Antineoplastic agents target the 25-hydroxyvitamin D₃ 24-hydroxylase messenger RNA for degradation: implications in anticancer activity

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Abstract
Calcitriol or 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] has antitumor activity and hence its levels in patients may play an important role in disease outcome. Here, we report that the antineoplastic agents, daunorubicin hydrochloride, etoposide, and vincristine sulfate inhibited the ability of 1,25(OH)₂D₃ to cause the accumulation of mRNA for kidney 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24), an enzyme which catalyzes this hormone. This was not due to a drug-induced cytotoxic effect, reduction in the expression of the vitamin D receptor or inhibition of the vitamin D receptor–mediated activation of the mitogen-activated protein kinases or CYP24 promoter activity. Interestingly, there was selective degradation of CYP24 mRNA in the presence of the drugs. This was accompanied by an enhancement in the levels of 1,25(OH)₂D₃ in cells incubated with 25-hydroxy vitamin D₃. These data identify a novel mechanism of action of some commonly used antineoplastic agents which by decreasing the stability of CYP24 mRNA would prolong the bioavailability of 1,25(OH)₂D₃ for anticancer actions. [Mol Cancer Ther 2007;6(12):3131–8]

Introduction
Calcitriol or 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and analogues have received much interest as anticancer agents because the secosteroid hormone has shown antitumor activity (1–3). Furthermore, 1,25(OH)₂D₃ and analogues can enhance, either synergistically or additively, the antitumor activities of several classes of antineoplastic agents (3, 4). These findings have led to clinical trials to evaluate the efficacy of combining 1,25(OH)₂D₃ and drugs such as docetaxel (taxotere) in the treatment of androgen-independent prostate cancer (3, 5, 6). How such combinations enhance the antitumor activity of each other is poorly understood.

The steady state level of circulating 1,25(OH)₂D₃ is determined by a balance between 1,25(OH)₂D₃ bioactivation and degradation (7). Bioactivation of vitamin D involves firstly the 25-hydroxylation of inactive vitamin D₃ in the liver to form 25(OH)D₃ (7). This is followed by 1α-hydroxylation catalyzed by the cytochrome P450 enzyme, 25-hydroxyvitamin D₃ 1α-hydroxylase (CYP27B1), in the kidneys to produce the biologically active 1,25(OH)₂D₃ in the circulation. Metabolic inactivation of 1,25(OH)₂D₃ is catalyzed by the C23/C24-oxidation pathway (7), where multiple steps are performed by another cytochrome P450 enzyme, 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24), that is also expressed in the kidney. To prevent the excessive accumulation of 1,25(OH)₂D₃ and its toxic hypercalcemic effects, CYP24 expression is up-regulated by 1,25(OH)₂D₃ in a negative feedback mechanism (8). CYP24 also catalyzes the removal of 25(OH)D₃ from the body (9).

Previous studies have shown that mitogen-activated protein (MAP) kinases play important roles in mediating the stimulatory effect of 1,25(OH)₂D₃ on CYP24 expression (10, 11). Interestingly, antineoplastic agents are known to stimulate the activity of MAP kinases (12–15). Thus, a regulatory effect of antineoplastic agents on CYP24 expression seemed likely. Our data show that daunorubicin hydrochloride, etoposide, and vincristine sulfate, three well-characterized antineoplastic agents, caused the accumulation of 1,25(OH)₂D₃ in kidney cells. Under appropriate conditions, they inhibited, to varying degrees, the ability of 1,25(OH)₂D₃ to induce the accumulation of the CYP24 mRNA and hence the bioactivation of 1,25(OH)₂D₃. A drug-directed enhancement in the degradation of CYP24 mRNA is the most likely cause. This may constitute a novel mode of antitumor action of the drugs.

Materials and Methods

Chemicals
1,25(OH)₂D₃, 25(OH)D₃, anticancer drugs, protein A sepharose, and anti-ERK5 antibody were purchased from...
Sigma-Aldrich Pty. Ltd. The anti-ERK2 antibody was from Santa Cruz Biotech. The anti–vitamin D receptor (VDR) antibody (9A7) was a kind gift from Dr. E.M. Gardiner (Garvan Institute of Medical Research, New South Wales, Australia). The Dual Luciferase Assay System was from Promega. TRIzol and SuperScript III reverse transcriptase were purchased from Invitrogen. Random hexamer primers, deoxynucleotide triphosphates, and PCR primers were purchased from Genereworks. Primers designed to amplify cDNA for CYP24 (F, 5′-ctcgagcagatccatacttgc-3′; R, 5′-atatacttctggtattcag-3′), CYP27B1 (F, 5′-gggccgagtcctttcctgaa-3′; R, 5′-atcttctgtcttctgcc-3′), VDR (F, 5′-ggacggcgccttgggtctaact-3′; R, 5′-ctccctccacatcattcaca-3′), and GAPDH (F, 5′-accgagaagcttgctgcag-3′; R, 5′-cagtgaacctgccctg-3′) were based on Genebank sequences (CYP24 accession no., NM000782; CYP27B1 accession no., NM000785; VDR accession no., X67482; GAPDH accession no., NM002046). The iQ SYBR Green Supermix was purchased from Bio-Rad Laboratories.

