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Nucleoside analog inhibits microRNA-214 through targeting heat-shock factor 1 in human epithelial ovarian cancer

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The important functions of heat shock factor 1 (HSF1) in certain malignant cancers have granted it to be an appealing target for developing novel strategy for cancer therapy. Here, we report that higher HSF1 expression is associated with more aggressive malignization in epithelial ovarian tumors, indicating that targeting HSF1 is also a promising strategy against ovarian cancer. We found that a nucleoside analog (Ly101-4B) elicits efficient inhibition on HSF1 expression and potent anticancer activity on epithelial ovarian cancer both in vitro and in vivo. Moreover, by targeting HSF1, Ly101-4B inhibits the biogenesis of microRNA-214, which has been revealed to be overexpressed and to promote cell survival in human ovarian epithelial tumors. These findings demonstrate that Ly101-4B is a promising candidate for ovarian cancer therapy, and expand our understanding of HSF1, by revealing that it can regulate microRNA biogenesis in addition to its canonical function of regulating protein-coding RNAs. (Cancer Sci 2013: 104: 1683-1689)

O varian cancer is the third most common as well as the most lethal gynecologic malignancy in women.^(1,2) Among the multiple histotypes of ovarian cancer, epithelial ovarian cancer accounts for 90% of all ovarian tumors and is a leading contributor to ovarian cancer deaths. Although initial treatment with platinum compounds is effective in approximately 70% of patients, most patients will have a relapse in less than 5 years with development of drug resistance, and the 5-year survival rate is, unfortunately, below 30%.^(1–3) Consequently, development of targeted therapies and novel drug candidates to combat epithelial ovarian cancer is in urgent need. Among various tumor therapy targets, heat shock factor 1 (HSF1) appears to be an appealing novel target for drug development for treatment of malignant tumors.^(4–7)

Heat shock factor 1 was originally identified as a heat shock-induced transcription factor, promoting cell survival against a range of patho-physiological conditions. Recent studies reveal that HSF1 also orchestrates a broad network of cellular functions.^(4,7) Its dysregulation is associated with tumor pathogenesis, progression and poor prognosis.^(4,6,8–13) It is worth noting that cancer cells of diverse origins depend more on HSF1 to maintain proliferation and survival than their corresponding non-tumorigenic cells,^(4–6) and HSF1 defection does not alter the proliferation and cell-cycle progression of normal cells.⁽⁴⁾ Consequently, targeting HSF1 has the potential to be an efficient strategy for cancer therapy.

In our previous work, we reported that a novel nucleoside analog, Ly101-4B, was able to target HSF1 in drug-resistant pancreatic cancer models.⁽¹⁴⁾ Ly101-4B is a novel triazole nucleoside analog bearing aromatic moieties on the triazole nucleobase^(14–16) and exhibited efficient anti-tumor activity both *in vitro* and *in vivo* in drug-resistant pancreatic cancer models, without adverse effects.⁽¹⁴⁾ Targeting HSF1 is a completely novel mechanism for the anti-tumor effect of a nucleoside analog.

Beyond protein targets, non-coding microRNA (miRNA) is another important class of gene that shows promise as a new generation target of molecular-targeted anti-cancer therapies.⁽¹⁷⁻¹⁹⁾ Over the past decade, a number of important miR-NA have been identified to be involved in the pathogenesis and development of ovarian cancer.^(20–23) Therefore, it is important to study possible miRNA regulatory functions to reveal the action mechanism of anti-cancer drugs.

In this work, we first examine the expression of HSF1 in primary human epithelial ovarian tumors, and reveal that HSF1 expression is significantly higher in malignant than in benign ovarian tumors. Then we demonstrate that Ly101-4B can be applied to successfully downregulate the expression of HSF1 and inhibit the proliferation of epithelial ovarian cancer. In addition, Ly101-4B is able to suppress the biogenesis of a critical miRNA (miR-214), which has been demonstrated to promote cell survival in ovarian cancer, via downregulation of HSF1 in ovarian cancer, implying that Ly101-4B constitutes a promising candidate for ovarian cancer therapy with a novel mechanism of action.

