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ORIGINAL ARTICLE

A novel isocoumarin derivative induces mitotic phase arrest and apoptosis of human multiple myeloma cells

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Abstract
Purpose The isocoumarin NM-3 reverses resistance of human multiple myeloma (MM) cells to dexamethasone and is in clinical trials. In the present work, the NM-3 analog, 185322, has been studied for activity against MM cells.
Methods Human U266, RPMI8226 and primary MM cells were analyzed for the effects of 185322 on cell cycle distribution, tubulin polymerization and induction of apoptosis.
Results We show that, in contrast to NM-3, treatment with 185322 is associated with a marked arrest of MM cells in M phase. The results also demonstrate that treatment with 185322 is associated with a rapid decrease in tubulin assembly and an increase in Bcl-2 phosphorylation, consistent with disruption of mitosis. Our results further demonstrate that mitotic failure induced by 185322 results in activation of an apoptotic response in MM cell lines and primary MM cells. By contrast, 185322 had little if any effect on growth and survival of human carcinoma cells.
Conclusion These findings identify a novel inhibitor of microtubule assembly that induces mitotic arrest and apoptosis of MM cells.

Keywords Isocoumarins · NM-3 analog · Multiple myeloma · Mitotic arrest · Apoptosis

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Abbreviations
MM Multiple myeloma
DEX Dexamethasone

Introduction

NM-3 is a small molecule isocoumarin derivative that has recently completed Phase I evaluation as an orally bioavailable anticancer agent [1, 2]. The initial demonstration that NM-3 inhibits proliferation of human vascular endothelial cells and downregulates vascular endothelial growth factor expression indicated that this agent could be effective as an inhibitor of angiogenesis [3–5]. Subsequent work showed that NM-3 also has direct cytotoxic effects against malignant cells [4, 6–9]. The available evidence indicates that NM-3 increases intracellular levels of reactive oxygen species and thereby contributes at least in part to the apoptotic response of transformed cells [6, 10]. These findings and the favorable toxicity profile in Phase I studies have indicated that NM-3 could be effective in targeting tumor cells and the tumor vasculature. Notably, the isocoumarin class of compounds have not exhibited anticoagulant activity. Recent work has also shown that NM-3 acts synergistically with dexamethasone (DEX) in inducing apoptosis of DEX-resistant human multiple myeloma (MM) cells [7].

A series of NM-3 analogs have been synthesized to screen for enhanced pharmacokinetic properties and antitumor activity [11]. The analogs, like NM-3, have been well tolerated in preclinical studies [11] and are being tested for activity against cells that are sensitive to NM-3. In this regard, previous studies demonstrated that MM cells exhibit a partial loss of viability when treated with NM-3 alone at concentrations achieved in

Phase I trials [7]. In the present work, one NM-3 analog, designated 185322, has been further studied for activity against MM cells. We show that, unlike NM-3, 185322 functions as a novel inhibitor of tubulin assembly. The results also show that 185322 induces mitotic arrest and apoptosis of MM cells.

Materials and methods

Cell culture

Human U266 and RPMI8226 cell lines were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Bone marrow mononuclear cells from patients with MM were isolated by Histopaq 1077 gradient separation. CD138 positive cells were isolated with CD138 MicroBeads (Miltenyi Biotec) and cultured in RPMI1640 medium containing 10% FBS, antibiotics and 2 ng/ml interleukin 6 (Roche Applied Science). Cells were treated with NM-3 [3] and 185322 [11] (provided by ILEX Oncology).

Cell cycle analysis

Cells were fixed in 70% ethanol at −20°C for 1 h, washed with PBS, incubated in DNA extraction buffer (2.5 mM citric acid, 120 mM Na_2HPO_4, pH 7.8) for 5 min and then treated with 20 μg/ml RNase A for 15 min. After incubation with propidium iodide (PI; Sigma), DNA content was analyzed by flow cytometry. Cell cycle distribution was determined using Modfit software. In certain experiments, cells were also stained with the MPM-2 antibody (Upstate Biotechnology) to assess distribution in M phase as described [12].

