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Anti-Leukemia Selectivity in Actinomycin Analogues

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Abstract—An excellent anti-leukemia activity has been found in a group of actinomycin D analogues derivatized at the 2,2′- or 5,5′-position of the depsipeptides. On the basis of the water solubilities, the DNA binding affinities, the RNA synthesis inhibitory activities, the anticancer activities of actinomycin D (AMD), and the crystal structures of DNA–AMD complexes, it becomes clear that AMD is extremely well designed as an effective poison produced by micro-organisms. The anticancer activity of AMD is mainly due to its selective inhibition of RNA synthesis. We have hypothesized that a modification on the AMD structure at a site not involved in DNA interaction can either increase or decrease the diffusion rate of the analogue into certain cancer cells. Since the *i*-propyl groups of the D-valine residues at the 2,2′-positions and *N*-methyl-L-valine residues at the 5,5′-positions in the depsipeptides do not participate in interaction with DNA, these amino acid residues were replaced with other D-amino acid residues and *N*-methyl-L-amino acid residues, respectively. The cancer screen tests have indicated that AMD analogues 2,2′-D-PheAMD, 2,2′-D-OmeAMD, 5,5′-L-TyrAMD, 5,5′-D-ValAMD, 5,5′-D-TyrAMD, 5,5′-D-PheAMD, and 5,5′-D-OmeAMD, inhibit selectively the growth of leukemia cell lines at about 100- to 500-fold lower drug concentrations than those required to inhibit other cancer cell lines.

Introduction

A large number of compounds are known that bind to DNA in various ways. A subset of those bind in the minor groove via non-covalent interactions, intercalate between base pairs, and form covalent bonds with DNA.[1] The physical and biological characteristics of these compounds have been studied, since some of them act as anticancer agents and others as carcinogens.

As shown in Figure 1, actinomycin D (AMD) is composed of a phenoxazone ring as an intercalating part and two cyclic penta-depsipeptides as DNA minor groove binding parts. The drug has a pseudo-2-fold symmetry around the N10-O5 line of the phenoxazone ring. AMD binds to DNA intercalatively with a relatively high association constant ($2.3\times10^6\ M^{-1}$) compared to those of the simple intercalators,[2] and selectively inhibits DNA-directed RNA synthesis.[3,4] AMD is a potent anticancer agent, and its anticancer activity has been believed to be due to its selective inhibition of transcription.[2] However, the clinical usefulness is limited by its extreme cytotoxicity (i.e., its anticancer spectrum is very broad and there is no distinguishable selectivity against specific cancers). AMD has been modified by directed biosynthesis, partial synthesis, and total synthesis in order to reduce its cytotoxicity by increasing the selectivity against certain cancers.[2,5] In many cases, replacements of amino acid residues in the cyclic depsipeptides of AMD have been found to make such analogues inactive or less active. For that reason, the modification of AMD has not been a very attractive approach.

We have determined the crystal structures of the two complexes between d(GAAGCTTC)$_2$ and AMD crystallized in the space groups $C2^6$ and $F222$.[7] The crystal structures of the AMD analogue N8AMD and F8AMD with d(GAAGCTTC)$_2$ have also been determined.[8,9] In these DNA–AMD complex structures, AMD intercalates into the middle sequence 5′-GC-3′ from the minor groove of DNA. The two cyclic depsipeptide rings lie on both sides of the minor groove and cover four base-pairs of DNA. The AMD molecule is tightly connected to the DNA at the middle portion of the molecule by forming four threonine–guanine hydrogen bonds and two additional hydrogen bonds between the N2 amino group of phenoxazone and the DNA backbone. The four threonine–guanine hydrogen bonds appear to recognize the DNA sequence (5′-GC-3′). These essential hydrogen bonds are covered by the cyclic depsipeptides, which are

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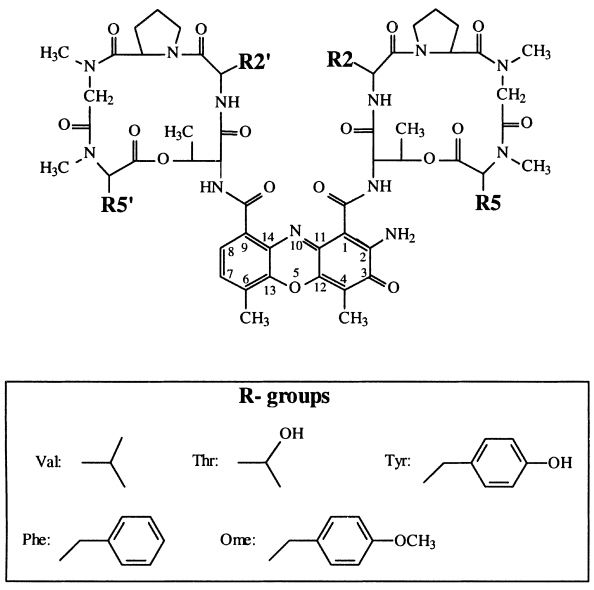

Figure 1. Chemical formula of AMD analogues.

composed of mainly hydrophobic amino acid residues. The depsipeptide rings are relatively rigid, but they are readily movable so that they can fit into the variously shaped minor groove of DNA without breaking essential drug–DNA hydrogen bonds.