Materials and Methods

Tissue collection and immunohistochemical assay. Ovarian tumor samples were collected at debulking surgery or purchased from Alenabio (Xi'an, China). A total of 30 serous adenocarcinomas, 1 mucinous adenocarcinoma, 3 endometrioid adenocarcinomas, 3 clear cell carcinomas and 37 primary benign serous cystadenomas were included. Tumor tissues were formalin fixed, paraffin embedded and sectioned for immunohistochemical assay. HSF1 was detected by rabbit polyclonal antibodies (Cell Signaling Technology, Boston, MA, USA) using a histostain-plus IHC kit (MRBiotech, Shanghai, China). After visualization by 3,3-diaminobenzidine (DAB) staining and counterstaining with hematoxylin, more than 10 fields were observed under a microscope at 200× magnification. Staining extent was semi-quantified by a subjective scoring system: the percentage of stained cells was scored as: 1 (<25%), 2 (25–49%), 3 (50–75%) and 4 (>75%). The staining intensity was subjectively estimated as: 1 (+), 2 (++), 3 (+++). The scores were calculated as "percentage of stained cells" \times "staining intensity." Statistical analyses were performed by *t*-test, and *P* < 0.05 was considered significant.

Cell culture. SKOV3 and HO8910 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Science

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(Shanghai, China). SKOV3 and primary ovarian cancer cells were maintained in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco). HO8910 cells were cultured in RPMI 1640 medium (HyClone) supplemented with 10% FBS.

Cell proliferation assessment. Approximately 1×10^4 cells were seeded per well in a 96-well plate, and allowed to attach for 24 h before exposure to Ly101-4B. Cells were treated in 100 µL fresh medium containing Ly101-4B (or DMSO as mock) for 48 h at 37°C. The number of viable cells remaining was determined by MTT assay. For each assay, test was repeated in at least 6 wells, and every experiment was independently repeated three times.

Flow cytometry. A cell apoptosis assay was performed with the AnnexinV-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, China), according to the manufacturer's instructions. Cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter).

Western blot assay. Cells were harvested and subjected to routine western blot assay, as described previously.⁽¹⁶⁾ The antibodies used in the present study included: anti-human HSF1 rabbit polyclonal antibody (Cell Signaling Technology); anti-human HSP27 rabbit polyclonal antibody (GeneTex); antihuman HSP70 rabbit monoclonal antibody (Epitomics); anti-human HSP90 rabbit monoclonal antibody (Cell Signaling Technology); anti-human phosphatase and tensin homologue (PTEN) rabbit monoclonal antibody (Epitomics); and antihuman β -actin mouse monoclonal antibody (Proteintech). This was followed by incubation with HRP-linked secondary antibodies for 1 h at room temperature. Immunoblot signals were visualized with Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology).

Quantitative PCR. RNA preparation and quantitative PCR were performed as described previously.⁽²⁴⁾ The sequences of primers are as following: 18s rRNA-F: CGGCGACGACCC ATTCGAAC, 18s rRNA-R: GAATCGAACCCTGATTCCCC GTC; *HSF1*-F: CATGAAGCATGAGAATGAGGCT, *HSF1*-R: ACTGCACCAGTGAGAATCAGGA. The comparative concentration was calculated from cycle thresholds, using 18s rRNA

as the internal standard control. Three independent experiments were performed. MiRNA quantification was performed as described previously⁽²⁵⁾ with the specific forward primer for miR-214: GTCGACAGCAGGCACAGACAGGCAGGT.

RNA interference and protein overexpressing. SiRNA set against *HSF1* (siHSF1), *HSP27* (siHSP27), *HSP70* (siHSP70), *HSP90* (siHSP90), miR-214-mimics and 2'-O-me-anti-miR-214 inhibitors were designed and synthesized by GenePharma (Shanghai, China); and the scramble oligonucleotides were validated by the manufacturer. Using Lipofectamine 2000 reagent (Invitrogen), 40 nM siRNA was transfected into SKOV3, according to the manufacturer's recommended protocol. HSF1 overexpressing plasmid (GV-HSF1) was purchased from Genechem (Shanghai, China), and transfected using Lipofectamine 2000 reagent (Invitrogen).

