

intraperitoneally, based on the tolerance dose. The total dose of 1,10-DAC was 400 mg/kg because 1,10-DAC showed more toxicity than 4,10-DAC and chrysene in a preliminary test. The mutant frequencies observed with the DNA preparations extracted from the six organs at 7 days after the last injection are shown in Table 1. More than 10 mutant plaques were analyzed in all organs.

The spontaneous mutant frequencies observed in the control group were similar among the six organs in both *lacZ* and *cII* assays, in the rate ranges of 46×10^{-6} to 88×10^{-6} and 21×10^{-6} to 37×10^{-6} , respectively. These results were comparable to our previous studies [5,8–10,15,25].

All the test compounds significantly increased the mutant frequencies in all the tested organs in the *lacZ*

Table 3
Sequences of *cII* mutations in the liver of 4,10-DAC-treated MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
D1	113	C to T	AAG	TCG	CAG	Ser to Leu
D2	86	C to A	AAG	ACA	GCG	Thr to Lys
D3	65	C to A	ATC	GCA	ATG	Ala to Gln
D4	106	G to T	GTT	GAT	AAG	Asp to Tyr
D5	101	G to T	GTG	GGC	GTT	Gly to Val
D6	101	G to C	GTG	GGC	GTT	Gly to Ala
D7	29–30	CT to TC	GAG	GCT	CTA	Ala to Val
D8	74	G to T	CTT	GGA	ACT	Gly to Val
D9	115	C to A	TCG	CAG	ATC	Gln to Lys
D10*	113	C to T	AAG	TCG	CAG	Ser to Leu
D11	42	G to T	ATC	GAG	AGT	Gln to Asp
D12	119	T to A	CAG	ATC	AGC	Ile to Asn
D13	150	G to T	CCA	AAG	TTC	Lys to Asn
D14	190	G to T	GTT	GAC	GAC	Asp to Tyr
D15	103	G to T	GGC	GTT	GAT	Val to Phe
D16	106	G to T	GTT	GAT	AAG	Asp to Tyr
D17	101	G to A	GTG	GGC	GTT	Gly to Asp
D18	196	G to A	GAC	GAC	ATG	Asp to Asn
D19	226	G to C	GCT	GCG	ATT	Ala to Pro
D20	214	C to T	GCG	CGA	CAA	Arg to Stop
D21	47	C to G	AGT	GCG	TTG	Ala to Gly
D22	91	G to T	GCG	GAA	GCT	Gln to Stop
D23	86	C to G	AAG	ACA	GCG	Thr to Arg
D24	132	G to T	TGG	AAG	AGG	Lys to Asn
D25	123	C to A	ATC	AGC	AGG	Ser to Arg
D26	196	G to C	GAC	GAC	ATG	Asp to His
D27	52	C to T	TTG	CTT	AAC	Leu to Phe
D28	64	G to C	ATC	GCA	ATG	Ala to Pro
D29	163	C to T	CTG	CTT	GCT	Leu to Phe
D30*	132	G to T	TGG	AAG	AGG	Lys to Asn
D31	125	G to A	AGC	AGG	TGG	Arg to Lys
D32	196	G to A	GAC	GAC	ATG	Asp to Asn
D33	179–184	–G	TGG	GGG	GTC	Frameshift
D34	148	A to C	CCA	AAG	TTC	Lys to Gln
D35	117	G to T	TCG	CAG	ATC	Gln to His
D36	141	G to T	GAC	TGG	ATT	Trp to Cys
D37	103	G to T	GGC	GTT	GAT	Val to Phe
D38	160	C to A	ATG	CTG	CTT	Leu to Met
D39	125–126	GG to TT	AGC	AGG	TGG	Arg to Ile
D40	166	G to C	CTT	GCT	GTT	Ala to Pro

* Ascribable to the same mutation obtained in an identical mouse.

assay and/or *cII* assay. The 4,10-DAC showed the highest mutagenicity among the test compounds, and the highest *lacZ* mutant frequency of 4,10-DAC, observed in the lung, was 19-, 6- and 4-fold over the spontaneously, chrysene- and 1,10-DAC-induced frequencies, respectively. The highest *lacZ* mutant frequency of 1,10-DAC was also observed in the lung. Mutant frequencies obtained with chrysene were not different between the organs examined and ranged from 1.5- to 3-fold over the spontaneous frequency. The mutant fre-

quencies in the *cII* assay showed a tendency similar to those in the *lacZ* assay.

3.2. Mutation spectra of DACs and chrysene in the liver and lung

Thirty-six and 38 control mutants in the liver and lung, respectively, were subjected to sequence analysis, together with 37 and 39 chrysene-induced mutants, 40 and 43 induced mutants of 1,10-DAC and 40

Table 4
Sequences of *cII* mutations in the liver of chrysene-treated MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
B1	210	G to T	CGA	TTG	GCG	Leu to Phe
B2	214	C to T	GCG	CGA	CAA	Arg to Stop
B3	107	A to G	GTT	GAT	AAG	Asp to Gly
B4	150	G to T	CCA	AAG	TTC	Lys to Asn
B5	113	C to T	AAG	TCG	CAG	Ser to Leu
B6	220	G to T	CAA	GTT	GCT	Val to Phe
B7	132	G to T	TGG	AAG	AGG	Lys to Asn
B8	34	C to T	CTA	CGA	ATC	Arg to Stop
B9	40	G to A	ATC	GAG	AGT	Glu to Lys
B10	272–273	–A	TCT	GAA	CAA	Frameshift
B11	190–198	–GAC	GAC	GAC	GAC	Deletion
B12	107	A to G	GTT	GAT	AAG	Asp to Gly
B13	42	G to T	ATC	GAG	AGT	Glu to Asp
B14	125	G to T	AGC	AGG	TGG	Arg to Met
B15	57	C to A	CTT	AAC	AAA	Asn to Lys
B16	141	G to C	GAC	TGG	ATT	Trp to Cys
B17 ^a	190–198	–GAC	GAC	GAC	GAC	Frameshift
B18	74	G to A	CTT	GGA	ACT	Gly to Glu
B19	86	C to G	AAG	ACA	GCG	Thr to Arg
B20	196	G to C	GAC	GAC	ATG	Asp to His
B21	91	G to T	GCG	GAA	GCT	Glu to Stop
B22	178	T to G	GAA	TGG	GGG	Trp to Gly
B23	212	C to A	TTG	GCG	CGA	Ala to Glu
B24	127	T to A	AGG	TGG	AAG	Trp to Arg
B25	88	G to C	ACA	GCG	GAA	Ala to Pro
B26	196	G to A	GAC	GAC	ATG	Asp to Asn
B27	129	G to T	AGG	TGG	AAG	Trp to Cys
B28	88	G to A	ACA	GCG	GAA	Ala to Thr
B29	99–101	–G	GTG	GGC	GTT	Frameshift
B30	212	C to A	TTG	GCG	CGA	Ala to Glu
B31	124	A to T	AGC	AGG	TGG	Arg to Trp
B32	179–184	–G	TGG	GGG	GTC	Frameshift
B33	91	G to T	GCG	GAA	GCT	Glu to Stop
B34	101	G to A	GTG	GGC	GTT	Gly to Asp
B35	89	C to T	ACA	GCG	GAA	Ala to Val
B36	89	C to A	ACA	GCG	GAA	Ala to Glu
B37	163	C to G	CTG	CTT	GCT	Leu to Val

^a Ascribable to the same mutation obtained in an identical mouse.

Table 5
Sequences of *cII* mutations in the liver of control MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
A1	40	G to A	ATC	GAG	AGT	Glu to Lys
A2	205–213	–CGATTGGCG				Deletion
A3	34	C to T	CTA	CGA	ATC	Arg to Stop
A4	115	C to A	TCG	CAG	ATC	Gln to Lys
A5	212	C to T	TTG	GCG	CGA	Ala to Val
A6	89	C to A	ACA	GCG	GAA	Ala to Glu
A7	119–208	–ATGGCTCGAT				Deletion
A8	122	G to T	ATC	AGC	AGG	Ser to Ile
A9	233	T to C	ATT	CTC	ACC	Leu to Pro
A10	212	C to T	TTG	GCG	CGA	Ala to Val
A11 ^a	89	C to A	ACA	GCG	GAA	Ala to Glu
A12	150	G to T	CCA	AAG	TTC	Lys to Asn
A13	205	C to T	GCT	CGA	TTG	Arg to Stop
A14	95	C to A	GAA	GCT	GTG	Ala to Asp
A15	89	C to T	ACA	GCG	GAA	Ala to Val
A16	212	C to T	TTG	GCG	CGA	Ala to Val
A17	122	G to T	ATC	AGC	AGG	Ser to Ile
A18 ^a	150	G to T	CCA	AAG	TTC	Lys to Asn
A19	113	C to T	AAG	TCG	CAG	Ser to Leu
A20	210	G to T	CGA	TTG	GCG	Leu to Phe
A21	212	C to T	TTG	GCG	CGA	Ala to Val
A22	34	C to T	CTA	CGA	ATC	Arg to Stop
A23	110	A to T	GAT	AAG	TCG	Lys to Met
A24	241–246	–A	AAA	AAA	CGC	Frameshift
A25	196	G to A	GAC	GAC	ATG	Asp to Asn
A26 ^a	34	C to T	CTA	CGA	ATC	Arg to Stop
A27	107	A to C	GTT	GAT	AAG	Asp to Ala
A28	89	C to T	ACA	GCG	GAA	Ala to Val
A29	196	G to A	GAC	GAC	ATG	Asp to Asn
A30	212	C to T	TTG	GCG	CGA	Ala to Val
A31	3	G to A	–	ATG	GTT	Met to Ile
A32	179–184	–G	TGG	GGG	GTC	Frameshift
A33	95	C to G	GAA	GCT	GTG	Ala to Gly
A34	110	A to C	GAT	AAG	TCG	Lys to Thr
A35 ^a	89	C to T	ACA	GCG	GAA	Ala to Val
A36 ^a	110	A to C	GAT	AAG	TCG	Lys to Thr

