Aureobasidins as New Inhibitors of P-Glycoprotein in Multidrug Resistant Tumor Cells

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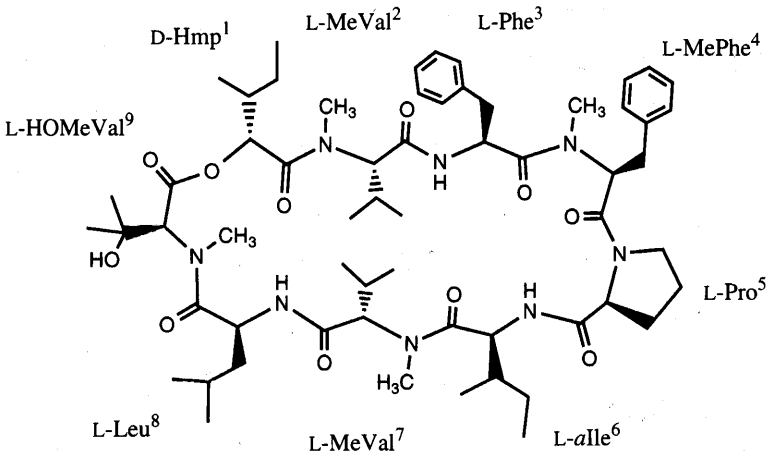
Cyclic depsipeptide antibiotic aureobasidin A (AbA) and its analogs were tested for the inhibitory activity of P-glycoprotein in multidrug resistant cancer cells as well as for the antifungal activity. Some analogs with lower antifungal activity than AbA showed higher inhibition of P-glycoproteins indicating difference of the structure-activity relationships between the two activities. Among AbA analogs tested, [D-β-hydroxy-methylvalyl[9]]-AbA newly prepared by chemical synthesis, which had much lower antifungal activity than AbA, showed 10-fold higher inhibitory activity of P-glycoprotein than AbA.

Aureobasidin A (AbA, Fig. 1) was isolated as an antifungal antibiotic produced by *Aureobasidium pullulans* R106 as the major component and contains eight amino acids and one hydroxy acid.[1,2] Over 20 aureobasidins have been isolated as metabolites and chemical derivatives. We have shown that AbA and some aureobasidins are highly active against a variety of pathogenic fungi including *Candida albicans*.[1,3] Structure-activity relationships of the analogs in the antifungal activity have indicated the highest importance of the β-OH group of residue 9, L-β-hydroxy-*N*-methylvaline (L-βHOMeVal).[1,4] We have established a method to prepare AbA by total chemical synthesis, synthesized new AbA analogs by the method, and carried out further studies on the

Fig. 1. Structure of aureobasidin A.



Abbreviations: D-Hmp, (2*R*, 3*R*)-hydroxy-methylpentanoic acid; L-MeVal, L-*N*-methylvaline; L-MePhe, L-*N*-methylphenylalanine; L-*a*Ile, L-*allo*-isoleucine.

structure-activity relationship in the antifungal activity.[5,6]

The resistance of tumor cells to several structurally unrelated chemotherapeutic drugs is a serious barrier to the treatment of human cancers. The multidrug resistance of tumors is caused through overexpression of a 170 kDa plasma membrane protein, the P-glycoprotein belonging to ATP-binding cassette (ABC) transporters superfamily.[7] There are various types of inhibitors of P-glycoprotein, including Ca-channel blockers, calmodulin antagonists such as verapamil and *cis*-flupenthixol, and immunosuppressive agents including cyclosporin A and its derivatives.[8~10] It is shown that AbA is a substrate of MDR1 pump, the P-glycoprotein, and MDR2 pump.[11] We also have shown that AbA causes accumulation of anticancer drug vincristine in multidrug resistant tumor cells.[12]

In this paper, we report results of determination of the inhibitory activity of P-glycoprotein of some AbA analogs in comparison with their antifungal activity. We also show discovery of an AbA analog showing much stronger inhibitory activity than AbA and verapamil.

