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Natural and Synthetic Analogues of Actinomycin D as Grb2-SH2 Domain Blockers

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Abstract—Natural analogues (D, C2, and VII) of actinomycin inhibit Grb2 SH2 domain binding with phosphopeptide-derived from Shc in vitro and in intracellular system. To study structure–activity relationships, 13 actinomycin analogues were synthesized and we found that the inhibition activity depended on the substituents of cyclic peptide groups in actinomycin and two analogues with Tyr residue are the most potent inhibitors with IC_{50} value of 0.5 and 0.8 μM, respectively.

Overexpression of the epidermal growth factor receptor (EGFR) and ErbB-2 has been found in a number of human cancers, including colon, lung and bladder cancers.[1] The HER-2/neu (also known as erbB-2) protooncogene encodes a 185 kDa transmembrane glycoprotein (p185) with intrinsic tyrosine kinase activity homologous to the EGFR.[2] Shc is phosphorylated in response to a variety of receptor tyrosine kinases such as EGFR and ErbB-2[3] and also by cytoplasmic tyrosine kinases such as Lck, Src, Fps or Sea.[4,5] Phosphorylated Shc then acts as a linker or adapter for other SH2 domain-containing proteins. One such protein is a 23-25 kDa Grb2 composed of two SH3 domains and one SH2 domain.[6] The SH2 domain binds to specific phosphotyrosine motifs on receptor or other adaptor proteins such as Shc, whereas the SH3 domains associate with proline-rich motifs in the C-terminal part of SOS, a guanine nucleotide exchange factor for Ras proteins. Recruitment of the Grb2-SOS complexes results in relocalization of SOS to the membrane, in which the event is considered sufficient to induce Ras activation, then the activated Ras in turn leads to Raf and mitogen-activated protein (MAP) kinase activation.[7]

In the course of screening for Shc/Grb2 interaction inhibitors, we discovered actinomycin D, C2 and VII as inhibitors from microbial origins.[8,9] Because of a DNA-intercalating activity, actinomycins have been used as biochemical reagent for the study of molecular and cell biology, and clinically as antineoplastic drug in different tumors. Whereas the concentration of DNA intercalation is high doses (5–10 μM), cytotoxicity in different tumor cell lines appears at low doses (< 10 nM). The mechanism of inhibition of tumor growth at low doses is not well defined. In a recent study, we reported that actinomycin D as a novel SH2 domain ligand inhibited Shc/Grb2 interaction in B104-1-1 and SAA cells.[10] In this report, we compare inhibitory activity of natural (actinomycin C2, VII) and synthetic analogues with actinomycin D to study the structure–activity relationships in vitro binding assay of Shc-derived phosphopeptide to Grb2-SH2 and in growth-inhibition assay of B104-1-1 cells (Figure 1 and Table 1).

To compare the inhibitory activity of Shc/Grb2 interaction in vitro of analogues with actinomycin D, we used the Scintillation Proximity Assay (SPA) procedure. This assay measured binding of a [^{3}H] labeled phosphopeptide derived from the sequence around Tyr317 residue in the human Shc (Ac-SpYVNVK-NH_2) to the SH2 domain of Grb2 fused to glutathione S-transferase (GST). Binding of the phosphopeptide to the Grb2-SH2 domain was quantified using protein A-SPA beads (Amersham) and

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an anti-GST antibody to bind the fusion protein. To learn the growth-inhibition assay of B104-1-1 cells by actinomycin D analogues, we used colorimetric method with WST-1. Table 1 and Figure 1 show synthetic analogues of actinomycin D substituted at R_1 and/or R_2 positions, which were synthesized by the reported method.[11,12] Analogues (**5**, **7** and **8**) substituted with D-Thr or L-Thr at R_1 or R_2 positions show lower activity than other amino acids in both in vitro binding assay and growth-inhibition assay. Analogues substituted with Phe, Ome and Val show $IC_{50} < 2$ μM in vitro and several analogues (**2**, **3**, **4** and **6**) of them appear $GI_{50} \leq 10$ nM.