On the basis of the crystal structures of AMD–DNA complexes and the available physical and biological data, it is quite clear that the tight binding of AMD to DNA inhibits RNA synthesis. Thus, AMD is a strong poison, and this cytotoxicity accounts for its inhibition of cancer-cell growth. Therefore, it is impossible to reduce the cytotoxicity of AMD without losing anticancer activity. At this stage, we have hypothesized that a certain modification of the AMD structure can increase or decrease the diffusion rate of the analogue into certain cancer cells since cell membranes of different types of cells are built slightly differently.[10] If a portion of the AMD molecule involved in DNA binding was modified, then the analogue would lose anticancer activity. On the other hand, if a portion of the AMD molecule not involved in any DNA interaction was modified, the modification would change the analogue's physical characteristics (such as water solubility and lipophilicity) but not anticancer activity. Changes of these physical characteristics might create selectivity against certain cancer cells. In the crystal structures of DNA–AMD complexes, the *i*-propyl groups of the D-valine residues at (**R2**, **R2′**) and *N*-methyl-L-valine residues at (**R5**, **R5′**) in the depsipeptides are pointed to the outside of the complex, and thus these groups are considered not to participate in interaction with DNA (Fig. 2). Therefore, AMD analogues, in which the D-valine and *N*-methyl-L-valine residues are replaced with other D-amino acid residues and *N*-methyl-L-amino acid residues, respectively, are expected to bind to DNA as strongly as AMD does and to inhibit RNA synthesis as strongly as AMD does. However, if the cyclic depsipeptides of AMD play an unexpected role in inhibition of RNA synthesis, replacement of the D-valine residue or *N*-methyl-L-valine residue with another residue would affect the inhibition activity of the AMD analogue. On the basis of this rather unconventional hypothesis, we have synthesized AMD analogues by replacing amino acid residues at the (**R2**, **R5′**) and (**R5**, **R5′**) positions in the depsipeptides.[11,12] Our studies have indicated that modification of these residues does affect the physical and biological characteristic of the analogues.[11,12]

Now we have examined the effects on anticancer activities of replacing the D-valine residue with other D-amno acid residues, or the *N*-methyl-L-valine residues with

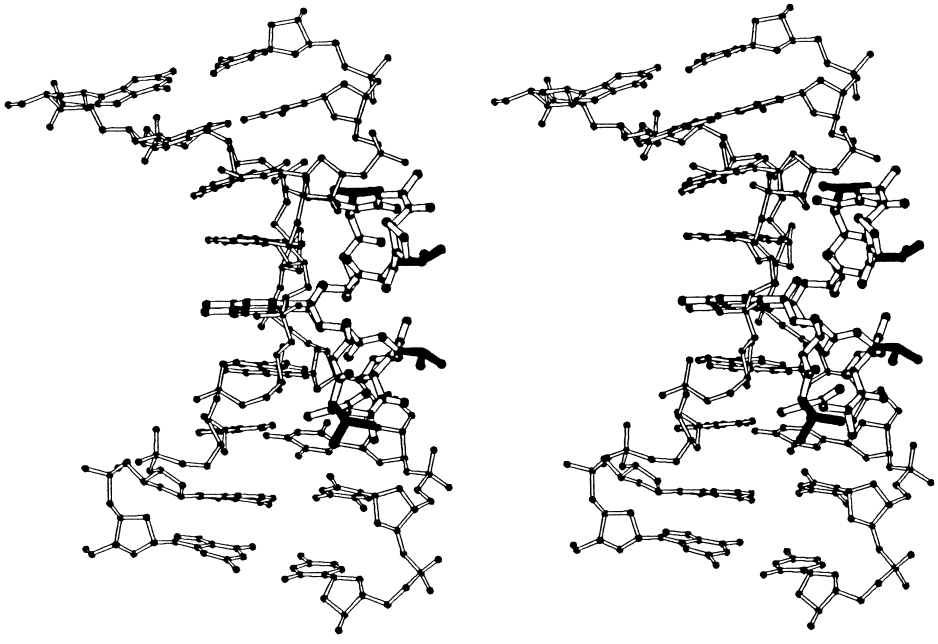

Figure 2. Stereo drawing of the d(GAAGCTTC)$_2$-AMD complex.[7] The AMD molecule is illustrated with thick open bonds while the DNA octamer has thin open bonds. The *i*-propyl groups of D-valine and *N*-methyl-L-valine residues, which are considered not to participate in interaction with DNA, are shown with thick solid bonds.

other *N*-methyl amino acid residues, respectively. Here we report an excellent anti-leukemia selectivity found in a group of AMD analogues, along with a brief summary of the water solubilities, the DNA binding characteristics, and the RNA synthesis inhibitory activities of the AMD analogues.

