Farnesyl and Geranylgeranyl Transferase Inhibitors Induce G1 Arrest by Targeting the Proteasome

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Abstract

Isoprenoid inhibitors are being evaluated as agents for the treatment of cancer. Their antitumor activity is attributed to inhibition of post-translational modification of Ras, which is crucial for its translocation and attachment to the plasma membrane, and ultimate involvement in signal transduction. However, whether blocking of Ras is solely responsible for the observed antitumor activity is unresolved. In this report, we propose an alternate mechanism. Using breast tumor models, we show that agents possessing a lactone moiety, including statins (such as lovastatin) and the isoprenoid inhibitors (such as FTI-277 and GGTI-298), mediate their cell cycle inhibitory activities by blocking the chymotrypsin activity of the proteasome in vitro. This results in the accumulation of cyclin-dependent kinase inhibitors p21 and p27 with subsequent G1 arrest. Cells devoid of p21 were refractory to the growth-inhibitory activity of lovastatin, FTI-277, and GGTI-298. However, in these p21 null cells, isoprenylation of key substrates of farnesyl transferase (such as Ras) and of geranylgeranyl transferase (such as RAP-1) were inhibited by FTI-277 and GGTI-298, respectively, suggesting that although both these isoprenoid inhibitors reached and inhibited their intended targets, inhibition of the isoprenylation of Ras and RAP-1A are not sufficient to mediate G1 arrest. We also show that the cell cycle effects can be attributed to the functional lactone moiety of the aforementioned agents. Collectively, our data suggest that FTI and GGTI and other agents containing an active lactone moiety mediate G1 arrest via inhibition of the proteasome and up-regulation of p21, independent of the inhibition of isoprenylation of Ras or RAP-1. (Cancer Res 2006; 66(2): 1040-51)

Introduction

Isoprenoid inhibitors, such as farnesyl transferase inhibitors (FTI), were developed originally as anti-Ras agents for the treatment of many types of solid tumors whose proliferation is dependent on Ras. To be functional, the Ras-like proteins require post-translational modification, translocation, and attachment to the plasma membrane. Sequentially, these proteins are isoprenylated followed by proteolytic cleavage of the COOH terminus at the CAAX motif and then carboxymethylation at the modified cysteine residue and/or a palmitoylation of certain Ras forms, such as H-Ras and N-Ras (1). RAP-1 is a 21-kDa Ras-related human gene product and shares a similar COOH-terminal CAAX domain with the Ras proteins. The CAAX motif on RAP-1 and Ras is the substrate for geranylgeranyl transferase–mediated geranyl geranylation and farnesyl transferase–mediated farnesylation, respectively. Therapeutically, isoprenoid inhibitors have been used to target constitutively active Ras or other small GTP-binding proteins that are mutated in a variety of tumors (2, 3). Until now, their mode of action has focused exclusively on inhibition of the Ras signaling pathway. However, when these were used in the clinic, it became clear that their antitumor activity could not solely be through Ras inhibition (4–8). For example, the tumor sensitivity of SC66336, an FTI in clinical trial, was more inhibitory than dominant-negative Ras in assays of soft agar colony formation and cell proliferation, suggesting activity against targets other than, or in addition to, Ras (9).

Although several candidates from the group of Ras-related molecules have been identified (10), their dominant target, for the most part, has remained elusive. We approached the search for their alternate target by asking whether these agents could function via a Ras-unrelated pathway, solely based on their structures. Several years ago, it was reported that lactacystin, a pure inhibitor of the proteasome, is functional due to its active lactone moiety (11, 12). Moreover, biochemical analyses on the mechanism of proteasome inactivation by lactacystin strongly suggest that the lactone moiety is the key structural species responsible for proteasome inhibition, whereas the dihydroxy acid and the N-acetyl-l-cysteine intermediate are not inactivating (11, 13). Additionally, we have shown previously (14) that the dihydroxy acid forms of lovastatin and pravastatin (structurally similar to the active, open-ring form of lactacystin) failed to inhibit the proteasome. In this report, we questioned whether FTIs and GGTIs, which contain a lactone moiety, could also inhibit the proteasome with resulting G1 arrest. We show that the lactone-containing compounds, such as FTI-277 and GGTI-298, are capable of modulating cell cycle progression in breast cancer by inhibiting the proteasome. Additionally, these inhibitors inhibit the proteasome at physiologic concentrations. Complimentary evidence for the role of lactone agents in causing cell arrest is provided by a panel of agents grouped into three classes according to the following criteria: structural similarity to the active lactone, cell cycle inhibition, induction of cyclin-dependent kinase inhibitors (CDKs), and inhibition of the proteasome activity. Together, the results suggest that the functional lactone moiety of the statins and the isoprenoid inhibitors may be essential for the observed proteasome inhibition and G1 arrest.