Materials and Methods

Preparation of Natural Aureobasidins and New AbA Analogs

Natural aureobasidins, AbA, AbB, AbC, AbD, AbE, AbF, AbG, AbI, AbJ, and AbR were prepared as described previously.[1] Preparations of new AbA analogs used having new amino acids at positions 3 and 4 will be published elsewhere. Briefly, H-L-Phe^3-L-MePhe^4-L-Pro^5-L-aIle^6-L-MeVal^7-L-Leu^8-L-βHOMeVal^9-D-Hmp^1-L-MeVal^2-OH, a fragment derived from AbA by hydrogen fluoride treatment, was digested with proline-specific endopeptidase, yielding H-L-aIle^6-L-MeVal^7-L-Leu^8-L-βHOMeVal^9-D-Hmp^1-L-MeVal^2-OH.[13] The hexapeptide was coupled with a tripeptide composed of residues 3, 4 amino acids and Pro, and the resulting linear nonapeptide was cyclized as described.[5] The new amino acids of residue 3 include *O*-benzyl-L-tyrosine (L-Tyr(Bzl)) and *O*-cyclohexylmethyl-L-tyrosine (L-Tyr(Chm)). The new amino acids of residue 4 are *N*-methyl-L-alanine (L-MeAla) and *N*-methyl-D-alanine (D-MeAla).

[D-βHOMeVal^9]-AbA was synthesized by the same scheme described previously, in which Boc-L-Leu-D-βHOMeVal-D-Hmp-OH was used in place of Boc-L-Leu-L-βHOMeVal-D-Hmp-OH.[5] It had ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.81, 8.70, 7.94, and 7.86 (total 2H, br. d, NH), 7.32~7.15 (total 9H, m, ar.), 6.58 (1H, d, J=7.0 Hz, MePhe ar.), 5.66 (1H, d), 5.39 (s), 5.32 (m), 5.21 (m), 4.91 (m), 4.79 (m), and 4.52 (m) (total 7H, α), 4.22 (m, 1H, D-HOMeVal OH), 3.32, 3.29, 3.19, 3.13, 3.02, 2.99, 2.60, and 2.50 (total 12H, s, N-CH_3), 2.08 (2H, m), 1.04~0.71 (total 36H, m, CH_3), and FAB-MS m/z 1101 $[MH]^+$.

Intracellular Accumulation of [^{3}H]Vincristine (VCR) and Rhodamine 6G

Aureobasidins and verapamil were dissolved in dimethylsulfoxide and diluted with phosphate-buffered saline (PBS, pH 7.2). We used adriamycin-resistant human cell lines, ovarian A2780 tumor cells AD_{10} and breast tumor cells MCF-7/mdr.

a) One ml of AD_{10} cell suspension (10^6 cells/ml) in the medium composed of RPMI-1640 and 5% fetal calf serum were plated in a well of 24-well tissue culture dishes. After incubation at 37°C for 24 hours, [^{3}H]VCR (222 GBq/mmol, Amersham) was added at the final concentration of 20 nM. Then the indicated concentrations of drugs (5 μl) or saline were added. After incubation at 37°C for 2 hours, the intracellular VCR accumulation was determined as described previously.[14]

b) Two hundred μl of MCF-7/mdr cell suspension (10^5 cells/ml) in the medium composed of MEM supplemented with 10% fetal bovine serum were plated in a well of 96-well tissue culture dishes. After incubation at 37°C for 48 hours, the medium was replaced with 150 μl of RPMI-1640 and rhodamine 6G was added at the final concentration of 5 μM. Then the indicated concentrations of drugs (25 μl), or saline were added. After incubation at 37°C for 1 hour, cells were washed twice with PBS and the intracellular rhodamine 6G accumulation was determined by fluorescence densitometer Cytofluor 2300. An IC_{50} value of [D-βHOMeVal^9]-AbA was determined by duplicate measurement of the inhibitions caused by the compound 1000, 300, 100, 30, 10, and 3 nM. The IC_{50} of the reference compound verapamil was 23 μM. The assays using MCF-7/mdr cells were carried out by Panlabs Inc. (Bothell, WA).

Antifungal Activity of Aureobasidins

Aureobasidins were tested for the antifungal activity to *Candida albicans* TIMM 0136, *C. glabrata* TIMM 1062, *Cryptococcus neoformans* TIMM 0354, and *Saccharomyces cerevisiae* ATCC 9763. The activity was shown as the minimum inhibitory concentration (MIC)

determined by the conventional agar dilution method using Sabouraud-dextrose medium.

