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Susceptibility of myelomonocytic leukemia U937 cells to the induction of apoptosis by the non-peptidic Bcl-2 ligand HA14-1 is cell cycle phase-dependent

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Abstract

Pharmacological approaches employing agents that bind to the Bcl-2 surface pocket have been used successfully to neutralize the activities of anti-apoptotic Bcl-2 family members, and induce apoptosis. Several reports suggest that Bcl-2 expression/function is cell cycle-dependent. Hence, is killing by Bcl-2 surface pocket binding agents also cell cycle-dependent? In the current study, centrifugal elutriation was used to generate cell cycle phase-enriched preparations of the human myelomonocytic leukemia cell line U937. Elutriated fractions were treated with sub-optimal cytotoxic concentrations of the pro-apoptotic, non-peptidic Bcl-2 pocket-binding agent HA14-1. A concentration of HA14-1 sufficient to kill ~30–35% of asynchronous cultures minimally affected the progressions of elutriated post-checkpoint G₁, S, G₂/M phase cells, but completely suppressed the progression of pre-G₁ checkpoint G₁ cells. Analyses of trypan blue exclusion, morphology, nuclear condensation, mitochondrial membrane potential, sub-diploid DNA contents, and caspase-3 indicated preferential killing and induction of apoptosis in pre-G₁ checkpoint G₁ and G₂/M phase cells.

Keywords

Apoptosis; Bcl-2; Cell cycle; HA14-1; U937

1. Introduction

Susceptibility to inducers of apoptosis is influenced by the cellular content of members of the Bcl-2 super family. Family membership is determined by the presence of 1–4 Bcl-2 homology domains designed BH1,2,3 or 4 [1]. Members of the Bax and BH3-only sub-families contain three and one BH domain, respectively. Both sub-families encode proteins that have pro-apoptotic properties [1–6]. Conversely, members of the Bcl-2 sub-family contain four BH domains, and prevent/counteract the activities of pro-apoptotic BH proteins [1,4–10]. Two of the principal members of the Bcl-2 sub-family are Bcl-2 and Bcl-X_L [1].

Several approaches have been used to document the anti-apoptotic properties of Bcl-2. For example, cell lines engineered to over express Bcl-2 are often more resistant than the parental lines to several different types of apoptotic inducers [4–10]. Conversely, down regulation of Bcl-2 content by antisense or siRNA approaches generally enhances susceptibility to apoptotic inducers [10]. Similarly, susceptibility to apoptotic inducers is often enhanced in cells treated

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with cell permeable peptides or non-peptidic agents that bind to, and neutralize the protective activities of Bcl-2 [11–13].

HA14-1 (ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate) is a non-peptidic agent that binds to, and inactivates Bcl-2/Bcl-X_L [13]. It was discovered by computational approaches that screened for agents that could occupy the surface cleft on Bcl-X_L normally bound by BH3-only proteins and BH3 peptides [13].

Several studies have demonstrated that HA14-1 induces Ca²⁺ release from the ER, Ca²⁺ uptake by the mitochondria, uncoupling of mitochondrial electron transport, generation of reactive oxygen species, loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome *c* release, and activation of the apoptosome [13–16].

Although limited, data exist suggesting that Bcl-2 expression and function may be regulated as a function of the cell cycle. Specifically, Gao and Dou [17] reported that the Bcl-2 contents of several cell lines synchronized by serum starvation or aphidicolin treatment were the highest in G₁ phase cells. Conversely, G₁ phase cells in the same study were the least sensitive to the induction of apoptosis by a variety of agents [17]. With respect to Bcl-2 function, most studies on the issue have focused on the fact that Bcl-2 phosphorylation represents an inactivating post-translational modification [18–20].

In Jurkat T cells, in the absence of any pro-apoptotic stimulus, Bcl-2 is normally phosphorylated in G₂/M phase cells [20]. Studies employing sub-populations of untreated Jurkat T cells prepared by centrifugal elutriation have indicated that G₂/M cells are more sensitive than cells in other phases of the cell cycle to the pro-apoptotic effects of exogenously added FasL [19]. Similarly, Bcl-2 phosphorylation occurs in a variety of cell types arrested in G₂/M as a consequence of exposure to microtubulin disruptors/stabilizers [18–20].

