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ORIGINAL ARTICLE

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EM012, a microtubule-interfering agent, inhibits the progression of multidrug-resistant human ovarian cancer both in cultured cells and in athymic nude mice

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Abstract Drug resistance, in particular multidrug resistance, is a serious problem that impedes the effectiveness of chemotherapy. Multidrug resistance results mainly from an enhanced efflux of drugs by drug pumps located on the cell membrane such as P-glycoprotein. In the study reported here we showed that EM012, a microtubule-interfering agent, is a weak substrate for P-glycoprotein and inhibited the proliferation of A2780/ADR human ovarian cancer cells, which possess multidrug resistance due to P-glycoprotein overexpression. A2780/ADR cells treated with EM012 exhibited pronounced mitotic arrest, developed large multilobed nuclei, and eventually died through the initiation of apoptosis. Intraperitoneal treatment of A2780/ADR xenograft tumors in athymic nude mice with EM012 significantly inhibited tumor progression through triggering apoptosis and conferred an apparent survival advantage. Furthermore, EM012 treatment did not cause detectable toxicity to normal tissues. These findings suggest that EM012 may serve as a novel chemotherapeutic agent for the treatment of multidrug-resistant human ovarian cancer.

Keywords Ovarian cancer · Multidrug resistance · EM012 · Cell cycle

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Introduction

Chemotherapy is the treatment of choice for a number of metastatic cancers. For example, paclitaxel is widely used in the treatment of metastatic breast and ovarian cancers [1]. The effectiveness of chemotherapy, however, is seriously limited by the development of drug resistance [2]. Although patients show significant response to chemotherapeutic drugs during treatment, most relapse and fail to respond to the same drug at a later stage. Multidrug resistance, by which cancer cells quite often become resistant to many structurally and mechanistically unrelated drugs, makes successful chemotherapy more complex and difficult to achieve [2]. This phenomenon is primarily due to an enhanced efflux of drugs from the inside to the outside of cells by drug pumps, a family of ABC transporter proteins located on the cell membrane [3]. P-glycoprotein, for example, is one such drug pump and is encoded by the multidrug resistance 1 gene. Overexpression of P-glycoprotein has been found to affect drug accumulation in the cell and correlates with the multidrug resistance phenotype in cancer cells [4–6].

Noscapinoids (Fig. 1a), including noscapine, EM011 (5-bromonoscapine), and EM012 (the reduced form of 5-bromonoscapine), are a class of microtubule-interfering agents that exhibit antitumor activity [7–10]. A common structural feature of this group of agents is the presence of an isoquinoline ring system and an isobenzofuran ring system (Fig. 1a). These agents bind

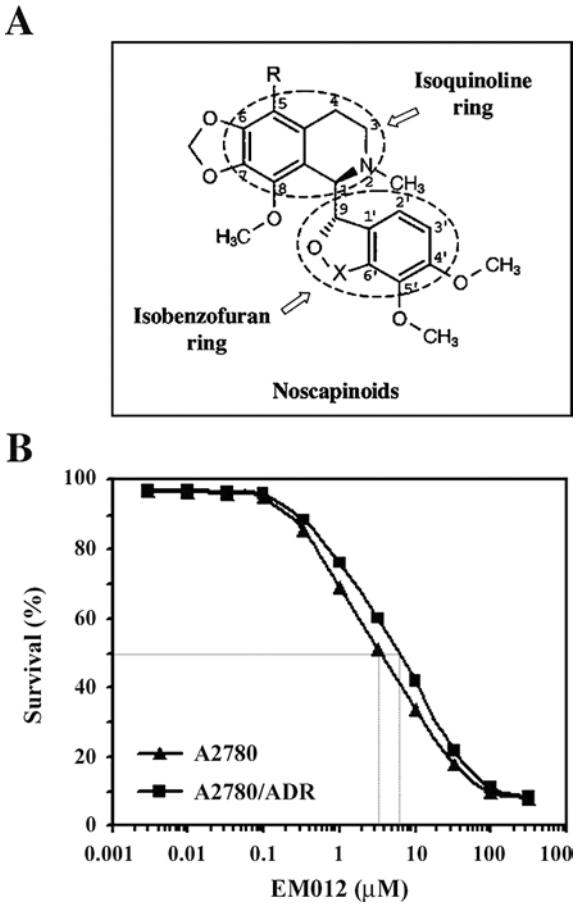

Fig. 1 Noscapinoids are weak substrates for P-glycoprotein and effectively inhibit the proliferation of A2780 human ovarian cancer cells and A2780/ADR multidrug-resistant cells derived from A2780. **a** Chemical structures of noscapinoids, including noscapine (R = H, X = CO), EM011 (R = Br, X = CO), and EM012 (R = Br, X = CH_2). **b** A plot of cell survival versus EM012 concentration showing the method used for the determination of IC_{50} (the drug concentration needed to inhibit cell proliferation by 50%). The IC_{50} values for the noscapinoids in A2780 and A2780/ADR cells are presented in the text