Results

We tested natural known aureobasidins and new synthesized AbA analogs for the inhibitory activity of efflux of a substrate, anticancer compound VCR or rhodamine 6G, out of the cell by the P-glycoprotein by determining the amount of the substrate accumulated intracellularly. All aureobasidins tested showed no cytotoxicity to the cells at the concentrations tested. AbA and most of natural aureobasidins caused increase of accumulation of VCR into AD_{10} cells (Table 1) as much as or little less than verapamil did. The inhibitory activity of P-glycoprotein of MCF-7/mdr cells by aureobasidins was superior to that of AD_{10} cells. Aureobasidins inhibited the P-glycoprotein of AD_{10} cells as much as verapamil did, whereas aureobasidins were more inhibitory to MCF-7/mdr than verapamil.

It seems that there is some positive correlation between the antifungal activity and the inhibitory activity of P-glycoprotein. For instance, AbG without the OH group at residue 9 was less active than AbA in the inhibitory activity of P-glycoprotein as well as in the antifungal activity. AbJ having a COOH group instead of the OH group at residue 9 almost lost the inhibitory activity of P-glycoprotein and less active against fungi than AbA. These results indicate importance of the OH group for the both activities.

However, AbR with little antifungal activity inhibited the efflux as much as AbA. Among new aureobasidins

Table 1. P-Glycoprotein inhibitory activity and antifungal activity of natural aureobasidins.

	Intracellular increase (%) at the conc of the drugs (μg/ml)			Antifungal activity (MIC μg/ml)		
	AD_{10}		MCF7/mdr	*C.a.*[a]	*C.g.*	*C.n.*
	1	10	10			
AbA	48	68	112	<0.05	0.05	0.78
[D-Hiv[1]]-AbA (AbB)	66	87	99	0.10	0.20	1.56
[L-βHOMePhe[4]]-AbA (AbE)	48	69	63	<0.05	0.20	3.12
[L-Leu[6]]-AbA (AbI)	48	71	79	0.10	0.20	1.56
[L-Val[6]]-AbA (AbC)	58	79	97	<0.05	0.39	25
[L-Val[7]]-AbA (AbF)	37	62	33	0.78	1.56	25
[L-MeVal[9]]-AbA (AbG)	44	45	36	3.12	>25	>25
[L-γHOMeVal[9]]-AbA (AbD)	63	93	77	0.20	1.56	25
[L-N,βMeAsp[9]]-AbA (AbJ)	35	19	27	0.78	3.12	25
[L-βHOMePhe[4], L-MeVal[9]]-AbA (AbR)	103	107	129	25	>25	>25
Verapamil	100	100	100			

[a] *C.a.*: *Candida albicans* TIMM 0136; *C.g.*: *C. glabrara* TIMM 1062; *C.n.*: *Cryptococcus neoformans* TIMM 0354.

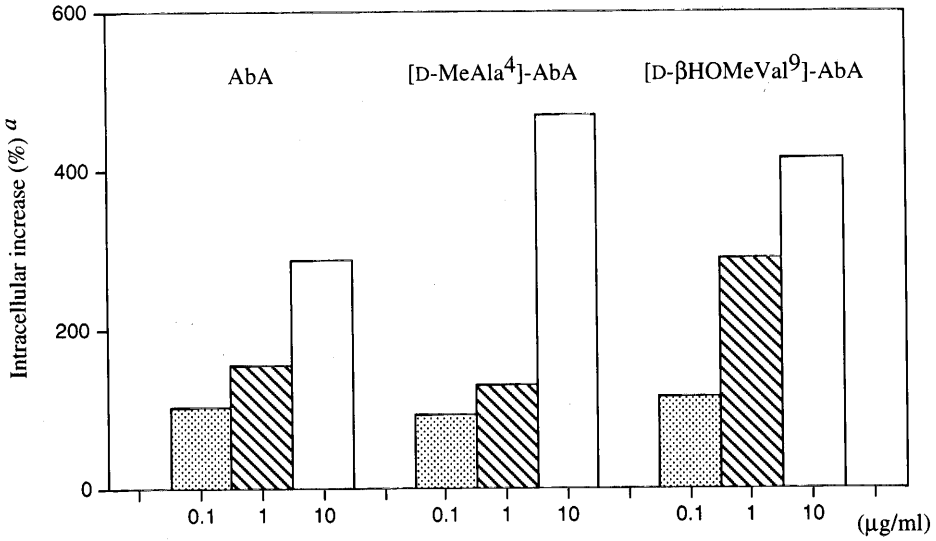
Table 2. P-Glycoprotein inhibitory activity and antifungal activity of new AbA analogs.