Recently, several studies have appeared in which combinations of HA14-1 and other agents synergize with one another in the induction of apoptosis [16,21–26]. In at least two cases the non-HA14-1 component of the combination was capable of inducing an accumulation of cells in G₂/M [23,27]. These findings, in conjunction with the studies described above, raise the issue of whether the pro-apoptotic effects of HA14-1 maybe cell cycle specific. As an approach to this problem we used centrifugal elutriation to separate U937 myelomonocytic leukemia cells into highly enriched, cell cycle phase-specific populations. Such preparations were subsequently treated with HA14-1 and monitored for progression through the cell cycle, and the development of apoptosis. Our results clearly indicate that G₂/M phase and pre-G₁ checkpoint G₁ phase U937 cells are preferentially susceptible to the pro-apoptotic effects of HA14-1.

2. Materials and methods

2.1. Chemicals

HA14-1 was purchased from Ryan Scientific, Inc. (Isle of Palms, SC). Ac-DEVD-AMC was purchased from BD Transduction Laboratories (San Diego, CA). AMC was from Calbiochem (La Jolla, CA). Hoechst H033342 (HO) and tetramethylrhodamine methyl ester (TMRM) were purchased from Molecular Probes (Eugene, OR).

2.2. Cell culture and treatment

U937 cells were obtained from the American Type Culture Collection (Manassas, VA), and grown in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in logarithmic growth at a density between 0.1 and 1×10⁶ cells/ml at 37 °C in a humidified atmosphere consisting of 5%

CO₂. Cells were passaged at densities that assured exponential growth for at least 4 days. Culture treatments were generally initiated 1 day after the passaging of cells. The ability to exclude trypan blue was used to assess cell viability. HA14-1 was dissolved in DMSO and stored at -80 °C as single use samples.

2.3. Elutriation of U937 cells

A Beckman JE-6B elutriation system and rotor were used to enrich for populations of U937 cells in different phases of the cell cycle. A detailed description of the elutriation protocol has been published [28]. For simplicity, cells recovered at a specific % of maximum flow rate capacity are referred to as that elutriation fraction (i.e. cells eluted at 30% of the maximum flow rate capacity constitute elutriation fraction 30). Debris and dead cells were removed in elutriation fractions 20 and 25. Elutriation fractions were pelleted by centrifugation and subsequently washed twice with PBS. Prior to the second washing samples were removed for estimation of trypan blue permeability, DEVDase assays, flow cytometry, and fluorescence microscopy. After the second washing the cell pellets were suspended in growth medium, adjusted to a density of 0.5×10^6 cells/ml, treated with HA14-1 or DMSO, and returned to a culture incubator. Samples for caspase analyses were frozen in liquid nitrogen and stored at -80 °C until the time of assay.

2.4. Flow cytometry

Suspensions of U937 cells were pelleted by centrifugation, and washed twice with PBS before being fixed and subsequently processed for FACs analyses of DNA content as described by Reiners et al. [29]. DNA analyses were made with a Becton Dickinson FACScalibur instrument (BD Biosciences, San Jose, CA). Percentages of cells in the G₁, S and G₂/M stages of the cell cycle were determined with a DNA histogram-fitting program (MODFIT, Verity Software, Topsham, ME). A minimum of 104 events/sample was collected for subsequent analyses.

2.5. DEVDase assay

Washed pellets of U937 cells were suspended in lysis buffer (10 mM Tris, pH 7.5; 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, 10 mM NaPP_i, 1 mM NaVO₄), frozen in liquid nitrogen, and stored at -80 °C until time of assay. On the day of assay the lysates were thawed, sonicated for 1 s, and centrifuged at $13,000 \times g$ for 10 min. DEVDase activity in the supernatant fluids was assayed by monitoring the cleavage of Ac-DEVD-AMC [30]. Release of AMC was monitored at 37 °C with a fluorescence plate reader. Changes in fluorescence over time were converted into pmol of product by comparison to standard curves made with AMC. DEVDase specific activities are reported as nmol product/min per mg protein. The bicinchoninic acid protein assay, using BSA as a standard, was used to estimate protein concentrations.

2.6. Fluorescence microscopy

Asynchronous and freshly isolated, elutriated fractions of U937 cells were diluted to $\sim 2.5 \times 10^5$ cells/ml, treated with HA14-1 or DMSO, and returned to a culture incubator. Thereafter, samples were withdrawn and incubated with HO33342 (5 μM) and TMRM (5 μM). After ~10 min labeled cells were pelleted by centrifugation, washed once with PBS, and resuspended in fresh medium. HO33342 and TMRM fluorescence were used to monitor chromatin condensation and mitochondrial membrane potential, respectively, as previously described [30].

