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Isolation of cDNAs Encoding Cellular Drug-Binding Proteins Using a Novel Expression Cloning Procedure: Drug-Western

HIDEKI TANAKA, NOBUKO OHSHIMA and HIROYOSHI HIDAKA

Department of Pharmacology, Nagoya University School of Medicine, Showa-ku, Nagoya, Japan

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ABSTRACT

A rapid and convenient new method for isolating the genes encoding cellular drug-binding proteins is described. This method, drug-western, is based on the use of the drug conjugated with a marker molecule as a probe for the screening of a cDNA library. Unlike the other methods, this method allows us to identify the genes for trace amounts of cellular drug-binding proteins without purification. We have used this approach to isolate human cDNA clones encoding binding proteins of HMN-154 ((E)-4-[2-[2-(*p*-methoxy-benzene-sulfonamide) phenyl]ethenyl] pyridine), a novel benzenesulfonamide anticancer compound (Katoh and Hidaka, 1997). The proteins encoded by two of the isolated clones are identical to NF-YB, B subunit of nuclear transcription factor NF-Y, and thymosin β-10, respectively. Recombinants of both proteins bind specifically to HMN-154 in vitro. Comparison of amino acid sequences between these proteins shows the sequence similarity in a short amino acid stretch [K(X)AKXXK]. Deletion or mutation of this region causes the significant loss of binding of both proteins to HMN-154. Furthermore, HMN-154 inhibits DNA binding of NF-Y to the human major histocompatibility complex class II human leukocyte antigen DRA Y-box sequence in a dose-dependent manner. Interestingly, other binding proteins identified by this method also possess the same or a similar motif. These results clearly demonstrate that NF-YB and thymosin β-10 are specific cellular binding proteins of HMN-154 and that this shared region is necessary for the binding to HMN-154. Hence, this new method is thought to be useful for the identification of drug-binding proteins.

The biological effects of drugs in target cells are thought to be produced by the specific interactions between drugs and physically important proteins. Recent advances in molecular biology allow us to detect protein-protein interaction (e.g., far-western method and two-hybrid system) or DNA-protein interaction (e.g., southwestern method) more quickly than before (Lelong et al., 1989; Chien et al., 1991; Salehzada et al., 1991). On the other hand, none of the molecular biological methods unfortunately has applied to detect drug-protein interaction until the present time.

Many biochemical efforts have been made to identify cellular proteins binding to drugs of interest. Typically, these interactions have been studied by drug-affinity chromatography in which the drug is used as affinity ligand. By this drug-affinity column method, many cellular binding proteins of various compounds have been identified successfully. For instance, W-77 (calmodulin inhibitor) affinity chromatography is utilized for the purification of calmodulin and S-100 (Endo et al., 1981). H-9 [inhibitor of cAMP-dependent protein kinase, Ca^{++}-activated phospholipid-dependent protein kinase (PKC), and cGMP-dependent protein kinase (PKG)] affinity chromatography is utilized for PKC purification (Inagaki et al., 1985). However, if proteins of interest are at low concentration in cells such as nuclear transcription factors, the purification of those proteins is very laborious work. Therefore, we report here a novel method to isolate genes for drug-binding proteins directly from a cDNA expression library by the use of the drug conjugated with a marker molecule.

In our test case, we used HMN-154, our novel benzenesulfonamide anticancer compound, as a probe. HMN-154 is extremely effective against various cancer cell lines including drug-resistance cell lines in vitro (Katoh and Hidaka, 1997; unpublished observation). However, the molecular mechanism of the action of HMN-154 has remained to be elucidated. The interesting characteristics of HMN-154 strongly suggested that novel mechanisms mediating its anticancer activity may exist.

As the marker molecule, we chose BSA. HMN-154 was chemically cross-linked with BSA. This BSA-conjugated HMN-154 (HMN-154/BSA) was used as a probe for the screening of the cDNA expression library. By this method, we show here that NF-YB, one component of nuclear transcription factor NF-Y (transcriptionally active form composed of YA, YB, and YC subunits) (Dorn et al., 1987a,b; Hooft van Huijsduijnen et al., 1990; Li et al., 1992; Maity et al., 1992), and thymosin β-10 (McMahon et al., 1986; McCreary et al., 1988) are binding proteins of HMN-154. Recombinants of both proteins specifically bound to HMN-154 in vitro. Furthermore, we demonstrate that "HN domain", the shared amino acid sequence between these two proteins, is necessary for the binding to HMN-154.

ABBREVIATIONS: MHC, major histocompatibility complex; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility-shift assays.

The HN-domain of NF-YB is located within the domain that is necessary for DNA binding of NF-Y heterotrimer. We demonstrate finally that the interaction between HMN-154 and NF-YB leads to the inhibition of DNA binding of NF-Y complex to its recognition sequence.

Through these results, it is clearly demonstrated that this method is useful for direct identification and cloning of genes encoding cellular drug-binding proteins without purification. It is likely that this novel method has obvious applications upon identifying the binding factors of other drugs.

Materials and Methods

Drugs. Chemical structures of benzenesulfonamide compounds used here are shown in Fig. 1. HMN-154 showed strong anticancer activity with very broad spectra in vitro and in vivo (Katoh and Hidaka, 1987; M. Matsuda, T. Honmura, H.T., F. Fujita, M. Fujita, K. Kimura, and H. Hidaka, submitted). HMN-154 was solubilized in phosphate-buffered saline (PBS) containing 10% (v/v) of dimethyl sulfoxide (DMSO) to a concentration of 10 mM and stored at 4°C in the dark. Before each experiment each stock solution was diluted with PBS to the appropriate concentration for the experiment.

