Synergistic activity of the histone deacetylase inhibitor suberoylanilide hydroxamic acid and the bisphosphate zoledronic acid against prostate cancer cells in vitro

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Abstract
Bisphosphonates are widely used agents for the treatment of malignant bone disease. They inhibit osteoclast-mediated bone resorption and can have direct effects on cancer cells. In this study, we investigated whether the anticancer activity of the third-generation bisphosphate zoledronic acid (ZOL) could be enhanced by combination with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). We found that ZOL and SAHA cooperated to induce cell death in the prostate cancer cell lines LnCaP and PC-3. The effect was synergistic, as evidenced by combination index isobologram analysis. ZOL and SAHA synergized to induce dissipation of the mitochondrial transmembrane potential, to activate caspase-3, and to trigger DNA fragmentation, showing that the combination of ZOL and SAHA resulted in the initiation of apoptosis. Because ZOL acts by inhibiting the mevalonate pathway, thereby preventing protein prenylation, we explored whether the mevalonate pathway was also the target of the cooperative action of ZOL and SAHA. We found that geranylgeraniol, but not farnesol, significantly reduced ZOL/SAHA-induced cell death, indicating that the synergistic action of the agents was due to the inhibition of geranylgeranylation. Consistently, a direct inhibitor of geranylgeranylation, GGTL-298, synergized with SAHA to induce cell death, whereas an inhibitor of farnesylation, FTI-277, had no effect. In addition, SAHA synergized with mevastatin, an inhibitor of the proximal enzyme in the mevalonate pathway.

Introduction
Prostate cancer is the most frequent malignancy and the second leading cancer-related cause of death among men in the United States and Western Europe. Throughout the natural course of the disease, patients are at risk for bone metastases, which are often associated with severe skeletal complications, including devastating bone pain and pathologic fractures (1). Thus, the reduction of skeletal-related events is an important clinical challenge in the treatment of prostate cancer patients. In several clinical trials, bisphosphonates (BP) have been proven useful in the management of bone metastases from different cancers and have become a treatment modality for malignant bone lesions in patients with various tumors (2, 3). In patients with bone metastases from prostate carcinoma, particularly the nitrogen-containing BP (N-BP), zoledronic acid (ZOL) has been shown to significantly reduce skeletal morbidity (4–6).

N-BPs affect osteoclast activity, and thus bone resorption, by inhibiting farnesyl pyrophosphate (FPP) synthase, a key enzyme in the mevalonate pathway (7, 8). The latter provides cells with FPP and geranylgeranyl pyrophosphate, which are required for the posttranslational modification (i.e., prenylation) of GTP-binding proteins, such as Ras, Rho, and Rac (9). Prenylation is essential for the proper function of these proteins that are relevant for osteoclast function and survival. In addition to their effect on osteoclasts, N-BPs have also been shown to exert a direct cytotoxic action on tumor cells in vitro: they have been shown to inhibit cell growth and induce cell death in a variety of cancer cell lines, including prostate carcinoma cell lines (8, 10, 11). Again, the inhibition of FPP synthase seems to be central to the direct activity of N-BPs against tumor cells.

Histone deacetylase inhibitors (HDI) are another class of antineoplastic agents that hold promise to improve cancer therapy. HDIs function by inhibiting histone deacetylases, resulting in the accumulation of acetylated histones, in turn leading to an increase in transcriptionally active chromatin. Many in vitro and animal studies have shown that HDIs can induce differentiation and apoptosis, inhibit cell proliferation, and exert immunostimulatory and antiangiogenic
activities in tumor cells (12). Importantly, HDIs have been shown both in vitro and in vivo to affect cancer cells while leaving normal cells comparatively unscathed. The clinical potential of these agents has been documented by several early-phase clinical trials in a variety of solid and hematologic malignancies (13).

