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Selective Inhibition of the Tumor Marker Aldo-keto Reductase Family Member 1B10 by Oleanolic Acid

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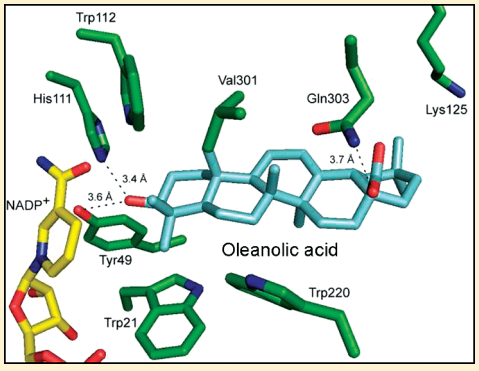
ABSTRACT: A human member of the aldo-keto reductase (AKR) superfamily, AKR1B10, was recently suggested as a therapeutic target in the treatment of several types of cancer. Due to its high sequence identity with human aldose reductase (AKR1B1), selective inhibition of AKR1B10 compared with AKR1B1 is required for the development of anticancer agents. In this study, we have examined AKR1B10 inhibition by seven pentacyclic triterpenes (**1**−**7**) that show potential anticancer properties. Among them, oleanolic acid (**1**) was found to be the most potent competitive inhibitor (inhibition constant, 72 nM) with the highest AKR1B10/AKR1B1 selectivity ratio of 1370. Molecular docking of **1** with AKR1B10 and AKR1B1 and site-directed mutagenesis studies suggested that the nonconserved residues Val301 and Gln303 in AKR1B10 are important for determining its inhibitory potency and selectivity. Oleanolic acid (**1**) also inhibited the cellular metabolism by AKR1B10 (IC_{50}, 4 μM) and decreased mitomycin C tolerance of colon cancer HT29 cells. Thus, the selective and potent inhibition of AKR1B10 by **1** may be related to a possible cancer inhibitory role.



A human member of the aldo-keto reductase (AKR) superfamily, AKR1B10, is a NADPH-dependent reductase, which was originally identified as a human aldose reductase (AKR1B1)-like protein that is up-regulated in hepatocellular carcinomas.[1] AKR1B10 is overexpressed in lung carcinomas,[2] uterine carcinomas,[3] and cholangiocarcinomas,[4] as well as hepatocellular carcinomas. The silencing of the AKR1B10 gene results in growth inhibition of cancer cells[5−7] and suppression of growth of hepatocellular carcinoma xenografts in mice,[7] indicating that the enzyme participates in the tumor development. In addition, AKR1B10 is suggested to be involved in the development of mitomycin C resistance in human colon cancer cells.[8] Thus, this enzyme is not only a potential diagnostic and/or prognostic marker but also a target for the prevention and treatment of the above types of cancer.[9]

AKR1B10 is a NADPH-dependent monomeric reductase that reduces a variety of aldehydes, including endogenous substrates such as acrolein, 4-hydroxynonenal, and retinals.[1,5,6,10−12] The enzyme is suggested to participate in cell carcinogenesis and tumor development by detoxifying cytotoxic carbonyls,[5,6,10] mediating retinoic acid homeostasis,[11] and regulating cellular fatty acid synthesis and lipid metabolism.[6,12] However, because of its high amino acid sequence identity with AKR1B1, the properties of AKR1B10 are similar to those of AKR1B1,[1,12] which plays important roles in glucose metabolism and detoxification of lipid peroxidation products and precursors of advanced glycation end products.[13,14] In this respect, selective inhibition of AKR1B10 over AKR1B1 is required for the development of anticancer agents targeting AKR1B10.