Analysis of microtubule polymerization

Cells were lysed in microtubule stabilizing buffer (20 mM Tris–HCl, pH 6.8, 2 mM EDTA, 0.5% NP-40, 1 mM $MgCl_2$, 0.5 μg/ml paclitaxel, protease inhibitor mixture (Complete; Roche Diagnostics) and 100 μg/ml phenylmethylsulfonyl fluoride) and centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants containing soluble tubulin and the pellets containing polymerized tubulin were subjected to immunoblot analysis with anti-β-tubulin (Sigma) and anti-β-actin (Sigma) antibodies.

Immunoblot analysis

Cells were lysed in lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, protease inhibitor mixture and 100 μg/ml phenylmethylsulphonyl fluoride). Cell lysates were subjected to immunoblotting with anti-phospho-Bcl-2 (Cell Signaling Technology), anti-Bcl-2 (Cell Signaling Technology) or anti-β-actin (Sigma). Reactivity was detected with horseradish peroxidase-conjugated second antibodies and chemiluminescence (ECL Plus Kit; Amersham Biosciences).

Assessment of apoptosis

MM cell lines were stained with PI and analyzed by flow cytometry as described above for detection of cells with sub-G1 DNA content. Primary MM cells were stained with PI and Hoechst dye (bis-benzimide; Sigma) as described [13]. Apoptotic cells were identified under a fluorescence microscope based on fragmented nuclei and cell membrane permeability [13]. Five hundred cells were scored for apoptosis in each of three independent experiments.

Results and discussion

185322 induces G2/M phase arrest of MM cells

NM-3 [3-(2-methylcarboxymethyl)-6-methoxy-8-hydroxyisocoumarin] was modified to the isocoumarin analog designated 185322 [8-methoxy-6-methyl-3-(3-pyridylmethyl)isocoumarin] (Fig. 1a). Previous work showed that NM-3 decreases viability of IL-6-dependent U266 and IL-6-independent RPMI8226 MM cells at clinically achievable concentrations of 100–200 μg/ml [7]. To determine if 185322 affects MM cell growth, U266 cells were analyzed by flow cytometry after treatment with 185322 at 3 or 30 μg/ml. Exposure to 185322 at 3 μg/ml had no detectable effect on cell cycle distribution (data not shown). However, treatment with 185322 at 30 μg/ml was associated with a marked time-dependent increase of U266 cells in G2/M phase (Fig. 1b). By contrast, NM-3 had little if any effect on U266 cell cycle distribution at 100 or 200 μg/ml (Fig. 1b and data not shown). Similar results were obtained when RPMI8226 cells were exposed to these agents (Fig. 1c), indicating that 185322 is functionally distinct from NM-3 by inducing G2/M arrest of MM cells. Notably, treatment of human A549 lung cancer and LNCaP prostate cancer cells with 185322 had little effect on the percentage in G2/M phase (Fig. 1d). These findings indicated that 185322 induces G2/M arrest of MM cells.

185322 arrests MM cells in M phase by inhibiting tubulin polymerization

To distinguish between distribution in G2 and M phases, cells were incubated with the MPM-2 antibody