In Vitro Cytotoxicity of Various HMN-154 Derivatives. Cells were seeded into a 96-well microplate at a cell density of 1×10^4/well. Drug was added on the next day, and the plate then was incubated for 72 h at 37°C (5% CO_2). The growth inhibitory concentration was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Scudiero et al., 1988), and 50% growth inhibitory concentration (IC_{50}) was calculated by Scansoft 96 software program (Dainippon Pharmaceutical Co., Osaka, Japan).

Preparation of Drug Probe. For the ligation between HMN-154 and BSA by chemical cross-linker, the functional amino group was chemically introduced into HMN-154 (HMN-154m in Fig. 2). This

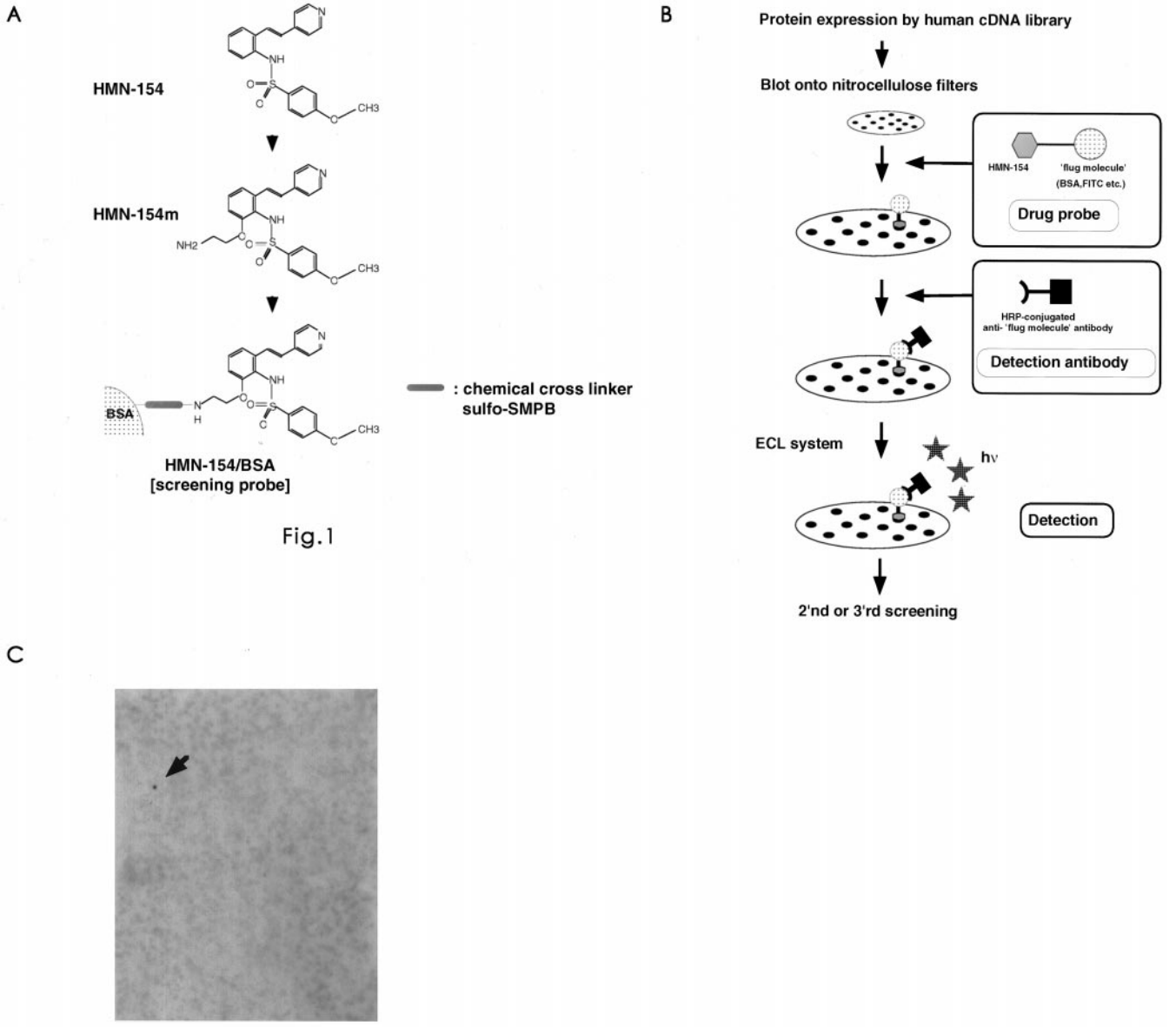


Fig. 1. A, preparation of BSA-conjugated drugs. HMN-154 is a novel benzenesulfonamide anticancer drug (Katoh and Hidaka, 1997). HMN-154m was synthesized and conjugated with BSA by the chemical cross-linker, sulfo-SMPB (see *Materials and Methods*). B, summary of the drug-western procedures. The detailed protocol is described in the text. C, enhanced chemiluminescence detection of plaques expressing drug-binding proteins. Proteins expressed by the λTriplEx cDNA library were blotted onto nitrocellulose membrane and incubated with BSA-conjugated HMN-154. One of the typical positive clones expressing HMN-154-binding proteins is indicated by the arrowhead.

derivative was conjugated with BSA by a chemical cross-linker, sulfo-succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) (Pierce, Rockford, IL), according to the supplier's instruction. Briefly, 40 mmol of HMN-154m was dissolved in 3 ml of PBS and sulfo-SMPB solution (20 mmol in 0.3 ml of PBS) was added by dropping. Then, 4 mmol of BSA (fraction V; Sigma Chemical, St. Louis, MO) in 7 ml of PBS was mixed and incubated for 2 h with moderate stirring. All reactions mentioned were carried out at room temperature (RT). To remove excess amounts of cross-linker, the reaction mixture was gel-filtrated (RG-10 column; Pierce).

