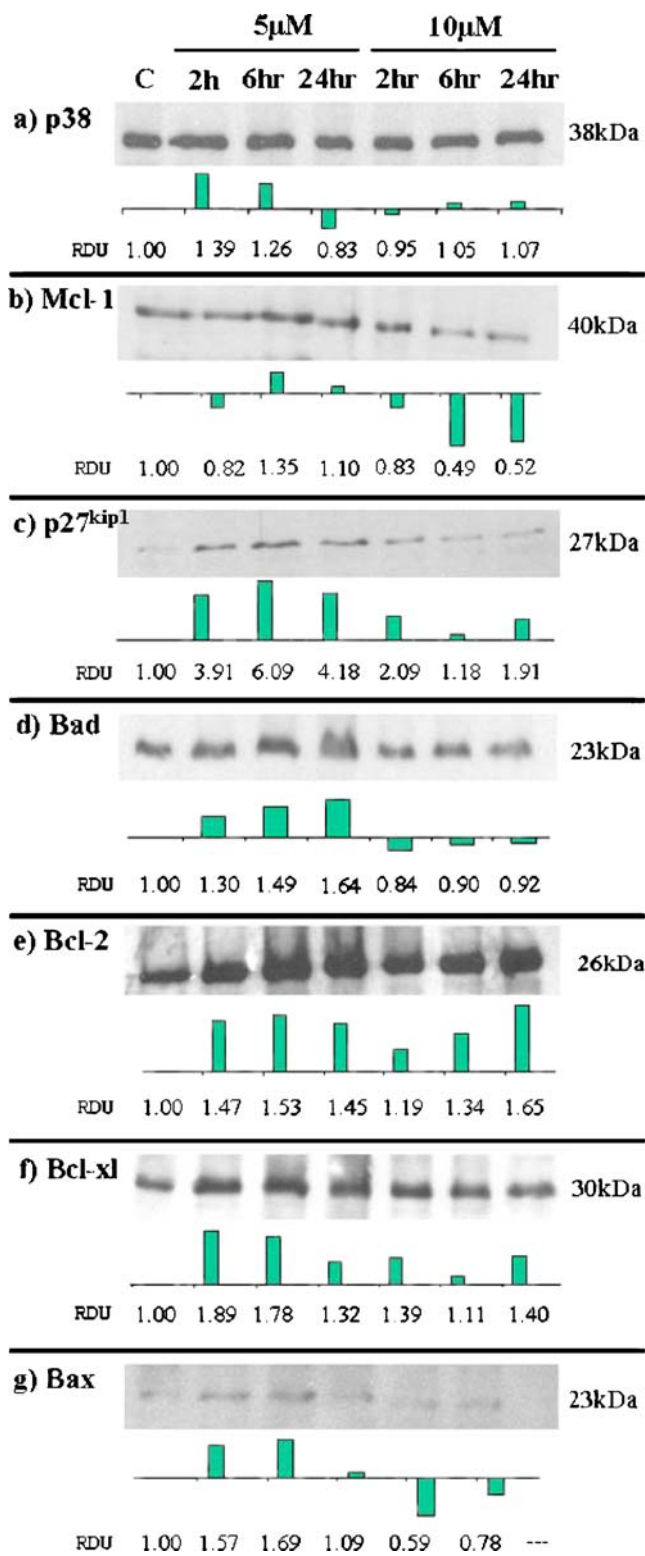
#### Effect of HA14-1 on cell cycle and signaling proteins

Western blot analyses of Bcl-2 family proteins, and other cell cycle associated proteins, were performed to determine the molecular sequelae of HA14-1-induced apoptosis. There was minimal change in the levels of either phospho-p38 or phospho-p44/42, demonstrating that HA14-1 did not affect p38 MAPK or p44/42 MAPK pathways. From our experiments and other reports [26, 27],



