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Identification of organoselenium compounds that possess chemopreventive properties in human prostate cancer LNCaP cells

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ABSTRACT

The process of cancer development consists of three sequential stages termed initiation, promotion, and progression. Oxidative stress damages DNA and introduces mutations into oncogenes or tumor suppressor genes, thus contributing to cancer development. Cancer chemoprevention is defined to prevent or delay the development of cancer by the use of natural or synthetic substances. In the present study, we synthesized a series of organoselenium compounds and evaluated their possible chemopreventive properties in human prostate cancer LNCaP cells. Among 42 organoselenium compounds tested, two compounds, 3-selena-1-dethiacephem **13** and 3-selena-1-dethiacephem **14** strongly activated the Nrf2/ARE (antioxidant response element) signaling and thus markedly increased expression of heme oxygenase-1 (HO-1), a phase II antioxidant enzyme. Translocation of Nrf2 to the nucleus preceded HO-1 protein induction by two compounds. The intracellular ROS level was strongly reduced immediately after treatment with these compounds, showing that they are potent antioxidants. Finally, both compounds inhibited cell growth via cell cycle arrest. Our findings suggest that compounds **13** and **14** could not only attenuate oxidative stress through Nrf2/ARE activation and direct ROS scavenging but also inhibit cell growth. Thus, these compounds possess the potential as pharmacological agents for chemoprevention of human prostate cancer.

1. Introduction

The process of cancer development is divided into three sequential stages: initiation, promotion, and progression.[1] During the initiation stage, carcinogens or their active metabolites damage DNA and induce mutations in oncogenes or tumor suppressor genes, leading to immortalization of normal cells. This is followed by the promotion stage in which immortalized cells are encouraged to expand. During the promotion stage, as a result of accumulation of genetic damages due to carcinogens, immortalized cells acquire malignant features and enter the progression stage. The initiation occurs frequently and quickly, whereas the promotion stage is more lengthy. Thus, carcinogens cause genomic transformation and affect both the initiation and promotion stages of cancer development.

Chemoprevention refers to the use of synthetic or naturally-occurring agents to prevent, reverse or suppress the process of cancer development, which has been increasingly appreciated as an effective approach for the management of a number of types of cancer.[2] In prostate cancer, various dietary factors such as selenium, vitamin E and lycopene have been subjected to clinical trials.[3] Several studies revealed that vitamin E and selenium were associated with a reduction in the incidence of prostate cancer.[4–6] These findings lead to the Selenium and Vitamin E Chemoprevention Trial (SELECT) sponsored by the National Institutes of Health.[7] In 2008, however, this clinical study was prematurely discontinued due to a lack of benefit from either selenium or vitamin E in reducing prostate cancer risk.[8]

Oxidative stress is defined as an imbalance between the cellular antioxidant defense systems and endogenously or exogenously

Abbreviations: ARE, antioxidant response element; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; Keap1, kelch-like ECH-associated protein 1

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generated pro-oxidants. Excess amounts of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical damage DNA, protein, and lipid, leading to cellular dysfunction. Similarly to carcinogens, oxidative stress causes gene mutations and contributes to cancer development. Thus, alleviation of oxidative stress could be beneficial for chemoprevention.[9]

Nrf2 (nuclear factor E2-related factor 2)/ARE (antioxidant response element) signaling regulates expression of phase II antioxidant and detoxifying enzymes such as glutathione peroxidase (GPX), γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase (NQO-1), and glutathione S-transferase (GST).[10] Transcription factor Nrf2 binds to and activates the ARE enhancer present within the promoter region of genes encoding for phase II enzymes. Under normal conditions, Nrf2 associates with cytoplasmic Keap1 (kelch-like ECH-associated protein 1) and undergoes constitutive proteasome-dependent degradation. When the cysteine sulfhydryl groups are covalently or oxidatively modified by electrophiles or ROS, Keap1 alters its conformation, resulting in Nrf2 release and its translocation into the nucleus. Phytochemicals such as curcumin and sulphoraphane activate the Nrf2/ARE signaling via interacting with Keap1.[11,12] Modification of Nrf2 phosphorylation by protein kinases such as Akt and ERK enhances its translocation into the nucleus.[13] ROS are scavenged by GPX and the cellular antioxidant, glutathione, which is synthesized by the consecutive actions of γ-GCS and glutathione synthetase. HO-1 catalyzes heme to biliverdin, which is then converted to bilirubin that acts as a potent antioxidant. In addition, NQO-1 and GST are capable of detoxifying carcinogens. Therefore, activation of Nrf2/ARE signaling and induction of phase II enzymes could be a promising strategy to control the initiation and promotion stages of cancer development.[2,14]

In the present study, we synthesized a series of organoselenium compounds and evaluated possible chemopreventive properties in human prostate cancer LNCaP cells. Using firefly luciferase reporter assays, we identified that two compounds, 3-selena-1-dethiacephem **13** and 3-selena-1-dethiacephem **14**, strongly activate Nrf2/ARE signaling and increase HO-1 expression. These two compounds also possessed a potent antioxidant activity. Furthermore, both compounds **13** and **14** were capable of inhibiting cell growth via cell cycle arrest. Our findings suggest that these organoselenium compounds could attenuate oxidative stress through Nrf2/ARE activation and direct ROS scavenging and inhibit cell growth, playing multiple roles in chemoprevention.