Materials and Methods

Reagents, cell lines, and culture conditions. Lovastatin and simvas-tatin were purchased from LKT Laboratories, Inc. (St. Paul, MN), FTI-277, GGTI-298, Ftsase Inhibitor II, lactacystin, MG-132, epoxomicin, and succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (7-AMC) were purchased from Calbiochem (La Jolla, CA), and curcumin, trichostatin A, ascorbate, aspirin,
<table>
<thead>
<tr>
<th>Group</th>
<th>Compound</th>
<th>IC₅₀* (µmol/L)</th>
<th>Cell cycle arrest</th>
<th>(Hs578T/MDA-MB-231/MDA-MB-436)</th>
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<tbody>
<tr>
<td>I</td>
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<td>G₂/G₂/G₂</td>
<td></td>
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<tr>
<td></td>
<td>MG-132</td>
<td>2.5</td>
<td>G₂/G₂/G₂</td>
<td></td>
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<tr>
<td></td>
<td>Lactacystin</td>
<td>2</td>
<td>G₂/G₂/G₂</td>
<td></td>
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<tr>
<td></td>
<td>Trichostatin A</td>
<td>10</td>
<td>G₂/G₂/G₂</td>
<td></td>
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<td></td>
<td>Curcumin</td>
<td>8</td>
<td>G₂/G₂/G₂</td>
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<td></td>
<td>Proteasome</td>
<td>2.5</td>
<td>G₂/G₂/G₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibitor I</td>
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<td>G₂/G₂/G₂</td>
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<th>Group</th>
<th>Compound</th>
<th>IC50* (µmol/L)</th>
<th>Cell cycle arrest (^\dagger) (Hs578T/MDA-MB-231/MDA-MB-436)</th>
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<td><strong>Group II</strong></td>
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<td></td>
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<td>Simvastatin</td>
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<td>G1/G1/none</td>
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<td></td>
<td>FTI-277</td>
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<td>G1/G1/none</td>
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<td></td>
<td>GGTI-298</td>
<td>15</td>
<td>G1/G1/none</td>
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<td><strong>Group III</strong></td>
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<td></td>
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<td></td>
<td>Acetylsalicylic acid (aspirin)</td>
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</tr>
<tr>
<td></td>
<td>Butyrolactone</td>
<td>35</td>
<td>None/None/None</td>
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(Continued on the following page)
butyrolactone, Na-butyrate, and chymotrypsin (C-3142) were purchased from Sigma (St. Louis, MO). Serum was purchased from Hyclone Laboratories (Logan, UT), and cell culture media was purchased from Life Technologies, Inc. (Grand Island, NY). All other chemicals were reagent grade. All cell lines were purchased from the American Type Culture Collection (Rockville, MD), and the culture conditions for MDA-MB-231, MDA-MB-436, and Hs578T breast cancer cell lines were described previously (15).

**Cell cycle analysis.** For DNA content analysis, harvested cells were centrifuged at 1,000 × g for 5 minutes, fixed by the gradual addition of ice-cold 70% ethanol, and washed with PBS. Cells were then treated with RNase (10 μg/mL) for 30 minutes at 37°C, washed once with PBS, and resuspended and stained in 1 mL of 69 μmol/L propidium iodide in 38 mmol/L sodium citrate for 30 minutes at room temperature. The cell cycle phase distribution was determined by analytic DNA flow cytometry as described previously (16). The percentage of cells in each phase of the cell cycle was calculated using FlowJo software (TreeStar, Ashland, OR).

**Antibodies and Western blot analysis.** Cells were treated with different doses of Epoxomicin, MG-132, Lactacystin, Trichostatin A, Curcumin, Lovastatin, Simvastatin, FTase II, ascorbate, aspirin, butyrolactone, and Na-butyrate (in water) for 36 hours as indicated in the figure legends and dose-response changes were monitored by flow cytometry. The phase of the cell cycle where arrest was mediated is indicated for each drug. The experiment was repeated three times.