^a Ascribable to the same mutation obtained in an identical mouse.

and 42 induced mutants of 4,10-DAC in the respective two organs. The mutations are characterized in Tables 2–9 and summarized in Tables 10 and 11. In Tables 10 and 11, the same mutations from an identical mouse were treated as a single event.

In the liver, spontaneous mutations consisted mainly of G:C to A:T transitions (14/30) followed by G:C to T:A transversions (7/30) as shown in the previous report on the *cII* mutant spectrum in the liver of control MutaTM Mouse [9,26].

The majority of chrysene-induced mutations were G:C to T:A transversions (13/36), followed by the G:C to A:T transitions (9/36). Both 1,10-DAC- and 4,10-

DAC-induced mutations also consisted mainly of G:C to T:A transversions (17/38 and 18/38, respectively). The *cII* mutant spectra by all the test compounds in the lung showed a tendency similar to those in the liver.

4. Discussion

1,10-DAC and 4,10-DAC, the tetracyclic azaPAHs and diaza-analogs of chrysene, could not be converted to the bay-region diol epoxide form because of their nitrogen atoms in the benzene rings of the bay-region

Table 6
Sequences of *cH* mutations in the lung of 1,10-DAC-treated MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
c1	163–164	CT to AA	CTG	CTT	GCT	Leu to Asn
c2	215	G to C	GCG	CGA	CAA	Arg to Pro
c3	132	G to T	TGG	AAG	AGG	Lys to Asn
c4	79	G to T	ACT	GAG	AAG	Glu to Stop
c5	160–161	CT to AG	ATG	CTG	CTT	Leu to Arg
c6	178	T to A	ATG	CTG	CTT	Trp to Arg
c7	100–101	GG to AT	GTG	GGC	GTT	Gly to Ile
c8	65	C to A	ATC	GCA	ATG	Ala to Glu
c9	150	G to T	CCA	AAG	TTC	Lys to Asn
c10 ^a	79	G to T	ACT	GAG	AAG	Glu to Stop
c11	132	G to T	TGG	AAG	AGG	Lys to Asn
c12	34	C to T	CTA	CGA	ATC	Arg to Stop
c13	140	G to C	GAC	TGG	ATT	Trp to Ser
c14	179–184	–G	TGG	GGG	GTC	Frameshift
c15	212	C to T	TTG	GCG	CGA	Ala to Val
c16	141	G to T	GAC	TGG	ATT	Trp to Cys
c17	62	T to C	AAA	ATC	GCA	Ile to Thr
c18	196	G to T	GAC	GAC	ATG	Asp to Tyr
c19	212	C to G	TTG	GCG	CGA	Ala to Gly
c20	42	G to T	ATC	GAG	AGT	Glu to Asp
c21	160	C to A	ATG	CTG	CTT	Leu to Met
c22	215	G to C	GCG	CGA	CAA	Arg to Pro
c23	127	T to G	AGG	TGG	AAG	Trp to Gly
c24	64	G to T	ATC	GCA	ATG	Ala to Ser
c25	100	G to A	GTG	GGC	GTT	Gly to Ser
c26	111	G to T	GAT	AAG	TCG	Lys to Asn
c27	62	T to G	AAA	ATC	GCA	Ile to Ser
c28	196	G to T	GAC	GAC	ATG	Asp to Tyr
c29	179–184	–G	TGG	GGG	GTC	Frameshift
c30	122	G to T	ATC	AGC	AGG	Ser to Ile
c31	126	G to C	AGC	AGG	TGG	Arg to Ser
c32	128	G to T	AGG	TGG	AAG	Trp to Leu
c33	161	T to A	ATG	CTG	CTT	Leu to Gln
c34	129	G to T	AGG	TGG	AAG	Trp to Cys
c35	179–184	–G	TGG	GGG	GTC	Frameshift
c36	164–165	–T	CTG	CTT	GCT	Frameshift
c37	150	G to T	CCA	AAG	TTC	Lys to Asn
c38	178	T to G	GAA	TGG	GGG	Trp to Gly
c39	167	C to G	CTT	GCT	GTT	Ala to Gly
c40	106	G to T	GTT	GAT	AAG	Asp to Tyr
c41	51	G to C	GCG	TTG	CTT	Leu to Phe
c42	133	A to T	AAG	AGG	GAC	Arg to Trp
c43	169	G to C	GCT	GTT	CTT	Val to Leu

^a Ascribable to the same mutation obtained in an identical mouse.

epoxide or diol moiety. Nevertheless, 1,10-DAC and 4,10-DAC showed *in vitro* mutagenicity in the Ames tests using rat liver S9 or human liver microsomes in our previous study [17]. Therefore, these DACs might be converted to ultimate mutagenic forms by a mechanism different from that of chrysene. We have

investigated the *in vivo* mutagenicity of some aza-PAHs, such as quinoline and 10-azaBaP [5,8–10,15]. The 1,10-DAC and 4,10-DAC consist of two quinoline moieties and also have a structure similar to 10-azaBaP. In the present study, we attempted to investigate the *in vivo* mutagenicity of DACs compared with chrysene

Table 7
Sequences of *cII* mutations in the lung of 4,10-DAC-treated MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
d1	123	C to A	ATC	AGC	AGG	Ser to Arg
	138	C to A	AGG	GAC	TGG	Asp to Glu
d2	29	C to A	GAG	GCT	CTA	Ala to Asp
d3	117	G to T	TCG	CAG	ATC	Gln to His
d4	212	C to T	TTG	GCG	CGA	Ala to Val
d5	125	G to T	AGC	AGG	TGG	Arg to Met
d6	160	C to A	ATG	CTG	CTT	Leu to Met
d7	128	G to T	AGG	TGG	AAG	Trp to Leu
d8	293	G to T	TTC	TGA	–	Stop to Leu
d9	220	G to T	CAA	GTT	GCT	Val to Phe
d10	57	C to A	CTT	AAC	AAA	Asn to Lys
d11	111	G to T	GAT	AAG	TCG	Lys to Asn
d12	115	C to A	TCG	CAG	ATC	Gln to Lys
d13	51	G to T	GCG	TTG	CTT	Leu to Phe
d14	179–184	–G	TGG	GGG	GTC	Frameshift
d15	115	C to T	TCG	CAG	ATC	Gln to Stop
d16	88	G to C	ACA	GCG	GAA	Ala to Pro
d17	132	G to T	TGG	AAG	AGG	Lys to Asn
d18	117	G to T	TCG	CAG	ATC	Gln to His
d19	65	C to A	ATC	GCA	ATG	Ala to Glu
d20*	115	C to A	TCG	CAG	ATC	Gln to Lys
d21*	88	G to C	ACA	GCG	GAA	Ala to Pro
d22	145	C to G	ATT	CCA	AAG	Pro to Ala
d23	103	G to T	GGC	GTT	GAT	Val to Phe
d24	167	C to G	CTT	GCT	GTT	Ala to Gly
d25	212	C to G	TTG	GCG	CGA	Ala to Gly
d26	163	C to T	CTG	CTT	GCT	Leu to Phe
d27	224	C to A	GTT	GCT	GCG	Ala to Asp
d28	175	G to T	CTT	GAA	TGG	Glu to Stop
d29	40	G to A	ATC	GAG	AGT	Glu to Lys
d30	160	C to A	ATG	CTG	CTT	Leu to Met
d31*	103	G to T	GGC	GTT	GAT	Val to Phe
d32	89	C to T	ACA	GCG	GAA	Ala to Val
d33	179–184	–G	TGG	GGG	GTC	Frameshift
d34	95	C to A	GAA	GCT	GTG	Ala to Asp
d35	134	G to T	AAG	AGG	GAC	Arg to Met
d36	101	G to T	GTG	GGC	GTT	Gly to Val
d37	34	C to T	CTA	CGA	ATC	Arg to Stop
d38	129	G to T	AGG	TGG	AAG	Trp to Cys
d39	89	–C	ACA	GCG	GAA	Frameshift
d40	125	G to T	AGC	AGG	TGG	Arg to Met
d41	65	C to A	ATC	GCA	ATG	Ala to Glu
d42	122	G to T	ATC	AGC	AGG	Ser to Ile

* Ascribable to the same mutation obtained in an identical mouse.

to understand the nature of the *in vivo* mutagenicity of azaPAHs.

