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Graphical Abstract

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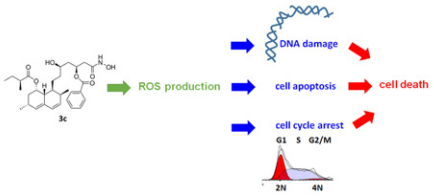

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Synthesis and biological evaluation of lovastatin-derived aliphatic hydroxamates that induce reactive oxygen species

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ABSTRACT

Some hydroxamate compounds induce cancer cell death by intracellular reactive oxygen species (ROS). This study introduced the hydroxamate core into lovastatin, a fungus metabolite clinically used for the treatment of hypercholesterolemia. The resulting compounds were evaluated for the activity for inducing ROS production. Most compounds exhibited higher activity than original lovastatin. Of these compounds, compound **3c** had the most potent activity. Test of cytotoxicity in a panel of human cancer cell lines indicated compound **3c** had activities superior to cisplatin in prostate cancer PC-3 cells and breast cancer T47D cells. In contrast, it in amounts up to 40 μM had a much lower cytotoxic effect on normal human IMR-90 cells. Further profiling of cell cycle progression, cell apoptosis, and DNA damage activated checkpoint signaling pathway revealed the important role of compound **3c**-mediated cytotoxicity in ROS generation.

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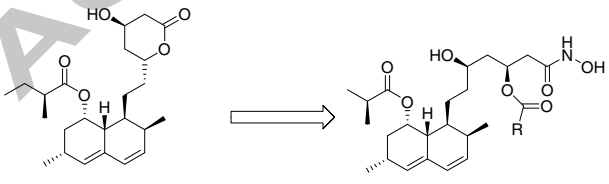
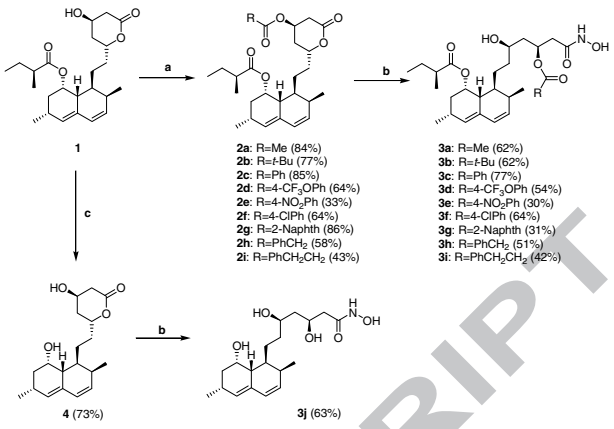
Reactive oxygen species (ROS), which are chemically reactive molecules containing oxygen, have an essential role in maintaining the homeostasis of physiological function.[1] Moderate ROS levels regulate cell growth and differentiation, control enzyme activity, and modulate inflammation.[2, 3] In contrast, excess ROS cause oxidative damage leading to cell death by reacting with intracellular biomolecules such as DNA, proteins and lipids.[4] Many tumor cell types reportedly have higher ROS levels compared to their normal counterparts.[5-7] Enhanced ROS production in cancer cells can cause ROS levels to reach the toxic threshold at which malignant cells death is preferentially induced. Therefore, agents that promote ROS production may have therapeutic applications in anticancer strategies.[8, 9]

Lovastatin (**1**), a fungal metabolite originally isolated from *Aspergillus terreus* and from *Monascus rubber*,[10-12] is widely used to treat hypercholesterolemia. It competitively inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the reduction of HMG-CoA into mevalonate. This effect then lower cholesterol.[13] Lovastatin (**1**) also blocks the synthesis of mevalonate-related downstream isoprenoids such as geranylpyrophosphate and farnesylpyrophosphate, both of which bind to Ras and its related proteins, and thus perturbs the function of G-proteins.[14, 15] These studies suggest that lovastatin (**1**) may have various cell physiological activities that affect cell growth, including cell proliferation, apoptosis, invasion and differentiation.[16-19] These physiological activities are further described below. In several tumor cell types, lovastatin (**1**) is known to inhibit proliferation, induce apoptosis, and arrest the cell cycle in G1/S phase.[15, 20] Studies reveal that lovastatin (**1**) combined with chemotherapeutic drugs synergistically sensitizes cancer cells to anticancer agents in several tumor cell lines as well as *in-vivo* models.[21-25] Despite its many anticancer studies reported, attempts for applying chemical synthesis to improve lovastatin activity are few.[26] Various hydroxamate-containing compounds reportedly can not only inhibit histone deacetylase (HDAC), but also induce intracellular ROS production. The combinatorial effect causes cancer cell death.[27-29] To enhance the antiproliferative activity of lovastatin (**1**), the hydroxamate motif that is associated with the ROS production was experimentally incorporated into the chemical structure of lovastatin (Scheme 1). We hypothesized that this modification would enhance the ROS promoting activity of lovastatin (**1**) in tumor cells. Evaluations of ROS-inducing ability of all synthesized compounds (**3a-j**) showed that compound **3c** significantly induced ROS production in cancer cells, but had no HDAC enzyme inhibitory activity. Notably, the cytotoxicities of compound **3c** in several cancer cells were higher than those of lovastatin (**1**). This study then examined how compound **3c**-mediated ROS production affects cell cycle progression, cell apoptosis, and DNA damage in the checkpoint signaling pathway.