tubulin stoichiometrically and alter its conformation [7, 8]. In addition, they inhibit microtubule dynamics without significantly altering the polymer/monomer ratio of tubulin even at high concentrations [8–10]. In the present study, we showed that EM012 is a weak substrate for P-glycoprotein and inhibits the progression of multidrug-resistant human ovarian cancer both in cultured cells and in athymic nude mice by blocking cell-cycle progression at mitosis, causing multinucleation, and triggering apoptotic cell death.

Materials and methods

Materials

Noscapine was purchased from Sigma-Aldrich. EM011 and EM012 were prepared as described previously [8]. All the drugs were dissolved in dimethyl sulfoxide (DMSO).

Cell culture

The multidrug-resistant human ovarian cancer cell line A2780/ADR was derived from A2780 [11, 12]. Both A2780 and A2780/ADR cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a 5% CO_2/95% air atmosphere.

Measurement of IC_{50} values

Cells were seeded in 96-well plates at a density of 2×10^3 cells per well. They were treated with increasing concentrations of drugs the next day while in log-phase growth. After 72 h of drug treatment, cells were fixed with 50% trichloroacetic acid and stained with 0.4% sulforhodamine B dissolved in 1% acetic acid. Cells were then washed with 1% acetic acid to remove unbound dye. The protein-bound dye was extracted with 10 m*M* Tris base and the optical density determined at a wavelength of 564 nm. The percentage cell survival was then plotted as a function of drug concentration to determine the IC_{50} value (see Fig. 1b). Fold resistance factor was defined as the ratio of IC_{50} value obtained for the A2780/ADR cell line to that obtained for A2780.

Flow cytometric analysis

Flow cytometric evaluation of the cell-cycle status was performed on a Coulter Elite flow cytometer (Beckman Coulter) by staining cellular DNA with propidium iodide as described previously [9].

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with cold (−20°C) methanol, incubated with a mouse monoclonal antibody against α-tubulin (clone DM1A, Sigma-Aldrich) and a fluorescein isothiocyanate-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch), and then stained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). All images were acquired using a Zeiss Axiovert 135 fluorescence microscope with ×100/1.3 oil lens (Plan-Neofluar, Carl Zeiss).

Apoptosis assays

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) and annexin V staining assays were performed using an in situ cell detection kit (Roche Molecular Biochemicals) and an annexin V

apoptosis detection kit (BD Biosciences), respectively, following the manufacturers' instructions. Trypan blue (0.4% solution, Sigma-Aldrich) staining was also used to assess cell viability.

Analysis of ovarian cancer progression in mice

Two million A2780/ADR cells were injected subcutaneously into female BALB/c athymic nude mice 6–7 weeks of age. After the tumors in the implanted mice had become palpable (about 5 weeks after cell injection), the mice were treated intraperitoneally every 3 days with 30 mg EM012/kg body weight in saline water or saline alone as control. Each treatment group comprised 12 mice. The progression of the tumors was monitored by measuring three perpendicular diameters using calipers, and tumor volume was calculated by the formula: 1/2(length×width×height) [13]. Mice were killed when tumors ulcerated, or when the mice exhibited other criteria of morbidity as defined by our Institutional Animal Care and Use Committee guidelines. For survival studies, we defined survival up to the date on which the mice were killed.

Histopathological analysis

After the mice were killed, gut, liver, spleen, and tumor were removed. Tissues were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin as described previously [10].