Fluorescence was detected with a Nikon E600 fluorescence microscope equipped with a Photometrics SenSys CCD camera cooled to 10 °C.

2.7. 2.8. Statistical analyses

The Tukey's Honestly Significant Difference Test was used for analyses of data (Statistica 5.0 software; StatSoft, Tulsa, OK). Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. Cytostatic and cytotoxic effects of HA14-1

Treatment of asynchronous cultures of U937 cells with HA14-1 caused a concentration-dependent suppression of proliferation, and cytotoxicity (Fig. 1A and B, respectively). The cytostatic/cytotoxic effects of HA14-1 occurred over a very narrow concentration range. Whereas, 5 μM HA14-1 was not cytotoxic and only slightly cytostatic, 25 μM HA14-1 killed every cell within 24 h. An intermediate concentration (10 μM) of HA14-1 strongly suppressed proliferation (Fig. 1A), but was only modestly cytotoxic (Fig. 1B).

Light microscopy suggested that cultures treated with 25 μM HA14-1 died by apoptosis. Specifically, within 4 h of treatment all cells had shrunken and were decorated with blebs (data not presented). Similar changes occurred in a portion of the culture population treated with 10 μM HA14-1. In contrast, cultures treated with 5 μM HA14-1 appeared normal. DEVDase activity assays demonstrated that the observed morphological changes were accompanied by the activation of pro-caspases-3/7 (Fig. 1C). Maximal DEVDase activities were observed within 1–2 h of treatment with ≥ 10 nM HA14-1. Caspase activation did not occur in cultures treated with a non-cytotoxic concentration of HA14-1 (5 μM ; Fig. 1C).

We recently reported that elutriation could be used to prepare cell cycle phase-specific populations of U937 cells [28]. In particular, very pure preparations of early G_1 phase (fraction 30) and middle/late G_1 phase (fraction 35) cells could be obtained by elutriation (Fig. 2A). In addition, highly enriched preparations of S and G_2/M phase cells could also be generated by elutriation (fractions 40–50). Cells in elutriation fractions 35 and 40 collectively constituted ~65% of the total elutriated population (Fig. 2B). Cells in elutriation fraction 30 constituted ~15 percent of the total elutriated population. Fraction 50 was highly enriched in G_2/M phase cells, and constituted <10% of the total population recovered after elutriation. All cell fractions prepared by elutriation demonstrated high viability (>96%, Fig. 2C), and immediately resumed progression through the cell cycle when recultured (compare panels in row 1 to panels in rows 4 and 5 in Fig. 3; unpublished studies).

In order to determine whether susceptibility to HA14-1 was cell cycle phase-dependent, elutriated U937 fractions were recultured after treatment with different concentrations of HA14-1. In agreement with the trypan blue exclusion data generated with asynchronous cells, all cells in all the elutriation fractions were killed following exposure to 25 μM HA14-1 (Fig. 2C); whereas, virtually no toxicity was seen in any cell fraction treated with 5 μM HA14-1. However, differential susceptibilities were noted when elutriated fractions were treated with 10 μM HA14-1, a sub-optimal concentration of toxicant. Maximum cytotoxicity, as monitored by trypan blue permeability, was observed in fractions 30 and 50 (early G_1 and G_2/M enriched populations). Survival in these fractions was significantly less than that observed in either asynchronous cultures, or late G_1 and S phase cells (fractions 35, 40, 45). This differential sensitivity was observed irrespective of whether analyses were performed 5–6 or 22–24 h after HA14-1 treatment.

3.2. Flow cytometric analyses of DNA contents in HA14-1 treated cultures

Flow cytometry was used to monitor cell cycle progression and apoptosis following HA14-1 exposure (Fig. 3). Exposure to 10 μM HA14-1 potentially arrested most of the G_1 cells in elutriation fraction 30 (first column in Fig. 3), but affected only a portion of the G_1 population

in elutriation fraction 35 (second column in Fig. 3). Presumably, fraction 30 contains primarily G₁ cells that have not traversed the G₁ checkpoint (pre-G₁ checkpoint); whereas, fraction 35 contains a mixture of G₁ cells in which some have traversed the G₁ checkpoint (post-G₁ checkpoint). In contrast, HA14-1 had little effect on the progression of non-G₁ cells in elutriation fractions 40–50 (3rd, 4th and 5th columns in Fig. 3). Indeed, cell counting studies confirmed that the coordinated appearance of G₁ cells and loss of G₂/M cells in fractions 45 and 50, within 5.5 h of HA14-1 treatment, reflected the progression and cytokinesis of the time zero G₂/M population (data not presented).

