Bioorganic & Medicinal Chemistry Letters 19 (2009) 959–961

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# HY251, a novel cell cycle inhibitor isolated from *Aralia continentalis*, induces $G_1$ phase arrest via p53-dependent pathway in HeLa cells

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ARTICLE INFO

*Article history:*
Received 24 June 2008
Revised 17 November 2008
Accepted 24 November 2008
Available online 27 November 2008

*Keywords:*
*Aralia continentalis*
HY251
Cell cycle progression
HeLa cells
p53
G1 phase arrest

ABSTRACT

This study was aimed to elucidate the novel structure of HY251 isolated from the roots of *Aralia continentalis* and to evaluate its detailed inhibition mechanisms on cell cycle progression in HeLa cells. The structure of HY251 was elucidated based on the interpretation of the NMR spectra, as 3-propyl-2-vinyl-1,2,3,3a,3b,6,7,7a,8,8a-decahydrocyclopenta[*a*]indene-3,3a,7a,8a-tetraol. The flow cytometric analysis revealed an appreciable $G_1$ phase arrest in HeLa cells treated with 100 μM of HY251. This HY251-induced $G_1$ phase arrest is associated with decreased expression of cyclin D3 and up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$, via p53 phosphorylation at Ser-15 by transcriptional up-regulation of *ATM*, which resulted in increased hypophosphorylated pRb in HeLa cells.

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Serine/threonine protein kinases play pivotal roles in the signal transduction pathways that control the proliferation and differentiation of eukaryotic cells.[1,2] The cyclin-dependent kinases (CDKs), which belong to this Ser/thr protein kinase family, are regulators of eukaryotic cell cycle progression in cooperation with various endogenous cyclins and cyclin-dependent kinase inhibitors (CKIs), including the $p21^{CIP1}$, $p27^{KIP1}$, and $p16^{INK4}$.[3,4] Achieving cell cycle control is an important goal in the treatment of diseases characterized by uncontrolled cell proliferation, such as a cancer, that results from some fault in cell cycle progression. Consequently, the CDKs could be important molecular targets for such therapeutic intervention, and inhibition of CDK activity may be particularly useful in the treatment of cancer.[5–12]

In the course of our screening for a novel inhibitor of cell cycle progression as an anticancer drug candidate, we found HY251 from the roots of *Aralia continentalis*, which is called *Dokwhal* (獨活), the traditional medicinal herb spread widely in northeastern Asian region. Many constituents from its root extracts, including essential oils and diterpene acid, have been isolated as active components for antioxidant, anti-inflammatory, analgesic, sedative, antifungal, anti-thrombotic, and growth inhibition.[13,14]

To isolate an active compound as a cell cycle inhibitor from the 70% methanol extract of the roots (dry weight: 100 g), the extract was filtrated and concentrated in vacuo and the remnant subsequently extracted with ethyl acetate (EtOAc). The EtOAc fraction, which showed potent anti-proliferative effect on the HeLa cells, was further fractionated by silica-gel column chromatography (DAVISIL® 60–200 μm, Grace Vydac, Hesperia, CA, USA) eluted with hexane, EtOAc, and methanol. The fraction eluted with hexane:EtOAC (8:2) showed potent anti-proliferative effect and further fractionated using the preparative-HPLC ($C_{18}$ column, 250 × 22 mm, Waters, Oregon, USA) eluted with 57% acetonitrile/water (flow rate: 20 ml/min). As a result, 5.5 mg of HY251 was purified (total yield: 0.005%) and used for the structure elucidation.

The structure of HY251 was determined using NMR spectroscopy.[15] Seventeen peaks were observed in the $^{13}C$ NMR spectrum of HY251. Their types were determined by DEPT experiments: one methyl, seven methylene, five methine, and four quaternary carbons. The correlations between $^{1}H$ and $^{13}C$ were decided from the HMQC spectrum. Based on the interpretation of the COSY spectrum, the correlations among five $^{1}H$ peaks at 1.39, 2.12, 5.15, 5.46, and 5.56 ppm, and their partial structure could be determined. This result was confirmed by the long-ranged couplings obtained from HMBC. Because the $^{1}H$ peak at 5.15 ppm was long-range coupled with the $^{13}C$ peak at 66.9 ppm in the HMBC spectrum, it was determined to be C-3a. Likewise, the backbone structure of HY251 was

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doi:10.1016/j.bmcl.2008.11.087

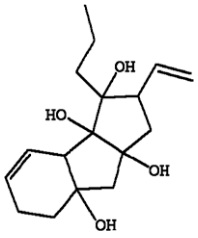


**Figure 1.** The chemical structure and nomenclature of HY251, 3-propyl-2-vinyl-1,2,3,3a,3b,6,7,7a,8,8a-decahydrocyclopenta[*a*]indene-3,3a,7a,8a-tetraol.