**Plasmids**

The CYP24 promoter firefly luciferase constructs containing either proximal −298 or −1401 bp of the CYP24 promoter sequence together with 74 bp of the 5′-untranslated region in pGL3 basic vector have been described previously (10). The pRL-null vector containing the Renilla luciferase was purchased from Promega Corp.

**Transfection and CYP24 promoter activity**

COS-1 African green monkey kidney interstitial fibroblast or human embryonic kidney (HEK293T) cells were seeded (40,000/well) in a 24-well tray and grown in DMEM containing 5% FCS and antibiotics (10, 11). After an overnight culture, the medium was replaced with serum-free medium and the cells were treated with a drug in the presence or absence of 1,25(OH)2D3 (10 nmol/L). Control cells received vehicle (<0.1% v/v). After 24 h, the cells were harvested and lysates were assayed for dual luciferase activity using a TD20/20 Luminometer (Turner Design Instruments).

**RNA Extraction and Real-time PCR**

Total RNA (1 μg), extracted using TRIzol, was reverse-transcribed using Random hexamers and SuperScript III reverse transcriptase. Real-time PCR reactions were carried out in duplicate in a final volume of 10 μL. The cDNA samples were amplified using iQ SYBR Green Supermix primers (250 nmol/L) as described above. Relative expression between samples was calculated using the comparative cycle threshold (Ct) method (ΔCt; ref. 16).

**Estimation of 1,25(OH)2D3 Production from 25(OH)D3**

HEK293T cells (1.25 × 105/well) were grown to confluence. The cells were serum-starved overnight, incubated with 25(OH)D3 (8 μmol/L) in the presence or absence of daunorubicin, etoposide, vincristine, or vehicle. After 24 h, the medium was harvested for 1,25(OH)2D3 estimation by RIA after immunoextraction (ImmunoDiagnostics Systems Ltd.) and the cell pellets were processed for protein estimation. The assay detects down to 8 pmol/L of 1,25(OH)2D3. Cross-reactivity with 1,25(OH)2D3, 1,25(OH)2D2, and 24,25-(OH)2D3 is 100%, 91%, 0.001%, and 0.0123%, respectively.

**ERK1/ERK2 and ERK5 Activity Assays**

The activities of immunoprecipitated ERK1/ERK2 and ERK5 were assayed as described previously using myelin basic protein as a substrate (17, 18).

**Western Blots**

Western blotting was conducted as described previously (17). Briefly, lysate proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-VDR antibody. The immune complexes were detected by enhanced chemiluminescence. The blots were stripped and reprobed with anti–β-actin antibody. The films were scanned using a Canon Canoscan 9900F scanner.

**Statistical Analysis**

Statistical analysis was done using Student’s unpaired t test or the Tukey-Kramer multiple comparisons test.

**Results**

**Antineoplastic Agents Caused the Accumulation of 1,25(OH)2D3**

To gain an idea of whether antineoplastic agents could modulate the levels of 1,25(OH)2D3, we first incubated HEK293T cells with 25(OH)D3 which was converted to 1,25(OH)2D3 as expected (Fig. 1), validating this as an
in vitro model for investigation. We selected daunorubicin hydrochloride and etoposide, two well-characterized topoisomerase II inhibitors, and the Vinca alkaloid, vincristine sulfate, to study. These agents were investigated at concentrations similar to those found in the plasma of patients (19, 20). As shown in Fig. 1, all three drugs increased the accumulation of 1,25(OH)2D3 when the cellswere incubated with 25(OH)D3. These results show that the drugs could directly affect the levels of 1,25(OH)2D3 and argue against a toxic effect of the drugs. Indeed, we have shown that the drugs did not affect the viability of the kidney cells, either in the presence or absence of 1,25(OH)2D3, and in the presence or absence of serum (data not shown). In contrast, HL60 cells were readily killed.