Results and discussion

We have previously found that noscapine and its two derivatives, EM011 and EM012, are potent antitumor agents that target microtubules [7–10]. Since multidrug resistance is a serious issue for the existing chemotherapeutic drugs, we examined the effect of noscapinoids on the proliferation of the A2780/ADR human ovarian cancer cell line, which was derived from the A2780 cell line and exhibits multidrug resistance due to overexpression of the drug pump P-glycoprotein [11, 12]. The IC_{50} values of noscapine, EM011, and EM012 were 39, 6.9, and 6.1 μM, respectively, in A2780/ADR cells, and 23, 3.4, and 3.2 μM, respectively, in A2780 cells. A2780/ADR cells therefore had 1.7-fold, 2.0-fold and 1.9-fold resistance to noscapine, EM011, and EM012 respectively, when compared with A2780 cells. By contrast, A2780/ADR cells showed 852-fold resistance to the widely used chemotherapeutic drug paclitaxel, which also targets microtubules. This is not unexpected because paclitaxel is known to be a substrate for P-glycoprotein [6]. Our results thus suggest that noscapinoids are rather weak substrates for P-glycoprotein. The IC_{50} values of EM012 in A2780 and A2780/ADR cells appeared much lower than those of noscapine and slightly lower than those of EM011, indicating that EM012 had the highest activity against the proliferation of these ovarian cancer cells. We therefore selected EM012 for the following studies in A2780/ADR cells.

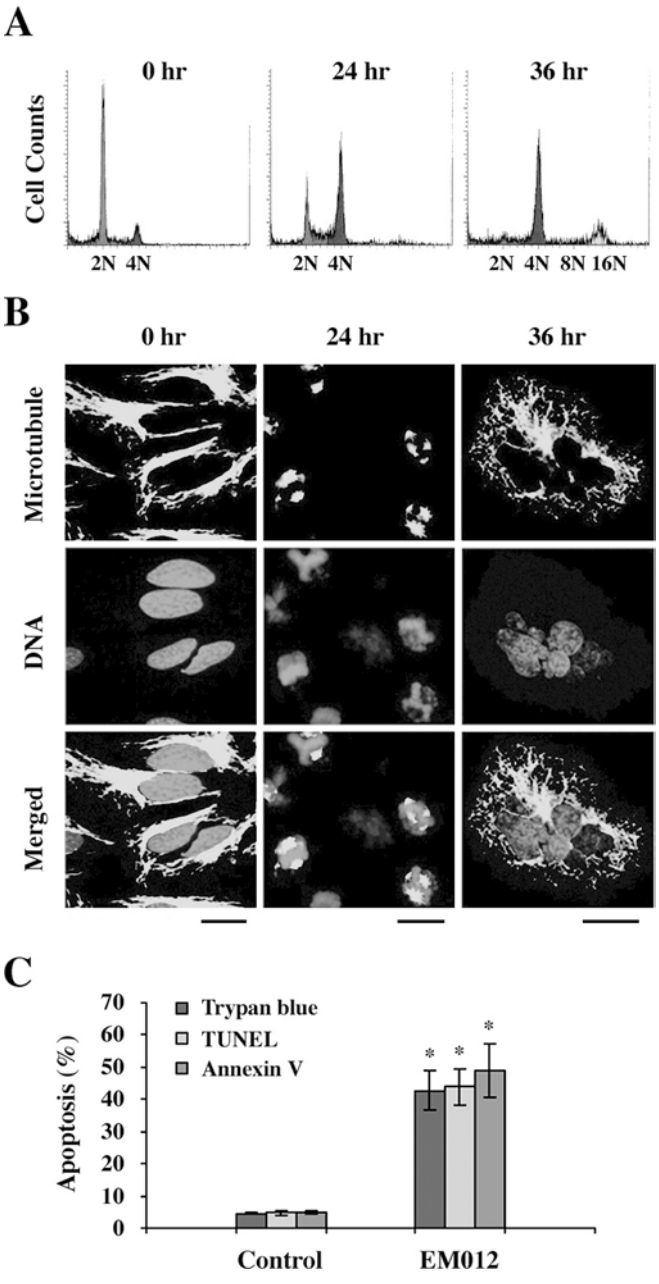

Fig. 2 EM012 inhibits the cell-cycle progression of A2780/ADR cells at mitosis, causes the formation of large multilobed nuclei, and then triggers apoptosis. **a** Flow cytometric analysis of DNA content in A2780/ADR cells after treatment with 6 μM EM012 for 0, 24, and 36 h. **b** Representative immunofluorescence images of microtubules (*green*) and DNA (*blue*) in A2780/ADR cells treated with 6 μM EM012 for 0, 24, and 36 h. *Bar* 10 μm. **c** Apoptosis percentages measured by trypan blue staining, TUNEL, and annexin V staining assays in A2780/ADR cells treated with 6 μM EM012 or the solvent DMSO (control) for 48 h. $^*P < 0.05$ vs control