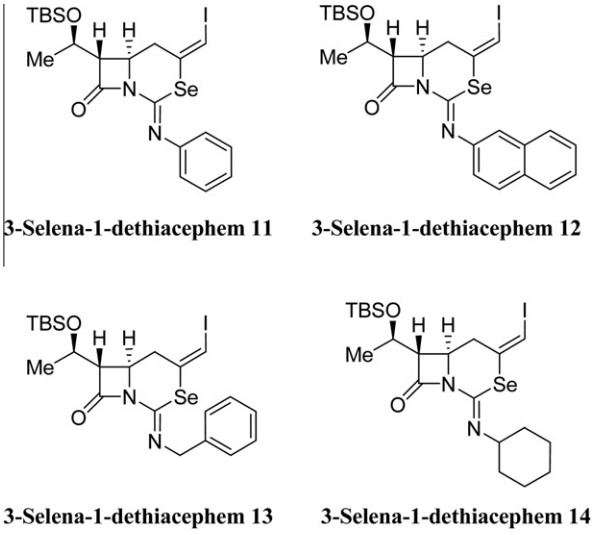

Figure 1. Chemical structures of organoselenium compounds. Chemical structures of 3-selena-1-dethiacephem **11**, **12**, **13**, and **14** are shown.

2. Results

2.1. Identification of organoselenium compounds capable of increasing ARE enhancer activity and HO-1 protein expression

First of all, we determined the ARE enhancer activity of 42 organoselenium compounds (Supplementary Fig. 1) in hormone-sensitive prostate cancer LNCaP cells using firefly luciferase reporter assays. Among all compounds tested, 3-selena-1-dethiacephem **13** and **14** (Fig. 1) increased the ARE enhancer activity by about sixfold (Fig. 2A). However, ARE activation by these compounds was less than twofold in hormone-refractory prostate cancer PC3 and DU145 cells. Consistent with the ARE enhancer activities, induction by compounds **13** and **14** of protein expression of HO-1, one of target genes regulated by Nrf2/ARE signaling, was much higher in LNCaP cells than in PC3 and DU145 cells (Fig. 2B). These results indicate that the organoselenium compounds **13** and **14** are capable of activating Nrf2/ARE signaling in prostate cancer cells, especially in hormone-sensitive LNCaP cells. Thus, we further analyzed these two compounds in LNCaP cells in the subsequent studies.

2.2. Structure–function relationships among organoselenium compounds

Among 42 organoselenium compounds we synthesized, most of the organoselenium compounds possess either no exocyclic double bond or only one exocyclic double bond, that is, carbon–nitrogen double bond. The presence of the *exo*-olefin in the compounds **11–14** or *endo*-olefin in the compound **32** (Supplementary Fig. 1) is found to be important to activate Nrf2/ARE signaling. 3-Selena-1-dethiacephem **11** and **12** were most similar to compounds **13** and **14** in chemical structures (Fig. 1) and possesses two exocyclic carbon–carbon and carbon–nitrogen double bonds with *E*- and *Z*-configurations, respectively. The compounds **11** and **12** possess aromatic substitution at the imine nitrogen whereas the compounds **13** and **14** have aliphatic substitution at the imine nitrogen. As shown in Fig. 3, ARE enhancer activation by compounds **11** and **12** was much lower than that by compounds **13** and **14**, suggesting that the presence of the *exo*-olefin (carbon–carbon double bond) as well as the aliphatic substitution at the imine part is critical for these compounds to activate Nrf2/ARE signaling.

2.3. Nuclear translocation of Nrf2 by compounds 13 and 14

Both compounds **13** and **14** increased HO-1 expression in a dose-dependent manner (Fig. 4A). As shown in Fig. 4B, HO-1 induction by compounds **13** and **14** was first seen 6 h after treatment and lasted until 24 h. After nuclear translocation, Nrf2 binds to the ARE enhancer located in the promoter region of genes encoding phase II enzymes, resulting in up-regulation of their expression. We determined the Nrf2 levels in nuclear and cytoplasmic fractions after treatment with compounds **13** and **14**. Translocation of Nrf2 to the nucleus was first seen 0.5 and 1 h after treatment with compounds **13** and **14**, respectively, and then nuclear Nrf2 levels gradually increased until 6 h (Fig. 5A). Furthermore, co-transfection with a dominant-negative mutant of Nrf2 diminished the ARE enhancer activity induced by compounds **13** and **14** (Fig. 5B). These results suggest that HO-1 protein induction by compounds **13** and **14** is preceded by Nrf2 nuclear translocation and requires Nrf2 binding to the ARE.