Table 12 summarizes the *in vivo* mutagenicity of a series of azaPAHs with the pyridine moiety, investigated in our previous and present studies using MutaTM Mouse. One of the authors (T.S.) reported that

the median toxic dose (TD₅₀) of chemicals as used for numerical description of their carcinogenic potency is well correlated with *in vivo* mutagenic potency, which was calculated by division of the chemical-induced mutant frequency by the total injection dose of the chemical in the transgenic mouse [27]. We calculated

Table 8
Sequences of *cII* mutations in the lung of chrysene-treated MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
b1	112	T to C	AAG	TCG	CAG	Ser to Pro
b2	89	C to T	ACA	GCG	GAA	Ala to Val
b3	34	C to T	CTA	CGA	ATC	Arg to Stop
b4	141	G to A	GAC	TGG	ATT	Trp to Stop
b5	94	G to C	GAA	GCT	GTG	Ala to Pro
b6	126	G to C	AGC	AGG	TGG	Arg to Ser
b7	214	C to T	GCG	CGA	CAA	Arg to Stop
b8 ^a	34	C to T	CTA	CGA	ATC	Arg to Stop
b9	190–198	–GAC	GAC	GAC	GAC	Deletion
b10	106	G to T	GTT	GAT	AAG	Asp to Tyr
b11	113	C to A	AAG	TCG	CAG	Ser to Stop
b12	101	G to T	GTG	GGC	GTT	Gly to Val
b13	122	G to T	ATC	AGC	AGG	Ser to Ile
b14	196	G to T	GAC	GAC	ATG	Asp to Tyr
b15 ^a	113	C to A	AAG	TCG	CAG	Ser to Stop
b16 ^a	190–198	–GAC	GAC	GAC	GAC	Deletion
b17	34	C to T	CTA	CGA	ATC	Arg to Stop
b18	215	G to C	GCG	CGA	CAA	Arg to Pro
b19	113	C to T	AAG	TCG	CAG	Ser to Leu
b20	233	T to A	ATT	CTC	ACC	Leu to His
b21	104	T to G	GGC	GTT	GAT	Val to Gly
b22	42	G to T	ATC	GAG	AGT	Glu to Asp
b23	25	G to T	AAC	GAG	GCT	Glu to Stop
b24	117	G to T	TCG	CAG	ATC	Gln to His
b25	233	–T	ATT	CTC	ACC	Frameshift
b26	123	C to G	ATC	AGC	AGG	Ser to Arg
b27	169	G to C	GCT	GTT	CTT	Val to Leu
b28 ^a	215	G to C	GCG	CGA	CAA	Arg to Pro
b29	155	C to T	TTC	TCA	ATG	Ser to Leu
b30	26	A to G	AAC	GAG	GCT	Glu to Gly
b31	34	C to T	CTA	CGA	ATC	Arg to Stop
b32	40	G to A	ATC	GAG	AGT	Glu to Lys
b33	117	G to T	TCG	CAG	ATC	Gln to His
b34	179–184	–G	TGG	GGG	GTC	Frameshift
b35	163	C to A	CTG	CTT	GCT	Leu to Ile
b36	150	G to T	CCA	AAG	TTC	Lys to Asn
b37	94	G to C	GAA	GCT	GTG	Ala to Pro
b38	221	T to C	CAA	GTT	GCT	Val to Ala
b39	73	G to A	CTT	GGA	ACT	Gly to Arg

^a Ascribable to the same mutation obtained in an identical mouse.

the mutagenic activity by the following formula to compare the mutagenicity between azaPAHs:

fold-increase in *lacZ* MF (%) = $\frac{\text{lacZ MF obtained by test chemical}}{\text{spontaneous lacZ MF}} \times 100$.

Mutagenic activity = $\frac{\text{fold-increase in lacZ MF}}{\text{total dose of test chemical}}$.

The organs given in capital letters in Table 12 indicate those that showed significant mutation and those given in small letters indicate negative organs. The

underlined organs are those which showed the highest increase in *lacZ* MF. When the test chemical induced mutation in multiple organs, the mutagenic activity was calculated using the data of the underlined organs.

Although the five azaPAHs tested in our previous reports (quinoline, benzo[*f*]quinoline, benzo[*h*]quinoline, 1,7-phenanthroline and 10-azaBaP) induced mutation only in the specific organ such as the liver, 1,10-DAC and 4,10-DAC showed significant mutant frequencies in all of the six organs examined and the

Table 9
Sequences of *cII* mutations in the lung of control MutaTM Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
a1	146	C to T	ATT	CCA	AAG	Pro to Leu
a2	196	G to A	GAC	GAC	ATG	Asp to Asn
a3	113	C to T	AAG	TCG	CAG	Ser to Leu
a4	217	C to T	CGA	CAA	GTT	Gln to Stop
a5	180	G to A	GAA	TGG	GGG	Trp to Stop
a6	73	G to A	CTT	GGA	ACT	Gly to Arg
a7	132	G to T	TGG	AAG	AGG	Lys to Asn
a8	58–61	–A	AAC	AAA	ATC	Frameshift
a9	241–246	–A	AAA	AAA	CGC	Frameshift
a10	149–164	–AGTTCTCAATGCTGCT				Deletion
a11	25	G to T	AAC	GAG	GCT	Glu to Stop
a12	214	C to T	GCG	CGA	CAA	Arg to Stop
a13	197	A to G	GAC	GAC	ATG	Asp to Gly
a14	111	G to T	GAT	AAG	TCG	Lys to Asn
a15	206	G to C	GCT	CGA	TTG	Arg to Pro
a16	212	C to T	TTG	GCG	CGA	Ala to Val
a17	38	T to G	CGA	ATC	GAG	Ile to Ser
a18	40	G to A	ATC	GAG	AGT	Glu to Lys
a19 ^a	40	G to A	ATC	GAG	AGT	Glu to Lys
a20	34	C to T	CTA	CGA	ATC	Arg to Stop
a21	90–91	GG to T	GCG	GAA	GCT	Frameshift
a22	113	C to T	AAG	TCG	CAG	Ser to Leu
a23	140	G to A	GAC	TGG	ATT	Trp to Stop
a24	110	A to T	GAT	AAG	TCG	Lys to Met
a25	175	G to A	CTT	GAA	TGG	Glu to Lys
a26	172	C to T	GTT	CTT	GAA	Leu to Phe
a27	89	C to T	ACA	GCG	GAA	Ala to Val
a28 ^a	34	C to T	CTA	CGA	ATC	Arg to Stop
a29	221	T to G	CAA	GTT	GCT	Val to Gly
a30	273–275	ACA to CGATGCACG				Insertion
a31	212	C to T	TTG	GCG	CGA	Ala to Val
a32	193	G to A	GAC	GAC	GAC	Asp to Asn
a33	34	C to T	CTA	CGA	ATC	Arg to Stop
a34	31	C to G	GCT	CTA	CGA	Leu to Val
a35	155	C to A	TTC	TCA	ATG	Ser to Stop
a36	128	G to A	AGG	TGG	AAG	Trp to Stop
a37	88	G to T	ACA	GCG	GAA	Ala to Ser
a38 ^a	34	C to T	CTA	CGA	ATC	Arg to Stop

^a Ascribable to the same mutation obtained in an identical mouse.

highest *lacZ* mutant frequency in the lung. Chrysene also significantly induced mutation in all of the six organs examined, although chrysene showed similar mutant frequencies in all the six organs. These results suggest that nitrogen substitution in the chrysene skeleton may enhance the inducibility of mutation in the mouse lung.

4,10-DAC showed the highest mutagenic activity, followed in order by: quinoline > 1,7-phenanthroline ≈ 1,10-DAC > benzo[*h*]quinoline ≈ 10-azaBaP = ben-

zo[*f*]quinoline. The number of mutagenic activity of chrysene is the same as that of benzo[*f*]quinoline, which shows the lowest mutagenic activity among the series of azaPAHs tested. The 4,10-DAC gave mutant frequencies higher than 1,10-DAC in all the test organs and the increase in *lacZ* MF obtained by 4,10-DAC is more than twice that by 1,10-DAC in most of the organs tested. Therefore, these results suggest that the nitrogen-substituted position affects the strength of mutagenicity of the DAC structure.