Two analogues **1** and **12** with Tyr residue strongly inhibited the binding between GST-Grb2 SH2 domain and phosphopeptide-derived from Shc with IC_{50} value of 0.5 and 0.8 μM, respectively, but not proliferation of B104-1-1 cells (see Figure 2b for compound **1**). We thought that because these analogues have hydrophilic group, therefore, we examined whether an analogue **1** inhibited protein–protein interaction of real proteins (Shc/Grb2) in B104-1-1 homogenates. Associated protein complexes containing Shc were immunoprecipitated with polyclonal anti-Shc antibody from homogenates treated with DMSO (control) or 10 μM of **1**. The association with Grb2 was assessed by immunoblotting with monoclonal anti-Grb2 antibody. The inserted box in Figure 2b shows that 10 μM of **1** inhibited Shc/Grb2 interaction about 80%. The result supported that the analogue **1** with high binding affinity in vitro did not inhibit cell proliferation because it has a problem of impermeability through plasma membrane.

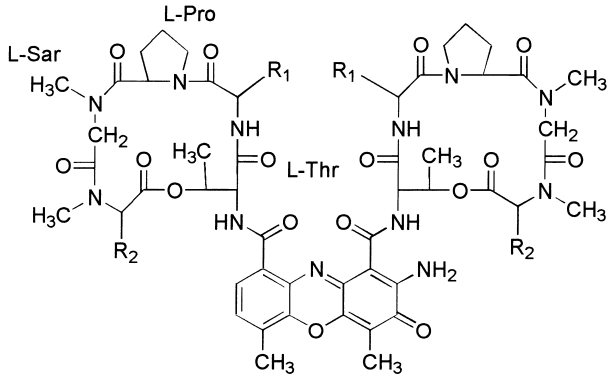

Figure 1. The structure of actinomycin D. Analogues are substituted at R_1 and R_2 positions.

The natural analogues (actinomycin C2 and VII) of actinomycin D showed similar inhibitory activity to actinomycin D in vitro and in cell lines. We investigated that these analogues blocked Shc/Grb2 interactions in cell-based experiments using B104-1-1 cells, because a large number of the Shc/Grb2 complexes were detected. Associated protein complexes containing Shc were

Table 1. Comparison of inhibitory activity in vitro binding assay and in growth-inhibition assay for actinomycin D analogues

Compound	R_1[a]	R_2[a]	Inhibition (%)			
			5 μM	2 μM	IC_{50} (μM)	GI_{50}[e] (nM)
AMD	D–Val	L–MeVal	50.5	40.1	5	0.7
AMC2[b]	D–Ile	L–MeVal	40.8	30.9	5.9	0.8
AMVII[b]	D–Ile	D–Ile	25.3	15.1	7.6	1
1[c]	D–Tyr	L–Val	90.7	84.3	0.5	>
2	D–Phe	L–Val	82.8	73.6	1.2	8
3[d]	D–Ome	L–Val	80.6	69.7	1.5	10
4	D–Val	D–Val	86.0	72.0	1	3
5	D–Val	D–Thr	27.4	3.5	>10	>
6	D–Val	D–Phe	87.7	75.1	0.9	3
7	D–Thr	L–Val	56.7	42.9	4.5	n
8	D–Val	L–Thr	28.5	15.9	>10	n
9	D–Val	L–Phe	78.8	56.8	1.8	n
10	D–Val	L–Tyr	89.7	75.9	0.8	n
11	D–Val	L–Ome	73.8	55.1	2	n
12	D–Val	D–Tyr	90.2	79.9	0.8	n
13	D–Val	D–Ome	73.0	46.9	2	n

[a]R_1 and R_2 are side chains of amino acid in Table 1.
[b]AMC2 and AMVII are natural analogues of actinomycin D.
[c]Compounds **1**–**13** are synthesized analogues of actinomycin D;.
[d]Ome; O-Methyl-Tyrosine.
[e]n = not measured; > = inactive.