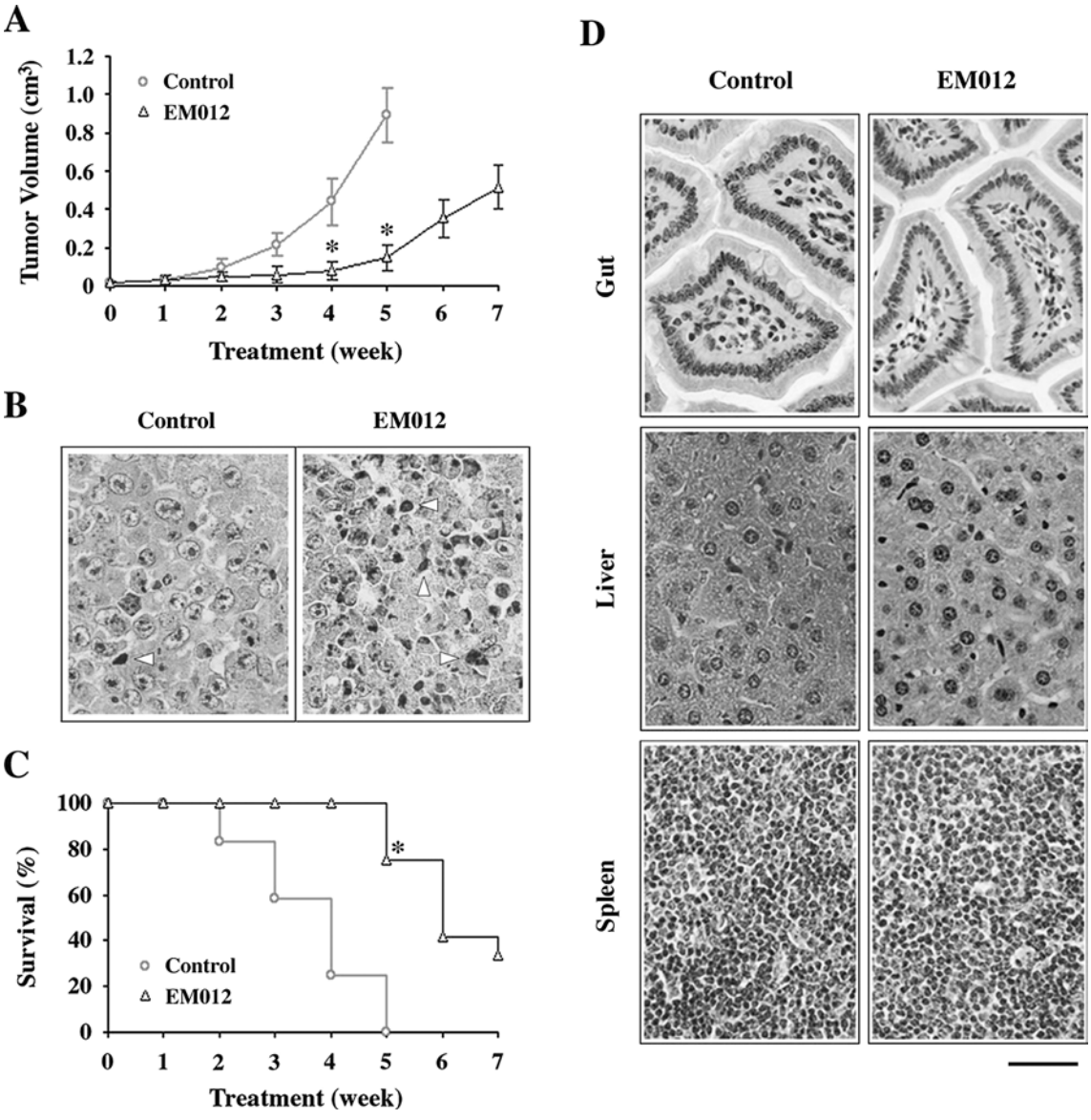

Fig. 3 EM012 inhibits the progression of A2780/ADR human ovarian cancer in an athymic mouse model and confers a survival advantage, without causing obvious toxicity to normal tissues. Palpable tumors had become established in athymic mice about 5 weeks after subcutaneous injection of A2780/ADR cells. Mice were treated intraperitoneally every 3 days with 30 mg EM012/kg body weight in saline water or saline water alone as control. Each treatment group comprised 12 mice. **a** EM012 inhibits tumor progression. $*P < 0.05$ vs control. **b** EM012 induces apoptosis in tumor cells. Shown are representative micrographs of tumor sections with apoptotic figures indicated by *arrowheads*. **c** EM012 treatment confers a survival advantage. $*P < 0.05$ vs control. **d** EM012 treatment does not cause obvious pathological abnormalities in normal tissues. Shown are representative micrographs of 10-μm thick sections of gut, liver, and spleen stained with hematoxylin and eosin. *Bar* 50 μm