2.4. Suppression of intracellular ROS levels by compounds 13 and 14

One of the major mechanisms of Nrf2/ARE activation is modification of the cysteine sulfhydryl groups of Keap1 by ROS, which

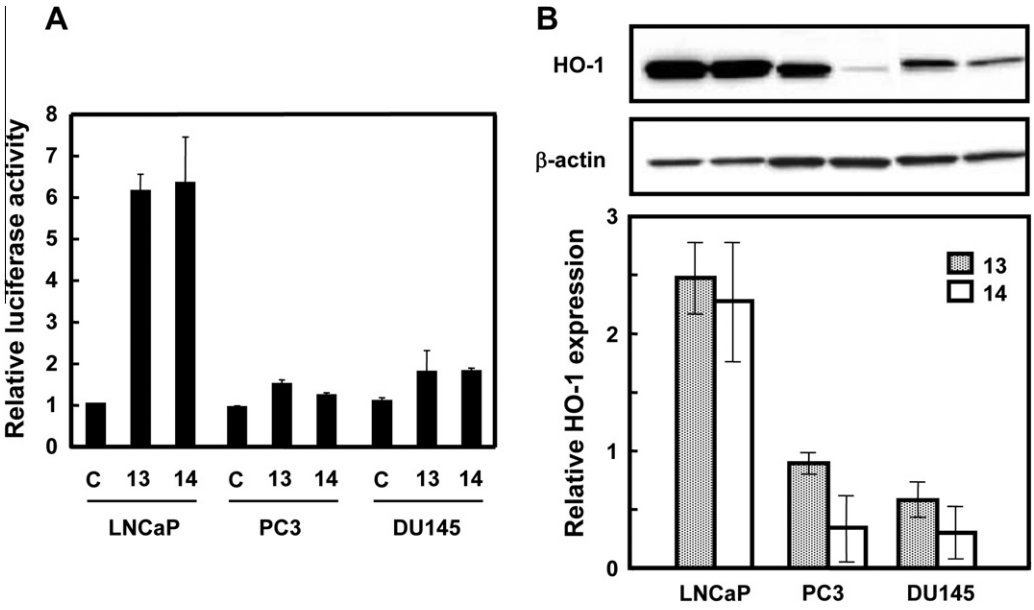

Figure 2. ARE enhancer activation and HO-1 induction by compounds **13** and **14**. (A) LNCaP, PC3 and DU145 cells were transfected for 24 h with the ARE firefly luciferase reporter vector (500 ng) along with the Renilla luciferase control vector (5 ng) and then treated without (DMSO) or with compound **13** or **14** (5 μM) for 24 h, followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to the DMSO control (C). Data are expressed as mean ± SEM (n = 3). (B) LNCaP, PC3, and DU145 cells were treated without (DMSO) or with compound **13** or **14** (5 μM) for 24 h. Then, cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin. The HO-1 expression was normalized to β-actin expression. Data are expressed as mean ± SEM (n = 3).

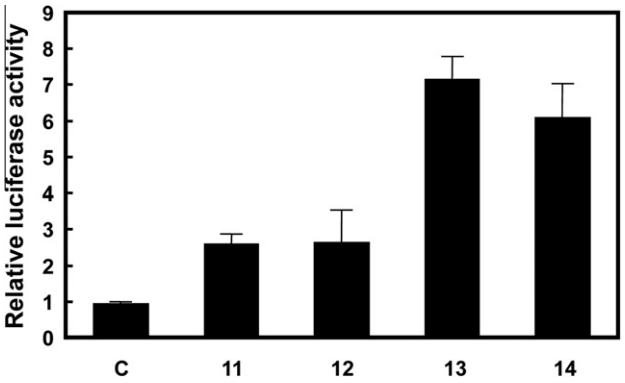

Figure 3. ARE enhancer activation by compounds **13** and **14** and their structurally-related compounds **11** and **12**. LNCaP cells were transfected for 24 h with the ARE firefly luciferase reporter vector (500 ng) along with the Renilla luciferase control vector (5 ng) and then treated without (DMSO) or with compound **11**, **12**, **13**, or **14** (5 μM) for 24 h, followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to the DMSO control (C). Data are expressed as mean ± SEM (n = 3).

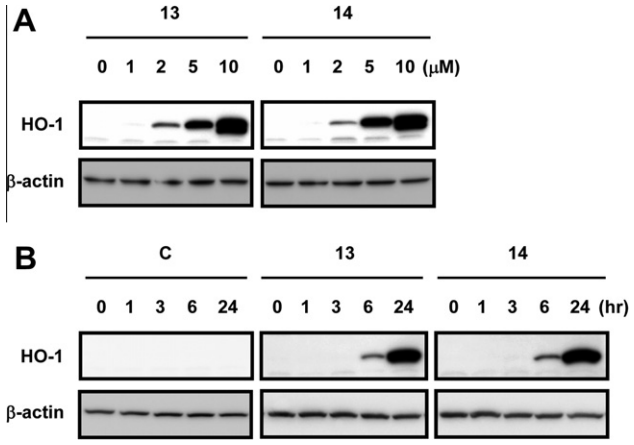

Figure 4. Dose- and time-dependent expression of HO-1 by compounds **13** and **14**. (A) LNCaP cells were treated for 24 h without (DMSO) or with increasing concentrations of compound **13** or **14** (1, 2, 5, and 10 μM). (B) LNCaP cells were treated for indicated times (0, 1, 3, 6, and 24 h) without (DMSO) or with compound **13** or **14** (5 μM). After treatments, cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown.

leads to Nrf2 release and its translocation into the nucleus. We investigated whether compounds **13** and **14** might increase ROS production. However, the intracellular ROS level was markedly decreased 5 min after treatment with compounds **13** and **14**, which remained suppressed until about 1 h and then returned to the basal level at 12 h (Fig. 6). These results suggest that compounds **13** and **14** possess a strong antioxidant activity and that ROS generation is not involved in Nrf2/ARE activation by these compounds.