Results and Discussion

Some physical and biological characteristics of AMD analogues

The water solubilities, DNA association constants, and the drug concentrations at 50% inhibition of RNA synthesis (IC_{50}) in HeLa cells are summarized in Table 1. The water solubilities of the AMD analogues are very peculiar and are almost impossible to predict. For example, the water solubility of 2,2′-D-ThrAMD is only 31 μg/mL while that of AMD is 2900 μg/mL. However the differences between AMD and 2,2′-D-ThrAMD, [-CH$(CH_3)_2$ versus −CH(OH)CH_3], are slight even though the −CH(OH)CH_3 group is generally more hydrophilic than the −CH$(CH_3)_2$ group. In contrast to 2,2′-D-ThrAMD, 5,5′-L-ThrAMD is more water-soluble (>4000 μg/mL) than AMD. It is noted that both 2,2′-D-ThrAMD and 5,5′-L-ThrAMD have threonine residues substituted for the valine residues at the **R2**, **R2′** and **R5**, **R5′** positions of the depsipeptides, respectively. The other example is that the water solubility of 5,5′-D-ValAMD is only 165 μg/mL whereas the solubility of the L-form AMD is 2900 μg/mL. The unusually high water solubility of AMD might be related to its unusual large negative temperature dependence.[13] As will be discussed below, 5,5′-D-ValAMD is a more potent poison than 5,5′-L-ValMAD (AMD itself), but the D-amino acid analogue may have been eliminated by nature because of its low water solubility. It is noted that AMD is the only compound which has hydrophobic amino acid residues at the **R2**, **R2′** and **R5**, **R5′** sites but still has a relatively high water solubility and strong RNA synthesis inhibitory activity. As will be described below, water solubilities of the analogues have some correlation with the RNA synthesis inhibitory activities. In general, less water-soluble compounds have higher RNA synthesis inhibitory activities in vivo, suggesting that they diffuse better into living cells.

The DNA binding characteristics of several AMD analogues have been examined (Table 1).[11,12] In general, the DNA binding capacities of the 2,2′-analogues are lowered by one order of magnitude, whereas those of the 5,5′-analogues are at the same level as AMD. The crystal structures of the DNA–AMD complexes suggested that substitution of the *N*-methyl-L-valine residues at (**R5**, **R5′**) in the cyclic depsipeptides with *N*-methyl-D-valine residues might increase the hydrophobic interaction with the minor groove of the DNA.[12] The newly created interaction between the side chain of the D-amino acid residue and the DNA minor groove might extend the sequence specificity from two bases to four bases. Therefore, we have further characterized the binding properties of 5,5′-L- and 5,5′-D-amino acid residue analogues using longer oligonucleotides containing four different AMD binding sequences, TGCA, CGCG, AGCG, and GGCC. The results are summarized in Table 2. The L-Val (AMD) and L-Thr aliphatic analogues have a clear sequence preference (i.e., TGCA > CGCG > AGCT > GGCC). Our result agrees with Chen's observation of AMD binding strength.[14] Although the

Table 2. Association constants ($k \times 10^{-6}\,M^{-1}$) of AMD analogues to the oligonucleotides (DNA-1, DNA-2, DNA-3, and DNA-4) containing the TGCA, CGCG, AGCT, and GGCC sequences, respectively: DNA-1: d (T-A-T-A-T-A-**T-G-C-A**-T-A-T-A-T-A)$_2$: DNA-2: d (T-A-T-A-T-A-**C-G-C-G**-T-A-T-A-T-A)$_2$: DNA-3: d (A-T-A-T-A-T-**A-G-C-T**-A-T-A-T-A-T)$_2$: DNA-4: d (A-T-A-T-A-T-**G-G-C-C**-A-T-A-T-A-T)$_2$

Compound	DNA-1- -TGCA-	DNA-2- -CGCG-	DNA-3- -AGCT-	DNA-4- -GGCC-
5,5′-L-ValAMD	5.0	2.4	0.9	0.1
5,5′-L-ThrAMD	3.9	2.0	0.7	0.1
5,5′-L-TyrAMD	4.3	3.2	1.0	0.2
5,5′-L-PheAMD	4.0	5.4	1.4	0.4
5,5′-L-OmeAMD	3.3	2.4	0.8	0.3
5,5′-D-ValAMD	3.7	3.0	0.8	0.3
5,5′-D-ThrAMD	2.3	1.4	0.5	0.1
5,5′-D-TyrAMD	2.3	0.7	0.2	—
5,5′-D-PheAMD	2.2	0.9	0.3	—
5,5′-D-OmeAMD	1.0	0.9	0.2	—

Table 1. Water solubility (μg/mL) at 22 °C, DNA association constants (M^{-1}), and IC_{50} (drug concentration (nM) at 50% inhibition of RNA synthesis in HeLa cells). The units of the association constant with calf thymus DNA are bp M^{-1}. The sequences of oligonucleotides are: DNA-1 = d(GAAGCTTC)$_2$; DNA-2 = d(GTTGCAAC)$_2$; CT DNA = calf thymus DNA