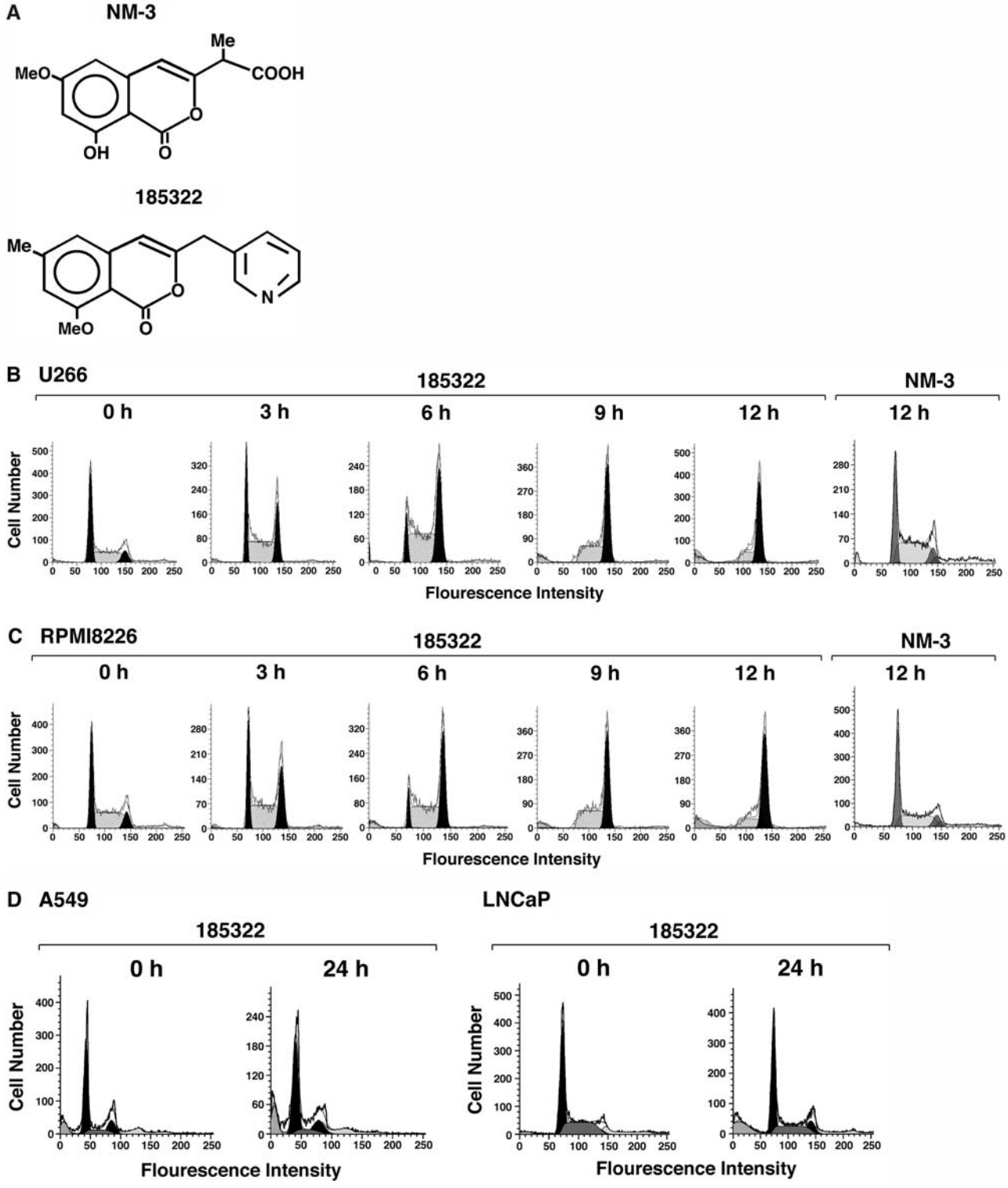

Fig. 1 185322 induces G2/M arrest of MM cells. **a** Structures of NM-3 and 185322. U266 (**b**) and RPMI8226 (**c**) cells were treated with 30 μg/ml 185322 for the indicated times or 200 μg/ml NM-3 for 12 h. The cells were stained with PI and DNA content was analyzed by flow cytometry. **d** A549 and LNCaP cells were treated with 30 μg/ml 185322 for 24 h and analyzed for cell cycle distribution

that detects proteins phosphorylated at the onset of mitosis [12, 14]. Compared to control U266 cells, treatment with 185322 for 12 h was associated with a marked increase in the distribution of cells in M phase (Fig. 2a). Similar effects were observed when RPMI8226 cells were treated with 185322 (Fig. 2a). An analysis of U266