2.5. Inhibition of cell growth by compounds 13 and 14

Several studies indicate that some organoselenium compounds inhibit cell cycle and/or induce apoptosis, contributing to inhibition of promotion and progression of cancer.[15] Here we investigated whether compounds **13** and **14** could inhibit cell growth and induce apoptosis. Both compounds changed the cell morphology to a flattened and enlarged shape and significantly decreased cell number at 24 and 48 h after treatment (Fig. 7A). However, no apoptotic cells were detected as determined by Hoechst 33342 staining (data not shown). Consistent with cell growth inhibition observed, expression of cyclins B1 and D1 was markedly decreased 48 h after treatment with compounds **13** and **14**, while that of p21 was not affected, suggesting that cell cycle arrest was induced by these compounds (Fig. 7B). These results indicate that compounds **13** and **14** have a capacity to inhibit cell growth through induction of cell cycle arrest. Finally, we studied the possible interaction between HO-1 induction and cell growth inhibition caused by compounds **13** and **14**. As shown in Fig. 8A,

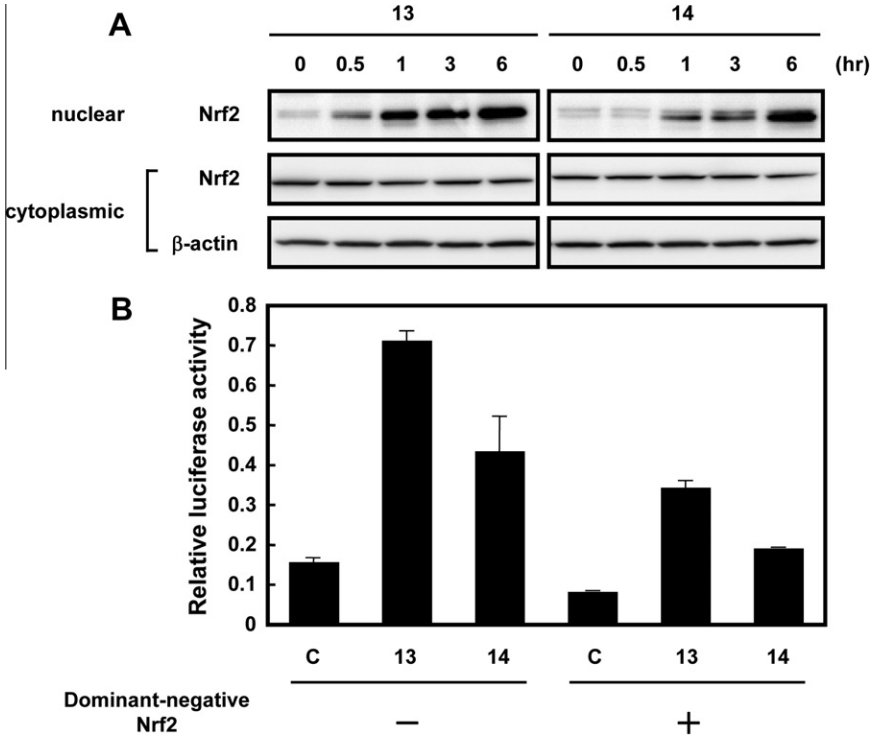

Figure 5. Effects of compounds **13** and **14** on nuclear translocation of Nrf2. (A) At indicated times after treatment with compound **13** or **14** (5 μM), nuclear and cytoplasmic fractions were prepared from LNCaP cells and subjected to Western blot analysis for Nrf2 and β-actin. A representative blot from three independent experiments is shown. (B) The ARE firefly luciferase reporter vector (500 ng) and the Renilla luciferase control vector (5 ng) were transfected into LNCaP cells for 24 h with either empty vector or Nrf2 dominant-negative mutant expression vector (200 ng). Then, cells were treated without (DMSO) or with compound **13** or **14** (5 μM) for additional 24 h, followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. Data are expressed as mean ± SEM (n = 3).

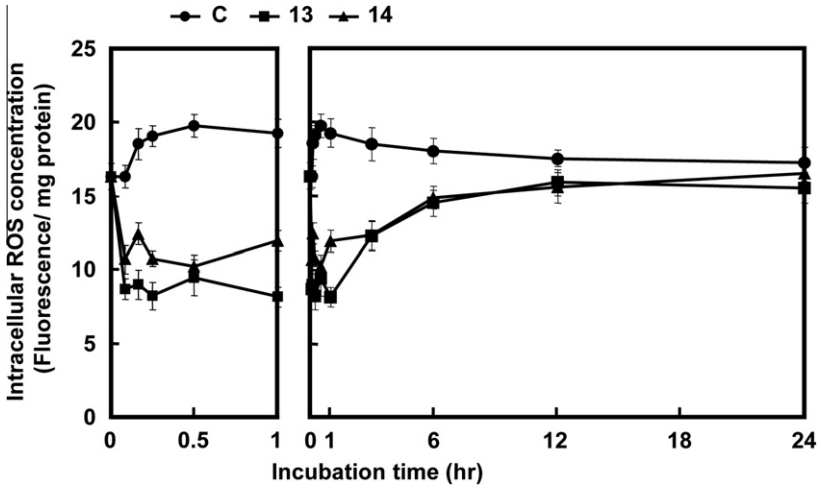

Figure 6. Effects of compounds **13** and **14** on intracellular ROS levels. (A) At indicated times after treatment without (DMSO) or with compound **13** or **14** (5 μM), LNCaP cells were subjected to measurement for intracellular ROS levels by using the fluorescent probe CM-H_2DCFDA. The ROS levels were expressed as fluorescence per mg of protein. Data are expressed as mean ± SEM (n = 9).