Screening of the Library. Human placenta λTriplEx cDNA expression library (Clontech, Palo Alto, CA) was screened by BSA-conjugated HMN-154 (HMN-154/BSA) as a probe. A single colony of XL1-blue was picked and inoculated into Luria-Bertani (LB) medium containing 10 mM $MgSO_4$ and 0.2% maltose. After overnight incubation at 37°C, cells were transfected with the library. Approximately 2×10^4 transformants were plated per 137-mm LB agar (LB medium containing 1.5% agar and 10 mM $MgSO_4$) plate. Nitrocellulose membrane (Hybond-C; Amersham International, Little Chalfont, UK) soaked in 10 mM isopropyl β-D-thiogalactopyranoside solution was placed onto the plate to induce the protein expression. After incubating at 37°C for 4 to 5 h, membrane was washed with TBST [10 mM Tris, pH7.5, 400 mM NaCl, 0.05% Tween 20, 50 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mg/ml aprotinin] five times. Membrane blocking was performed with 1% gelatin in TBST at RT for 30 min. Then, membrane was incubated with 10 ml of TBST containing 10 μl of drug (HMN-154/BSA) for more than 6 h at RT. After washing with TBST, membrane was subjected to the incubation with horse radish peroxidase (HRP)-conjugated anti-BSA antibody (Cappel, Turnhout, Belgium) for 2 h at RT. After washing with TBST three times, plaques expressing the protein that bound drug were detected by the enhanced chemiluminescence system (Amersham).

Protein Expression. *Kpn*I-*Apa*I-ended full-length NF-YB (618 bp) or thymosin β-10 (129 bp) cDNA was cloned into pBluescript SK(−) (Stratagene, La Jolla, CA) or pcDNA3.1(−)/Myc-His Xpress vector (Invitrogen, NV Leek, the Netherlands), respectively. To express each of the proteins, in vitro transcription and translation were performed by using the TNT Coupled Wheat Germ Extract System (Promega, Madison, WI) in the presence of [^{35}S]methionine (Amersham) according to the manufacturer's protocol. Recombinant thymosin β-10 is expressed as the protein fused with c-myc-epitope at the C terminus.

Generation of Mutants of NF-YB and Thymosin β-10. All internal deletion and amino acid substitution mutations were generated by oligonucleotide-directed dual-amber method (Hashimoto-Gotho, 1995). All mutants were verified by DNA sequencing and then expressed by TNT Coupled Wheat Germ Extract System as before.

Immunoprecipitation Assay. For immunoprecipitation assay, 2 μl of transcription and translation reaction mixture was incubated with 2 μg (estimated as the amount of BSA) of HMN-154/BSA at 4°C for 12 h. Then, 1 μg of anti-BSA antibody was added and incubated further at 4°C for 6 h. After incubating the reaction mixture with 10 μl of Protein G Plus/Protein A agarose (50% suspension; Calbiochem, San Diego, CA) at 4°C for 6 h, the reaction mixture was centrifuged and the agarose bead was washed three times with 500 μl of TBST. The immunoprecipitate was then resuspended in Laemmli sample buffer (Laemmli, 1970), boiled for 5 min, and resolved by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) (NF-YB) or tricine-buffered SDS-PAGE (thymosin β-10). Subsequent to electrophoresis, the gels were fixed, dried, and exposed. In competition experiments, immunoprecipitation was performed in the presence of an indicated concentration of HMN-154 as the competitor. In this experiment, agarose bead was washed three times with the buffer containing 20 mM HEPES (pH 7.0), 300 mM NaCl, 0.1% Tween 20, 50 μM PMSF, and 0.5 mg/ml aprotinin.

Electrophorectic Mobility-Shift Assays (EMSA). The purified oligonucleotide 5′-ATTACTCTTTGGCCAATCAGAAAAATATTT-3′ (30 mer), which contains the human major histocompatibility complex (MHC) class II DRA Y-box element (Benoist and Mathis 1990), was end-labeled by using T4 polynucleotide kinase in the presence of [γ-^{32}P]dATP (>3000Ci/mol) and made double-stranded by annealing to the antisense oligonucleotide.