But the greatest potential of HDIs may lie in their capability to augment the antitumor efficiency of other therapeutic regimens. Indeed, they have been shown in vitro to significantly enhance the cytotoxic activity of ionizing radiation or chemotherapy (14). Likewise, the combination of BPs with antineoplastic compounds has been shown to result in synergistic effects against cancer cell lines (10, 11). However, a possible favorable interaction between HDIs and BPs has not yet been studied. To explore this issue, we examined whether ZOL and a prototypic HDI, suberoylanilide hydroxamic acid (SAHA; vorinostat; ref. 15), would cooperate in exerting cytotoxic effects on prostate cancer cells. We found that SAHA did synergize not only with ZOL but also with mevastatin and a specific inhibitor of protein geranylgeranylation, GGTI-298, to induce cell death in two prostate cancer cell lines: LNCaP and PC-3.

Materials and Methods

Reagents

ZOL was a gift from Novartis Pharma. SAHA, MS-275, and the pan-caspase inhibitor z-VAD-fmk were purchased from Alexis. Sodium butyrate, farnesol (FOH), geranylgeraniol (GGOH), the farnesyltransferase inhibitor FTI-277, the geranylgeranyltransferase inhibitor GGTI-298, and mevastatin were purchased from Sigma.

Cell Culture

LNCaP and PC-3 prostate cancer cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 or DMEM, respectively, supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. The medium for LNCaP cells was additionally supplemented with 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate (media and supplements were purchased from Biochrom). Cells were harvested after the indicated treatments, centrifuged, and re-suspended in PBS containing 1% glucose, 50 μg/mL RNase A (Roche), and 50 μg/mL propidium iodide and incubated in the dark at room temperature for 30 min. Flow cytometry analysis was done on a FACS Calibur. Cells (20,000) were analyzed in each sample; results were analyzed by the combination index (CI) method according to Chou and Talalay (16) using CalcuSyn software from Biosoft. CI values >1.1 indicate antagonism, values between 0.9 and 1.1 indicate additivism, and values >0.9 indicate synergism.

Flow Cytometric Analysis of Mitochondrial Transmembrane Potential ($\Delta \psi_m$)

$\Delta \psi_m$ was determined by assessing the accumulation of the cationic lipophilic fluoro chromome 3,3‘-dihexyloxacarbo cyanine iodide [DiOC6(3)] in the mitochondrial matrix. After the indicated treatments, cells were incubated with 50 nmol/L DiOC6(3) (Molecular Probes) at 37°C for 30 min before harvesting. After washing, 10,000 cells were analyzed using a FACSCalibur. Data were gated to exclude debris.

Caspase-3 Activity

Caspase-3 activity was measured using the synthetic fluorogenic substrate Ac-DEVD-AFC (Bachem). After the indicated treatments, cells were lysed in 10 mmol/L Tris-HCl, 10 mmol/L NaH2PO4/NaHPO4 (pH 7.5), 130 mmol/L NaCl, 1% Triton X-100, and 10 mmol/L Na2P2O7 and then incubated with 20 mmol/L HEPES (pH 7.5), 10% glycerol, 2 mmol/L DTT, and 25 μg/mL Ac–DEVD–AFC at 37°C for 2 h. The release of trifluoromethylcoumarin was analyzed on a Wallac Victor fluorometer (Perkin-Elmer) using an excitation/emission wavelength of 390/510 nm. Relative caspase-3 activities were calculated as a ratio of emission of treated cells to untreated cells.

Statistical Analysis

Statistical significance of differences between experimental groups was determined using the paired two-tailed Student’s t test.
Results
SAHA and ZOL Synergize to Induce Cell Death in Prostate Cancer Cells
To test a possible favorable cytotoxic interaction between SAHA and ZOL in prostate carcinoma cells, we initially monitored cell death by flow cytometric analysis of propidium iodide uptake. Four hours after treatment with SAHA (1–4 μmol/L), LNCaP and PC-3 cells were exposed to ZOL (10–100 μmol/L) for another 48 h. As shown in Fig. 1, nonpretreated LNCaP cells were marginally sensitive to ZOL under these conditions. However, when cells were pretreated with SAHA, ZOL evoked cell death in a concentration-dependent manner. For example, after pretreatment with 2 μmol/L SAHA, ZOL-induced cell death ranged from 23.4% to 32.8%. In PC-3 cells, treatment with ZOL alone resulted in cell death in up to 25.1% of cells. In combination with 2 μmol/L SAHA, ZOL-triggered cell death ranged from 23.2% to 56.1%. To determine whether these observations could be extended to HDIs in general, we did similar experiments with two structurally unrelated HDIs: sodium butyrate and MS-275. For both compounds, we observed a cooperative interaction with ZOL in LNCaP and PC-3 cells (data not shown). Because BPs are strong chelators of divalent cations, we conducted control experiments with EDTA replacing ZOL to exclude the physicochemical effect of sequestration of Ca²⁺, Mg²⁺, and Fe²⁺. We found that EDTA at concentrations up to 100 μmol/L did not affect cell viability neither in the absence nor in the presence of SAHA (data not shown).