	Intracellular increase (%) at the conc of the drugs (μg/ml)			Antifungal activity (MIC μg/ml)		
	AD_{10}		MCF7/mdr	*C.a.*	*C.g.*	*C.n.*
	1	10	10			
AbA	83	88	157	<0.05	0.05	0.78
[L-MeAla[4]]-AbA	65	70	193	0.05	0.78	12.5
[D-MeAla[4]]-AbA	71	144	322	0.05	0.20	3.12
[L-Tyr(Bzl)[3], D-MeAla[4]]-AbA	75	67	224	0.10	1.56	1.56
[L-Tyr(Chm)[3], D-MeAla[4]]-AbA	59	60	150	0.05	0.39	0.39
[D-βHOMeVal[9]]-AbA	157	129	322	0.39	1.56	>25
Verapamil	100	100	100			

synthesized, [D-MeAla^4]-AbA and [D-βHOMeVal^9]-AbA showed higher P-glycoprotein inhibitory activity than AbA at 10 μg/ml (Table 2). [D-βHOMeVal^9]-AbA having much less antifungal activity than AbA showed higher inhibition of P-glycoprotein than it.[4] Replacement of Phe at residue 3 of [D-MeAla^4]-AbA with Tyr derivatives caused reduction of the P-glycoprotein inhibitory activity regardless of enhancing the activity against *C. neoformans*. These results indicate difference of the structure-activity relationships between the antifungal activity and the inhibition of P-glycoprotein.

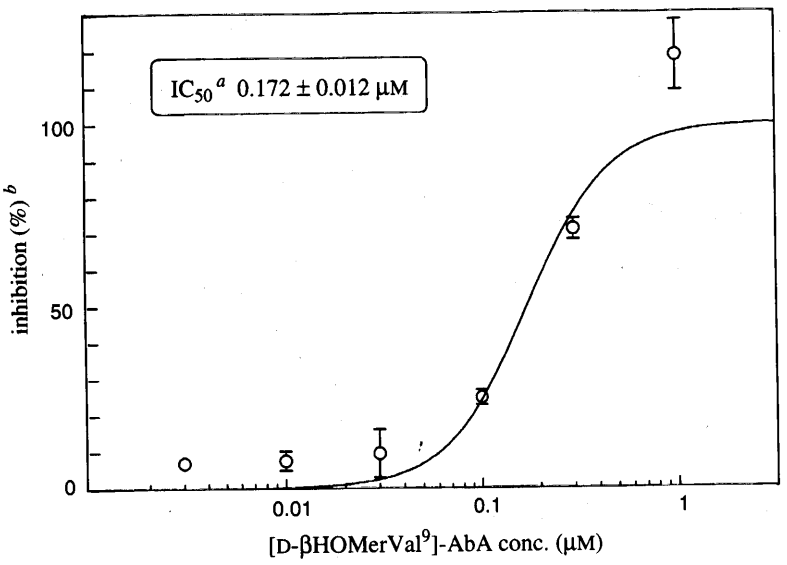
The inhibition at 1 μg/ml of [D-βHOMeVal^9]-AbA was as strong as that at 10 μg/ml of AbA, showing ten-fold higher activity of [D-βHOMeVal^9]-AbA than AbA (Fig. 2). We determined its IC_{50} value of P-glycoprotein inhibition using MCF-7/mdr. The IC_{50}

Fig. 2. Dose dependency of P-glycoprotein inhibitory activity of aureobasidins.



Adriamycin-resistant ovarian tumor cells AD_{10} were used for the assay (See Materials and Methods).
[a] The intracellular accumulation of [^{3}H]VCR into the cells without a drug was calculated as 100%.

Fig. 3. Control of MDR pump on MCF-7/mdr cells by [D-βHOMeVal^9]-AbA.