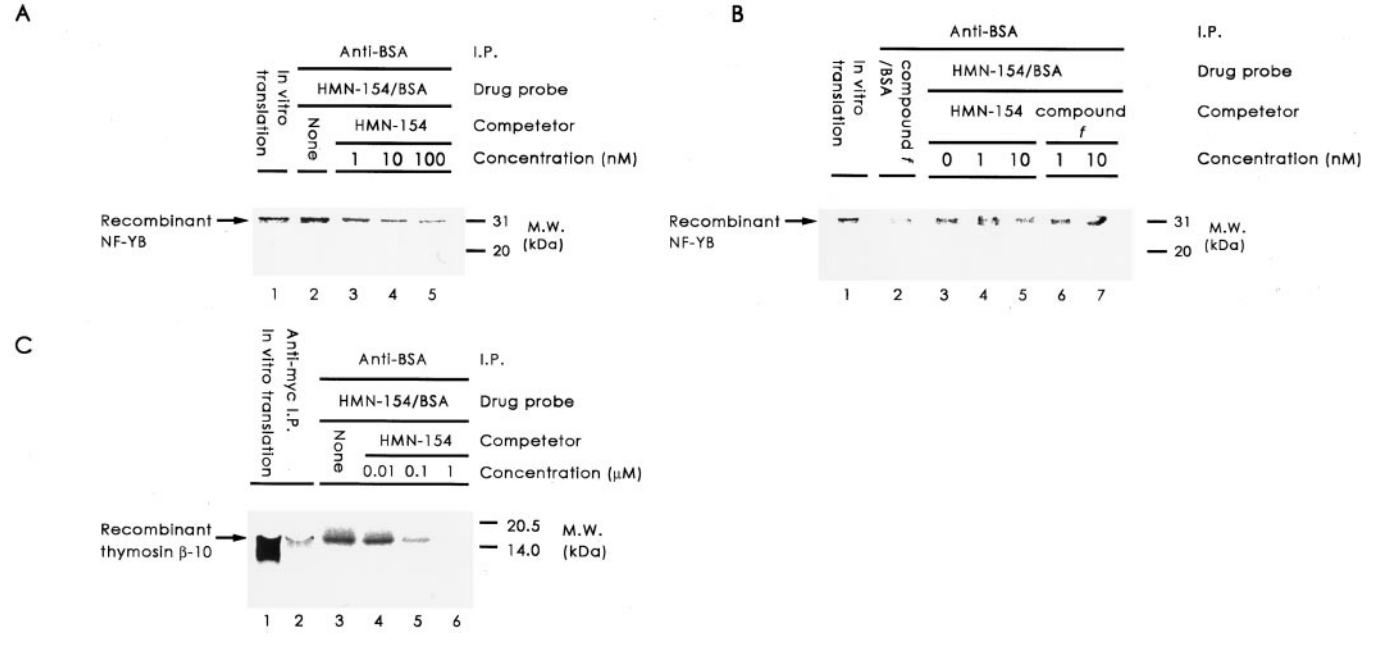


Fig. 2. Specific binding between HMN-154 and recombinant NF-YB (A) or thymosin β-10 (B). Recombinants of NF-YB and thymosin β-10 were generated by transcription of their cDNAs by T7 RNA polymerase in vitro followed by translation of the transcripts by wheat germ extract in the presence of [^{35}S]methionine (lane 1). A, recombinant NF-YB was incubated with HMN-154/BSA. NF-YB-drug complex was immunoprecipitated with anti-BSA antibody and resolved by 12.5% SDS-PAGE (lane 2). The immunoprecipitation assay also was performed in the presence of HMN-154 (lanes 3–5) at an indicated concentration as the competitor. As reported previously (Hooft van Huijsduijnen et al., 1990), the apparent molecular mass estimated by SDS-PAGE (32~35 kDa) was higher than the deduced molecular mass (23 kDa). B, immunoprecipitation assay using recombinant thymosin β-10 in the presence (lanes 4–6) or absence (lane 3) of a competitor. Immunoprecipitates were resolved by tricine-buffered SDS-PAGE. Myc epitope-tagged thymosin β-10 was identified by immunoprecipitation with anti-Myc epitope antibody (Invitrogen) (lanes 1 and 2). I.P., immunoprecipitation.

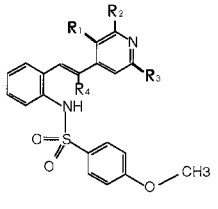
Binding reactions were performed as follows: 2.5 μg of HeLa nuclear extract (Promega) was incubated in 18 μl of a buffer containing 10 mM Tris-HCl (pH 7.3), 0.05 mM EDTA, 40 mM NaCl, 3 mM $MgCl_2$, 10 mM 2-ME, 0.25 mM dithiothreitol, and 2 μg poly(dI/dC) for 60 min. Antibody supershift of NF-Y complexes was performed by including 2 μg of anti-NF-YA or YB antibody (kindly provided by Dr. Robert Mantovani) in the reaction mix. ^{32}P-labeled probe (10,000 cpm) was added and the reaction mixture was further incubated for 30 min. All reactions were performed at RT. Samples were run on 5% nondenaturing polyacrylamide gels in 0.5× TBE at RT and 10 V/cm. The gels were dried and exposed by a BAS image analyzer (Fuji Film, Tokyo, Japan).