The appearance of cells having sub-diploid DNA contents is commonly used as an indicator of apoptosis. MODFIT analyses of the cytometry data obtained with different elutriation fractions showed dramatic differences in the frequencies of cells having sub-diploid DNA contents (Fig. 3, frequency percentage is in upper left hand corner of panels). Within 2 h of treatment 40 and 20% of the cells in elutriation fractions 30 and 50 had sub-diploid DNA contents, respectively. Lower percentages of apoptotic cells were scored in elutriation fractions 35, 40 and 45. The percentage of apoptotic cells in elutriation fractions 35, 40 and 45 did not markedly change during the following 3.5 h. In contrast, the percentages of apoptotic cells in fractions 30 and 50 appeared to decrease during the same period. There are two explanations for the latter result. In the case of fraction 30, cell counting analyses at 5.5 h revealed markedly fewer cells than the number initially treated, and the presence of numerous small apoptotic bodies.

Presumably, the apoptotic cells scored at 2 h had already disintegrated, and hence were undetectable at 5.5 h. In the case of elutriation fraction 50, counting analyses demonstrated that the overall cellularity of this fraction had increased by at least 40% within 5.5 h of treatment. Indeed, the coordinate loss of G₂/M cells and appearance of G₁ cells in fraction 50 are consistent with this increase in cellularity. Division of the starting G₂/M population would increase the relative proportion of living cells in the total population, and thus decrease estimates of apoptotic cells. It should be noted that the percentage of apoptotic cells scored in fraction 45 after 5.5 h of HA14-1 also underestimates the effects of HA14-1 on the starting population. This is because the starting G₂/M population in this fraction had also divided during the 5.5 h treatment period.

A considerable percentage of cells in all of the elutriation fractions had sub-diploid DNA contents after 22 h of 10 μ M HA14-1 (bottom row of Fig. 3). However, the highest percentage of cells with sub-diploid DNA contents occurred in elutriation fractions 30 and 50. In addition, there were notable absences of both S and G₂/M phase cells in all elutriation fractions. A second independent study with 10 μ M HA14-1 (employing flow cytometric analyses of DNA contents) confirmed the preferential sensitivities of cells in elutriation fractions 30 and 50 to the pro-apoptotic effects of HA14-1 (data not presented).

3.3. Analyses of $\Delta\Psi_m$ and condensed chromatin

In order to quantify the relationship between cell cycle phase and susceptibility to HA14-1, we employed fluorescence microscopy to monitor mitochondrial membrane potential ($\Delta\Psi_m$) and chromatin condensation following short-term exposure to 10 μ M HA14-1. In a variety of cell types HA14-1 induces the loss of $\Delta\Psi_m$, which is accompanied by the release of cytochrome *c*, and the subsequent activations of pro-caspases-9 and -3, and condensation of chromatin [14–16]. The panels in Fig. 4 depict representative fields of solvent- and HA14-1-treated elutriated cells stained with HO33342 to monitor chromatin condensation and TMRM to measure $\Delta\Psi_m$. Virtually every cell in solvent-treated cultures had detectable $\Delta\Psi_m$, but very few exhibited condensed chromatin. In contrast, cells having condensed chromatin were frequently seen in HA14-1-treated asynchronous cultures, and HA14-1-treated elutriation fractions 30 and 50. In every case, cells having condensed chromatin also failed to stain with

TMRM. It should be noted that solvent-treated fraction 50 had numerous cells in various stages of mitosis. In contrast, such cells were relatively absent in the corresponding HA14-1-treated fraction (compare panels across the bottom row of Fig. 4). These missing cells most likely represent those cells most susceptible to the pro-apoptotic effects of HA14-1, and represent that population lost between the second and fifth hour of treatment.

Fig. 5 summarizes quantitative analyses of chromatin condensation (panel A) and $\Delta\Psi_m$ (panel B) data generated in two independent experiments. Solvent treatment had no effects on either $\Delta\Psi_m$ or chromatin condensation in any elutriation fraction. In contrast, HA14-1 treatment significantly increased the percentages of cells in elutriation fractions 30 and 50 having condensed chromatin and non-detectable $\Delta\Psi_m$.