Previously reported inhibitors of AKR1B10 are bile acids,[12] synthetic aldose reductase inhibitors,[9,15] flavonoids, anti-inflammatory agents,[15] additional plant phenols,[16] 9-methyl-2,3,7-trihydroxy-6-fluorone,[17] chromene-3-carboxamide derivatives,[18] and fibrates.[19] Among them, tolrestat, an aldose reductase inhibitor, and the chromene-3-carboxamide derivatives are potent inhibitors, but almost equally inhibit AKR1B1. In contrast, bile acids and glycyrrhetic acid (**7**), an anti-inflammatory agent, show relatively high selectivity to AKR1B10 over AKR1B1. Compound **7** is a plant-derived pentacyclic triterpenoid, of which some are cytotoxic against various cancer cell lines.[20−23] The pentacyclic triterpenoids also exhibit other multiple actions such as anti-inflammatory, anti-HIV, hepatoprotective, hypoglycemic, and immunomodulatory activities, and herbs containing these triterpenoids have been used widely for medicinal purposes in many Asian countries. In order to find more selective inhibitors

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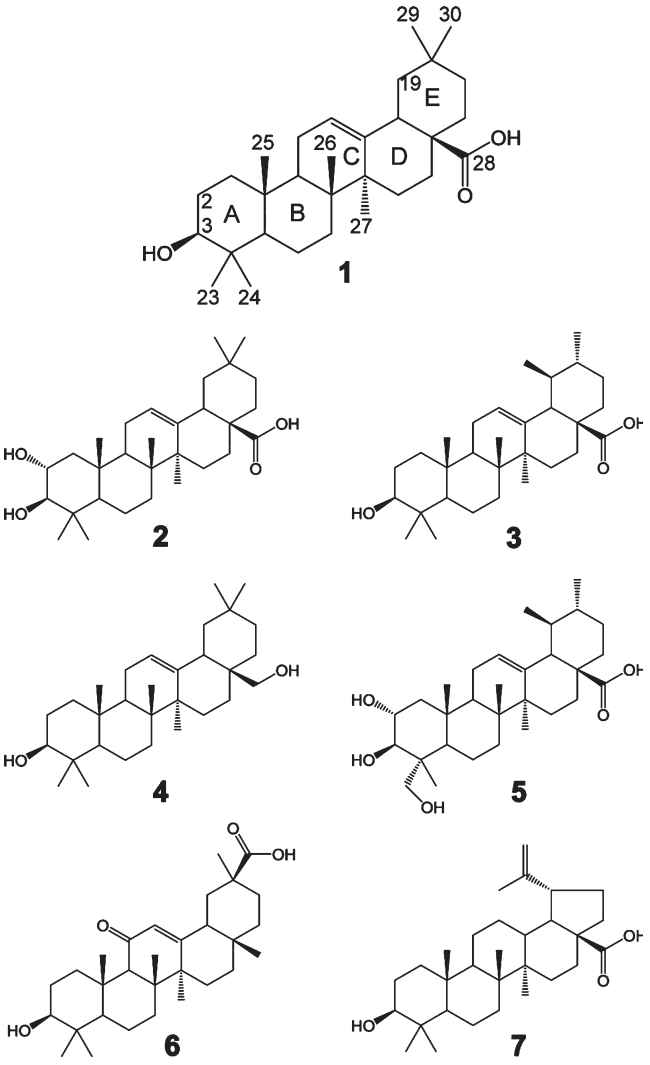
of ARK1B10, we have compared the inhibitory potencies against AKR1B10 and AKR1B1 of oleanane, ursane, and lupane triterpenoids, which are structurally distinct from **7**. Among them, oleanolic acid (**1**) was found to be the most potent and selective, showing an inhibition constant (K_i) of 72 nM for AKR1B10 and 1370-fold less inhibition for AKR1B1. Since the selectivity of **1** was much higher than those of the above known AKR1B10 inhibitors, we also investigated its binding mode in the active site of the enzyme by docking simulations for both AKR1B10 and AKR1B1 and site-directed mutagenesis of the binding-site residues of AKR1B10.