Results

Determination of the Position on HMN-154 at Which the Linkage to BSA Is Introduced. In vitro cytotoxicity of various derivatives of HMN-154 against KB (human nasopharyngeal cancer) and colon38 (human colorectal carcinoma) cells tested is summarized in Table 1. HMN-154 showed very strong cytotoxicity against KB and colon38 cells with an IC_{50} value of 0.0026 and 0.003 μg/ml, respectively. It was demonstrated clearly that the introduction of methyl group ([bond]CH_3) at a positions R2 and R3 in a pyridine ring

TABLE 1
In vitro cytotoxicity of various derivatives of HMN-154 against KB (human nasopharyngeal cancer) and colon38 (human colorectal carcinoma) cells

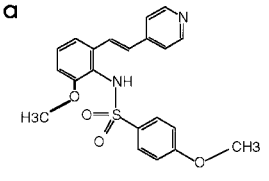
A



	R1	R2	R3	R4	IC50 (μg/ml) KB	colon38
(HMN-154)	-H	-H	-H	-H	0.0026	0.003
	-CH3	-H	-H	-H	0.039	0.043
	-H	-CH3	-CH3	-H	7.94	>10
	-H	-H	-CH3	-H	1.67	2.97
	-H	-H	-H	-CH3	0.024	0.052

B

a



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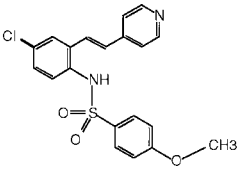

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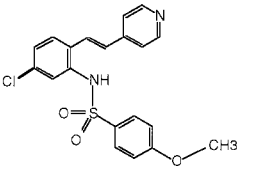
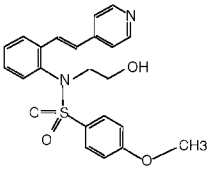


IC50 (μg/ml)		a	b	c
	KB	0.0033	0.018	0.38
	colon38	0.002	0.021	1.05

d



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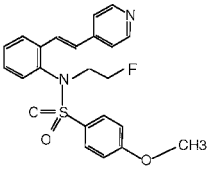
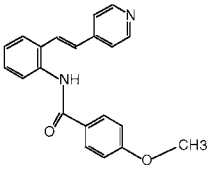
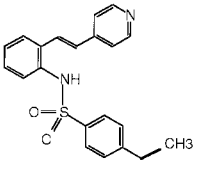


f



IC50 (μg/ml)		d	e	f
	KB	0.089	0.036	>10
	colon38	0.10	0.041	>10

g



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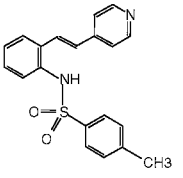

IC50 (μg/ml)		g	h
	KB	0.031	0.037
	colon38	0.037	0.079

on HMN-154 significantly reduced the cytotoxicity (Table 1A). In addition, sulfonamide portion also was shown to be necessary for cytotoxicity (Table 1B, compound **f**). Other derivatives, except compound **a**, showed the reduced cytotoxicity with an IC_{50} range of 0.018 to 1.05 μg/ml. Compound **a** (Table 1B) was the most cytotoxic among the derivatives tested, and the IC_{50} values toward KB and colon38 cells were comparable to those of HMN-154. This result suggested that the methoxy group (-*O*-CH_3) on compound **a** could be used for the linkage to BSA without reduction of the cytotoxicity.

Drug-Western Screening. An outline of this screening method is illustrated in Fig. 1B. The principle of this screening method is almost identical to those of other expression cloning methods except for the use of the drug probe. Of approximately 2×10^6 transformants analyzed, 10 plaques producing significant signals (positive clone) were selected. A photograph representing the typical positive clone is shown in Fig. 1C. Library-derived phagemids were recovered by transfecting each of those phages to *Escherichia coli* strain BM25.8 according to the manufacturer's instruction. The sequence of the cDNA insert was determined by dideoxynucleotide termination method. DNA sequences of six clones showed no significant homology to any known sequences. Sequences of other four clones were identical with the coding region of NF-YB, thymosin β-10, growth hormone, and gonadotropin-releasing hormone, respectively. We found that growth and gonadotropin-releasing hormones were not the specific binding proteins of HMN-154, because genes for these proteins often were isolated when other drugs (W-77, calmodulin inhibitor; H-9, cAMP-dependent protein kinase inhibitor) (Endo et al., 1981; Inagaki et al., 1985) were used as the screening probes (T. Niwa, A. Matsuura, and H.H., unpublished observation). Hence, we examined further the specificity of the binding of NF-YB and thymosin β-10 to HMN-154 in vitro.

Specific Interaction between HMN-154 and NF-YB or Thymosin β-10. To confirm the specific interaction between HMN-154 and NF-YB or thymosin β-10 in vitro, immunoprecipitation assay was performed. Recombinant NF-YB and thymosin β-10 was generated by an in vitro transcription and translation system in the presence of [^{35}S]methionine. Labeled proteins were incubated with HMN-154/BSA and then immunoprecipitated with anti-BSA antibody. As shown in Fig. 2, A and B, HMN-154/BSA bound recombinant NF-YB or thymosin β-10 and the binding was inhibited by the addition of HMN-154 as the competitor. The inhibition occurred in a dose-dependent manner (Fig. 2A, lanes 3–5, and C, lanes 4–6). In contrast, compound **f**, which shows much less cytotoxicity against KB and colon38 cells (Table 1B), did not bind to NF-YB (Fig. 2B, lane 2). Furthermore, compound **f** could not inhibit the binding between HMN-154/BSA and NF-YB (Fig. 2B, lanes 4 and 5). These results clearly show that the binding between HMN-154 and NF-YB or thymosin β-10 is specific and cytotoxicity-dependent.