treatment with Zn-protoporphyrin (ZnPP), a HO-1 inhibitor, did not affect cell growth inhibition caused by these compounds. Furthermore, HO-1 knockdown by siRNA transfection did not influence cell growth inhibitory effects of compounds **13** and **14** (Fig. 8B).

3. Discussion

Activation of the Nrf2/ARE signaling has been proposed to be a promising strategy for chemoprevention, since phase II enzymes that are encoded by Nrf2/ARE target genes are capable of reducing oxidative stress and detoxifying carcinogens.[2,14] In an attempt to identify organoselenium compounds that are effective in chemoprevention, we synthesized 42 compounds (Supplementary Fig. 1) by the reactions shown in Supplementary Figures 2–4. Using firefly luciferase reporter assays, we searched for activators of the ARE enhancer in human prostate cancer cells. As a result of screening, we identified compounds **13** and **14** (Fig. 1) as potent Nrf2/ARE activators (Fig. 2A). The ARE enhancer activity of these two compounds was much higher in LNCaP cells than in hormone-refractory

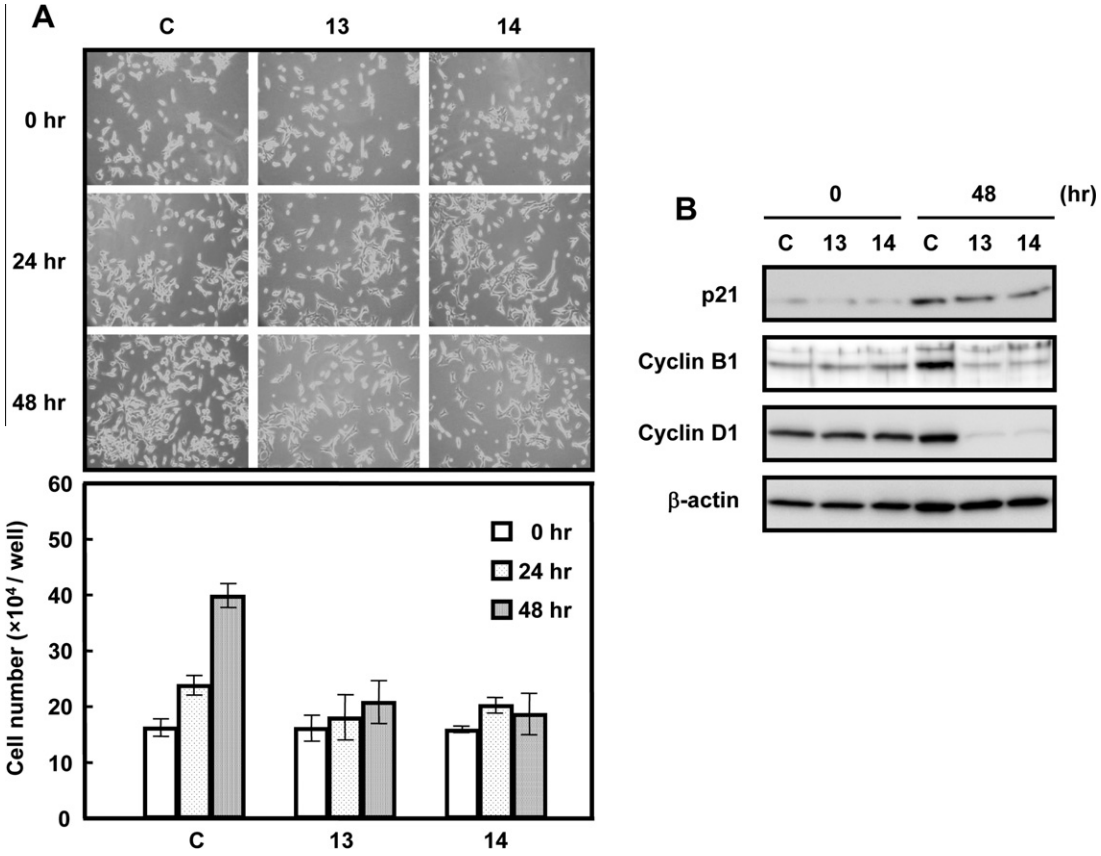

Figure 7. Effects of compounds **13** and **14** on cell growth. LNCaP cells were treated without (DMSO) or with compound **13** and **14** (5 μM). (A) At indicated times after treatment, pictures were taken (upper column) and the number of viable cells was determined using the trypan blue dye exclusion assay (lower column). Data are expressed as mean ± SEM (n = 3). (B) Forty-eight hours after treatment, cell lysates were prepared and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