RESULTS AND DISCUSSION

Inhibitory Potency and Selectivity of Triterpenoids. The compounds evaluated in this study were the oleanane triterpenoids **1**, **2**, **4**, and **6**, the ursane triterpenoids **3** and **5**, and the lupane triterpenoid **7**. All the triterpenoids inhibited AKR1B10 more potently than AKR1B1 (Table 1). Among them, oleanolic acid (**1**) showed the lowest IC_{50} value for AKR1B10. In contrast, the inhibitory potency of the hydroxylated oleanolic acid derivatives [maslinic acid (**2**) and erythrodiol (**4**)] was low, suggesting that the introduction of a 2α-hydroxy group into the A ring of **1** and replacement of the 28-carboxyl group with an alcohol group interfere with the binding of **1** to the inhibitor binding site of the enzyme. Betulinic acid (**7**), ursolic acid (**3**), and asiatic acid (**5**) were also less potent inhibitors for AKR1B10, suggesting that the six-carbon E ring with 29,30-dimethyl substituents, together with the presence of the 28-carboxyl group, is an important structural prerequisite for potent inhibitors. Although **1** showed the second most potent IC_{50} value in the inhibition of AKR1B1, its inhibitory selectivity to AKR1B10 (IC_{50} ratio of AKR1B1/AKR1B10) was the greatest. The ratio of 1370 was also much higher than that of a bile acid, isolithocholic acid, which was previously known as the most selective inhibitor of AKR1B10.[12]

Table 1. Effects of Pentacyclic Triterpenoids on Reductase Activities of AKR1B10 and AKR1B1

	IC_{50} (μM)		ratio
inhibitor	AKR1B10	AKR1B1	1B1/1B10
oleanolic acid (**1**)	0.090 ± 0.009	124 ± 25	1370
maslinic acid (**2**)	0.63 ± 0.05	72 ± 14	114
betulinic acid (**7**)	2.0 ± 0.2	11 ± 2	6
ursolic acid (**3**)	4.0 ± 0.8	41 ± 1	10
asiatic acid (**5**)	5.9 ± 0.3	34 ± 5	6
glycyrrhetic acid (**6**)[a]	4.9	280	57
erythrodiol (**4**)	30 ± 3	84 ± 20	3

[a] IC_{50} values are taken from ref 12.

The inhibition patterns of **1** and **3** were noncompetitive with respect to pyridine-3-aldehyde substrate in the reduction reaction by AKR1B10 and were competitive with respect to the alcohol substrate geraniol in the reverse reaction (the K_i values were 0.072 ± 0.12 and 2.0 ± 0.2 μM, respectively). Different inhibition patterns in the forward and reverse reactions have been reported for other AKR1B10 inhibitors,[12,15−18] including tolrestat, which binds to the substrate-binding site in the crystal structure of the enzyme−$NADP^+$−inhibitor ternary complex.[24] This is explained by the kinetic reaction mechanism that has a rate-limiting step at the release of the oxidized coenzyme, $NADP^+$.[12,24] Thus, **1** and **3** may bind to the substrate-binding site of AKR1B10. The K_i value for **1** is higher than isolithocholic acid (15 nM),[12] chromene-3-carboxamide derivatives (2.7−24 nM),[18] and tolrestat (50 nM),[12] but is lower than those for other known inhibitors.[15−17,19]

Structural Insight into Inhibitory Selectivity of Oleanolic Acid (1) to AKR1B10. The K_i values indicated that the affinity of **1** to AKR1B10 was 28-fold higher than that of **3** despite their structural difference only in the positions of the methyl substituents on the E ring. The underlying structural reasons for the high affinity of **1** were examined by constructing models of docked **1** and **3** in the AKR1B10−$NADP^+$ complex (Figure 1A,B). In these models, the two inhibitors occupied the substrate-binding site of the enzyme, in which their 3β-hydroxy groups are in close proximity to catalytically important residues (Tyr49 and His111). There were differences in the orientation of the other parts of the two molecules. The 28-carboxyl group of **1** formed a hydrogen-bond interaction with the side chain of Gln303 (3.7 Å), whereas that of **3** interacted with the side chain of Lys125 (2.8 Å). While the side chain of Trp220 was close to the B and D rings of **1** (3.2−4.1 Å) and can form hydrophobic interactions, it is too far from **3** (>4.6 Å). Furthermore, Val301, a hydrophobic