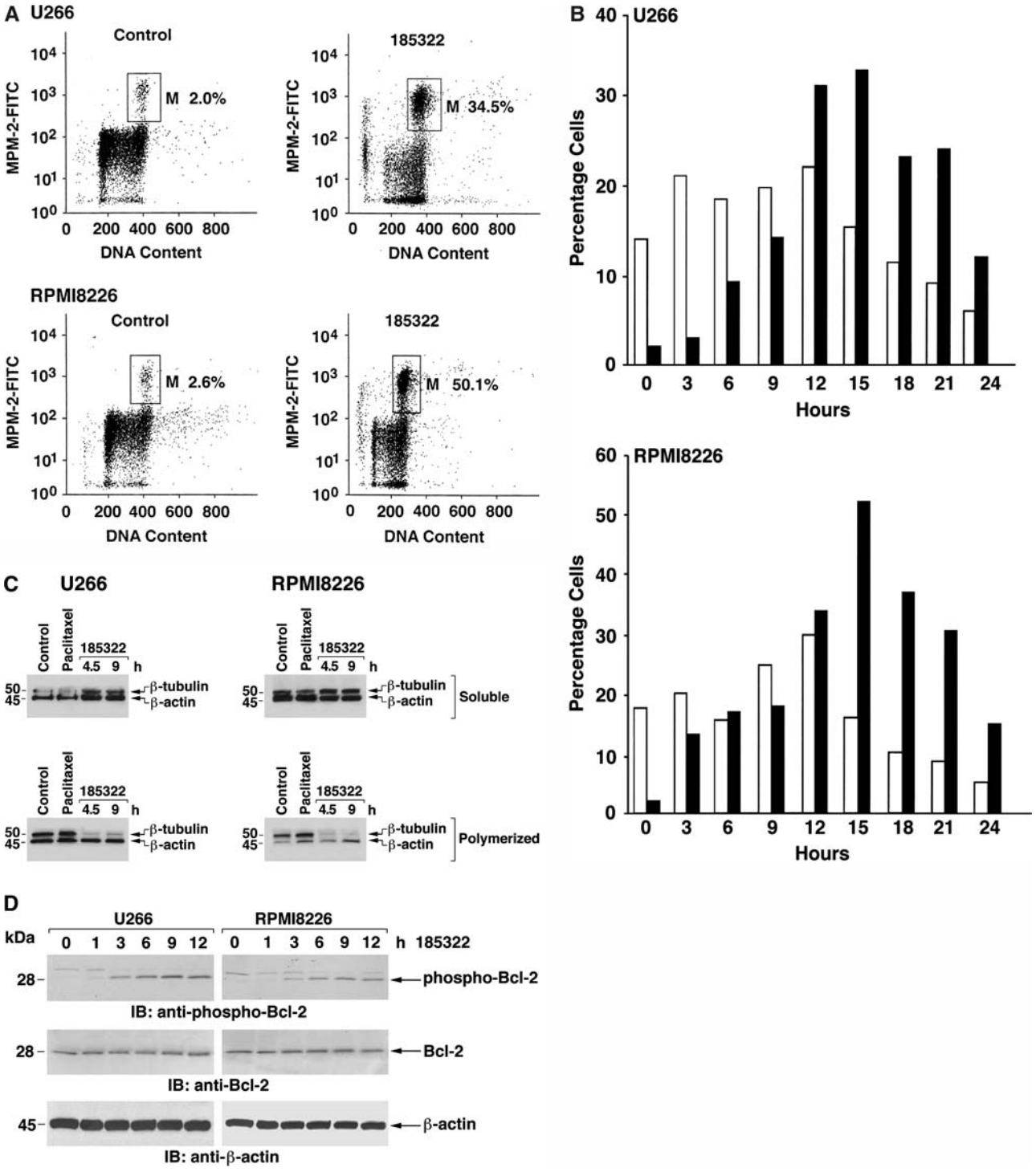

Fig. 2 185322 induces M phase arrest by inhibiting tubulin polymerization. **a** U266 and RPMI8226 cells were treated with 30 μg/ml 185322 for 15 h, fixed and stained with MPM-2-FITC conjugate and PI. As analyzed by bidimensional flow cytometry, cells with increased fluorescence above baseline are captured in the boxes and are represented as the percentage in M phase. **b** U266 and RPMI8226 cells were treated with 30 μg/ml 185322 for the indicated times and stained with MPM-2-FITC and PI. The results are presented as the percentage of cells in G2 (*open bars*) and M (*solid bars*) phase. **c** U266 and RPMI8226 cells were treated with 8.54 μg/ml paclitaxel for 4.5 and 9 h or 30 μg/ml 185322 for the indicated times. The cells were lysed in microtubule stabilizing buffer and tubulin in the soluble (*upper panels*) and insoluble (*lower panels*) fractions was analyzed by immunoblotting. The fractions were also immunoblotted with anti-β-actin as a control. **d** Lysates from U266 and RPMI8226 cells treated with 30 μg/ml 185322 for the indicated times were immunoblotted with anti-phospho-Bcl-2, anti-Bcl-2 and anti-β-actin