We tested the combination of SAHA and ZOL for synergy by the CI isobologram method (CI < 0.9 is indicative for a synergistic interaction; ref. 16). In LNCaP cells, synergy was seen in particular after pretreatment with 2 μmol/L SAHA (CI, 0.54–0.73); but also after 1 μmol/L SAHA (CI, 0.69–0.99) and 4 μmol/L SAHA (CI, 0.77–0.92), moderate synergy to additivity was noted (Supplementary Table S1). An even more pronounced synergistic interaction between SAHA and ZOL was observed in PC-3 cells. Concentrations of 25 to 100 μmol/L ZOL produced strong synergy (CI, 0.22–0.55) with SAHA at any concentration applied.

The Synergistic Activity of SAHA and ZOL Involves Apoptosis
Next, we examined whether the synergistic anticancer action of SAHA and ZOL involves apoptosis. Apoptosis was evaluated in LNCaP cells by using the broad-spectrum irreversible caspase inhibitor z-VAD-fmk, by assessing caspase-3 activity, and by determining DNA fragmentation with cell cycle analysis. First, the effect of z-VAD-fmk was determined by propidium iodide uptake analysis. Figure 2A shows that the pan-caspase inhibitor significantly reduced cell death induced by the combination of SAHA and ZOL. Second, we measured caspase-3 activity; the activation of caspase-3 is a typical feature of apoptotic cell death and thus considered a suitable measure of apoptotic responsiveness (17). ZOL alone activated caspase-3 only very weakly, whereas in cells preexposed to SAHA, ZOL caused caspase-3 activation in a dose-dependent fashion (Fig. 2B). Third, cells were assessed for apoptosis by staining the nuclei of ethanol-fixed cells with propidium iodide and determining the DNA content by flow cytometry. Typically, sub-G₁ cells

3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
are indicative of apoptosis. As illustrated in Fig. 2C, the results reflect those of the cell death assay: whereas treatment with ZOL alone induced only marginal apoptosis, 28.7% of cells became apoptotic after exposure to the combination of 2 μmol/L SAHA and 100 μmol/L ZOL. Synergistic induction of apoptosis by SAHA and ZOL could be largely prevented by z-VAD-fmk (Fig. 2D). We observed no significant effect of the SAHA/ZOL treatment on the distribution of cells in the G1, S, and G2-M phase of the cell cycle; the increase of sub-G1 cells occurred on the expense of both G1 and G2-M phase cells (data not shown).

SAHA and ZOL have both been shown to harness the mitochondrial pathway of apoptosis (18, 19). We thus analyzed in LNCaP and PC-3 cells whether these agents could interact at the mitochondrial level to trigger apoptosis. As presented in Fig. 3A, ZOL alone had little effect on Δψm in LNCaP cells. When the same experiment was carried out in cells pretreated with SAHA, ZOL evoked Δψm dissipation in up to 52.1% of cells. Nonpretreated PC-3 cells showed some responsiveness to ZOL (i.e., 18.3% loss of Δψm after 100 μmol/L ZOL). Pretreatment with SAHA augmented the ZOL-mediated effect on mitochondria, with a decay of Δψm being observed in up to 53% of...
SAHA and Zoledronic Acid Synergize to Kill Cancer Cells

To determine whether the combined action of SAHA and ZOL on mitochondrial function depended on caspases, we applied z-VAD-fmk. Figure 3B shows that the loss of Δψm in the combined drug group could be significantly inhibited by the pan-caspase inhibitor.