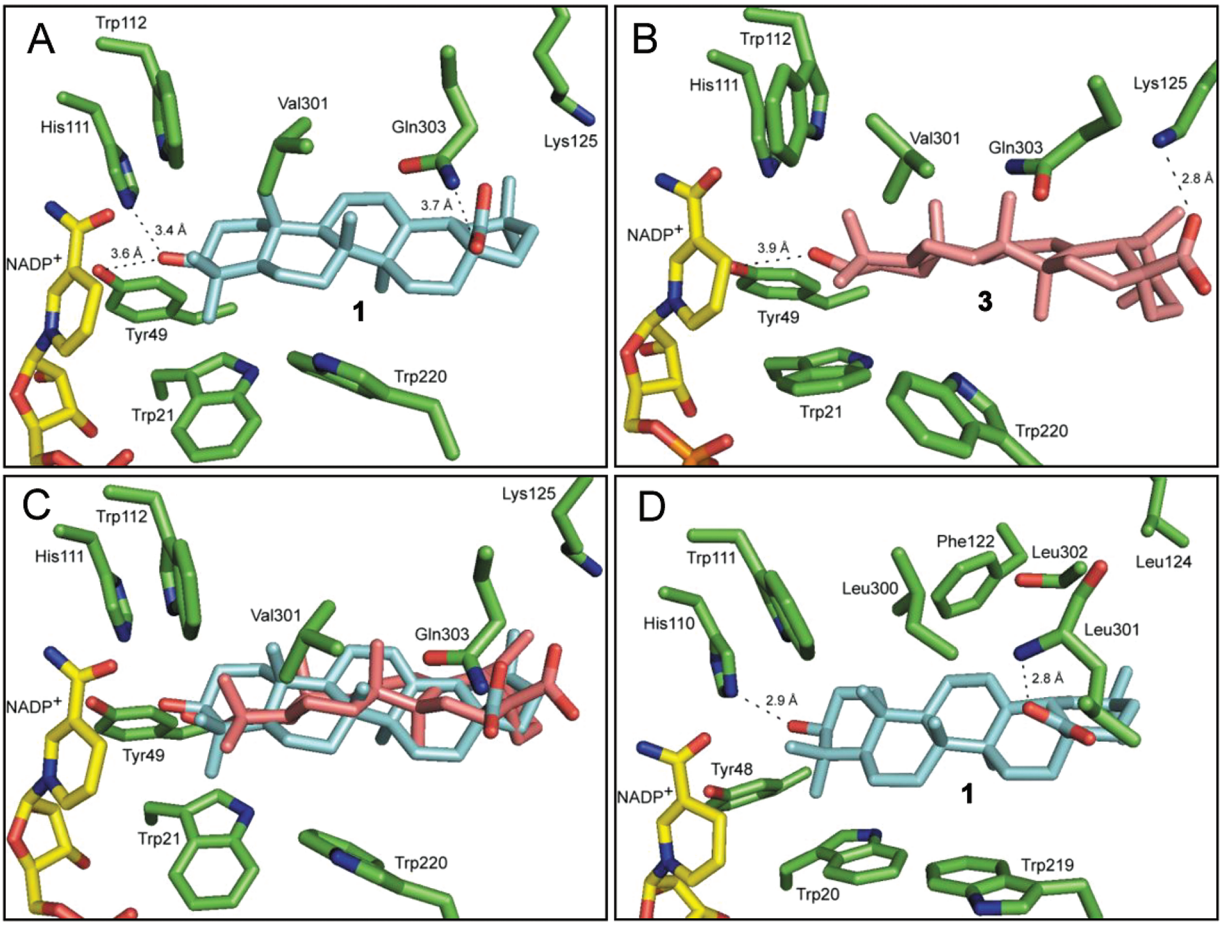


Figure 1. Binding models of oleanolic acid (**1**) and ursolic acid (**3**) in the NADP$^+$ complexes of AKR1B10 and AKR1B1. (A) **1**-docked AKR1B10 model. (B) **3**-docked AKR1B10 model. (C) Difference in orientation of **1** (sky-blue) and **3** (purple-pink), in which **3** was superimposed onto the **1**-docked AKR1B10 model. (D) **1**-docked AKR1B1 model, in which its amino acid numbers correspond to those +1 of AKR1B10. The nicotinamide portion of NADP$^+$ (yellow) and residues interacting with the triterpenoids are depicted with possible hydrogen bonds. Among the residues within 4.0 Å from the triterpenoids, some residues of AKR1B10 (Val48, Trp80, Phe123, and Cys299) and AKR1B1 (Val47, Trp79, Phe218, Cys298, and Ala299) of AKR1B1 are not shown.

amino acid in the substrate-binding site of the enzyme,[24] was also close to the 25- and 26-methyl groups of **1** (both 4.1 Å), compared to the distances between the residues and the two methyl groups of **3** (>5.4 Å). As evident by the superimposed structures of the models of **1** and **3**, oleanolic acid (**1**) sits deeper in the active site of the enzyme than ursolic acid (Figure 1C).

Oleanolic acid (**1**) was modeled into the active site of AKR1B1 to compare its binding mode with that in AKR1B10. In this model (Figure 1D), the hydrogen-bond interaction of the 3β-hydroxy group of the inhibitor with His110 and hydrophobic interactions of the inhibitor with Trp219 were similarly observed. This model was different from that of the AKR1B10−**1** complex in the following three interactions: (1) the distance between the 3β-hydroxy group of **1** and Tyr48 was far (4.1 Å), (2) the 28-carboxyl group of **1** formed a hydrogen-bond interaction with the main chain nitrogen of Leu301 (2.8 Å), and (3) the side chain of Leu301 interacted with the 25- and 26-methyl groups of **1**. Therefore, the high selectivity of **1** toward AKR1B10 compared with AKR1B1 might be due to the interactions of the inhibitor molecule with Tyr49, Gln303, and Val301 of AKR1B10, which are lacking or replaced by the interactions with Leu300 and Leu301 in the AKR1B1−**1** modeled structure.