**Fig. 4** **a** Western blot analysis of caspase-3 expression in HA14-1 treated SudHL-4 cells. SudHL-4 cells were exposed to 10 µM of HA14-1 for 2, 6, and 24 h and expression of caspase-3 evaluated by Western blot analysis as described in the “Materials and methods” section with equivalent protein loading determined by BCA protein concentration assay. Densitometric analysis was performed to determine relative expression of caspase-3 compared to control (C) and represented in relative densitometric units (RDU). **b** Caspase activity of HA14-1-treated SudHL-4 cells measured by the CaspACE Assay System. SudHL-4 cells were exposed to 10 µM of HA14-1 for 23 h and caspase-3 activity was evaluated. A pan-caspase inhibitor Z-VAD-FMK was used to inhibit caspase-3 induction. The data represent the mean ± standard error of mean of triplicate measurements. Induction of caspase-3 activity observed at 23 h with 10 µM HA14-1 was increased by 1.6-fold, but was not statistically significant





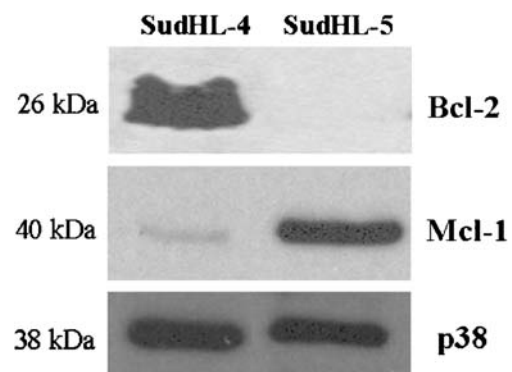
p38 was shown to have consistent expression regardless of exposure; thus, Western blots for p38 MAPK were used as protein loading control (Fig. 5). In contrast, a twofold down-regulation of Mcl-1 was seen when cells were treated with 10 μM HA14-1 as early as 6 h, which was sustained

◀ **Fig. 5** Expressions of Bcl-2 family and cell cycle-associated proteins in response to HA14-1. SudHL-4 cells were exposed to 5 and 10 μM HA14-1 and total cell lysates were collected at 2, 6, and 24 h. The expression of **a** p38, **b** Mcl-1, **c** p27<sup>kip1</sup>, **d** Bad, **e** Bcl-2, **f** Bcl-xl, and **g** Bax were determined by Western blot analysis, as described in the “Materials and methods” section, using appropriate antibodies listed in Table 1, with equivalent protein loading determined by BCA protein concentration assay. Densitometric analysis was performed to determine relative expression of the proteins compared to control (C) and represented in relative densitometric units (RDU)

through 24 h (Fig. 5). Our results also show a sixfold up-regulation of p27<sup>kip1</sup> at concentrations of 5 μM spanning from 2 to 24 h, but less significant up-regulation was observed at 10 μM (Fig. 5). Bad also demonstrated an increase in expression at 5 μM, with relatively minimal change seen at 10 μM (Fig. 5). Bcl-xl and Bcl-2 proteins both showed significant increase in expression throughout all concentrations of drug treatment (Fig. 5). Bax levels fluctuated throughout the treatments, with a slight increase at short exposures and low dosage, but a slight decrease of activity at longer exposures and higher doses of the drug (Fig. 5).

#### Inverse relationship between Bcl-2 and Mcl-1 levels

To evaluate whether HA14-1 affects the expression of other antiapoptotic proteins, we analyzed the basal levels of Bcl-2 and Mcl-1 in two cell lines (Fig. 6). Expression of Bcl-2 and Mcl-1 showed an inverse relationship. The cell line exhibiting a high level of Bcl-2 expression (SudHL-4) exhibited a low level of Mcl-1 expression. The cell line with minimal Bcl-2 expression (SudHL-5) demonstrated a high level of Mcl-1 expression. This trend was also observed during HA14-1-induced apoptosis where levels of Bcl-2 increased with concomitant decrease in Mcl-1,



**Fig. 6** The inverse relationship between the expression of Bcl-2 and Mcl-1 in SudHL-4 and SudHL-5 cells, shown by Western blot as described in the “Materials and methods” section with equivalent protein loading determined by BCA protein concentration assay. p38 was used as loading control

suggesting that the expression of the two antiapoptotic proteins may be inversely regulated (Fig. 5b, e).