Compound	Water solubility	DNA-1×10^6	DNA-2×10^6	CT DNA×10^4	IC_{50} (nM)	Log_{10} (IC_{50})
AMD	2900	0.9	5.0	1.3	30	−7.52
2,2′-D-ThrAMD	31	0.5	2.2	0.9	470	−6.33
2,2′-D-TyrAMD	3.7	0.4	0.7	0.7	1000	−6.00
2,2′-D-PheAMD	2.6	0.5	0.9	0.5	10	−8.00
2,2′-D-OmtAMD	1.8	0.3	0.6	0.6	10	−8.00
5,5′-L-ThrAMD	4700	0.7	3.9	1.5	>5000	>−5.30
5,5′-L-TyrAMD	40	1.4	4.0	1.5	80	−7.10
5,5′-L-PheAMD	23	1.0	4.3	1.6	10	−8.00
5,5′-L-OmtAMD	10	0.8	3.3	0.9	8	−8.10
5,5′-D-ValAMD	165	0.8	3.7	2.3	1	−9.00
5,5′-D-ThrAMD	180	0.5	2.3	1.6	100	−7.00
5,5′-D-TyrAMD	40	0.3	2.2	1.5	80	−7.10
5,5′-D-PheAMD	30	0.2	2.3	1.7	20	−7.70
5,5′-D-OmtAMD	30	0.2	1.0	1.4	80	−7.10

Table 3. Numerical mean graphs (deviations from either the mean log(GI_{50}) or the mean log(TGI)*)