Among the residues lining the inhibitor-binding site of AKR1B10, Lys125, Val301, and Gln303 of AKR1B10 are different from the corresponding residues (Leu124, Leu300, and Ser302, respectively) in AKR1B1. To investigate the participation of the residues in the binding and selectivity of the inhibitors, we prepared mutant AKR1B10s, in which the three residues were replaced with the corresponding residues in AKR1B1. In addition, Trp220 was replaced by a smaller aromatic residue, Tyr, in order to evaluate the role of its hydrophobic interaction with **1**. The effects of the mutations on the K_i values for **1** and **3** are summarized in Table 2. With exception of the K125L mutation, other mutations decreased the affinity for **1** by more than 9-fold compared to the wild-type enzyme. In particular, the W220Y mutation resulted in the largest decrease in the affinity of **1**. The results not only support the involvement of at least Val301 and Gln303 in the high inhibitory selectivity of **1** toward AKR1B10 predicted by the inhibitor docking studies but also suggest the importance of Trp220 in the binding of this triterpenoid. In contrast, the effects of the mutations on the affinity for **3** were low

Table 2. Effects of Mutations of AKR1B10 on K_i Values for Oleanolic Acid (1) and Ursolic Acid (3)

	1[a]		3[a]	
enzyme	K_i (nM)	Mu/Wt	K_i (μM)	Mu/Wt
wild type	72 ± 12		2.0 ± 0.2	
K125L	160 ± 21	2	2.8 ± 0.3	1.4
V301L	940 ± 100	13	2.1 ± 0.2	1.1
Q303S	660 ± 40	9	3.7 ± 0.5	1.9
W220Y	3560 ± 240	49	6.0 ± 0.5	3.0

[a] The inhibition patterns of the triterpenoids in the $NADP^+$-linked geraniol dehydrogenase were all competitive with respect to the substrate. Mu/Wt represents the ratio of the K_i value for the mutant enzyme to that for the wild-type enzyme.