Xenograft model construction and tumor treatment. Animal maintenance and experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiaotong University, Shanghai, China. For the present study, 1×10^6 SKOV3 cells were injected s.c. into the flanks of 4-week old female nude athymic mice (BALB/c-nu /nu; Harlan). After 1 week, tumors were well established and mice were grouped randomly into a treatment group and a mock group (six per group). Ly101-4B (100 mg/kg weight) was administered to the treatment group by i.p. injection twice a week. The mock group received an equal volume of DMSO. At the end of the experiment, mice were killed by cervical dislocation. Organs were excised and fixed in 10% phosphatebuffered formalin and embedded in paraffin. Paraffin sections (5 µm) were analyzed after H&E staining. The Ki-67 protein was detected by immunohistochemical analysis using the Ki-67 cell proliferation detection kit (KeyGEN, Nanjing, China) according to the product manual. HSF1 protein in xenografted tumors was also detected by immunohistochemical analysis with specific antibodies and a histostain-plus IHC kit (MRBiotech, Shanghai, China) according to the manual. After visualization by DAB staining, more than 10 fields were observed under a microscope at 200× magnification.



Fig. 1. Expression of heat shock factor 1 (HSF1) in primary epithelial ovarian cancer. (a) HSF1 expression was examined by immunohistochemical analysis. Photographs indicate the representative results of HSF1 immunostaining in different ovarian tumors. Scale bar, 100 μ m. Imagines in black panes are enlarged to show the staining details. (b) HSF1 expression was evaluated by a semi-quantification scoring system considering both positive percentage and staining intensity. Bars, median values. Statistical significance between malignant and benign groups is indicated (***, P < 0.001).



Fig. 2. Ly101-4B inhibited the proliferation, suppressed the expression of heat shock factor 1 (HSF1) and increased the apoptosis in SKOV3 cells. (a) Structure of Ly101-4B. (b) Quantitative PCR was performed to detect transcripts of *HSF1* in SKOV3 cells. The internal standard of 18s rRNA was used and the relative transcript concentration was normalized to mock cells, which were treated with DMSO. Results represent the average of three independent experiments, and error bars indicate the standard deviations (***, P < 0.001). (c) HSF1 protein was detected by western blot assay. "Mock" represents cells treated with DMSO and "Ly101-4B" represents cells treated with 60 μ M for 48 h. β -actin was applied as a reference. (d) MTT assay was performed to determine the cell viability. An equal amount of DMSO was used as mock control. Each experiment was performed in triplicate, and error bars indicate the standard deviations (***, P < 0.001). (e) SKOV3 cells were treated with 60 μ M Ly101-4B for 48 h, followed by flow cytometry analysis. AnnexinV-FITC(+)/PI(-) indicated the early apoptotic cells, while annexinV-FITC(+)/PI(+) labeled the late apoptotic cells. Numbers indicate the percentages of each cell type. Three independent experiments were performed, and similar results were obtained. The images represents cells treated with DMSO, and "Ly101-4B" represents cells treated with 60 μ M Ly101-4B for 48 h. β -actin was detected by western blot assay. "Mock" represents cells treated with DMSO, and "Ly101-4B" represents cells treated with 60 μ M Ly101-4B for 48 h. β -actin was detected by the performed in triplicate, and error bars indicate the percentages of each cell type. Three independent experiments were performed, and similar results were obtained. The images represents cells treated with DMSO, and "Ly101-4B" represents cells treated with 60 μ M Ly101-4B for 48 h. β -actin was applied as loading control. (g) HSP27, HSP70 and HSP90 protein were analyzed by western blot assay, using β -actin as a referen

Results

Elevated heat shock factor 1 expression in primary human epithelial ovarian tumors. Hyper-expression of HSF1 has been reported and is an aggressiveness marker in certain malignant carcinomas;^(4,6,7,11–13) however, to our knowledge, correlation between HSF1 expression and malignization in ovarian neoplasms has not yet been documented. To investigate the HSF1 expression in ovarian tumors, we examined and compared its expression in malignant (n = 37) and benign (n = 37) tumors. Both the percentage of HSF1-positive cells and the staining intensity were higher in the malignant than in the benign group (Fig. 1a). The expression of HSF1 was semi-quantified using a scoring system (Fig. 1b). Although the scores between the two groups are overlapped in some cases, the medium value for the malignant group (5.73) is significantly higher than for the benign tumors (2.75)