Table 10
Summary of *cII* mutation spectra in the liver of MutaTM Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	30 (100)	38 (100)	38 (100)	36 (100)
Base substitution	26 (87)	35 (92)	35 (92)	32 (89)
Transitions				
GC to AT	14 (47)	7 (18)	8 (21)	9 (25)
AT to GC	1 (3)	2 (5)	0 (0)	2 (6)
Transversions				
AT to TA	1 (3)	3 (8)	1 (3)	2 (6)
AT to CG	2 (7)	1 (3)	1 (3)	1 (3)
GC to TA	7 (23)	17 (45)	18 (47)	13 (36)
GC to CG	1 (3)	5 (13)	7 (18)	5 (14)
-1 Frameshifts	2 (7)	1 (3)	1 (3)	3 (8)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	2 (7)	1 (3)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	0 (0)	1 (3)	2 (5)	0 (0)

The same mutations from an identical mouse were counted as a single event.

We further attempted *cII* mutant spectrum analysis in the liver and lung, which were shown to give the highest *lacZ* mutant frequencies by chrysene and DACs, respectively. In both organs, the spontaneous mutations consisted mainly of G:C to A:T transitions and the mutant spectrum in the control MutaTM Mice showed no difference between the liver and lung. The G:C to A:T transitions in the control mice are likely to be ascribable to the deamination of 5-methylcytosine

at the CpG site as previously reported [28]. DACs predominantly induced the G:C to T:A transversion in both organs. It was especially noteworthy that there was no difference in the *cII* mutant spectrum of 4,10-DAC between the lung and liver in spite of the quantitative difference showed by 4,10-DAC with its 3.9-fold higher *cII* mutant frequency in the lung than in the liver. These results suggest that the high mutagenicity in the lung of 4,10-DAC-treated mice might not arise from

Table 11
Summary of *cII* mutation spectra in the lung of MutaTM Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	35 (100)	41 (100)	39 (100)	35 (100)
Base substitution	30 (86)	34 (83)	35 (90)	32 (91)
Transitions				
GC to AT	19 (54)	3 (7)	6 (15)	10 (29)
AT to GC	1 (3)	1 (2)	0 (0)	3 (9)
Transversions				
AT to TA	1 (3)	3 (7)	0 (0)	1 (3)
AT to CG	2 (6)	3 (7)	0 (0)	1 (3)
GC to TA	5 (14)	16 (39)	25 (64)	11 (31)
GC to CG	2 (6)	8 (20)	4 (10)	6 (17)
-1 Frameshifts	2 (6)	4 (10)	3 (8)	2 (6)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	1 (3)	0 (0)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	2 (6)	3 (7)	1 (3)	0 (0)

The same mutations from an identical mouse were counted as a single event.

Table 12
In vivo mutagenicity of a series of aza-PAHs

Chemical	Total dose (mg/kg)	Tested organ ^a		Mutagenic activity ^b	Sequence analysis	Ref.
Quinoline	250	<u>LIVER</u>	spleen	1.76	Increase of GC to CG	[8–10]
Benzo[<i>k</i>]quinoline	400	<u>LIVER</u>	spleen	0.37	Featureless	[15]
Benzo[<i>h</i>]quinoline	400	liver	<u>LUNG</u>	0.45	Featureless	[15]
1,7-Phenanthroline	200	<u>LIVER</u>	spleen	1.14	Increase of GC to CG	[15]
10-azaBaP	625	<u>LIVER</u>	spleen	0.39	Featureless	[5]
1,10-DAC	400	<u>LIVER</u>	<u>SPLEEN</u>	1.08	Increase of GC to TA	From this study
4,10-DAC	800	<u>LIVER</u>	<u>SPLEEN</u>	2.32	Increase of GC to TA	From this study
Chrysene	800	<u>LIVER</u>	<u>SPLEEN</u>	0.37	Increase of GC to TA	From this study

Superscript (c) in footnote (b) stands for fold-increase in *lacZ* MF (%) = *lacZ* MF obtained by test chemical/spontaneous *lacZ* MF × 100.

^a The organs in capital letters are those that showed significant induction of mutation and those in small letters indicate negative organs. The underlined organ showed the highest increase in *lacZ* MF. BM(bm), bone marrow; gs, glandular stomach; fs, forestomach.

^b Mutagenic activity = fold-increase in *lacZ* MF^c/total dose of test chemical.

the increase of organ-specific adduct(s). In our previous studies, quinoline and 1,7-phenanthroline induced the G:C to C:G transversion [9]. On the other hand, 1,10-DAC and 4,10-DAC induced the G:C to T:A transversion. These results suggest that the enlargement of the molecular size in azaPAHs might change the major mutation pattern from the G:C to C:G transversion to the G:C to T:A transversion. Chrysene also increased the G:C to T:A transversion like DACs. Therefore, it was suggested that the nitrogen substitution in the chrysene skeleton may give no influence on the mutation spectrum.

We have previously reported that quinoline, a partial structure of DACs and one of simplest aza-PAHs, showed mutagenicity in both in vitro [14,29] and in vivo [8] and that metabolic activation of quinoline might take place in the pyridine moiety to form the ultimate genotoxic form, an enamine epoxide (1,4-hydrated 2,3-epoxide) [10–14]. Moreover, benzo[*h*]quinoline and 1,7-phenanthroline, tricyclic azaPAHs with the quinoline moiety as a partial structure, may be activated in the pyridine moiety to show the mutagenicity as reported in our previous paper [16]. DACs, which consist of two quinoline moieties as a partial structure, might be also converted to the enamine epoxide structure in the pyridine moiety to be able to induce mutation. We are trying to investigate further to clarify the metabolic activation pathway of DACs.

In conclusion, it is suggested that the two types of nitrogen substitution in the chrysene structure enhances the mutagenicity in the mouse lung, although they have no influence on the organ specificity and mutation spectrum.

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HX531, a retinoid X receptor antagonist, inhibited the 9-cis retinoic acid-induced binding with steroid receptor coactivator-1 as detected by surface plasmon resonance

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Abstract

HX531 is a retinoid X receptor (RXR) antagonist that inhibits 9-cis retinoic acid-induced neutrophilic differentiation of HL-60 cells. In order to elucidate the inhibitory mechanism of HX531, we have developed a novel ligand sensor assay for RXR in which the receptor-coactivator interaction is directly monitored using surface plasmon resonance (SPR) biosensor technology. A 20-mer peptide from steroid receptor coactivator-1 (SRC-1), containing nuclear receptor interaction motif LXXLL was immobilized on the surface of a BIAcore sensor chip. Injection of human recombinant RXR with or without 9-cis retinoic acid resulted in ligand-dependent interaction with the SRC-1 peptide. Kinetic analysis revealed dissociation constants (KD) of 9-cis RA-preincubated RXR to SRC-1 was 5.92×10^{-8} M. Using this technique, we found that 1 μ M HX531 reduced the k_a value of liganded-RXR with SRC-1, suggesting that HX531 reduced the affinity of RXR to SRC-1. This SPR assay system was applied to obtain quantitative kinetic data of RXR ligand binding to the SRC-1 peptide and the alteration of these data by antagonists.

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Keywords: Retinoid X receptor; Steroid receptor coactivator-1; 9-cis Retinoic acid; HX531; PA024; HL-60 cells; Surface plasmon resonance

1. Introduction

Retinoids have a chemopreventive and therapeutic potency in oncology and dermatology. All-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA) are well-known natural retinoids. Retinoidal activities are mainly achieved through the transcriptional regulations of specific genes via two kinds of nuclear receptors (NRs): (a) RAR α , β and γ , and (b) RXR α , β and γ . ATRA binds RARs and 9-cis RA to both RARs and RXRs.

Abbreviations: 9-Cis RA, 9-cis retinoic acid; ATRA, all-trans retinoic acid; NRs, nuclear receptors; RXR, retinoid X receptor; rhRXR, recombinant human RXR; SRC-1, steroid receptor coactivator-1; SPR, surface plasmon resonance; RU, resonance unit; KD, dissociation constants; k_a , association rate constant

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HL-60 cells, which are an established cell line from an acutely promyelocytic leukemia patient [1], differentiate into neutrophilic cells in response to both ATRA [2] and 9-cis RA [3]. This differentiation ability of HL-60 cells has been useful for screening of chemically synthesized agonists or antagonists of NRs; HX531 was discovered as an antagonist of RXRs from the synthetic chemical library in this screening system [4]. Since HX531 inhibits the activation of both RXR homodimer and the RAR/RXR heterodimer, HX531 is expected to modulate the gene expression through these NRs. The precise mechanism of antagonistic effects by this compound, however, has not been fully elucidated.