SAHA Synergizes with GGTI-298, But Not with FTI-277, to Induce Cell Death in LNCaP Cells

N-BPs, including ZOL, have been reported to act on cancer cells via the mevalonate pathway by inhibiting FPP synthase, thereby preventing the biosynthesis of FPP and geranylgeranyl pyrophosphate (8). To investigate whether the synergistic induction of apoptosis by SAHA and ZOL also occurred via the mevalonate pathway, we applied the isoprenols FOH and GGOH, which have been shown to bypass the inhibition of FPP synthase (20). We found that the synergistic action of SAHA and ZOL could be significantly suppressed by GGOH but not by FOH (Fig. 4). The addition of GGOH reduced the cell death induced by the combination of SAHA and ZOL to almost exactly the percentage of cell death induced by SAHA alone.

We also studied the effect of specific inhibitors of protein farnesylation and geranylgeranylation, FTI-277 (21) and GGTI-298 (22), respectively, in combination with SAHA. As shown in Fig. 5, FTI-277 had little effect on the viability of LNCaP cells both when applied alone or in conjunction with SAHA. In contrast, GGTI-298 induced cell death in a dose-dependent manner. When administered alone, GGTI-298 elicited cell killing in up to 12.5% of cells, and when coadministered with SAHA, it evoked cell death in up to 44.8% of cells. These data were tested for synergy by the CI isobologram method. The calculated CI values (0.54–0.96) indicated a synergistic to additive effect (Supplementary Table S2).3

SAHA and Mevastatin Synergize to Induce Cell Death in LNCaP Cells

Cellular FPP and geranylgeranyl pyrophosphate levels can also be reduced by statins, such as mevastatin, inhibitors of the proximal and rate-limiting enzyme in the mevalonate pathway (23). We thus assessed whether SAHA could also cooperate with mevastatin to induce cell death in LNCaP cells. Figure 6A shows that treatment with mevastatin alone evoked cell death in 7.7% of cells. In cells pretreated with SAHA, exposure to mevastatin resulted in up to 45.4% of cells. These results were in turn analyzed by CI isobologram method, revealing moderate synergy to additivity (CI, 0.69–0.97; Supplementary Table S3).3 When mevastatin or the combination of SAHA and mevastatin was coapplied with the isoprenols, FOH had no effect, whereas GGOH significantly reduced cell death (Fig. 6B).

Discussion

BPs are well-recognized inhibitors of osteoclast-mediated bone resorption (7, 8, 10). They also have been shown to exert direct antitumor effects in cell lines derived from either primary bone cancers, such as osteosarcoma (24, 25) and Ewing’s sarcoma (26), or from cancers metastatic to bone, such as multiple myeloma (27), renal cell carcinoma (28), lung cancer (29), breast cancer (30, 31), and prostate cancer (32–35). In addition, the N-BP ZOL has been reported to cooperate with different anticancer agents (e.g., docetaxel, imatinib, doxorubicin, ifosfamide,
gemcitabine, and cisplatin) in affecting cancer cells in vitro or in xenograft models (36–42). In the present study, we show that the combination of ZOL with the HDI SAHA produced synergistic cytotoxic effects in prostate cancer cell lines.