Scheme 1. Design of lovastatin-derived hydroxamates **3a-i**

Scheme 2



Reagents and condition : (**a**) Ac_2O, Pyr, RT, for **2a**, ; RCOCl, Pyr, $PhCH_3$, RT, for **2b-i**; (**b**) 50% NH_2OH, THF, N_2, RT; (**c**) (1) LiOH, MeOH-THF, Δ; (**2**) $PhCH_3$, Δ.

Lovastatin-based aliphatic hydroxamates were synthesized as shown in Scheme 2. Lovastatin (**1**) reacted with the appropriate acyl chlorides gave **2a-i**, respectively. Reaction of compounds **2a-i** with NH_2OH provided the corresponding hydroxamates **3a-i**. Basic saponification of lovastatin (**1**) in the presence of LiOH and subsequent lactonization gave **4**. Compound **4** was reacted with NH_2OH to provide **3j**.

Figure 1 shows that flow cytometric analysis was performed using DCFH-DA fluorescent probe in human breast cancer MDAMB-231 cells to test all lovastatin-derived compounds **3a-j** synthesized in this study for activity that induced ROS production. Additionally, two positive controls doxorubicin[30] and cisplatin[31] that reported increased ROS production in cells were included in the test. Comparisons of compounds **3a-j** and lovastatin indicated that the activities of all compounds were improved compared to that of original lovastatin. However, these compounds exhibited no HDAC-inhibiting activity (Figure 1S). Most compounds, except for compound **3j**, had higher ROS production activity than did cisplatin. Notably, the activities of compound **3c** and **3g** were strongest. The activities of benzoyl substituted series **3c-g** were higher than those of both compounds **3a** with acetyl group and **3b** with *t*-butanoyl group, which suggested that the benzoyl moiety positively contributed to activity. Comparisons of substituted benzoyl aliphatic hydroxamates **3d-g** showed that compound **3g** had the highest potency. However, its potency was comparable to that of compound **3c**, which indicated that the substituent on phenyl ring only slightly increased ROS production. Next, compounds **3h-i** with one to two carbons chain-length of linker attached to phenyl ring were synthesized. These compounds had equally potent activities. Moreover, compound **3j** had only weak activity. These experimental results speculated that the weak ROS-inducing activities of compounds **3a, 3b,** and **3j** were possibly caused by their poor cell membrane permeability and the weak electron-donating ability of the substituent on the β-position of these tree compounds.

The results of growth inhibition against human prostate PC-3 cells indicated that the cytotoxicities of three of these compounds, **3c**, **3h** and **3i**, equaled or exceeded that of lovastatin (Table 1S). Moreover, their activities were much higher than that of cisplatin. In particular, the antiproliferative activity of compound **3c** was highest.