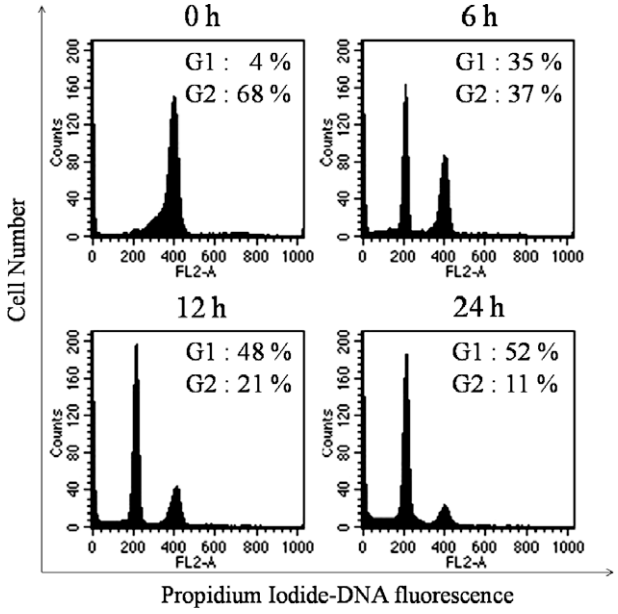


**Figure 2.** Flow cytometric analysis of the HeLa cells treated with 100 μM of HY251 with synchronization of the cells at $G_2$ phase using nocodazole. The HeLa cells were treated with 50 ng/ml nocodazole for 12 h. The cells were then washed three times and treated with 100 μM of HY251. The cells were stained with Propidium Iodide (PI) and the nuclei analyzed for their DNA content by flow cytometry using Cell Quest software. A total of 10,000 nuclei were analyzed from each sample.

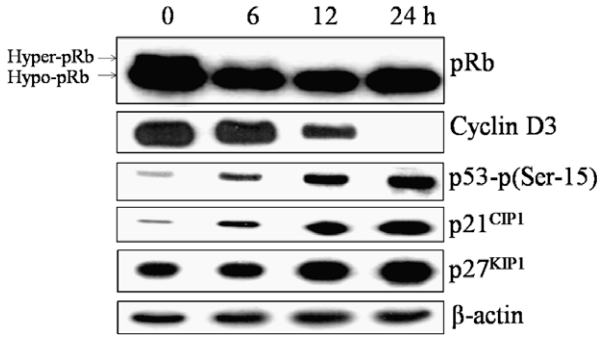


**Figure 3.** Western blot analysis of the HeLa cells treated with 100 μM of HY251 on the cell cycle regulatory proteins. β-Actin served as the internal control.

determined based on the interpretation of the HMBC spectrum. The propyl group attached to C-3 and the vinyl group attached to C-2 could be confirmed by the $^1H$–$^1H$ correlation obtained from the COSY spectrum. All NMR data and their complete assignments are shown in the Supplementary materials.[16] As a result, HY251 is a novel compound as 3-propyl-2-vinyl-1,2,3,3a,3b,6,7,7a,8,8a-decahydrocyclopenta[*a*]indene-3,3a,7a,8a-tetraol (Fig. 1).

To investigate the effects of HY251 on cell cycle progression in the HeLa cells, we measured DNA content of the HeLa cell treated with 100 μM of HY251 using flow cytometric analysis. However, to rule out the possibility that HY251 could be associated with specific apoptotic induction on the cells arrested only at $G_2$/M phase, we used nocodazole, a microtubule polymerization inhibitor, which arrests cells in $G_2$/M phase without any cytotoxicity at the concentration 50 ng/ml used in current study. If nocodazole is treated to the cells, the $G_2$/M phase population is dramatically enhanced (typically, more than 70%). Thus, nocodazole is used to quantitatively measure cells escaping a $G_2$/M checkpoint, if cells treated with chemicals induced $G_1$ phase arrest of the cell cycle.

As shown in Figure 2, the percentage of cell population in $G_1$ phase was increased from 4% at 0 h to 52% at 24 h, and the cells in the $G_2$/M phase were decreased dramatically from 68 to 11% at 24 h. Therefore, the HeLa cells treated with 100 μM of HY251 showed an appreciable $G_1$ phase arrest in a time-dependent manner.

The kinase activity of CDKs and activation of cyclins is a driving force in the progression of the cell cycle. Therefore, to determine if HY251 can selectively inhibit CDK kinases of $G_1$ phase, we used endogenous kinetic assays for CDK2, CDK4 and GSK3β obtained using immunoprecipitation from the HeLa cells. GSK3β kinase was used as a control Ser/thr protein kinase. However, the treatment of HY251, interestingly, showed no inhibition on in vitro kinetic assays of CDKs and GSK3β tested so far.

Furthermore, we also examined whether this compound can bind at ATP-binding pocket of Ser/thr protein kinases. We assessed a computational in silico docking study using the X-ray crystallographic structure of CDK2 with HY251, because the crystal structure of CDK4 is not available. This binding mode study of HY251 with CDK2 demonstrated no interaction in the ATP-binding pocket of CDK2 (data not shown).