(1.1−3.0-fold), reflecting the low inhibitory potency of **3**. It still remains unknown how the small structural difference in the methyl substituents on the E ring between **1** and **3** affects the binding modes of the two inhibitors. Since the methyl substituents on the E rings of **1** and **3** were similarly located at the entrance of the binding cleft of AKR1B10 in their docked models (Figure 1A−C), the presence of the 19-methyl group in **3** (which is not present in **1**) might prevent it from sitting deeply in the binding site.

The contribution of Val301 and/or Gln303 to the inhibitory selectivity is also suggested in the binding of other relatively selective AKR1B10 inhibitors such as isolithocholic acid,[12] anti-inflammatory *N*-phenylanthranilic acids,[15] curcumins,[16] and 9-methyl-2,3,7-trihydroxy-6-fluorone.[17] In contrast, the site-directed mutagenesis of V301L and Q303S does not affect their inhibitory potency of nonselective inhibitors, tolrestat,[24] and chromene-3-carboxamide derivatives.[18] Thus, Val301 and/or Gln303 may be important selectivity determinants of inhibitor binding to AKR1B10.

Effect of Oleanolic Acid (1) on Cellular Metabolism and Mitomycin C-Resistant Cancer Cells. The inhibitory effect of **1** on AKR1B10 at a cellular level was examined in the metabolism of an isoprenyl aldehyde, farnesal, which is efficiently reduced to farnesol by AKR1B10 in vitro and in the enzyme-overexpressed HeLa cells.[12] Since **1** was cytotoxic to the HeLa cells like other cancer cells,[20] we treated the cells for 6 h with concentrations of **1** lower than 30 μM, in which more than 80% of the cell viability was retained. Oleanolic acid (**1**) inhibited the farnesal metabolism more potently than **6** and was effective from 1 μM, showing an IC_{50} value of 4 μM (Figure 2A). The IC_{50} value is similar to that of tolrestat and lower than those for curcumins (11−61 μM) determined under the same conditions.[16]

We also examined the inhibitory effect of **1** on growth of mitomycin C-resistant colon cancer HT29 cells, in which the overexpression of AKR1B10 (3.7-fold) is reported to be involved in drug resistance.[8] When the cells were cultured in the medium containing 0.5 μM mitomycin C for 96 h, **1** dose-dependently decreased the growth of the resistant cells (Figure 2B). The efficacy of **1** was evident at 10 μM, and at 30 μM its effect was similar to that of the same concentration of tolrestat. As reported previously,[25] **1** was not highly cytotoxic to nonresistant HT29 cells, which were not affected at its concentration of 30 μM and showed a LD_{50} of 180 μM for **1**. Recently, with treatment of rats, **1** has been reported to down-regulate the expression of mRNA for renal aldose reductase, which is similar structurally to