Antineoplastic Agents Inhibited the Ability of 1,25(OH)2D3 to Up-regulate the Level of CYP24 mRNA

The above data could have been caused by an increased conversion of 25(OH)D3 to 1,25(OH)2D3 or an inability to degrade the 1,25(OH)2D3 that was formed. We therefore investigated the expression of CYP27B1 and CYP24. The data in Table 1 and Fig. 2 suggest that inhibition of the negative feedback control was the more likely cause because the drugs inhibited the ability of 1,25(OH)2D3 to up-regulate the level of CYP24 mRNA without affecting the level of CYP27B1 mRNA. Etoposide and daunorubicin hydrochloride were more effective than vincristine sulfate at inhibiting the 1,25(OH)2D3-mediated accumulation of CYP24 mRNA level (Fig. 2A and B). Similar inhibitory effects were observed at 4 or 6 h (data not shown). None of the drugs tested affected basal mRNA levels. These data suggest that antineoplastic agents could interrupt the negative feedback regulation of 1,25(OH)2D3 by suppressing the ability of 1,25(OH)2D3 to up-regulate the level of CYP24 mRNA.

Effects of Antineoplastic Agents on the Expression of VDR

To investigate how the drugs inhibited the up-regulation of CYP24 mRNA by 1,25(OH)2D3, we incubated COS-1 and HEK293T with daunorubicin hydrochloride or etoposide (Fig. 3A and B) and examined the expression of the VDR.

### Table 1. Daunorubicin hydrochloride and etoposide did not alter the expression of CYP27B1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP27B1 expression [2^−(CYP27B1 CT-GAPDH CT)]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.0043 ± 0.0011</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.0033 ± 0.0012</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.0036 ± 0.0015</td>
</tr>
</tbody>
</table>

NOTE: HEK293T cells in serum-free medium were incubated with vehicle (0.1% v/v), daunorubicin hydrochloride (1 μmol/L), or etoposide (10 μmol/L) in the presence of 1,25(OH)2D3 (10 nmol/L) for the indicated times. Total RNA was extracted and the level of CYP27B1 mRNA was determined by qRT-PCR. Results, calculated using the comparative cycle threshold method (ΔCT), are mean ± SE (n = 3). The level at time 0 h was 0.0046 ± 0.0002. No significant differences were observed between control and drug-treated cells (P > 0.05, Tukey-Kramer multiple comparisons test).
The drugs caused a modest but clear increase in the level of cell-associated VDR (Fig. 3A). Surprisingly, this increase was not associated with an increase in VDR mRNA levels (data not shown). Previous studies have shown that 1,25(OH)2D3, by increasing the half-life of the VDR protein, can increase VDR protein levels without increasing the VDR mRNA (21, 22). Thus, a similar mechanism might be involved in the action of the drugs on VDR protein expression. These data thus exclude a decrease in VDR expression as a cause for the inhibitory effect of the drugs on 1,25(OH)2D3-stimulated up-regulation of CYP24 mRNA.

**Effects of Antineoplastic Agents on the Activities of the ERK1/ERK2 and ERK5 MAP Kinases**

We have previously showed that ERK1/ERK2 and ERK5 MAP kinases were required for 1,25(OH)2D3 to stimulate the activity of the CYP24 gene promoter in VDR-transfected COS-1 cells (10). We therefore investigated whether the drugs prevented 1,25(OH)2D3 from stimulating the MAP kinases. Daunorubicin hydrochloride and etoposide alone (data not shown) caused a modest increase in the activities of ERK1/ERK2 (Fig. 3B) and ERK5 (Fig. 3C). However, neither daunorubicin (Fig. 3D) nor etoposide (data not shown) prevented 1,25(OH)2D3 from stimulating the activity of ERK5. Thus, it is highly unlikely that the drugs prevented 1,25(OH)2D3 from stimulating the MAP kinases.

**Effects of Antineoplastic Agents on the Activity of the CYP24 Promoter**

The above data raised the possibility that the drugs could inhibit gene transcription downstream of the MAP kinases. To investigate this, COS-1 cells were transiently cotransfected with a construct that contained the firefly luciferase gene driven by the proximal −298 bp CYP24 promoter. This promoter region contains two well-characterized vitamin D response elements that mediate the genomic actions of 1,25(OH)2D3 (23, 24). Interestingly, daunorubicin hydrochloride per se stimulated the activity of the CYP24 promoter (Fig. 4A), consistent with its effect on MAP kinases. The threshold of stimulation was 0.5 μmol/L and an EC50 of 0.8 μmol/L. At lower doses (0.1 and 0.3 μmol/L), the drug also caused a synergistic increase in CYP24 promoter activity in the presence of 1,25(OH)2D3, but this effect was not observed at higher doses (Fig. 4A). In the combined presence of 1,25(OH)2D3 and daunorubicin...
hydrochloride, the EC50 was ~0.09 μmol/L. These data show that the drug can stimulate basal CYP24 promoter activity as well as cause a synergistic induction with 1,25(OH)2D3. Etoposide and vincristine sulfate per se also caused a small increase in CYP24 promoter activity (Fig. 4B). In the presence of 1,25(OH)2D3, both drugs caused a synergistic increase in promoter activity. A synergistic interaction between the drugs and 1,25(OH)2D3 on CYP24 promoter activity was also observed in HEK293T cells (data not shown).