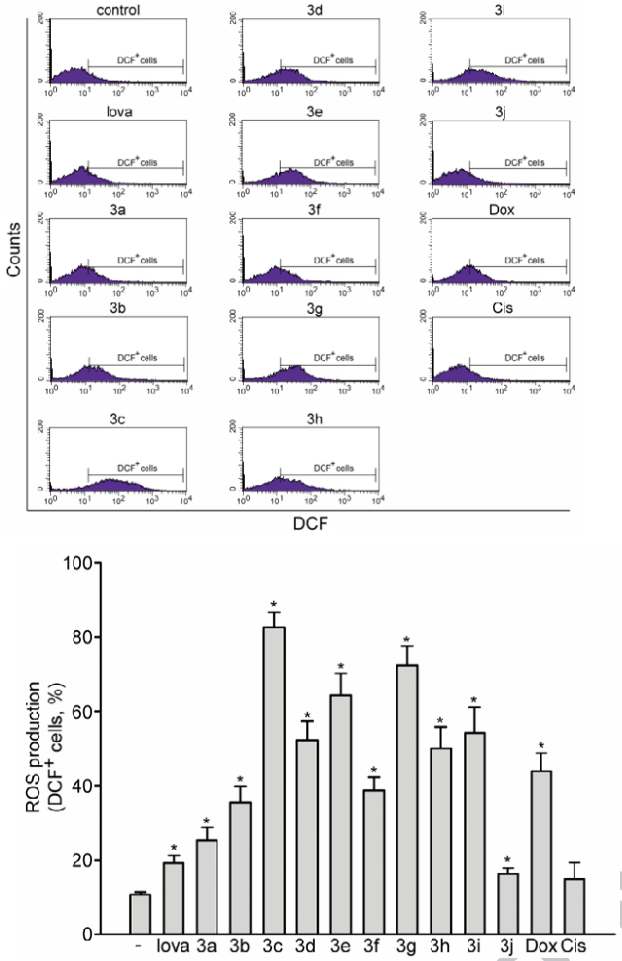


Figure 1. Effects of compounds **3a-j** on intracellular ROS concentrations. MDAMB-231 cells were treated with vehicle, lovastatin (30 μM), compounds **3a-j** (10 μM), cisplatin (10 μM) or doxorubicin (5 μM) for 24 h. The intracellular ROS level was then analyzed by flow cytometry performed with a DCFH-DA fluorescent probe. Results shown in the upper panel are representative of at least four independent experiments. Complied results are show in the bottom panel of the chart. Each column represents the mean ± S.E.M. of at least four independent experiments (*$p < 0.05$, compared with the control group).

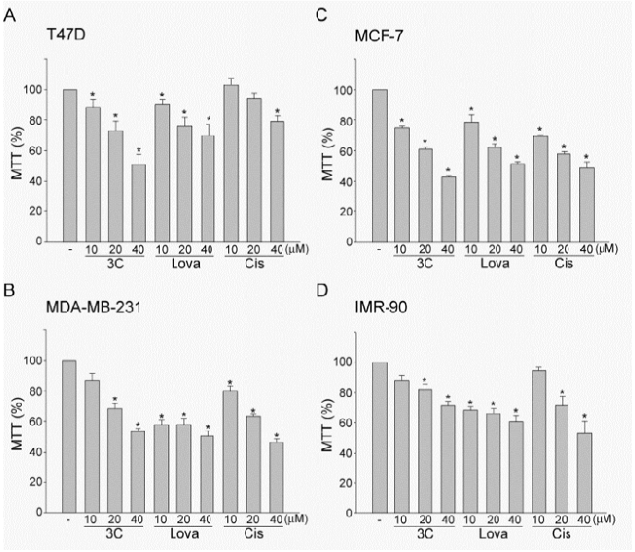


Figure 2. Cell viability of the breast cancer cell lines MCF7, T47D, MDA-MB-231, and the normal cell line IMR-90 treated with compound **3c** lovastatin, and cisplatin. The MTT assay was used to measure growth in the T47D (A), MCF7 (B), and MDA-MB-231 (C) cancer cell lines and in the IMR-90 normal lung cell line (D) after the cells had been treated with drugs for 48 hr. The data were presented as the means ± s.d. All experiments were performed with three technical replicates.

In further experiments to identify potential anti-breast cancer agents, compound **3c** with significant growth inhibition was selected for the evaluation of antiproliferative activity against a panel of human breast cancer cell lines such as MDA-MB-231, MCF7 and T47D cells using cisplatin as a positive control. In T47D and MCF7cancer cell lines, compound **3c** exhibited higher cytotoxicities (IC_{50}=30-40 μM) than did lovastatin (IC_{50}>40 μM) (Figure 2A-C). Compared to cisplatin, the antiproliferative activities of compound **3c** against MDA-MB-231 and MCF7 were comparable, but its activity against T47D cells was higher. In contrast, compound **3c** even in amounts up to 40 μM exhibited lower cytotoxicity for normal IMR-90 cells compared to cisplatin (Figure 2D). These experimental results indicated that compound **3c** may have the therapeutic index superior to cisplatin.

Reactive oxygen species are a major source of DNA damage because they can modify DNA, indirectly induce lesions, and affect cell viability. Therefore, we investigated whether compound **3c** promotes DNA damage in MDA-MB-231 cells by using phosphorylation of γH2AX as a DNA damage marker.[32] Figure 3A shows that compared to lovastatin, compound **3c** significantly increased the severity of DNA damage for 48 h (Figure 3A). This further confirmed that compound **3c** markedly enhanced phosphorylation for chk1 as well as chk2, two protein kinases in the checkpoint signaling pathways associated with DNA damage.[33] Figure 3B shows that the effects are superior to those of doxorubicin, a known topoisomerase II inhibitor used to induce DNA damage.[34] However, lovastatin revealed no activity in these two proteins.