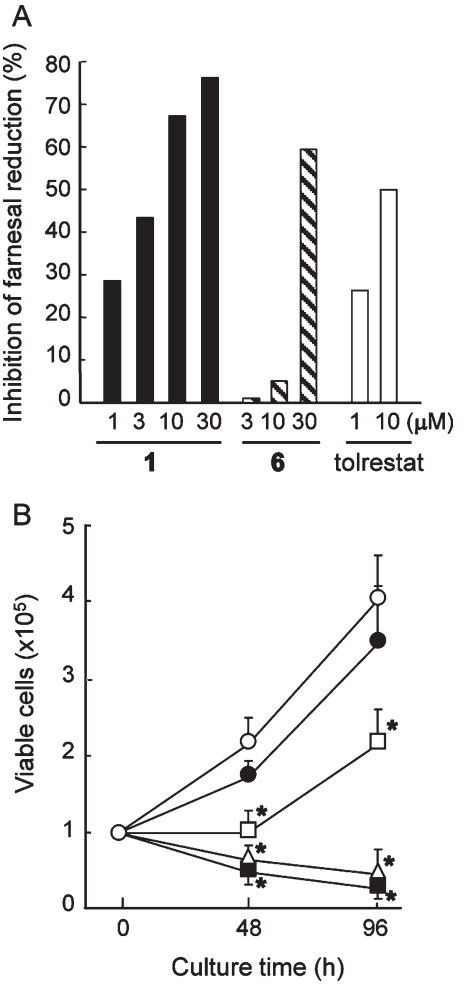


Figure 2. Inhibition of oleanolic acid (**1**) in cellular AKR1B10. (A) Effect on cellular farnesal reduction. The AKR1B10-expressed HeLa cells were pretreated with the indicated concentrations of **1**, glycyrrhetic acid (**6**), and tolrestat for 2 h and then incubated with 20 μM [1-^{14}C] farnesol for 6 h. The inhibition percentages of the farnesal reduction by the inhibitors are expressed as the mean of duplicate experiments. (B) Effect of **1** on reversal of mitomycin C-resistant HT29 cells. The cells were cultured in the medium containing 0.5 μM mitomycin C, and the viable cell numbers were estimated at the indicated times after the addition of the inhibitors. Inhibitors: 3 μM **1** (●), 10 μM **1** (□), 30 μM **1** (■), and 30 μM tolrestat (△). *Significant difference from the control cells (without inhibitor: ○). $p < 0.05$ (by statistical evaluation using the unpaired Student's *t*-test).

AKR1B10.[26] Reverse transcription-PCR analysis showed that **1** (3−30 μM) did not influence the expression of the mRNA for AKR1B10 in HT29 cells (data not shown). Thus, the growth suppression of the mitomycin C-resistant cells by **1** may be predominantly due to the inhibition of AKR1B10 activity.

Comparison with Other Enzymes Inhibited by Oleanolic Acid (1). Like other triterpenoids, **1** exhibits multifunctional properties such as anti-inflammatory, anti-HIV, antineoplastic, cytotoxic, hepatoprotective, and hypoglycemic activities by modulating activities and expression of multiple intracellular target proteins and signaling pathways.[20−23] Such target proteins include several enzymes that are inhibited by **1**. These are cyclooxygenases,[27] DNA polymerase β,[28] DNA ligase,[29] topoisomerase,[30] protein kinases,[31] cytochrome P450s,[32]

HIV-1 protease,[33] glycogen phosphorylase,[34] α-glucosidase,[35] phospholipase A_2,[36] and protein tyrosine phosphatase 1B.[37] Among these enzymes, DNA polymerase β and phospholipase A_2 are the most sensitive to **1**, but the IC_{50} values (3−8.5 μM) for the two enzymes are much higher compared to that for AKR1B10 (0.09 μM), which is also 2 orders of magnitude lower than those for the other enzymes. Thus, AKR1B10 is highly sensitive to **1**, and its inhibition may contribute to the antineoplastic action of **1**, in which the enzyme is highly expressed. The overexpression of AKR1B10 is reported not only in mitomycin C-resistant HT-29 cells[8] but also in medulloblastoma cell lines resistant to cyclophosphamide.[38] Oleanolic acid (**1**) has potential as adjuvant therapy for cancer chemotherapeutic drug resistance, in which AKR1B10 is overexpressed.