Tumor cell lines\Drug	AMD	2DThr	2DTyr	2DPhe	2DOme	5LThr	5LTyr	5LPhe	5LOme	5DVal	5DThr	5DTyr	5DPhe	5DOme
Leukemia														
CCRF-CEM	0.35	−0.18	—	−0.87	−0.35	−0.13	**< −1.70**	—	—	**< −2.51**	0.08	−1.53	**−1.65**	**−1.64**
HL-60(TB)	0.53	−0.28	−0.81	**< −1.77**	**< −1.97**	0.30	−0.75	−0.73	—	**< −2.51**	> 0.10	**−1.07**	−0.32	−1.53
K-562	−0.43	−0.82	−0.49	**−1.27**	−0.12	−0.52	**−1.26**	< −0.74	—	**−1.48**	> 0.10	−0.99	**−1.13**	−1.49
MOLT-4	−0.13	−0.81	−0.63	**< −1.77**	**< −1.97**	−0.35	−0.81	—	—	**−1.37**	> 0.10	−0.88	**−1.42**	**−1.19**
SR	0.41	**< −1.39**	**< −2.04**	**< −1.77**	**< −1.97**	−0.79	**−1.35**	—	—	−0.66	−0.63	**−1.06**	**−1.42**	**−1.20**
RPMI-8226	—	−0.83	−0.59	**< −1.77**	**< −1.97**	−0.40	—	—	—	—	—	—	—	—
Non-small cell lung cancer														
A549/TCC	−0.06	0.61	−0.08	−0.37	1.79	0.47	0.26	1.26	0.41	> 1.49	> 0.10	0.30	−0.98	−0.32
EKVX	0.32	> 2.61	1.81	1.63	1.54	0.41	1.13	1.03	−0.61	> 1.49	> 0.10	0.82	0.87	0.98
HOP-62	−0.19	0.68	1.08	0.24	−0.36	−0.13	—	−0.49	−1.04	—	> 0.10	—	—	—
HOP-92	0.19	—	—	—	—	0.02	0.25	0.62	−0.85	0.25	> 0.10	0.62	0.41	0.61
NCI-H226	−0.07	−1.38	−0.51	−0.44	−0.61	−0.24	1.01	< −0.74	−1.46	> 1.49	> 0.10	0.02	0.69	0.43
NCI-H23	0.11	−0.10	−0.41	−0.46	−0.46	0.14	0.05	< −0.74	—	−0.84	> 0.10	0.18	0.24	−0.11
NCI-H322M	−0.16	0.14	—	0.08	0.12	0.64	0.47	0.53	−0.24	0.30	> 0.10	0.24	1.01	0.96
NCI-H460	−0.56	−0.77	−0.71	1.53	> 2.03	−0.58	−0.74	—	—	—	> 0.10	−0.63	−0.83	−0.95
NC1-H522	−0.86	−0.47	0.43	−0.43	−0.70	−1.03	−0.64	< −0.74	< −1.48	−1.29	−0.49	−1.01	−0.90	—
Colon cancer														
COLO 205	−0.50	−0.67	−0.19	−0.72	−0.73	0.07	−0.08	< −0.74	—	−1.08	0.00	−0.24	−0.30	−0.53
HCC-2998	0.22	0.83	1.17	0.38	1.04	0.68	0.12	< −0.74	—	−0.19	> 0.10	0.48	0.24	−0.23
HCT-116	−1.00	−0.73	−0.75	−0.90	—	−0.36	−0.63	0.55	—	—	−0.23	−0.75	< −1.76	—
HCT-15	0.94	1.84	0.90	1.51	> 2.03	1.16	1.09	3.26	—	> 1.49	> 0.10	> 1.46	1.02	1.10
HT29	−0.21	0.03	−0.16	−0.74	−0.73	0.23	−0.27	−0.70	—	−1.25	−0.04	−0.57	−0.74	−0.61
KM12	−0.22	−0.33	−0.28	1.25	1.06	0.74	−0.03	—	< −1.48	0.58	0.07	0.08	−0.08	−0.34
SW-620	—	−0.65	−0.52	−0.16	1.03	−0.03	0.11	1.36	—	0.54	> 0.10	0.51	0.20	0.33
CNS cancer														
SF-268	0.03	−0.69	−0.84	0.31	0.10	0.39	−0.11	−0.55	—	0.03	> 0.10	0.12	0.30	−0.14
SF-295	−0.62	0.16	−0.46	−0.10	−0.18	0.69	0.50	< −0.74	—	0.42	−0.07	0.78	0.27	0.40
SF-539	−0.25	−0.43	0.05	−0.51	−0.56	−0.54	−0.36	< −0.74	< −1.48	−0.64	−0.17	0.04	0.03	−0.15
SNB-75	0.45	−0.83	−0.90	−0.14	−0.44	−0.08	−0.65.	—	—	−2.21	−0.40	−0.84	0.69	—
U251	−0.43	−0.70	−0.51	1.26	—	−0.64	−0.66	0.54	—	—	−0.21	−0.71	−0.47	−1.20
SNB-19	—	−0.47	−0.48	1.44	1.57	0.17	−0.09	—	1.58	> 1.49	−0.14	−0.42	0.05	0.09
Melanoma														
LOXIMVI	−0.88	−0.83	−1.11	−1.44	−0.81	−0.36	−0.79	0.42	—	—	−0.15	−0.88	−0.94	—
MALME-3M	−0.04	−0.66	−0.45	−0.50	−0.40	−1.29	—	< −0.74	—	—	−0.04	—	—	—
M14	0.43	0.70	0.99	−0.17	−0.18	0.50	0.85	< −0.74	—	−0.17	> 0.10	0.76	0.52	0.71
SK-MEL-2	−0.61	−0.54	−0.71	−1.13	−1.23	−0.68	−0.13	< −0.74	—	−0.68	> 0.10	−0.13	0.25	0.22
SK-MEL-28	0.14	−0.47	−0.09	−0.30	−0.38	−0.05	0.02	—	—	−0.35	−0.13	−0.18	0.16	0.01
SK-MEL-5	−0.25	0.04	−0.50	−0.49	−0.52	−0.51	−0.56	—	—	−0.48	> 0.10	0.10	−0.10	−0.05
UACC-257	0.32	−0.29	−0.08	−0.52	−0.43	0.05	0.23	< −0.74	—	−0.04	> 0.10	0.40	0.28	0.38
UACC-62	−0.05	−0.47	0.59	−1.40	−1.17	−1.00	−0.39	< −0.74	—	−1.23	—	−0.17	−0.13	−0.11
Ovarian cancer														
IGROV1	−0.36	−0.52	−1.14	−0.55	−0.52	−0.43	0.36	< −0.74	—	> 1.49	−0.19	−0.62	−0.31	−0.68
OVCAR-3	−0.02	−0.24	−0.67	−0.37	−0.57	0.08	−0.26	< −0.74	—	—	> 0.10	−0.69	—	—
OVCAR-4	0.43	0.32	0.16	0.81	0.72	0.52	0.25	−0.67	—	< −2.51	> 0.10	−0.45	−0.31	—
OVCAR-5	−0.03	0.71	0.36	−0.03	0.65	0.56	0.93	1.56	1.73	—	> 0.10	0.77	0.88	1.23
OVCAR-8	−0.09	−0.45	−0.30	1.41	—	−0.14	0.10	1.30	—	−0.97	> 0.10	0.48	0.25	−0.51
SK-OV-3	0.57	1.02	1.22	—	—	0.43	1.05	−0.20	—	> 1.49	> 0.10	0.80	0.52	0.98
Renal cancer														
786-0	−0.66	−0.74	−0.69	−0.30	−0.57	−0.17	−0.30	0.70	0.13	> 1.49	−0.12	0.16	−0.25	−0.20
A498	0.23	0.50	1.16	−0.38	−0.59	−1.08	0.37	< −0.74	—	−0.30	> 0.10	0.79	0.18	0.28
ACHN	0.20	0.24	−0.38	1.31	1.25	0.24	−0.16	1.36	1.55	> 1.49	> 0.10	0.67	0.18	0.29
RXF-393	−0.05	−0.82	−0.59	−0.79	−0.42	−0.36	0.22	—	—	−0.42	−0.24	0.74	0.40	0.59
SN12C	0.08	−0.47	0.13	1.12	0.59	−0.01	−0.03	< −0.74	—	−0.71	> 0.10	−0.57	−0.71	−0.40
TK-10	0.52	0.55	1.08	1.45	1.44	0.94	1.35	< −0.74	—	> 1.49	> 0.10	> 1.46	0.69	1.18
UO-31	1.21	0.98	0.76	0.90	0.69	0.34	0.69	−0.59	−1.00	0.47	> 0.10	1.18	1.08	0.89
CAKI-1	—	1.22	0.51	0.55	0.52	0.29	0.76	1.71	2.52	> 1.49	> 0.10	> 1.46	0.70	0.76
Prostate cancer														
PC-3	−0.16	0.68	0.57	0.67	0.58	0.15	0.00	−0.09	—	0.24	> 0.10	−0.02	0.45	−0.09
DU-145	0.18	0.73	0.45	−0.03	−0.04	0.54	0.45	—	—	—	> 0.10	0.31	0.05	0.00
Breast cancer														
MCF7	−1.14	−0.89	−1.20	> 2.23	—	−0.30	−1.12	—	0.63	—	−0.25	−1.02	−0.81	−0.59
MCF7/ADR-RES	1.97	> 2.61	1.91	> 2.23	> 2.03	1.17	1.65	3.26	2.28	> 1.49	> 0.10	> 1.46	1.85	> 1.86