prostate cancer PC3 and DU145 cells. In support of these findings, HO-1 induction by these compounds was markedly elevated in LNCaP cells than in PC3 and DU145 cells (Fig. 2B). These results suggest a possibility that Nrf2/ARE activation is higher in hormone-sensitive cells than in hormone-refractory cells. Indeed, HO-1 induction by compounds **13** and **14** was also found to be elevated in estrogen-sensitive MCF-7 cells than in estrogen-insensitive MDA-MB-231 cells (data not shown). Nevertheless, this hypothesis remains to be tested. Since the major focus of the present study was to identify compounds effective for chemoprevention during the early stages of carcinogenesis, we used hormone-sensitive LNCaP cells but not hormone-refractory PC3 and DU145 cells in the subsequent studies. Nrf2/ARE signaling regulates expression of an array of phase II enzymes. Both compounds **13** and **14** induced NQO-1 expression, but induction of this enzyme was much smaller than that of HO-1 (data not shown). Furthermore, expression of γ-GCS and GST-π was not at all up-regulated by compounds **13** and **14**, suggesting that these compounds have characteristics to relatively specifically induce HO-1 expression. Nevertheless, induction of NQO-1 by compounds **13** and **14**, although limited in extent, may contribute to detoxification of carcinogens. Structure–function relationship studies including compounds **11** and **12** indicated that the presence of *exo*-olefin (carbon–carbon double bond) as well as the aliphatic substitution at the imine part is critical for the compounds **13** and **14** to activate Nrf2/ARE signaling (Fig. 3). Nuclear translocation of Nrf2, a hallmark of Nrf2/ARE activation, initiated 0.5 and 1 h after treatment with compounds **13** and **14**, respectively, and gradually increased thereafter (Fig. 5A), which preceded HO-1 protein induction that was first seen at 6 h (Fig. 4). Furthermore, over-expression of a dominant-negative mutant of Nrf2 diminished ARE activation by compounds **13** and **14**, indicating that HO-1 induction by these compounds requires Nrf2 binding to the ARE (Fig. 5B).

Several mechanisms have been shown to confer Nrf2/ARE activation including phosphorylation of Nrf2 and oxidative or covalent modification of the sulfhydryl groups of Keap1.[10] Both compounds **13** and **14** profoundly reduced the intracellular ROS levels immediately after treatment (Fig. 6), suggesting that these compounds are potent antioxidants. It is therefore unlikely that oxidative modification of the sulfhydryl groups of Keap1 is involved in Nrf2/ARE activation by these compounds. We also examined effects of compounds **13** and **14** on signal transduction. However, these compounds showed little or no effects on phosphorylation of Akt, ERK, JNK, and p-38 (data not shown). Thus, Nrf2/ARE activation by these compounds cannot be ascribed to Nrf2 phosphorylation. Covalent modification of the sulfhydryl groups of Keap1 by electrophiles has been proposed as another mechanism of Nrf2/ARE activation. In the case of curcumin, it has been previously demonstrated that two α,β-unsaturated carbonyl groups interact with the sulfhydryl groups of Keap1 as Michael reaction acceptors, leading to activation of Nrf2/ARE signaling.[11,12,16] Compounds **13** and **14**, however, do not contain α,β-unsaturated carbonyl groups. On the other hand, ebselen, an organoselenium compound (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) that mimics the activity of glutathione peroxidase,[17] has a direct ROS-scavenging activity and HO-1 induction activity.[18] It has been also reported that ebselen is capable of activating Nrf2/ARE signaling via acting as electrophiles.[19,20] Thus, similarly to ebselen, compounds **13** and **14** might covalently modify the sulfhydryl groups of Keap1 and thereby activate Nrf2/ARE signaling. The precise