We first checked the cell-cycle status of EM012-treated A2780/ADR cells by flow cytometric analysis of DNA content. Untreated cells (0 h treatment) showed a typical distribution of cell populations in G_1 with 2N (unduplicated) DNA content and in G_2/M with 4N (duplicated) DNA content (Fig. 2a). Treatment with EM012 at 6 μ*M* for 24 h significantly increased the population of cells with 4N DNA content (Fig. 2a). Immunofluorescence microscopy further revealed that, whereas untreated cells exhibited normal radial microtubule arrays, cells treated with EM012 at 6 μ*M* for 24 h showed pronounced multipolar spindles and condensed chromosomes indicating mitotic arrest (Fig. 2b). This was probably due to the activation of the spindle assembly checkpoint, a cellular surveillance mechanism that monitors the integrity of the mitotic spindle [14]. After 36 h of treatment, the population of cells with 2N DNA content was significantly decreased and a small population of cells (31%) accumulated more than 8N DNA content (Fig. 2a). This correlated with the appearance of cells with large multilobed nuclei (multinucleation) as shown in Fig. 2b. EM012-treated A2780/ADR cells eventually died through the initiation of apoptosis as revealed by trypan blue staining,

TUNEL, and annexin V staining assays (Fig. 2c). Whereas only about 5% of cells underwent apoptosis after treatment with the solvent alone (control), more than 40% of cells became apoptotic after treatment with EM012 at 6 μ*M* for 48 h. The percentage of apoptosis obtained from the annexin V staining assay was slightly higher than that obtained from the other two assays. This is reasonable since annexin V staining assay detects the loss of phosphatidylserine asymmetry of the plasma membrane, which is an early event during the process of apoptosis.

We then sought to determine whether EM012 could inhibit the progression of multidrug-resistant human ovarian cancer in vivo. Tumor growth was initiated by subcutaneously injecting 2×10^6 A2780/ADR cells into the back of female immunodeficient mice and treated with EM012 as described in "Materials and methods". Treatment with EM012 for 5 weeks inhibited the progression of tumor volume by 83% compared with that in the control group (average ± SD tumor volume 0.89 ± 0.14 and 0.15 ± 0.07 cm^3 in the control and EM012-treated groups, respectively; Fig. 3a). EM012-treated tumor sections showed increased apoptotic activity when compared with the control-treated sections (Fig. 3b), indicating that this drug inhibited tumor progression through triggering apoptosis. EM012 treatment also conferred an apparent survival advantage on the mice (Fig. 3c). By week 5, all the mice in the control group had to be killed due to tumor ulcerations or extreme morbidity. However, only 25% of mice treated with EM012 had to be killed at this time point.

We also examined the gut, liver, and spleen after 5 weeks of treatment to determine whether EM012 had caused toxicity to normal tissues. As shown in Fig. 3d, EM012 treatment had not caused obvious pathological abnormalities in these tissues. It remains unclear at present why this drug does not affect normal tissues while inhibiting tumor progression. Our previous studies showed that noscapinoids suppress microtubule dynamics without significantly affecting the polymer/monomer ratio of tubulin even at high concentrations [8–10]. Thus cellular functions that do not require exquisite control of microtubule dynamics may not be interrupted. This may have contributed to the observed specificity of EM012 in inhibiting tumor progression in the mouse model.

In summary, we demonstrated in this study that the microtubule-interfering agent EM012 is a weak substrate for the drug pump P-glycoprotein. It inhibits the progression of P-glycoprotein overexpressing, multidrug-resistant human ovarian cancer by arresting cell-cycle progression at mitosis, causing multinucleation, and triggering apoptosis. Considering the fact that multidrug resistance is a major limiting factor in chemotherapy, our results indicate EM012 may be an effective chemotherapeutic agent for treating refractory ovarian cancers.

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