(*continued on next page*)

Table 3 (*continued*)

Tumor cell lines\Drug	AMD	2D-Thr	2D-Tyr	2D-Phe	2D-Ome	5L-Thr	5L-Tyr	5L-Phe	5L-Ome	5D-Val	5D-Thr	5D-Tyr	5D-Phe	5D-Ome
MDA-MB-231/ATCC	>0.09	1.19	1.08	0.64	0.89	0.76	0.47	<−0.74	−1.13	>1.49	>0.10	0.59	1.07	1.27
HS 578T	0.26	0.88	1.18	−0.10	0.02	−0.47	−0.52	—	—	−1.04	>0.10	>1.97	−0.21	−0.86
MDA-MB-435	0.20	0.43	1.00	−0.42	−0.55	0.05	−0.15	—	—	−0.46	>0.10	0.07	0.06	−0.20
MDA-N	−0.23	0.64	0.29	−0.12	−0.03	−0.02	−0.08	<−0.74	—	−0.67	>0.10	0.12	−0.15	−0.16
BT-549	0.28	—	—	—	—	—	0.09	—	—	−0.04	—	−0.36	0.52	0.18
A34T-47D	−0.27	−0.13	0.31	0.04	−0.12	0.27	−0.01	<−0.74	—	>1.49	>0.10	−0.34	−0.04	−0.02
Mean log(GI_{50}) or log (TGI)*	−8.79	−6.61	−5.96	−7.23*	−7.03*	−5.73	−7.85	−7.26*	−6.52*	−7.49*	−6.10	−7.46	−8.24	−7.86

binding preference order of the L-aromatic analogues is similar to that of the aliphatic analogues, there is no significant reduction in binding affinity between TGCA and CGCG. With the L-Tyr analogue, the binding affinities to the TGCA and CGCG sequences are inverse (i.e., CGCG > TGCA > AGCT > GGCC). Interestingly, the D-Val substitution increases the DNA binding capacity of the analogue to CGCG and GGCC sites over that of the natural L-Val compound (AMD itself). On the other hand, the D-aromatic analogues bind well only to DNA with the TGCA sequence. There are significant reductions in their binding capacities with other DNAs. These binding characteristics indicate that the substitution of the D-aromatic residues creates a unique four-base sequence-specificity (TGCA).

The RNA polymerase inhibitory activities of the AMD analogues, which are apparently directly related to the cytotoxicity and anticancer activities of the analogues, were examined by using living human cells (HeLa cells).[11,12] The concentrations of the AMD analogues that gave 50% inhibition of RNA synthesis (IC_{50}) in HeLa cells ranged from 5 μM to 1 nM (Table 1). The RNA synthesis inhibitory activities have good correlation with the anticancer activities (see below). However, the relative abilities to inhibit RNA synthesis in living cells (HeLa cells) do not correlate perfectly with the DNA binding capacities of the AMD analogues observed in in vitro experiments, suggesting that the AMD analogues have different diffusion rates into the cells. The analogues with hydrophobic substitutions have slightly increased RNA synthesis inhibitory activities, whereas the analogues with hydrophilic substitutions have drastically reduced inhibitory activities. The reduction of the inhibitory activities of analogues having hydrophilic groups is probably due to slow diffusion into the nucleus of the living cell. As predicted by the crystal structures of the complexes, the *N*-methyl-D-amino acid substitutions at the *N*-methyl-L-valine sites (**R5**, **R5′**) significantly increase the inhibitory activities. The RNA synthesis inhibitory activity of 5,5′-D-ValAMD has been reproducibly determined to be 20-fold greater than that of AMD.

Anticancer activities

Since the AMD analogues displayed a relatively large range of inhibitory activities against the 60 cancer cell lines, some of the drug activities did not fall within the five-order concentration range (i.e., a 10^{-4} to 10^{-8} M or a 10^{-5} to 10^{-9} M or a 10^{-6} to 10^{-10} M concentration range was used for the screening tests). For this reason, the drug selectivity is shown by a mean graph of either the 50% growth inhibition values (GI_{50}) or the total growth inhibition values (TGI). These mean graphs indicate that the AMD analogues inhibit selectively the leukemia cell lines. The numerical mean graphs (deviations from either the mean log(GI_{50}) or the mean log(TGI)) are summarized in Table 3, and the mean graphs of the leukemia cell lines are presented in Figure 3. The mean GI_{50}, TGI and LC_{50} values of the AMD analogues are listed in Table 4.

The mean concentrations of GI_{50} and TGI values indicate that substitution with hydrophobic amino acid residues does not affect the inhibitory activity against cancer cell growth. Notably 5,5′-D-ValAMD is more potent than AMD itself (5,5′-L-ValAMD). On the other hand, the substitution with a hydrophilic amino acid residue reduces substantially the inhibitory activity. The GI_{50} and IC_{50} (drug concentration at 50% inhibition of RNA synthesis in HeLa cells) values agree quite well, suggesting that the anticancer activities of AMD and AMD analogues are due to the inhibition of RNA synthesis.