The p160 steroid receptor coactivator (SRC) gene family contains three homologous members, which serve as transcriptional coactivators for NRs and certain other transcription factors [5]. These coactivators interact with ligand-bound NRs to recruit histone acetyltransferases (HAT) and methyl-

transferases to specific enhancer/promotor regions, which in turn facilitates chromatin-remodeling [6,7], assembly of general transcription factors, and transcription of target genes. The relatively conserved central region of the SRC family contains three LXXLL motifs that are responsible for interaction with ligand-bound NRs [8–11]. Recently, it was reported that 9-cis RA-liganded RAR/RXR showed enhanced binding with SRC-1 [12]. Hence, we suspected that a binding assay utilizing liganded-RXR with SRC-1 would be useful for the analysis of RXR kinetic parameters.

HX531 inhibited the increase in the nitro blue tetrazolium (NBT) reduction ability of HL-60 cells induced by 9-cis RA [4]. In this study, we have examined the antagonistic effect of HX531 against 9-cis RA on cell growth and on expression of the formyl-Met-Leu-Phe receptor (fMLP-R) and CD11b on HL-60 cells. Furthermore, we have developed an analytical method for agonist or antagonist effects on RXR using surface plasmon resonance (SPR) biosensor technology. Here, we also discuss the interaction of SRC-1 and RXR incubated with 9-cis RA and HX531 using this new method.

2. Materials and methods

2.1. Materials

Biotinylated peptides (>95% of purity) were purchased from Biologica (Nagoya, Japan). Recombinant human RXR β (rhRXR β) was obtained from Affinity BioReagents (Golden, Co). 9-Cis RA and anti-human CD11b monoclonal antibody were from Sigma Chemical (St. Louis, MO) and DAKO (Glostrup, Denmark), respectively.

2.2. Cell culture and neutrophilic differentiation in relation to neutrophilic granulocytes lineage

HL-60 cells were kindly supplied by the Japanese Collection of Research Resources Cell Bank. Cells were maintained in RPMI 1640 medium, containing 10% heat-inactivated FBS and 30 mg/L kanamycin sulfate at 37 °C under moisturized air, containing 5% CO₂. HL-60 cells were differentiated into neutrophilic cells by addition of 9-cis RA. HX531 was preincubated with cells for 30 min before addition of 9-cis RA. Five days after addition of the differentiating agent, expressions of both fMLP-R and CD11b, and cell numbers were measured.

2.3. fMLP-R and CD11b expression

The differentiated cells were collected and incubated with FITC-conjugated fMLP or phycoerythrin-conjugated anti-CD11b antibody, and then labeled cells were subjected to flow cytometric analysis (FACSCalibur, Becton and Dickinson).

2.4. SPR assay

The measurements were performed using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) at 25 °C in a running buffer comprising 0.2% bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂ and 0.1% Tween 20. Streptavidin chips (BIAcore AB) were first treated with three 1-min pulses of 50 mM NaOH, containing 1 M NaCl at a flow rate of 10 μ l/min. Wild-type peptide from human SRC-1 (wtSRC-1, CPSSHSSLTARHKILHRLLEQEGSPS-CONH₂, residues 676–700), containing the consensus NR interaction motif (LXXLL), and the consensus-mutated SRC-1 peptide (mtSRC-1, CPSSHSSLTARHKIAHRALQEGSPS-CONH₂) were biotinylated and immobilized on individual surfaces. Recombinant human RXR β dissolved in the running buffer was preincubated with 9-cis RA or PA024, an agonist of RXRs, in either the presence or absence of HX531 at room temperature for 1 h, and then injected over the surfaces at a flow rate of 10 μ l/min. Ligands were dissolved in ethanol and the final concentration of ethanol was adjusted to 1.0%. After the injection was completed (300 s), the formed complex was washed with the running buffer for an additional 300 s. The chip surfaces were regenerated down to the peptide level by subsequent washing for 60 s pulses with 0.1% SDS and 3.3 mM EDTA solution and 10 mM NaOH solution. Ligand-treated RXR interaction with SRC-1 was represented by resonance unit (RU). Both the KD and *k*_a values were calculated from the experimental curve with BIAcore evaluation 3.1 software package. The formation of surface-bound complexes was analyzed according to the interaction type of A + B \leftrightarrow AB.

2.5. Statistical analysis

Each experiment was repeated three or more times, and representative data are indicated. Statistical analysis was performed using the unpaired t-test. Values of *P* < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Effects of HX531 on proliferation and CD11b and fMLP-R expression of 9-cis RA-treated HL-60 cells.

To confirm the antagonistic effects of HX531, we examined the effects of 1.0 μ M HX531 on the growth of 0.1 μ M 9-cis RA-treated HL-60 cells 5 days after cultivation. As shown in Fig. 1A, HX531 could not completely restore but partially antagonized the inhibitory effect of 9-cis RA on proliferation of HL-60.

The expression of fMLP-R is one of suitable maker, since we have previously reported that it positively correlated the differentiation of HL-60 cells, i.e. migration and superoxide producing abilities [13–15]. Next, the expression of both fMLP-R and CD11b was analyzed by flowcytometry 5 days

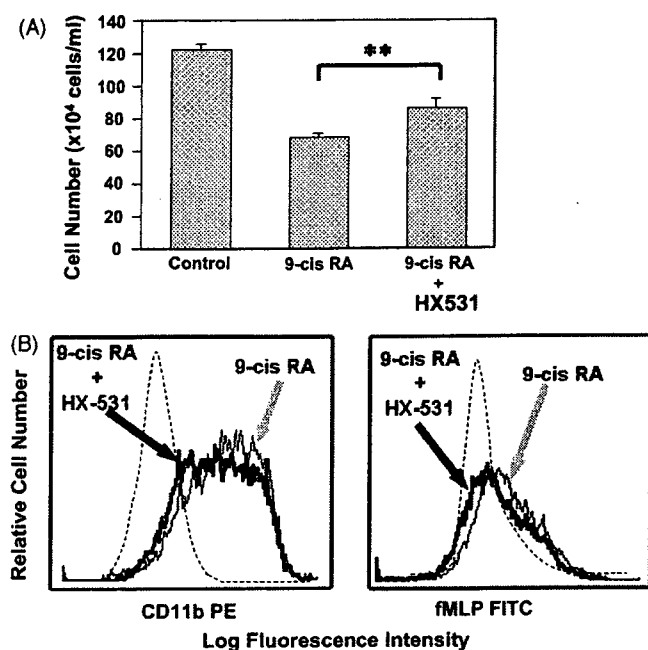


Fig. 1. Effects of HX531 on 9-cis RA-induced-proliferation and differentiation. (A) HL-60 cells were treated HX531 and 9-cis RA. Five days after treatment, cellular number was determined. Columns and bars represent the mean \pm S.D. of triplicate wells (**, $P < 0.01$). (B) fMLP-R and CD11b expression were analyzed by flow cytometry. Non-treated cells are indicated by dotted lines. The gray arrow indicates cells cultured with 9-cis RA (gray lines) and the black arrow the cells cultured with 9-cis RA in the presence of HX531 (black lines).

after addition of 9-cis RA in the presence or absence of HX531 (Fig. 1B). HX531 reduced not only CD11b expression but also fMLP-R expression compared with 9-cis RA-treated HL-60 cells. These results confirmed the antagonistic effects of HX531 on 9-cis RA-induced neutrophilic differentiation and growth arrest.

3.2. Establishment of SPR assay and inhibitory effects of HX531

To elucidate the antagonistic effects of HX531 against 9-cis RA, we undertook establishment of an SPR assay system using the ligand-dependent interaction of NRs and SRCs. First, we tried to detect the signals of ligand-dependent RXR binding with SRC-1 using SPR. When rhRXR β was injected over the sensor chip surface immobilized with a 20-mer peptide, containing the NR interaction consensus motif LXXLL of SRC-1 (wtSRC-1), a small response was observed in the absence of 1 μ M 9-cis RA (Fig. 2A, upper panel; 0.225 μ M (-)). The signal observed from the injection of 0.225 μ M rhRXR β was markedly enhanced by preincubation with 9-cis RA (+) compared with control (-), and the signal increased with rhRXR β in a dose-dependent fashion. On the other hand, when mutated SRC-1 peptide (mtSRC-1), containing alanine substitutions in the core motif (AXXAL) was immobilized on the sensor chip surface as a control, only a

Table 1

Treatment (μ M)	HX531 ($\pm 1 \mu$ M)	k_a ($\times 10^3 \text{ M}^{-1} \text{ s}^{-1}$)
Control	-	0.50
	+	0.18
PA024	-	4.89
	1.0	3.41
	1.0	2.40
9-cis RA	-	2.16
	1.0	1.45
	1.0	1.08

The k_a values of rhRXR β treated with PA024 and 9-cis RA in the presence or absence of HX531.

very weak signal was observed (Fig. 2A, lower panel) compared to that of wtSRC-1 peptide. Thus, these data indicated that the 9-cis RA-elicited signal observed in the injection of RXR with the SRC-1 peptide was specific to the LXXLL motif.