We found that SAHA and ZOL interacted cooperatively to induce cell death in LNCaP and PC-3 prostate carcinoma cells, as judged by flow cytometric propidium iodide uptake analysis. CI isobologram analysis indicated that the interaction between SAHA and ZOL was synergistic, particularly at higher concentrations of ZOL. LNCaP and PC-3 cells differed in their responsiveness to ZOL alone: under the conditions applied, ZOL was only faintly cytotoxic in LNCaP cells, whereas it induced cell death in up to 25% of PC-3 cells. When ZOL was combined with SAHA, a higher synergy was achieved in the ZOL-sensitive PC-3 cells (CI, 0.22–0.99) than in the LNCaP cells (CI, 0.54–0.99); nonetheless, the combination of SAHA and ZOL was clearly synergistic in both the cell lines. Experiments with two other HDIs, sodium butyrate and MS-275, revealed that this combinatorial effect was not specific for SAHA. It should be noted that the response to ZOL in our study differed from that reported in previous studies on the effect of ZOL in LNCaP and PC-3 cells, as did the results in these studies among each other (32–34). But, as already proposed by Corey et al. (33), the differences between the results “may be due to differences in the sensitivity of the methods used for determination of apoptosis, and/or to variations in the cultured cell lines”. Interestingly, in preliminary experiments with another N-BP, pamidronate, no synergistic effect was observed in any cell line. This observation suggests that synergy with SAHA may not occur with all N-BPs despite the structural and pharmacologic similarities among the members of this compound class.
Like the majority of antineoplastic agents, N-BPs and HDIs trigger cell death through the induction of apoptosis (10–12, 14). Our results presented here show that the coadministration of SAHA and ZOL also resulted in the initiation of apoptosis. We found that SAHA in conjunction with ZOL potently activated caspase-3, a finding that is consistent with apoptosis. We also found that exposure to SAHA/ZOL led to the accumulation of sub-G_1 cells, another feature characteristic of apoptosis. Apoptosis can proceed via two pathways: the death receptor (extrinsic) or the mitochondrial (intrinsic) pathway; typically, chemotherapeutic drugs harness the mitochondrial pathway of apoptosis (43). SAHA and ZOL synergistically affected mitochondrial function, indicating that the activities of these agents converge upstream of mitochondria. In addition, experiments using the polycaspase inhibitor z-VAD-fmk revealed that caspase activation was required for the cytotoxic effects exerted by the combination of SAHA and ZOL, as judged by measuring propidium iodide uptake and by quantifying DNA fragmentation, further substantiating that SAHA/ZOL-induced cell death was brought about by apoptosis. Of note, z-VAD-fmk also protected against ΔΨ_m dissipation, suggesting that the mitochondrial apoptotic function was caspase dependent.

A large number of studies have shown that N-BPs, including ZOL, act on osteoclasts as well as on cancer cells by inhibiting FPP synthase, an enzyme of the mevalonate pathway (8). Several lines of evidence presented in our study indicate that the enhanced cytotoxicity of the combination of SAHA and ZOL stemmed from the cooperative action of these agents on the mevalonate pathway. First, our results show that the SAHA/ZOL-mediated loss of cell viability was significantly reduced by GGOH. In fact, the addition of GGOH fully reversed the proportion of synergistic cell death that was due to ZOL, suggesting that the contribution of ZOL to the synergistic effects arose entirely from inhibiting geranylgeranylation. Second, we found that SAHA interacted cooperatively with the specific geranylgeranyltransferase inhibitor GGTI-298 to elicit cancer cell death. Third, the effect of the combination of SAHA and ZOL on cell viability was mimicked by the combination of SAHA and mevastatin, an inhibitor of the proximal enzyme in the mevalonate pathway.

By inhibition of the mevalonate pathway, N-BPs, as well as statins, decrease the cellular levels of both FPP and geranylgeranyl pyrophosphate. However, with respect to the in vitro effects of ZOL on prostate cancer cells, inhibition of geranylgeranylation seems to be more critical than the inhibition of farnesylation (34, 35, 44). Here, we have presented evidence that the synergistic action of SAHA and ZOL was also conferred through inhibiting geranylgeranylation rather than farnesylation. This conclusion is supported by the following findings: SAHA/ZOL-induced cell death was reduced by GGOH but not by FOH. Consistently, GGTTI-298 synergized with SAHA to induce cell death, whereas FTI-277 had no effect. Likewise, the cytotoxic activity of mevastatin, when applied alone or in combination with SAHA, was attenuated by GGOH but not by FOH. This finding is in concordance with the notion that the anticancer effects of statins stem from reduced geranylgeranylation rather than reduced farnesylation (23).

Both HDIs and BPs are promising anticancer agents. Here, we have shown that their antineoplastic efficacy can
be considerably increased by cotreatment. Moreover, we have shown that SAHA also interacts synergistically with a specific inhibitor of protein geranylgeranylation, GGTI-298, and a statin, mevastatin, to enhance anticancer activity. Given that statins as monotherapy are ineffective anticancer agents (23, 45), the combination of HDIs and statins now warrants a more in-depth exploration for the treatment of cancer. In conclusion, our study suggests that the combination of HDIs and BPs as well as other inhibitors targeting the mevalonate pathway may be an effective strategy for anticancer therapy.

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