Accordingly, to confirm the detailed mechanism of cell cycle inhibition at $G_1$ phase, we assessed Western blots to check the correlation between the effect of HY251 on $G_1$ phase arrest and the regulation patterns of cell cycle regulators, such as cyclins and CKIs. We examined CKI gene expression and the levels of phosphorylation of pRb after HY251 treatment. As shown in Figure 3, we found that induction of $G_1$ phase arrest was associated with the appreciable decreased expression of cyclin D3, and up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$, which have key roles in regulating the entry of cells at the G1/S transition checkpoint, and followed apparent increase of hypophosphorylation form of pRb in a time-dependent manner at 6–24 h in the HeLa cells. In opposite, cyclin E and other notable CKI, such as $p16^{INK4}$, were not affected by HY251 treatment (data not shown). Therefore, $G_1$ phase arrest induced by HY251 is involved in CDK4 and CDK2 inactivation through down-regulation of cyclin D3 and up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$, respectively.

Recently, it has been reported that chemical agents that damage DNA act through posttranslational modifications of p53 and activate its downstream targets in various human cancer cells.[17–19] These p53 modifications, such as phosphorylation at Ser-15, were induced through activation of an ATM signaling pathway including up-regulation of ataxia telangiectasia-mutated (*ATM*) gene.[18] Furthermore, this specific p53 phosphorylation at Ser-15 in turn results in acetylation of p53. Finally, these posttranslational modifications of p53 are directly responsible for $p21^{CIP1}$ expression because the binding activity of acetylated p53 to the $p21^{CIP1}$ promoter.

As shown in Figure 3, we found that increased phosphorylation of p53 at Ser-15 by HY251 represents an early response to a variety of genotoxic stresses and displays reduced binding to the inhibitor protein, MDM2, which was targeted for ubiquitination that inhibits its transactivating function.[20,21]

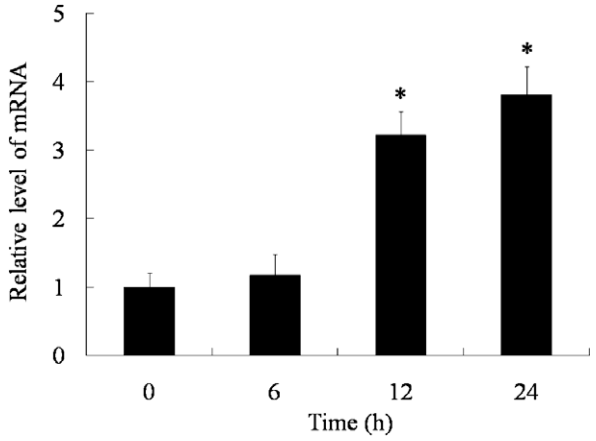


**Figure 4.** Effect of HY251 on *ATM* gene expression examined by the quantitative real-time PCR. Data indicated the relative Ct value for *ATM* gene when compared to the reference gene (*GAPDH*). Data represent means±SD of three different experiments and evaluated by Student's *t*-test. Values of $^{*}p < 0.05$ were considered to be significant.

In addition, p53 activation could be explained as a reason for the increased cellular level of $p21^{CIP1}$ due to the almost identical pattern of increased protein levels in a time-dependent manner in the HeLa cells (Fig. 3).

Furthermore, to examine the involvement of *ATM* gene in phosphorylation of p53 at Ser-15,[22] we performed to check the *ATM* gene expression level using quantitative real-time PCR (Fig. 4). In general, steady-state levels of ATM protein varied from undetectable in most AT (ataxia telangiectasia) cells to highly expressed in the HeLa, U2OS, and normal human fibroblasts. However, ATM protein level remained constant throughout the cell cycle progression and did not change in response to serum stimulation and ionizing radiation.[23]

Interestingly, we found that *ATM* gene expression at 12 and 24 h after HY251 treatment were significantly increased 3.1- and 3.8-fold than that of control, respectively (Fig. 4). These data show that HY251 induces transcriptional up-regulation of *ATM* gene, which elicits a specific p53 phosphorylation at Ser-15 to induce $p21^{CIP1}$ expression in the HeLa cells.

In conclusion, the current study showed that HY251, a novel cell cycle inhibitor isolated from the roots of *A. continentalis*, induces the $G_1$ phase arrest in the HeLa cells. We suggest that the cell cycle arrest may be mediated by the decreased expression of cyclin D3, a $G_1$ phase specific cyclin, caused CDK4 inactivation and phosphorylation of p53 at Ser-15 by ATM kinase, as well as the increased expression of $p21^{CIP1}$ and $p27^{KIP1}$, caused inactivation of CDK2. These results indicate that HY251 can be a candidate for novel anticancer agent, therefore, in vivo study, such as xenograft animal model test, and *p53* and *ATM* gene knock-out using *si*RNA technique should be performed in a future.

## Acknowledgments

This work was partly supported by Seoul R&BD Program (10580) and by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea Government (MEST) (R13-2008-026-01000-0). Ha Lim Oh and Haeyoung Lim contributed equally to this work.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.087.

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