To obtain the sensorgrams showing the LXXLL motif-specific interaction, we subtracted the sensorgrams using mtSRC-1 peptide from those produced by the wtSRC-1 peptide in the following experiments. The result by sensorgrams (Fig. 2B) clearly shows the specific binding of liganded-RXR to the SRC-1 peptide, and the kinetic analysis established an association constant (K_A) for 9-cis RA-preincubated RXR of $1.69 \times 10^7 \text{ M}^{-1}$, which was three-fold greater than its dissociation constants (K_D , $5.92 \times 10^{-8} \text{ M}$).

Next, rhRXR was incubated with PA024 (1 μ M or 10 μ M) or 9-cis RA (1 μ M or 10 μ M), and then the incubation mixture was subjected to SPR analysis subtracting the sensorgrams using mtSRC-1 peptide from those produced by the wtSRC-1. In this analysis, the concentration of the rhRXR protein was fixed at 0.9 μ M (Fig. 3A), and the association rate constant (k_a) from liganded-RXR binding with SRC-1 was calculated. The addition of 1 μ M and 10 μ M of PA024 markedly increased the k_a from $0.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in the control to $3.41 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $4.89 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively. 9-cis RA (1 μ M and 10 μ M) also increased the k_a value to $1.45 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $2.16 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively, although the K_D value of RXR did not change. These data indicate that RXR agonists caused an increase in the binding of RXR to SRC-1 in this system. The binding of RXR to the SRC-1 peptide may increase due to ligand presence in a dose-dependent manner.

When rhRXR β was preincubated with (HX) or without (control) 1 μ M HX531 alone, no signal difference was observed (Fig. 3B), suggesting that HX531 dose not affect the ligand-independent binding of rhRXR β to SRC-1 peptide. When rhRXR β was incubated with either 1 μ M PA024 or 1 μ M 9-cis RA in the presence of HX531, the signals were reduced compared with those in the absence of HX531 (Fig. 3B). All k_a values are shown in Table 1. These results indicated that it is possible to analyze the kinetics param-

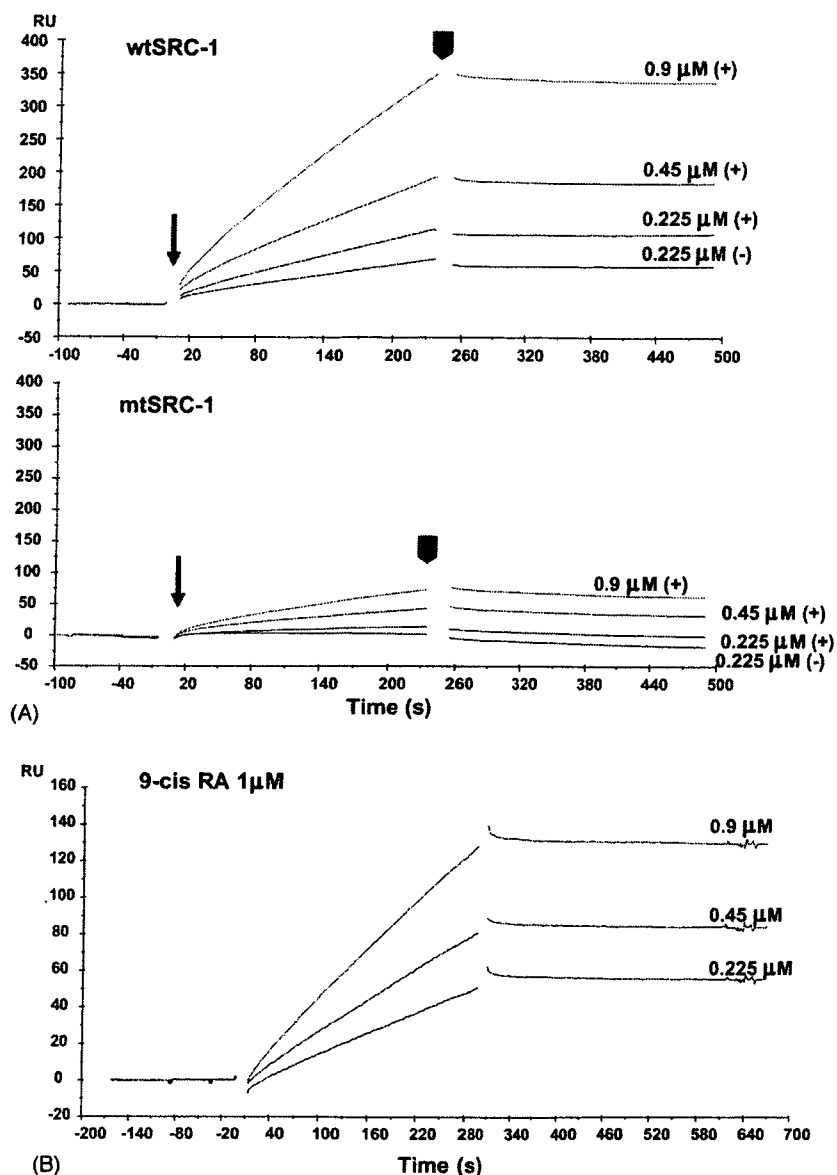


Fig. 2. (A) 9-cis RA caused specific interaction between RXR and SRC-1 peptides, containing a consensus LXXLL motif. Each concentration of rhRXR preincubated with (+) or without (-) 1 μ M 9-cis RA was injected over the surfaces immobilized with wtSRC-1 peptide (upper panel) or mtSRC-1 peptide (lower panel). The arrow and the arrowhead indicate the beginning and end of the injections, respectively. (B) Kinetic analysis of 9-cis RA-liganded-rhRXR β binding to the SRC-1 peptide. Subtraction sensorgrams of mtSRC-1 from those of the wtSRC-1 peptide represent the consensus-specific interaction of rhRXR with the SRC-1 peptide in the presence of 1 μ M 9-cis RA. KD value was calculated by BIAcore evaluation 3.1.

ters of agonists or antagonists in this system. As shown in Table 1, the k_a value of rhRXR β treated with either PA024 or 9-cis RA was reduced by the addition of HX531, indicating that HX531 lowers the affinity of liganded-rhRXR β to SRC-1.

Finally, rhRXR β was preincubated in the presence or absence of HX531 with 0.1 μ M 9-cis RA, a sufficient but minimal dose to promote differentiation of HL-60 cells (Fig. 1), and the signals were compared. In this SPR assay, the 9-cis RA-dependent signal was completely inhibited by the preincubation with 1.0 μ M HX531 (Fig. 3C). These data indicated the possibility that the antagonistic mechanism of

HX531 is the inhibition of the binding of liganded-RXR with SRC-1.

4. Discussion

Many synthetic agonists or antagonists of NRs have been developed to control specific gene expression [7]. These agonists or antagonists are expected to be used as medical treatment for patients through the control of etiologic gene expression related to specific diseases. The search for the candidate agonists and antagonists for NRs have been performed us-