4. Discussion

The cytotoxicity of HA14-1 towards asynchronous U937 cells was characterized by a very steep concentration curve. While 5 μ M HA14-1 was not cytotoxic and only weakly cytostatic, >99% of the culture was killed by a 5-fold higher concentration of the agent. Comparable results have been reported with other cell types over a similar concentration range [13,22]. Whereas cell cycle-dependent effects were not obvious in such protocols, treatment of cell cycle phase specific populations of U937 cells with a sub-optimal concentration of HA14-1 revealed two agent-related, cell cycle-dependent activities. First, a sub-optimal cytotoxic concentration of HA14-1 induced a G₁ arrest. Second, G₂/M and pre-G₁ checkpoint G₁ phase populations were preferentially susceptible to the pro-apoptotic effects of the agent.

The basis for the cell cycle-dependent susceptibility of U937 cells to HA14-1 is not known. The simplest explanation would be if U937 Bcl-2 contents fluctuated during the cell cycle. Gao and Dou [17] have reported studies suggesting that the Bcl-2 contents of several cell lines fluctuate markedly during the cell cycle. Specifically, their data showed the highest and lowest Bcl-2 contents in cycling G₁ and G₂/M phase cells, respectively. Alternatively, the function of Bcl-2 may be regulated by post-translational modification in a cell cycle-dependent fashion. Indeed, Bcl-2 is normally phosphorylated in non-treated human Jurkat T cells during G₂/M [20]. Transfection analyses employing Bcl-2 constructs in which the N-terminal phosphorylation loop domain has been deleted [19], or the phosphorylation sites have been mutated [9,20], indicate that phosphorylation of Bcl-2 represents an inactivating post-translational modification. Bcl-2 phosphorylation enhances susceptibility to various classes of apoptotic inducers [9,20], and markedly alters ER Ca²⁺ homeostasis [9]. If Bcl-2 was normally phosphorylated in G₂/M populations of U937 cells, the phase specific killing noted in the current study may partially reflect a lowering of the threshold necessary for the induction of apoptosis.

Recent studies suggest that the triggering of apoptosis by HA14-1 is more complex than its simple association, and neutralization of Bcl-2 or Bcl-X_L. Specifically, Chen et al. [24] reported that HA14-1 treatment activates Bax and induces its association with mitochondria. Furthermore, these authors demonstrated that Bax null fibroblasts are resistant to the pro-apoptotic effects of HA14-1 [24]. We have observed a similar resistance in DU145 cells (Kessel, unpublished observation), a human prostate tumor line that is Bax deficient [31]. It is conceivable that the cell cycle-dependent susceptibility of U937 cells to HA14-1 reflects phase specific expression of BAX. There is precedent for cell cycle regulation of Bax protein levels [17]. However, the patterns of expression appear to be dependent upon the cell type, p53 status, and protocol used for synchronization [17]. Alternatively, the cell cycle-dependent susceptibility of U937 cells to HA14-1 may reflect phase-specific differences in the threshold that must be overcome to activate Bax. An et al. [14] recently reported that HA14-1 causes the release of ER Ca²⁺ stores, and that mitochondrial uptake of the released Ca²⁺ was necessary

for Bax translocation to the mitochondria. Since Bcl-2 modulates the release of ER Ca^{2+} stores [4–6], and this activity is affected by Bcl-2 phosphorylation, our data are consistent with the observation of An et al. [14] if Bcl-2 is phosphorylated in pre- G_1 checkpoint G_1 and G_2/M phase U937 cells.