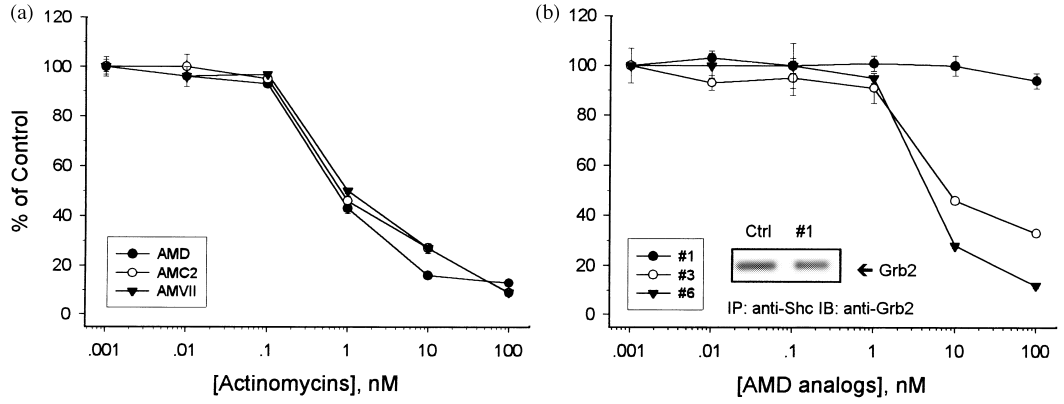

Figure 2. The growth-inhibition assay using colorimetric method with WST-1. B104-1-1 cells were seeded at a density of 5000 cells/well in a 96-well microtiter plate. One day after seeding, cells were replenished with fresh complete medium containing actinomycins or 0.1% DMSO. After incubation for 48 h, cell proliferation reagent WST-1 (Roche Biochemicals) was added to each well. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad). In inset in (b), B104-1-1 cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EGTA, 1 mM sodium vanadate, 50 mM NaF, 30 mM $Na_4P_2O_7$, 10% glycerol, 1 mM PMSF, 5 μg/mL aprotinin, 10 μg/mL leupeptin), and then lysates were treated with DMSO or **1**. The Shc/Grb2 complexes were immunoprecipitated with rabbit antiserum against Shc. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using anti-Grb2 antibody.

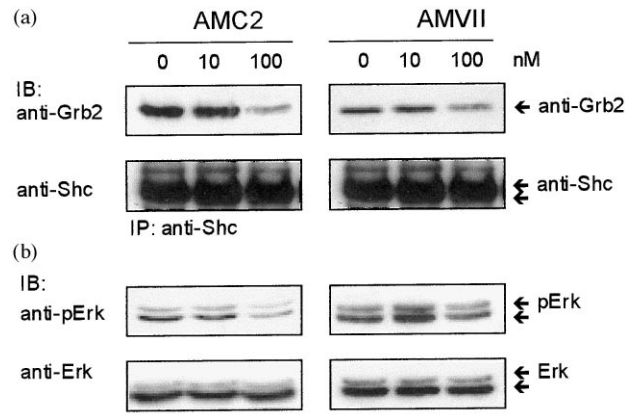

Figure 3. (a) Inhibition of Shc/Grb2 protein–protein interaction by actinomycin C2 and VII in B104-1-1 cells. B104-1-1 cells were maintained in growth medium with increasing concentrations of actinomycins for 48 hrs. Cells were lysed in lysis buffer and then the Shc/Grb2 complexes were immunoprecipitated with rabbit antiserum against Shc. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using anti-Grb2 antibody. The blot was stripped and then probed with anti-Shc antibody to ensure equal loading of immunoprecipitated protein; (b) Phosphorylation of Erk1/Erk2 in B104-1-1 cells treated with actinomycin C2 and VII. B104-1-1 cells were starved in the presence of actinomycins, stimulated with serum and then lysed in lysis buffer. Cell lysates were resolved by SDS-PAGE and then immunoblotting was performed with polyclonal anti-phospho-Erk1 or monoclonal anti-Erk1 antibody.