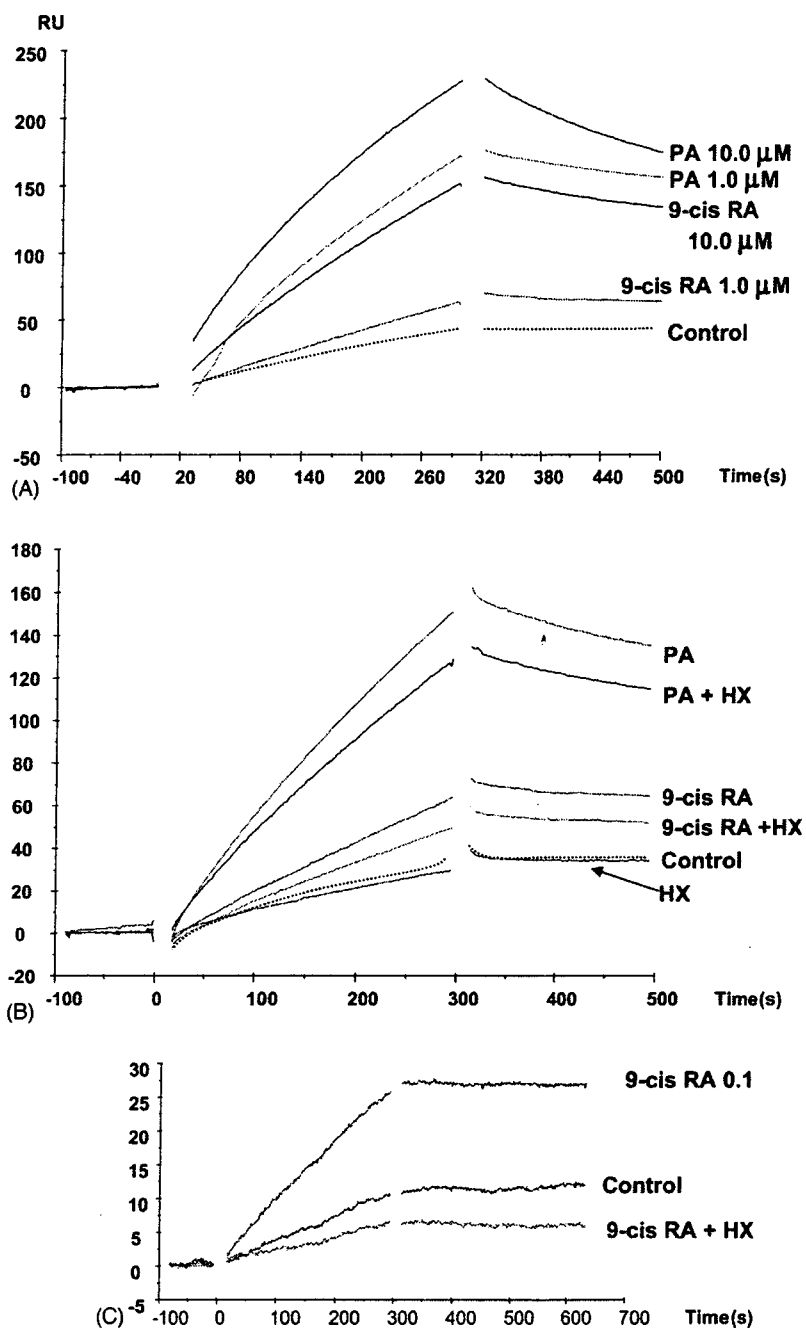


Fig. 3. Comparison of 9-cis RA and PA024 on the binding of rhRXR with SRC-1 and the inhibition of HX531 on these binding interactions. (A) 1.0 μM and 10 μM of 9-cis RA or PA024 (PA) were added to rhRXR, and the solution was subjected to SPR assay system. (B) 1.0 μM HX531 (HX) and agonists were added to rhRXR simultaneously. (C) 0.1 μM 9-cis RA was preincubated with rhRXRβ in the presence or absence of HX531.

ing specific cell culture assays or reporter assays. Using nitro blue tetrazolium (NBT) reduction assay of neutrophilic differentiation in HL-60 cells, either PA024 [16], an agonist for RXR, or HX531 [4], an antagonist for RXR, could be detected. The inhibition of cell growth and the stimulatory expression of fMLP-R and CD11b by 0.1 μM 9-cis RA were antagonized by 1.0 μM HX531 (Fig. 1A and B); these results corresponded with those of previous reports [4, 16]. From this assay, however, it was impossible to evaluate or assess the molecular mechanism producing these agonistic or antago-

nistic effects. In the present study, in order to elucidate the kinetics of the HX531 antagonist, we developed an SPR assay system using the interaction of NR with SRC. We have investigated here the effect of agonists and antagonists on the apparent affinity of RXRβ for an SRC peptide. As shown in Table 1, the apparent k_a of RXRβ to the SRC-1 peptide in the presence of PA024 was higher than that of 9-cis RA, confirming that PA024 is stronger than the natural agonist. Furthermore, the addition of 1 μM HX531 markedly reduced the apparent k_a of RXRβ to the SRC-1 peptide in the pres-

ence of either 1 μM PA024 or 1 μM 9-cis RA. In this context, using SPR assay systems with SRC-1 peptides, HX531 was confirmed to possess the ability to reduce the affinity of RXR β to a SRC.

Farnesoid X receptor (FXR) is a NR that forms a heterodimer with RXR. FXR is activated by natural ligands, such as bile acids, and we previously established SPR assay system for FXR [17]. The binding of liganded-RXR β with the SRC-1 peptide (Fig. 2) is thought to be also dependent to the LXXLL motif as described previously [6]. In the present SPR assay, 1 μM HX531 completely inhibited 0.1 μM 9-cis RA-induced signal, the concentration of which was a sufficient but minimal dose for promoting differentiation in HL-60 cells (Fig. 1A and B) [18]. Therefore, the present SPR assay using this concentration of 9-cis RA was easily adapted to the screening of RXR β antagonists. The present SPR assay can also be utilized in screening for agonists that possess the ability to provide sufficient binding affinity of RXR β to the SRC-1 peptide.

The p160 SRC complex contains histone acetyltransferases (HAT), including the CREB-binding protein (CBP), p300 and the p300/(CBP)-associated factor (p/CAF) and methyltransferases, including CRAM1 and PRMT1. These chromatin-remodeling enzymes are recruited to promoters through interaction between NRs and SRC coactivators in a ligand-dependent manner [7,19]. Although the C-terminal domains of SRC-1 and SRC-3 contain HAT activities [8,20], these activities are much weaker than those in CBP, p300 and p/CAF and inactivation of HAT in SRC-1 does not significantly affect its coactivation function [20,21]. These reports indicate that it may be more important for transcription of their target genes that SRC-1 recruits other coactivators that have high HAT or methyltransferase activity rather than that SRC-1 exhibits its HAT activity. Poujol et al. [12] reported that 9-cis RA caused increase in binding of RAR/RXR with SRC-1. Moreover, in our SPR assay system, the function of agonists and antagonists could be distinguished, and HX531 inhibited the 9-cis RA- or PA024-induced binding of RXR with SRC-1 (Fig. 3).

Recently, new agonists and antagonists of NRs have been synthesized, and simple assay systems are needed immediately. Our SPR assay system is a simple and useful technique for analyzing the binding of NRs and coactivators.

Acknowledgments

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REGULAR ARTICLE

Proteomic analysis of sera from hepatocellular carcinoma patients after radiofrequency ablation treatment

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Comparative proteomic analysis was used to search for characteristic alterations in the sera of hepatocellular carcinoma (HCC) patients who had undergone curative radiofrequency ablation treatment. Serum samples collected from eight patients before and after treatment were subjected to 2-DE. Eighty-eight protein spots differentially expressed with the treatment were selected by clustering analysis, and the proteins were identified by MS based on MALDI-TOF/TOF analysis and public database searches. The statistical analysis suggested that four proteins decreased after treatment (pro-apolipoprotein, α 2-HS glycoprotein, apolipoprotein A-IV precursor, and PRO1708/PRO2044, which is the carboxy terminal fragment of albumin) and that seven proteins were increased after treatment, including leucine-rich α 2-glycoprotein and α 1-antitrypsin. These data facilitate the identification of differentially expressed proteins that are involved in HCC carcinogenesis and provide candidate biomarkers for the development of diagnostic and therapeutic tools.

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1 Introduction

The current methods for the diagnosis of hepatocellular carcinoma (HCC) rely on serological markers, such as alpha-fetoprotein (AFP) and certain liver enzymes, together with

physical assessments and imaging technologies (e.g., ultrasound and computed tomography) [1]. Increases in the serum levels of des-gamma-carboxyprothrombin (DCP) and the fucosylated isoform of AFP have also been associated with HCC. Nevertheless, particularly in hepatitis B virus (HBV) infection, a diagnosis of HCC is sometimes made when the disease is too far advanced for effective treatment, and thus the 5-year survival rates are very low [2]. Clearly, better methods for screening and early diagnosis are important for improved prognosis.

Radiofrequency ablation treatment (RFA) has recently been introduced in the therapeutic modality of HCC [3]. Assuming that the tumor is diagnosed as HCC at an early stage, i.e., at a tumor diameter of less than 3 cm and with restricted growth in several nodules, it can be curatively treated with RFA. In RFA treatment, heat is generated locally

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Abbreviations: AFP, alpha-fetoprotein; AHS2G, alpha-2-HS glycoprotein; DCP, des gamma carboxyprothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RFA, radiofrequency ablation therapy; TAE, transcatheter arterial embolization

by a high-frequency alternating current that flows from the electrodes. A probe is inserted into the lesion in the liver and local heat is generated to melt the tissue adjacent to the probe, causing tissue coagulative necrosis. In the case of RFA, as compared with percutaneous ethanol injection therapy [3, 4], the area of necrosis can be controlled precisely by adjusting the duration of the radiofrequency emission, which increases concentrically from the electrode. Because RFA can shorten the period of hospitalization, it is rapidly gaining popularity as a treatment modality for HCC in Japan and elsewhere [5].