**Grouping of lactone containing compounds based on chymotrypsin inhibition and cell cycle modulation (Cont’d)**

<table>
<thead>
<tr>
<th>Structure</th>
<th>IC50* (μmol/L)</th>
<th>Cell cycle arrest† (Hs578T/MDA-MB-231/MDA-MB-436)</th>
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<tr>
<td>Sodium butyrate</td>
<td><img src="image" alt="Structure" /></td>
<td>40</td>
</tr>
<tr>
<td>FTase II</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt;50</td>
</tr>
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</table>

*IC50* was determined by *in vitro* chymotrypsin assay where 0.03 unit of purified chymotrypsin was incubated in the presence of increasing concentrations of the indicated drugs for 2 hours at 37°C at which point the fluorogenic peptide substrate (Suc-LLVY-AMC) was added. The fluorescence assays (excitation, 360nm; emission, 465 nm) were conducted at 37°C for 2 hours in microtiter plates. Each experiment was repeated at least three times, and the value shown is the average of these experiments.

†Hs578T, MDA-MB-231, and MDA-MB-436 were treated with increasing concentrations of the indicated drugs for 36 hours. The cell cycle distribution changes were monitored by flow cytometry. The phase of the cell cycle where arrest was mediated is indicated for each drug. The experiment was repeated three times.
volume of 200 μL. Substrate hydrolysis was measured by continuous monitoring of fluorescence (excitation at 360 nm, emission at 465 nm) on a microtiter plate reader (Victor², Perkin-Elmer, Shelton, CT) of the liberated 7-amido-4-methylcoumarin for 15 minutes. The emitted fluorescence of the experimental and control samples (after background subtraction; i.e., buffer alone) plotted against the amount of the inhibitor shows the extent of inhibition of the proteasome or chymotrypsin activity in the cell lysate. In the control lysate (without inhibitor), the plot depicts the inherent proteasome activity in the cell lysate.

In vitro chymotrypsin activity assay. The method used to measure chymotrypsin activity was essentially the same as that used for the proteasome activity assay except that 0.03 unit of purified chymotrypsin (C-3142) was used in place of the cell extract. Briefly, 0.03 unit of chymotrypsin was incubated with increasing concentrations of the indicated inhibitors, or 0.03 unit of the enzyme was incubated with a single amount of the inhibitors in the presence of increasing concentrations of the substrate. The activity of the enzyme was then measured fluorometrically as in the proteasome activity assay protocol.

Results

Induction of G₁ arrest by isoprenoid inhibitors. To assess the inherent role of the lactone moiety in mediating G₁ arrest, we chose three isoprenoid inhibitors FTI-277, GGTI-298, and FTase II based on their structural similarity to lactone containing agents with known inhibitory activity toward proteasome inhibitors MG-132 and lovastatin (see Table 1 for structures). We questioned whether these three lactone moiety–containing isoprenoid inhibitors could also inhibit the proteasome. We used a model system composed of three different breast cancer cell lines (MDA-MB-231, MDA-MB-436, and Hs578T), with different patterns of expression of p21. All cell lines are estrogen receptor negative, p53 mutant or null, and Rb mutant or null. However, the protein p21 is expressed only in MDA-MB-231 and Hs578T cells and not in MDA-MB-436 cells (17). Cells from the three cell lines were treated for 36 hours with the indicated concentrations of the aforementioned isoprenoid inhibitors and also with two lactone containing statins, the prodrug forms of lovastatin (which, we have previously shown to cause G₁ arrest; ref. 14) and simvastatin and analyzed by flow cytometry (Fig. 1). The flow cytometric data revealed that lovastatin and simvastatin induced G₁ arrest in both MDA-MB-231 and Hs578T cells but only minimal arrest in MDA-MB-436 cells. FTI-277 and GGTI-298 effectively arrested MDA-MB-231 and Hs578T in the G₁ phase of the cell cycle, but FTase II had no effect on the cell cycle of the three cell lines examined. Overall, the cell arrest was most significant in the MDA-MB-231 and Hs578T cells treated with GGTI-298 followed by FTI-277. FTase II had much less effect. In the MDA-MB-436 cell line, which is devoid of the p21 protein, no significant cell cycle arrest was observed at any of the concentrations of the isoprenoid inhibitors.