The present study has revealed that oleanolic acid (**1**) is the most selective inhibitor of AKR1B10 among the existing inhibitors and raises the intriguing possibility that the enzyme's inhibition is a novel mechanism of the antineoplastic action of **1**. Due to the high selectivity of **1** to AKR1B10 versus AKR1B1, structural knowledge of the type of interactions between the enzyme and triterpenoids suggested by molecular docking and site-directed mutagenesis analyses can be used to design compounds that are tailored to selectively inhibit AKR1B10, in order to avoid their possible side effects.

EXPERIMENTAL SECTION

Compounds and Materials. Oleanolic acid (**1**) and betulinic acid (**7**) were isolated from the calyces of *Diospyros kaki*,[39] and asiatic acid (**5**) was purified from the resin of *Vateria indica*.[40] Ursolic acid (**3**), glycyrrhetic acid (**6**), maslinic acid (**2**), and erythrodiol (**4**) were obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan), Sigma-Aldrich, Cayman Chemical (Ann Arbor, MI), and Extrasynthése (Genay, France), respectively. [1-^{14}C]Farnesol was purchased from American Radiolabeled Chemicals. Human HeLa and HT29 cells were obtained from American Type Culture Collection (Manassas, VA).

Preparation of Recombinant Enzymes. Recombinant AKR1B1,[41] wild-type AKR1B10 with the N-terminal 6-His tag,[12] and its mutant forms (K125L, W220Y, V301L, and Q303S)[15,16] were expressed in *Escherichia coli* BL21 (DE3) pLysS cells transformed with expression plasmids harboring their cDNAs and purified to homogeneity, as described previously.

Assay of Enzyme Activity. The reductase and dehydrogenase activities of the enzymes were determined at 25 °C by measuring the rate of change in NADPH absorbance (at 340 nm) and fluorescence (at 455 nm with an excitation wavelength of 340 nm), respectively.[12] The IC_{50} values for inhibitors were determined in the reaction mixture that consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, 0.2 mM pyridine-3-aldehyde, and enzyme, in a total volume of 2.0 mL. Kinetic studies in the presence of inhibitors were carried out in both pyridine-3-aldehyde reduction and $NADP^+$-linked geraniol oxidation over a range of five substrate concentrations (0.2−5 × K_m) at a saturating concentration of coenzyme, and vice versa. The IC_{50} and K_i values are expressed as the means of at least three determinations.

Molecular Modeling and Energy Minimization. The coordinates for AKR1B10 (PDB code: 1ZUA)[24] and AKR1B1 (PDB code: 1PWM)[42] were obtained from the RCSB Protein Data Bank. The structure was prepared using the Maestro (Schrödinger, LLC) software package Version 8.5, as described previously.[12] In order to eliminate any bond length and bond angle biases, the ligand, oleanolic acid (**1**) or ursolic acid (**3**), was subjected to a full minimization prior to the docking. The docking calculations were performed using the program Glide 5.0[43] on a Linux workstation under the conditions described previously.[12] Figures were generated using PyMOL (DeLano Scientific).

Cell Culture Experiments. HeLa and HT29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO_2. The transfection of the pGW1 plasmids harboring the cDNA for AKR1B10 into the HeLa cells, the activity assay of the expressed enzyme, and analysis of the metabolism of [1-^{14}C]farnesol in the cells were carried out as described previously.[12]

A subpopulation of HT29 cells resistant to mitomycin C was prepared by continuous exposure to increasing concentrations of this drug as described previously.[8] The cells showed 2.5-fold higher LD_{50} (50% lethal dose) for mitomycin C than the parental cells and were cultured to a density of 1 × 10^5 cells/well in a 48-well plate, in the medium containing 0.5 μM mitomycin C. The cells were treated for 96 h in the medium without or with an AKR1B10 inhibitor.

Cell viability was evaluated by the trypan blue dye exclusion method.[44] The expression of the mRNA for AKR1B10 in the cells was analyzed by reverse transcription-PCR analysis,[8] in which HT29 cells were treated for 24 h with 3, 10, and 30 μM **1** prior to the extraction of total RNA.

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