HMN-154 Binding Motif in Identified Molecules: HN Domain. Between residues 75 and 81 some weaker homologies are seen with residues 14 to 19 of thymosin β-10 (Fig. 3A). This homologous region is predicted to form an α-helix structure in each molecule and contains lysin residues that appeared periodically. Further library screening by this method has identified many other binding proteins as listed in Table 1. More interestingly, these proteins also possessed the same or similar motif. These facts made us speculate that this conserved motif among these identified proteins is the HMN-154 binding motif. We designated this motif "HN domain". To confirm this hypothesis, we performed an immunoprecipitation assay using various mutants of NF-YB and thymosin β-10. Amino acid sequence of each mutant was also shown in Fig. 3A.

Among the amino acid substitution mutants of NF-YB, m3 showed much less binding to HMN-154/BSA compared with the wild type (Fig. 3B, lanes 1 and 4). Furthermore, m2 and deletion mutant, dm, which lacks whole HN domain, failed to bind to HMN-154 (Fig. 3B, lanes 3 and 5). In contrast, mutation of the first lysine residue (m1) showed much less effect on the binding to HMN-154 (Fig. 3B, lane 2). These results indicate that HN domain, especially second and third lysine residues in the domain, is necessary for the binding to HMN-154.

Similarly, immunoprecipitation assay using mutants of thymosin β-10 was performed (Fig. 3C). Mutants m2, m3, and m123 showed much weaker binding to HMN-154 (Fig. 3C, lanes 3–5), but mutant m1, somehow, showed slightly stronger binding than the wild type (Fig. 3C, lane 2).

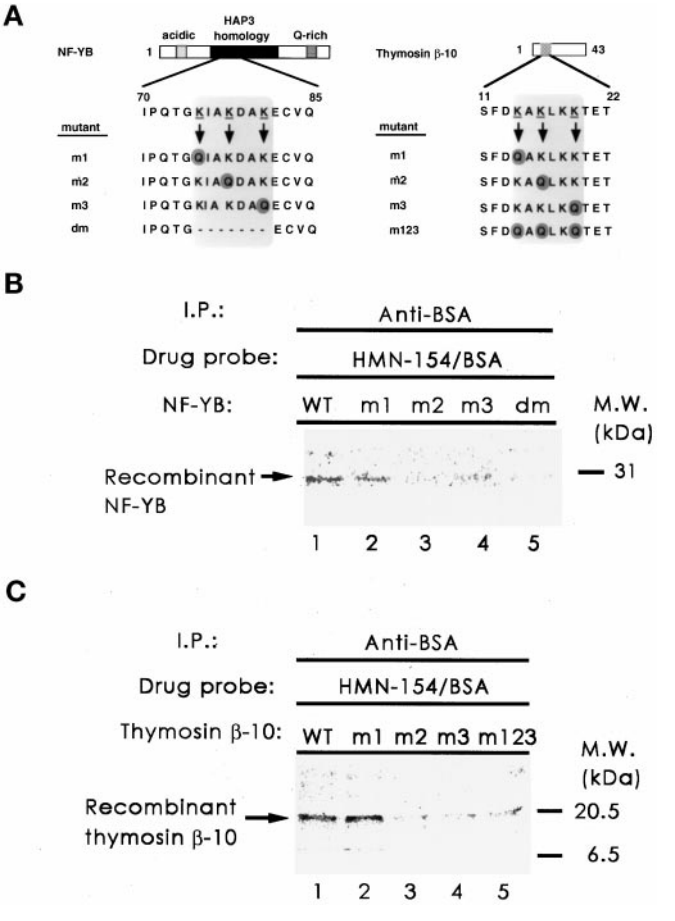

Fig. 3. The HMN-154-binding motif: HN domain. A, alignment of the amino acid sequences of the HN domains in NF-YB (left) and thymosin β-10 (right). The positions of amino acid substitution(s) in each mutant is indicated by a shaded circle. The amino acid deletion mutant dm of NF-YB has no HN domain. B and C, various mutants of NF-YB (B) or thymosin β-10 (C) were incubated with HMN-154/BSA. Drug-protein complexes were immunoprecipitated with anti-BSA antibody and resolved by 12.5% SDS-PAGE (NF-YB) or tricine-buffered SDS-PAGE (thymosin β-10). I.P., immunoprecipitation.

The Interaction between HMN-154 and NF-YB Causes the Loss of DNA Binding of NF-Y Complex. Because the HN- omain of NF-YB resides within the domain that is necessary for DNA binding of NF-Y heterotrimer (Fig. 4A; Kim et al., 1996), we examined the effect of HMN-154 on DNA binding of NF-Y complex. EMSA was performed using a nuclear extract from HeLa cells and an oligonucleotide probe containing human MHC class II HLA Dra Y-box (Benoist and Mathis, 1990). As shown in Fig. 4B, one major, retarded DNA-protein complex was observed (Fig. 4B, lane 1). The anti-NF-YA monoclonal antibody inhibited the complex formation, but the supershifted band was not observed as reported previously (Fig. 4B, lane 2; Mantovani et al., 1992). The anti-NF-YB polyclonal antibodies supershifted the complex (Fig. 4B, lane 3). No specific DNA-protein complexes were observed when the CCAAT core sequence in the oligonucleotide was mutated (data not shown). HMN-154 inhibited NF-Y-DNA complex formation in a dose-dependent manner (Fig. 4B, lanes 4–6). On the other hand, control compound **f** did not affect the NF-Y-DNA complex formation (Fig. 4B, lanes 7 and 8). HMN-154 also showed no significant effect on the NF-κB and AP-1-DNA complex formation even at a concentration of 3 μM (data not shown). These results clearly demonstrate that the binding between HMN-154 and NF-YB is specific and depends on its cytotoxicity.