p21/p27, m-Ras, and RAP-1 accumulate upon treatment with isoprenoid inhibitors. To determine whether the cell cycle
G1 arrest mediated by FTI-277 or GGTI-298 was due to changes in the levels of p21 and p27, we treated the three cell lines with the indicated concentrations of FTI-277, GGTI-298, and FTase II for 36 hours and analyzed the expression of p21 and p27 by Western blot analysis (Fig. 2). The induction of p21 was most prominent in MDA-MB-231 and Hs578T cell lines treated with FTI-277 and GGTI-298. Both inhibitors showed ~6-fold and 3-fold increases of p21, respectively, in MDA-MB-231 and Hs578T. Treatment with FTase II minimally induced the CDKIs (Fig. 2), which was consistent with its inability to arrest the cell cycle in G1, as indicated by flow cytometric (fluorescence-activated cell sorting) data (Fig. 1). p27 was also induced in MDA-MB-231 and Hs578T cells in response to FTI-277 and GGTI-298; however, the induction of p27 was less pronounced than that of p21 (Fig. 2). These studies suggest that the cell cycle arrest observed in our model cell lines in response to FTI-277, GGTI-298 was mediated predominantly by the accumulation of the p21 protein.

To confirm that the substrates of FTase and GGTase are affected by the treatment of cells with FTIs and GGTIs, we examined the isoprenylation of Ras and RAP-1 in the FTI- and GGTI-treated cells. Cells treated with a range of concentrations of FTI-277 or GGTI-298 showed inhibition of post-translational isoprenylation processing of m-Ras by FTI-277 and RAP-1 by GGTI-298, resulting in a shift of the processed form to a slower-migrating, unprocessed form, even at the lowest concentrations of the drugs used (Fig. 2). FTase II had no effect on RAP-1 or m-Ras. In MDA-MB-436 cells, which are resistant to the growth-inhibitory effects of FTI and GGTI, RAP-1 and m-Ras processing was also completely inhibited by both GGTI-298 and FTI-277 (Fig. 2). The levels of both Ras and RAP-1 were also elevated in all three cell lines in response to treatment, most likely because these two proteins were being degraded via the proteasome pathway (18–20). These results suggested that although FTI-277 and GGTI-298 reached and inhibited their intended targets (i.e., FTase and GGTase), inhibition of the isoprenylation of Ras and RAP-1 did not mediate G1 arrest.

**Inhibition of CDK2 activity by FTI-277 and GGTI-298.** Next, we examined the ability of these inhibitors to modulate the activity of CDK2 kinase, a key enzyme in G1-S transition, by measuring the phosphorylation of histone H1 in CDK2 immunoprecipitates prepared from FTI-277-, GGTI-298-, and FTase II-treated cells.
Treatment of the MDA-MB-231 and Hs578T cells resulted in marked inhibition of the CDK2 kinase activity in a dose-dependent fashion (Fig. 3). GGTI-298 produced the most dramatic decrease (about one twelfth of that in the control cells) in kinase activity at the 10 μmol/L drug concentration compared with the untreated control cells followed by FTI-277, which showed a decrease to about one sixth of the control kinase activity. Similar to the Western blot analysis results regarding p21 and p27, there was no significant change in the kinase activity of these two cell lines at any concentration of FTase II. In MDA-MB-436 cells, which lack the p21 protein, treatment with any of the inhibitors resulted in only minimal inhibition at the highest drug concentration (Fig. 3).

To determine whether the inhibition of CDK2 kinase activity is due to the accumulation of p21 and p27 and subsequent increased binding to CDK2, we assessed the components of CDK2 complexes following the treatment of cells with FTI-277 and GGTI-298. A dose-dependent increase of the CKIs (p21 and p27) in complex with CDK2 was observed (Fig. 3) in MDA-MB-231 and Hs578T cells. MDA-MB-436 lacks p21; thus, no changes were observed, as expected. Treatment with FTase II produced no changes in p21 in any of the three cell lines examined. These data suggest that the induction of p21 and, to a lesser extent, p27 following treatment of cells with FTI-277 and GGTI-298 leads to their increased binding to CDK2 and hence inhibition of CDK2 activity and thus subsequent G1 arrest. Collectively, these results raise the possibility that GGTI-298 and FTI-277 effectively reach their bona fide targets (i.e., GGTase and FTase) in these human breast cancer cell lines, but this is not sufficient to inhibit cell growth implying that inhibition of the proteasome is likely to play a role in mediating G1 arrest by these agents.