Fig. 3. Ly101-4B inhibited cell proliferation and downregulated heat shock factor 1 (HSF1) in HO8910 and human primary ovarian cancer cells. (a) MTT assay was performed after Ly101-4B treatment for 48 h. An equal amount of DMSO was used as vehicle control. Each sample was assessed in triplicate, and error bars indicate the standard deviations (***, P < 0.001; **, P < 0.01; *, P < 0.05). (b) The mRNA levels of *HSF1* were assessed by quantitative PCR, using 18s rRNA as a reference. The values are the mean \pm SE of three experiments (**, P < 0.01; *, P < 0.05).



Fig. 4. Ly101-4B inhibited the expression of miR-214. (a) MiRNA were detected by quantitative PCR in SKOV3 cells. "Mock" represents cells treated with DMSO; "Ly101-4B" represents cells treated with Ly101-4B. Results represent the average of at least three independent experiments, and the error bars indicate the standard deviations (**, P < 0.01). (b) Mimic RNA was used to mimick miR-214 (mimics); 2'-O-me-anti-miR-214 inhibitor (inhibitor) was used to inhibit miR-214; scramble siRNA or scramble 2'-O-me oligonucleotides (inhibitor control) was used as control. Phosphatase and tensin homologue (PTEN) was analyzed by western blot assay, using β actin as a reference. (c) Mimic RNA was used to mimick miR-214 in SKOV3 cells, and scramble siRNA was used as control. Cell viability was analyzed by MTT assay. (d) SKOV3 cells were transfected with 2'-O-me-anti-miR-214 inhibitor or scramble 2'-O-me oligonucleotides (inhibitor control). Cell proliferation was examined with MTT assay. (e) Cell viability of SKOV3 cells with combination treatment of mimics and Ly101-4B (**, P < 0.01; *, P < 0.05).

(P < 0.001). The correlation between HSF1 expression and malignization of ovarian cancer suggests that HSF1 has a biological significance in ovarian cancer, and could constitute a promising new therapy target.

Anticancer activity and heat shock factor 1-suppressing effect of Ly101-4B in epithelial ovarian cancer cells. In our previous work, we successfully developed a novel 1,2,4-triazole nucleoside analog named Ly101-4B (Fig. 2a), which has been shown to downregulate HSF1 and elicit potent anticancer activities in a human drug-resistant pancreatic cancer model.⁽¹⁴⁾ For the purpose of developing a novel drug candidate against epithelial ovarian cancer, we investigated the anticancer activities and the HSF1-suppressing effect of Ly101-4B in a human epithelial ovarian cancer model.

We first examined HSF1 expression in a human epithelial cisplatin resistant ovarian cancer cell line (SKOV3) following Ly101-4B treatment. After being treated for 24 h, the mRNA level of *HSF1* in SKOV3 cells clearly decreased (Fig. 2b). In addition, the protein expression was considerably depleted after Ly101-4B incubation for 48 h (Fig. 2c). This indicated that Ly101-4B could downregulate HSF1 in epithelial ovarian cancer cells.

Then, we evaluated the anti-proliferative activity of Ly101-4B in SKOV3 cells. As shown in Figure 2d, Ly101-4B treat-