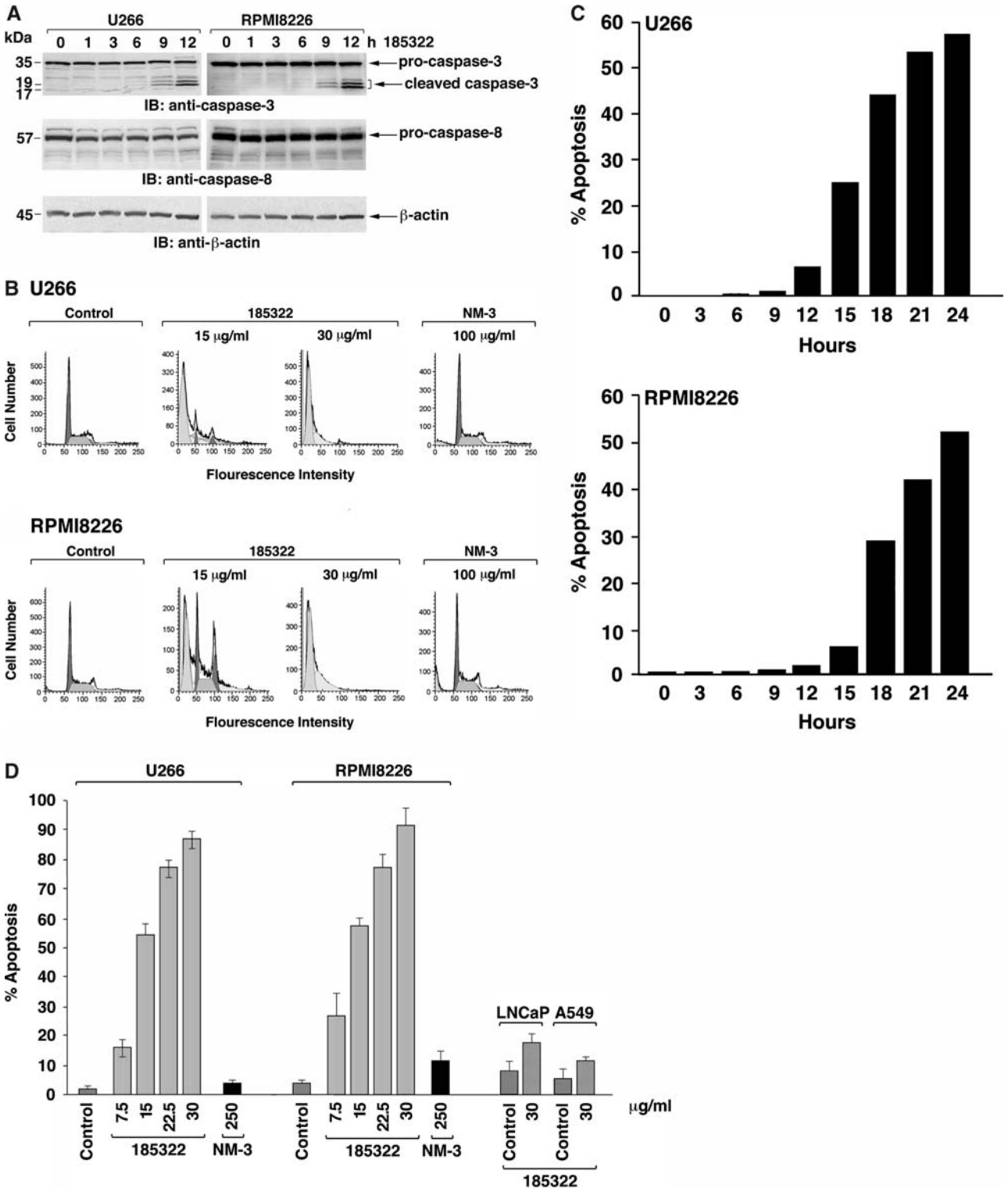

Fig. 3 185322 induces apoptosis of MM cell lines. **a** Lysates from U266 and RPMI8226 cells treated with 30 μg/ml 185322 for the indicated times were immunoblotted with anti-caspase-3, anti-caspase-8 and anti-β-actin. **b** U266 and RPMI8226 cells were treated with 15 or 30 μg/ml 185322 or with 100 μg/ml NM-3 for 24 h and then analyzed for cell cycle distribution. **c** U266 and RPMI8226 cells were treated with 30 μg/ml 185322 for the indicated times and then analyzed by flow cytometry. The results represent the percentage of cells with sub-G1 DNA. **d** U266 and RPMI8226 cells were treated with the indicated concentrations of 185322 or NM-3 for 24 h. A549 