Inhibition of proteasome activity by isoprenoid inhibitors. Both FTI-277 and GGTI-298 have a reactive lactone moiety and are structurally similar to a number of proteasome inhibitors, including the specific and potent inhibitors, such as MG-132, lactacystin, and PS341 (21–31). The ability of FTI-277 and GGTI-298 to inhibit the proteasome activity was assessed to validate whether the mechanism by which they mediate G1 arrest and accumulate p21 or p27 or both is at least in part through inhibition of the proteasome. To this end, we assessed the ability of FTI-277, GGTI-298, and FTase II to modulate the proteasome in cell extracts isolated from the three breast cancer cell lines (Fig. 4). Proteasome complexes prepared from MDA-MB-231, MDA-MB-436, and Hs578T cells were used to examine the chymotrypsin activity of the proteasome by using the fluorogenic substrate 7-AMC, as described in Materials and Methods. For these proteasome preparations from the three cell lines, the proteasome activity was inhibited in a dose-dependent fashion by FTI-277 and GGTI-298. FTI-277 and GGTI-298 inhibited the proteasome to about one sixth of the level seen in control extracts (without inhibitor). In contrast, FTase II minimally inhibited the proteasome, the same as in the control extracts. These findings therefore suggest that the isoprenoid inhibitors FTI-277 and GGTI-298 inhibit the proteasome activity in vitro at physiologic concentrations very similar to those required to cause G1 arrest and mediate the induction of p21 and p27 in vivo in MDA-MB-231 and Hs578T cells (Fig. 2).

Although these two inhibitors were effective in inhibiting the proteasome in MDA-MB-436, such inhibition had no effect on G1 cell arrest (Fig. 1) most likely because p21 is not expressed in this cell line. This observation therefore strongly suggests that p21 is the major “biological” effector of the proteasome for mediating G1 arrest.

Inhibition of purified chymotrypsin activity by isoprenoid inhibitors. The proteasome has three enzymatic activities: trypsin-like, chymotrypsin-like, and peptidyl glutamyl peptide hydrolyzing (32–34). Although crude cell extracts were used in the proteasome activity assay, as indicated in Fig. 4, to show that we were indeed measuring the chymotrypsin activity in the cell extracts, we examined the ability of FTI-277, GGTI-298, FTase II, and simvastatin (as positive control) to inhibit the pure chymotrypsin enzyme. This was examined by two different means, and both methods showed that these agents can indeed inhibit chymotrypsin activity (Fig. 5). In the first method, a constant amount (0.03 unit) of purified chymotrypsin was added to varying concentrations of simvastatin, FTI-277, GGTI-298, and FTase II, and the enzyme activity was measured based on the level of emission of the fluorogenic substrate for chymotrypsin, 7-AMC. We found that as the concentration of the inhibitors increased, the chymotrypsin activity was inhibited with the following sensitivity: simvastatin, GGTI-298 > FTI-277, and minimal inhibition by FTase II (Fig. 5A). In the second method, the levels of both the enzyme and the inhibitors were kept constant, as the concentration of the substrate (AMC)
were treated with the indicated concentrations of drugs for 36 hours followed by Western blot analysis with p21 and p27 (Fig. 6A) and flow cytometric analysis (Fig. 6B). Table 1 depicts the cell cycle phase arrest mediated by every agent in each of the three cell lines. Figure 6 shows dose-dependent treatments of each cell line with a representative agent form each group of agents. The results showed that all the agents in groups I I arrested all three cell lines in the G2 phase of the cell cycle (Table 1), and the arrest was more pronounced in MDA-MB-231 and Hs578T and less so in MDA-MB-436 (Fig. 6B). The group II agents arrested only the MDA-MB-231 and Hs578T in the G1 phase of the cell cycle, whereas the cell cycle of the MDA-MB-436 was unperturbed by these agents (Table 1; Fig. 6B). The group III agents did not produce any cell cycle arrest in any of the three cell lines examined (Table 1; Fig. 6B). We also examined the expression of p21 and p27 treated with each class of agent in all three cell lines (Fig. 6A), and results reveal that treatment of Hs578T and MDA-MB-231 results in very strong accumulation of p21 and p27 protein with group I agents and mainly of p21 with group II agents. In MDA-MB-436 cells (which are devoid of p21), p27 was significantly induced upon treatment with group I agents only. The levels of the p21 and p27 protein did not change upon treatment with the group III agents in all cell lines. These results show that based on structural similarities, lactone-containing drugs can be grouped into those mediating G2, G1, or no cell cycle arrest. Additionally, these results also suggest that very strong inhibition of the proteasome mediates G2 arrest (i.e., group I agents), whereas moderate inhibition of the proteasome results in G1 arrest (i.e., group II agents) in the cell lines examined in this study.