In an effort to identify protein markers that have pathological significance for various cancers, proteomic analysis with 2-DE and MS has been used. Presently, proteomic analysis is widely used for the molecular analysis of various tumors and for the identification of new targets for cancer therapy [6, 7]. Proteomic analysis of disease is based on the profiling of the differential expression levels of various proteins between healthy and diseased states. Thus, identified disease markers may be involved in the etiology of the disease. Recently, two groups applied proteomic analysis to the sera of HBV-infected patients in a search for serological biomarkers of the disease [8, 9]. They found that apolipoprotein AI was associated with the HBV-infected status or HCC of HBV-infected patients. In order to identify candidate biomarkers, they used a 2-DE gel separation technique and MALDI-TOF MS. These findings are attributable to the development of MS/MS. Moreover, one of the latest advances in the accurate, sensitive resolution of mass is TOF/TOF technology [10, 11]. Therefore, in the present study, we used the new high-resolution MALDI-TOF/TOF mass spectrometer (Applied Biosystems), in combination with gel-based proteomic analysis, to profile the proteins in the sera of HCC patients. Eight pairs of pre- and post-treatment serum samples were analyzed for alterations in protein expression.

2 Materials and methods

2.1 Materials

Eight human serum samples were collected with the informed consent of patients with HCC, who had been admitted to the University of Tokyo Hospital. The samples were analyzed in accordance with procedures approved by the ethics committee of the hospital. The collection interval after treatment was 1 wk.

CHAPS, DTT, SDS, iodoacetamide, TEMED, ammonium bicarbonate, Tris, glycine, methanol, urea, and TFA of HPLC grade were purchased from Wako Pure Chemicals (Osaka, Japan). The IEF system (IPGphor), Immobiline DryStrips (18 cm, pH 4–7), and Protein A-Sepharose Fast Flow were purchased from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK). Acrylamide was obtained from Bio-Rad (Hercules, CA, USA). The MS experiments were carried out in a AB4700 MALDI-TOF/TOF mass spec-

trometer (Applied Biosystems, Framingham, MA, USA). A 99% grade CHCA was purchased from Sigma-Aldrich (St. Louis, MO, USA), sequence-grade trypsin was obtained from Promega (Madison, WI, USA), and the standard peptide mixture used for calibration was from Applied Biosystems.

2.2 Sample preparation

Each serum sample of 200 μ L was absorbed with 200 μ L volume of Protein A-Sepharose Fast Flow, which had been previously equilibrated with sodium phosphate buffer (pH 7.1). After the flow-through fractions were collected, the resin was washed with an equal volume of sodium phosphate buffer (pH 7.1), and each wash solution was collected, together with the previously described flow-through fraction. The protein concentration was estimated using Bradford reagent (Bio-Rad), and the samples were stored at 80°C until use.

2.3 2-DE and gel staining

IEF was carried out with 18-cm, pH 4–7 IPG strips according to the manufacturer's instructions, with minor modifications. The 2-DE gels were run on two separate occasions for each sample. The IPG strips were located in the IPG ceramic holders and rehydrated overnight with 300 μ g of the sample, which was solubilized in 340 μ L of a solution that contained 8 M Urea, 2% CHAPS, 18.3 mM DTT, 0.5% v/v IPG buffer (pH 4–7), and a trace of bromophenol blue. The proteins were separated by the IEF system using a programmed voltage gradient at 20°C. After overnight rehydration, the voltage was set at 500 V for 1 h, increased to 4000 V with a 3-h linear gradient, and then increased further to 8000 V for 5.5 h. The samples were maintained at 8000 V until a total run of 47.5 kVh was accomplished (about 9.5 h in total). After IEF, the strips were equilibrated for 15 min in 10 mL of equilibration buffer (6 M Urea, 2% SDS, 0.05 M Tris-HCl (pH 8.8), 30% glycerol, 1% DTT) and then transferred for a further 15 min into equilibration buffer that contained 2.5 iodoacetamide instead of DTT. The equilibrated IPG strips were transferred onto 12.5% uniform second-dimensional polyacrylamide gels, and electrophoresis was carried out in vertical uniform slab SDS-PAGE at 30 mA per gel for about 4.5 h. Each gel measured 20 \times 18 cm. Protein spots in the gels were visualized by silver staining using conventional protocols [12], with minor modifications. Briefly, the gels were fixed overnight in 40% ethanol and 10% acetic acid in water. After the gels were washed twice with 30% ethanol in water, they were incubated for exactly 1 min in 0.05% w/v Na₂S₂O₃. The gels were then washed twice with water for 20 s each and incubated for 20 min in 0.2% w/v AgNO₃. The gels were washed three times for 20 s each in water and developed with 3% w/v Na₂CO₃ in 0.05% v/v formaldehyde in water until the desired contrast was reached (usually after 2.5 min). The reaction was stopped by the addition of 0.5% acetic acid and incubation for 20 min.

2.4 Gel image and data analyses

After silver staining, the 2-DE gels were scanned with an ES-2200 image scanner (Epson, Tokyo, Japan). The intensity of each spot was calculated in ppm using the PDQuest software (Bio-Rad). The volume of each spot was normalized for the total density of the gel. To exclude spots that showed variable intensity in duplicated experiments, we calculated the SD of each spot; spots that showed deviations of more than $2 \times$ SD and spots with missing values in over 25% of the samples were eliminated (Fig. 2) [13]. The averages of the intensity values from duplicate experiments for the remaining 812 spots were used for further analysis. Agglomerative hierarchical clustering of protein spots was performed using the Cluster and TreeView programs (<http://rana.lbl.gov/EisenSoftware.htm>) [14]. The changes in spot intensities after RFA treatment were evaluated using the *t*-statistic. The level of statistical significance was determined by the random permutation test with false discovery rate correction. Corrected *p* values less than 0.05 were considered significant.

2.5 In-gel digestion

Protein spots were excised from gels as 1×1 -mm pieces and were digested with trypsin using a previously published procedure [15, 16], which was modified as described in this section. Gel slices were destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, and then washed with water. After the addition of ACN and complete drying in a SpeedVac[®], the gels were reswollen in 2 μ L of trypsin solution (20 μ g/mL in 25 mM ammonium bicarbonate) and 0.1% *n*-octyl glucoside. After the solvents had penetrated into the gels, 10 μ L of 25 mM ammonium bicarbonate that contained 0.1% *n*-octyl glucoside was added, and the mixture was incubated at 37°C overnight. After in-gel digestion with trypsin, the tryptic peptides were extracted, first with 50 μ L and then with 25 μ L of extraction buffer (50% ACN/0.1% TFA). The supernatants were collected and dried in the SpeedVac[®], and the samples were dissolved in 25 μ L of 0.1% TFA.

2.6 Data examination and protein identification

Using ZipTip[®] μ C18, the tryptic peptides were desalted and concentrated, and then eluted with 1 μ L of matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA) onto a MALDI-TOF/TOF 2×96 -well target plate. MS/MS analysis was performed on a model 4700 Proteomics Analyzer (Applied Biosystems), and air was used as the collision gas. The spectra were obtained by the accumulation of 2000–3500 consecutive laser shots.

Proteins that were expressed differentially in the pre- and post-treatment samples were analyzed by MALDI-TOF, which permitted the identification of peptide masses for subsequent MS/MS analysis. Spectrum peak harvesting and baseline correction were carried out automatically. The query

was made for all species, with the minimum number of matched masses being set at four. The maximum tolerance level for the peptide masses was 50 ppm, and the modifications accepted were carbamidomethyl cysteines and artifactual oxidation of methionines. The NCBI non-redundant databases were used for the searches, and the MS/MS studies were carried out with the same parameters as those described previously for the PMF research, using the MASCOT PMF search tools (Matrix Science, London, UK; http://www.matrixscience.com/search_form_select.html). The precursor peak error was set at 2 Da, and fragment tolerance was defined as 0.6 Da. Internal calibration of the MS/MS data was not performed.

2.7 Western blotting

Western blotting was performed as described previously [17]. Three paired samples were separated on 10–20% gradient SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 100% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at 4°C overnight and incubated with sheep anti-AHSG antibody (ab8939) (Abcam, Cambridgeshire, UK) in TBST that contained 10% Block Ace at 4°C overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies, followed by chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences).

3 Results

3.1 Sample preparation and gel image analysis

We characterized the proteomic serum patterns of eight HCC patients following treatment with RFA or RFA plus transcatheter arterial embolization (TAE). A total of 16 serum samples (before and after treatment for eight patients) were analyzed by 2-DE; an image of a 2-DE gel is shown in Fig. 1. The clinical characteristics of the patients are shown in Table 1. Eight of the patients were infected with HBV or HCV and suffered from HCC (maximum diameter 3.2 cm). These patients were treated with curative RFA or curative RFA plus TAE. The changes in the levels of the HCC markers (AFP/L3/DCP), owing to the therapeutic procedure, are indicated in Table 1 (before vs. after treatment).

The serum samples were first prepared using Protein A-Sepharose Fast Flow beads to deplete the immunoglobulins, which are proteins that are abundant in the serum. Another abundant serum protein, albumin, was not depleted from the samples in this study, as albumin is also a carrier protein that conveys a large variety of compounds, including hormones, lipoproteins, and amino acids [18, 19]. Therefore, the removal of albumin from serum might result in the specific removal of low-abundance cytokines, peptide hormones, and lipoproteins of interest [20].