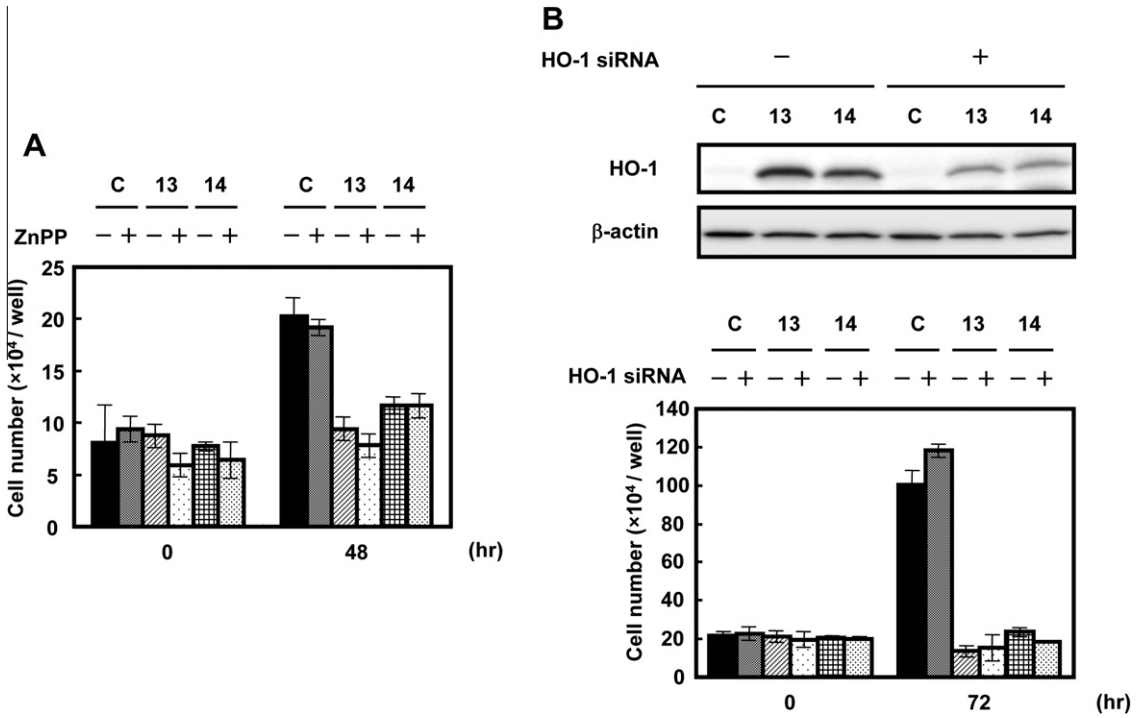

Figure 8. Effects of suppression of HO-1 activity and expression on cell growth inhibition by compounds **13** and **14**. (A) LNCaP cells were treated for 48 h without (DMSO) or with compound **13** or **14** (5 μM) in the absence or presence of ZnPP (5 μM). Then, the number of viable cells was determined using the trypan blue dye exclusion assay. Data are expressed as mean ± SEM (n = 3). (B) LNCaP cells were transfected for 6 h with a negative control or HO-1 siRNA (10 nM) and cultured in normal medium for 18 h. Then, cells were treated without (DMSO) or with compound **13** and **14** (5 μM) for 72 h and the number of viable cells was determined using the trypan blue dye exclusion assay (lower column). Data are expressed as mean ± SEM (n = 3). At the end of the experiments, cell lysates were harvested and subjected to Western blot analysis for HO-1 and β-actin (upper column). A representative blot from three independent experiments is shown.

mechanisms underlying Nrf2/ARE activation by these compounds remain to be explored.

Finally, compounds **13** and **14** significantly inhibited cell growth through induction of cell cycle arrest, but not caused apoptosis (Fig. 7). Similar cell growth inhibitory effects by these compounds were also observed in hormone-refractory PC3 and DU145 cells (Supplementary Fig. 5). Since it has been very recently reported that HO-1 induces cell cycle arrest and inhibits cell proliferation in vascular smooth muscle cells, pancreatic stellate cells and oral cancer cells,[21–23] we have hypothesized a possible interaction between HO-1 induction and cell growth inhibition in prostate cancer cells. However, neither inhibition of HO-1 activity by ZnPP nor of HO-1 expression by siRNA affected cell growth inhibition caused by compounds **13** and **14** (Fig. 8). These results suggest that compounds **13** and **14** could inhibit expansion of immortalized cells during the promotion stage of cancer development by inducing cell cycle arrest.

Taken all together, our findings suggest that compounds **13** and **14** could attenuate oxidative stress indirectly through Nrf2/ARE activation and directly through ROS scavenging, thereby alleviating DNA damage during the initiation and promotion stages of cancer development. They could also inhibit proliferation of immortalized cells via cell cycle arrest during the promotion stage. Thus, these compounds are expected to exert chemopreventive effects through multiple mechanisms in hormone-sensitive prostate cancer.

4. Conclusion

We synthesized 42 organoselenium compounds and identified two compounds, 3-selena-1-dethiacephem **13** and 3-selena-1-dethiacephem **14**, which were capable of attenuating oxidative stress and inhibiting cell growth in hormone-sensitive prostate cancer LNCaP cells. These compounds possess the potential as pharmacological agents for chemoprevention of prostate cancer.

5. Experimental

5.1. Cell culture and treatment

LNCaP, PC3, and DU145 human prostate cancer cells were purchased from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Zn-protoporphyrin (ZnPP) was purchased from Sigma (St. Louis, MO, USA).

5.2. Synthesis of organoselenium compounds

Forty-two organoselenium compounds (Supplementary Fig. 1) were prepared according to procedures previously reported (Supplementary Figs. 2–4).[24] Their chemical structures were confirmed by nuclear magnetic resonance (NMR) and mass spectrometric analyses. The physical and spectroscopic characteristics of compounds **13** and **14** were as follows. Typical procedure for the synthesis of 3-selena-1-dethiacephem **13** and **14** using 1.25 equiv of iodine: to a solution of alkyne-selenourea (50 mg, 0.10 mmol) in CH_2Cl_2 (2 mL) was added I_2 (33 mg, 0.13 mmol) at room temperature. After stirring at this temperature (6.5–11.5 h), the reaction mixture was extracted with CH_2Cl_2 and washed with saturated $Na_2S_2O_3$ and $NaHCO_3$. The organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was chromatographed on silica gel using ether/hexane (2:8) as eluent to give corresponding product **13** or **14**. 3-Selena-1-dethiacephem **13**: yield: 82%; melting point: 96.2–98.5 °C; IR (KBr): 3061, 2928, 2345, 1775, 1630, 1584, 1452, 1369, 1323, 1145 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.09 (s, 3H), 0.10 (s, 3H), 0.88 (s, 9H), 1.28