Discussion

Statins, such as simvastatin, marketed under the brand name Zocor traditionally have been prescribed for the treatment of high blood cholesterol. The rationale for such treatment is because these agents inhibit hydroxymethylglutaryl CoA reductase (HMGCR), the enzyme that converts HMG-CoA to mevalonate in the cholesterol biosynthetic pathway. Statins are considered...
very safe, with few side effects. They are administered in a prodrug, lactone (inactive) form, and are subsequently metabolized into the hydroxy acid (active) form by the cytochrome P450 liver enzymes (40, 41). Previously, we have shown that whereas the hydroxy acid form of statins directly inhibits HMGR in the cholesterol pathway, the lactone form functions through modulation of the proteasome (14).

Like statins, isoprenoid inhibitors, such as FTI-277 and GGTTI-298, also possess functional lactone moieties. The main objective of this report was to determine whether lactone compounds (FTI-277 and GGTTI-298) cause a G1 block through proteasome inhibition as a result of increases in p21/p27 in breast tumors. In the current study, we found that treatment of cells with FTI-277 and GGTTI-298 resulted in G1 arrest, accumulation of the CKIs (p21/p27), and inhibition of CDK2 kinase activity. In addition, the lactone-containing FTI-277 and GGTTI-298 inhibited the proteasome activity. Collectively, our findings suggest that one mechanism by which FTI-277 and GGTTI-298 mediate growth inhibition is through inhibition of the proteasome, independent of their actions on farnesyl or geranylgeranyl transferase enzymes.

Complimentary evidence for the role of lactone agents in causing cell arrest is provided by a panel of agents grouped into three classes (Table 1) according to the following criteria: structural similarity to the active lactone, flow cytometry, Western blot analyses, and in vitro chymotrypsin assays. Together, the results show that the lactone-containing agents fall into three different categories of compounds: group I, those that inhibit the proteasome with high specificity and high sensitivity. These agents completely inhibit the proteasome activity and result in severe induction of p21 and p27 (and other proteins degraded by the proteasome; data not shown) and also result in cells arresting in G2 phase of the cell cycle. Group II agents, those that inhibit the proteasome only partially and do so at higher concentrations than those in group I. Treatment of cells with these agents result in mainly the induction of p21 (and

Figure 6. Cell cycle perturbation mediated by lactone-containing agents. Hs578T, MDA-MB-231, and MDA-MB-436 were treated with the indicated concentrations of epoxomicin, simvastatin, and ascorbate for 36 hours. A, after treatments, cells were harvested, and cell lysates prepared and subjected to Western blot analysis with the indicated antibodies. B, the cell cycle distribution changes were monitored by flow cytometry. Relative percentages of cells in each phase of the cell cycle. I, II, and III refer to the three groups of lactone-containing agents described in Table 1.
minimally of p27) and G1 arrest. Statins and isoprenoid inhibitors fall into group II agents. Lastly, group III agents, with an inactive lactone moiety, result in very low inhibition of the proteasome, no induction of p21, p27, or any of the proteins degraded by the proteasome pathway and no perturbation of cell cycle. Together, these results suggest that lactone-containing drugs can be divided into different groups of agents based on their structural similarities and degree by which they inhibit the proteasome.

The model in Fig. 7 shows that the dihydroxy acid (open ring) form ofLovastatin and simvastatin inhibit HMGR, blocking cholesterol biosynthesis. By contrast, closed-ring lactone compounds and isoprenoid inhibitors inhibit both prenyl transferases and the proteasome. Inhibition of prenyl transferases leads to accumulation of Ras and RAP-1, prevents their translocation to the plasma membrane (PM), and potentially blocks signal transduction. Because Ras, Rap-1, p21, and p27 are all degraded by the proteasome, inhibition of the proteasome results in the accumulation of these proteins and ultimately cell cycle arrest. As indicated by the question marks, the closed lactones and isoprenoid inhibitors might affect the RAF and nuclear factor-κB (NF-κB) pathways, modulating both transcription and cell cycle progression. In addition, whether proteolysis of p21 and p27 occurs by mechanism(s) different from or in addition to proteasome inhibition is not known.