We also tested a longer promoter construct that contained 1401 bp of the CYP24 promoter. The data show that the extra 1100 bp had no significant effect on the ability of either the cytotoxic agents or 1,25(OH)2D3 per se to stimulate promoter activity in COS-1 cells (P > 0.05; Fig. 4C and D). However, the synergistic effect of 1,25(OH)2D3 and daunorubicin hydrochloride was reduced with the ~1401 bp promoter. Thus, whereas 1,25(OH)2D3 and daunorubicin hydrochloride alone gave a 1.8- and 2.1-fold increase over controls, respectively, with the longer promoter, the combination produced a 5-fold increase. This compared with an approximate 13-fold increase with the ~298 bp promoter construct. Thus, the coupling between the VDR and the CYP24 transcriptional machinery remained intact in the presence of the drugs.

Effect of Antineoplastic Agents on CYP24 mRNA Stability

The above observations suggest that the drugs could affect CYP24 mRNA at a posttranscriptional level. When HEK293T cells were incubated with 1,25(OH)2D3 for 24 h, washed and actinomycin D added to stop transcription, the addition of daunorubicin hydrochloride, etoposide (Fig. 5A), or vincristine sulfate (data not shown) enhanced the rate of decay of CYP24 mRNA. This effect was clearly evident within 1 to 2 h of drug treatment (Fig. 5A). A destabilizing effect of the drugs on CYP24 mRNA was also observed in COS-1 cells (data not shown). These results imply that a drug-promoted instability of CYP24 mRNA nullified their promoter-enhancing action. Neither daunorubicin hydrochloride nor etoposide affected the stability of either the VDR or CYP27B1 mRNA within the same samples (Fig. 5B and C).

Discussion

Our investigations into the effects of antineoplastic agents on the expression of CYP24 revealed interesting novel results. Daunorubicin hydrochloride, etoposide, and vincristine sulfate caused the accumulation of 1,25(OH)2D3 and this was associated with the inhibition of 1,25(OH)2D3-mediated increase in the level of the CYP24 mRNA. These effects were unlikely to involve the inhibition of topoisomerase II by daunorubicin hydrochloride and etoposide because vincristine sulfate, unlike anthracycline and etoposide, does not inhibit this enzyme. A suppressive effect of the drugs on VDR expression or signaling by 1,25(OH)2D3 can be excluded because the drugs per se increased the level of VDR protein and the 1,25(OH)2D3-mediated

Figure 4. Antineoplastic agents stimulated the activity of CYP24 promoter. COS-1 cells in serum-deficient medium were transiently transfected with a vector containing either a ~298 bp (A–D) or ~1401 bp (C and D) CYP24 promoter-luciferase construct. After 24 h, the cells were incubated with vehicle, daunorubicin hydrochloride (1 μmol/L or as indicated), etoposide (10 μmol/L), vincristine sulfate (5 μmol/L), and/or 1,25(OH)2D3 (10 nmol/L). Promoter activity was assayed 24 h later as described under Materials and Methods. Columns, mean fold induction over the basal value from four experiments conducted in triplicate determinations; bars, SE. Significance of difference between vehicle and a drug or between vehicle and 1,25(OH)2D3: *, P < 0.05; **, P < 0.001; between 1,25(OH)2D3 and 1,25(OH)2D3 + drug: *, P < 0.05; **, P < 0.01; between the ~298 and ~1401 bp constructs (C): *, P < 0.01 (Tukey-Kramer multiple comparisons test). Basal levels of promoter activity (mean ± SE; firefly/renilla luciferase) in vehicle-treated cells were: 0.14 ± 0.02 (A), 0.11 ± 0.01 (B), and 0.10 ± 0.01 (C and D) for the ~298 bp promoter and 0.03 ± 0.002 for the ~1401 bp promoter (P < 0.001).
activation of ERK5, a MAP kinase that is involved in regulating CYP24 promoter activity (10), was unaffected. Daunorubicin hydrochloride and etoposide per se also increased the activities of the MAP kinases.