Fig. 5. Ly101-4B could inhibit the expression of miR-214 via suppressing heat shock factor 1 (HSF1). (a) Schematic representation of HSF1 overexpressing plasmid (GV-HSF1). The expression of HSF1, which conjugated with an EGFP tag, is driven by the CMV promoter. (b) HSF1 was detected by western blot assay, using β -actin as a reference. (c) MiR-214 was detected by quantitative PCR after plasmid transfection for 48 h. Results represent the average of at least three independent experiments, and the error bars indicate the standard deviations (**, P < 0.01). (d) HSF1 was knocked down in SKOV3 cells by specific siRNA (siHSF1). The protein expression of HSF1 in SKOV3 cells was detected by western blot assay. β -actin was used as a loading control. (e) Following treatment with siHSF1 and Ly101-4B, the concentration of miR-214 was relatively quantified by quantitative PCR. Scramble siRNA was used as a mock control. Results represent the average of at least three independent experiments, and the error bars indicate the standard deviations (**, P < 0.01). (f) Cell viability in the absence (mock) or with Ly101-4B for SKOV3 cells that were transfected with siHSF1 or scramble siRNA (**, P < 0.01; *, P < 0.05). (g) HSP27, HSP70 and HSP90 were knocked-down by RNAi, and detected by western blot assay. (h) MiR-214 was detected by quantitative PCR after siRNA transfection.



ment efficiently inhibited cell proliferation, whereas cisplatin, the clinical reference control, exhibited only a moderate effect in SKOV3 cells. The apoptosis-inducing activity of Ly101-4B was also investigated. After Ly101-4B treatment the percentage of early apoptotic cells increased remarkably, from 5.0 to 19.0%, and the late apoptotic percentage was slightly increased, from 1.8 to 4.8% (Fig. 2e). The induced apoptosis by Ly101-4B was confirmed by caspase9 protein detection, which showed that the cleaved form of caspase9 (p35 segment) accumulated after incubation with Ly101-4B (Fig. 2f).

As HSF1 regulates the transcription of heat shock protein (HSP) genes we also wanted to inspect the expression of HSP27, HSP70 and HSP90 after Ly101-4B treatment. Similar to the results previously obtained in pancreatic cancer cells,⁽¹⁴⁾ considerably decreased protein expression of HSP27, HSP70 and HSP90 was detected in SKOV3 cells (Fig. 2g). The simultaneous decrease in expression of these HSP following downregulation of HSF1 highlights the direct consequence of the downregulation of the HSF1-mediated HSR pathway.

Encouraged by the above promising results, we further assessed the anticancer activity of Ly101-4B in another ovarian cancer cell line (HO8910) and in primary human ovarian cancer cells (hOVCC). HOVCC were separated from three patients who had been diagnosed with stage III grade 2-3 serous adenocarcinoma according to the International Federation of Gynecology and Obstetrics classification. Figure 3a shows that 48 h treatment with Ly101-4B led to a significant reduction in viable cells in both HO8910 and hOVCC. Due to the diversity of patients, the inhibiting efficiencies of both cisplatin and Ly101-4B were not uniform among individual primary cell samples; however, overall the efficiency of Ly101-4B was consistently much greater than that of cisplatin (Fig. 3a). To study the effect of Ly101-4B on HSF1, we examined the RNA expression of HSF1 in HO8910 and hOVCC that were treated with Ly101-4B. Similar to the result in SKOV3 cells, Ly101-4B treatment led to a decrease in *HSF1* mRNA concentrations in both HO8910 and hOVCC (Fig. 3b). These data revealed that Ly101-4B could efficiently downregulate HSF1 in both immortalized cell lines and primary hOVCC.

Ly101-4B suppressed miR-214 through a heat shock factor 1-mediated mechanism. There is increasing evidence that certain miRNA play critical roles in ovarian cancer pathogenesis and progression, indicating that miRNA are potential targets for ovarian cancer therapy.^(20–22) To gain insight into the anti-cancer mechanism of Ly101-4B, we investigated whether Ly101-4B could affect the expression of miRNA in ovarian cancer. Following Ly101-4B treatment, we quantified several miRNA, which have been reported to be dysregulated in ovarian cancer (Fig. 4a).^(20–23,26) Among these miRNA, we identified an attractive miRNA (miR-214), the expression of which decreased the most following Ly101-4B treatment. MiR-214 has been reported to be overexpressed in human ovarian epithelial tumors and its aberrant expression appears to be associated with highgrade and late-stage ovarian tumors.⁽²³⁾ Considering its important cellular functions in epithelial ovarian cancer, in the present study we focused our attention on miR-214.