## Discussion

The antiapoptotic protein Bcl-2 represents a potential target for therapy in many neoplasms including B-cell lymphomas. In this study, we demonstrate the potential utility of HA14–1 as an inducer of cellular apoptosis in cell lines derived from mature B-cell NHLs exhibiting varying levels of Bcl-2 expression. We demonstrate that HA14–1 induced the most significant degree of apoptosis in the SudHL-4 cell line, which represents a transformed follicular lymphoma that overexpresses Bcl-2 as a result of the t(14;18) chromosome translocation.

The small molecule, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14–1), functions as a Bcl-2 inhibitor by competitively blocking the binding of proapoptotic molecules such as Bak and Bax [16]. The therapeutic potential of HA14–1 has been demonstrated for a variety of hematologic malignancies including acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia, and multiple myeloma [16, 17, 21–23]. Furthermore, synergistic effects with MEK/MAPK inhibitors [21, 28], cytarabine and CDK inhibitors [24], and flavopridol, as well as the ability to sensitize tumor necrosis factor-related apoptosis-inducing ligand-resistance cell lines [28], has been demonstrated. Importantly, it does not affect the viability of peripheral blood lymphocytes [29].

Our studies demonstrated that HA14–1 induced cellular apoptosis of Bcl-2(+) NHL cells, exhibiting both dose- and time-dependent characteristics. Cellular apoptosis was mediated by caspase-3 activation as demonstrated by both the caspase-3 activity assay and the Western blot analysis. These data are consistent with previous observations [17].

Analysis of various cell cycle and signaling proteins in HA14–1-treated SudHL-4 cells showed the up-regulation of Bcl-2, Bcl-xL, p27<sup>Kip1</sup>, and Bad, while a down-regulation of Mcl-1 was observed. The up-regulation of p27<sup>Kip1</sup> may indicate the presence of cells in G<sub>0</sub>/G<sub>1</sub> growth arrest, which accompanies cellular apoptosis. On the other hand, overexpression of p27<sup>Kip1</sup> itself has been shown to induce apoptosis [16] and may be a mediator of HA14–1 activity. The up-regulation of Bcl-2 was surprising and may be explained by two possible events: First, Bcl-2 up-regulation during apoptosis can be seen as a response to cellular apoptotic signals, suggesting that even cells that constitutively overexpress the Bcl-2 protein can further up-regulate its expression in response to certain stimuli. Secondly, the increased levels of Bcl-2 may represent the displaced Bcl-2 protein that may have a longer half-life within the cell.

Indeed, others have reported a similar increase in Bcl-2 during troglitazone (high-affinity ligand for peroxisome proliferators-activated receptor-gamma) -mediated apoptosis [30].

Our data also showed that HA14–1 decreased the cell viability of lymphoma-derived cell lines that did not express Bcl-2 (SudHL-5), suggesting that HA14–1 may inhibit other protein members of the Bcl-2 family. The mechanism by which this occurs is unknown; however, it has been speculated to involve the low-affinity interactions with other members of the Bcl-2 family that possess the BH3-binding groove such as Bcl-xL [16]. In this regard, the SudHL-5 cells do express Bcl-xL (data not shown), which was down-regulated by similar doses of HA14–1. Alternatively, the effect of HA14–1 on SudHL-5 cells may be Bcl-2-independent and involve cellular necrosis, an observation made by other investigators in acute leukemic blasts [17, 23].

We observed an inverse relationship between the expression of Bcl-2 and Mcl-1 proteins in the cell lines analyzed. This reciprocal relationship between Bcl-2 and Mcl-1 has been previously noted in both nonneoplastic lymphocytes and in malignant lymphomas of B- and T-cell derivation [2]. Of interest, our results suggest that NHL cells may use one predominant member of the Bcl-2 family for regulation of cell survival. Additional studies to evaluate the interactions of HA14–1 and Bcl-2 family proteins, along with a more comprehensive study of the relationship between Bcl-2 and Mcl-1, may prove beneficial in developing better therapies for B-cell NHLs.

**Conflict of interest** The authors declare that they have no conflicts of interest.

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