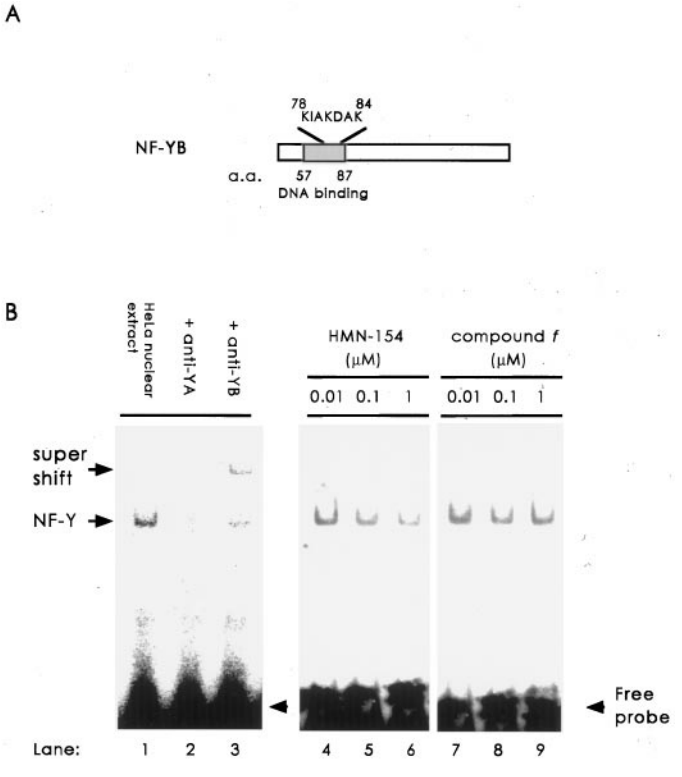

Fig. 4. A, HN domain is located within the context of minimal domain of NF-YB that is necessary for DNA binding of NF-Y heterotrimer (Kim et al., 1996). B, HMN-154 inhibits DNA binding of NF-Y heterotrimer to the human MHC class II Dra Y-box probe (−56 to −85; Benoist and Mathis 1990). Radiolabeled Dra probe (10,000 cpm) was incubated with HeLa nuclear extract (5 μg) and subjected to EMSA analysis. Nuclear extract was incubated with HMN-154 at the indicated concentration for 60 to 90 min before the addition of the ^{32}P-Dra probe (lanes 1 and 3–5). Antibody supershift experiment also was performed (lane 2), and the supershifted band is indicated by an arrow.

Discussion

In this report, we demonstrate first that expression screening by a BSA-conjugated drug is a rapid and convenient method to identify genes for cellular drug-binding proteins. In our test case, we measured the cytotoxicity of various derivatives of HMN-154 in vitro (Table 1). Such structure-activity analysis will be necessary to determine the position at which the linkage to BSA was introduced.

There are some reasons why BSA is used as the marker molecule. 1) A highly purified protein is easily supplied. 2) Specific antibody against BSA is commercially available. 3) Major serum protein is thought to interact with any other cellular proteins at the minimum level; the isolation of genes for growth hormone and gonadotropin-releasing hormone may be the result from the binding between these hormones and albumin. iv) The solubility of drugs in inorganic solvent would be improved by the conjugation with BSA, whereas HMN-154 is difficult to dissolve in the solution containing no organic solvent (i.e., DMSO), BSA-conjugated HMN-154 is soluble in PBS. However, unspecific interactions of BSA to blotted plaques sometimes produced relatively high background signals. In such cases, extended membrane wash with buffer containing a higher concentration of salt (up to 600 mM NaCl) was necessary.

Our method seems to be a similar approach to the far-western and southwestern methods except for the use of the marker-conjugated drug as the screening probe. Therefore, we designate this method “drug-western”. The major advantage of this drug-western method is the immediate availability of the cloned genes for drug-binding proteins without protein purification, which may allow us to detect the trace amount of drug-binding proteins. Indeed, we successfully identified nuclear transcription factor NF-YB as one of the binding proteins of our novel anticancer compound HMN-154 and demonstrated that the binding was specific. Thymosin β-10, identified as another HMN-154 binding protein, also specifically bound to HMN-154 in vitro. Moreover, HN domain [K(X)AKXXK] in both proteins is shown to be necessary for the binding to HMN-154.

From further screening, we identified some other HMN-154 binding proteins as listed in Table 2. Interestingly, those proteins shared HN(-like) domain. However, some binding proteins had slight differences in the sequence from that of typical HN-motif (KXKXXK, thymosin β-10; KXXKXK, Fte-1) (Kho and Zarbl, 1992; Takagi and Manley, 1994; Fisicaro et al., 1995). It seems quite possible that this partial

TABLE 2
HN(-like) domains present in other HMN-154-binding proteins
Further screening by our drug-western method identified other HMN-154-binding proteins (Fte-1, Csa-19, and cleavage stimulation factor 77-kDa subunit). These proteins also shared an HN- or HN-like domain.