[a] Mean ± S.E.M. on 3 separate experiments (n=2x3).
[b] The increase of accumulation in the presence of *cis*-flupenthixol (final 80 μM) was calculated as 100%.

value 0.172 ± 0.012 μM of [D-βHOMeVal^9]-AbA (Fig. 3) was 100-fold higher than that of verapamil and seemed to be comparable to those of cyclosporin derivative SDZ PSC 833 and cyclic peptolide SDZ 280-446, which are most powerful inhibitors of P-glycoprotein.[9,10]

Discussion

Aureobasidins showed high P-glycoprotein inhibitory activity and caused accumulation of substrates, anticancer agent VCR and rhodamine 6G. Some analogs with lower antifungal activity than AbA showed higher inhibition of P-glycoproteins indicating difference of the structure-activity relationships between the two activities. However, the OH group of βHOMeVal^9 was important for the inhibition of P-glycoprotein as well as for the antifungal effect. The much higher inhibitory activity of [D-βHOMeVal^9]-AbA than AbA having L-βHOMeVal^9 suggests the steric requirement of the OH group for the inhibition of P-glycoprotein and the antifungal activity. The conformation of OH group of AbA is unstable and can be stably fixed by the hydrogen bonding with either the carbonyl group of L-βHOMeVal^9 or of L-Leu^8.[4,15] The ability to make the hydrogen bonding will be involved in the interaction with a target to show high antifungal activity. In contrast, the OH group of [D-βHOMeVal^9]-AbA forms an internal hydrogen bonding more easily than that of AbA and thus is less active against fungi than AbA.[4] The stable conformation formed by the hydrogen bonding by the OH group may be important to show the P-glycoprotein inhibition or to be competitive to its substrates. The result that AbR, [L-βHOMePhe^4, L-MeVal^9]-AbA, was inhibitory as much as AbA to P-glycoprotein may indicate that the OH group at residue 4 substitutes for the OH group of L-βHOMeVal^9 of AbA. The lower activity of [D-MeAla^4]-AbA analogs having replacement at residue 3 than [D-MeAla^4]-AbA may be due to reduced affinity to P-glycoprotein *via* steric bulk of the new residue with retaining the affinity to the target of fungi.

The inhibitory activity to MCF-7/mdr cells of most of aureobasidins was superior to that of AD_{10} cells. This may be due to difference in the expression levels or the type of P-glycoproteins between the two cells, or in the substrates used for the assays. Mammalian cells have several ABC transporters and the multidrug resistance of tumor cells is caused by overexpression of either of two types of ABC transporters, MDR1 P-glycoprotein or multidrug resistance-associated protein (MRP1). Many multidrug resistance of tumor cells has been shown to be caused by the overexpression of MDR1 pump. Additonally it is shown that several other multidrug resistant cancer cells including small cell lung cancer cells overexpress MRP1 protein, which causes resistance to several different anticancer agents including doxorubicin (adriamycin).[16] Among various inhibitors of the multidrug resistance caused by overexpression of MDR1 P-glycoprotein, cyclosporin A derivative PSC 833 have the highest inhibitory activity of P-glycoprotein. The compound is much less inhibitory to MRP1 pump than to MDR1 pump.[17] *S. cerevisiae* has both types of ABC transporters, pleiotropic drug resistance gene (PDR5) like MDR1 and oligomycin resistance gene (YOR1/YRS1) like MRP1, which are isolated as the resistance genes to antifungals.[18,19] In our screening for a gene conferring the AbA resistance to *S. cerevisiae* through its multicopy, we have isolated YOR1/YRS1 and not PDR5.[12] We have also isolated a PDR1 mutant overexpressing YOR1/YRS1 protein to confer the AbA resistance.[12] This suggests a possibility of aureobasidins to inhibit MRP1 pump more effectively than MDR1 pump in some types of multidrug resistant tumor cells. The expression of MRP1 pump in MCF-7/mdr cells, that showed higher sensitivity than AD_{10} cells to aureobasidins, remains to be tested.

Recent frequent use of azole antifungal agents for the treatment of oropharyngeal candidiasis in AIDS patients caused the appearance of resistant *C. albicans* strains to these azoles.[20] It is reported that some of these resistant strains overexpress PDR5-like ABC transporter gene, CDR1, to give the resistance to azoles.[21] Thus, an use of an antifungal aureobasidin will give a benefit to suppress the resistance to azoles in addition to its high antifungal effect, and may add an advantage to inhibit a YOR1/YRS1-ilke ABC transporter of *C. albicans*[12], that will cause resistance to some antifungals.

[D-βHOMeVal^9]-AbA, one of the most active inhibitor of ABC transporters having a new type of chemical structure, may be useful to inhibit appearance of the multidrug resistance in the treatment of cancers and microbial infections by chemical pharmaceuticals. We will need further experiments to determine the reversing activity of the sensitivity to anticancer and antifungal drugs *in vitro* and *in vivo* including an experiment using mice.

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