immunoprecipitated from actinomycin C2 or VII-treated cell lysates with polyclonal anti-Shc antibody and then the association with Grb2 analyzed by immonoblotting with anti-Grb2 antibody. The result of immunoblotting experiment revealed that actinomycin C2 and VII inhibited Shc/Grb2 interaction at higher dose than actinomycin D reported previously (Fig. 3a). The inhibition of Shc/Grb2 interaction by actinomycin C2 and VII in B104-1-1 cells also reduced phosphorylation of MAP kinase (Erk1/Erk2), one of the major components in the Ras-MAP kinase signaling pathway (Fig. 3b). Actinomycin VII, however, inhibited Erk1/Erk2 activity with low inhibitory activity than actinomycin C2.

In conclusion, we report here that synthetic and natural analogues of actinomycin D inhibit Shc/Grb2 protein–protein interaction in a structure-dependent manner. Actinomycin D in comparison to synthetic and natural analogues may be the most potent inhibitor for blocking Shc/Grb2 interaction in B104-1-1 cells and compound **1** and **12** have strong binding affinity in vitro assay system, but not in cell proliferation assay. Our results suggest that these studies on structure–activity relationship provide a clue for the design and development of new SH2 domain antagonists.

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References

1. Verbeek, B. S.; Adriaansen-Slot, S. S.; Vroom, T. M.; Becker, T.; Rijksen, G. *FEBS Lett*. **1998**, *425*, 145.
2. Ben-Levy, R.; Paterson, H. F.; Marshall, C. J.; Yarden, Y. *EMBO J*. **1994**, *13*, 3302.
3. Xie, Y.; Li, K.; Hung, M.-C. *Oncogene* **1995**, *10*, 2409.
4. Xie, Y.; Pendergast, A. M.; Hung, M.-C. *J. Biol. Chem*. **1995**, *270*, 30717.
5. Migliaccio, E.; Mele, S.; Salcini, A. E.; Pelicci, G.; Lai, K.-M. V.; Superti-Furga, G.; Pawson, T.; Fiore, P. P. D.; Lanfrancone, L.; Pelicci, P. G. *EMBO J*. **1997**, *16*, 706.
6. Matuoka, K.; Shibata, M.; Yamakawa, A.; Takenawa, T. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 9015.
7. Aronheim, A.; Engelberg, D.; Li, N.; Al-Alawi, N.; Schlessinger, J.; Karin, M. *Cell* **1994**, *78*, 949.
8. Nam, J.-Y.; Kim, H.-K.; Son, K.-H.; Kim, S.-U.; Kwon, B.-M.; Han, M. Y.; Chung, Y. J.; Bok, S. H. *Bioorg. Med. Chem. Lett*. **1998**, *8*, 2001.
9. Koh, W. S.; Yoon, S. Y.; Lee, E. K.; Kwon, B.-M.; Kim, J. W.; Han, M. Y. *Cancer Lett*. **1997**, *120*, 1.
10. Kim, H.-K.; Nam, J.-Y.; Han, M. Y.; Lee, E. K.; Choi, J.-D.; Bok, S. H.; Kwon, B.-M. *FEBS Letters* **1999**, *453*, 174.
11. Chu, W.; Kamitori, S.; Shinomiya, M.; Carlson, R. G.; Takusagawa, F. *J. Am. Chem. Soc*. **1994**, *116*, 2243.
12. Takusagawa, F.; Wen, L.; Chu, W.; Li, Q.; Takusagawa, K. T.; Carlson, R. G.; Weaver, R. F. *Biochemistry* **1996**, *35*, 13240.