Motif	HMN-154 binding protein	Sequence	Function
KXXKXXK	NF-YB	KIAKDAK	transcriptional regulation
	cleavage stimulation factor 77kDa subunit	KVKKAEK	translational regulation?
	Csa-19	KLNKNKKLVK	transcriptional regulation
	v-fos transfomation effector protein (Fte-1)	KMLKKPK	translational regulation?
KXKXXK	Thymosin β-10	KAKLKK	actin sequestering?

deviation produced the different affinity to HMN-154. This idea is supported by competition experiments (Fig. 2, A and B) demonstrating that the binding of NF-YB and thymosin β-10 to HMN-154/BSA is inhibited by different concentrations of competitor, HMN-154 (Fig. 2, A and B). So far, we have no exact explanation of the enhanced binding between thymosin mutant m1 and HMN-154, but it is possible that substitution of the first lysine residue may cause the conformational change in this small peptide that facilitates the accession of HMN-154 to other lysine residues.

In all binding proteins identified, HN(-like) domain resides within an α-helical structure predicted by secondary structure analysis. A regular 3-amino acid spacing would place all lysine residues on the same side of the α-helix. On the HN domain of thymosin β-10, first and second lysine would orient on opposite sites of the helix due to only 2-amino acid spacing. This might cause the different binding mode between mutant m1 and HMN-154.

The molecular modeling of the HN domain of NF-YB and the binary complex with HMN-154 suggested that the second and third lysine residues could interact with sulfonamide portion and methoxy group on HMN-154, respectively. The hypothetical model also suggested that two spacer regions among three lysine residues did not participate in the binding with HMN-154, which might explain why the spacer regions are not conservative among the binding proteins (T. Niwa, A. Matsuura, and H.H., unpublished observations). These results would further support the importance of sulfonamide portion on HMN-154 demonstrated by structure-activity relationship analysis.

NF-Y is known to be a nuclear protein complex that is composed of at least three subunits, YA, YB, and YC, and that recognizes CCAAT core sequence (Dorn et al., 1987; van Huijsdvijen et al., 1990; Kim et al., 1996). This nuclear factor regulates expression of many eukaryotic growth-associated genes including cyclin A and cdc25C (Zwicker et al., 1995a,b). Moreover, recent reports demonstrate that Tax protein of human T-cell leukemia virus type I (HTLV-I) activates the expression of MHC class II DQβ gene through the specific interaction with NF-Y complex in HTLV-I transformed cells (Pise-Masion et al., 1997). This gene has been thought to be involved in cellular differentiation and activation (Zeleznik-Le et al., 1991). Thus, it is likely that NF-Y complex plays important roles for cancer cell growth and is the target molecule for the novel anticancer agent.

We have found recently that HMN-154 suppresses the NF-Y-dependent transcription of the human multidrug resistance (*h*MDR) gene (Gottesman and Pastan, 1993; Sundseth et al., 1997) in vitro. Consistently, HMN-154 restores the sensitivity of multidrug-resistant K2/ARS cells (human ovarian cancer) against adriamycin via the down-regulation of MDR1 (H. Tanaka, N. Ohshima, F. Katoh, H. Hidaka, in preparation).

In contrast to NF-YB, physiological roles for thymosin β-10 largely remain to be elucidated, but some data suggest that this small peptide acts as an actin-sequestering factor as well as other family molecules (McCreary et al., 1988) and is overexpressed in various carcinoma cells (Yu et al., 1993). Additionally, the degree of its expression level is shown to correlate with the grade of tumor malignancy (Hall, 1991). Thymosin β-10 might be involved in tumor metastasis through the alteration of cell adhesion (Verghese-Nikolakaki et al., 1996).

Here, we identified several HMN-154-binding proteins that are not functionally related to each other. NF-Y, one of the binding proteins, has shown to be functionally interrupted by HMN-154. However, the effects of HMN-154 on the function of other binding proteins remain to be elucidated. It is quite possible that HMN-154 interacts with multiple targets that play important roles in tumor cell growth.

By the screening of 3×10^6 clones, we isolated three clones encoding thymosin β-10 and only one clone encoding each of the other binding proteins. Thymosin β-10 is more abundant than nuclear transcription factors such as NF-YB. Frequency of the isolation of drug-binding proteins seems to correlate with the abundance of the binding proteins. Compared with DNA-DNA, DNA-protein, and protein-protein interactions, drug-protein interaction is thought to be weak. Therefore, one must be aware of the critical requisite for carefully controlling an optimal condition (e.g., amount of drug probe, membrane wash after incubation of drug probe) to efficiently detect such a weak interaction with a minimum unspecific background.

In this report, we used a novel expression cloning method, drug-western, to isolate the genes for HMN-154-binding proteins. We also tested the availability of this method when other drugs were used as the probe. When using W-77, which binds calcium-binding proteins such as neurocalcin, calcyclin, and S100P (Endo et al., 1981) as a probe, one cDNA clone that displayed 48% sequence similarity to rat calcium-binding protein 2A (CABP2A) (Van et al., 1993) was isolated. It is possible that this clone encodes the novel human calcium-binding protein. Hence, this method should be applicable for cDNA cloning using other drugs.

If the drug probe of interest is available, the drug-western method will give us the information of drug-binding proteins of interest without purification. This excellent feature of our drug-western method allows more rapid and convenient identification of cellular drug-binding proteins than the other methods described so far. We believe that this method become a major strategy for analyzing the molecular mechanism of drug action.

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Send reprint requests to: Hiroyoshi Hidaka, M.D., Ph.D., Department of Pharmacology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: hhidaka@tsuru.med.nagoya-u.ac.jp