Several recent studies have demonstrated the ability of HA14-1 to synergize with other pro-apoptotic agents in the killing of tumor cells [16,21–26]. The cell cycle, phase specific preferential killing noted in the current investigation may be germane to the issue of why synergy occurred in some of these studies. For example, HA14-1-induced apoptosis of the AML cell line OCI-AML3 was enhanced by protracted co-treatment with the MEK inhibitor PD184352 [25]. The expression of survivin in AML cells having constitutively active MAPKs, such as OCI-AML-3, is dependent upon MAPK activity [26,32]. Very recent studies have shown that depletion of survivin by siRNA results in mitotic arrest [33]. Although speculative, it is conceivable that the synergism obtained with PD184352 and HA14-1 in AML lines is a consequence of a larger number of cells being in G_2/M . Agent-facilitated accumulation of cells in G_2/M may also be responsible for the pro-apoptotic synerism observed in combinational treatments involving HA14-1 and the epothilone B analogue (BMS-2475500) [23] or proteasome inhibitors [21]. Specifically, by itself epothilone B induces G_2/M arrest [23], and markedly potentiated the cytotoxicity of HA14-1 in human breast tumor cell lines when added 12 h prior to, but not simultaneously with HA14-1 [23]. Similarly, potentiation of HA14-1-induced multiple myeloma apoptosis by the proteasome inhibitors MG132 and bortezomib/PS-341 was dependent upon the timing and order of agent treatments [21]. Potentiation only occurred when cultures were pre-treated with proteasome inhibitors for 10 h prior to HA14-1 addition. No potentiation occurred if HA14-1 and proteasome inhibitors were added at the same time. Bortezomib/PS-341 has been reported to induce G_2/M arrest in various tumor cell lines [27,34].

In summary, our current investigation, in conjunction with the above studies, suggest a rationale for designing combinational drug treatments involving Bcl-2 antagonists such as HA14-1. Specifically, one would predict that the efficacy of HA14-1 could be markedly enhanced by combined use with an agent that would promote a G_1 or G_2/M arrest.

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Fig. 1.

Cytostatic and cytotoxic activities of HA14-1. Approximately 24 h after sub-culture U937 cells were treated with either DMSO or different concentrations of HA14-1. Cultures were harvested at various times after treatment for analyses of (A) cell numbers, (B) trypan blue permeability, and (C) DEVDase activities. Symbols represent cultures treated with DMSO (●), 5 μM HA14-1 (□), 10 μM HA14-1 (○) and 25 μM HA14-1 (Δ). Cell number and viability data represent the means±SD of 3–4 plates. DEVDase activities represent means±SD of quadruplicate analyses. Results similar to those presented in the three panels were obtained in two additional independent experiments.

Fig. 2.

Cytotoxic effects of HA14-1 on cell cycle phase-specific populations of U937 cells. Asynchronous cultures of U937 cells were separated by centrifugal elutriation into sub-populations that were processed for analyses of DNA contents by flow cytometry (A) or counted (B). Sub-populations were subsequently treated with DMSO (●), 5 μM HA14-1 (□), 10 μM HA14-1 (× and ○), or 25 μM (△) HA14-1. Non-elutriated (NE) cultures were treated similarly. Cells were maintained in culture for an additional 5–6 h (×) or 22–24 h (●, ○, □, △) before being counted and analyzed for trypan blue permeability (C). Data represent means ±SD of values obtained in a minimum of 3 experiments. *Significantly lower than viability of non-elutriated population and elutriation fractions 35 and 40, at both time points, $P < 0.05$.

Fig. 3.

Flow cytometric analyses of DNA contents of cell cycle phase-specific populations of U937 cells treated with HA14-1. Asynchronous cultures of U937 cells were separated by centrifugal elutriation into sub-populations which were immediately treated with DMSO or 10 μ M HA14-1. Cells were subsequently cultured for the indicated lengths of time before being processed for analyses of DNA contents by flow cytometry. Numerical values in each panel represent % of gated cell population having sub-G₁ DNA contents. Data similar to that depicted in the figure were obtained in a second independent experiment.

Fig. 4.

Fluorescence microscopy of mitochondrial membrane ($\Delta\Psi_m$) potential and chromatin condensation in cell cycle phase-specific populations of U937 cells treated with HA14-1. Asynchronous and elutriated populations of U937 cells were treated with DMSO or 10 μ M HA14-1 for ~3.5 h prior to being loaded with HO33342 or TMRM for analyses of nuclear condensation and $\Delta\Psi_m$, respectively. Images were captured ~4 h after HA14-1 treatment.

Fig. 5. Quantitative analyses of mitochondrial membrane potential ($\Delta\Psi_m$) and chromatin condensation in cell cycle phase-specific populations of U937 cells treated with HA14-1. Cultures were treated and analyzed as described in the legend to Fig. 4 for (A) condensed chromatin, and (B) $\Delta\Psi_m$. Data represent analyses of a minimum of 300 cells from each elutriation fraction, and non-elutriated (NE) cultures, derived from two independent experiments.