The possibility that the drugs could inhibit CYP24 gene transcription induced by 1,25(OH)2D3, downstream of the VDR and MAP kinases, is also unlikely because the drugs not only stimulated the transcriptional activity of the CYP24 promoter on their own but also caused a synergistic increase in CYP24 promoter activity in the presence of 1,25(OH)2D3. The latter observation is most likely to have been caused by the drugs inhibiting the up-regulation of CYP24 by 1,25(OH)2D3, resulting in the loss of negative feedback control. The data further show that signaling from the VDR to the CYP24 promoter remained intact in the presence of the drugs.

All these data point to the possibility that the drugs promoted the degradation of CYP24 mRNA. Indeed, this was confirmed by our data. Concurrent investigations on the stability of the VDR and CYP27B1 mRNA species showed that the drugs did not affect the levels of the latter two mRNA species that have longer half-lives than the CYP24 mRNA. However, it is unlikely that mRNA half-life was a determinant of susceptibility to the drug-induced degradation. Studies on the stability of protein kinase Cδ (25), Hox 2.1 (26), and vascular cell adhesion molecule (27) mRNA have shown a lack of correlation between mRNA half-life and the action of etoposide. Our observation that daunorubicin hydrochloride, etoposide, and vincristine sulfate promoted CYP24 mRNA degradation places these antineoplastic agents in the same category as parathyroid hormone (28) and the anti-HIV protease inhibitor, ritonavir (29), which have been reported to promote CYP24 mRNA instability, thus identifying a novel action of the cytotoxic agents.

The enhanced rate of CYP24 mRNA degradation caused by the drugs most likely counteracted their positive effects on the CYP24 promoter. Thus, the overall reduction in 1,25(OH)2D3-mediated accumulation of CYP24 would be expected to preserve the levels of vitamin D metabolites and enhance or prolong their biological actions. Indeed, this concept is supported by a number of observations. Ritonavir, which also inhibits CYP24, increased the intracellular levels of 1,25(OH)2D3 and potentiated the ability of 1,25(OH)2D3 to induce growth arrest and differentiation of HL60 cells (29). Similarly, genistein, ketoconazole, and liarozole, by inhibiting the activity of CYP24 or its expression, can amplify the antiproliferative effects of 1,25(OH)2D3 in DU145 prostate cancer cells (30–32). These enhancing effects are consistent with our observation that the antineoplastic agents potentiated 1,25(OH)2D3-mediated activation of the CYP24 promoter. Furthermore, we have found the drugs to not only inhibit 1,25(OH)2D3-mediated increase in CYP24 mRNA in DU145 cells but the drug + 1,25(OH)2D3 combination also caused an enhanced loss of viability of these cells.8 These data suggest that our observations in cells of the kidney, the

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primary organ for bioactivation and inactivation of 1,25(OH)_{2}D_{3} are also relevant to other cell types.

Our observations have important medical repercussions for anticancer therapy because the ability of the drugs to suppress 1,25(OH)_{2}D_{3}-mediated accumulation of CYP24 differed between them. In combination chemotherapy involving 1,25(OH)_{2}D_{3} and an antineoplastic agent, relatively high doses of 1,25(OH)_{2}D_{3} are generally required with the concomitant increase in risk of adverse side effects. Indeed, a major hurdle to achieving a favorable clinical response is the need for doses that are just below those that cause hypercalcemia and hypercalciuria (3). Our findings suggest that the choice of drugs is important in achieving the greatest suppression of CYP24 expression. As these anticancer drugs also increased VDR protein, it may be possible to reduce the dose of 1,25(OH)_{2}D_{3} to achieve a favorable clinical response because these effects would likely increase the sensitivity and responsiveness of cancer cells to 1,25(OH)_{2}D_{3}, in addition to prolonging its bioavailability as discussed above. Future studies could test this in a preclinical in vivo model. For example, it would be possible to treat mice with an antineoplastic agent for a few days in the presence or absence of 1,25(OH)_{2}D_{3} and examine the levels of CYP24 mRNA and VDR protein in the kidneys or another tissue, combined with the measurement of vitamin D metabolites. Given that different drugs exhibit different abilities at suppressing CYP24 mRNA accumulation, the ability of a range of antineoplastic agents to suppress CYP24 mRNA accumulation could be compared to identify those with the greatest suppressive ability.

In summary, this study has revealed a previously unknown mechanism of action of daunorubicin hydrochloride, etoposide, and vincristine sulfate and this new understanding is likely to contribute to approaches for improved anticancer therapy involving 1,25(OH)_{2}D_{3} and an antineoplastic agent.

Acknowledgments
The authors thank Dr. Y.Q. Li for technical assistance.

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Mol Cancer Ther 2007;6(12): December 2007

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