As shown in Table 3, AMD is quite potent and kills most cells at less than 50 nM concentration, but its mean graph does not show any selectivity for certain cell lines. This indicates that AMD should have very limited use as an anticancer agent. On the other hand, 2,2′-D-PheAMD, 2,2′-D-OmeAMD, 5,5′-L-TyrAMD, 5, 5′-D-ValAMD, 5,5′-D-TyrAMD, 5,5′-D-PheAMD and 5,5′-D-OmeAMD have an excellent selectivity against leukemia cell lines. For example, 5,5′-D-ValAMD inhibits 50% of CCRF-CEM and HL-60(TB) leukemia cell growth at less than 0.1 nM whereas ~500-fold higher drug concentrations are required to inhibit by 50% the growth of the rest of the cancer cell lines. It should be noted that the GI_{50} values of 5,5′-D-ValAMD for CCRF-CEM and HL-60(TB) leukemia cells are ~150-fold lower than the GI_{50} values of AMD. Similar selectivity against the leukemia cell growth has been found in F8AMD and N8AMD, which are AMD analogues modified at C8 of the chromophore.[9,15] It is difficult to explain why the AMD analogues have such strong selectivity against leukemia cells. As discussed above, the AMD analogues that exhibit the selective anti-leukemina activity have three characteristic features: (1) RNA:DNA hybrid binding capability observed in F8AMD and N8AMD. (2) Longer sequence specificity observed in the 5,5′-D-aromatic amino acid analogues. (3) Significant reduction of water solubility (i.e., increase of the lipophilic property) observed in all the AMD analogues.

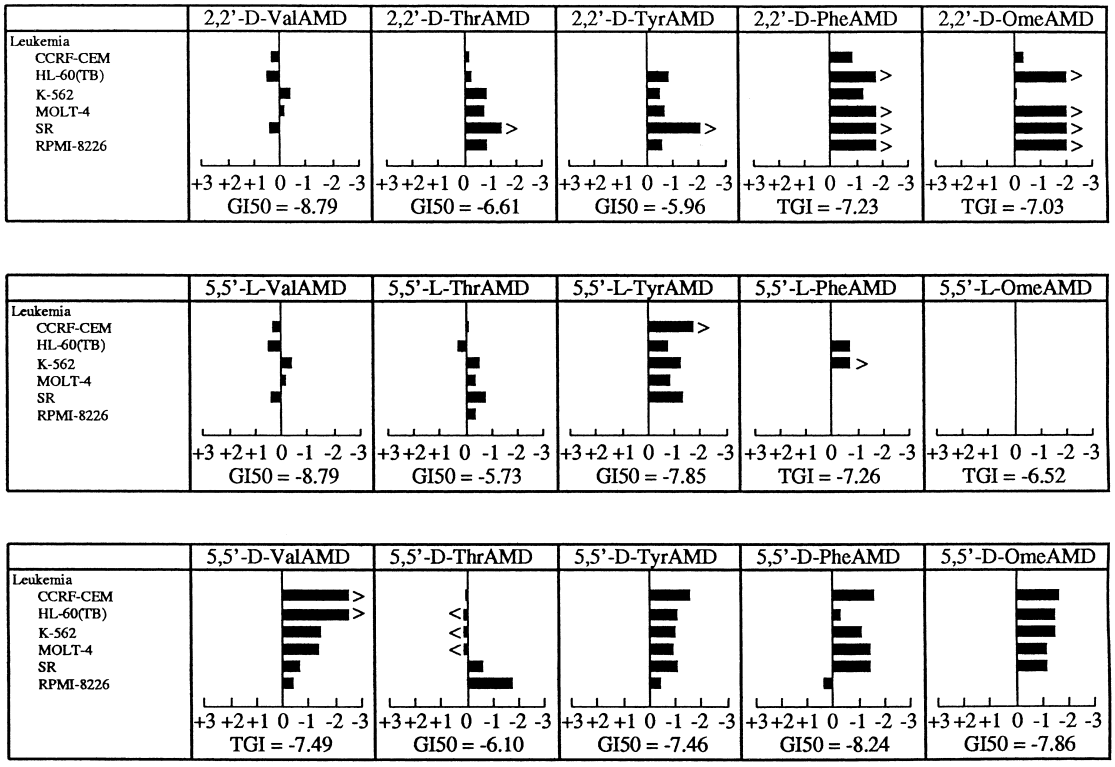

Figure 3. Mean graphs of either 50% growth inhibition (GI_{50}) or total growth inhibition (TGI) of AMD, and AMD analogues showing the selectivity against leukemia cell lines. The units of the graphs are either $\log_{10}(GI_{50})$ or $\log_{10}(TGI)$. Either the mean $\log_{10}(GI_{50})$ or $\log_{10}(TGI)$ is given under the scale. For example, the graph of 5,5′-D-ValAMD indicates that the leukemia cell growths are totally inhibited at about a 100- to 1000-fold lower drug concentration than the mean drug concentration required to inhibit the rest of the cancer cells. Symbols '>' and '<' indicate the bar graphs would be farther extended to right and left, respectively. Note that 2,2′-D-ValAMD and 5,5′-L-ValAMD are AMD itself. Due to a high cytotoxicity to the leukemia cell lines, the data of 5,5′-L-OmeAMD is not available.