and LNCaP cells were treated with 30 μg/ml 185322 for 24 h. Cells were analyzed for sub-G1 DNA. The results are presented as the percentage apoptosis (mean ± SD of three separate determinations)

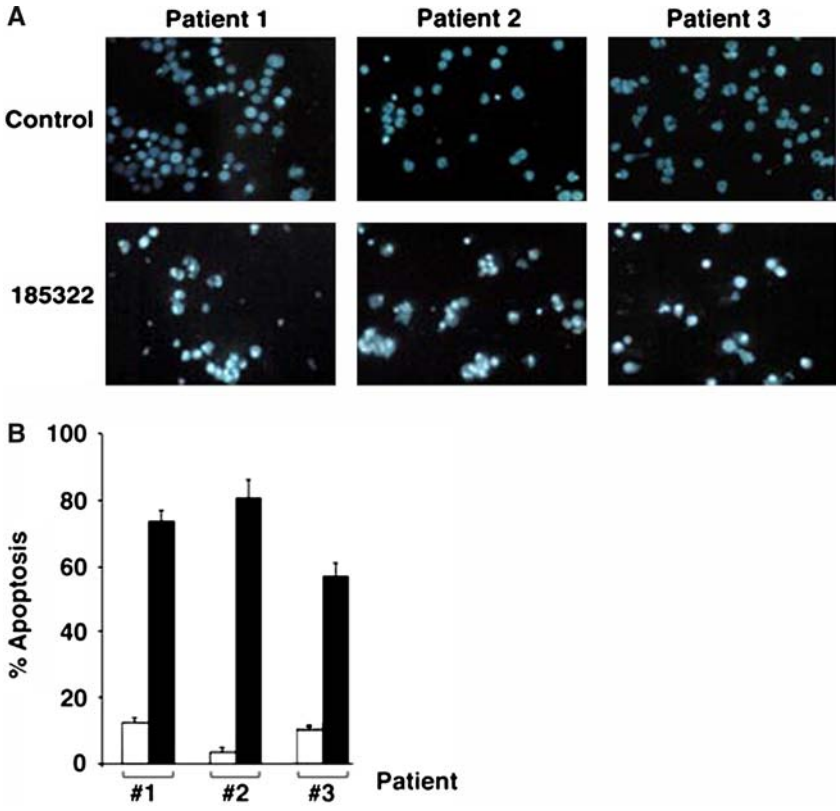

Fig. 4 185322 treatment of primary MM cells is associated with induction of apoptosis. **a** Mononuclear cells were isolated from bone marrow aspirates of 3 MM patients and selected with CD138 beads. The CD138+ cells were stimulated with IL-6 for 48 h, treated with 30 μg/ml 185322 for 24 h and then stained with PI and Hoechst dye. **b** The percentage apoptosis was determined by fluorescence microscopy for control (*open bars*) and 185322-treated (*solid bars*) cells