(d, J = 6.3 Hz, 3H), 2.32–2.39 (m, 1H), 2.97–3.00 (m, 1H), 3.35 (dd, J = 2.9 and 16.0 Hz, 1H), 3.94 (dt, J = 2.9 and 12.0 Hz, 1H), 4.25–4.31 (m, 1H), 4.49 (d, J = 14.8 Hz, 1H), 4.75 (d, J = 14.8 Hz, 1H), 6.49 (d, J = 1.2 Hz, 1H), 7.20–7.32 (m, 5H); ^{13}C NMR ($CDCl_3$): δ −5.05, −4.12, 17.9, 22.6, 25.7, 40.7, 53.5, 56.7, 64.7, 65.4, 74.1, 126.8, 127.4, 128.3, 132.6, 137.7, 138.4, 163.0; ^{77}Se NMR ($CDCl_3$): δ 467.0; MS (EI): m/z = 590 [M^+]. 3-Selena-1-dethiacephem **14**: yield: 84%; melting point: 66.5–67.7 °C; IR (KBr): 3049, 2377, 1782, 1639, 1337, 1133 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.08 (s, 3H), 0.09 (s, 3H), 0.88 (s, 9H), 1.17–1.33 (m, 6H), 1.48–1.67 (m, 4H), 1.70–1.81 (m, 3H), 2.31–2.38 (m, 1H), 2.91–2.94 (m, 1H), 3.07–3.14 (m, 1H), 3.32 (dd, J = 3.0 and 15.2 Hz, 1H), 3.87 (dt, J = 3.0 and 12.0 Hz, 1H), 4.19–4.25 (m, 1H), 6.49 (s, 1H); ^{13}C NMR ($CDCl_3$): δ −5.02, −4.06, 17.8, 22.6, 24.6, 25.4, 25.8, 33.2, 31.5, 40.8, 53.8, 63.2, 64.4, 65.8, 73.7, 132.5, 132.8, 162.8; ^{77}Se NMR ($CDCl_3$): δ 464.0; MS (EI): m/z = 582 [M^+].

5.3. Transfection and luciferase assays

The ARE reporter gene contained two copies of the ARE enhancer sequences found in the promoter region of glutathione S-transferase Ya subunit gene[25] upstream of thymidine kinase minimal promoter fused to a firefly luciferase gene (pGL3-ARE-TK-luc) (Promega, Madison, WI, USA).[26] Cells seeded onto 24-well plates were transfected for 24 h with the ARE firefly luciferase reporter vector and the Renilla luciferase expression vector (phRL; Promega) by using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA, USA). Luciferase activities were measured 24 h after treatment with compounds using the Pikkagene Dual luciferase assay system (TOYO B-Net, Tokyo, Japan). In specified experiments, the ARE firefly luciferase reporter vector and the Renilla luciferase expression vector were transfected for 24 h with either empty vector (pEF) or Nrf2 dominant-negative mutant expression vector (pEF-dominant-negative Nrf2) using Trans-IT transfection reagent (Mirus, Madison, WI, USA). These expression vectors are kind gifts from Dr. Jeffrey A. Johnson, University of Wisconsin–Madison.[27]

5.4. Western blot analysis

Anti-HO-1 (SPA-896) and -β-actin (A5441) antibodies were purchased from Stressgen Bioreagents (Ann Arbor, MI, USA) and Sigma, respectively. Anti-Nrf2 (sc-722), -cyclin B1 (sc-752), and -p21 (sc-817) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against cyclin D1 (#2926) was obtained from Cell Signaling Technology (Beverly, MA, USA). Whole cell extracts were prepared using the RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing protease inhibitor cocktails and phosphatase inhibitor cocktails 1 and 2 (Sigma). Nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Rockford, IL, USA). Proteins were separated by SDS–PAGE and then transferred onto PVDF membranes (Perkin Elmer, Northwalk, CT, USA). After blocking with 5% skim milk for 1 h, membranes were incubated overnight at 4 °C with a primary antibody and then reacted with a HRP-conjugated secondary antibody for 1 h. The immunoreactive proteins were visualized using the ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) and the LAS 4000 imaging system (Fuji Film, Tokyo, Japan). Densitometric analysis was performed with the Multi Gage software (Fuji Film).

5.5. Measurement of ROS levels

The intracellular ROS level was determined using a ROS-sensitive fluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA). After incubation with 10 μM CM-H_2DCFDA for 30 min at 37 °C, cells were washed twice with cold PBS and then re-suspended in cold lysis buffer. Cell lysates were subjected to fluorescence measurement at the excitation wavelength of 490 nm and the emission wavelength of 530 nm using the fluorescence microplate reader, MTP-600F (Corona Electric, Ibaragi, Japan).

5.6. Measurement of cell viability

The viable cell number was determined using the trypan blue dye exclusion assay.

5.7. Gene knockdown by siRNA

Cells seeded onto six-well plates were transfected for 6 h with siGENOME SMARTpool Human HO-1 (Thermo Scientific Dharmacon, Waltham, MA, USA) using Lipofectamine 2000 (Invitrogen). As a negative control, Stealth RNAi negative control medium GC duplex (Invitrogen) was used.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.019.

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