Figure 7. Model for inhibition of the proteasome by isoprenoid inhibitors. Open-ring lactones act through HMGR, blocking cholesterol biosynthesis. By contrast, closed-ring lactone compounds and isoprenoid inhibitors inhibit both prenyl transferases and the proteasome. Inhibition of prenyl transferases leads to accumulation of Ras and RAP-1, prevents their translocation to the plasma membrane (PM), and potentially blocks signal transduction. Because Ras, Rap-1, p21, and p27 are all degraded by the proteasome, inhibition of the proteasome results in the accumulation of these proteins and ultimately cell cycle arrest. As indicated by the question marks, the closed lactones and isoprenoid inhibitors might affect the RAF and nuclear factor-κB (NF-κB) pathways, modulating both transcription and cell cycle progression. In addition, whether proteolysis of p21 and p27 occurs by mechanism(s) different from or in addition to proteasome inhibition is not known.
Our results showed that the lactone compounds investigated (FTI-277 and GGTI-298) inhibit the isoprenylation of Ras and RAP, resulting in the accumulation of the unprenylated forms of Ras and RAP. These compounds also inhibit the proteasome and chymotrypsin as measured by in vitro proteosome and chymotrypsin assays. This suggests that the FTIs and GGTIs may indeed have dual targets, one target being their intended FTase and GGTase enzymes and the other being the proteasome. Protein degradation by the ubiquitin-proteasome pathway plays a fundamental role in determining the turnover of important regulatory proteins in diverse physiology, including inflammation, protein folding, and cell cycle regulation (42–45). RAP-1 A and c-Raf1 have an ubiquitin fold similar to that of Ras, as predicted from X-ray crystallography studies, thereby implicating their degradation via the proteasome. Such evidence lends support to the notion that Ras/Rho/Rac and other small GTP-binding proteins are metabolized via several different pathways, including the proteasome.

Several in vitro and clinical observations also support our in vitro findings that FTIs and GGTIs target the proteasome. The first evidence is a phase II clinical trial for the treatment of acute myeloid leukemia. In this trial, cotreatment with FTIs and GGTIs led to synergistic cytotoxicity effects, coinciding with apoptosis in both myeloid cell lines and primary amyloid myeloma leukemia (46). In another study, such cotreatment was accompanied by a decrease in growth rate and regression in pancreatic xenographs in vivo (47). In a recent review by Brunner et al. (48), the mechanism of tumor response in a Ras/p53−/− tumor (having the same status as in our model cell lines MDA-MB-231 and Hs578T), following FTI and GGTI treatment, was attributed to increased apoptosis and a decrease in the S-phase fraction. The results presented in the above studies mirror our observations on the treatment of breast tumor cells with FTIs and GGTIs, which depict increased accumulation of unprocessed Ras and RAP (Fig. 3). This would suggest that these inhibitors operate via several mechanisms in Ras/RAP processing, one of which is through the proteasome and might, in fact, represent a common modus operandi in the various cell types investigated, such as leukemic, pancreatic, and breast cancer cells.

Taken together, the most compelling evidence to date resulting from the in vitro and in vivo studies shows that the action of isoprenoid inhibitors on tumor growth is not tightly linked to Ras status: implicating other pathways, such as the proteasome. Our proposed alternative mode of action of the isoprenoid inhibitors, through the ubiquitin-proteasome pathway, should provide a better understanding of the mechanism of action of these inhibitors and ultimately in the design of additional targeted strategies for using this class of agents as cytostatic or chemopreventive agents or both in breast cancer.

Acknowledgments

Received 9/22/2005; accepted 11/8/2005.

Grant support: NIH/National Cancer grant ROICA105066 (E.T. Efuet) and National Cancer Institute grant ROI-CA87548 (K. Keyomarsi).

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We thank Dr. Isabelle Bedrosian for critical analysis of this article.

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Cancer Res 2006; 66: (2). January 15, 2006 1050 www.aacrjournals.org

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