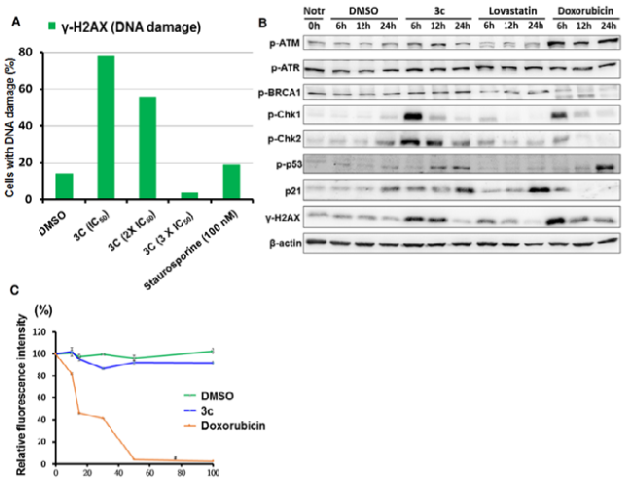


Figure 3. Compound **3c** induced DNA damage without DNA binding. (A) The DNA damage, (B) protein levels of cell signaling pathways for DNA damage and cell cycle, and (C) drug-DNA binding were analyzed in breast cancer MDA-MB-231 cells treated with compound **3c** (10 μM), lovastatin (10 μM) or doxorubicin (5 μM). The Notr was the control with no treatment medium, and DMSO was the solvent control.

Figure 3B shows that compound **3c** was further evaluated for the phosphorylation of upstream ATR and ATM as well as of downstream p53 associated with the activation of chk1 and chk2 signaling. The experimental results indicated that the phosphorylation of ATM and p53 induced by compound **3c** was significantly increased compared to that of lovastatin. Figure 3B shows that compound **3c** was comparable to lovastatin in terms of promoting p53 phosphorylation. Compound **3c** also enhanced the expression of p21 protein that is regulated by its counterpart gene $p21^{WAF1}$ serving as a downstream of p53. To investigate whether compound **3c**-induced DNA damage by directly binding to DNA, this study further analyzed DNA minor groove-binding in compound **3**c. Figure 3C shows that compound **3c** revealed no DNA binding activity even at 100 μM, which indicated that its DNA damage effect was independent of its interaction with DNA.

Different concentrations of compound **3c** were analyzed by flow cytometry and by cleaved PARP detection to investigate whether it induces cancer cell apoptosis. Cell apoptosis caused by compound **3c** was higher than that caused by lovastatin, and the effect was concentration-dependent (Figure 4A). Compound **3c** at 32 μM was then examined by flow cytometry combined with PI staining at various time courses (6, 12, 24, 48h) in MDA-MB-231 cells. Figure 4B shows that compound **3c** caused cell arrest in the S phase of the cell cycle progression at 6h and 12h.

In conclusion, this study synthesized a series of novel aliphatic hydroxamates derived from lovastatin and disclosed their SAR in ROS-inducing activity. Of these, the cytotoxicities of compound **3c** were improved compared to those of lovastatin in several human cancer cells. The effects of compound **3c** mediated-ROS on cell cycle arrest, cell apoptosis, and DNA damage activated by checkpoint signaling pathway indicated that compound **3c** induced significant DNA damage in MDA-MB-231 cell, which then caused cell death. Further studies of structural modifications that enhance antiproliferative activity are now underway.

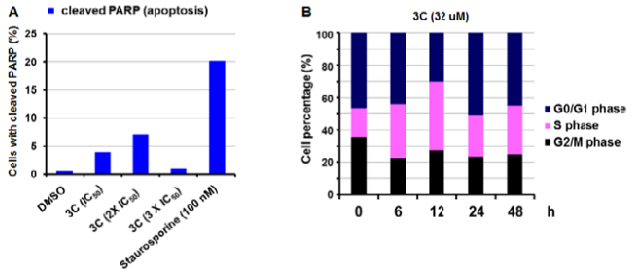


Figure 4. Compound **3c** induced apoptosis and cell cycle arrest. (A) Cell apoptosis and (B) cell cycle distribution were analyzed in breast cancer MDA-MB-231 cells treated with compound **3c** (32μM). The Notr was the control with no treatment medium, and DMSO was the solvent control.

Acknowledgments

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Supplementary Material

Supplementary data (the experimental procedure, the physical data of the presented compounds, HDAC enzyme-inhibiting data, ^{1}H and ^{13}C NMR spectra of **3a-j** and HPLC chromatogram of all synthesized compounds, Table 1S) associated with this article can be found in the online version.

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