and RPMI8226 cells at 3 h intervals over 24 h showed that the accumulation in M phase was maximal at 12–15 h and then declines through 24 h (Fig. 2b). Declines in the percentage of cells in G2 phase were also observed at 15–24 h of 185322 treatment (Fig. 2b). To determine if 185322 induces M phase arrest by affecting tubulin polymerization, we assayed MM cells for soluble and polymerized tubulin. As a control, treatment of MM cells with paclitaxel, an inhibitor of tubulin depolymerization, was associated with an increase in polymerized β-tubulin (Fig. 2c). By contrast, 185322 treatment resulted in an increase in soluble β-tubulin and a decrease in polymerized β-tubulin, consistent with inhibition of tubulin polymerization (Fig. 2c). Immunoblot analysis of β-actin levels was used as a control for protein loading (Fig. 2c). In concert with these results, phosphorylation of Bcl-2, a marker of M phase arrest by microtubulin inhibitors [15], was increased in association with the 185322-induced accumulation of cells in M phase (Fig. 2d). These findings indicate that 185322 induces arrest of MM cells in M phase by inhibiting tubulin polymerization.

Prolonged 185322 treatment is associated with MM cell apoptosis

The failure to complete mitosis can culminate in the activation of an apoptotic default pathway [16]. To determine if treatment of MM cells with 185322 is associated with induction of apoptosis, immunoblot analysis was performed to assess cleavage of pro-caspase-3. Activation of pro-caspase-3 to the cleaved forms was detectable in U266 cells at 9–12 h (Fig. 3a). Similar results were obtained when RPMI8226 cells were treated with 185322. By contrast, there was no detectable cleavage of pro-caspase-8 (Fig. 3a). Consistent with activation of the intrinsic apoptotic pathway, exposure to 185322, but not NM-3, was associated with a marked increase in U266 and RPMI8226 cells with sub-G1 DNA (Fig. 3b). The percentage of cells with sub-G1 DNA increased progressively from 12–24 h (Fig. 3c). Repetitive experiments at different concentrations of 185322 further showed that the pro-apoptotic effects of this agent are dose-dependent (Fig. 3d). Conversely, 185322 had little effect on induction of apoptosis of LNCAP and A549 carcinoma cells (Fig. 3d). These findings indicate that 185322 induces apoptosis of MM cell lines.

185322 induces apoptosis of primary MM cells

To determine if 185322 induces apoptosis of primary MM cells, bone marrow aspirates were obtained from three patients with MM. Following isolation with CD138 MicroBeads, the MM cells were stimulated with IL-6 for 48 h. Staining with an anti-CD138 antibody demonstrated CD138 positivity of 80–90%. Microscopic

assessment of membrane permeability and fragmented nuclei demonstrated a low level of apoptosis for the untreated MM cells (Fig. 4a). By contrast, treatment with 185322 was associated with a marked increase in both membrane permeability and fragmented nuclei (Fig. 4a). Quantitation of cells with fragmented nuclei demonstrated 4–12% apoptosis in the untreated cells and 56–80% apoptosis in the 185322 MM cell preparations (Fig. 4b). These findings indicate that, like MM cell lines, 185322 induces apoptosis of primary MM cells.

185322 is a novel inhibitor of MM cell mitosis

Microtubules are the targets of chemically diverse antimitotic agents that include the vinca alkaloids and the taxanes. The vinca alkaloids inhibit tubulin polymerization, while the taxanes stabilize microtubules [17]. The present studies demonstrate that, like microtubule-targeted drugs, 185322 induces arrest of MM cells in M phase. Our results also show that 185322 induces M phase arrest by inhibiting tubulin polymerization. 185322 is structurally distinct from the vinca alkaloids, taxanes and other agents, such as the combretastatins and epothilones, that have antitumor activity. However, as found for other agents that induce M phase arrest [16], 185322 treatment of MM cells was associated with phosphorylation of Bcl-2, activation of caspase-3 and induction of apoptosis. In addition and in concert with the decreased response of A549 and LNCaP cells to 185322-induced mitotic arrest, MM cells were substantially more sensitive than these carcinoma cells to 185322-induced apoptosis. The basis for this selectivity is not clear, but could reflect differences in 185322 metabolism in MM and carcinoma cells. Importantly, like MM cells in culture, primary MM cells also responded to 185322 with the induction of apoptosis. These findings indicate that 185322 is a novel inhibitor of tubulin polymerization and that this agent may be effective in MM treatment by inducing M phase arrest and apoptosis.

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