To further validate the effects of miR-214 on ovarian cancer cell proliferation, we used miR-214-mimics to elevate the miR-214 concentration, and used a 2'-O-me-anti-miR-214 inhibitor to knock down miR-214 in SKOV3 cells. First, the expression of PTEN, a target gene of miR-214,⁽²³⁾ was evaluated to validate the function of miR-214-mimics and 2'-O-me-anti-miR-214 inhibitor (Fig. 4b). Cell viability analysis showed that miR-214 overexpression promoted cell proliferation (Fig. 4c), while inhibiting miR-214 reduced cell survival (Fig. 4d). This result is consistent with what is reported previously in the literature,⁽²³⁾ confirming the proliferation-regulating role of miR-214 in ovarian cancer cells. Interestingly, we found that the reduced growth of Ly101-4B-treated cells was partially recovered by overexpressing miR-214 (Fig. 4e).



Fig. 6. Ly101-4B significantly reduced the growth of xenograft tumors in vivo. (a) Tumor volume was calculated as length \times width² \times 0.5236. Results represent the mean sizes of xenografted tumors (n = 6). Error bars indicate standard deviations. "Ly101-4B" represents the testing group; "mock" indicates the control group receiving an equal volume of DMSO. A photo of the tumors is also included. The uppermost tumors were separated from the control group, and the tumors below were separated from the treatment group. *, P < 0.05; **, P < 0.01. (b) The weights of mice were recorded during the Ly101-4B therapy. Results indicate the mean weight (n = 6), and error bars represent the standard deviations. (c) Ki-67 immunohistology was detected by analysis. Ki-67-positive cells were visualized with 3,3diaminobenzidine (brown). The slides were counterstained with hematoxylin. Scale bar, 100 $\mu\text{m}.$ (d) The expression of heat shock factor 1 (HSF1) was detected by an immunohistological assay. For each group, three tumors were included for analysis and more than 10 fields were observed. Photographs indicate the representative results. Scale bar, 100 µm. (e) The concentrations of HSF1 and miR-214 in xenografted tumors were relatively quantified by quantitative PCR. Results represent the mean expression level of two tumors for each group (*, P < 0.05). (f) The mouse organs including heart, lung, spleen, liver and kidney were sectioned and stained by H&E. The organs from the control group are shown in the first column (mock), and the organs from the treatment group are shown in the second column (Ly101-4B). Scale bar, 100 μm.

As mentioned above, Ly101-4B could downregulate HSF1. We wondered whether the suppression of miR-214 by Ly101-4B is HSF1-mediated. An HSF1 overexpressing plasmid (GV-HSF1, Fig. 5a) was constructed to express exogenous HSF1 protein, which is conjugated with an EGFP tag in SKOV3 cells (Fig. 5b). As is evident in Figure 5c, the miR-214 concentration was simultaneously increased after HSF1 overexpression. In contrast, we artificially knocked down HSF1 in SKOV3 cells (Fig. 5d), and then examined the expression of miR-214 (Fig. 5e). Results revealed that miR-214 concentration was decreased following HSF1 knockdown, consistent with what obtained after Ly101-4B treatment. When cells were treated with Ly101-4B after HSF1 knockdown, both HSF1 expression and miR-214 concentration was depleted even more (Fig. 5d,e). At the same condition, proliferation was most efficiently impaired by the combination treatment of Ly101-4B and siHSF1 (Fig. 5f).

Given the fact that Ly101-4B could inhibit HSP simultaneously, we wanted to check the correlation between HSP and miR-214 expression. As shown in Figure 5g and h, we knocked down those proteins by RNAi (RNA interfere) but did not find significance alteration of miR-214 expression. Consequently, the regulation function of HSF1 on miR-214 expression is independent on those HSP.

Anti-tumor activity of Ly101-4B in vivo. To further assess its activity in vivo, we evaluated the anti-tumor effect of Ly101-4B in a xenograft model. Athymic mice bearing SKOV3-xenografted tumors were treated with Ly101-4B twice a week at a dose of 100 mg/kg by i.p. injection. After 4 weeks' treatment, the tumor growth was significantly retarded compared with the control group, which was administered with the vehicle DMSO (Fig. 6a). These data revealed that Ly101-4B could efficiently inhibit ovarian tumor growth *in vivo*. In addition, we did not

observe any discernible weight alterations of treated mice (Fig. 6b).