Table 4. GI_{50}, TGI and LC_{50} of AMD and AMD analogues deduced from the anticancer screening tests

Compound	$Log_{10}(GI_{50})$	$Log_{10}(TGI)$	$Log_{10}(LC_{50})$
AMD	−8.79	> −7.32	> −6.69
2,2′-D-ThrAMD	−6.61	> −4.97	> −4.22
2,2′-D-TyrAMD	−5.96	> −4.25	> −4.00
2,2′-D-PheAMD	< −8.76	−7.23	> −5.65
2,2′-D-OmeAMD	< −8.69	−7.03	> −5.42
5,5′-L-ThrAMD	−5.73	> −4.59	> −4.06
5,5′-L-TyrAMD	−7.85	> −6.56	> −6.14
5,5′-L-PheAMD	< −7,98	< −7.26	> −4.36
5,5′-L-OmeAMD	< −7.89	< −6.52	> −4.17
5,5′-D-ValAMD	< −9.36	> −7.49	> −6.50
5,5′-D-ThrAMD	> −6.10	> −6.00	> −6.00
5,5′-D-TyrAMD	−7.46	> −6.32	> −6.03
5,5′-D-PheAMD	−8.24	> −6.95	> −6.28
5,5′-D-OmeADM	−7.86	> −6.46	> −6.06

Initially we thought that an RNA:DNA hybrid binding capability might be related to the unique anti-leukemia selectivity since F8AMD and N8AMD are potential RNA:DNA hybrid binding agents.[9] However, on the basis of the crystal structures of DNA–AMD complexes, the side chains of 2,2′-D-amino acid analogues and 5,5′-L-amino acid analogues cannot interact with the sugar and base moieties of DNA. Therefore, the selectivity against leukemia cells found in the AMD analogues are not due to the RNA:DNA binding capability. The sequence specificity could explain the selectivity of 5,5′-D-TyrAMD, 5,5′-D-PheAMD and 5,5′-D-OmeAMD, but not the other AMD analogues. Therefore, we have hypothesized that the modification on the AMD structure increases the analogue's lipophilicity and somehow improves the diffusion rate of the analogues into the leukemia cells.

Conclusion

AMD that binds to DNA and inhibits RNA synthesis is a potent anticancer agent, but its anticancer spectrum is very broad and there is no distinguishable selectivity against specific cancers. Modification at the sites ((**R2**, **R2′**) and (**R5**, **R5′**)) that are not involved in any DNA interaction creates a unique selectivity against certain cancer cells. This rather unconventional approach might be useful for the improvement of selectivity of other potent DNA binding drugs.

Experimental

Chemistry

The AMD analogues examined were synthesized according to procedures already described.[11,12] The NMR spectra were obtained with 300 MHz Varian XL-300 NMR

Spectrometer. For the mass spectrum measurements, electron ionization (EI) and chemical ionization (CI) spectra were obtained on a Nermag R10-10 quadrupole GC/MS system with SPEC-TRAL 30 data system. Fast-atom bombardment mass spectra (FABMS) were obtained on a ZAB HS mass spectrometer. The homogeneity of the products was checked by thin-layer chromatography on silica-gel plates. The intermediates and the final products of the AMD analogues were confirmed by NMR and mass analysis.

Water solubility measurements

The AMD analogues were dissolved in a buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM KCl) until the compounds were saturated. After centrifugation, the concentrations of AMD analogues were determined spectrophotometrically at 22±1 °C using an extinction coefficient of $2.45\times10^4\ M^{-1}\ cm^{-1}$ at 440 nm.[16]

DNA binding measurements

Self-complimentary oligonucleotides used were synthesized by a Cruachem PS-250 DNA synthesizer using the protocol provided from the company. After the deprotection procedure, the crude oligonucleotides were purified by reverse-phase HPLC using a C_{18} column. Calf thymus DNA (Sigma) was sonicated and purified to eliminate residual protein by repeated phenol extraction and then ethanol precipitation. The absorbance of 1.0 mL of drug solution (~1.0 μM) in a buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM KCl) was measured from 600 to 350 nm at 1 °C. Then a small aliquot of a known concentration of the DNA solution was placed in the sample cuvette and spectra were measured 10 min later. This procedure was repeated six times so that six spectra were measured at six different DNA concentrations. The absorbances of visible spectra were read at 1 nm intervals. Dilution factors were applied to all spectra. The association constants of AMD analogues were calculated based on the spectra using the method developed in our laboratory.[12]

RNA polymerase inhibition

Human (HeLa) cells were cultured as previously described.[11,12] The cells were incubated in duplicate cultures with various concentrations of the drugs for 30 min. Then 10 μCi of [^{3}H] uridine were added to label the RNA synthesized during a 30 min incubation at 37 °C. The labeled RNA was isolated by the rapid procedure of Chomczynski and Sacchi.[17] The label incorporated into RNA was determined by liquid scintillation counting.

Anticancer screening test

Anticancer screening tests were carried out at the National Cancer Institute of the National Institutes of Health. The cell panel consists of 60 lines against which compounds are tested at a minimum of five concentrations at 10-fold dilution. A 48-h continuous drug exposure protocol was used, and a sulforhodamine B protein assay was used to estimate cell viability or growth.[18]

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