After drug treatment, the expression of Ki-67, a cell proliferation marker,⁽²⁷⁾ was determined by immunohistological assay in the separated tumors (Fig. 6c). In the Ly101-4B-treated group, few Ki-67 positive cells were detected, whereas in mock treated tumors, a high proportion of Ki-67 positive cells was clearly detected (Fig. 6c). This result further validates the anti-tumor activity of Ly101-4B against ovarian cancer.

As demonstrated *in vitro*, Ly101-4B downregulated HSF1 and, consequently, suppressed the expression of miR-214. To verify whether this anti-tumor mechanism is retained *in vivo*, we examined HSF1 expression and miR-214 biogenesis following treatment with Ly101-4B. As revealed in Figure 6d, both HSF1 positive cells and staining intensity of HSF1 were sharply decreased in tumors issued from mice treated with Ly101-4B. The mRNA of *HSF1* was also depleted in Ly101-4B-treated tumors, in addition to a consequent expression reduction of miR-214 (Fig. 6e), consistent with the *in vitro* result.

We also examined the acute toxicity of Ly101-4B by pathological examination of major mouse organs using H&E staining. As shown in Figure 6f, we did not observe any noticeable histopathological changes in the corresponding organs, illustrating that Ly101-4B did not present acute toxicity in mice. Altogether, these data demonstrate that Ly101-4B significantly contributes to the inhibition of tumor progression without adverse effects *in vivo*.

Discussion

Heat shock factor 1 is regarded as a promising candidate for targeted cancer therapy, $^{\rm (4-7)}$ and agents that are capable of tar-

geting HSF1 to elicit anticancer activity have raised considerable interest recently.⁽⁵⁾ Here, we revealed that HSF1 expression was significantly higher in malignant than in benign human epithelial ovarian tumors, suggesting that HSF1 also could be a therapy target for ovarian cancer treatment.

Ly101-4B is a novel triazole nucleoside analog and has been identified as a HSF1 repressor in drug-resistant pancreatic cancer cells.⁽¹⁴⁾ In the present study, we investigated the HSF1suppressing effect and the anti-tumor activity of Ly101-4B in a human epithelial ovarian cancer model. In both immortalized cells (SKOV3 and HO8910) and hOVCCs, Ly101-4B efficiently suppressed the expression of HSF1 and significantly depleted cell viability. Finally, the anti-tumor activity and HSF1-suppressing effect of Ly101-4B were validated in a xenografted mouse model without adverse effect, suggesting that Ly101-4B provides a safe clinical application against ovarian cancer.

Recent studies have highlighted that HSF1 controls the expression of genome-wide genes belonging to multiple facets of oncogenic processes.^(6,7) These studies provide insight into the critical cellular roles of HSF1; nevertheless, the revealed regulatory functions of HSF1 are restricted to protein-coding genes. Besides coding genes, the expression of non-coding miRNA is also under tight control, and their dysregulation plays a critical role in tumor pathogenesis and development. Considering the extensive regulatory role of HSF1 and the significant function of miRNA in tumors, we wondered whether Ly101-4B could alter the expression of miRNA through a HSF1-mediated pathway. To answer this question, we examined the expression of a series of miRNA whose

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dysregulation has been reported in ovarian cancer and identified miR-214 as being repressed by Ly101-4B in both cultured SKOV3 cells and xenografted mouse tumors. Interestingly, when HSF1 was knocked down by RNAi, a depleted concentration of miR-214 was observed, indicating that the miR-214 suppressing effect of Ly101-4B was HSF1mediated. This result suggests that HSF1 also possesses the ability to regulate miRNA biogenesis.

Based on the results discussed above, we conclude that the novel nucleoside analog Ly101-4B is an appealing drug candidate for ovarian cancer therapy. Furthermore, we have demonstrated that the regulatory functions of HSF1 extend to coordinating the biogenesis of non-coding miRNA in addition to orchestrating the transcriptional program of coding mRNA.

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Disclosure statement

The authors have no conflict of interest.

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